ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
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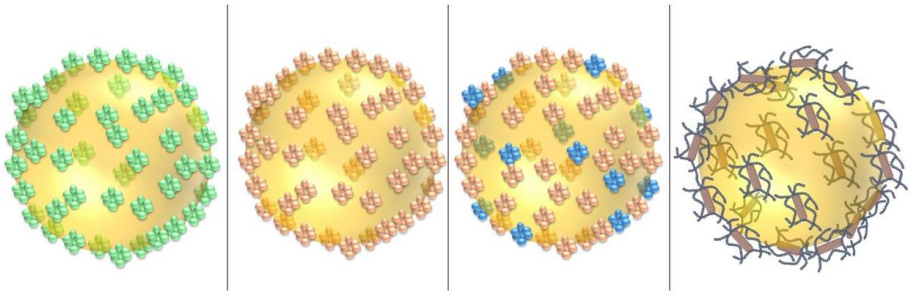
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF DAIRY RESEARCH

Doctoral Thesis

Incorporation of natural bioactive components into matrices to enhance food functionality and shelf-life



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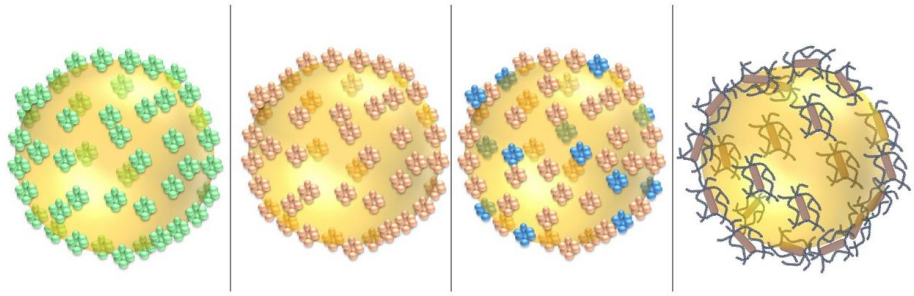
Ενσωμάτωση φυσικών βιοδραστικών ουσιών σε μήτρες για τη βελτίωση της λειτουργικότητας και του χρόνου ζωής των τροφίμων



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Incorporation of natural bioactive components into matrices to enhance food functionality and shelf-life

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Abstract

The past decades, the scientific community has been leading the food industry to take long strides into providing new or improved products for consumers and solve issues concerning food safety, promote sustainability and result in functional products that promote consumers' well-being beyond basic nutrition. Consumers' demands have grown since they are more informed and alert about their health and how to promote it. Colloids have been widely used in foods to improve their texture, encapsulate and deliver bioactive compounds and improve the solubility of, mainly, oil soluble compounds in aqueous media.

The main objective of the present dissertation was the preparation of edible, and functional Pickering emulsions (PEs), stabilized by particles of natural origin without the aid of any synthetic or chemical emulsifiers, such as surfactants. The role of those PEs was to carry and deliver oil soluble biologically active ingredients in aqueous based food matrices. The emulsifiers used were mainly of plant origin and they were carefully chosen so that apart from their vegan-friendly approach, they would also enrich the final products with desirable characteristics. Pickering emulsions were used as an inspiration for the systems presented in this project because of their lack of surfactants, their increased stability against coalescence and their higher oil content, which can increase the encapsulation degree. As ingredients for the preparation of colloidal particles, Pea Protein Isolate (PPI), Soy Protein Isolate (SPI), phycocyanin (PC), fungal chitosan (FC), as well as three probiotic lactic acid bacteria strains namely, *Lactobacillus fermentum* ACA-DC 179 (*Lb. fermentum* ACA-DC 179), *Lactobacillus delbrueckii* subsp. *bulgaricus* ACA-DC 87 (*Lb. bulgaricus* ACA-DC 87), and *Streptococcus thermophilus* ACA-DC 26 (*S. thermophilus* ACA-DC 26) were used. PC was extracted from *Spirulina platensis,* and it was used alongside PPI, as a co-emulsifier, natural color agent, and antioxidant molecule. The bacteria belong to the ACA-DC collection of the Laboratory of Dairy Research of the Agricultural University of Athens. The two most used edible oils in Greece, namely Extra Virgin Olive Oil (EVOO) and Sunflower Oil (SFO) were employed as the inner oil phase. As model ingredients for the encapsulation, a-tocopherol and squalene were tested.

Colloidal particles of spherical shape were successfully formulated through the pH-shifting method for FC, PPI, and SPI. Ultrasonication or High-Pressure Homogenization (HPH) were used to

optimize the size of the produced particles for better stability and homogeneity of the final product. Furthermore, for the preparation of the emulsions High-Shear Homogenization (HSH) alongside HPH, in the cases that was necessary, were employed to improve droplet size homogeneity, stability and protein adsorption at the water-oil interface.

Both the particles used, and the resulting emulsions were fully characterized. For the PPI, SPI, and FC particles, Dynamic Light Scattering (DLS) was used for their size determination and surface charge by ζ-potential, while Freeze Fracture Transmission Electron Microscopy (FFTEM) was employed to depict their morphology. For the emulsions characterization, Static Light Scattering (SLS) was used to determine their droplet size, optical microscopy, Confocal Laser Scanning Microscopy (CLSM) and Cryogenic Scanning Electron Microscopy (Cryo-SEM) were used to structurally characterize them in terms of morphology and ingredient distribution. The resulting particles were of spherical shape and a wide size range, leading to polydisperse emulsions. Nevertheless, the emulsions were able to incorporate oil up to 30% w/w but remained stable for 15 days. PPI emulsions proved to be more stable, so PPI was chosen for further experimentation.

After applying the pH-shifting technique, PPI particles were also treated with high pressure leading to smaller and monodispersed particles that increased stability of the emulsions formed. For the formation of the emulsions HSH was used alongside HPH. Oil could be fully incorporated up to 50% w/w and the droplet size decreased significantly. Thus, PC was introduced in those systems as a co-emulsifier, natural coloring agent, and antioxidant molecule. The resulting emulsions were tested macroscopically for destabilization, and microscopically to observe the oil droplets and protein rings around them using CLSM. Pendant drop tensiometry was employed to determine the effect of the proteins/particles on their own and when combined with each other. ζ-potential measurements were used as a means to examine the stability of the particles/proteins to predict the stability of the resulting emulsions. The combination of PC and PPI formed stable emulsions of blue color that were able to encapsulate the model bioactive ingredients and have antioxidant properties of their own. The antioxidant activity of the emulsions was tested by the DPPH colorimetric assay.

Another interesting ingredient used in the present PhD as a particle for the stabilization of Pickering emulsion was the polymer FC. FC was able to form spherical particles in the nanoscale by pH-shifting and ultrasonication. As mentioned above, FFTEM was used for the depiction of the particles and ζ-potential measurements to measure their surface charge in different concentrations that would give an idea for their stability, hence the overall stability of the formulated emulsions. Emulsions formed successfully incorporated up to 50% w/w of oil and were stable for more than 60 days. Apart from

the structural and antioxidant characteristics of the formulated Pickering emulsions, their antimicrobial activity was also tested. Two pathogenic bacteria strains were used as targets, namely, *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). It was proven that the emulsions were effective on both pathogens in two different offered doses namely, 10 and 50% v/v.

In order to further functionalize the FC emulsions a novel kind of particle was formulated, a conjugate of probiotic lactic acid bacteria and FC For this reason, the three bacteria strains mentioned above (*L. fermentum* ACA-DC 179, *L. bulgaricus* ACA-DC 87, and *S. thermophilus* ACA-DC 26) were tested for this purpose. FC was used as the suspension medium for the bacteria and the final solution as the aqueous phase of the emulsions. The bacteria when suspended in FC solution formed bacteria-fungal chitosan conjugates (BFCs). For the conjugates preparation, no external force was applied other than the mixing resulting to their electrochemical interaction because of their opposite charge. After a series of tests including, emulsion preparation (through HSH), emulsion stability, cell viability, *L. fermentum* ACA-DC 179 was chosen as the most promising strain for further assessment. Initially, the interaction between FC and *L. fermentum* ACA-DC 179 was measured while they were in solutions on their own and in the form of BFCs by ζ-potential. They were also visualized by CLSM and so were the emulsion droplets. Additionally, the antimicrobial activity of the formulated Pickering emulsions was tested as previously described. Finally, since *L. fermentum* ACA-DC 179 is a microorganism with proven probiotic activities, it was examined whether it maintains this ability as conjugate and adsorbed at an oil-water interface. For this, Enzyme-Linked Immunosorbent Assays (ELISAs) and Untargeted Metabolomics were applied. It was found that not only these novel conjugates produce emulsions that remain stable for over 60 days but also have dual functionality as antimicrobial and anti-inflammatory agents. The proposed systems are novel and offer plenty of opportunities for the preparation of various carries for bioactives that at the same time have their own functional properties.

Scientific area: Food Chemistry

Keywords: Pickering emulsions, encapsulation, functional foods, plant proteins, fungal chitosan, probiotics

Ενσωμάτωση φυσικών βιοδραστικών ουσιών σε μήτρες για την βελτίωση της λειτουργικότητας και του χρόνου ζωής των τροφίμων

Τμήμα Επιστήμης Τροφίμων & Διατροφής του Ανθρώπου
Εργαστήριο Γαλακτοκομίας

Περίληψη

Τα τελευταία χρόνια, η επιστημονική κοινότητα έχει βοηθήσει τη βιομηχανία τροφίμων να κάνει σημαντικά βήματα στην παροχή νέων ή βελτιωμένων προϊόντων για τους καταναλωτές και να επιλύσει προβλήματα που αφορούν την ασφάλεια των τροφίμων, να προάγει τη βιωσιμότητα και να έχει ως αποτέλεσμα λειτουργικά προϊόντα που προάγουν την ευημερία των καταναλωτών πέρα από τη βασική διατροφή. Οι απαιτήσεις των καταναλωτών έχουν αυξηθεί καθώς είναι πιο ενημερωμένοι και προσεκτικοί σχετικά με την υγεία τους και πώς να την προάγουν. Τα κολλοειδή έχουν χρησιμοποιηθεί ευρέως στα τρόφιμα για να βελτιώσουν την υφή τους, να εγκλωβίσουν βιοδραστικές ενώσεις και να βελτιώσουν τη διαλυτότητα, κυρίως, των λιποδιαλυτών ενώσεων σε υδατικά μέσα. Ο κύριος στόχος της παρούσας διατριβής ήταν η παρασκευή βρώσιμων και λειτουργικών Pickering γαλακτωμάτων (PEs), σταθεροποιημένων από σωματίδια φυσικής προέλευσης χωρίς τη βοήθεια οποιωνδήποτε συνθετικών ή χημικών γαλακτωματοποιητών, όπως οι επιφανειοδραστικές ουσίες. Ο ρόλος αυτών των PE ήταν να μεταφέρουν βιολογικά ενεργά συστατικά διαλυτά σε έλαιο, σε υδατικές βάσεις τροφίμων. Οι γαλακτωματοποιητές που χρησιμοποιήθηκαν ήταν κυρίως φυτικής προέλευσης και επιλέχθηκαν προσεκτικά, ώστε εκτός από την προσέγγιση φιλική προς τους vegans, να εμπλουτίζουν και τα τελικά προϊόντα με επιθυμητά χαρακτηριστικά. Τα γαλακτώματα Pickering χρησιμοποιήθηκαν ως έμπνευση για τα συστήματα που παρουσιάζονται σε αυτό το έργο λόγω της έλλειψης επιφανειοδραστικών, της αυξημένης σταθερότητάς τους και της υψηλότερης περιεκτικότητας σε έλαιο, που μπορεί να αυξήσει την συγκέντρωση των εγκλωβισμένων βιοδραστικών. Ως συστατικά για την παρασκευή κολλοειδών σωματιδίων χρησιμοποιήθηκαν απομονωμένη πρωτεΐνη αρακά (PPI), απομονωμένη πρωτεΐνη σόγιας (SPI), φυκοκυανίνη (PC), μυκητιακή χιτοζάνη (FC), καθώς και τρία προβιοτικά στελέχη βακτηρίων γαλακτικού οξέος, συγκεκριμένα το *Lactobacillus fermentum* ACA-DC 179 (*Lb. fermentum* ACA-DC 179), το *Lactobacillus delbrueckii* subsp. *bulgaricus* ACA-DC 87 (*Lb. bulgaricus* ACA-DC 87) και το *Streptococcus thermophilus* ACA-DC 26 *(S. thermophilus* ACA-DC 26). Η φυκοκυανίνη απομονώθηκε από *Spirulina platensis* και χρησιμοποιήθηκε μαζί με την PPI ως γαλακτωματοποιητής, φυσική χρωστική ουσία και αντιοξειδωτικό μόριο. Τα βακτήρια ανήκουν στη συλλογή ACA-DC του Εργαστηρίου Γαλακτοκομίας του Γεωπονικού Πανεπιστημίου Αθηνών. Τα δύο πιο συχνά χρησιμοποιούμενα εδώδιμα έλαια στην Ελλάδα, συγκεκριμένα το εξαιρετικό παρθένο ελαιόλαδο (EVOO) και το ηλιέλαιο (SFO), χρησιμοποιήθηκαν ως η εσωτερική φάση του ελαίου. Ως εγκλωβισμένα μόρια, δοκιμάστηκαν η α-

