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Abstract:

Model emulsions were developed with or without commercial titanium dioxide nanoparticles (NP) carrying various surface treatments in order to get close physicochemical properties whatever the NP surface polarity (hydrophilic and hydrophobic). Rheology and texturometry highlighted that the macroscopic properties of the three formulated emulsions were similar. However, characterizations by optical microscopy, static light scattering and zetametry showed that their microstructures reflected the diversity of the incorporated NP surface properties. In order to use these model emulsions as tools for biological evaluations of the NP in use, they had to show the lowest initial microbiological charge and, specifically for the NP-free emulsion, the lowest bactericidal effect. Hence, formulae were developed preservative-free and a thermal sterilization step was conducted. Efficiency of the sterilization and its impact on the emulsion integrity were monitored. Results highlighted the effect of the NP surface properties: only the control emulsion and the emulsion containing hydrophilic NP fulfilled both requirements. To ensure the usability of these model emulsions as tools to evaluate the 'NP effect' on representative bacteria of the skin microflora (*S. aureus* and *P. fluorescens*), impact on the bacterial growth was measured on voluntary inoculated formulae.

1. Introduction

Titanium dioxide (TiO_2) NP are commonly used in Cosmetics, especially as UV filters in sunscreen emulsions. Their tiny size, enabling transparency in the visible range, makes it possible to obtain end products without any residual traces on skin in addition to a good UV-protection (Serpone et al. 2007). However, if compared to micrometric oxides, nanometric ones are more reactive and in particular photo-reactive. Therefore, such nano-objects receive a first coating with *silica* or *alumina* as it allows quenching photo-produced radicals (Serpone

et al. 2006); in addition, a second coating is sometimes added in order to improve their dispersibility in formula. Whereas European Regulation (European Parliament, Council of the European Union 2014) clearly mentions the obligation to demonstrate the safety of even coated NP, very few studies took interest on their behavior in use. *Santaella et al.*(Santaella et al. 2014) compared the eco-toxicity of NP with degraded coating in aqueous environment (Auffan et al. 2010) to that of pure TiO_2. Other studies dealt with the impact of zinc oxide NP on bacteria according to their size (Pasquet et al. 2014). However, aggregation state and physicochemical behavior of NP in formula have to be taken into account and their safety in emulsions when they are layered on skin remained a critical question. Moreover, these questions are closely related to the large variety of surface treatments commercially available (polar, non-polar, hydrophilic, hydrophobic…).

To evaluate the only effect of NP in formula, emulsions need to be developed in order to respect three main criteria:

- Being stable and exhibiting similar microstructure and macroscopic properties whatever the coating of the incorporated NP ;
- Exhibiting the lowest initial microbiological charge ;
- Giving the control emulsion (without NP) the lowest bactericidal effect.

Therefore emulsions need to be developed without preservative. Hence, in order to fulfill the second criterion, alternative process, like sterilization procedures, consisting in the elimination of germs and spores in a product, has to be used. However, cosmetic form does not allow the use of technics such as filtration of emulsion systems because it could lead to a demixing phenomenon (Kocherginsky et al. 2003) or UV or gamma radiations because of presence of UV filters in this study. Sterilization by high temperature seemed the best solution especially as this kind of methods begins to be used on cosmetic emulsions (Delaunay & Legendre 2013) associated to an adapted packaging (Devlieghere et al. 2015). Nevertheless,

high temperatures could accelerate aging (Waterman & Adami 2005) and, recently *Rossano et al.* (2014) demonstrated that aging of emulsions containing hydrophobic NP induced particles aggregation, adsorption of formula's compounds and coatings deterioration. Thus, as it is still relatively unexplored, it is essential to bring data concerning the effect of coatings and sterilization temperature on the emulsion properties. Our study focuses on the characterization of model emulsions in order to ensure the similarity in terms of microstructure and physicochemical properties of a control emulsion without nanoparticle and formulae containing either hydrophilic/hydrophobic NP.

Finally, microbiological evaluations were conducted on two skin bacteria strains to check the low initial microbiological charge of the model emulsions and the low bactericidal impact of the control one.

2. Experimental Section

2.1. Chemical and reference materials.

Water with a resistivity of 18 mΩ.cm used during the emulsion preparation and zetametry was purified, filtrated on 0.2 µm and treated by UV in order to eliminate germs, after described as ultrapure. Otherwise distilled water with a resistivity of 18 mΩ.cm was used for other emulsion characterizations. Two different grades of TiO_2-NP were kindly given by Kobo and Merck; one hydrophilic, with a simple *silica* coating named N1 (EUSOLEX® TAVO, Merck Chimie SAS, France) and a second, hydrophobic, composed of *alumina (and) triethoxycaprylylsilane* named N2 (A10-TiO2-11S7, Kobo Products, France). Other cosmetic grade ingredients, introduced in the Table 1 were chosen to obtain simplified sunscreen, oil-

in-water emulsions type, and to improve NP dispersions. Ingredients were used as received. To achieve emulsions with the lowest bactericide impact, no preservative was added.

2.2. Emulsion preparation.

During preservative-free emulsion preparation, precautions were taken in order to minimize the risks of microorganisms contamination: all stainless steel materials were steamed for 45 min at 190°C and glass bottles were rinsed with ethanol prior to drying (INRS 2014). For technical reasons it was impossible to make all operation under laminar flow hood in sterile conditions. But controls revealed that aerial contaminants in final formulations remained under the detection limit.

2.2.1. Emulsion without nanoparticle.

Emulsion without NP was adapted from the protocol described by *Rossano et al.* (2014). It was developed to obtain similar emulsions, with or without NP, and to improve the NP dispersion. The gelling agent was sprinkled in water and hydrated 20 min without stirring. This mix corresponding to the phase B and the phase A were separately homogenized at 80°C under mechanical stirring for 10 min. After water loss compensation, phase A was added to phase B off the heat and under vigorous stirring (11000 rpm for 1.5 min), by using a rotor-stator type homogenizer (Ultra-Turrax, stator diameter 25 mm, rotor diameter 18 mm, IKA). Then, the mixture was put under stirring (Turbotest, radial flow turbine of 55 mm diameter, VMI Raynerie) at 750 rpm and triethanolamin (TEA) was immediately added. After 5 min, the stirring rate was raised to 1000 rpm. A cold water bath was used once cooled below 38°C. When the temperature of emulsion was close to 25°C, pH was adjusted at 6.6 – 6.8 using HCl 0.4 M. The mixture was kept under stirring for 5 additional minutes. It was then put under vacuum to remove air bubbles incorporated during the previous process.

