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# Fungi-derived chitosan as an emulsion stabilizer for the encapsulation of bioactives

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## Abstract

In recent years, an increasing number of industries have been embracing a more sustainable and environmentally friendly approach by adopting materials that are more eco-conscious compared to their previous choices. The food industry is at the forefront of this movement, striving to introduce novel biocompatible and biodegradable materials that can serve various purposes, such as protection agents, supplements, and carriers. Fungal chitosan (FC), has numerous characteristics, such as the lack of allergen animal proteins, consistency in physicochemical characteristics and easy production, while at the same time being an excellent emulsifier. This is why it has gained significant attention from scientists for various applications. In this study, FC particles were formulated using the pH-shifting method, while emulsions were prepared through high-shear homogenization. The emulsions were examined both macroscopically and microscopically. Their droplet size was determined using Static Light Scattering, revealing an average size of approximately 75  $\mu\text{m}$ . Furthermore,  $\alpha$ -tocopherol or squalene was successfully encapsulated in these emulsions. Cryogenic Scanning Electron Microscopy (Cryo-SEM) was utilized to visualize the surface of the droplets, and Confocal Laser Scanning Microscopy allowed for the observation of the droplet core. To assess their antioxidant properties, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) colorimetric assay was employed. Additionally, their antimicrobial potency was evaluated. In the presence of the bioactive compounds, both emulsions exhibited scavenging activity against the DPPH radical, with emulsions loaded with  $\alpha$ -tocopherol demonstrating the highest antioxidant capacity. Most importantly, all tested samples led to a reduction in the bacterial population of pathogen target bacteria *Escherichia coli* C1845 (*E. coli*) and *Staphylococcus aureus* DSM 21705 (*S. aureus*) proving they possess antimicrobial properties.

**Keywords:** fungi-derived chitosan, emulsions, antioxidant,  $\alpha$ -tocopherol, squalene

## 1. Introduction

Food safety has become an urgent matter for discussion universally due to the increasing globalization of the food supply chains, which are associated with potential health risks because of unsafe or contaminated food. Climate change, food fraud, allergens and emerging pathogens are only some of the issues the scientific community has been employed to confront [1,2]. The means for protection that have been used vary from proper food packaging, temperature control for storage, addition of preservatives and antibiotics as well as antioxidants [3,4]. Unfortunately, food packaging materials are mostly materials that are expensive, non-biodegradable and in some cases have proven to be harmful because of their chemical interactions with the contained food [5,6]. On the other hand, the excessive use of antibiotics and antimicrobial agents can lead to the development of antimicrobial-resistant bacteria that can impact the human health, making infections harder to treat [7,8]. Finally, preservatives have also raised concerns for their excessive use due to their chemical formula and low consumption tolerance. New findings lead to constant withdrawal of preservatives considered as safe, leading to consumers concerns [9].

The main concern of the modern food industry is to introduce novel techniques for food safety, such as in food packaging, while at the same time maintaining the quality of the foods and leading to a more environmentally friendly and sustainable path. Biopolymers have been recently used as an alternative to chemicals for food protection since they are biocompatible, biodegradable, and highly bioactive [10], [11]. Biopolymers are divided into three different classes based on the origin and synthesis method, namely natural, synthetic ones, and biopolymers. In contrast to other materials, such as plastic, biopolymers are degraded relatively fast by natural microorganisms under suitable conditions such as oxygen, moisture, and temperature without any environmental issues. Chitosan is a natural biopolymer, a cationic polysaccharide, the second most abundant on Earth and comes from crustaceans, invertebrates, and fungi. All kinds of chitosan can find numerous applications in the food industry many of which are directly linked to food safety and the upgrading of their nutritional value [12], [13]. To start with, chitosan can form edible films to act as coatings for perishable foods, such as fruits and vegetables [14]. It can improve the texture of certain foods through its gelation ability and form structures (emulsions, nano-capsules etc.) for the encapsulation and delivery of nutrients and bioactive substances [15], [16]. Furthermore, chitosan can act as an antimicrobial agent making it possible to reduce or eliminate the use of antibiotics or preservatives [17], [18]. The main mechanism for chitosan's antimicrobial potency is the electrostatic interactions between chitosan and the bacterial cell walls, which allows chitosan to form a "film" around the bacteria cells, not allowing them to interact with their surroundings or "feed" in order to grow. [19] Many factors play a role in the effectiveness of chitosan's antimicrobial action such as pH, molecular weight, concentration, even the kind of the microorganism target. It is claimed that chitosan acts best in acidic conditions and against Gram (+) bacteria [20]. In order to verify this claim and to test the behaviour of chitosan in our system we chose to test two microorganisms, a Gram (-) and a Gram (+), namely *Escherichia coli* (Gram -) and *Staphylococcus aureus* (Gram +), to see which would be mostly affected by chitosan in the conditions we use it. Finally, chitosan can act as an excellent emulsifier for forming especially Oil-in-Water (O/W) emulsions including food applications [21].

When focusing on the sources of chitosan, fungal, has certain advantages over the one deriving from crustaceans. Fungal chitosan (FC) is produced by fermenting fungal biomass, usually from species like *Aspergillus niger*. These fungi naturally synthesize chitin, which can be converted into chitosan through deacetylation [22]. FC is often considered more sustainable as it is produced without shellfish harvesting. This also answers to concerns about overfishing and ecosystem impact [23]. Although fungal chitosan does not differ much, chemically, or structurally from the chitosan that derives from crustaceans there are several other differences between the two that can be detected. First of all, chitosan from fungi is free of heavy metals like nickel and copper, that are found in crustaceans and it is also without allergenic animal proteins [24], [25]. Furthermore, the seasonal variation in crustaceans leads to variation of source materials. This fact alongside the harsh treatment of the exoskeleton that is needed produces chitosan with inconsistent physicochemical characteristics. At the same time when using fungi as a source, chitosan with lower molecular weight and viscosity can be obtained with better results. Moreover, the fungi can be readily grown in the laboratory on cheap nutrients, waste material can be recovered by simple chemical procedures and constant quality and supply of the raw material is possible [26]. Finally, the production of chitin, chitosan's precursor, is similar but with certain characteristics that has, it is considered to be easier done in fungi. Fungal chitin sources are generally easily homogenized using a kitchen blender, while the harder and more brittle crustacean shells must be crushed. The high mineral content of crustacean shells also requires an acidic demineralization step. This step is not required when processing fungal chitin. Deproteination is then completed for either fungal or crustacean chitin in mild alkaline conditions, before the final material is decolorized, using a bleaching procedure, if required. Chitin is obtained from crustacean shells as a final product, whereas fungal chitin sources yield a chitin- $\beta$ -glucan complex following extraction. Pure chitin can be derived from this complex, using acid treatments [27]. Furthermore, FC can be used in vegan applications, and it is more accepted as plant-based carrier. Finally, a comparative measurement between the crab- and fungi- derived chitosan is given in 2.7 A comparison between fungal and crustaceans derived chitosan, 3.6 A comparison between fungal and crustaceans derived chitosan. For the aforementioned reasons, fungi-derived chitosan was chosen for further investigation in the context of this study.

In the present study, we are introducing an innovative particle-stabilized emulsion based on FC. This emulsion serves as a platform for encapsulating lipophilic bioactive molecules, such as  $\alpha$ -tocopherol and squalene. The proposed carrier exhibits great promise as effective candidates for novel applications in the food industry. This approach not only shows potential for contributing to a more sustainable future but also holds the promise of promoting better health outcomes. It is an alternative to the more conventional crustaceans-derived chitosan that gives an outlet for people that either choose not to consume animal products or suffer from allergies. The main advantage to this proposed system is that it carries all previously mentioned beneficial characteristics without lacking any of the properties found in crustaceans-derived chitosan. To our knowledge, this is the first time that FC is being explored for its potential to form edible emulsions and to efficiently encapsulate lipophilic bioactives.

