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Modulating the release of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure

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ABSTRACT

Double water-in-oil-in-water ($W_1/O/W_2$) emulsions have the potential to encapsulate bacteria and their structure if destabilised can facilitate bacterial release in a time controlled manner. The description of release mechanisms of bacteria in $W_1/O/W_2$ emulsions is limited to hyper-osmotic conditions; however, the mechanism responsible for bacterial release in hypo-osmotic conditions is different and the presence of bacteria may affect the emulsion and *vice versa*. In this work, the stability and release properties of $W_1/O/W_2$ emulsions were studied with or without the presence of GFP-tagged *Escherichia coli* (*E. coli*-GFP) in the inner aqueous phase (W_1). The impact of altering the osmotic balance by diluting the emulsion in a hypo-osmotic solution was also determined. $W_1/O/W_2$ 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%), lipophilic surfactant (PGPR, 1% to 8%) 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 at high amounts of W_1 and low concentrations of Tween80 or PGPR. Bacterial release occurred due to oil globule bursting and the viability of bacteria was unaffected by the release mechanism. The concentration of bacteria affected the double emulsion structure during the first step of emulsification but had no effect on emulsion structure during osmotic balance alteration.

1. Introduction

Water-in-oil-in-water ($W_1/O/W_2$) emulsions are systems created by dispersing water-in-oil (W_1/O) emulsion in a second outer aqueous phase (W_2).[1] In recent years $W_1/O/W_2$ emulsions have gained a lot of interest as they have the ability to encapsulate and release hydrophilic substances in a time controlled manner making them promising for drug delivery, cosmetics and food products.[2]

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The purpose of encapsulating microbial species in $W_1/O/W_2$ emulsions vary widely from trying to protect bacteria such as *Lactobacillus* spp. probiotics from gastric juices,[3,4] biles salts,[5] prolonged chilled storage[6] and during cheese manufacturing and melting[7] to utilising them as a 3D microenvironment for biofilm formation[8] and as analytical tools for cultivation, genetic activation and quorum sensing.[9] In such applications the formulation of a highly stable $W_1/O/W_2$ emulsion is desirable. However, $W_1/O/W_2$ emulsions are kinetically unstable due to the presence of two thermodynamically unstable interfaces[10] and their stability require a balance between the Laplace and osmotic pressures.[11,12] By destabilising their structure release of hydrophilic compounds is possible and this can either be thermally-induced[13,14] or osmotically-induced.[15]

The alteration of osmotic pressure in $W_1/O/W_2$ emulsion will destabilise the structure of the emulsion and can either lead to swelling or shrinkage of the oil globules depending on whether the concentration of solutes is higher in W_1 or W_2. Manipulating the osmotic pressure in $W_1/O/W_2$ emulsions can be used for delivering microbial species *in vitro* or *in vivo* which have great potential for applications within the fermentation, agricultural and medical industries. Recently we have reported the mechanism by which *Escherichia coli* is released from $W_1/O/W_2$ emulsion globules in the presence of NaCl in W_2 and we found that by changing the emulsion structure this mechanism can be controlled.[16] However, there is a lack of studies investigating the release of bacteria from the W_1 phase of $W_1/O/W_2$ emulsions in hypo-osmotic conditions which as opposed to hyper-osmotically triggered release formulations provides a sustained release of salts as well as bacterial cells over a period of time in a controlled manner which is desirable in certain applications.

It was shown that by diluting $W_1/O/W_2$ emulsions in hypo-osmotic solution they swell and breakdown to release antiseptics.[17] The concentration of the lipophilic (in the oil phase stabilising the W_1/O interface) and hydrophilic (in the W_2 phase stabilising the O/W_2 interface) surfactants play a major role in controlling the release of the water-soluble substances from the W_1 phase of $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure.[18,19] When the W_1 volume fraction was increased the release of solutes from the oil globules in hypo-osmotic solution become higher.[15] Also in a hypo-osmotic solution the release of solutes from $W_1/O/W_2$ globules was higher when the osmotic pressure gradient between the W_1 and W_2 phases was increased.[15,18,19] However, in these studies the mechanism behind the release has not been clear and was described as "thinning of the oily phase" until breakdown of the oil globules occured.[20] Furthermore, it is difficult to relate the release of water-soluble substances to one mechanism and many processes can be involved including:

- coalescence of the W_1 droplets with O/W_2 interface,[21]
- rupture of the O/W_2 interfacial film,[18]
- migration of the water and water-soluble substances through the oil phase via surfactant-facilitated transport

through reverse micelles,[22] hydrated surfactant and/or diffusion through thin surfactant lamellae.[23]

We have recently shown that the release of bacteria from $W_1/O/W_2$ globules under hyper-osmotic pressure resulted from bursting of the oil globules independent of diffusion mechanisms.[16] Moreover, investigating the mechanism of release by swelling using insoluble particles such as bacteria as opposed to water-soluble substances in W_1 is advantageous when trying to understand how release occurs during the swelling process of the $W_1/O/W_2$ globules under hypo-osmotic pressure. This is because the release of water-soluble substances from $W_1/O/W_2$ globules can be the result of various mechanisms whereas bacterial release will only result from the rupturing of the interfacial film and bursting of the oil globules. We therefore need to investigate the mechanism responsible for the release of bacteria from $W_1/O/W_2$ globules in a hypo-osmotic solution and subsequently try to control this mechanism by changing the structure of the $W_1/O/W_2$ emulsion.

Bacteria can either destabilise[24,25] or stabilise[26,27] the structure of a single emulsion. Bacterial cells possess physiochemical surface properties which are determined by the chemistry of the cell surface important in cell attachment to surfaces.[24] Moreover these properties can have an impact on the stability of an emulsion. For example, bacteria that have surface charge opposite to the oil globules were shown to localise at the O/W_2 interface of the oil globules destabilising the structure of the emulsion.[26] On the other hand bacteria were shown to prevent coalescence of the oil globules and increase the stability of the emulsion.[26,27] Also the structure of emulsion can affect the growth of bacteria. For example, pathogenic *E. coli* 0157:H7 cells grown in emulsions as opposed to liquid media produced more curli (surface structures involved in bacterial attachment to surfaces) and were more heat resistant.[28] Also it was shown that Gram negative and Gram positive bacteria were constrained to grow as colonies instead of as single cells when the volume of the oil phase was increased[29] and when the oil globule size was small.[30] When encapsulated in water droplets of a single W/O emulsion microbial cells can excrete waste products leading to osmotic mismatch that causes droplet shrinkage at rates depending on the strain, species and number of the microbial cells.[31] Moreover, all these studies involved single emulsions and there is a lack in studies investigating the stability of double emulsions with bacteria.

By altering the osmotic balance between the W_1 and W_2 phases the stability and physical properties of the $W_1/O/W_2$ emulsion change. Deliberate destabilisation of $W_1/O/W_2$ emulsions is desirable in certain applications and if controlled can be useful for the release of water-soluble substances from the W_1 phase at the desired time. Thus, $W_1/O/W_2$ emulsion oil globules need to be sufficiently stable so that the encapsulated water-soluble substances stay within the W_1 phase but at the same time breakable so that release can be triggered.[15] However, there is a lack of studies investigating how insoluble particles such as bacteria can be delivered from the inner to the outer phase of $W_1/O/W_2$ emulsions in hypo-osmotic

conditions. In this study, the effect of varying the structure of $W_1/O/W_2$ emulsion on the release of *E. coli* from the oil globules facilitated by osmotic pressure was investigated and the mechanism of this release was microscopically visualised. Also the amount of inner W_1 phase, hydrophilic surfactant (in W_2 phase) and lipophilic surfactant (in oil phase) 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, prolonged storage and chemistry of emulsion on *E. coli*. We also studied the effect of the presence of *E. coli* (in W_1 phase) on stability of the double emulsion and the encapsulation efficiency of the bacteria was monitored during storage.