2.2.2. Nanoparticle dispersions.

Because rotor-stator type homogenization was not sufficient to obtain an acceptable dispersion in emulsion, NP powders were first pre-dispersed in one of the emulsion phases, leading to a NP paste. Hydrophilic NP and water with a ratio of 1/2 (w/w) were sonicated 10 min (Ultrasonic cleaner, VWR, 45 kHz, 120 W). Hydrophobic NP and cetearyl ethylhexanoate with a ratio of 2/3 (w/w) were mixed. Then, this pre-dispersion was grounded on a three-cylinder mill for three times to reduce the size of agglomerates.

2.2.3. Emulsions with nanoparticles.

The protocol remained the same as without NP except that just after the addition of TEA, NP paste was added under a stirring of 1000 rpm to reach the optimal dispersion in emulsion. The end of the preparation was the same than without NP.

2.2.4. Sterilization.

Each emulsion was poured into several 50 mL glass autoclave-resistance bottles (VWR). After maturation at 20 ± 2°C for 24h, a half of them was sterilized during 20 min at 120°C and 1 bar relative pressure in an autoclave (Laboster, Subtil-Crepieux, Chassieu, France). The second part was let at the same temperature. After heating and when the temperature cooled below 90°C, the autoclave was opened and the bottles were let at ambient air in order to accelerate the cooling. Thus, for instance, ST-F-0 was the autoclaved version of F-0.

Efficiency of the heat treatment can be evaluated by calculation of the sterility value (SV):

$SV = \int 10^{(T(t)-T_{ref})/Z} . dt$

- T(t) is the core temperature in the time interval,
- T_{ref} is the reference temperature,

- Z is the difference of temperature required to accelerate tenfold the germs destruction reaction.

In the case of a sterilization step, T_{ref} and Z are respectively equal to 121.1°C and 10°C (Bimbenet et al. 2007). A temperature probe (DataTrace, CMI) was introduced into a product bottle in order to obtain the core temperature essential for the SV calculation.

2.3. Emulsions characterization.

Once prepared, emulsions were characterized after a period of maturation for two days or if so, sterilization.

2.3.1. Microstructural characterization.

2.3.1.1. Optical Microscopy.

Qualitative observations were performed using an optical microscope (DMLP/DC 300, Leica Microsystems, Wetzlar, Germany) equipped with a camera and the IM1000 software (version 1.20 Release 19). In order to visualize emulsion microstructures, droplets sizes and quality of NP dispersion, a tip of emulsion plus a water drop were placed between slide and coverslip. Pictures were obtained at a magnification of x200.

2.3.1.2. Particle size measurement.

Particles sizes measurements were performed by using a laser diffractometer SALD 7500 Nano (405 nm, Shimadzu, Marne-la-Vallée, France) combined with the measure cell SALD BC 75 consisting in a 7 mL batch and a stirrer and equipped with the WingSALD II-7500 software (version 3.1). In order to reach an absorption value of the samples between 1.2 and 1.5 and the best quality of the obtained signal, particle dispersions had to be diluted and homogenized according to the following procedures. Approximately 0.1 g of NP pastes was

pre-dispersed in a test tube with 5 mL of distilled water for coating 1 or of cetearyl ethylhexanoate for coating 2. This pre-dispersion was then sonicated 5 min (120 W). In the case of emulsion particle sizing measurement, approximately 0.1 g of emulsion were pre-diluted in a test tube with 5 mL of distilled water. The mix was vortexed 10 sec at 1000 rpm. Finally, pre-dispersed samples were added by drop until reaching the expected absorption. All measurements were performed at least in triplicate on two different batches. D10, D50 and D90 were thereafter expressed in particle volume. By this way, for instance, D10 represented the threshold value in micrometer for which 10% of particles had a smaller size.

2.3.1.3. Zetametry.

Mean zeta potentials of emulsions were performed using a Zeta Sizer Nano-Z and folded capillary cells (Malvern instruments, Malvern, UK). Emulsions were diluted at 0.5% w/w in ultrapure water containing NaCl at 10^{-4} M (conductivity of 1.70 µS/cm). Six samples of each emulsion were adjusted at pH 2, 4, 6, 8, 10 and 12, respectively, with using NaOH or HCl (0.4 or 0.04M).

Measurements were performed at least three times.

2.3.2. Macroscopic properties.

2.3.2.1. Rheology.

Rheological properties were achieved with a controlled-stress rheometer (Discovery HR1, TA instrument, Guyancourt, France) using an aluminum cone-plate geometry (40 mm diameter, cone angle of 1.994° and truncation of 47 µm). Data analyses were conducted with the software TRIOS® 3.0. Stress sweep was carried out at 25°C, temperature controlled by Peltier effect, with an imposed stress gradient from 0.01 to 150 Pa and a fixed frequency of 1

Hz. This analysis highlighted the linear viscoelastic region and permitted to obtain the viscoelastic parameters, independent of the applied stress as listed below:

- tan (δ), where δ is the phase shift between strain and stress and is equal to G"/G'.
- G', the elastic modulus (Pa),
- G", the viscous modulus (Pa),
- γ_c, the critical strain, corresponds to the percentage strain at 90% of the plateau G' value.

All measurements were performed at least twice on two different batches.

2.3.2.2. Texture analyses.

Texture analyses were performed by using a TA.XT Plus (Stable Micro Systems, Cardiff, UK) and the software Texture Exponent 32 (version 5,0,6,0, 2010). It allows the characterization of mechanical properties of emulsions. The protocol was adapted from *Gilbert et al.* (2013). Effect of the compression was studied with the P/35 probe (35 mm diameter, aluminum). 750 µL of emulsion were deposited on the analyzer base by using a Microman® M250 (Gilson) and compressed by the probe until a gap of 0.5 mm with a rate of 1 mm/s. Three parameters were recovered:

- the positive area, corresponding to the work required during the product compression (kg.sec),
- the maximum force reached (kg),
- the negative area, corresponding to the work required during the probe removal (kg.sec).

Ability to spreading was lead with the friction modulus A/FR. A polypropylene (PP) sheet was fixed on the analyzer base and the probe was covered with a Helioplate™ HD 2 (Helioscreen, Creil, France) consisting of a PMMA plate. Four lines of each 50 µL of

emulsion were deposited using a Microman® M250 (Gilson, Villiers-le-Bel, France) in the displacement direction. The probe was pulled on a distance of 120 mm with a speed of 3 mm/s. Pictures were taken from spreading traces on the PP sheet. In order to evaluate the corresponding traces thickness, the lightness evolution (L* factor in the CIE L*a*b* color model) was measured every 2 cm, starting to the beginning of the trace (Spectrophotometer CM-5, Konica Minolta, Nieuwegein, Netherlands).

All measurements were performed at least twice on two different batches.