## **2. Materials and Methods**

### **2.1 Materials**

Fungi-derived chitosan (20–100 cps, 98% deacetylation) was sourced from Qingdao Chibio Biotech, (Qingdao, Shandong, China) and chitosan C2396 (20–100 cps, 98% deacetylation) from TCI chemicals (Zwijndrecht Belgium). Acetic acid, methanol, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, Nile Red and Fluorescein-5-isothiocyanate (FITC) were purchased from Sigma- Aldrich (Chemie GmbH Munich, Germany). Extra Virgin Olive Oil (EVOO) and Sunflower Oil (SFO) was obtained from a local supermarket in Athens, Greece. Medium Chain Triglycerides (MCT) were purchased from IOI Oleochemical (Hamburg, Germany). Buffered Peptone Water (ISO) was purchased from LAB M (Heywood, Lancashire, United Kingdom), Brain Heart Infusion broth (BHI) from Biokar Diagnostics (Allone, France) and Bacteriological agar from Condalab (Madrid, Spain). Sodium dodecyl sulphate (SDS) was purchased from MP Biomedicals (Eschwege, Germany). For the interfacial tension experiments ultra-pure water was used.

### **2.2 Particles preparation, size determination, and morphology observation**

#### *2.2.1. Preparation of chitosan particles*

Chitosan particles were prepared at four different concentrations, namely 0.1, 0.2, 0.3 and 0.5% w/v. This was to evaluate the ability of the particles to formulate emulsions and choose the optimum to proceed with the rest of the experiments. At first, a stock solution of chitosan (1% w/w) was prepared in 1% v/v acetic acid. The stock solution was diluted with distilled water to prepare the four desired concentrations. The pH of the solutions was set to approximately 6.5 with the aid of NaOH 0.1M. Finally, the particles were sonicated for 20 minutes, to obtain smaller particles using the protocol described elsewhere [28].

#### *2.2.2. Determination of particles' size using Dynamic Light Scattering (DLS)*

An initial measurement of the FC droplet sizes was conducted using a Zetasizer Nano ZS (ZEN3600) analyzer from Malvern Instruments Ltd., Malvern, UK. This analyzer was equipped with a He-Ne laser operating at 633 nm wavelength and employed Non-Invasive Backscatter (NIBS) optics. The Polydispersity Index (PDI) of the emulsions was also determined. To ensure consistent conditions, all samples were diluted with deionized water to a concentration of 0.01% v/v, and pH adjustments were made as needed. Data analysis was performed using Malvern Zetasizer Nano software, version 6.32 (Malvern Instruments Ltd., Malvern, UK), which employs a spherical model for diffusing particles with low polydispersity. These measurements were conducted in triplicate at a temperature of 25 °C [29].

### 2.2.3 Morphology observation of particles using Freeze Fracture Transmission Electron Microscopy (FFTEM)

The Freeze Fracture (FF) replica preparation procedure involved placing a drop of the sample onto a gold planchette, followed by rapid freezing by immersing the holder into liquid propane held at the temperature of liquid nitrogen. This swift freezing step was crucial to vitrify the sample, preventing structural disruption caused by crystallization. The frozen samples were then introduced into the freeze-fracture enclosure of a BAF 060 Leica Microsystems apparatus, maintained at a temperature of  $\approx 150$  °C and a pressure of  $10 \times 10^{-8}$  mBar. Subsequently, a metal knife, cooled to  $\approx 200$  °C, was employed to fracture the samples. The freshly fractured surface was immediately coated with successive depositions of platinum at a  $45^\circ$  angle and carbon at a  $90^\circ$  angle. Outside the BAF060 apparatus, the gold planchettes were immersed in the sample solution to detach the replicas from the samples. These replicas then underwent several sequential baths of ethanol and 1 M NaOH to eliminate any remaining sample material. Finally, they were soaked in pure water for a few additional hours. The replicas were ultimately collected on 400 mesh copper grids and dried before undergoing Transmission Electron Microscopy (TEM) imaging. TEM was carried out using a HITACHI H 600 microscope operating at 75 kV [30].

## 2.3 Assessment of the emulsions stability

### 2.3.1 Determination of the Creaming Index Percentage (CI%)

To evaluate the stability of the emulsions, we conducted storage tests under two distinct conditions. Emulsions (of O/W later referred to as “empty” and of oil loaded with an active-in-water later named “loaded”) were prepared into 12 mL flat bottom glass tubes (15 mm in diameter and 50 mm in height) with screw caps. In one set of experiments, these tubes were stored at room temperature, while in the second set, they were refrigerated at 4 °C. Upon preparation, the emulsions exhibited phase separation, forming two distinct layers (cream and subnatant) within a few minutes. The evolution of the cream layer during storage was carefully monitored, and data were recorded at intervals of 5 days. The stability of these emulsions was assessed through the creaming index (CI%), which is a measure of the cream layer height ( $H_c$ ) expressed as a percentage of the total sample height ( $H_t$ ) within the tube. This index was calculated using the formula (1) [31]. The procedure was carried out three times.

$$CI (\%) = \left( \frac{H_c}{H_t} \right) * 100 \quad (1)$$

## 2.4. Emulsions preparation, droplets size determination and morphology observation

In this study, our primary focus was on the creation of O/W emulsions, formulated and stabilized by FC particles. To accomplish this, we introduced chitosan particle dispersions at varying concentrations (0.1%, 0.2%, 0.3%, and 0.5% w/v) as the aqueous phase, while EVOO, SFO, and MCT were tested as potential oil phases. The addition of oil in each case was done

gradually under constant stirring. Additionally, we included  $\alpha$ -tocopherol or squalene in the oil phase before emulsification at a concentration of 1% w/w, aiming to investigate the emulsion's capability to incorporate and stabilize model bioactive substances. Furthermore, we explored various water-to-oil ratios, specifically 80:20, 70:30, and 60:40 w/v, to assess the emulsions' ability to encapsulate increasing amounts of oil. This was carried out to determine the maximum quantity of oil that could be efficiently incorporated into the systems. By systematically examining these factors, our goal was to gain insights into the properties of chitosan derived from fungi, the stability of the formulated emulsions, and their effectiveness as carriers for lipophilic bioactive compounds.

#### 2.4.1. Preparation of emulsions

The preparation method was based on the previously published work. More specifically, the aqueous phase (chitosan particles dispersion) was measured in a glass vial followed by the dropwise addition of oil, with or without the lipophilic additives. The addition of the oil was made under high-speed homogenization using a high-speed homogenizer (X1000D Unidrive, Ingenieurbüro CAT, Ballrechten-Dottingen, Germany) with a 10 mm Diameter Generator, Teflon Bearing, Immersion Depth 150 mm, at 10 000 rpm. Following the addition of the oil, the homogenization was carried on for 3 more minutes at 20 000 rpm. Afterwards, emulsions were allowed to reach steady state for a few hours before any further experiments or observations were made [28]. A schematic representation of the process followed is presented in Fig. 1.