τοκοφερόλη και το σκουαλένιο. Κολλοειδή σωματίδια σφαιρικού σχήματος σχηματίστηκαν επιτυχώς μέσω της μεθόδου αλλαγής pH για τα FC, PPI και SPI. Η υπερήχηση ή η ομογενοποίηση υψηλής πίεσης (HPH) χρησιμοποιήθηκαν για τη βελτιστοποίηση του μεγέθους των παραγόμενων σωματιδίων για καλύτερη σταθερότητα και ομοιογένεια του τελικού προϊόντος. Επιπλέον, για την παρασκευή των γαλακτωμάτων χρησιμοποιήθηκαν η Ομογενοποίηση Υψηλής Ταχύτητας Διάτμησης (HSH) μαζί με την Ομογενοποίηση Υψηλής Πίεσης (HPH), όπου ήταν απαραίτητο, για να βελτιώσουν την ομοιογένεια του μεγέθους των σταγόνων, τη σταθερότητα και την προσρόφηση πρωτεΐνης στην επιφάνεια νερού-ελαίου. Τόσο τα σωματίδια που χρησιμοποιήθηκαν όσο και τα προκύπτοντα γαλακτώματα χαρακτηρίστηκαν πλήρως. Για τα σωματίδια PPI, SPI και FC, χρησιμοποιήθηκε η τεχνική της Δυναμικής Σκέδασης Φωτός (DLS) για τον προσδιορισμό του μεγέθους τους και το ζ-δυναμικό για το επιφανειακό φορτίο, ενώ η Ηλεκτρονική Μικροσκοπία Διέλευσης (FFTEM) χρησιμοποιήθηκε για την απεικόνιση της μορφολογίας τους. Για την χαρακτηρισμό των γαλακτωμάτων, χρησιμοποιήθηκε Στατική Σκέδση Φωτός (SLS) για τον προσδιορισμό του μεγέθους των σταγόνων τους, μικροσκοπία οπτική, η Συνεστιακή Μικροσκοπία Σάρωσης με Λέιζερ (CLSM) και η Κρυογονική Ηλεκτρονική Μικροσκοπία Σάρωσης (Cryo-SEM) χρησιμοποιήθηκαν για τον δομικό τους χαρακτηρισμό σε όρους μορφολογίας. Τα προκύπτοντα σωματίδια είχαν σφαιρικό σχήμα και ευρύ φάσμα μεγέθους, οδηγώντας σε πολυδιεσπαρμένα γαλακτώματα. Παρόλα αυτά, τα γαλακτώματα ήταν σε θέση να ενσωματώσουν έλαιο μέχρι 30% w/w και παρέμειναν σταθερά για 15 ημέρες. Τα γαλακτώματα PPI αποδείχθηκαν πιο σταθερά, επομένως το PPI επιλέχθηκε για περαιτέρω πειραματισμούς. Μετά την εφαρμογή της αλλαγής pH, τα σωματίδια PPI υποβλήθηκαν επίσης σε υψηλή πίεση, οδηγώντας σε μικρότερα και μονοδιεσπαρμένα σωματίδια που αύξησαν τη σταθερότητα των σχηματισμένων γαλακτωμάτων. Για το σχηματισμό των γαλακτωμάτων, χρησιμοποιήθηκε η HSH μαζί με την HPH. Το έλαιο μπορούσε να ενσωματωθεί πλήρως μέχρι 50% w/w και το μέγεθος των σταγόνων μειώθηκε σημαντικά. Έτσι, η PC εισήχθη σε αυτά τα συστήματα ως γαλακτωματοποιητής, φυσική χρωστική και αντιοξειδωτικό μόριο. Τα προκύπτοντα γαλακτώματα ελέγχθηκαν μακροσκοπικά για αποσταθεροποίηση και μικροσκοπικά για την παρατήρηση των σταγόνων ελαίου και των πρωτεϊνικών δακτυλίων γύρω από αυτές χρησιμοποιώντας την CLSM. Η τεχνική pendant drop tensiometry χρησιμοποιήθηκε για να καθοριστεί η επίδραση των πρωτεϊνών/σωματιδίων στην επιφανειακή τάση νερού-ελαίου. Οι μετρήσεις ζ-δυναμικού χρησιμοποιήθηκαν ως μέσο για την εξέταση της σταθερότητας των σωματιδίων/πρωτεϊνών για να προβλεφθεί η σταθερότητα των προκύπτοντων γαλακτωμάτων. Ο συνδυασμός PC και PPI σχημάτισε σταθερά γαλακτώματα μπλε χρώματος που ήταν σε θέση να εγκλείσουν τα πρότυπα βιοενεργά συστατικά και είχαν τις δικές τους αντιοξειδωτικές ιδιότητες. Η αντιοξειδωτική δραστηριότητα των γαλακτωμάτων δοκιμάστηκε με τη μέθοδο DPPH. Ένα άλλο ενδιαφέρον συστατικό που χρησιμοποιήθηκε στην παρούσα διατριβή ως σωματίδιο για τη σταθεροποίηση των γαλακτωμάτων Pickering ήταν το πολυμερές FC. Το FC ήταν σε θέση να σχηματίσει σφαιρικά

σωματίδια σε νανοκλίμακα με αλλαγή pH και υπερήχηση. Όπως αναφέρθηκε παραπάνω, η FFTEM χρησιμοποιήθηκε για την απεικόνιση των σωματιδίων και οι μετρήσεις ζ-δυναμικού για τη μέτρηση του φορτίου επιφάνειας σε διαφορετικές συγκεντρώσεις που θα έδιναν μια ιδέα για τη σταθερότητά τους, άρα και για τη συνολική σταθερότητα των σχηματισμένων γαλακτωμάτων. Τα γαλακτώματα που σχηματίστηκαν ενσωμάτωσαν επιτυχώς έως και 50% w/w έλαιο και ήταν σταθερά για πάνω από 60 ημέρες. Εκτός από τα δομικά και αντιοξειδωτικά χαρακτηριστικά των σχηματισμένων γαλακτωμάτων Pickering, δοκιμάστηκε και η αντιμικροβιακή τους δράση. Χρησιμοποιήθηκαν δύο στελέχη παθογόνων βακτηρίων ως στόχοι, συγκεκριμένα το *Escherichia coli* (E. coli) και το *Staphylococcus aureus* (S. aureus). Αποδείχθηκε ότι τα γαλακτώματα ήταν αποτελεσματικά και στα δύο παθογόνα σε δύο διαφορετικές προσφερόμενες δόσεις, δηλαδή 10 και 50% v/v. Για να αυξηθεί περαιτέρω η λειτουργικότητα των γαλακτωμάτων σταθεροποιούμενων από FC, σχηματίστηκε ένα νέο είδος σωματιδίου, ένα σύμπλοκο προβιοτικών βακτηρίων γαλακτικού οξέος και FC. Για το σκοπό αυτό, δοκιμάστηκαν τα τρία στελέχη βακτηρίων που αναφέρθηκαν παραπάνω (*L. fermentum* ACA-DC 179, *L. bulgaricus* ACA-DC 87, και *S. thermophilus* ACA-DC 26). Τα βακτήρια, όταν επαναιωρούνταν σε διάλυμα FC, σχημάτισαν σύμπλοκα βακτηρίων-FC (BFCs). Για την παρασκευή των συμπλόκων, δεν προσφέρθηκε επιπλέον ενέργεια πέραν της ανάμιξης, οδηγώντας σε ηλεκτροχημική αλληλεπίδραση λόγω του αντίθετου φορτίου τους. Μετά από μια σειρά δοκιμών, συμπεριλαμβανομένων της παρασκευής γαλακτώματος (μέσω HSH), της σταθερότητας του γαλακτώματος, της βιωσιμότητας των κυττάρων, επιλέχθηκε το *L. fermentum* ACA-DC 179 ως το πιο υποσχόμενο στέλεχος για περαιτέρω αξιολόγηση. Αρχικά, η αλληλεπίδραση μεταξύ FC και *L. fermentum* ACA-DC 179 μετρήθηκε ενώ ήταν σε διαλύματα μόνα τους και με τη μορφή BFCs μέσω του ζ-δυναμικού. Επίσης, απεικονίστηκαν με την CLSM, όπως και οι σταγόνες του γαλακτώματος. Επιπλέον, η αντιμικροβιακή δραστηριότητα των σχηματισμένων γαλακτωμάτων Pickering δοκιμάστηκε όπως περιγράφηκε προηγουμένως. Τέλος, δεδομένου ότι το *L. fermentum* ACA-DC 179 είναι ένας μικροοργανισμός με αποδεδειγμένες προβιοτικές ιδιότητες, εξετάστηκε αν διατηρεί αυτή την ικανότητα ως συζευγμένο και προσροφημένο στην επιφάνεια ελαίου-νερού. Για το σκοπό αυτό, εφαρμόστηκαν ELISAs και Μεταβολομική. Βρέθηκε ότι αυτά τα νέα συμπλοκα όχι μόνο παράγουν γαλακτώματα που παραμένουν σταθερά για πάνω από 60 ημέρες αλλά έχουν και διπλή λειτουργικότητα ως αντιμικροβιακοί και αντιφλεγμονώδεις παράγοντες. Τα προτεινόμενα συστήματα είναι καινοτόμα και προσφέρουν πλήθος ευκαιριών για την παρασκευή διάφορων φορέων για βιοενεργές ουσίες ενώ ταυτόχρονα διαθέτουν την δικιά τους λειτουργικότητα.

Επιστημονική περιοχή: Χημεία Τροφίμων
Λέξεις κλειδιά: Γαλακτώματα Pickering, εγκλωβισμός, λειτουργικά τρόφιμα, φυτικές πρωτεΐνες, χιτοζάνη, προβιοτικά

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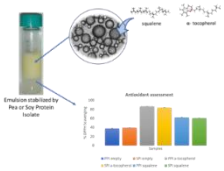
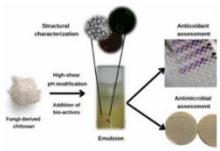
Thesis Workflow

Paper 1



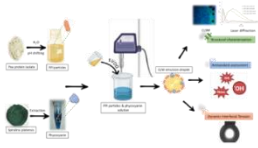
- Emulsions stabilized by PPI or SPI
- Particles' characterization (DLS, FFTEM)
- Encapsulation of bioactives
- Emulsions' structural characterization (LD, Cryo-SEM, BM)
- Emulsions' antioxidant assessment (DPPH assay)

Paper 2



- Fungal chitosan as an emulsion stabilizer
- Emulsions' structural characterization (LD, Cryo-SEM, BM, Confocal microscopy)
- Antioxidant and antimicrobial assessment (DPPH assay, KA)

Paper 3



- PPI & PC for the co-stabilization of emulsions
- Use of PC as a stabilizing, coloring & antioxidant agent
- Proteins' characterization (ζ-potential, pendant drop tensiometry)
- Emulsions' structural characterization (LD, Confocal)
- Emulsions' antioxidant assessment (DPPH assay)

Paper 4

- Preparation of multifunctional emulsions
- Probiotic bacteria-fungal chitosan conjugates
- Emulsions' structural characterization (LD, confocal microscopy, pendant drop tensiometry)
- Emulsions' anti-inflammatory assessment (ELISA)

Contents

List of abbreviations

2'-azinobis-3-ethylbenzthyazolin-6-sulfonic acid	ABTS
2,2-diphenyl-1-picryl-hydrazyl	DPPH
ABTS/Trolox equivalent antioxidant capacity	TEAC
androst-4-ene-3,17-dione	AD
androst-1,4-diene-3,17-dione	ADD
Alcaligenes faecalis	*A. faecalis*
Angiotensin I-Converting Enzyme Inhibitory	ACE-I
Atomic Force Microscopy	AFM
Bile Salt Hydrolase	BSH
Confocal Laser Scanning Microscopy	CLSM
Conjugate of probiotic bacteria and FC	BFCs
Cryogenic Scanning Electron Microscopy	Cryo-SEM
Differential Scanning Calorimetry	DSC
Dynamic Interfacial Tension	DIT
Dynamic Light Scattering	DLS
Electron Paramagnetic Resonance	EPR
Enzyme-Linked Immunosorbent Assays	ELISAs
Escherichia coli	*E. coli*
Extra Virgin Olive Oil	EVOO
Ferric Reduction of Antioxidant Power	FRAP
Folin–Ciocalteu Method	FCM

Fourier Transform Infrared Spectroscopy	FTIR
Freeze Fracture Transmission Electron Microscopy	FFTEM
Fungal Chitosan	FC
Gas Chromatography	GC
Gastrointestinal Tract	GIT
Helium-Neon	He-Ne
High Internal Phase	HIPE
High-Pressure Homogenization	HPH
High-Shear Homogenization	HSH
Hydrogen Atom Transfer	HAT
Lactic acid bacteria	LAB
Laser Diffraction	LD
Layered Double Hydroxide	LDH
Liquid Chromatography	LC
Mass Spectroscopy	MS
Mass-to-Charge Ratio	m/z
Microemulsion	ME
Minimum Bactericidal Concentration	MBC
Minimum Inhibitory Concentration	MIC
Mycobacterium neoaurum	*M. neoaurum*
N,N-dimethyl-p-phenylenediamine dihydrochloride	DMPD
Nanoemulsion	NE

Oil-in-Water	O/W
Oil-in-Water-in-Oil	O/W/O
Oxygen Radical Absorbance Capacity	ORAC
Partially Hydrogenated Oils	PHOs
Pea Protein Isolate	PPI
Phase Inversion Temperature	PIT
Photochemiluminescence	PCL
Phycobiliproteins	PBPs
Phycocyanin	PC
Pickering Emulsions	PEs
Pickering Particles	PPs
Polydispersity Index	PDI
Cupric Antioxidant Capacity	CUPRAC
Single Electron Transfer	SET
Soy Protein Isolate	SPI
Spirulina platensis	*S. platensis*
Staphylococcus aureus	*S. aureus*
Static Light Scattering	SLS
Sunflower Oil	SFO
Three-Dimensional	3D
Total Oxyradical Scavenging Capacity	TOSC
Total Peroxyl Radical Trapping Antioxidant Parameter	TRAP

Water-in-Oil	W/O
Water-in-Oil-in-Water	W/O/W

Chapter 1
Introduction and Aim of the study

Table of Contents

1. Emulsions - types and emulsification methods

1.1 Emulsions

Nanocarriers of specific compounds are numerous (Figure 1.1a) and they vary from emulsions to liposomes, dendrimers and even nanoparticle networks (Torchilin, 2007). They can find diverse applications across industries, ranging from pharmaceuticals and cosmetics to food, agriculture, and industrial manufacturing (Subhan et al., 2021; Zeb et al., 2020). They possess unique properties that make them appealing as means for targeted delivery, enhanced performance, and improved efficacy in various sectors (Oliveira et al., 2022; Torchilin, 2006).