2.4. Micrbiological assays.

2.4.1. Bacteria strain and culture conditions.

Pseudomonas fluorescens MFP05 and *Staphyloccocus aureus* MFP 03 are normal human skin bacteria strains collected by swabbing and characterized by metabolic, proteomic and genomic analysis (Hillion et al. 2013). These strains are stored on cryobeads at -140°C. Before use, they were sub-cultured over-night in 50 mL of Luria Bertani (LB) broth at 28 ± 2°C for MFP05 or 37 ± 2°C for MFP03. The cultures were then diluted again in LB broth to reach the appropriate initial bacterial density corresponding to an $OD_{580nm} = 0.08$. OD values were measured using a spectrophotometer (Helios Epsilon, ThermoSpectronics, Cambidge, UK). Bacteria were used when the cultures reached the end of the exponential growth phase.

2.4.2. Counting and detection of mesophilic aerobic bacteria.

Mesophilic aerobic bacteria in emulsions were counted and detected by using the norm NF EN ISO 21149:2006 (AFNOR 2009). It includes three culture tests. To base the study on solid grounds, we chose to correlate two of them. The first one, without enrichment, consists in diluting approximately 1 g of emulsion in ten times this exact mass in peptone water and then vortexing at 1000 rpm for 20 sec. 100 µL of this mix was spread on a TSA Petri dish and

incubated at 30 ± 2 °C for four days. The second test, with enrichment, was the dilution of approximately 1 g (exactly weighed) of emulsion in ten times the mass in EUGON LT 100 broth. The liquid mix was incubated at least 20h at 30 ± 2 °C under orbital stirring at 180 rpm. Then the mix was vortexed at 1000 rpm and 100 µL were spread on a EUGON LT 100 agar Petri plate. They were then incubated at 30 ± 2 °C for four days. Three Petri dishes were prepared for each condition and emulsion. The number of mesophilic aerobic bacteria (N) was determined by the equation:

- N = m / (V x d) where,
- m is the mean number of colonies on Petri dishes,
- V is the inoculated volume on a Petri dish (mL),
- D is the dilution of the emulsion (1/10 in this case).

2.4.3. Evolution of willingly inoculated bacteria in model emulsions.

Approximately 5 g of each emulsion were diluted to the third in physiologic water (NaCl 0.9%) or LB broth. Obtained solutions were inoculated with MFP03 or MFP05 at an initial concentration between 10^5 and 10^6 UFC/mL. The exact bacterial concentration was controlled by plating 100 µL of solutions and serial dilutions on TSA Petri Dishes immediately after bacterial inoculation. Inoculated solutions were incubated at 28 ± 2°C in the case of MFP05 or 37 ± 2°C for MFP03 under orbital agitation at 180 rpm. After different period of time (24h, 48h and 72h, respectively), 100 µL of solutions were spread on TSA Petri dishes in order to monitor bacteria growth evolution. All Petri dishes were incubated 24h at 28 ± 2°C for MFP05 or 37 ± 2°C for MFP03 before counting.

2.5. Data analyses.

Results were expressed as mean ± standard deviation (SD). Statistical analyses of collected data were performed on XLSTAT software (version 2012.1.01, Addinsoft, France). Single-way analyses of variance (ANOVA) were applied to data series in order to test significance of parameters. When a significant difference was revealed between emulsions ($p<0.05$), groups of emulsions were formed using Tukey multiple comparison test.

3. Results and Discussion

3.1. Comparative study of emulsions with or without nanoparticles.

Three emulsions were obtained by the lab-scale process depicted in the Material and Methods section: a control emulsion without NP (F-0), an emulsion (F-N1) with hydrophilic NP (N1) and an emulsion (F-N2) with hydrophobic NP (N2).

3.1.1. Microstructural characterization.

- **Characterization of nanoparticles dispersions.**

When working with NP, emulsions and their mixtures, an important issue is to characterize size distributions, making sure that the techniques used, the operating conditions and analyses parameters fitted well.

Both NP primary sizes given by suppliers were around 10 nm diameters which correspond to the dimension of the particles measured just after manufacturing. However, at the nanometric scale, attractive forces are intense and NP in powder are known to form aggregates-like structures of much larger dimensions (larger than 1 µm) (Aldous & Kent 2013). Thus, in the present study, several processes were tested to improve the particles dispersions in emulsions. The best one was obtained when pre-dispersion was prepared separately and added after the emulsification step. Size distributions of NP in pre-dispersion

were monitored by laser granulometry as described in Materiel & Methods. Because of the thinness of the dispersions, determination of the refractive indexes (RIs) of the particles was required to use the Mie theory. Different parameters could have an impact on this RI and should be investigated. At the studied wavelength, calculation models showed that the crystallinity, the shape and the size did not impact the RI of TiO_2-particles (Auvinen et al. 2013). The coating could also have an effect on the particles refraction. Thus, calculations based on the RI of the TiO_2 or of the coating were made and gave the same results in term of size distribution.

Added to ester phase, N2 NP formed larger aggregates, with a D90 equal to 30 µm. Thanks to its high viscosity, the paste was refined using a three-cylinder mill. This method decreased the D90 under 4 µm. N1 NP in water were already well-dispersed and had a D90 already around 11 µm. Due to the low viscosity of the hydrophilic dispersion, an ultra-sonication treatment was used and reduced even more the D90 until 1.7 µm. Given in number of particles, the D50 of N2 was equal to 180 nm and the D50 of N1 to 60 nm. Consequently, N1 met the official definition of NP (Official Journal of the European Union 2011) while N2 was really close. However, in this study, size distribution was expressed in volume as it magnifies the impact of the biggest aggregates and allows a better evidencing of the dispersion heterogeneity.

- **Characterization of emulsions.**

For the same reasons as above, emulsions size distributions were also given in volume of colloids. All colloids present in emulsions, TiO_2-particles as well as oil droplets, were detected during measurements. Since these colloids had various sizes and optical properties, the use of a RI was not applicable. For this reason, the Fraunhofer's approximation was chosen in term of calculation model. This model is generally applicable for colloids owning

size larger than few micrometers; however *Jones* (1977) and *Seville et al.* (1984) demonstrated that the error made on the measured size is acceptable, when considering smaller and non-absorbing colloids like in our situation.

For F-0, even if this monomodal distribution was polydisperse (Figure 1), the D90 value, around 7 µm and the D50 value at 2.331 µm both proved that the blank emulsion was mostly composed of small droplets. F-N1 size distribution was very similar to the blank one: only the D10 was significantly lower. NP in the emulsion corresponded to a new population of colloids with a dimension around 0.6 µm. Size distribution obtained for F-N2 highlighted a large distance between the D10 and the D90 values and depicted a high polydispersity. Moreover, the D50 was five time higher than the mean size value for F-0: F-N2 distribution was thus essentially formed of a new larger population around 10 µm.