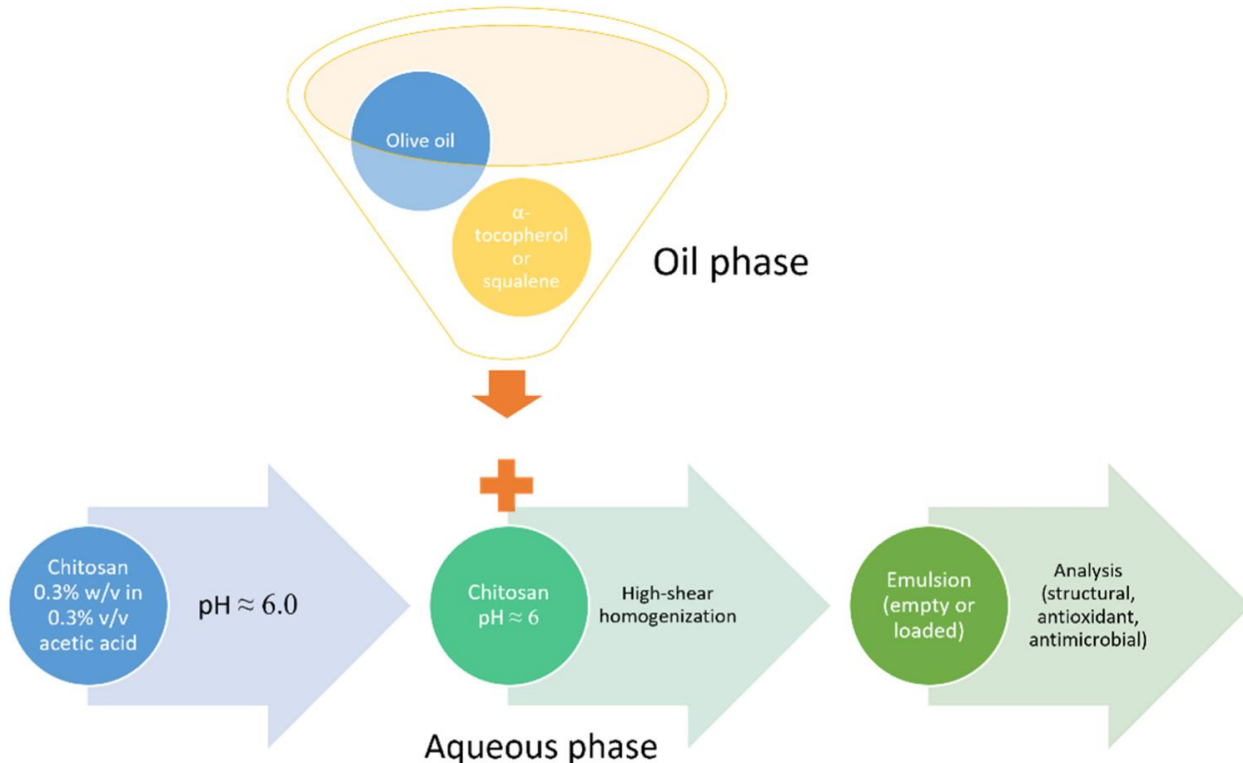


Figure 1: Schematic representation of the emulsions' preparation method.

#### 2.4.2. Droplet size determination by Static Light Scattering (SLS)

The Mastersizer 2000 granulometer from Malvern Instruments Ltd. in Malvern, UK, along with Mie theory, was utilized to measure the size distributions of emulsion droplets. To obtain these measurements, samples were introduced into the "small volume sample dispersion unit" following a 1:10 dilution. The size distribution was assessed based on two key parameters: the volume-weighted mean diameter, denoted as  $D$ , and the polydispersity index, often referred to as uniformity ( $U$ ). Polydispersity ( $P$ ) is defined as the volume-weighted average difference between the diameter and the median diameter, normalized by the median diameter itself. The median diameter represents the midpoint of the size distribution. In other words, it is the diameter at which half of the dispersed phase consists of droplets smaller than the median diameter, while the other half comprises droplets larger than the median diameter. These values are obtained through Eqs. (2) and (3), with  $N_i$  representing the total number of droplets having a diameter  $D_i$  and  $D_{50}$  signifying the median diameter, which is the diameter at which the cumulative undersized volume fraction equals 50% [32,33].

$$D = \frac{\sum_i N_i D_i^3}{\sum_i N_i D_i^2} \quad \text{and} \quad P = \frac{1}{D_{50}} \frac{\sum_i N_i D_i^3 |D_{50} - D_i|}{\sum_i N_i D_i^3} \quad (2)$$

#### 2.4.3. Droplets Morphology Observation using Cryogenic Scanning Electron Microscopy (Cryo-SEM), Confocal Laser Scanning Microscopy (CLSM) and Microscopic Observation

Examinations of the emulsion droplets were carried out using the Leica DM IRB inverted research microscope by Leica Microsystems GmbH, situated in Wetzlar, Germany. To prepare the samples for observation, they were appropriately diluted with distilled water and adjusted to pH 5.5. Following this, the samples were positioned on a microscope slide without a cover and examined under bright-field illumination. Cryo-scanning electron microscope (Cryo-SEM) evaluations were conducted utilizing a ZEISS GEMINI 300 Field Emission Scanning Electron Microscope operating at 1.5 kV. The SEM was equipped with the PP3010T cryo stage by Quorum Technologies, based in England. To initiate the procedure, a small amount of emulsion was applied to the specimen holder, and then it was swiftly frozen using a nitrogen freezing station, creating slushy freezing conditions. This rapid freezing method serves to reduce potential damage caused by ice crystals and enhances the preservation of the specimen. The frozen samples were subsequently moved to a cryogenic preparation chamber set at  $-140^\circ\text{C}$ , where they were fractured using a cold blade. Ultimately, the processed samples were positioned onto a stable SEM cold stage to facilitate thorough examination. [34]. Confocal fluorescence microscopy was employed for the visualization and depiction of the oil phase within the emulsions as well as the chitosan "ring" around them. For the chitosan staining the procedure followed was one previously described with some modifications. FITC was dissolved in methanol in an initial concentration of 0.3% w/v. After that, the right volume was added in a chitosan solution (0.3% w/v, pH 5.5) to achieve a final



FITC concentration 5 ppm. The mixture was left at room temperature in the dark for 1 h [35]. For the oil phase was stained with the lipophilic dye Nile Red (0.01% w/w). Subsequently, the emulsions were prepared following the previously described procedure (Section 2.3.1) using the same oil phase as mentioned before. Afterwards, emulsions were prepared as previously described. Emulsions were washed with deionized water until no FITC was present in the supernatant. Following, a droplet of the stained samples was placed onto a slide and covered with a cover slip to ensure the absence of any air bubbles. The samples were then mounted and subjected to examination using a confocal laser scanning microscope (Leica TCS SPE, Leica Microsystems, Heidelberg, Germany), employing an excitation wavelength of 488 nm and an emission wavelength of 500 nm with a x20 lens. The LAS AF software from Leica Microsystems was utilized for image acquisition [36].

## **2.5. Assessment of the emulsions antioxidant capacity using the DPPH free radical**

The antioxidant capacity of the emulsions was assessed using a colorimetric DPPH assay, which was conducted following a previously outlined procedure with minor adjustments. Briefly, 2 mL of a DPPH solution dissolved in ethyl acetate at a concentration of 0.108 mM was measured using UV–VIS spectroscopy within a plastic tube. Subsequently, 100  $\mu$ L of the sample being tested was added to this solution. The resulting mixture was vigorously agitated and then left in darkness for a duration of 30 min. Following this incubation period, the absorbance was measured at a wavelength of 515 nm. As a reference, ethyl acetate was employed as a blank, while a mixture comprising 2 mL of DPPH and 100  $\mu$ L of ethyl acetate served as the control sample. The percentage of inhibition of the free radical was calculated using the following formula (Formula 4), where A0 represents the absorbance of the control sample, and A1 denotes the absorbance observed after the 30-minute reaction period [37].

$$\%Inhibition = \left[ \frac{A_0 - A_1}{A_0} \right] * 100 \quad (3)$$

## **2.6. Assessment of the emulsions antimicrobial ability**

For the assessment of the emulsions' antimicrobial activity two bacterial strains were used as targets, namely, *Escherichia coli* C1845 (*E. coli*) and *Staphylococcus aureus* DSM 21705 (*S. aureus*), which were kindly provided by the Laboratory of Dairy Research of the Agricultural University of Athens. Both strains were routinely grown for 18 h in BHI broth reaching approximately 9 log cfu/mL. For the KA, the aforementioned overnight cultures were serially diluted in buffered peptone water to prepare bacterial suspensions of two different initial populations, namely 6 and 3 log cfu/mL for each tested target strain. After, three different types of emulsions, namely empty, loaded with  $\alpha$ -tocopherol and loaded with squalene, were mixed with the prepared bacterial suspensions in two ratios, 1:1 and 1:10 (emulsion: bacteria suspension). All mixtures were incubated under constant stirring at 37 °C for 24 h. After 4 and 24 h of incubation,

serial dilutions were performed in all mixtures and placed on BHI agar plates to enumerate surviving bacterial cells after incubation of plates for 48 h at 37 °C [38].