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 bis-(1,3-dibutylbarbituric acid) trimethaine oxanol [DiBAC$_4$ (3)] 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) 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°C. 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 or NaCl solution and used for encapsulation.

2.3. Rheological measurements

Rheological characterisation of emulsion separate components was done by measuring the viscosity of the oil phase without or with varying concentrations of PGPR (2%, 4%, 6% or 8%), W_2 phase without or with varying concentrations of Tween80 (0.5%, 1%, 5% or 10%) and W_1/O emulsion with different W_1 volume fraction (20% or 40% W_1) in the presence of *E. coli*-GFP was performed at 25°C using AR-G2 rheometer (TA instruments, New Castle, Delaware

USA) on a parallel plate geometry (diameter 40mm). The apparent viscosity was measured over a shear rate 0.1-100 s^{-1}.

2.4. 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 (Fig. S1). In the first step primary W_1/O emulsions were made. An oil phase was prepared by dissolving PGPR (1%, 2%, 4%, 6% or 8 wt%) 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 or NaCl solution (final concentration of 0.02 M, 0.04 M, 0.085 M or 0.17 M) containing the washed *E. coli* cells (5x10^7 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 a $W_1/O/W_2$ 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°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). From a volume of 50ml $W_1/O/W_2$ emulsion the cream layer containing the oil globules was separated using a syringe and the serum phase was discarded. The cream layer was transferred into a sterile 500 ml conical flask containing Tween80 (0.5%, 1%, 5% or 10 wt%) dissolved in either de-ionised water or NaCl solution (final concentration of 0.02 M, 0.04 M, 0.085 M or 0.17 M) and incubated at 25°C for 45 minutes or over a 180 minute period shaking at 100 rpm to ensure homogenised mixing.

2.5. 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 conditions of analysis conducted are described in Supplementary Methods S1.

Observation of phase separation

The cream height fraction of the $W_1/O/W_2$ emulsions was measured immediately after preparation and as a function of storage time in accordance to our previous method.[16]

2.6. 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. The light source used to excite the GFP was a

light-emitting diode (LED) and the emission was observed at 509 nm.

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 was observed using optical and fluorescent microscopy (Zeiss Axioplan) at room temperature as described in Supplementary Methods S2.

2.7. Quantifying the release of *E. coli*-GFP during storage

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.[33] 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.

2.8. Determining the total cell count 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 (4 µl/ml) and [DiBAC$_4$ (3)] (50 ng/ml) and incubated in the dark for 30 minutes. The serum phase was injected through a nucleopore black polycarbonate membrane filter with 0.2 µm pore size and 13mm diameter placed inside a Swinnex filter holder (Millipore) and the cells were counted using a fluorescent microscope (Zeiss Axiolab). The procedure is described in detail in Supplementary Methods S3.

2.9. Determining the viability of *E. coli* (MG1655) and *E. coli*-GFP during incubation

E. coli cell counts were made immediately after preparation and as a function of storage time using serial dilutions in PBS (phosphate buffered saline) buffer solution and plating on tryptic soy agar using the Miles & Misra technique.

2.10. Encapsulation efficiency (%) measurements

The encapsulation efficiency and release of *E. coli*-GFP were calculated by the following equations:

Encapsulation efficiency = ((N$_0$ - N) / N$_0$) x 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.

2.11. 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 size of the W$_1$ droplets can influence the stability of W$_1$/O/W$_2$ emulsions.[34] In this study we ensured that the homogenisation conditions were such that the average mean size distribution [D (4, 3)] of W$_1$ droplets was comparable (3-5µm; Fig. S2 and S3) in all primary W$_1$/O formulations. *E. coli*-GFP was successfully encapsulated within the W$_1$ droplets of the primary W$_1$/O emulsion (Fig. S4). When the primary W$_1$/O emulsion contained 40% W$_1$ and 2% PGPR, the presence of a high concentration of *E. coli*-GFP (10^9 CFU/ml) prevented emulsion at 40% W$_1$ and 2% PGPR W$_1$/O emulsion to form; however, primary W$_1$/O emulsions were able to form at low concentration of *E. coli*-GFP (10^8 CFU/ml) (Fig. 1A). Again the formation of primary W$_1$/O emulsion did not occur at a high concentration of *E. coli*-GFP (10^9 CFU/ml) with 40% W$_1$ but was able to form at 20% W$_1$ (Fig. 1B). When the PGPR concentration was reduced to 1%, the W$_1$/O emulsion did not form at low concentration of *E. coli*-GFP (10^8 CFU/ml) but was able to form without the presence of *E. coli*-GFP (Fig. 1C). To find out whether this phenomenon was due to the metabolic activity of bacteria, dead *E. coli*-GFP cells (10^9 CFU/ml) were added to 40% W$_1$ and homogenised with the oil phase containing 2% PGPR. In the presence of dead *E. coli*-GFP cells the formation of primary W$_1$/O emulsion did not occur at 10^9 CFU/ml but were able to form at 10^8 CFU/ml (Fig. 1D) showing that metabolic activity plays no role in destabilising the primary W$_1$/O emulsion. These results suggest that the presence of *E. coli* cells (live or dead) within the W$_1$ phase affects the formation of the primary W$_1$/O emulsion during the first step of the homogenisation process and this depends on the concentration of *E. coli*-GFP and/or PGPR.

3.2. Influence of osmotic balance alteration on release of *E. coli*-GFP

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To understand how the release of bacteria during osmotic pressure alteration is affected by varying the $W_1/O/W_2$ emulsion composition, the release of *E. coli*-GFP over time was quantified while altering the osmotic pressure gradient (Fig. 2A), the concentration of Tween80 (Fig. 2B), PGPR (Fig. 2C) and the W_1 phase (Fig 3A and B).

With the formulation containing 40% W_1 and stabilised with 1% Tween80 the release of *E. coli*-GFP was significantly (P<0.05) increased when osmotic balance was altered compared to when unaltered (Fig. 2A). The release of *E. coli*-GFP was significantly (P<0.05) increased at higher (0.17 M and 0.085 M) compared to lower concentrations (0.04 M and 0.02 M) of NaCl (Fig. 2A). During osmotic balance alteration 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%) (Fig. 2B). During osmotic balance alteration the release of *E. coli*-GFP was significantly (P<0.05) higher at low concentrations of PGPR (2% and 4%) compared to high concentrations of PGPR (6% and 8%) (Fig. 2C).