As sampling required for the size distribution measurement induced a dilution of emulsions and, as a consequence potential physical modifications compared to the initial droplets sizes in bulk, the microstructure of emulsions was also controlled by optical microscopy. Indeed, the lowest dilution necessary to this method allowed observations closer to the reality even if limited by the resolution around 1 µm. Microscopic observations of F-0 (Figure 2A) confirmed the thinness and the homogeneity of the emulsion and allowed to identify droplets network, appearing as a sort of spider web visible on the picture by alternation of dark areas, build of droplets, and white areas consisting of blank. This phenomenon depicted a highly flocculated system. It justified the sample preparation for the granulometry measurement that reduce the aggregation phenomena and allowed the measurement of primary sizes. Optical microscopy also confirmed that only few aggregates were formed in F-N1 and that the majority of the NP was well-dispersed once in emulsion (Figure 2B). The important droplets

network described for F-0 was no longer observed for F-N1. In this work, the high amount of tiny particles located in the continuous phase led to the formation of a new network, consisting of particles and droplets. Moreover TiO_2-particles coated with *silica* had negative surface charges (Junior & Baldo 2014) that might lead to a repulsion between droplets and prevented flocculation. The optical micrograph of F-N2 (Figure 2C) showed large dark objects, around 10-20 µm, masking droplets and corresponding to the aggregates characterized by the D90 value. Added to an oil-in-water emulsion, hydrophobic particles could not be as well dispersed as N1 and formed large vesicles of cetearyl ethylhexanoate and NP. Here again, F-N2 was less flocculated than F-0 surely because the initial emulsion was less concentrated in oil droplets: vesicles contained almost 40% of the total oil of the emulsion. To conclude, the blank emulsion and the emulsion with hydrophilic NP were very similar in term of size distributions, while F-N2 containing hydrophobic NP was rougher. In both case, the presence of NP seemed to avoid the flocculation phenomenon.

3.1.2. Macroscopic properties.

Emulsions are viscoelastic materials that have both solid and viscous liquid properties. In this study, dynamic oscillatory tests did through a stress sweep (Table 2) bring out these behaviors. On the linear viscoelastic region, the G' of F-0 was much higher than the G" demonstrating the elastic behavior of the emulsions. This led to a tan δ equal to 0.2; this low value corresponds to a strong viscoelastic system. Approaching γ_c, emulsion structure kept up until a strong decrease of both moduli. The observed G" overshoot is characteristic of a weak gel (not shown) usually obtained when any gelling agent is added in emulsion continuous phase.

With a similar G' value, F-N1 and F-N2 showed the same predominant elastic behavior as F-0. Even if it was demonstrated that a less flocculated networks or a higher mean size could lead to a lower elastic modulus (Tadros 2004; Pal 1996), in this study, the presence of a solid dispersion offset this fall because it strengthened the network. However, for both emulsions containing NP, tan δ was significantly higher and the G" overshoot was no more observed. The presence of NP in emulsion gave a system considered as a weaker gel compared to F-0, sign of a reduction of the network strength. Critical strains, γ_c, were reduced showing a more polydispersed system: finally, F-N2 appeared less homogenous than F-N1 which was less than F-0.

Texture analyses were performed on emulsions and brought out the same behaviors for F-0, F-N1 and F-N2 during compression test (Table 2). Those results highlighted that the consistency of the three emulsions was the same. Spreading tests performed in order to mimic the application of sunscreen on skin, also highlighted the same behaviors of emulsions. The presence of UV filters only raised the lightness profile of traces (Figure 3) but did not modify the spreading quality. Finally, the slight differences observed during the microstructural characterizations did not impact the macroscopic properties of these emulsions.

3.2. Sterilization by high temperature of emulsions.

In this study, a classical sterilization batch method, imitating Appertisation, was chosen and consisted in high temperature sterilization by autoclaving emulsions directly in its storage packaging (glass bottles). The calculated SV was superior to 22 min whereas the minimum value recommended by the European Pharmacopoeia (Delaunay & Legendre 2013) is only 15 min, meaning that the treatment was efficient. By this way, three new emulsions were obtained ST-F-0, ST-F-N1 and ST-F-N2. During sterilization, high temperatures decreased

the viscosity procured by the gelling agent. After cooling, ST-F-0 and ST-F-N1 restructured spontaneously and recovered a normal emulsion aspect without demixing. Sterilized formula containing N2 was no longer homogenous: a compact block stood in the middle of the glass bottle and was surrounded by a clearer liquid. After few hours cooling down, the viscosities of both phases equilibrated and the batch looked more homogenous even if lumps were still observable.

3.2.1. Microscopic characterization.

The blank emulsion, ST-F-0, and the emulsion with hydrophilic NP, ST-F-N1, had droplet size distributions similar to their non-sterilized versions: only the D90 significantly increased after thermal treatment. Some larger droplets could be observed on the micrograph of ST-F-0 (arrows on Figure 4A) confirming the presence of a new larger droplets population around 10-100 µm (Figure 5). Large objects around 10 µm were present in F-N1 after sterilization (Figures 4B and 5) and consisted in both droplets and particles aggregates.

Observations of ST-F-N2 phases (Figures 4C and 4D) showed that the block part was constituted of around 500 µm long dark objects probably corresponding to large NP aggregates. The liquid part contained mostly fine droplets and air bubbles which appeared in the formula during the sterilization step as a consequence of the gas dilatation. The sterilized emulsion looked like after an accelerated aging already described by *Rossano et al.* (2014). These observations highlighted the high heterogeneity of ST-F-N2 that constituted a real barrier to the physicochemical characterizations of the emulsion and particle size measurements were not conducted.

Zeta potential (ZP) measurement gave a mean value that took into account surfaces of both droplets and particles (curves not shown). At pH 7, corresponding to the pH in emulsions after maturation, ZP measured for F-0 was around -30 mV. Presence of NP in F-N1 and F-N2 slightly decreased the ZP intensity at respectively – 23 mV and -17 mV. After heat treatment, the measured mean zeta potential in emulsions significantly amplified for F-0 (-43 mV) and F-N1 (-33 mV) indicating that surface rearrangements occurred and increased electrostatic repulsion forces, favorable to the emulsion stability. To sum up, the thermal treatment did not impact the integrity of microstructures of the blank emulsion and of the emulsion containing hydrophilic NP while emulsion with hydrophobic NP was no longer homogeneous.