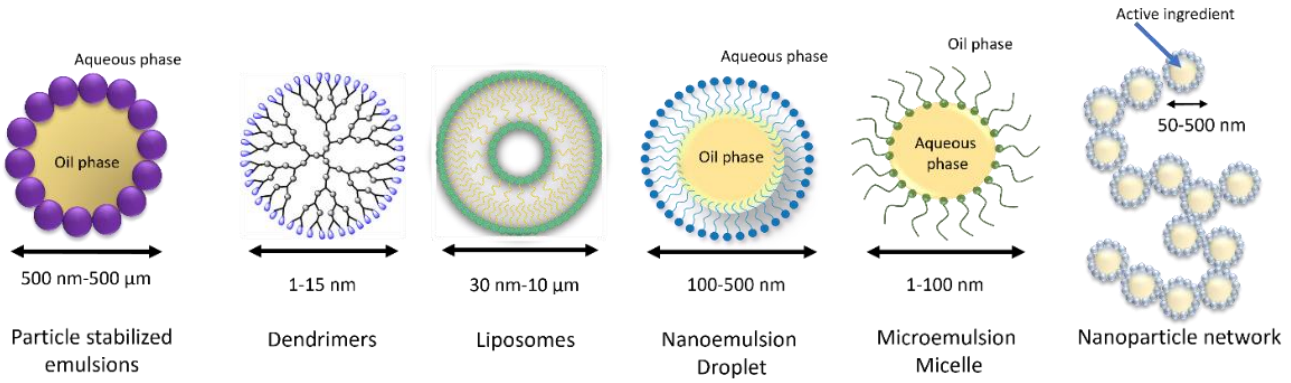


Figure 1.1a: Different types of nano-carriers.

Emulsions have been widely studied and used for many years and research efforts keep improving them while creating new ones. Emulsions are colloidal dispersions of two immiscible liquids stabilized by small molecules called surfactants, in the case of conventional emulsions, nano- (NEs) and micro-emulsions (MEs), or by solid particles, like in the case of Pickering emulsions (PEs). These two immiscible liquids are usually oil and water, or else an oil and an aqueous phase. Surfactants are amphiphilic molecules that when added to a mixture of liquids with different polarities orient themselves in a way to form droplets of one liquid into another (Pavoni et al., 2020). The resulting emulsions, depending on what are the continuous and the dispersed phase can be characterized either as water-in-oil (W/O) or as oil-in-water (O/W). Additionally, there can be multiple emulsions, namely water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O). Furthermore, depending on the size of the droplets formed (ranging from micrometers to nanometers) and the method of

formation (either spontaneous or induced by external energy), emulsions are further classified into conventional emulsions, nanoemulsions, or microemulsions (Figure 1.1b).

Besides using the right ingredients, energy is required for the emulsification process. Even microemulsions, that are spontaneously formed and thus thermodynamically stable, need the slightest energy application (McClements, 2012). When energy is required then the emulsification methods employed are divided in two general categories namely high- and low-energy methods. High-energy methods include mechanical methods, like high-pressure (HPH) and high-shear homogenization (HSH), ultrasonication, micro-fluidization and membrane emulsification, while low-energy methods are phase inversion temperature (PIT), solvent displacement, and cold emulsification methods (Jasmina et al., 2017). Understanding the types of emulsions and the method of emulsification allows for better design and application of these versatile systems. Each method has its advantages and is chosen based on the desired properties of the final emulsion, such as droplet size, stability, and the nature of the dispersed and continuous phases.

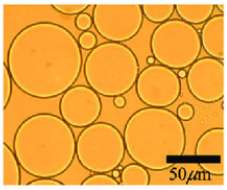
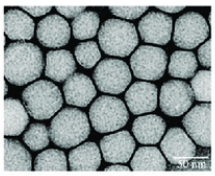
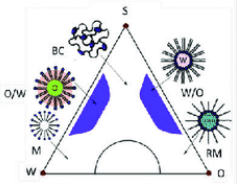
	macroemulsions	nanoemulsions	microemulsions
			
size	1-100 µm	20-500 nm	10-100 nm
shape	spherical	spherical	spherical, lamellar
stability	thermodynamically unstable, weakly kinetically stable	thermodynamically unstable, kinetically stable	thermodynamically stable
method of preparation	high & low energy methods	high & low energy methods	low energy method
polydispersity	often high (>40%)	typically low (<10-20%)	typically low (<10%)

Figure 1.1b: Macroemulsions vs Microemulsion vs Nanoemulsion (McClements, 2012)

1.2 Pickering emulsions

The concept of particle-stabilized emulsions was first introduced in the early 20th century. In 1907, S.U. Pickering, a British chemist, published a paper in which he described emulsions stabilized by solid particles in an attempt to substitute soaps, which at the time were used as emulsifiers. This discovery highlighted that finely

divided solids could be adsorbed at an oil-water interface and stabilize emulsions, preventing droplets' coalescence. Pickering's work was preceded by some earlier observations by Ramsden in 1903, who noted similar phenomena, where after mere agitation protein solutions would form structures that separated their ingredients. However, Pickering's more detailed and systematic study led to the emulsions being named after him (Pickering, 1907; Ramsden & Gotch, 1997).

PEs, as mentioned earlier, are, by definition, stabilized solely by solid colloidal particles and their size varies from a few hundred nanometers to a few micrometers depending on the size of the particles used for their stabilization, the oil volume fraction and the emulsification method (Tsabet & Fradette, 2015). Particles used for their stabilization are partially wettable from both phases and they can be adsorbed at the water-oil interface forming a steric barrier that prevents the two liquids from coming into contact while they also decrease the interfacial tension between them (Cui et al., 2023). The most simplified way to predict the required particles' concentration for the desired droplet size is depicted in Equation 1. In Equation 1, U is the velocity, η_c the viscosity of the continuous phase, σ_{12} the interfacial tension between the two phases, ε• the extension rate, and R the maximum size of the formed stable drops (Wu & Ma, 2016).

$$C_a = \frac{U\eta_c}{\sigma_{12}} = (\dot{\varepsilon} * R\eta_c)/\sigma_{12} \text{ (Equation 1)}$$

For the formation of PEs, one could also estimate the required energy for a particle to be attached or detached to or from the interface between the continuous and dispersed phase (also known as detachment energy, ΔE), which can be expressed as presented in Equation 2.

$$\Delta E = \gamma_{ow}\pi R_{sphere}^2(1 - |\cos\theta|)^2 \text{ (Equation 2)}$$

In Equation 2, γ_{ow} is the interfacial tension between the dispersed and continuous phase, R_{sphere} the radius of the spherical particle and θ the three-phase contact angle (Binks & Lumsdon, 2000). It should be noted that the previous Equation (2) applies only for spherical particles since there are different sizes and shapes (Figure 1.2a). The equations for disk- and rod-shaped particles are presented below as Equation 3 and 4, respectively, where R_{disk} is the radius for the disk-shaped particles and l and q represent the length and width of the rod-shaped ones (Peddireddy et al., 2016; Vis et al., 2015).

$$\Delta E = \gamma_{ow}\pi R_{disk}^2(1 - |\cos\theta|)^2 \text{ (Equation 3)}$$

$$\Delta E = \gamma_{ow} lq(1 - |\cos\theta|)^2 \text{ (Equation 4)}$$

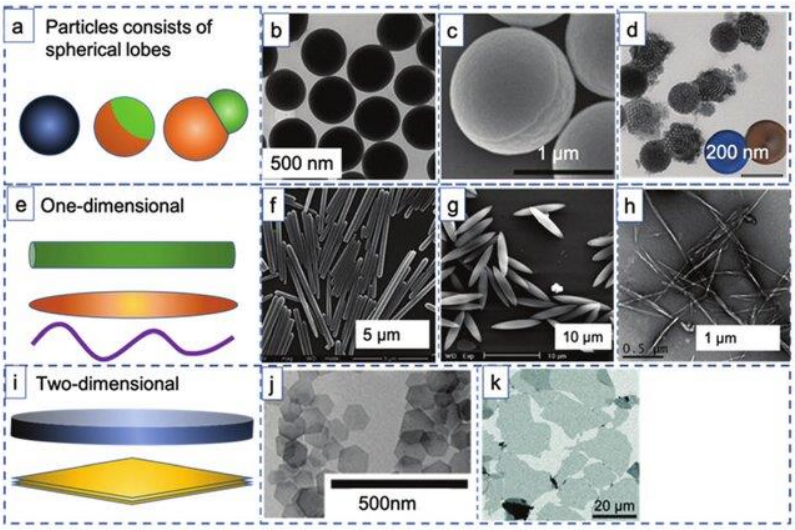


Figure 1.2a: Different sizes and shapes of particles for the stabilization of Pickering emulsions (Chang et al., 2021)

PEs present some advantages over other types of emulsions that make them much more appealing for many industries (Figure 1.2b). To start with, they exhibit increased stability that can be attributed to the robust barrier formed by the solid particles adsorbed at the water-oil interface that protects the droplets against coalescence and other destabilizing phenomena such as Ostwald ripening. The adsorption of solid particles also creates a high energy barrier for droplet coalescence meaning that the energy required to remove particles from the interface is much higher than that needed to desorb surfactants (Chevalier & Bolzinger, 2013; Melle et al., 2005). Additionally, there is a high number of ingredients to form particles that can endow systems desirable characteristics, such as conductivity, porosity, etc. Also, there is a large number of biocompatible, biodegradable and non-toxic ingredients, such as modified starch, cellulose and chitosan, that can be used and lead to safer products (Gonzalez Ortiz et al., 2020). Another attractive aspect of PEs is the large surface area they provide for the adsorption of molecules, enabling, thus, the encapsulation of compounds. At the same time, they are easily regulated by controlling the concentration and size of the particles stabilizing them (Fu et al., 2022). Finally, PEs can be reused/recycled multiple times without significant loss of their stability or efficacy, reducing the costs for certain applications and waste generation improving their environmental impact (Morais et al., 2023).

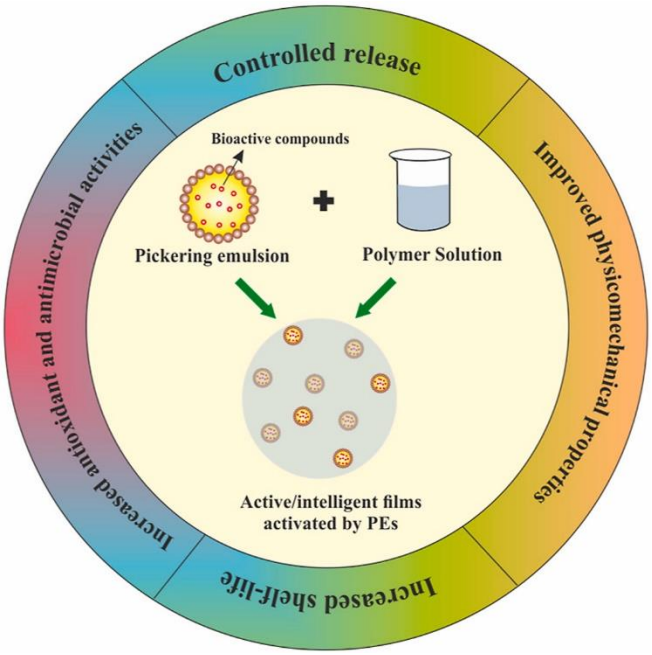


Figure 1.2b: Advantages of Pickering emulsions (Pan et al., 2023)

1.3 Functionalization and applications of Pickering emulsions

The functionalization of PEs includes the incorporation of additional ingredients, in their core or general structure, to enhance their stability, functionality and overall performance. There are several mechanisms and strategies through which such goals can be achieved. Probably the most common and widely used is the fortification of PEs through the encapsulation of active ingredients, such as vitamins, antioxidants, pharmaceutical compounds and several other ingredients. These are located either in the water or the oil phase of the emulsion depending on their nature, covered by particles that prevent their degradation. At the same time, they retain their functionality and endow the systems they are part of with their activities as antioxidants, antimicrobials or nutraceuticals especially for food applications (Cheon et al., 2023). Furthermore, solid particles can be modified to enhance their interaction with specific active ingredients, improving encapsulation efficiency and stability (Irina et al., 2023). PEs can act beyond their carrier capacity and be functional on their own with no need for encapsulating other molecules. Functional PEs can easily be formed by using as particles materials with special characteristics, such as chitosan that is known to possess antimicrobial properties (Devlieghere et al., 2004). Additionally, particles can be chosen or modified in a way that they facilitate the controlled release of any encapsulate substance or molecule so that they can be used as carriers of drugs towards specific targets (Dai et al., 2019). Finally, multiple encapsulations are possible with PEs both in the same phase and in different ones, for instance, a hydrophilic and a hydrophobic compound can be both encapsulated in double PEs (W/O/W or O/W/O) (Jiang et al., 2021; Tenorio-Garcia et al., 2022).