The effect of varying the concentration of W_1 at 1% and 5% Tween80 on the release of *E. coli*-GFP with and without altering the osmotic balance over time was quantified (Fig. 3A and B respectively). At 20% W_1 and 1% Tween80 there was no significant difference in release of *E. coli*-GFP when osmotic balance was altered compared when unaltered after 45 and 90 minutes but became significantly (P<0.05) higher after 180 minutes (Fig. 3A). However, at 40% W_1 and 1% Tween80 the release of *E. coli*-GFP was significantly higher when the osmotic balance was altered compared to unaltered after 45, 90 and 180 minutes. Also at 1% Tween80 the release of *E. coli*-GFP was significantly (P<0.05) higher at 40% W_1 compared to 20% W_1 after 45, 90 and 180 minutes (Fig.3B). In contrast, at 40% W_1 and 5% Tween80 there was no significant difference in release of *E. coli*-GFP when osmotic balance was altered compared to unaltered after 45 and 90 minutes but was significantly (P<0.05) higher after 180 minutes. Also there was no significant difference in release of *E. coli*-GFP at 20% W_1 and 5% Tween80 when osmotic balance was altered compared to unaltered after 45, 90 and 180 minutes. Also at 5% Tween80 the release of *E. coli*-GFP was significantly (P<0.05) higher at 40% W_1 compared to 20% W_1 after 90 and 180 minutes. These results suggest that the release of *E. coli*-GFP is affected by the concentration of NaCl, Tween80 and PGPR during osmotic balance alteration.

3.3. Changes in oil globule size during osmotic balance alteration and storage

To understand how the stability of $W_1/O/W_2$ globules encapsulating *E. coli*-GFP is affected during osmotic pressure alteration we measured the change in oil globule size (D (4, 3)) over time by varying the osmotic pressure gradient (Fig. 4A),changing the concentration of Tween80 (Fig. 4B) and PGPR (Fig. 4C). The effect of varying the concentration of W_1 and Tween80 on the size (D (4, 3)) of the oil globules with and without altering the osmotic balance in

the presence or absence of *E. coli*-GFP in W_1 (Table 1 and Table S2) was measured.

The presence of bacteria in W_1 had no effect on the D (4, 3) during the incubation period (Table 1 and S4). After alteration of the osmotic balance the D (4, 3) significantly (p<0.05) increased (Fig. 4A and Table 1) over time. When the concentration of NaCl in W_1 was high (0.085 M and 0.17 M) the increase in D (4, 3) was significantly (P<0.05) higher compared to when the concentration of NaCl in W_1 was low ((0.02 M and 0.04 M) (Fig. 4A). During osmotic balance alteration there was no significant difference in D (4, 3) at different concentrations of Tween80 (Fig. 4B) or PGPR (Fig. 4C) but the D (4, 3) was significantly (p<0.05) higher at 40% W_1 compared to 20% W_1 (Table 1). There was no significant difference in the D (4, 3) when the osmotic balance was unaltered in between W_1 and W_2 (Table S4). These results indicate that the D (4, 3) is affected by the concentration of NaCl and W_1 during osmotic balance alteration whilst the concentration of Tween80 and PGPR had no effect on D (4, 3).

The presence of *E. coli*-GFP in W_1 had no effect on the change in D (4, 3) of $W_1/O/W_2$ emulsions during 7 days storage. There was no significant difference in the D (4, 3) with *E. coli*-GFP compared to without *E. coli*-GFP after 3 and 7 days (Table S3).

3.4. Changes in creaming behaviour during osmotic balance alteration and storage

Percentage gain in cream layer thickness over time was measured in order to characterise the creaming behaviour of $W_1/O/W_2$ emulsions containing *E. coli*-GFP in W_1 as an effect of varying the osmotic pressure gradient (Fig. 4A), concentration of Tween80 (Fig. 4B), and PGPR (Fig. 4C). The effect of varying the concentration of W_1 and Tween80 on the percentage gain in cream layer thickness of the $W_1/O/W_2$ emulsions with and without altering the osmotic balance in the presence or absence of *E. coli*-GFP in W_1 (Fig. 5A, B and Table S4) was measured.

The presence of bacteria in W_1 had no effect on the cream layer thickness during the incubation period (Fig. 5A, B and Table S4). The percentage gain in cream layer thickness was significantly (P<0.05) increased over time when the osmotic balance was altered compared to when unaltered (Fig. 4A). With the formulation containing 40% W_1 and stabilised with 1% Tween80 and during osmotic balance alteration the gain in cream layer thickness was significantly (P<0.05) increased when the concentration of NaCl in W_1 was high (0.085 M and 0.17 M) compared to when the concentration of NaCl in W_1 was low ((0.02 M and 0.04 M) (Fig. 4A). During osmotic balance alteration there was no significant change in cream layer thickness at different concentrations of Tween80 (Fig. 4B) or PGPR (Fig. 4C). During no osmotic balance alteration and regardless of W_1 and Tween80 concentration there were no significant changes in cream layer thickness (Table S4). During osmotic balance alteration the gain in cream layer thickness was significantly (P<0.05) higher at 40% W_1 compared to 20% W_1

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regardless of Tween80 concentration (Fig. 5A and B). These results indicate that creaming behaviour is affected by the concentration of NaCl and W_1 during osmotic balance alteration whilst the concentration of Tween80 and PGPR had no effect.

The presence of *E. coli*-GFP in W_1 had no effect on the creaming behaviour of $W_1/O/W_2$ emulsions during 7 days storage. There was no significant difference on the cream layer thickness with *E. coli*-GFP compared to without *E. coli*-GFP after 3 and 7 days (Table S3).

3.5. Microscopic observation of $W_1/O/W_2$ emulsions and *E. coli*-GFP release

The $W_1/O/W_2$ emulsions were observed using fluorescence microscopy to investigate the effect of altering the osmotic balance and changing the concentration of W_1 and Tween80 on the structure of the $W_1/O/W_2$ emulsion and the release of *E. coli*-GFP from W_1 to W_2. After osmotic balance alteration an increase in size of the $W_1/O/W_2$ globules was observed as well as the presence of *E. coli*-GFP in both W_1 and W_2 phases. During osmotic balance alteration and at 40% and 1% Tween80 the oil globules showed swelling (Fig. S5A) and *E. coli*-GFP cells were present in the W_1 phase and also in the W_2 phase (Fig. S6A). During osmotic balance alteration and at 20% W_1 and 1% Tween80 the oil globules showed swelling (Fig. S5B) whilst *E. coli*-GFP cells were mainly present in the W_1 phase with a few present in the W_2 phase (Fig. S6B). During no osmotic balance alteration the oil globules showed no swelling (Fig. S5C) whilst *E. coli*-GFP cells mainly remained within the W_1 phase (Fig. S6C).

3.6. 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 culture on media and microscopic assessment of bacterial cells stained for injury. There was no significant difference in the number of *E. coli* cells obtained from the plating method compared to microscopic enumeration (Fig. 6). Also after release into the W_2 phase, there were no DiBAC$_4$ (3) positive *E. coli* cells (Fig. S7) suggesting that encapsulation and release had no effect on *E. coli* membrane potential.

4. Discussion

The stability of the primary W_1/O emulsion at different concentrations of *E. coli*-GFP cells was studied with varying PGPR and W_1 concentration. The primary W_1/O emulsion did not form at high concentration of *E. coli*-GFP cells (10^9 CFU/ml) when the concentration of W_1 was high (40%) and PGPR was low (1%). Moreover, the same also occurred when the cells were dead showing that the disruption of W_1/O emulsion formation was not due to any metabolic activity of the cells. It was also observed that when the W_1/O emulsions were not able to form, multiple W/O/W/O emulsion morphology appeared. This type of emulsion was described as a super multiple emulsion[35] and appeared as part

of the emulsion morphology evolution during catastrophic phase inversion which occurs when one of formulation variables (e.g. surfactant affinity) or composition variables (e.g. oil/water ratio) is changed during the homogenising process.[35] We believe that this occurred due to the lipophilic nature of PGPR allowing it to adsorb to the bacterial lipid membrane[16] reducing its concentration within the oil phase. However, more investigation is required to understand how the presence of *E. coli*-GFP could lead to a super emulsion morphology formation.