3.2.2. Macroscopic properties.

As illustrated on Table 2, the G' value measured for ST-F-0 was significantly lower than the previous value obtained for F-0, while the G" value remained the same; thus, the solid behavior of the emulsion dramatically decreased. Moreover, the tan δ increased indicating that the gel behavior was considerably reduced. Complementary manipulations on polymer solutions showed that the G' value was divided by two whereas the G'' remained similar after the sterilization step. The viscoelastic evolutions of the emulsion during heat treatment may result from a decrease of the flocculated network but also from polymer degradation. Compression tests did not allow differentiating F-0 and ST-F-0 (Table 2): therefore, apparent texture, like firmness of the emulsion, was not impacted by the heat treatment. For ST-F-N1, the G' value was the same as its non-sterilized version whereas the G" value was increased (Table 2). The rise of the tan δ reflected a reduction of the viscoelastic behavior of the emulsion. Moreover, γ_c decreased indicating a loss of homogeneity, in relation with the rise of the D90 value.

The maximum force reached during the ST-F-N1 compression test (Table 2) was significantly higher when compared to other emulsions. This result revealed an high firmness for the sterilized emulsion containing hydrophilic NP (Friedman et al. 1963), in line with the high value of the viscous modulus. As this phenomenon should be explained by water losses during sterilization inducing a concentration effect of both droplets and particles, the water content of emulsions was evaluated by desiccation before and after the sterilization step but no difference was observed. Then, the increase in consistency and changes in viscoelastic behaviors of ST-F-N1 should not be explained only by the slight changes in size distributions but also by the structural rearrangements as suggested by surface modifications evidence by zetametry.

However, the γ_c remained identical in control and sterilized emulsions highlighted that their homogeneity was not impacted by the heat treatment (Table 2). Moreover, spreading tests highlighted that the important consistency of the ST-F-N1 did not affect the spreading quality as seen by comparing F-N1 and ST-F-N1 traces (Figure 2). Nevertheless, the lightness measurement was higher along the ST-F-0 trace than along the F-0 trace. *Chantrapornchai et al.* already measured the bulk lightness of low concentrated emulsions with monitored flocculation. They demonstrated that in these conditions flocculated droplets decreased the L-value (Chantrapornchai et al. 2001). In this present work, as ST-F-0 and F-0 had the same granulometry profiles, ST-F-0 seemed less flocculated than the F-0 formula.

Although ST-F-N2 was not homogeneous, its spreading quality was tested in order to evaluate the impact of lumps in use. Experiments confirmed the heterogeneity of this emulsion: at the beginning of the trace, lightness was similar to that of F-N2 but L* quickly decreased. Previous studies have shown that the lightness of an emulsion decreases with the size of droplets (Chantrapornchai et al. 1998). Then, the presence of larger droplets in ST-F-N2 than in the other emulsions could explain the low value of measured lightness. It is also

interesting to note that the trace did not end before the maximum mobile displacement and was not well-spread (Figure 6). In fact, the emulsion seemed not to adhere on the hydrophobic surface of PP sheet but remained on the hydrophilic PMMA plate. This phenomenon should be regarded as a first sign of phase separation.

In conclusion, ST-F0 and ST-F-N1 showed macroscopic properties slightly different than their non-sterilized versions. Mainly, the gel behavior was considerably reduced for both emulsions. However, these emulsions remained homogeneous and their qualities in term of consistency and spreading ability were acceptable. In addition to the presence of lumps in ST-F-N2, the emulsion was less covering compared to other studied emulsions and did not match the properties of sun protection cream.

3.3. Assessment of the microbiological properties of model emulsions.

3.3.1. Initial microbiological charge: evaluation of aerobic mesophilic bacteria content.

The efficiency of the sterilization process was evaluated following NF EN ISO 21149:2006 (AFNOR 2009). Culture without enrichment gave the real number of mesophilic aerobic bacteria in emulsions. It was around 100 and 170 UFC/g of product and appears acceptable in regard of the acceptation limit recommended by the European Pharmacopoeia for the non-sterile cutaneous products fixed at 200 UFC/g of product (European Pharmacopoeia 8.0 2014). Petri dishes spread with enriched non-sterilized emulsions were covered with bacteria colonies after incubation. On the contrary, in the case of sterilized emulsions, Petri dishes were devoid of any bacteria development thus proving the efficiency of the heat treatment.

Hence, these results demonstrated that the initial emulsion microbiological charge of emulsions was acceptable and could not be an obstacle to future microbiological assays. For this purpose, only the non-sterilized versions were thereafter tested on bacteria strains.

3.3.2. Bactericidal effect of model emulsions.

Two normal human skin bacteria strains were used: *Pseudomonas fluorescens* MFP 05, a gram negative ubiquitous bacterium representing more than 90% of the microbial flora in humid skin areas (Grice et al. 2008), S*taphyloccocus. aureus* MFP03*,* a gram positive bacterium, usually considered as a member of transient human skin flora carried by 35 to 60% of the human population (Percival et al. 2012).

When the control emulsion was diluted in LB broth (Figure 7A) nutrient contribution let *P. fluorescens* and *S. aureus* to quickly develop. The growth of MFP03 (slope of the linear part of the curve) appeared more rapid than for MFP05 but this difference should only reflect their optimal growth temperature (37°C and 28°C respectively) and metabolism. In this culture condition, no bactericidal effect of the control emulsion was observed.

Thus, it appears interesting to evaluate the bactericidal effect of the presence of NP. The results highlighted that the NP did not significantly affect the growth of both bacteria. Finally, both strains grew as well in all emulsions, with or without NP. One hypothesis could be that the high nutrient contribution of LB broth hided the effect of the nanoparticle presence as well as their coatings. Therefore, dilution in physiological water was conducted as it only brings nutrients already present in the emulsion. In this second condition, the evolutions of bacteria in the diluted control emulsion were also similar and there was no NP effect (Figure 7B). The growth kinetic of *P. fluorescens* MFP05 was reduced with a plateau, corresponding to the total biomass formed which was decreased of ten times. *P. fluorescens* MFP05 is resistant and

highly adaptable to environment variations (Chapalain et al. 2008) as previously shown, emulsions are not appearing as a favorable media for bacteria development (Teixeira et al. 2007). This was confirmed by the behavior of *S. aureus* MFP03 that was unable to grow in emulsion diluted in physiological water. This bacterium could not adapt and metabolize emulsion components. Its population was undergoing a progressive decay due to the lack of adapted resources.

To conclude, the control emulsion fulfilled the defined criteria in the introduction and this test following the bacteria growth in emulsions highlighted the absence of NP effect.