## **2.7. A comparison between fungal and crustaceans derived chitosan**

In order to confirm the ability of fungal chitosan to fully being able to replace the one deriving from crustaceans, without missing out on any of its useful characteristics, we prepared emulsions with chitosan from crab shells, and we assessed the droplet size. Furthermore, using the pendant drop technique we examined the two chitosans' effect on the dynamic interfacial tension of the water oil interphase. The dynamic interfacial tension between oil and water was assessed through measurements conducted with a pendant-drop tensiometer (CAM 200, KSV, Biolin Scientific, Stockholm, Sweden). The obtained data were analyzed using drop-shape analysis software (Attension Theta Software, V. 4.1.9.8, Biolin Scientific, Stockholm, Sweden), and curve fitting was achieved through the application of the Young–Laplace equation. The experimental setup involved creating a pendant drop of the aqueous phase within the oil phase, contained in a quartz cell (Hellma Analytics, Müllheim, Germany) [39]. The oil droplet size distribution was determined employing a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern UK) and the Mie theory were used to measure the emulsions' droplet size. Samples were added in the “small volume sample dispersion unit” after proper dilution (1:20 in SDS). The size distribution was characterized in terms of the surface-averaged diameter  $D$  and uniformity  $U$  [40].

## **3. Results and discussion**

### **3.1. Particles preparation, dimensional determination and morphology observation**

Grafting chitosan particles can present challenges due to their susceptibility to a range of factors, including pH variations, application of sonication, and prevailing environmental conditions [41]. The proper modification of chitosan is of great importance, as it significantly impacts the stability, categorization (whether Oil-in-Water or Water-in-Oil), and overall structure of particle-based emulsions. This dependency arises primarily from the specific properties of solid particles, and especially their wettability by both the oil and aqueous phase [42]. It has been examined how pH affects the molecular structure of particles by extension the stability of emulsions, and it has been found that the optimum pH, in which particles formulate the most stable emulsions is at 6.5 [43]. Dynamic Light Scattering was applied in order to measure the size of the fungal chitosan-based particles after their pH modification and sonication. The spherical shape model that the DLS uses to extract the results was confirmed by the FFTEM images. Particles that appeared on those images were indeed of spherical shape and different sizes, and are in accordance with their conventional counterparts measured in previous studies [44]. Through our conducted experiments, we found that subjecting the particles to sonication treatment resulted in the generation of smaller particles also with a lower PDI. Data obtained, showed particles that were

quite big but relatively homogenous (Table 1). The major particles population was at 209 nm, while there was a second minor population at 21 nm. The second, smaller population is probably chitosan that has not been affected by the pH shifting or impurities and this is why the intensity signal on DLS is so small. These findings are in accordance with previous research using conventional chitosan from crustaceans for the formation of particles. In fact, in modifications that are based on ionic gelation and not on forms of coating, such as film formation, the sizes that have been reported varied from 150–400 nm. [21,45]. Furthermore, previous research explored the preparation of emulsions utilizing self-aggregated particles and compared the outcomes between those subjected to ultrasonication pretreatment and those without it [46]. Our findings corroborate with their results and further support the concept that sonication plays a crucial role in achieving emulsions with reduced droplet size and enhanced uniformity.

Table 1 Dynamic Light Scattering results of the FC particles hydrodynamic diameter and PDI. Each value in the Table is represented as mean  $\pm$  SD (n = 3).

	Peak 1 (nm)	Peak 2 (nm)	PDI
Before sonication	489 $\pm$ 12	247 $\pm$ 21	0.678 $\pm$ 0.097
After sonication	209 $\pm$ 18	21 $\pm$ 2	0.375 $\pm$ 0.049

### 3.2. Stability assessment against creaming and coalescence of the emulsions

The stability of the emulsions against creaming was examined by monitoring the CI (%) over time. Emulsions were stored at ambient temperature (25 °C) and in the fridge (4 °C), to compare the two conditions. Furthermore, the stability among emulsions prepared with EVOO, MCT or SFO was tested. MCT and SFO could not be fully incorporated in emulsions stabilized by less than 0.5% of chitosan. Even when fully incorporated, the emulsions formed were not stable for more than 7 days. After that oil would appear on the surface of the emulsions followed by creaming. Thus, all further experiments were conducted using EVOO which formed more stable emulsions. In Fig. 2 emulsions freshly prepared with EVOO are presented. It is evident from Figs. 2a and 2b that emulsions in every chitosan concentration and in 80:20 as well as 70:30 water:oil ratio underwent a fast-creaming process. Significantly less creaming can be observed for the samples containing 0.1% and 0.2% w/w chitosan at the 60:40 water: oil ratio (Fig. 2c). All other chitosan emulsions at the 60:40 water:oil ratio as well as every sample at 50:50 water:oil ratio did not present signs of creaming (Figs. 2c, 2d). Creaming occurs due to the density mismatch between oil and water and to the non-Brownian nature of the drops (larger than 1  $\mu$ m). The larger amount of oil reduces the CI% due to higher volume occupation of the dispersed phase explaining the difference between Fig. 2a to d at fixed chitosan concentration. The effect of the chitosan concentration at 70:30 is likely related to interactions between drops mediated by particles (at the interface and/or in the bulk). It has been found previously that with the increase of the two previously mentioned factors the creaming decreases [47,48]. All empty emulsions stability was

monitored regardless of which one was chosen to encapsulate  $\alpha$ -tocopherol or squalene. The emulsions occurring after the encapsulation were monitored as well, to observe whether the encapsulated substances would have any effect on the stability. Emulsions stored at ambient temperature, with 80:20 water:oil ratio were destabilized after 5 days, and an oil layer was visible on the top of the emulsion meaning a massive destabilization through coalescence. The same happened for emulsions with 0.3 oil volume fraction after 10 days. Every other emulsion remained stable for at least 20 days, with the ones containing 0.3% and 0.5% w/w of chitosan not showing any sign of destabilization by coalescence or creaming for 60 days. Furthermore, the addition of the bioactive substances did not appear to influence the stability. Finally, no microbial growth was observed on any of the samples. The only cases of appearance of microbial growth were in the stock chitosan emulsions in concentrations lower than 0.3% w/v (e.g., 0.1, 0.03% w/v), after 30 days of storage at ambient temperature. This is a promising first sign of antimicrobial performance on the emulsions' part, since in previous research we saw that emulsions stabilized by Pea or Soy protein Isolate showed the opposite [40]. In that case, the emulsions grew a microbial population after five days of storage at ambient temperature if no antimicrobial agent was added. When the least stable samples were stored at 4 °C, their stability highly increased, with no evidence of any kind of destabilization for at least 30 days [49,50]. The cream after the first few hours of storage was much firmer, which is expected since EVOO's viscosity at 4 °C is higher than at room temperature [51]. The CI% does not change for any of the samples.

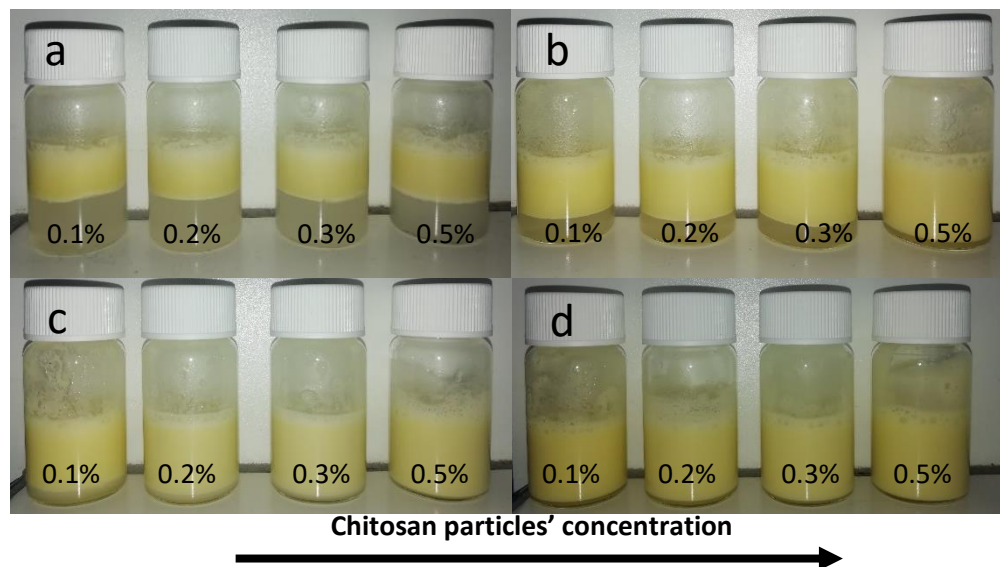


Figure 2: Emulsions prepared with chitosan particles at different concentrations (% w/w) presented from left to the right lowest to highest concentrations in (a) 80:20 water :oil ratio, (b) 70:30 water: oil ratio, (c) 60:40 water: oil ratio and (d) 50:50 water: oil ratio.