The functionalization of PEs does not come without challenges, such as ensuring the uniform distribution of active ingredients within the droplets, maintaining the emulsions' stability overtime and not losing ingredients' functionality upon their adsorption in the water-oil interfaces. When designing fortified PEs, one should also pay attention on the type of particles used, not only so that they are effective for the desired application, but also comply with regulatory compliments for safety and efficacy. PEs are included in the European Food Safety Authority (EFSA) under the Novel Foods Regulation for which new ingredients or novel uses of existing ones in PEs may require approval (*Novel Food | EFSA*, 2024). Finally, a great challenge that one cannot disregard is the development of scalable manufacturing processes to produce fortified PEs consistently and cost-effectively, an issue of great importance for every industry. To address these issues, research includes exploring new bioactive compounds and fortification methods to further enhance the stability and functionality of PEs, as well as further investigating their potential applications in various industrial applications.

Because of their characteristics, PEs present a certain versatility on their functions (Figure 1.3) and are used by many industries. Some of the areas that can be exploited are biomedicine, chemical and food industry, agriculture as well as oil and gas field (de Carvalho-Guimarães et al., 2022; Fu et al., 2022; Y. Yang et al., 2017). On top of that, PEs can also be designed in a way to respond to environmental stimuli like pH-shifting, temperature or even light exposure, thus controlling the release of encapsulated compounds (Tang et al., 2015). Finally, PEs when used in films and coatings offer an enhanced water vapor barrier. This can improve the overall performance and durability of those materials, where this ability is essential, making them very appealing for food packaging and protective coatings (Niro et al., 2021).

More specifically, food-grade PEs can be used in many ways by the food industry. For example, they can be used in dairy products to increase their stability by adding casein in the form of particles. Casein, a heat resistant protein can be combined with calcium or phosphorus and create nanoparticles. These particles can act as stabilizers of the milk emulsion enhancing the products' stability and at the same time act as carriers of micronutrients like calcium and phosphate (Patel, 2020). The food industry can also take advantage of PEs' ability of encapsulation and controlled release of compounds to produce functional foods while, at the same time, addressing the solubility issue for many of them. A notable example of this is the formulation of PEs not sensitive to the gastrointestinal tract (GIT) pH for the improvement of oral bio-accessibility of curcumin. These emulsions were stable in acidic conditions and they gradually de-emulsified at a neutral pH values (Li et al., 2023). The protection of the encapsulated ingredients from the external environment is also of great importance since it enhances their durability, hence, activity and bioavailability (Mwangi et al., 2020).

Obesity has risen lately as one of the most imminent health problems in developed countries and especially younger populations (*WOF, % Obesity by Country*, n.d.). The main goal for science is first to find an alternative way to process vegetable oils and make them solid without the use of hydrogenation and reduce the amount of fats used while maintaining foods' texture and mouthfeel. PEs have been found to be able to turn

liquid oils into solid-like fats avoiding partially hydrogenated oils (PHOs). This has been reported to be possible with the use of High Internal Phase Emulsions (HIPE) that consist of very dense structures creating a self-standing, viscoelastic emulsion able to mimic the solid state making them very attractive as alternatives for PHOs (Huang et al., 2019). Fat substitution is also possible while improving texture and mouthfeel. It has been reported that PEs stabilized by protein microgels were used as meat fat replacement in a food matrix produced by mechanically separated meat. Results were promising and revealed that complete substitution of meat fat with PEs not only maintained meat's texture but improved it (Rezaee & Aider, 2023). Research for the fortification mechanisms and applications of PEs is ever evolving socially, economically and environmentally shifting towards different needs.

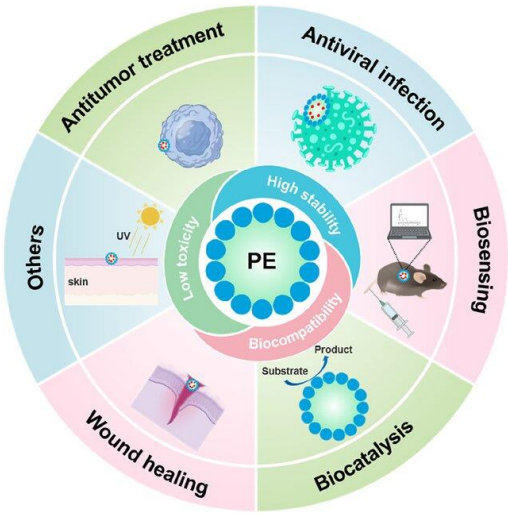


Figure 1.3: Applications of Pickering emulsions (Tavassoli et al., 2023)

1.4 Addition of bioactive compounds-Emulsions' fortification

As a "bioactive ingredient" is usually defined a compound found mostly in foods or dietary supplements that have a physiological effect on the human body, beyond basic nutrition (*NCI Dictionary of Cancer Terms*, 2011). They are naturally occurring in small quantities in plant, animal and microbial sources and they exhibit various activities, such as antioxidant, anti-inflammatory, antimicrobial anti-diabetic and more (Achilladelis et al., 2023; Ertosun et al., 2023; Machado et al., 2023). They are often sensitive to degradation and oxidation, and they have low bioavailability limiting their applications (Yadav et al., 2024).

PEs have been proven to be exceptionally useful and potent when it comes to the encapsulation and delivery of bioactive ingredients. Their increased stability against coalescence makes them desirable matrices for such applications and increases the encapsulated substances' bioavailability and effectiveness (Cahyana et

al., 2022). The durability of PEs against external conditions also protects the encapsulated ingredients from environmental situations that may be harmful to them (Boostani et al., 2024). There is a great number of different active ingredients for encapsulation to choose from depending on the application, the desired activity, solubility and availability. It must be kept in mind that although PEs offer a promising approach for improving the stability, bioavailability and controlled release of these molecules, the correct choice of carrier is of great importance to achieving one's goal.

Some of the molecules that are often encapsulated are vitamins, such as Vitamins A, D and E that are fat-soluble and can be incorporated in O/W systems, but also the water-soluble Vitamin C that can be used in W/O PEs. Vitamins can have antioxidant and anti-aging properties, like Vitamins A and E, but can also boost the immune system, like Vitamin C (Cuomo et al., 2020). When the antioxidant activity is the desired functionality several compounds like polyphenols and curcumin can be chosen as well as flavonoids and carotenoids. Oils possessing functional properties can also be structured as droplets in O/W emulsions to be protected and dispersed in aqueous media. Such oils could be omega-3 fatty acids that support heart health and brain function as well as essential oils like tea tree oil with antimicrobial properties or lavender essential oil with calming effects alongside antimicrobial activities (Rodríguez et al., 2016). Two of the most well-studied natural ingredients because of their beneficial activities are α-tocopherol and squalene. They are both oil-soluble and have been proven to act in a beneficial way in many conditions.

More specifically, α-tocopherol is the active form and one of the eight isoforms of Vitamin E, which is the name given to a family of eight different molecules, consisting of a chromanol ring with an aliphatic side chain (Figure 1.3.1a). They consist of two groups, tocopherols and tocotrienols, based on the side chain being saturated or unsaturated, respectively. Within each group, four isoforms exist, namely, α, β, γ, and δ named for specific methyl group substitutions at positions 5, 7 and 8 of the chromanol ring (Nakatomi et al., 2023). The name tocopherol derives from the Greek words "tókos" meaning "birth" and "phérein" meaning "to bear or carry," referring to its role in supporting fertility and reproduction which was the first to be studied on rats (Mason, 1977). The main sources for α-tocopherol are vegetable oils, like olive oil, various nuts such as almonds, leafy green vegetables like spinach, certain fruits like avocado and commercial fortified foods like breakfast cereals (Shahidi & De Camargo, 2016). The main method for obtaining α-tocopherol is by natural extraction from these sources (Coelho et al., 2021). Alternatively, the chemical or enzymatic synthesis is also possible and leads to a mixture of different stereoisomers that can be purified (Kundu & Sarkar, 2021; Zou et al., 2021). Naturally extracted α-tocopherol is considered to be more bioavailable and effective compared to its synthetic form. They both must meet certain strict criteria before use to ensure purity, safety and efficacy of the final product. The final choice between synthetic and natural α-tocopherol depends on various factors, like cost, expected or desired bioavailability and the exact application (Ranard et al., 2020; Viana da Silva et al., 2022).

α-Tocopherol exhibits certain biological activities with the most potent and studied being its antioxidant capacity as a free radical scavenging agent proved through various methods like the colorimetric

2,2-diphenyl-1-picryl-hydrazyl (DPPH), Oxygen Radical Absorbance Capacity (ORAC) and the Photochemiluminescence (PCL) methods, among others (Castro et al., 2006; Karmowski et al., 2015; Müller et al., 2010). It also possesses anti-inflammatory activity and can prove very helpful in a series of health conditions, like cardiovascular disease, rheumatoid arthritis, and neurodegenerative diseases, and possibly alleviate their symptoms (Tucker & Townsend, 2005). Complimentarily, α-tocopherol is well-established in the cosmetics industry since it aids skin and hair health through hydration and by repairing environmental related damages (Ahn et al., 2023; Dattola et al., 2020). The properties of α-tocopherol have been shown to get improved upon encapsulation. More specifically, studies presented results where encapsulated α-tocopherol demonstrated increased stability under gastric conditions and controlled release from the encapsulation matrix, and at the same time it retained its antioxidant properties (Shah, 2020; W. Xu et al., 2021). There are also studies reporting the improvement of α-tocopherol's antioxidant activity and bioavailability after encapsulation. This phenomenon is explained by the improved solubility, dispersibility and bioavailability of encapsulated molecules (Cheng et al., 2020; Inchingolo et al., 2021).

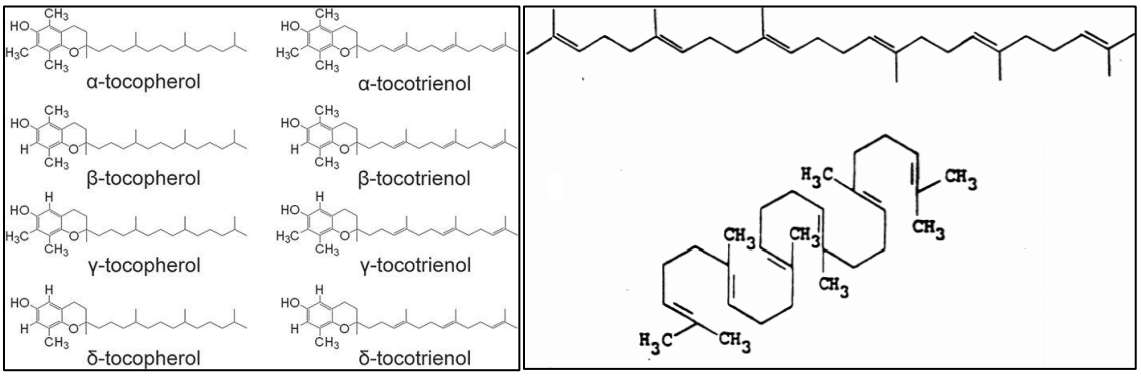


Figure 1.3.1: (a)The 8 different isoforms of Vitamin E (Fukui, 2019) (b) The chemical structure of squalene (Sayin Sakul et al., 2019)

Squalene is a triterpene colorless oil with the chemical formula $C_{30}H_{50}$, although impure samples can appear yellow (Figure 1.3.1b). The primary source of squalene for years has been sharks' liver, hence its name, derives from "Squalus" which is a genus of shark (Popa et al., 2015). Squalene is a precursor for the biosynthesis of various hormones and an intermediate in the cholesterol synthesis pathway in animals, phytosterols in plants, as well as other triterpenes of the cell membrane (Lozano-Grande et al., 2018). However, the use of these marine animals for squalene extraction is restricted as per animal protection regulations. At the same time, the co-extraction of organic sea pollutants may cause certain health problems, even severe ones such as cancer. Therefore, it was of imminent need to explore new natural sources of plant origin (Turchini et al., 2010). Considerable amounts of squalene can be found in plants like olive oil, palm oil, wheat-germ oil, amaranth oil,

and rice bran oil (Huang et al., 2009). Squalene is also produced endogenously in humans, where it is found in sebum accounting for almost 13% of the composition of human skin's surface lipids (Passi et al., 2002). Concerns over the sustainability of the aforementioned methods have led to further evolving production methods of squalene by microbial fermentation. Microorganisms like yeast or bacteria are genetically engineered to enhance their natural ability to produce squalene and genes responsible for squalene synthesis are inserted into them. The engineered microorganisms are cultured in large fermentation tanks and convert sugars and other substrates into squalene. Squalene is finally extracted from the fermentation broth and purified using different kinds of processes, like solvent extraction and filtration (Ghimire et al., 2016). Finally, in recent years there has been progress in the production of squalene from algae since there are microalgae species that naturally produce high levels of squalene. These algae are cultivated in controlled environments, such as photobioreactors, and the biomass is harvested and dried. The extraction and purification steps are similar to those used in plant oil extraction (Potijun et al., 2021).