4. Conclusion

In this study, formulation development was conducted to provide lab-scale preservative-free sunscreen emulsions that should contain the same major ingredients and differed only by the presence and nature of TiO_2-NP (NP), an inorganic UV-filter. This development phase showed that TiO_2-NP could be difficult to implement, depending on their coating. While hydrophilic NP, coated with *silica*, was easily integrated into the continuous phase of the emulsion and did not change markedly the physicochemical characteristics of the emulsion, hydrophobic NP, coated with *alumina (and) triethoxycaprylylsilane* induced formation of large aggregates with the other ingredients during the emulsion preparation. Process had therefore to be carefully optimized and controlled in order to succeed in obtaining well dispersed and smooth emulsion. Emulsions containing NP as well as the control one had been characterized by microscopic analyses (laser diffraction granulometry and optical microscopy) and macroscopic analyses (rheology and texturometry) in order to ensure that they shared the same physicochemical properties. Even if these technics highlighted slight

differences in terms of microscopic structures between emulsions, other macroscopic analyses such as rheology and texturometry did not showed bulk major differences. This should allow assessing the role of NP in use, regardless the global physicochemical properties of formulae.

We were able to show that the three emulsions fulfilled the main criteria for the evaluation of the effect of NP in formula on the microbiota. One interesting point is that non-sterile formula, developed with specific cautions taken with the formulation material and procedure, showed an initial bacterial charge well below the acceptable limit as defined by the European Pharmacopeia 8.0.

Applicability of high temperature sterilization to cosmetic emulsions and sunscreen emulsions containing inorganic UV-filters was as well evaluated. Although this treatment should be estimated sufficient to induce emulsion demixing, the control emulsion and the emulsion containing hydrophilic NP passed through it. For both emulsions, the change in gel behaviors could not be only explained by the presence of new larger but minor populations; polymer deterioration and structural rearrangements may also explain these phenomena. Unfortunately, for emulsions containing hydrophobic NP, sterilization induced the formation of two phases; one formed by aggregated NP and larger droplets, and the other by a liquid phase containing small droplets. Even starting from an emulsion with an optimized dispersion of hydrophobic NP, heat treatment induced dramatic changes for both the microstructure as well as the macroscopic properties of this emulsion. The polymeric coating of hydrophobic NP probably makes this kind of treatment more difficult to adapt and formulation development remains to be done. As an example, additional emulsifiers may be used to stabilize NP during the sterilization step. Nevertheless the high temperature sterilization appears applicable to cosmetic emulsions and sunscreen emulsions containing, or not, inorganic hydrophilic UV-filters.

Finally, in order to verify the usability of these model emulsions, an evaluation of the microbiological effect of NP was performed on non-sterile formula. It revealed that, when bacteria were voluntary inoculated in formulae, all emulsions had the same impact on the growth of two human skin bacteria representative strains, *S. aureus* and *P. fluorescens*.

The model emulsions developed in this article are well suited for the evaluation of the 'NP effect' in formula on microbiota, because they hide their other physicochemical aspects whatever the polarity of the coating.

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Table 1: Content, function and supplier of ingredients used in emulsions.

Phase	Content (%w/w)	Product INCI Name	Function	Supplier
Phase A	2,75	Steareth 2	Surfactant	Croda (UK)
	1,75	Steareth 21	Surfactant	Croda (UK)
	2,00	Paraffin	Emollient	Baërlocher (France)
	0,20	Beeswax	Emollient	Baërlocher (France)
	20	Cetearyl ethylhexanoate	Emollient	Stéarinerie Dubois
Phase B	0,10	Acrylates/C10-30 alkyl acrylate crosspolymer	Polymer gelling agent	Lubrizol (Belgium)
	Qs	Deionised water		
	0,13	Triethanolamin	Additive	BASF (Germany)
	5	TiO_2 NP	Inorganic UV filter	-

Table 2: Characteristic parameters of emulsions.

	Viscoelastic parameters				Compression parameters		
	G' ± SD (Pa)	G'' ± SD (Pa)	tan delta ± SD	γ_c ± SD (%)	Positive Area + SD	Maximum force (kg)	Negative Area + SD

					(kg.sec)		(kg.sec)
F-0	622 ^{A} ± 80	122 ^{CD} ± 13	0,20 ^{C} ± 0,01	2,21 ^{AB} ± 0,77	0,170 ^{A} ± 0,023	0,363 ^{BC} ± 0,034	-0,154 ^{A} ± 0,022
ST-F-0	384 ^{B} ± 28	106 ^{D} ± 13	0,28 ^{A} ± 0,02	2,80 ^{A} ± 0,28	0,166 ^{A} ± 0,012	0,337 ^{C} ± 0,026	-0,157 ^{A} ± 0,012
F-N1	648 ^{A} ± 60	143 ^{BC} ± 11	0,22 ^{B} ± 0,01	1,88 ^{BC} ± 0,20	0,194 ^{A} ± 0,030	0,427 ^{B} ± 0,051	-0,160 ^{A} ± 0,032
ST-F-N1	675 ^{A} ± 138	195 ^{A} ± 27	0,29 ^{A} ± 0,02	1,55 ^{C} ± 0,57	0,285 ^{B} ± 0,061	0,518 ^{A} ± 0,072	-0,247 ^{B} ± 0,038
F-N2	670 ^{A} ± 55	159 ^{B} ± 11	0,24 ^{B} ± 0,00	1,18 ^{C} ± 0,09	0,188 ^{A} ± 0,029	0,408 ^{B} ± 0,071	-0,163 ^{A} ± 0,026

Different letters in the same row means significant difference between emulsions for this parameter ($p<0.05$).