### **3.3. Emulsions preparation, droplets size determination and morphology observation**

#### *3.3.1. Selection of the preferred system for the further study*

During the emulsions preparation it was kept in mind that the formulated systems should meet certain requirements, such as stability against coalescence, homogeneity of the droplets size (Polydispersity lower than 0.6), and antioxidant ability. Thus, several factors were taken into consideration such as the kind of oil used and its volume fraction, the concentration of particles and the homogenization method that would lead to the systems with the desirable characteristics. These are all elements that affect the stability of an emulsion and its overall structure [50]. For this purpose, different combinations of particle concentrations and oil volume fractions were examined. Furthermore, various homogenization speeds and homogenization times were applied. Also, the kind of oil that was used, was also tested to see how it would affect the overall emulsion image. Fungal chitosan particles were used at 4 different concentrations, namely 0.1, 0.2, 0.3, & 0.5% w/v. All these concentrations were tested at the 80:20, 70:30 and 60:40 water:oil ratios. The tested homogenization durations were 1, 2 and 3 min. Homogenization speed ranged from 5 000 to 20 000 rpm. For the oil phase EVOO, SFO and MCT were examined. Macroscopically, it could be seen that the creaming was less pronounced with increasing particle concentration and oil volume fraction. The most stable emulsions against creaming were produced at 20 000 rpm for 3 min in all cases so that this was the chosen homogenization process in the following. The water:oil ratio that was finally selected was 60:40 since it was the one showing no creaming, and the easiest to handle since the emulsions prepared with 50:50 water:oil ratio was very thick and difficult to handle for further analysis. Indeed, this is linked to the fact that emulsions' viscosity increases when the dispersed phase volume fraction increases and the drop size decreases. EVOO was chosen as the oil phase since it is the most nutritious of the three oils mentioned above. On the other hand, MCT and SFO provided emulsions with lower stability. Our team delved into this matter in prior research, where we explored the underlying cause, the lower stability caused by the incorporation of MCT or SFO compared to the EVOO. The findings of this research revealed that during the formulation of oil-water mixtures (mixtures lacking the addition of particles), all types of oils could form emulsions. Notably, the "control emulsions" originating from EVOO exhibited a higher level of stability indicating that the EVOO itself probably has emulsification properties [40]. The explanation of this can be found in EVOO's composition, since it contains a mixture of triglycerides of several fatty acids and small quantities of other components that display surface active abilities [52–55]. Nevertheless, to ensure that the particles grant long term stabilization, meaning that these emulsions belong to the class of Pickering emulsions, they were observed over a period of time longer than the destabilization time of the control emulsions. Hence, all systems

that were used to encapsulate the bioactive compounds and that were further analyzed, had 0.3% w/v particle concentration and 60:40 water:EVOO ratio.

### 3.3.2. Droplet size determination by Static Light Scattering (SLS)

In Table 2, the droplet size of empty and loaded emulsions with  $\alpha$ -tocopherol or squalene is presented. The samples polydispersity P ranges from 0.46 to 0.63, indicating polydisperse samples that remain nonetheless monomodal with narrower drop size distribution widths for the loaded emulsions. The polydisperse character of the emulsions can be explained by the fact that the used particles are themselves not monodispersed. Particles size has been proven to affect the emulsions droplet size distribution [56] of the samples. The emulsion difference in diameter is not significant considering the polydispersity of the samples. Another useful conclusion that could be drawn from the measurements is that the encapsulation of both  $\alpha$ -tocopherol and squalene does not have any effect on the mean droplet size. Previous studies have also reported that no size differences were observed after the encapsulation of bioactive compounds [57]. This indicates that the two molecules are likely encapsulated in the core of the drops and do not perturb the interface. Finally, Fig. 3 shows the droplet size distribution of the samples which gives a direct view of the wide range of size population explaining the P values presented in Table 2.

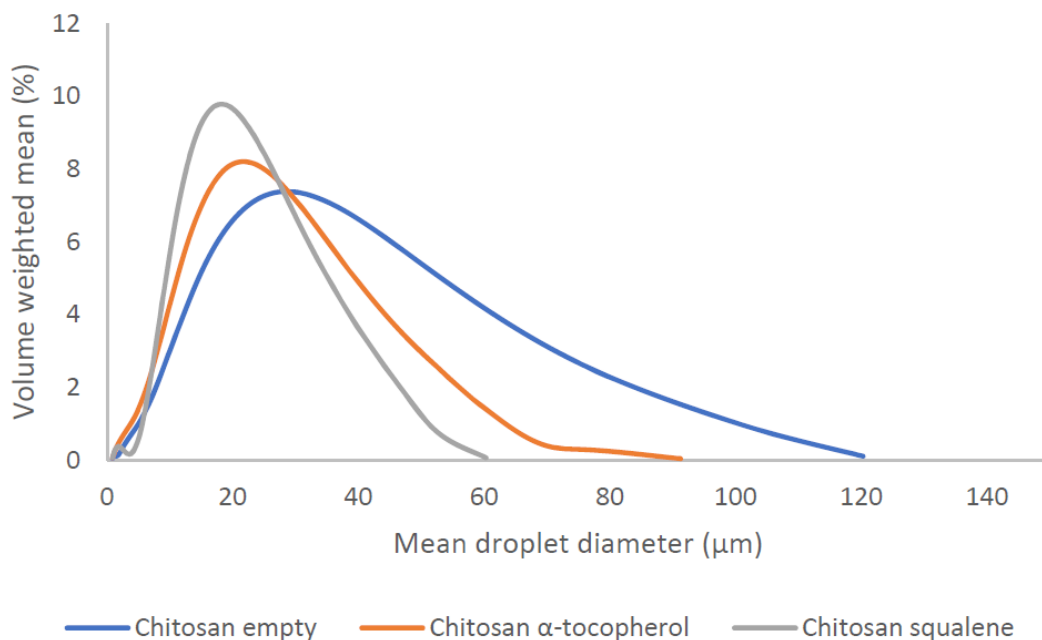


Figure 3: % Volume weighted mean diameter of chitosan emulsions droplets for empty emulsions, loaded with  $\alpha$ -tocopherol or loaded with squalene emulsions

Table 1: Determination of the emulsions' droplet diameter and polydispersity P by Static Light Scattering. Each value in the Table is represented as mean  $\pm$  SD (n = 3).

Sample	d ( $\mu\text{m}$ )	P
Chitosan empty	75.2 $\pm$ 4.2	0.63 $\pm$ 0.02
Chitosan $\alpha$ - tocopherol	61.6 $\pm$ 4.6	0.57 $\pm$ 0.03
Chitosan squalene	71.8 $\pm$ 2.7	0.46 $\pm$ 0.01

### 3.3.3. Droplets Morphology Observation by Cryogenic Scanning Electron Microscopy (Cryo-SEM), Confocal Scanning Laser Microscopy (CSLM) and Microscopic Observation

The emulsions' droplets were observed by Cryo-SEM, by optical microscope and with CLSM. The images obtained from the microscope showed spherical droplets with various sizes, which supports the polydispersity values obtained from the SLS measurements (Fig. 4a). The images are supported by previous publications that use conventional chitosan particles for emulsions stabilization [58]. Those smaller droplets during the SLS measurements were probably "hidden" from the dominant larger ones. When droplet size was measured manually from the microscopy images, the resulting size was slightly smaller than that obtained with the Mastersizer. This is probably due to how size was calculated since when measured manually the size was calculated as the sum of the sizes divided by the number of drops, while the Mastersizer gives the volume weighted mean of the drops' size. On the other hand, Cryo-SEM analysis was challenging to perform due to the thermal sensitivity of the samples which wouldn't allow a bigger zoom in to take place. Nevertheless, some concrete results can be collected, such as the spherical shape of the particles as well as of the droplets. Also, a clear image of a droplet covered by chitosan particles can be seen in Fig. 4b. Our observations are therefore in accordance with the images on similar polysaccharide-based stabilized emulsions reported in literature [59]. The images obtained from CLSM (Fig. 4c) shows the oil inside the droplets stained with Nile red. This image confirms the fact that we have an O/W emulsion and that all the oil is in fact incorporated successfully inside the droplets since there are no areas of red color in a non-spherical shape. Similar results have been provided by previous research on chitosan stabilized emulsions [60]. Furthermore, the fact that the red color is apparent only inside the droplets and there is none scattered outside of them, proves that both squalene and  $\alpha$ -tocopherol (oil soluble) are fully encapsulated, since they are also stained red due to their lipophilic nature. Finally, the "ring" of chitosan around the droplets is visible in Fig. 4d with green color after chitosan's staining with FITC. FITC has been used in numerous other studies for the depiction of chitosan with CLSM [61].