In agreement with previous studies,[36,37,38] we found that when the $W_1/O/W_2$ emulsion was diluted in a hypo-osmotic solution the oil globules became larger in size. We confirmed that this occurred not as a result of coalescence but due to actual migration water from W_2 to W_1 by following the evolution of a single oil globule in hypo-osmotic solution using microscopy (see Supplementary Results S1 and Supplementary Figure S8). As water migrates from W_2 to W_1 the swelling of the oil globules diminishes because of the reduction in the concentration gradient. The swelling of the oil globules due to the presence of NaCl in the W_2 phase was shown to be concentration dependent as the oil globules were larger at higher concentrations (0.085 M and 0.17 M) compared to lower concentrations of NaCl (0.04 M and 0.02 M). These findings correlate with a study by Chong-Kook *et al.* concluding that the oil layer of $W_1/O/W_2$ emulsion globules acts as a water-permeable membrane between the two aqueous phases of the $W_1/O/W_2$ emulsion under the osmotic pressure gradient.[39] This phenomenon can be related to the osmotic pressure gradient created between the W_2 and W_1 phases during osmotic balance alteration.[15] Thus, at concentrations of 0.085 M and 0.17 M NaCl in W_2 an osmotic pressure of 42 atm and 84.2 atm is obtained respectively, and at concentrations of 0.02 M and 0.04 M NaCl in W_2 an osmotic pressure of 10.52 atm and 21 atm is obtained respectively. It has been well demonstrated that water migration in $W_1/O/W_2$ emulsions occurs due to interfacial processes control the transport of water in $W_1/O/W_2$ emulsions rather than bulk diffusion.[38] When the W_1 droplets and oil globule interfaces are not physically in contact mechanisms that control the transport of water between W_1 and W_2 phases include spontaneous emulsification and reverse micellisation.[37] Another mechanism controls water transport when the W_1 and oil globule interfaces are physically in contact and that involves the hydration of the surfactant molecule at one interface, its diffusion through the oil phase to dehydrate at the other interface that is in contact with the phase of higher solute concentration.[40] In this study we found that the concentrations of Tween80 (in the W_2 phase) or PGPR (in the oil phase) had no significant effect on the oil globule size.

In the video-microscopy results (see Supplementary Results S2, Supplementary Figure S9 and Supplementary Video S1) we observed that after altering the osmotic balance with 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. We also observed that the W_1 droplets

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persisted within the W_2 phase upon release. In a recent study we have reported the same observation.[16] However; more investigation is needed to understand the nature of these W_1 droplets. In systems where the osmotic pressure was unaltered we found minimal release of *E. coli*-GFP probably due to mechanical breakdown (i.e. mixing) of the $W_1/O/W_2$ emulsion globules. The release of *E. coli*-GFP was higher when osmotic balance was altered compared to when unaltered which was NaCl concentration-dependent. During osmotic balance alteration and at low concentration of Tween80 release of *E. coli* cells from $W_1/O/W_2$ globules was increased regardless of W_1 concentration. Jager-Lezer *et al*. found that when $W_1/O/W_2$ emulsions were diluted in a hypo-osmotic solution the W_1 droplets increased in size leading to swelling of the oil globules until a critical size is reached.[38] Moreover, the authors claim that beyond the critical size the oil globules will burst and release their inner contents into W_2.

The mechanism of interfacial film rupture is determined by the characteristics of the continuous phase (e.g. viscosity) and the interfacial layers surrounding the oil globules (e.g. thickness, interfacial tension).[41,42] Above a certain critical film thickness, film rupture of the oil globule is improbable.[43] However, film rupturing becomes more probable once the film has thinned to the critical thickness.[43] The thinner the film the lower the energy of activation required to form a hole that grows leading to film rupture and oil globule bursting.[41] Tween80 molecules are able to keep the oil globules apart as well as provide a barrier against rupture. When present at higher concentrations in the W_2 phase, hydrophilic surfactants tend to form multilayer arrangement of interdigitated surfactant chains that surrounds and "wraps" the oil globules[44] resulting in an increased interfacial film strength.[38] When Tween80 is present in W_2 at high amounts it adsorbs at the O/W_2 interface creating more condensed interfacial films which are able to resist hole creation and propagation that leads to film rupture.[45] Gaps that are formed at the O/W_2 interface due to incomplete surface coverage of the interface by the surfactant molecules or due to uneven distribution of surfactant molecules on the interface will lead to hole formation.[46] If there are insufficient surfactant molecules in the system to saturate the O/W_1 interface there will be gaps in the interfacial film.[46] Also during swelling the interfaces of the oil globules are stretched and pressed against each other and as this happens the emulsifier molecules will be dragged along the interface leaving regions with more and regions with fewer surfactant molecules at the O/W_2 interface.[47] Moreover, if the adsorption of surfactant molecules to the O/W_2 interface to cover these gaps is slow the interfacial film becomes more susceptible to rupture.[46] During swelling of $W_1/O/W_2$ globules the expansion of the O/W_2 interface results in decreased interfacial film strength and therefore making it more susceptible to rupture.[18,19] We believe that as the interface expands the distance between the Tween80 molecules at the interface increases and gaps will form. Moreover, excess Tween80 molecules in W_2 start to adsorb at the interface to fill up the free spaces caused by swelling and therefore maintain the O/W_2 interface and prevent the oil globule from bursting. The

adsorption of Tween80 at the interface occurs more rapidly when the excess Tween80 concentration in W_2 is high which prevents or slows down the rupture of the interfacial film. In contrast when the concentration of excess Tween80 in W_2 is low the adsorption of Tween80 at the interface is slower resulting in an expanding film that becomes progressively thinner and more susceptible to rupture. Also an increase in continuous phase viscosity increases the time required for the interfacial films to drain to their critical thickness and therefore prolong its resistance to rupture.[41] The viscosity of the continuous phase was shown to increase when the amount of Tween80 was increased.[48] However, at the concentrations used in our study we found that increasing the amount of Tween80 had no significant effect on viscosity of the continuous phase.

The release of *E. coli*-GFP was significantly higher at lower concentrations of PGPR. Geiger *et al*. found that by increasing the lipophilic surfactant concentration in the oil phase more rigidified O/W_2 interface is formed measured as an increase in shear elastic modulus and interfacial tension.[19] Furthermore, the authors suggested that an increase in lipophilic surfactant concentration increases the swelling capacity of the oil globule which delays breakdown and release of solutes. They argued that this occurred as a result of excess lipophilic surfactant in the oil phase diffusing to the O/W_2 interface to fill up free spaces caused by the swelling.[19] The viscosity of the oil phase will increase when more lipophilic surfactant is present in the oil phase.[47] We found that the viscosity of the oil phase significantly ($P<0.05$) increased at higher amounts of PGPR (see Supplementary Results S3 and Supplementary Table S5). Jiao *et al*. showed that by increasing the concentration of Span80 (a lipophilic surfactant) the viscosity of the oil phase increased resulting in the ability of the oil globules to resist deformation upon applying force (by placing a coverslip on top of the $W_1/O/W_2$ emulsion).[49,50] Moreover, when present in excess in the oil phase, the lipophilic surfactant was found to increase the visco-elasticity of the interfacial film of the oil globule.[51]