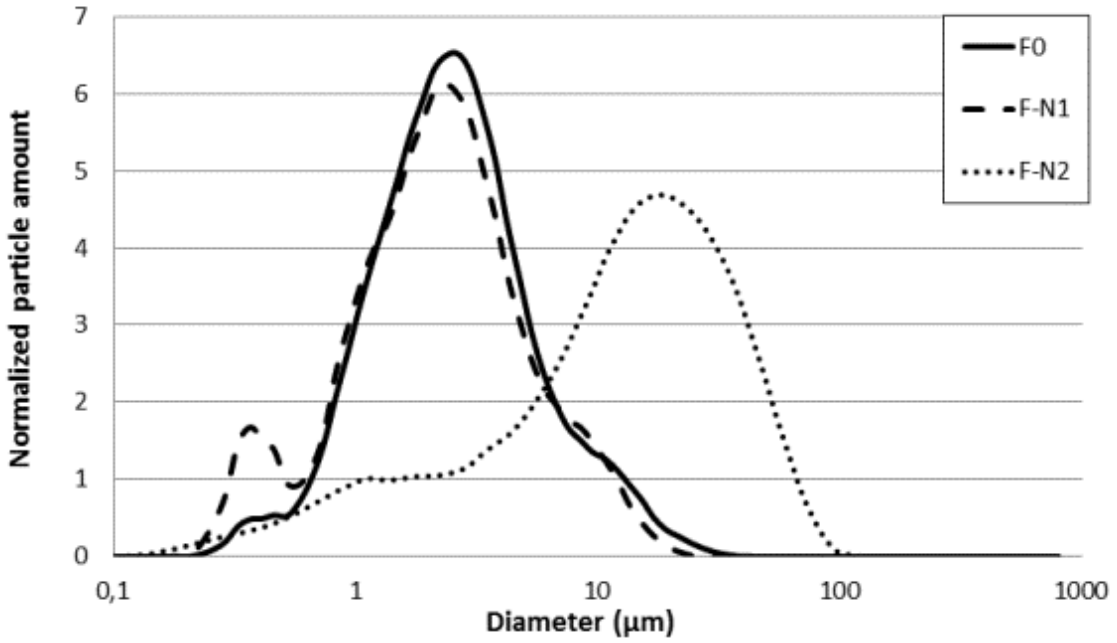

Figure 1: Particle size distributions given in volume particle of fresh emulsions.

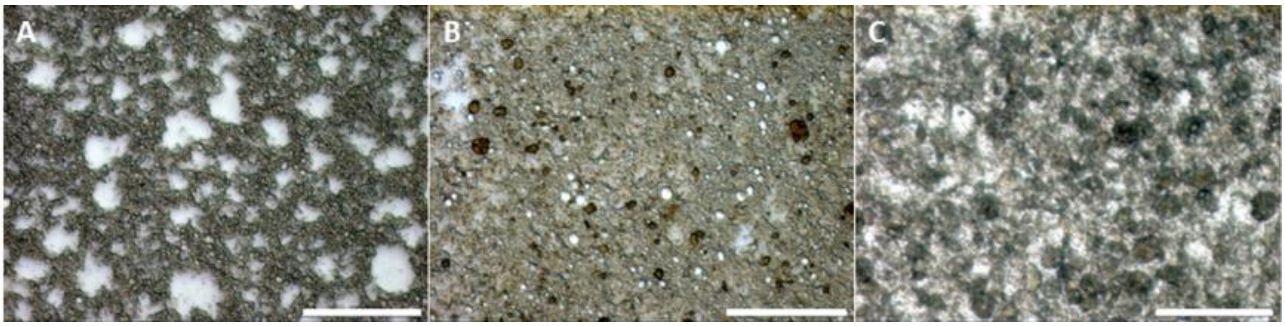

Figure 2: Optical micrograph of; A: F-0, B: F-N1 et C: F-N2. (The scale bar corresponds to 100 µm).

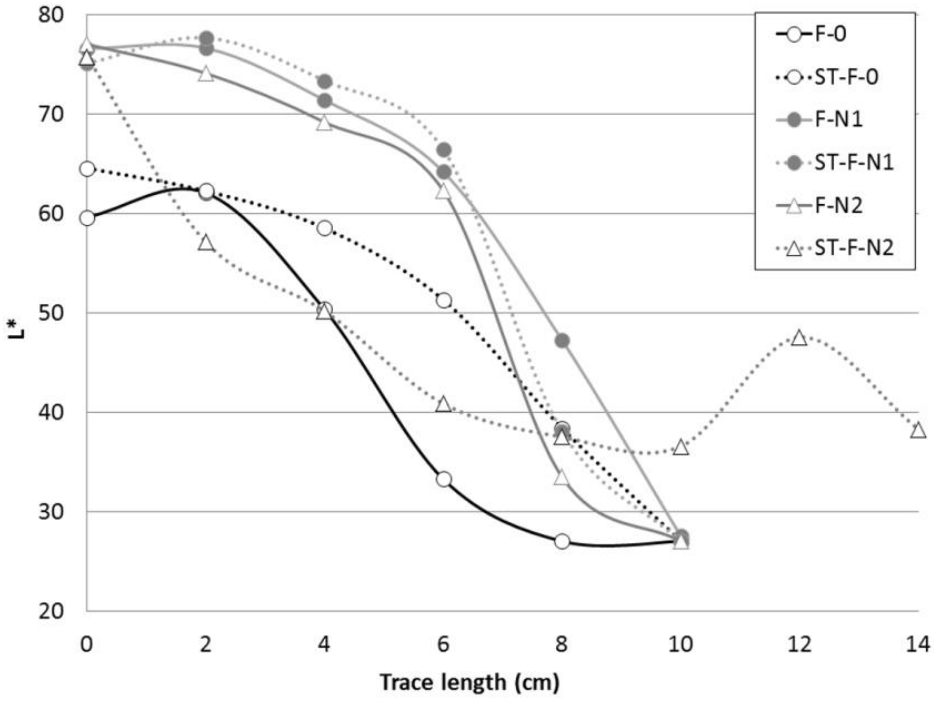

Figure 3: Evolution percentage of lightness (L*) along spreading traces.

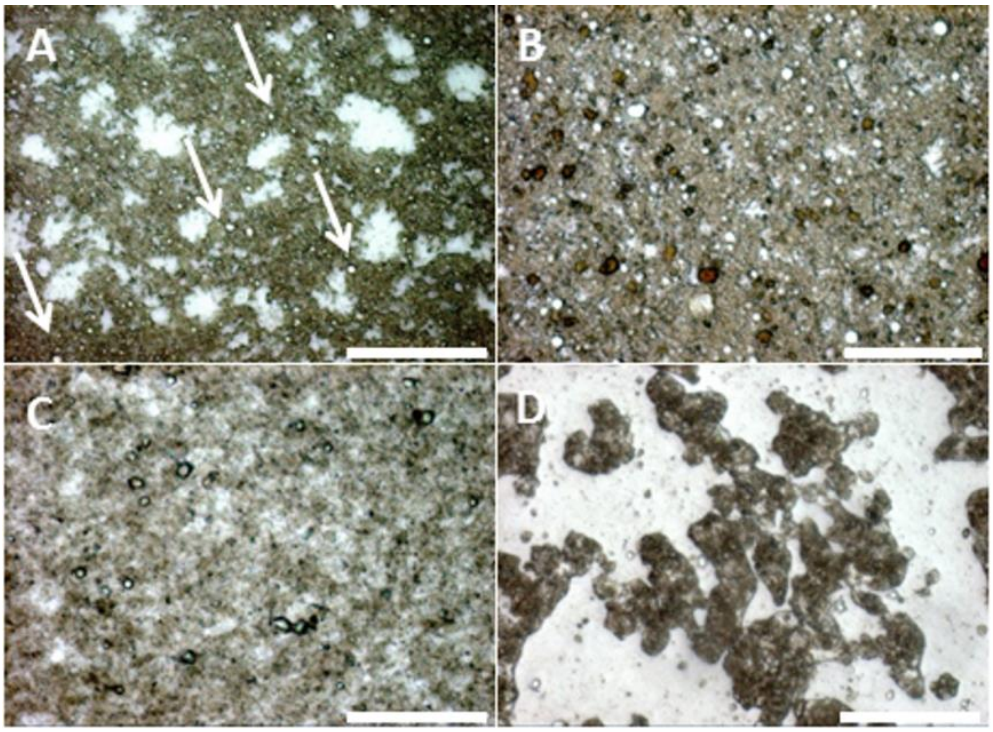

Figure 4: Optical micrograph of; A: ST-F-0, B: ST-F-N1, C: liquid part of ST-F-N2 and D: solid part of ST-F-N2. (The scale bar corresponds to 100 µm).