Squalene can exert its biological activity in many ways. Squalene acts as a potent antioxidant, protecting cells and tissues from oxidative damage caused by free radicals by neutralizing them. At the same time, it has been proven to be an anti-inflammatory agent since it has been shown to target pro- and anti-inflammatory mediators and pathways to modulate the over-activation of neutrophils, monocytes and macrophages (Ibrahim & Naina Mohamed, 2021; Zhang et al., 2023). Additionally, it has been used as an immune system enhancer in certain vaccines to increase the immune response and improve the vaccine's efficacy (Nguyen-Contant et al., 2021). It is also used in cosmetic products as a moisturizing, skin barrier repair and wound healing agent (Pavlou et al., 2021; Shanmugarajan et al., 2021). Encapsulation can significantly enhance the action of squalene by improving its stability, bioavailability, targeted delivery and controlled release. There are several studies concerning various applications that validate this. More specifically, it has been shown that squalene encapsulation by emulsification and freeze-drying using egg white protein nanoparticles improve its stability and bioavailability (Sponton et al., 2023). To improve its action, squalene has been used in an O/W emulsion as a vaccine adjuvant with promising results (Kim et al., 2020). The encapsulation of squalene in emulsions and nanoemulsions for targeted delivery has also been studied and it was shown to enhance its therapeutic effects and specific cell response (Nguyen-Contant et al., 2021; Zhang et al., 2021). Finally, it has been demonstrated that the incorporation of squalene in alginate microcapsules can temporarily modulate immediate immune response after the grafting procedure of encapsulated islets cells and reduce loss of islet cells (Navarro Chica et al., 2021).

2. Materials used for the preparation of particles and modification methods

2.1 Materials used as particles for the stabilization of Pickering emulsions

As mentioned in the previous chapter solid micro- or nanoparticles act as emulsifiers, stabilizing PEs and replacing classic surfactants especially since the use of the latter is raising more and more concern in recent years. There is a great number of materials that can be used for the preparation of particles including plant and animal proteins, inorganic substances, such as silicon dioxide and gold, polymers, polysaccharides, types of algae and even microbial cells (Yang et al., 2017). Among the numerous plant proteins, pea and soy proteins are the two most commonly used especially as their respective isolates, namely Pea Protein Isolate and Soy Protein Isolate (PPI and SPI) (Liang & Tang, 2014; Sarkar & Dickinson, 2020; Yang et al., 2020). In the case of animal proteins, the prevailing ones are milk whey proteins and caseins, which have been considered in numerous studies (Huang et al., 2019; Yang et al., 2024; Zhang et al., 2023). Silicon dioxide is the inorganic substance most frequently used as an inorganic particle while starch alongside chitosan constitute the most applicable polysaccharides in PEs' stabilization (Cui et al., 2021; Meng et al., 2023; Ribeiro et al., 2023). One of the most recent advancements in the field of PEs is the use of microbial cells, on their own or in combination with other ingredients, as PE stabilizers. It is a quite novel approach with a lot of ground to be covered and research is continuously ongoing and evolving (Shen et al., 2014).

Particles as a parameter in the design and formulation of PEs is of great importance as they can affect many of their properties. Thus, the appropriate particle must meet several key criteria to effectively be adsorbed at a water-oil interface, stabilize droplets of the dispersed phase and prevent their coalescence. First, particles should have an intermediate wettability, meaning that they are partially wettable by both the oil and aqueous phase as the respective surfactants are amphiphiles partially dissolved in both phases. This means that they can effectively be adsorbed at the interface around the droplets. The way wettability is measured is by estimating the so-called three-phase contact angle of the particles (Figure 2.1a). The contact angle is the intersection region between the dispersed phase, the continuous phase and the solid particles, and the optimal size depending on the system lies between 15 and 90^{o} (Figure 7). Hydrophilic particles favor the formation of O/W emulsions, while hydrophobic ones lead usually to W/O emulsions (Binks & Lumsdon, 2000)

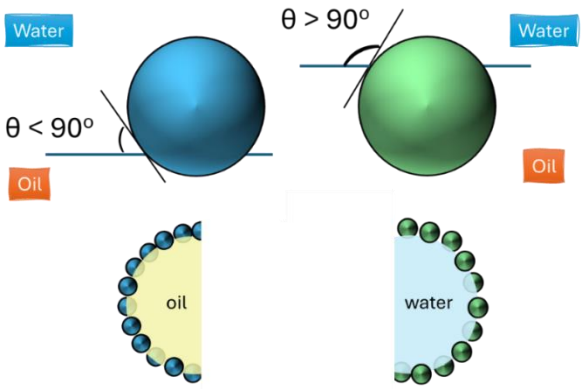


Figure 2.1a: Illustration of the three-phase contact angle for a hydrophilic (left) and a hydrophobic (right) particle forming O/W and W/O emulsions respectively

Of utmost importance, when it comes to the formation of PEs, are the particles' size and shape. As far as size is concerned, particles are used in sizes that range from 10 nm to several micrometers and these are generally effective in stabilizing PEs. Nevertheless, it has been found that smaller particles offer an increased surface area leading to higher surface coverage and better adsorption (Tarimala & Dai, 2004). Furthermore, it has been proven that smaller particles lead to the formation of smaller droplets, which favor emulsions' stability against coalescence and creaming (Tsabet & Fradette, 2015). In general, particles' size should be smaller than the desired droplet size (Xiao et al., 2016). The concentration of particles can greatly influence the stability of PEs. Higher concentrations of particles can lead to more stable emulsions due to the increased number of particles at the interface, which prevents droplet coalescence (Rodriguez et al., 2021) In addition, besides spherical, particles can be rod-shaped, disk-like and even linear, depending on their origin (Ortiz et al., 2020;. Li et al., 2022). Depending on the desired outcome, scientists have pointed out different effects for a variety of particle shapes. It has been reported that due to the higher aspect ratio of anisotropic particles used, the interfacial layer, the desorption energy, and the capillary force between droplets can increase leading to emulsions with increased stability. This happens because the different anisotropies in geometry form "puzzles" that favor the emulsified droplet state (Cossu et al., 2015; Jiang et al., 2022). The more novel-shaped solid particles, including ellipsoids (Kumar et al., 2021), nanofibrils (Lv et al., 2021), nanocages (Lim & Salentinig, 2021), plated-shape (Wang et al., 2022), and nanotubes (Lisuzzo et al., 2022), could even exhibit different stabilization mechanisms and some of them are presented in Figure 2.1b (Zhang et al., 2021).

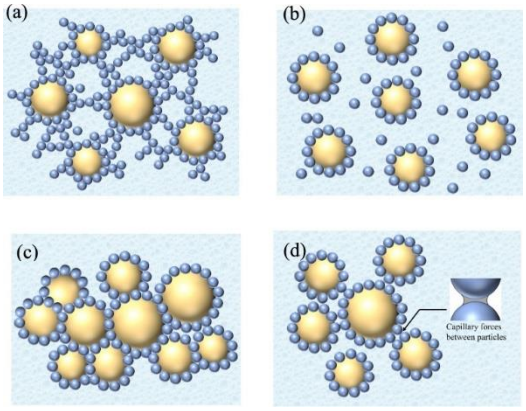


Figure 2.1b: Different stabilization mechanisms of PEs (a) Three-dimensional grid mechanism (b) Interfacial membrane barrier mechanism (c) Mechanism of bridging (d) Capillary force mechanism (Ming et al., 2023)

Another important aspect concerning particles is their surface characteristics including surface charge and functionalization. A suitable surface charge can enhance stability by providing electrostatic repulsion between droplets and reducing the likelihood of coalescence(Benyaya et al., 2023; . French et al., 2020). Additionally, functionalization in terms of the ability to modify the particle surface with functional groups or polymers can also enhance their stabilizing properties, improve biocompatibility, or impart additional functionalities like responsiveness to external stimuli, such as pH or temperature (Björkegren et al., 2020; Vasantha et al., 2020; Wang et al., 2022). The rigidity and robustness of particles that will ensure their mechanical stability directly affects the emulsions stability, since flexible or soluble particles may not provide sufficient barrier properties, thus forming a rather unstable layer at the water-oil interface (Xu et al., 2020).

Finally, two very important parameters that can determine whether the PEs can reach the upscaling phase to a final commercial product are the compatibility with the desired application and the economic feasibility. For food, pharmaceutical, or cosmetic applications, particles must be non-toxic and biocompatible while if an eco-friendly claim has to be made, particles should be biodegradable or derive from sustainable sources (Alison et al., 2016; Shabir et al., 2023). Additionally, the production of the particles should be cost-effective, thus the raw materials, synthetic process and scalability should be considered. Materials that are readily available decrease the cost and the difficulty of procurement (de Carvalho-Guimarães et al., 2022; Frelichowska et al., 2010).

2.2 Pea and Soy Proteins

Plant proteins, deriving from plant sources, such as pea, soy, faba and lentil, have several attributes that make them appropriate for stabilizing PEs. They have inherent surface-active properties due to their

amphiphilic nature and can be processed into particulate forms through methods, like pH shifting and enzymatic hydrolysis, and are biocompatible and biodegradable. Besides their proper fit as PEs stabilizers, they carry several other advantages including nutritional benefits, sustainability since plants are renewable sources, versatility when it comes to their applications and the fact that they are generally recognized as safe (GRAS) ingredients (Jafari et al., 2020; Sarkar & Dickinson, 2020). They can effectively replace animal proteins in creating products suitable for consumers with allergies or avoiding meat products, e.g. vegans or vegetarians. Proteins of plant origin can be divided into four major groups, namely albumins, globulins, prolamins and glutelins (Figure 2.2). In terms of solubility, they are categorized as water-soluble albumins or salt solution-soluble globulins. On the other hand, prolamins are soluble in alcohol and insoluble in water, while glutelins are soluble in acid or alkaline solutions and insoluble in water and alcohol. A more precise way of identifying proteins has been developed, based on sedimentation coefficient ($S_{20,\,w}$) values, where S stands for Svedberg units (offers a measure of a particle's size indirectly based on its sedimentation rate under acceleration) and higher numbers indicate larger proteins (Naismith, 1955; Wolf & Briggs, 1956).

Soy and pea protein isolates are the most studied proteins for emulsifying. Soy proteins are isolated from soybeans, and they contains all essential amino acids. It is one of the least expensive sources of dietary protein (Michelfelder, 2009). Soy proteins can be separated into the four major groups by ultracentrifugation and are designated as the 2S, 7S, 11S, and 15S fractions (Sui et al., 2021). Soy protein isolate (SPI) is a highly processed form of soy protein that derives from defatted soybean flakes. It is a concentrated form of protein with a high protein-to-fat ratio, and it is a good source of fibers, vitamins and minerals (van den Berg et al., 2022). Thus, it can be added to foods made from crops deficient in several essential amino acids, such as rice and wheat, thereby improving their nutritional value (Urade, 2011). Some of the health benefits that have been reported and linked to the consumption of SPI are its function as tyrosine kinase protein inhibitor, reduction of lipid and bile acid absorption from the GIT, improvement of anti-neoplastic enzyme activity and antioxidant activity (Rizzo & Baroni, 2018; L. Xu et al., 2015). Early research suggested that soy protein consumption was able to regulate serum cholesterol, (Low-Density Lipoprotein LDL), and triglycerides. Soy-based protein products were then considered to have potential beneficial effects on cardiovascular health (Jenkins et al., 2010). Other than certain health benefits, SPI carries also several functional characteristics that make it appealing for several applications. To start with, SPI has excellent emulsifying and foaming properties. These properties render them suitable for food applications, such as soups and whipped creams (Deng, 2021; W. Li et al., 2022). Furthermore, SPI can form gels when heated and then cooled down. Its gelation can be proven very useful especially when the need is to increase the viscosity in products like dressings (Totosaus et al., 2002). Despite its many favorable characteristics, SPI has some contradictions as well. It is listed among the top 8 food allergens, affecting about 0.4% of adults worldwide (Cordle, 2004). Nevertheless, it is still among the most used plant protein isolates in the food industry.