With the formulation containing 20% W_1 and 1% Tween80 There was no significant difference in the release of *E. coli*-GFP between the different $W_1/O/W_2$ emulsions when the osmotic balance was unaltered after 45 and 90 minutes, however the release became higher with compared to without NaCl in W_2 after 180 minutes (Fig. 3A). NaCl can interact unfavourably with Tween80 and reduce the interfacial film strength of the $W_1/O/W_2$ globule[50] and this was shown to be concentration dependent.[52] In comparison to sodium salicylate that was added to the W_2 phase of $W_1/O/W_2$ emulsions to alter the osmotic balance, NaCl led to a significant reduction in the strength of the interfacial film probably due to unfavourable interactions between NaCl and Tween80 and/or salting out.[50]

In addition to oil globule bursting other mechanisms may have resulted in the release of *E. coli*-GFP. We have recently shown that coalescence between oil globules does not result in release of *E. coli*-GFP.[16] The coalescence of W_1 droplets with the O/W_2 interface may result in release of *E. coli*-GFP but this mechanism was shown

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to occur more frequently with increasing concentrations of Tween80[21] which if was occurring in our study would have resulted in increased release of *E. coli*-GFP cells at high concentration of Tween80 but that would be in contrast to what we observed in this study. Further work is needed to find out if such mechanisms were responsible in the release of *E. coli*-GFP from the oil globules in hyper-osmotic solution.

We found that the release of *E. coli*-GFP significantly (P<0.05) increased at high amount of W_1. The release of solutes from $W_1/O/W_2$ emulsion globules in hypo-osmotic solution increased with higher W_1 fraction.[15] When the W_1 volume is higher we found a significant increase in D (4, 3) and creaming thickness. Therefore, we believe that the interfacial film will be expanding at a faster rate when the W_1 concentration is high compared to when it is low and this will lead to more gaps to form at the O/W_2 interface which increase the chances of hole formation and propagation resulting in film rupture. We also found that the viscosity of the W_1/O emulsion increases with higher amounts of W_1 (see Supplementary Results S3 and Supplementary Table S5). According to the Mooney equation[53,54] the increase in viscosity of $W_1/O/W_2$ emulsion globules at higher amounts of W_1 has a destabilising effect on the oil globules making them more susceptible to rupture.[49] However, the increase in $W_1/O/W_2$ emulsion globule viscosity due to higher amount of W_1 phase has an opposite effect on emulsion stability than does the increase in the globule's viscosity due to higher amounts of lipophilic surfactant present in the oil phase.[49]

When water migrates from W_2 to W_1 the volume of the continuous phase is reduced which can be observed as an increase in cream layer height. The stability of the cream layer was not affected by the presence of *E. coli*-GFP within the $W_1/O/W_2$ emulsion globules. Also 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. Moreover, the release mechanism had no effect on the viability of *E. coli* cells and there were no DiBAC$_4$ (3) stained *E. coli* cells. Since DiBAC$_4$ (3) is a membrane potential dye and will stain *E. coli* cells with a depolarised membrane[55] our results indicate that no injury was caused to the *E. coli* cells after encapsulation and release from $W_1/O/W_2$ globules. The encapsulation efficiency of $W_1/O/W_2$ emulsions was significantly (P<0.05) lower after 3 and 7 days of storage, however they still achieved high encapsulation efficiency (>90%) (see Supplementary Results S4 and Figure S10) and their viability was not affected (see Supplementary Results S5 and Figure S11) proving their suitability for industrial applications.

5. Conclusion

In this study the release of *E. coli* cells from $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure was investigated. The release of bacteria from the oil globules of $W_1/O/W_2$ emulsion was due to the bursting of the oil globules and this release can be modulated by changing the structure of the $W_1/O/W_2$ emulsion. Globule bursting under hypo-osmotic pressure allows controlling the release of bacteria as well as hydrophilic compounds from the

inner W_1 phase. More investigation is required to understand the release mechanism such as monitoring interfacial film strength during osmotic balance alteration. Moreover, bacterial viability was not affected by the structure of the $W_1/O/W_2$ emulsion. We demonstrate the potential use of $W_1/O/W_2$ emulsion in microbiological applications as they show high stability and encapsulation efficiency for up to 7 days of storage. The possibility of modulating bacterial release from $W_1/O/W_2$ emulsion globules by changing its structure could be useful for fermentation industry to control and/or inhibit bacterial release. For example, a formulation can be intelligently designed containing oil globules encapsulating various different bacterial species and/or growth factors that can be sequentially released during a fermentation process.