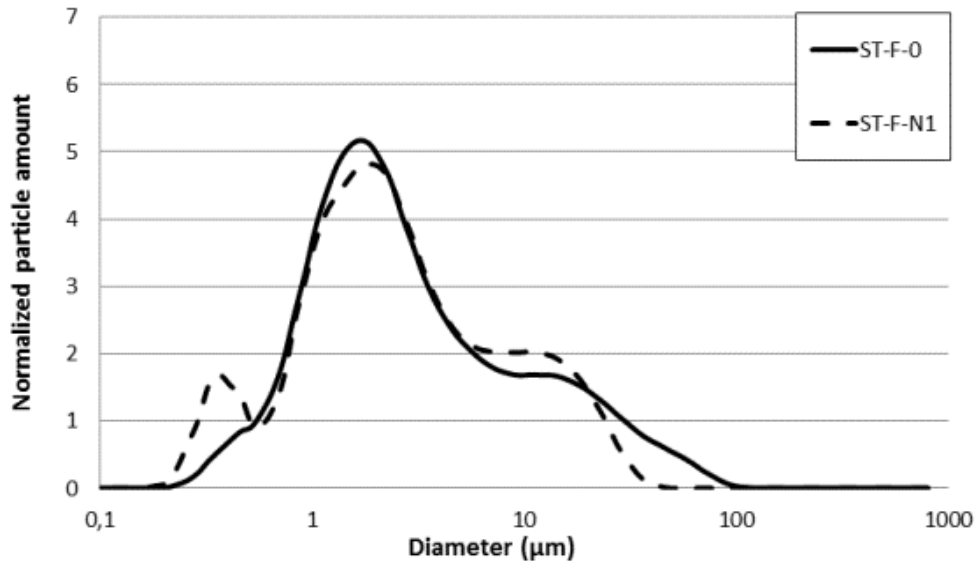

Figure 5: Particle size distributions given in volume particle of sterilized emulsions.

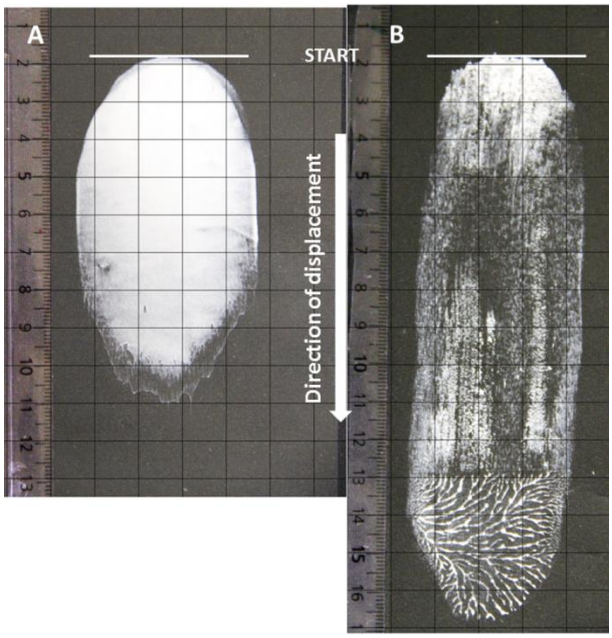

Figure 6: Example of spreading traces, A: for F-N2, B: for ST-F-N2.

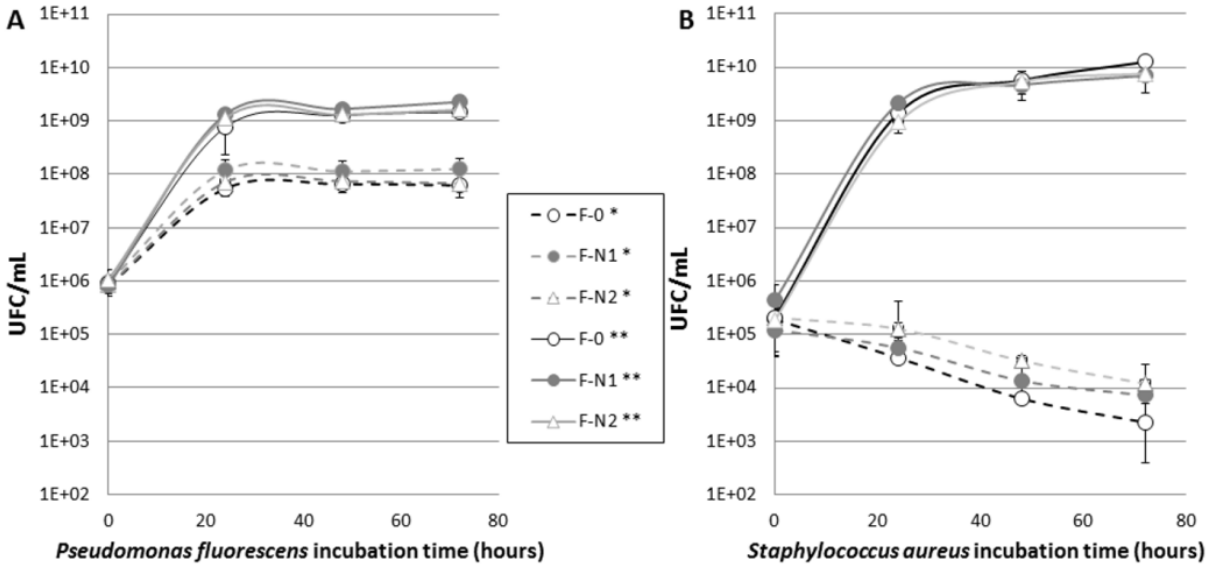

Figure 7: Bacteria growth in different diluted emulsions (n=3); *emulsions diluted in physiologic water, **emulsions diluted in LB.