PPI is a highly processed form of pea proteins deriving from yellow peas. PPI's structure is affected by the conditions and methods of extraction used and the mixture of protein groups, including albumins, globulins, and storage proteins legumin (11S), vicilin (7S) and convicilin. The group distribution of PPI can vary depending on the extraction conditions and methods used like salt- or pH-dependent extraction (Hansen et al., 2022). Furthermore, reports have suggested that PPIs have a broad molecular mass (MM) distribution ranging between 10-100 kDa. The MM, surface properties and denaturation of the proteins can also be affected by the extraction conditions including pH, the presence of salts and temperature (Lam et al., 2018). Pea protein has a well-balanced amino acid profile with high levels of lysine. Its availability, low cost, nutritional value and health benefits, render it to a novel and effective alternative for SPI or animal proteins in functional food applications. PPI offers several health benefits that make it appealing to the food industry. In contrast to SPI, it is hypoallergenic and less likely to cause immune responses (Stilling, 2020). It has also been reported to possess antioxidant and anti-inflammatory properties that may provide protective benefits, while its high protein content creates the feeling of saturation supporting weight management efforts (Shanthakumar et al., 2022). Additionally, the consumption of PPI promotes the synthesis of muscle proteins, leading to the building and repair of muscle tissue (Pearson et al., 2023). Peas are a sustainable crop, and the production of PPI has a lower environmental impact compared to animal-based proteins (S. Chandran et al., 2023). The functionality of PPI is yet another appeal for several industrial applications. PPI has good water solubility, especially at neutral and alkaline pH values, which can be improved by extraction methods that preserve the protein structure and minimize denaturation (Lam et al., 2018). It also has excellent gelling properties and forms soft gels that find applications in the development of dairy analogues, and it is influenced by pH, ionic strength and heat treatment (Shen et al., 2022). Furthermore, PPI has surface-active properties that enables the stabilization of O/W emulsions for applications, like mayonnaise and salad dressings. Its emulsification capacity can be controlled by the extraction process to preserve the native form of the proteins (Lu et al., 2020). Finally, PPI can bind and retain water and fat, a characteristic useful for improving texture and mouthfeel in foods. The water and fat binding properties are influenced by factors, like protein concentration and pH (Ge et al., 2020).

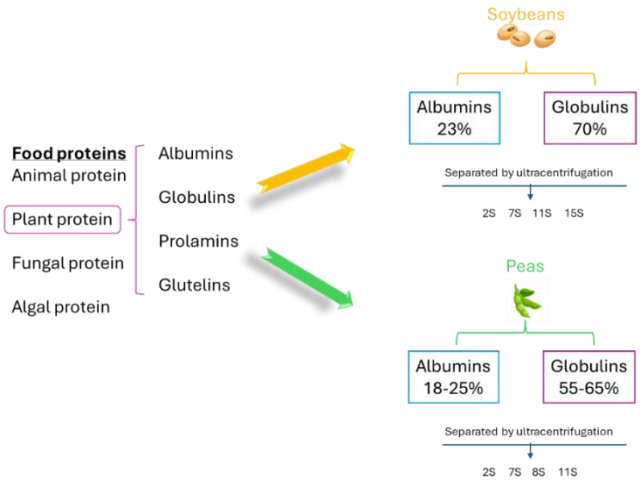


Figure 2.2: Classification of food proteins, soy, and pea proteins.

2.3 Phycocyanin

Over the past decades, the demand for food has increased, while the available arable agricultural lands are decreasing. Nevertheless, the demand for alternative protein sources continues to rise. Thus, recently, algae have been suggested and studied as a sustainable protein source. Other than that, algae comprise a broad spectrum of other nutrients that plants may lack, such as lipids, vitamins, peptides and pigments (Ijaola et al., 2024). Algae can be divided into two categories, namely micro- and macroalgae. Microalgae (microphytes) are represented by green (*Chlorophyta*), blue-green (*Cyanobacteria*), yellow-green (*Ochrophyta, Xanthophyta*), and golden (*Ochrophyta, Chrysophyta*) algae, and diatoms (*Bacillariophyta*). Macroalgae or simple algae include red (*Rhodophyta*), green (*Chlorophyta*), and brown algae (*Ochrophyta*). The primary metabolites of algae are lipids, proteins, carbohydrates, and water while the ratio of primary metabolites in microalgae depends on the type of algae and the cultivation conditions. For example, *Spirulina platensis* (*S. platensis*) is an excellent protein source, with 60–71% of its weight being protein (Babich et al., 2022).

Spirulina (*Arthrospira platensis*) is a marine microalga (cyanobacteria) of Indian origin. It can be cultured in liquid, surface and semi-solid cultures using mineral growth media. The growth of *S. platensis* can be estimated by measuring the pH during cultivation (AlFadhly et al., 2022). Although it is generally considered as safe for consumption, recent studies have demonstrated potential harmful effects due to high levels of cyanotoxins, heavy metals, pesticides, or polycyclic aromatic hydrocarbons (PAHs) (Grosshagauer et al., 2020). Nevertheless, *S. platensis* finds its place among various applications in the food industry as a supplement, in livestock farming to enhance poultry growth and in the pharmaceutical industry for the treatment of diabetes (Ahda et al., 2023; El-Shall et al., 2023; Maddiboyina et al., 2023).

A valuable component of *S. platensis* found in high quantities is phycocyanin (PC). PC is a water soluble, blue protein, part of a larger pigment protein group called phycobiliproteins (PBPs). These pigments are located in phycobilisomes, assembled on the thylakoid surface and divided according to pigment colors (Figure 2.3a) (Jaeschke et al., 2021). PC's structure consists of a monomer formed by two helix-shaped subunits, α and β, with one, bilin chromophore (phycocyanobilin), attached on the α subunit and two attached on the β subunit. The monomers gather forming a ring-shaped trimer $(\alpha\beta)_3$, resulting in a hexameric structure $[(\alpha\beta)_3]_2$ (Figure 2.3b) (Li et al., 2022). PC can be extracted with different methods, such as solvent extraction, Pulsed Electric Field (PEF) or ultrasound assisted extraction. The most common method is that of the solvent extraction, where as solvent can be used water, glycerol, or a water-glycerol mixture (Morya et al., 2023). The extraction yield and purity of the PC extract can be easily calculated spectrophotometrically as described in Equations 5 and 6 (C_{PC}= PC concentration, A_{620}=absorbance at 620 nm, A_{650}= absorbance at 650 nm, EP= extract purity, A_{280}= absorbance at 280 nm).

$$C_{PC} = \frac{A_{620} - 0.474 * A_{650}}{5.34} \text{ (Equation 5)}$$

$$EP = \frac{A_{620}}{A_{280}} \text{ (Equation 6)}$$

PC upon extraction is usually of food grade purity, while for analytical grade further purification, usually ion exchange chromatography, is needed. PC's stability highly depends on environmental parameters, like temperature, pH, and light exposure. It remains stable at temperatures up to 45°C and at pH values between 5.5 and 6.0. It is degraded under UV light exposure, with the degradation rate increases with light intensity, thus, it should be kept in the dark (Fabre et al., 2022). Although PC has been widely used as a food coloring agent, research lately focuses on its health properties and nutraceutical features. It has proven antioxidant activity as free radical scavenger that has been proven by antioxidant assays based on different mechanisms (Fratelli et al., 2021; Zhuang et al., 2022). PC has also been proposed as an anti-inflammatory agent for different diseases, such as inflammatory bowel disease (IBD), atherosclerosis and liver inflammation (Liu et al., 2022). After COVID-19, PC and PC-derived phycocyanobilin were tested as neuroprotective agents against major neurodegenerative disorders and COVID-19-induced damage of the nervous system. Although results do seem promising, further research is necessary to establish a clearer image of its action (Pentón-Rol et al., 2021). It has also been studied for its hepatoprotective activity protecting the liver from damage and disease, as well as anti-diabetic effect by regulating blood sugar (Citi et al., 2024; El-Sakhawy et al., 2023). PC finds plenty of space for application in the cosmetics field, and lately it has been studied as an SPF UV booster agent (Ashaolu et al., 2021; Galani et al., 2023). Finally, PC has been demonstrated to have anti-obesity effects, which can help reduce

body fat and prevent obesity-related conditions, while it offers cardiovascular protection and has anti-microbial properties (Castro-Gerónimo et al., 2023; Dranseikienė et al., 2022).

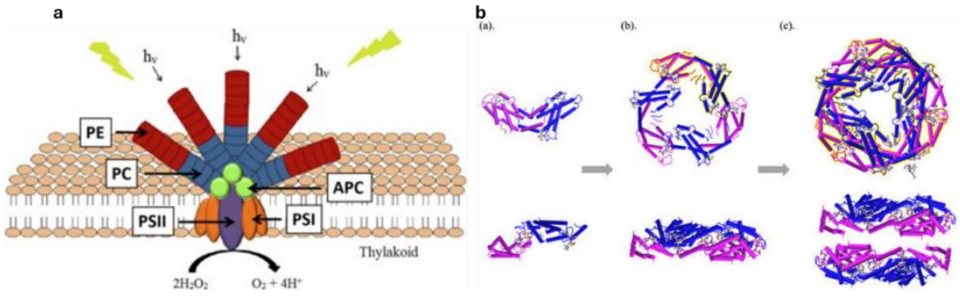


Figure 2.3: (a) Phycobiliproteins structural organization. PE = phycoerythrin; PC = phycocyanin; APC = allophycocyanin; PSII = photosystems II; PSI = photosystems I. (Jaeschke et al., 2021), (b) Cylinder and plate representation of PC monomer where (a), PC trimer (b), and PC hexamer (c) (Li et al., 2022)

With the continuous growth of demand for PC facilitated encapsulation, in various emulsion types, has been employed as a means to deliver and further stabilize it. There have been studies of PC's use in food emulsions as a natural coloring agent dissolved in the aqueous phase that report its effect on the rheological and textural properties of the emulsion (Batista et al., 2006). Additionally, it has been encapsulated in double W/O/W emulsions and results showed an improvement in photostability and solubility even during light exposure and non-advantageous pH values (Li & Abbaspourrad, 2022). Researchers were also able to co-deliver PC (hydrophilic) and astaxanthin (hydrophobic), by encapsulating them in pH sensitive emulsions and control their release (Yu et al., 2022). Proteins, because of the amino acid sequences with varying hydrophilic to hydrophobic properties, adsorb more or less strongly at interfaces, reducing interfacial tension and modifying mechanical properties of the interfacial layer thus providing or increasing emulsion stability. This is why apart from the health benefits that PC brings along to an emulsion, it can also play a double role, also as an emulsion stabilizer. Nevertheless, there are still not a lot of reports on this aspect. Proteins deriving from microalgae exhibit superior stabilizing properties as they manifest a distinct adsorption behavior and ensue elevated steric repulsion and viscoelasticity (Bertsch et al., 2021). Like other materials, PC can be treated in order to alter its hydrophobicity and tune its emulsifying properties. This can happen by grafting PC on another substance, by glycation or by denaturation. The transformation of PC into a natural blue, antioxidant emulsifier was possible, as reported reference, through denaturation with urea. Urea was used to improve the protein's emulsifying capacity and then it was removed by dialysis. The results were promising and indicated the potential use of modified PC in food products, like juices, low-temperature treated dairy products, and functional beverages, as a colorant, emulsifier, and antioxidant (Li et al., 2024). Ultra-high-pressure (UHP) treatment alongside glycation

also proved to be able to improve PC functionality and emulsifying ability. Results showed changes in the secondary structure of the modified PC, a loose molecular structure, and high content of hydroxyl groups. In addition, the solubility, emulsification, and foaming performance of modified PC were superior to those of non-modified PC (Zheng et al., 2020). Finally, it was found that grafting PC on other molecules, like chitosan, improves its functionality, leads to the formation of stable emulsions and aids digestibility of the systems (Zhong et al., 2024). Overall, the possible applications of PC are numerous, and the interest is continuously growing, thus, science follows this tendency and examines the deeper aspects of PC's activities.

2.4 Chitosan

The growing globalization of food supply chains has made food safety a critical issue, linked to potential health risks of unsafe or contaminated foods. The scientific community is actively addressing several concerns, including climate change, food fraud, allergens, and emerging pathogens (Henson & Caswell, 1999; R. Liu et al., 2020). An ongoing effort is being made to achieve the use of alternative, sustainable materials that could substitute polymers. Biopolymers are a group of natural or synthetic polymers that derive from renewable sources, like plants, animals and microorganisms. This group includes carrageenan, pectin, gelatins and chitosan (Van de Velde & Kiekens, 2002). They are often used to replace food packaging materials that are expensive, non-biodegradable and in some cases have been proven to be harmful (Raheem, 2013). Some of them also possess anti-microbial, antioxidant and other activities that render them proper to be used instead of antibiotics or chemical preservatives (Fabra et al., 2014; Merino et al., 2022).

Chitosan is a natural biopolymer, a cationic polysaccharide, the second most abundant on earth. It derives from chitin that can derive from crustaceans, invertebrates, fungi and algae (Figure 2.4) (Iber et al., 2021). Chitin is a polymer of N-acetyl-D-glucosamine, and when it is subjected to deacetylation and the repeating units in the polymer are predominantly without the acetyl functional group, then it turns into chitosan. Thus, chitosan is composed of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Two are the main chemical characteristics of chitosan that drastically affect its properties and functionality, namely MM and degree of deacetylation (DD). DD is defined as the percentage of the repeating units of β-1,4-D-glucosamine while its MM is defined as the mass of a single molecule of chitosan, typically measured in kilodaltons (kDa) (Kou et al., 2021). Both DD and MM affect directly its solubility, ability to interact with other molecules and form materials, digestibility, biodegradability and bioavailability, among others (Lizardi-Mendoza et al., 2016).