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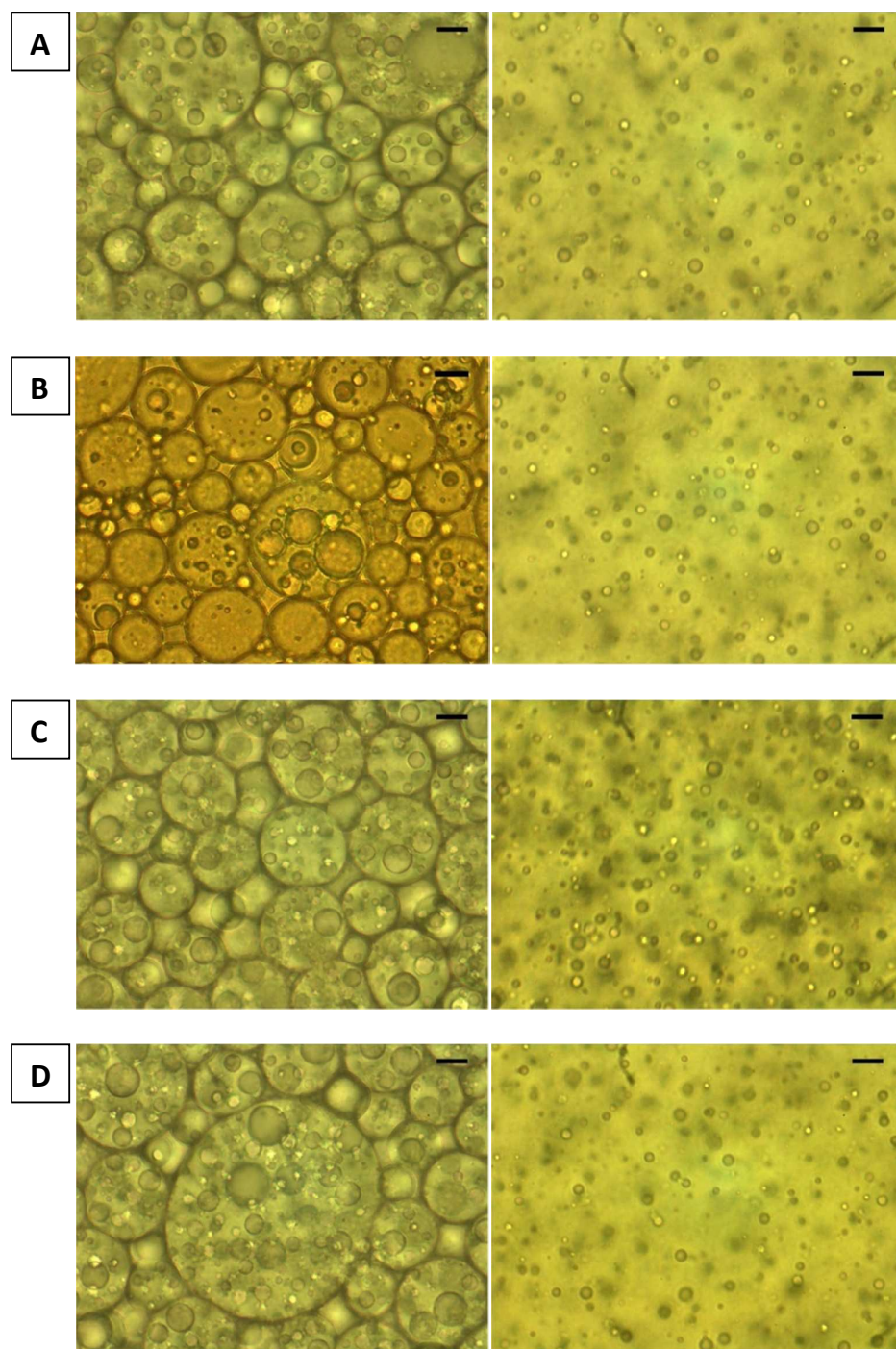
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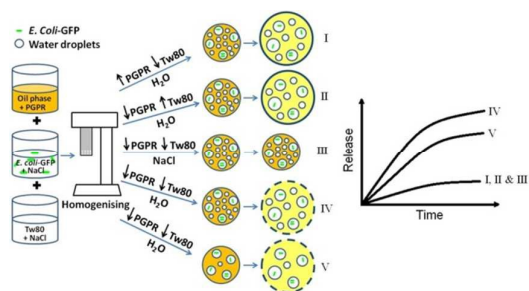
Figure 1. Single W_1/O emulsions prepared with different concentrations of PGPR or concentrations of *E. coli*-GFP. The W_1/O emulsions were prepared with inner-phase (W_1) volume percentage of 40% or 20% containing *E. coli*-GFP and stabilised with PGPR. The formulations were as follows: A) 2% PGPR containing live cells at 10^9 CFU/ml in 40% W_1 (left) and 10^8 CFU/ml in 40% W_1 (right); B) 2% PGPR containing live cells at 10^9 CFU/ml in 40% W_1 (left) and in 20% W_1 (right); C) 1% PGPR containing live cells at 10^8 CFU/ml in 40% W_1 (left) and no *E. coli*-GFP in 40% W_1 (right); D) 2% PGPR containing dead cells at 10^9 CFU/ml in 40% W_1 (left) and 10^8 CFU/ml in 40% W_1 (right); Scale bar: 10μm.
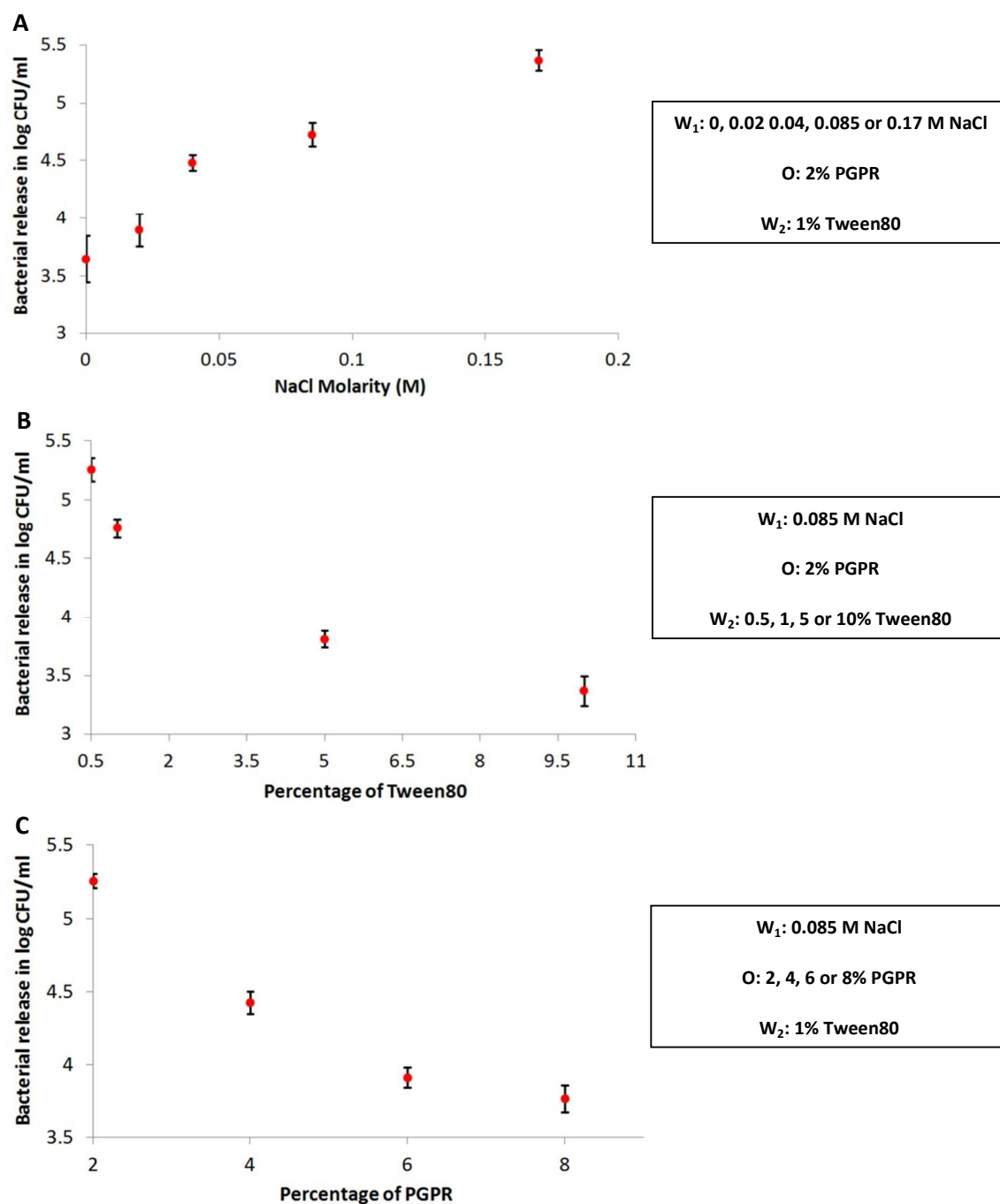
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Figure 2. Effect of varying the structure of the $W_1/O/W_2$ emulsion on the release of *E. coli*-GFP. The amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions was measured after 45 minutes incubation at 25°C was quantified. The $W_1/O/W_2$ emulsions were prepared: A) 40% W_1 containing *E. coli*-GFP and varying concentrations of NaCl (0.02 M, 0.04 M, 0.085 M or 0.17 M) in W_1 and stabilised with 1% Tween80 in W_2 and 2% PGPR; B) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and and stabilised with varying concentrations of Tween80 (0.5%, 1%, 5% or 10%) in W_2 and 2% PGPR in the oil phase; C) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and and stabilised with 1% Tween80 in W_2 and varying concentrations of PGPR (2%, 4%, 6% or 8%) in the oil phase. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