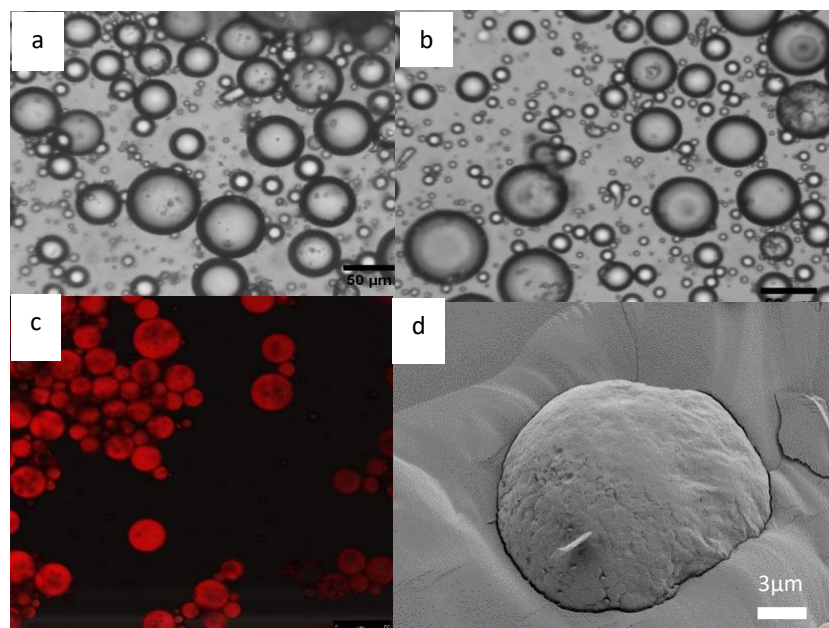


Figure 4: (a) brightfield image x10 magnification of chitosan empty emulsion with optical microscope (b) Cryo-SEM image of chitosan emulsion, (c) oil droplet confocal image x20 magnification of chitosan empty emulsion and (d) chitosan “ring” confocal image x20 magnification of chitosan empty emulsion.

### 3.4. Assessment of the emulsions antioxidant capacity by DPPH free radical scavenging

Chitosan-stabilized emulsions can encapsulate and deliver various antioxidant compounds, such as natural extracts, vitamins, or synthetic antioxidants. This encapsulation offers protection to the antioxidants, prevents their degradation, and enables controlled release, enhancing their overall antioxidant activity [62]. In this section we compare the antioxidant ability of chitosan and chitosan stabilized emulsions with and without encapsulated molecules. The antioxidant capacity of the samples was measured spectrophotometrically, with the DPPH assay over time to observe the reaction between the free radicals and the emulsions. Table 3 includes the % scavenging of the DPPH free radical 30 min after the addition of the sample in the radical’s solution. A graph of the results is also presented in Fig. 5, showing as does Table 3, the % scavenging ability of the emulsions against DPPH free radical. The 30-minute reaction is enough time for the inhibition kinetics to reach a plateau, so that the scavenging of each sample would be the maximum. It should be noted that a time related study of the antioxidant capacity was attempted but due to the nature of the sample (creamy) the DPPH-emulsion solution was not clear, so the spectrophotometer could not measure it. Thus, we resorted in measuring the absorbance after 30 min and after the DPPH-



emulsion solution was filtered to remove any obscurities. The aqueous solution of chitosan does not exhibit a significant antioxidant ability. Previous studies have shown that chitosan itself does not possess significant antioxidant properties [63]. However, it can potentially enhance the antioxidant capacity of certain food systems by acting as a carrier for antioxidants or by interacting with other antioxidant compounds. These forms of grafted chitosan are water soluble and less viscous than native chitosan is allowing better dispersion and better approach for aqueous soluble targets. Furthermore the activity of the grafted substances is not decreased with this kind of modification [64]. The results obtained show that all emulsions have a scavenging effect against DPPH, suggesting that they can be characterized as antioxidants, at least for this kind of antioxidant mechanism. The empty ones exhibit the lowest antioxidant effect compared to the loaded ones, but much higher than the chitosan solution. This can be inferentially attributed to the presence of EVOO. EVOO's antioxidant capacity is well-known and widely studied [57], while for chitosan, antioxidant capacity has been reported when it is previously hydrolyzed [65]. Emulsions loaded with squalene or  $\alpha$ -tocopherol exhibit higher antioxidant ability than the empty one. Similar results in terms of antioxidant activity have been obtained by our group in previous research. In that case bioactives were incorporated in emulsions stabilized by plant proteins. Proteins did not add to the emulsions' antioxidant ability and  $\alpha$ -tocopherol appeared to be more drastic than squalene. In that case empty emulsions' inhibition against DPPH ranged from 25 to 39% while for  $\alpha$ -tocopherol loaded ones inhibition reached 86% and squalene loaded ones showed inhibition that reached 62%. [40]. If we investigate the structure of each molecule, we can have a first explanation on the potency difference between them. The antioxidant ability of a certain substance against free radicals lies firstly upon the number and position of hydroxyphenyl groups. This fact already gives  $\alpha$ -tocopherol a precedence over squalene. The O/W type of the emulsion also can affect the activity of the encapsulated substances because even though they are both oil soluble the hydroxyphenyl group of  $\alpha$ -tocopherol could orient it closer to the interphase allowing it to interact more easily with the aqueous medium [66], [67]. Finally, studies have shown that over time, when  $\alpha$ -tocopherol is dispersed in an oil medium with squalene, then  $\alpha$ -tocopherol degrades with a quicker rate acting protectively over squalene [68], [69]. In Table 4 there are listed publications and their results about similar systems' or compounds' antioxidant activity against DPPH free radical.

Table 3: % Scavenging ability of empty chitosan emulsions and their respective loaded ones with  $\alpha$ -tocopherol or squalene. Each value in the Table is represented as mean  $\pm$  SD (n = 3).

% DPPH Scavenging			
Chitosan 0.3% w/w	Chitosan empty	Chitosan $\alpha$ -tocopherol	Chitosan squalene
5.3 $\pm$ 0.1	37.3 $\pm$ 0.7	86.1 $\pm$ 0.1	62.0 $\pm$ 01

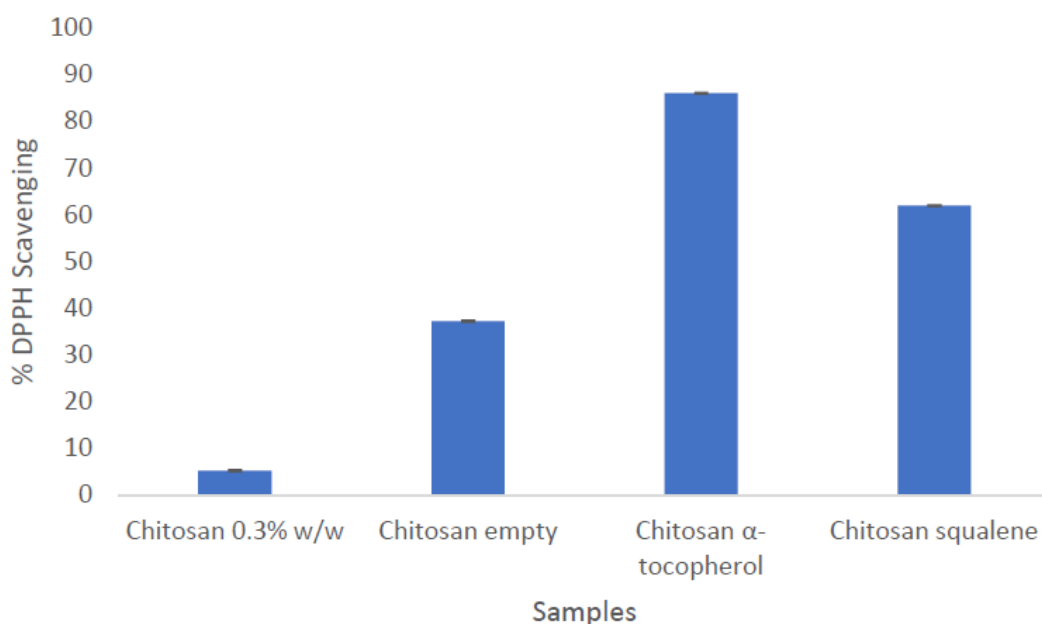


Figure 5: % Scavenging ability of empty chitosan emulsions and their respective loaded ones with  $\alpha$ -tocopherol or squalene.