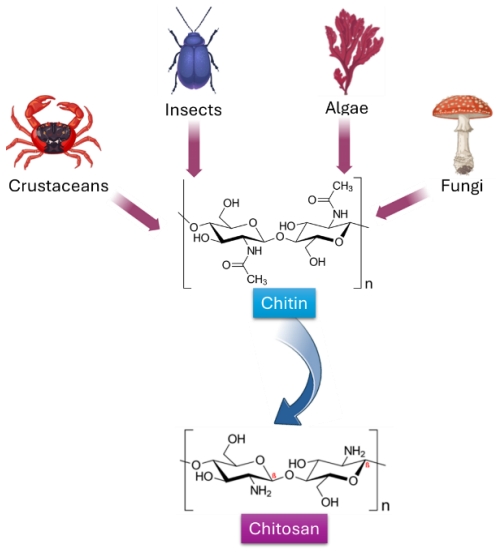


Figure 2.4: Natural sources of chitin and chitosan derived from the N-deacetylation of chitin

All types 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 (Hassan et al., 2023; Manigandan et al., 2018). To start with, chitosan can form edible films to act as coatings for perishable foods, such as fruits and vegetables (Duan et al., 2019). It can improve the texture of certain foods through its gelation ability and form structures for the encapsulation and delivery of nutrients and bioactive substances (Cheba, 2020; Y. Chen et al., 2023) Furthermore, chitosan can act as an anti-microbial agent making it possible to reduce or eliminate the use of antibiotics or chemical preservatives (Devlieghere et al., 2004; Hafdani & Sadeghinia, 2011). It is also considered to have anti-coagulant action, and anti-inflammatory ability, while it is non-toxic and it can be easily modified to alter/tune its properties (Islam et al., 2017).

Fungal-derived chitosan has certain advantages over the one deriving from crustaceans. Fungal chitosan (FC) is produced by fermenting fungi, usually from species like *Aspergillus niger*. FC is often considered more sustainable, as it is produced without shellfish harvesting, and does not differ much, chemically or structurally from chitosan that derives from crustaceans. Moreover, FC is free of heavy metals, and more consistent about its physicochemical characteristics, since it is not bound by the seasonal variation in crustaceans that leads to variation of source materials. Moreover, fungi can be grown in the laboratory on cheap nutrients, cell wall material can be recovered by simple chemical procedures and constant quality and supply of the raw material can be achieved (Ghormade et al., 2017).

On top of all the above, chitosan is an excellent emulsifier. It can act in different ways and stabilize oil or water droplets. Chitosan can form a viscoelastic film around emulsion droplets, acting as a physical barrier

against coalescence. This film is robust and can stabilize emulsions by preventing droplet aggregation (Pinem et al., 2022).First, one can exploit its charge and use it to electrostatically stabilize an emulsion. Positively charged chitosan molecules provide electrostatic repulsion between emulsion droplets, preventing coalescence and enhancing stability. Furthermore, it can act by increasing the viscosity of the emulsion's continuous phase, decreasing the droplets mobility. It can also act in PEs by being adsorbed at the water-oil interface after mechanical agitation (Klinkesorn, 2013; Payet & Terentjev, 2008). One of the most useful characteristics of chitosan is the ability to be modified thus leading to a change in its emulsifying properties. The simplest modification method of chitosan is the altering of pH. Higher pH values lead to the deprotonation of chitosan that can result in a more compact chain conformation and an increase of its hydrophobic character, which enhances emulsifying properties. Additionally, it can interact with other biomolecules, such as sodium caseinate, to form complexes that enhance emulsifying properties (Yang et al., 2023; Zinoviadou et al., 2012). Finally, chitosan's functional properties, such as its antioxidant and anti-microbial activities, can be tuned by adjusting the emulsion droplet size and composition.

2.5 Microbial cells

Among the many materials suitable for PEs' stabilization, microbial cells are of the latest and most promising additions to the list. Several types of food-grade and non-food-grade microorganisms have been suggested and used on their own or in combination with other materials. Like conventional PPs, the size, shape and surface characteristics of microbial cells affect their stabilizing capacity. Microorganisms can be of different geometric shapes (e.g. rods, cocci, and ellipsoid) that can influence the absorption on the interface, hence the emulsions' stability (Firoozmand & Rousseau, 2016). Size as well as shape greatly affect the stabilizing efficiency, since the smaller the particles the more stable the emulsions. This has been attributed to the higher packing density and homogeneity on the surface of the droplets (Hunter et al., 2008). Bacterial adhesion is influenced also by cell wall hydrophobicity, surface charge, and surface structure (Figure 2.5). Different bacterial species and strains adhere differently to surfaces due to variations in these physicochemical characteristics. Gram-positive bacteria have a thick peptidoglycan layer (≈30 nm) that acts as a durable and flexible barrier. In contrast, Gram-negative bacteria have a thinner peptidoglycan layer (≈10 nm) topped by an outer membrane containing proteins, lipopolysaccharides, and phospholipids (Habimana et al., 2014). In the case of microbial cells, biosurfactant, substances naturally produced by microorganisms can affect their surface activity and surface charge, thus altering their emulsifying properties (Hua et al., 2003). All parameters listed above, highly affect the resulting emulsion's stability, homogeneity and type.

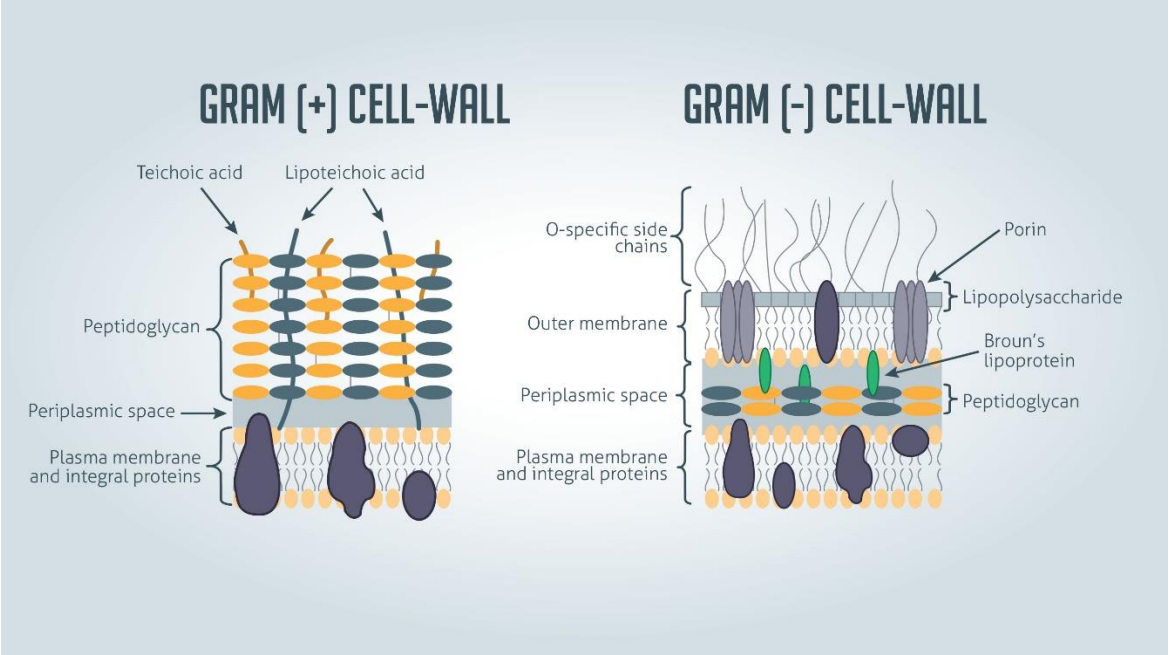


Figure 2.5: Gram positive vs Gram negative cell wall (Gram Positive vs Gram Negative, n.d.)

The earlier research used pathogenic bacteria like *Escherichia coli* (*E. coli*), *Mycobacterium neoaurum (M. neoaurum)* or *Alcaligenes faecalis (A. faecalis)*. These bacteria have been used on their own as whole-cell stabilizers or in combination with chitosan in the case of *E. coli*. When *E. coli* cells were combined with chitosan, they interacted electrostatically due to their opposite charges. The formed network of conjugated *E. coli*-chitosan particles was able to stabilize O/W PEs with increased stability against coalescence and by simple hand mixing, with no extra energy being required (Wongkongkatep et al., 2012). *M. neoaurum* and *A. faecalis* were also used as PE stabilizers exploiting at the same time their catalytic activities. Interfacial biocatalysis for microbial transformation of hydrophobic cholesterol into androst-4-ene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) was successfully implemented using *M. neoaurum*. The bacteria stabilized the PEs and consequently exhibited enhanced biocatalytic activities compared with the conventional aqueous system (Xie et al., 2021). On the other hand, while *M. neoaurum* was used untreated, *A. faecalis* cells were immobilized. At the same time, the hydrophobic/lipophilic balance of the encapsulating magnetic mineral elements used were optimized, helping the encased bacteria become interfacially active (Chen et al., 2015). This led to the enhancement of the bacteria bioconversion performances by minimizing internal and external diffusional resistances. An earlier study delt with the stabilization mechanisms behind bacteria cell adsorption and demonstrated that bacteria can produce the so called biosurfactants. Those can be classified into two principal groups based on their molecular weight, namely, low molecular weight (glycolipids and lipopeptides) and high molecular weight (polysaccharides, lipopolysaccharides, proteins, and lipoproteins). They also tested several

bacteria strains indicating the dependence of PE stability on the bacteria genus and strain(Dorobantu et al., 2004).

2.6 Modification methods of particles

As previously stated, PEs are stabilized by solid or soft micro- or nano-colloidal particles that being adsorbed at the water-oil interface. Those particles, in order to offer the maximum stabilizing effect, should meet certain criteria, such as appropriate wettability from the oil and aqueous phase, surface activity, suitable size and size homogeneity. If a material does not fulfill these requirements, then there are some modification methods that can be applied to modulate specific characteristics. The modification methods of Pickering Particles (PPs) involve tailoring their wettability by altering their surface chemistry and/or surface roughness (Figure 2.6). These methods are crucial for the formation and stability of PEs.

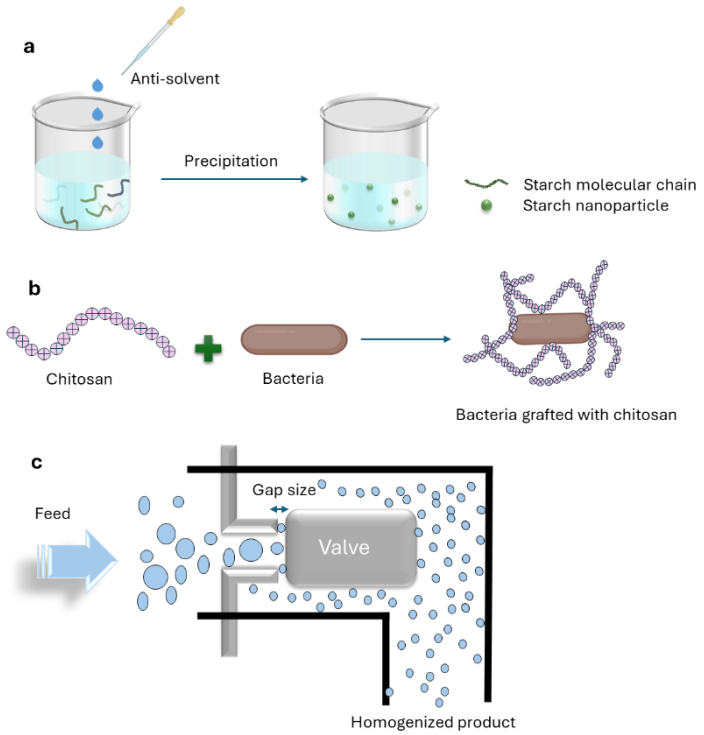


Figure 2.6: Different modification methods of PPs where (a) anti-solvent precipitation method, (b) chemical modification by grafting and (c) size reduction by high-pressure treatment

To change a material's surface chemistry (surface activity), there are several routes that can be followed. Firstly, the wettability of particles is possible to be tuned by *in situ* modification with amphiphilic molecules. This technique aims to tune the particle's three-phase contact angle, to acquire the optimum wettability, thus,

to form more stable emulsions. In this method, surfactants or small polymer molecules can be used (Xiao et al., 2018). Another very common technique is grafting of particles with other materials to change or add some specific property. There are reports of grafting thermally responsive polymer brushes onto nanoparticles to create PEs that can be controlled by temperature (Heidari et al., 2022). pH responsive PEs can also be formulated by grafted carboxymethyl starch nanoparticles (Xiao et al., 2020).

Surface roughness can be controlled by growing other material layers on the surface of particles. This method allows for the creation of particles with specific roughness that can stabilize both O/W and W/O emulsions. The most common suggestion in literature is the formation of silica layers, while there are also some more rare and anterior reports that used gold as layers (Graf et al., 2003; Lim et al., 2003). Electrostatic attraction or hydrogen bonding interactions can be also used to modify the surface roughness of inorganic nanoparticles, such as laponite, layered double hydroxide (LDH), aluminum oxide, and clay. Rough particles can be fabricated by electrostatically adsorption of oppositely charged particles. An example is the negatively charged poly(methacrylic acid-co-methyl methacrylate) nanoparticles adsorbed onto amine modified silica particles to change their topology and make their surface rougher (San-Miguel & Behrens, 2012). This can be applied even with oppositely charged particles of the same origin, such as silica (Zanini et al., 2017).