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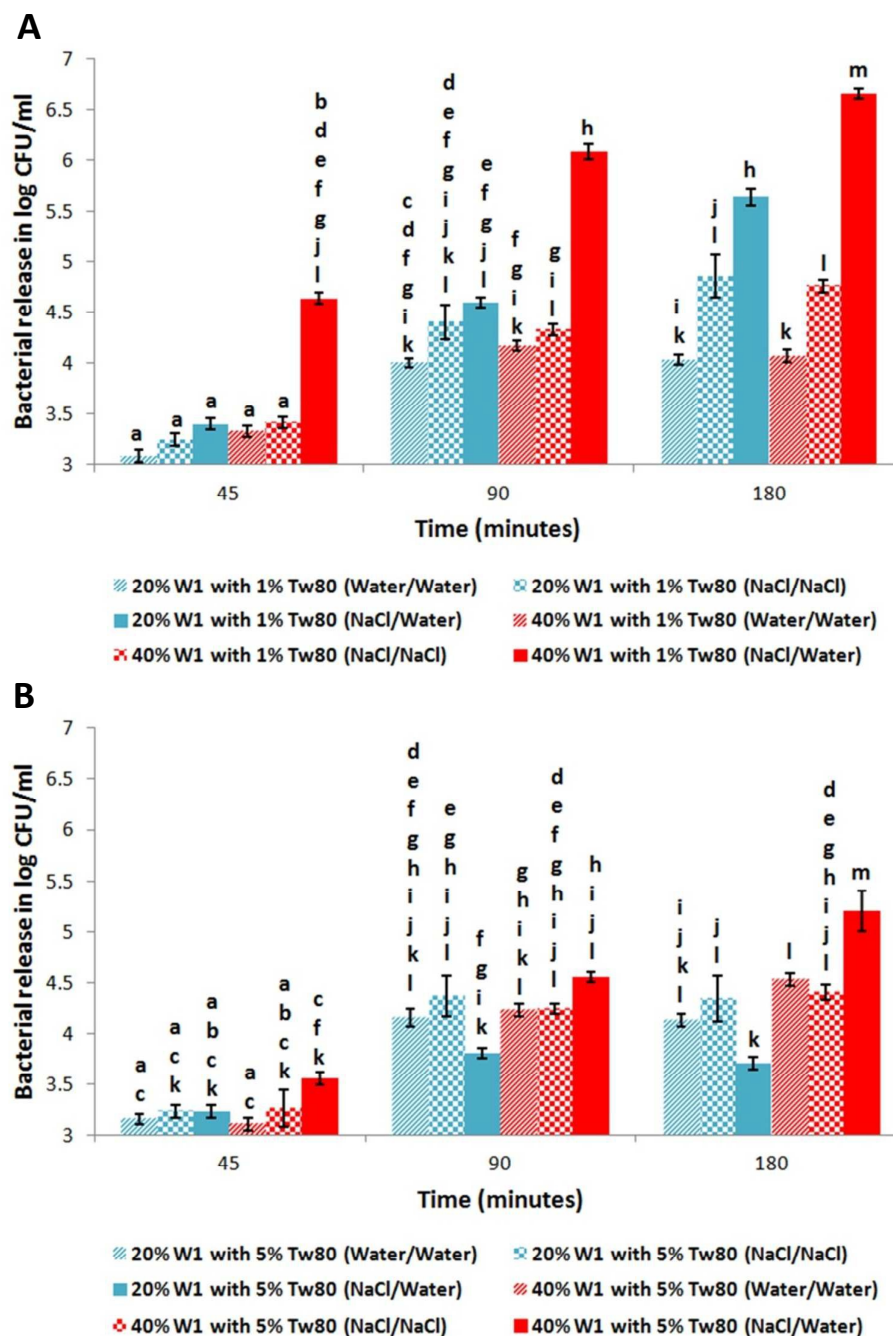
Figure 3. Effect of varying the structure of the $W_1/O/W_2$ emulsion on the release of *E. coli*-GFP. The amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 45, 90 and 180 minutes incubation at 25^oC was quantified. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 20% or 40% containing *E. coli*-GFP with or without 0.085 M NaCl in W_1 or W_2 and stabilized with 1% (A) or 5% (B) Tween80 in W_2 and 2% PGPR in the oil 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.
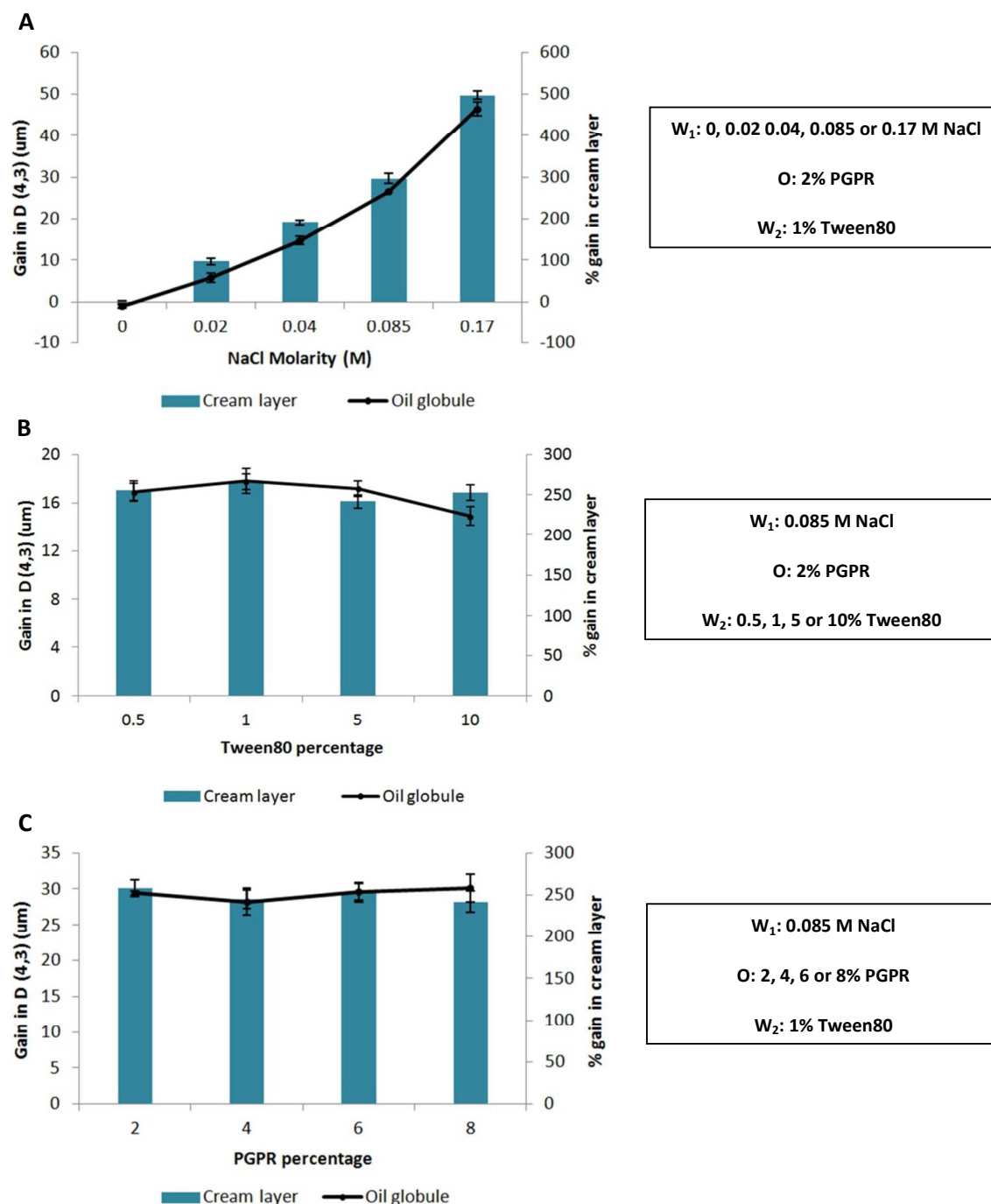
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Figure 4. Reduction in mean oil globule diameter size (µm) by light scattering [D (4, 3)] and percentage gain in cream layer thickness of $W_1/O/W_2$ emulsions in hypo-osmotic solution after 45 minutes relative to time 0 minutes incubation at 25°C. The $W_1/O/W_2$ emulsions were prepared: A) 40% W_1 containing *E. coli*-GFP and varying concentrations of NaCl (0.02 M, 0.04 M, 0.085 M or 0.17 M) in W_1 and stabilised with 1% Tween80 in W_2 and 2% PGPR; B) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and stabilised with varying concentrations of Tween80 (0.5%, 1%, 5% or 10%) in W_2 and 2% PGPR in the oil phase; C) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and and stabilised with 1% Tween80 in W_2 and varying concentrations of PGPR (2%, 4%, 6% or 8%) in the oil phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