### 3.5. Assessment of the emulsions' antimicrobial activity

Chitosan's antimicrobial activity has been previously assessed. The main focus was on chitosan's molecular weight, origin and pH, and on how those factors affect the antimicrobial ability [70–72]. In this study we assess the added value of chitosan as an antimicrobial agent, when at the same time it is used as an emulsion stabilizer and carrier of important bioactive compounds such as  $\alpha$ -tocopherol and squalene. For this purpose, *E. coli* C1845 and *S. aureus* DSM 21705 were used as targets using two different initial bacterial suspensions corresponding to approximately 3 and 6 log cfu/mL. Two emulsion: bacterial suspension mixing ratios were tested, 1:1 and 1:10. The surviving bacterial cells were enumerated after 4 and 24 h of incubation with the emulsions. The data presented in Table 5 show that when the emulsions were mixed at 1:1 ratio with the bacterial suspensions they exerted high antimicrobial activity against both pathogens tested. More specifically, microbial counts were only 0–3 log cfu/mL after the first 4 h of incubation for both *E. coli* and *S. aureus* strains when starting with initial population of approximately 2.7– 3 log cfu/mL depending on the mixing ratio (1:1 and 1:10, respectively). Moreover, microbial counts were only 1.6– 5.6 log cfu/mL, starting with initial population of approximately 5.7– 6 log cfu/mL depending on the mixing ratio emulsion:bacterial suspension (1:1 and 1:10, respectively). When bacterial suspensions and emulsions were mixed at 1:10 ratio different results were obtained depending on target strain. Interestingly, the 1:10 ratio had lower effect on *E. coli* strain, reducing its population by 1–2 log cfu/mL. On the other hand, the same emulsion had a much greater effect

on *S. aureus*, decreasing its microbial counts by 2–4 log cfu/mL. Additionally, it was observed that between 4 and 24 h of mixtures' incubation, no differences were observed, among the same emulsion:bacterial suspension for both targets indicating that the action of chitosan does not continue to progress after achieving the highest possible restraint on microbial growth. Furthermore, among the three different types of emulsions tested (empty, loaded with  $\alpha$ -tocopherol and loaded with squalene), no statistically significant differences were found regarding the same target strain. The reason behind the emulsions' lesser antimicrobial effect over *E. coli* could be attributed to the molecular mass (MM) of the used chitosan, which was approximately 50–100 kDa. It has been previously reported that decreased chitosan's MM is correlated with lower antimicrobial ability against *E. coli* [73]. The results presented in this study are also supported by findings that showed that lower concentrations of chitosan were more effective against *S. aureus* rather than *E. coli* [74]. Finally, studies have been previously published showing that chitosan in general is more effective against Gram positive bacteria rather than Gram negative ones [75–77].

Table 5 Microbial counts of Surviving cells of *E. coli* and *S. aureus* after 4 and 24 h incubation at 37 oC with emulsions empty, loaded with a-tocopherol and loaded with squalene. Bacterial suspensions and emulsions were mixed at ratios 1:1 and 1:10 (emulsion: bacterial suspension).

Emulsion:bacteria suspension	<i>E. coli</i> (3 log cfu/mL)				<i>E. coli</i> (6 log cfu/mL)			
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
Incubation time	4 h		24 h		4 h		24 h	
Empty	1.3	2.3	1.8	2.6	3	5.5	0	4.9
Loaded with $\alpha$ -tocopherol	0	2.8	0	2.3	2	5.5	0	4.8
Loaded with Squalene	1	2.3	1	2.3	1	5.6	1	5.3
	<i>S. aureus</i> (3 log cfu/mL)				<i>S. aureus</i> (6 log cfu/mL)			
Emulsion:bacteria suspension	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
Incubation time	4 h		24 h		4 h		24 h	
Empty	0	1.9	0	1.9	0	3.3	0	2.9
Loaded with $\alpha$ -tocopherol	1	1.8	1	1	1.5	4.0	1.3	3.9
Loaded with Squalene	1	1.6	0	0.6	1	3.6	1	3.3

### 3.6. A comparison between fungal and crustaceans derived chitosan

Chitosan stabilized emulsions fall under the more general category of particle stabilized or in other words Pickering emulsions. This type of emulsion present certain advantages over the

more classic approaches like nanoemulsions. Those advantages are mainly, the absence of emulsifiers, leading to products with lower toxicity, the increased stability, due to the irreversible adsorption of the particles on the water-oil interphase and the fact that one has the ability to choose as stabilizers compounds that are able to endow systems with desirable characteristics. The main characteristics that make chitosan so appealing is the fact that it is easily tunable in terms of solubility and structure, with a simple pH shifting. At the same time, it has excellent antimicrobial activities rendering the use of conservatives unnecessary or at the very least it can decrease the needed amount, thus expanding the final product's shelf-life naturally contributing to better sustainability and improved health outcomes. Chitosan has often been used as an emulsifier throughout the years. The most used chitosan is the one derived from crustaceans and in combination with other compounds such as silica, lecithin, surfactants and more [78–80]. Studies about the formulation of emulsions stabilized solely by chitosan have reported a very big range of droplet sizes (10–200  $\mu\text{m}$ ), depending on each chitosan's individual characteristics, such as molecular weight, viscosity, and pH. In this study, we have used two chitosans in the same range of viscosity and molecular weight and at the same pH so that the comparison would be accurate. Emulsions stabilized by fungi-derived chitosan were prepared and measured again so that we could have the exact same conditions as for the crab-derived chitosan. Results showed that, emulsions stabilized by fungal chitosan have a significantly smaller droplet size of  $78.9 \pm 1.2 \mu\text{m}$ , while crab-derived chitosan gave emulsions with larger droplets of  $157.3 \pm 2.6 \mu\text{m}$ . Furthermore, it can be observed in Fig. 6 that crab chitosan emulsions have a much wider diameter distribution in comparison to the much narrower size distribution of fungal chitosan emulsions. The uniformity values for the fungal and crab chitosan emulsions are 0.47 and 0.65 respectively, mirroring the image we see in Fig. 6. Finally, the size of the particles from crustacean-derived chitosan forms was measured with DLS. Results showed very large particles of different size populations ( $240.2 \text{ nm} \pm 10.2$ ,  $830 \text{ nm} \pm 3.7$ ) accompanied by a rather high PDI value of  $0.98 \pm 0.26$ . This is one more indication why crustacean-derived chitosan forms emulsions with large droplets and low uniformity. The results corroborate with previously published work with crustacean-derived chitosan in terms of particles and droplet size [81]. In Fig. 7 are presented the changes in the dynamic interfacial tension ( $\gamma$ ) between oil and water in the presence of the two chitosans. It is evident that both polymers rapidly decrease  $\gamma$  values with fungal chitosan having a small lead. The final values recorded after allowing the systems to reach an equilibrium were  $9.78 \pm 0.01$  for crab-derived chitosan and  $8.98 \pm 0.02$  for fungal chitosan. This means that fungal chitosan is adsorbed better at the oil-water interface giving smaller droplets and probably more stable emulsions. A similar decrease in the dynamic interfacial tension has been previously reported and supports the results of the present study [82].