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Table 1. Change in mean oil globule diameter size (µm) of $W_1/O/W_2$ emulsions during osmotic pressure alteration measured by light scattering [D (4, 3)] after 45, 90 and 180 minutes relative to time 0 minutes incubation at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner-phase (W_1) volume percentage of 20% or 40% in the presence or absence of *E. coli*-GFP with 0.085 M NaCl in the W_1 phase and stabilised with 1% or 5% Tween80 in W_2 and 2% PGPR in the oil phase. Results are taken from a minimum of 3 independent experiments.

	$W_1/O/W_2$ emulsion formulations	45 minutes	90 minutes	180 minutes
No *E. coli*-GFP	20% W_1, 1% Tw80, 0.085 M NaCl in W_1 only	7.44±0.4^a	13.66±0.3^a	24.58±1.2^b
	20% W_1, 5% Tw80, 0.085 M NaCl in W_1 only	9.6±2.6^a	11.7±1^a	21.8±0.3^b
	40% W_1, 1% Tw80, 0.085 M NaCl in W_1 only	22.6±1.3^b	42.8±2.5^c	50.94±2.6^d
	40% W_1, 5% Tw80, 0.085 M NaCl in W_1 only	24.8±0.7^b	40.72±0.8^c	50.38±1.6^d
With *E. coli*-GFP	20% W_1, 1% Tw80, 0.085 M NaCl in W_1 only	7.86±1^{ab}	13.21±1.4^{ab}	24.76±3^c
	20% W_1, 5% Tw80, 0.085 M NaCl in W_1 only	7.18±0.7^{ab}	10.25±1.3^a	21.11±1.2^c
	40% W_1, 1% Tw80, 0.085 M NaCl in W_1 only	21.2±1.1^d	37.2±0.9^e	50.9±2.6^f
	40% W_1, 5% Tw80, 0.085 M NaCl in W_1 only	19.9±1.9^{bd}	35.9±2^e	47.9±2^f

The data was analysed with one-way ANOVA.

$^{a, b, c, d, e, f}$ means ± standard deviation with different letters are significantly different

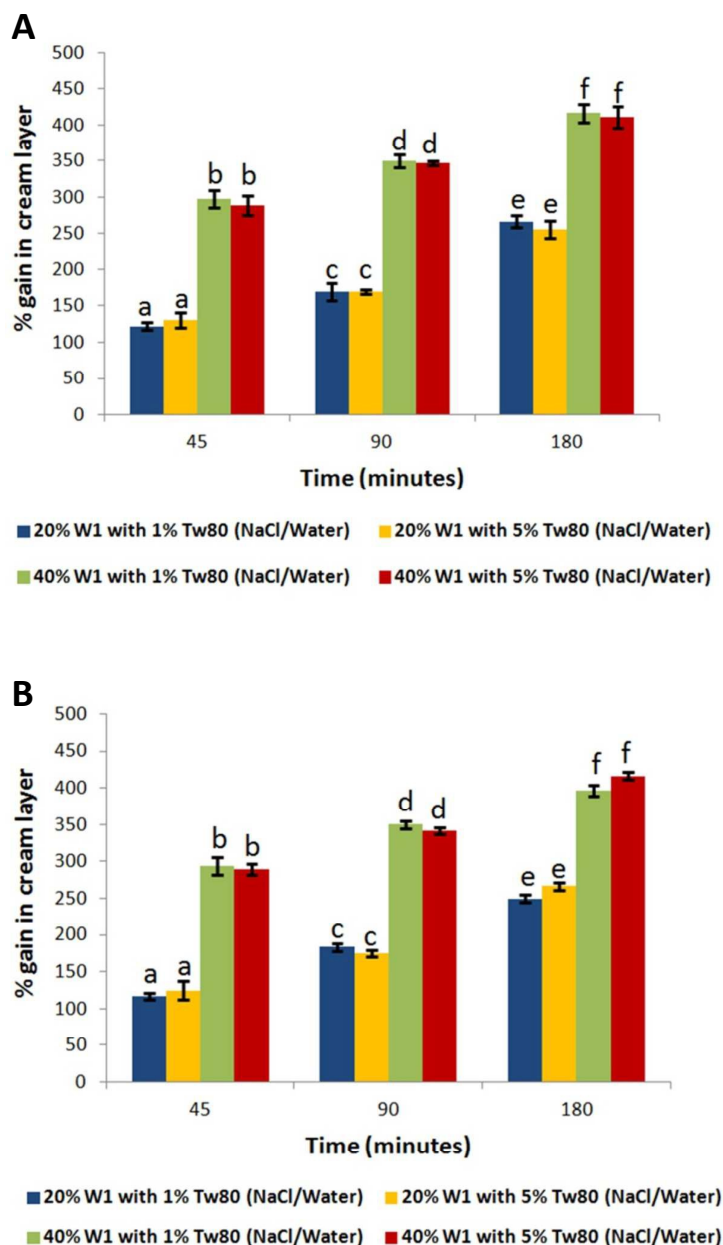**RSC Advances Accepted Manuscript**

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Figure 5. Change of percentage in cream layer thickness of $W_1/O/W_2$ emulsions during osmotic balance alteration after 45, 90 and 180 minutes relative to time 0 minutes incubation 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 with 0.085 M NaCl in the W_1 phase stabilised with 1% or 5% Tween80 in W_2 and 2% PGPR in the oil 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.
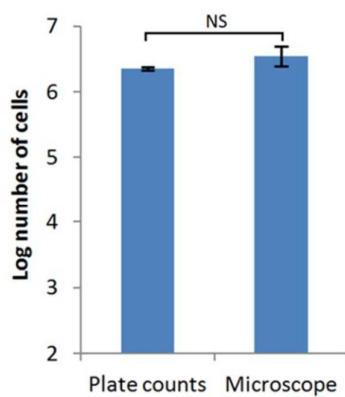
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Figure 6. Log number of released *E. coli* cells in serum phase quantified by plate counting and microscopic enumeration after 180 minutes incubation at 25°C. The W_1/O/W_2 emulsion was made with 40% W_1 with *E. coli* with 0.085 M NaCl in the W_1 phase stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. The data was analysed with Student's *t*-Test.