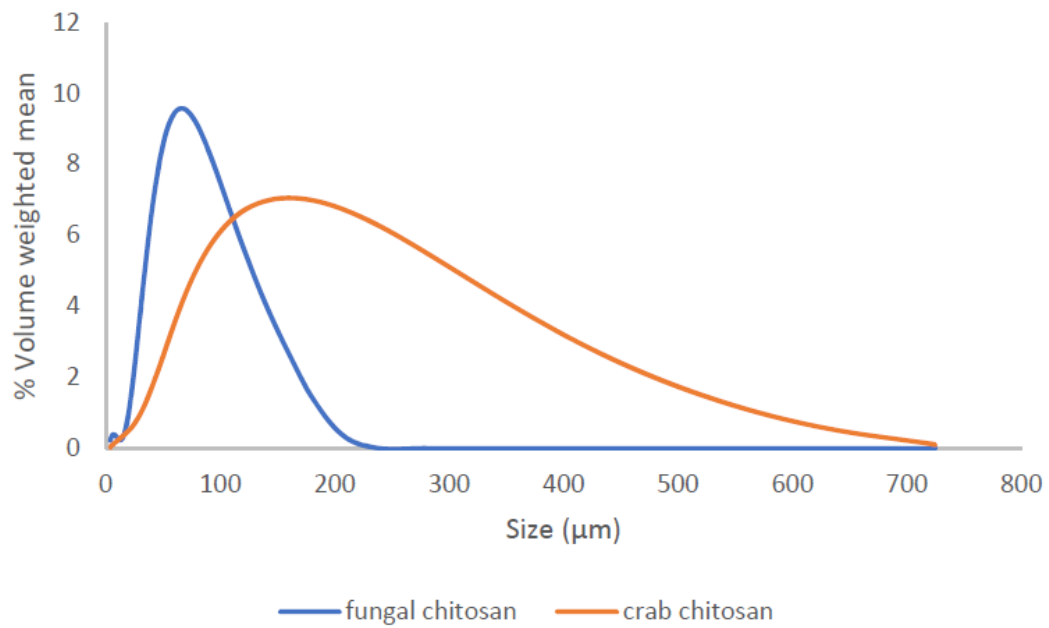


Figure 6: % Volume weighted mean droplet diameter of emulsions stabilized by mushroom or crab chitosan.

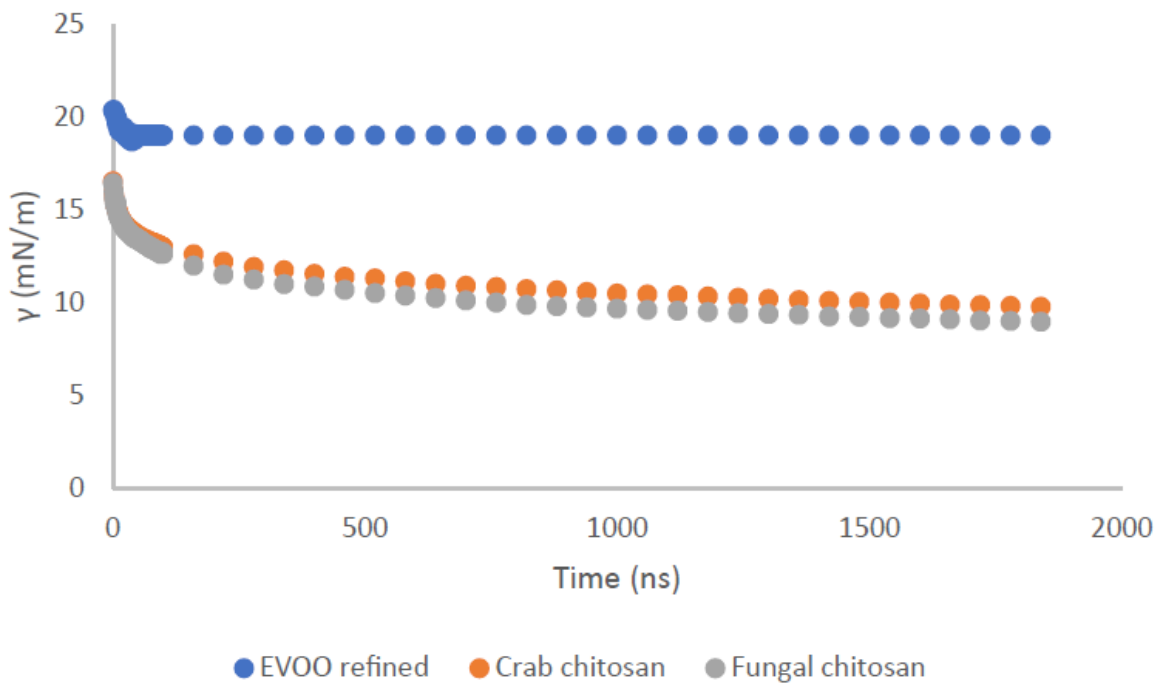


Figure 7: Dynamic interfacial tension  $\gamma$  of fungal and crab derived chitosan with refined olive oil as a control.

#### 4. Conclusions

In conclusion, this study investigated the preparation, structure, and stability of emulsions stabilized by chitosan particles with potential antioxidant and antimicrobial properties. The chitosan particles, derived from mushrooms, displayed spherical morphology. The obtained particle size at pH 6.5 is of the order of 210 nm with a certain polydispersity. They were successfully used to stabilize emulsions and to formulate so-called Pickering emulsions. The surface-average diameter and droplet size distribution of the emulsions was determined by static light scattering, and microscopy observations confirmed the spherical shape of the droplets. The emulsions exhibited stability against creaming and coalescence, with oil fractions above 40% and particles' concentration above 0.3% showing enhanced stability. The addition of bioactive compounds, such as  $\alpha$ -tocopherol and squalene, did not significantly affect the droplet size or stability of the emulsions showing the robustness of the formulation and process. Furthermore, the emulsions demonstrated antioxidant activity, with emulsions loaded with  $\alpha$ -tocopherol and squalene exhibiting higher scavenging ability compared to non-loaded emulsions. The antimicrobial activity of the emulsions against two foodborne pathogens, *E. coli* and *S. aureus*, was evaluated in this study. It was found that the emulsions do in fact present antimicrobial ability in a concentration dependent manner and in a greater degree against the Gram-positive *S. aureus*. Overall, these findings suggest that chitosan-stabilized emulsions have potential applications in the food industry as sustainable and healthier alternatives. They also give an alternative to crustacean-derived chitosan leading to plant-based and vegan friendly products, which could include a group of consumers that would otherwise be excluded. Furthermore, FC's lack in certain animal proteins gives people with allergies an alternative path towards food products that they would be otherwise not possible to consume. These novel systems could not only act as carriers for oil soluble nutrients in aqueous-based foods but also contribute to the foods increased shelf-life through their antimicrobial properties. Further research would be interesting to explore and evaluate the performance of these emulsions in various food formulations in specific applications. Foods that could be potential candidates for incorporating these emulsions would be plant-based creams/desserts or plant-based drinks, as well as meat alternatives. A very important aspect of fungal chitosan that should be highlighted is that it is free of allergens normally present in crustaceans-derived chitosan, thus opening a new market space for people that cannot consume such products, not by choice but because of certain health issues.

**CRedit authorship contribution statement** Galani Eleni: Investigation, Methodology, Writing – original draft. Xenakis Aristotelis: Data curation, Funding acquisition, Supervision, Validation, Writing – review & editing. Chatzidaki Maria D.: Conceptualization, Data curation, Supervision, Validation, Writing – review & editing. Tsakalidou Effie: Data curation, Supervision, Validation, Writing – review & editing. Schmitt Veronique: Data curation, Supervision, Writing – review &

editing. Laurichesse Eric: Investigation, Methodology. Zoumpopoulou Georgia: Data curation, Investigation, Methodology, Writing – review & editing. Ly Isabelle: Investigation, Methodology.

**Declaration of Competing Interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Data Availability Data will be made available on request.

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## GRAPHICAL ABSTRACT