Complementary methods to the ones described above are those of changing the size and size homogeneity of particles, divided in two major categories, namely mechanical and chemical breakdown methods. The most commonly used mechanical methods are cryogenic milling, wet milling, high-pressure and high-shear treatments (Xiao et al., 2016). Chemical modification most usually consists of a procedure of acid hydrolysis. This technique can reduce the size of particles, which are insoluble in water and most organic solvents due to the presence of crystalline structures. Hydrolysis using strong acids can remove the susceptible amorphous regions of materials and retain the crystal structure. It has been indicated that the acid hydrolysis combined with ultrasound can produce starch nanocrystals (Zhu, 2015).

Finally, anti-solvent precipitation is the most easy and successful method of modifying the solubility of materials. It presents several advantages, such as low cost and easy application, and it especially suits water-insoluble food materials. The first step in this technique is to dissolve the material in an appropriate solvent followed by the addition of this solution to another non-solvent, which is usually an aqueous phase. A rotary evaporator is then used to remove the "good" solvent dispersed in an aqueous phase under reduced pressure, and then the materials quickly reach saturation and crystallize out forming particles (Chen et al., 2020a).

In conclusion, modifying PPs through physical, chemical, or combined methods significantly affect their properties and can enhance their ability to stabilize emulsions. These modifications allow for the tailoring particles' properties to meet specific requirements, improving emulsion stability and functionality. As research progresses, the development of novel modification techniques will continue to expand the potential applications of PPs in various industries, including food, pharmaceuticals, cosmetics, and environmental remediation.

3. Particles' characterization

Comprehensive characterization of PPs is of great importance not only in terms of their chemical physicochemical properties (solubility, wettability) but also in terms of their structural characteristics (size and shape), and their interfacial properties. These parameters, significantly impact the formation, stability, and functionality of Pickering emulsions. Various techniques have been developed to analyze the size, shape, surface chemistry, wettability, and interfacial behavior of PPs. The most dominant are Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FTIR), three-phase contact angle measurement, Dynamic Interfacial Tension (DIT) measurement, rheometry and Differential Scanning Calorimetry (DSC) (Cherhal et al., 2016; Jiménez Saelices & Capron, 2018; Muiz et al., 2023; Nimaming et al., 2023). Each technique gives unique and valuable information about the particles, helping discover, alter or enhance their functionalities. The ones used for the present dissertation are presented below.

3.1 Dynamic Light Scattering (DLS)

DLS is a technique employed for the size and polydispersity determination of small colloidal particles or emulsion droplets. DLS measures the fluctuations in light intensity that are caused by particles in the solution subjected to the Brownian motion. A monochromatic light beam, usually a laser, illuminates the sample. Each particle acts as a secondary source by scattering the light with different intensities depending on their size. The intensity fluctuations are recorded randomly by the detector in different times because of the particles' relative position changes in the solution (Figure 3.1). The fluctuation time of the scattered light intensities depends on the diffusion coefficient of the particles. This means that larger particles diffuse more slowly than smaller ones. The scattered light is then analyzed using an autocorrelator, which compares the intensity of light at each spot over time (Figure 14). Furthermore, DLS uses spherical models for the results' analysis. For this reason, the hydrodynamic radius R_h of the particles is estimated by the Stokes-Einstein equation (Equation 7)

$$D = \frac{kT}{6\pi\eta R_h} \quad (7)$$

where, D is the diffusion coefficient, k is the Boltzmann's constant, T is the absolute temperature, and η is the viscosity of the solvent. When particles are not of spherical shape, R_h is considered to be the apparent hydrodynamic radius or equivalent sphere radius. Finally, the size distribution of the particles is also acquired as the polydispersity index (PDI), which is an output of the autocorrelation function. PDI values lie between 0 and 1, where 1 is the highly heterogeneous population and 0 is the highly homogeneous nanoparticle population (Kumar & Dixit, 2017).

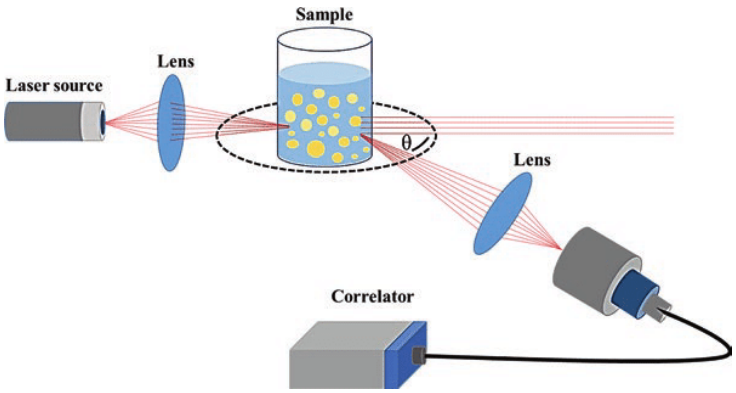


Figure 3.1: The principle of DLS (Choudhary et al., 2019)

Although most commercial DLS devices work on fixed angle, there are some that give the ability to choose the scattering angle, which significantly affects the results since there is an optimum one for each particle size. For smaller particles, the scattering is equal at all angles, so all are acceptable. However, when particles are above 20 nm, larger particles scatter more strongly at smaller angles, while smaller particles scatter more strongly at larger angles (Maguire et al., 2018). Measurements at multiple angles allow for more accurate results and helps to minimize errors due to particle size and shape variations. This approach is particularly useful for polydisperse samples where the scattering angle can affect the results (Petersen et al., 2014). Despite of whether single or multiple-angle measurements are performed, DLS is the most used technique for particle size determination for sizes ranging from 0.3 nm to 10000 nm. It is quick, easy to perform and non-destructive.

3.2 Freeze Fracture Transmission Electron Microscopy (FFTEM)

Transmission electron microscopy (TEM) is a microscopy technique that uses a beam of electrons to form an image of a specimen. It is the most widely applied structural analysis tool to date and has the power to visualize almost everything from the micrometer to the angstrom scale. The freeze-fracture (FF) technique is used as a pretreatment method when structural analysis through TEM or Cryo-TEM is challenged due to the nature of the sample. FFTEM gives useful information about the structure of molecular aggregates, colloidal dispersions, particles, and emulsions.

For this technique, the sample is initially rapidly frozen using liquid nitrogen or a cryogen, such as propane. The FF process prevents the formation of ice crystals that can damage the sample structure. The frozen samples are then inserted in a freeze-fracture apparatus at a very low temperature and under pressure and are being fractured using a microtome or a similar device. The fractured surface is then shadowed

immediately by a layer of platinum forming a replica of the exposed structures. This layer provides contrast and structural detail, while an extra carbon layer is added to secure the stability of the replica. These replicas are then immersed in several consecutive baths of ethanol and sodium hydroxide to destroy the remaining sample, and ultimately in pure water. Finally, the replicas are dried before TEM imaging (Figure 3.2a) (Kim & Yoon, 2023; Tsuchiya, 2019).

TEM is a microscopy technique that uses a beam of electrons to form a high-resolution image of a specimen. TEMs consist of three essential systems including the electron gun, the image-producing system, and the image-recording system. The electron gun produces the electron beam, and some of the waves of the beam interact with the sample and after that the resulting image is magnified by a series of lenses (Figure 3.2b). The image-producing system focuses the electrons through the specimen, and the image-recording system converts the electron image into a form perceptible to the human eye (Franken et al., 2020).

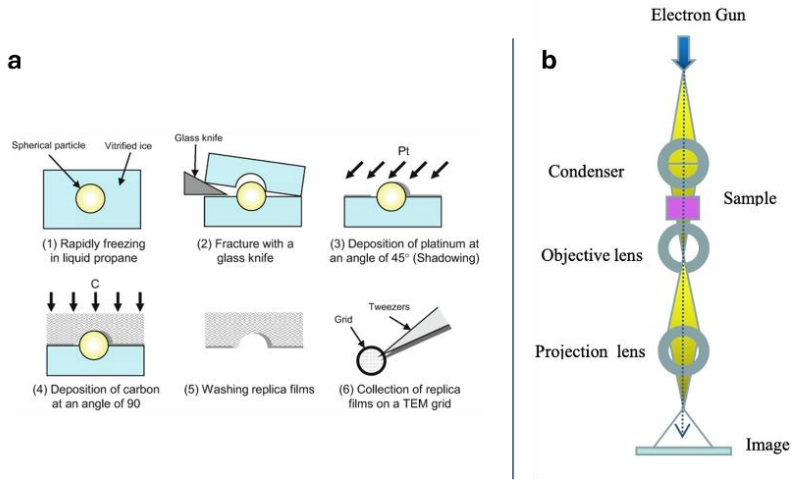


Figure 3.22: Schematic representation of (a)the freeze-fracture pretreatment technique, and (b) the TEM imaging (Transmission Electron Microscope (TEM), Tsuchiya, 2019)

No matter how useful TEM is it might bring along several challenges. First, it is of great importance that one understands the data collected. FFTEM gives information about the fractured surface, and not for the inherent morphology of the sample. The images obtained depend on how the frozen samples are fractured and sometimes, it is difficult to adequately interpret them. Interpreting TEM images can be challenging due to the complex interactions between the electron beam and the sample. This includes understanding the contrast mechanisms and the effects of sample thickness and composition on the image. For example, FFTEM images of O/W emulsions are similar to those of unilamellar vesicles. Furthermore, a challenge that can rise is the

possible interaction between the electron beam and the sample. This can lead to artifacts and distortions in the image including effects, such as beam damage, radiation damage, and melting (Pennycook et al., 2003).

3.3 Dynamic Interfacial Tension (DIT)

Dynamic interfacial tension (DIT) is a critical parameter when it comes to understanding and even controlling the behavior of interfaces. DIT describes the interfacial tension between two immiscible liquids as it changes over time. Interfacial tension is the force per length unit acting at the interface between the two immiscible liquids (usually oil and water) and arises due to the imbalance of molecular forces. It is different from static interfacial tension, which is measured after the system reaches an equilibrium. DIT is affected by the presence of surface-active ingredients (Nagarajan & Wasan, 1993). During the formation of an emulsion, surfactants or particles, in the case of PEs, are adsorbed at the interface, reducing the interfacial tension over time. The diffusion of molecules to the interface and their subsequent reorganization or their potential substitution by antagonistic molecules can also impact the DIT. All these processes are time-dependent contributing to the dynamic nature of the interfacial tension.

In order to measure the DIT of an interface several techniques can be used like the Oscillating Drop/Bubble method, Spinning Drop Tensiometry, the Wilhelmy Plate method, the Du Nouy Ring method, the capillary rise method, and the Pendant Drop method (Figure 3.3a). They are all dynamic techniques based on different principles. Specifically in the Pendant Drop method, a drop of liquid is suspended from a needle, and the shape of the drop is analyzed over time to determine the interfacial tension (Huang et al., 2022).

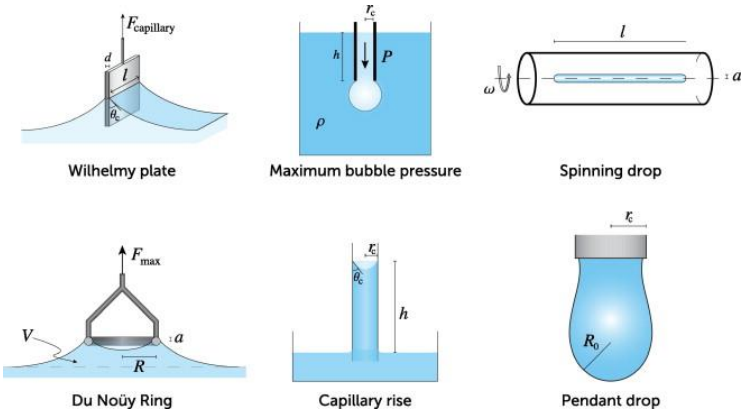


Figure 3.3a: Schematic representations of various experimental techniques used to determine interfacial tension (Berry et al., 2015)

Pendant drop tensiometry is a widely used technique for the determination of the DIT of and oil-water interface. A drop suspended from a needle has a characteristic shape due to the balance between the forces of gravity (pulling the drop down) and surface tension (trying to minimize the surface area of the drop). The shape of the pendant drop is described by the Young-Laplace equation (Equation 8), which relates the pressure difference across the interface to the curvature of the interface and the interfacial tension. ΔP is the Laplace pressure, the pressure difference across the fluid interface (the exterior pressure minus the interior pressure), γ is the surface tension , η is the unit normal pointing out of the surface, H_f is the mean curvature, and R_1 and R_2 are the principal radii of curvature. Note that only normal stress is considered, because a static interface is possible only in the absence of tangential stress.

$$\Delta_p = -\gamma \left(\frac{1}{R_1} + \frac{1}{R_2}\right) \quad (8)$$

By analyzing the shape of the pendant drop, the interfacial tension can be determined. The profile of the drop is typically captured using a high-resolution camera, and image analysis software is used to fit the drop shape to theoretical models with the Young-Laplace parameters to extract the interfacial tension value (Figure 3.3b) (Handschuh-Wang et al., 2023). Despite some limitations, the accuracy, precision, and versatility of pendant drop tensiometry make it an invaluable tool for studying interfacial phenomena and optimizing product formulations and processes.

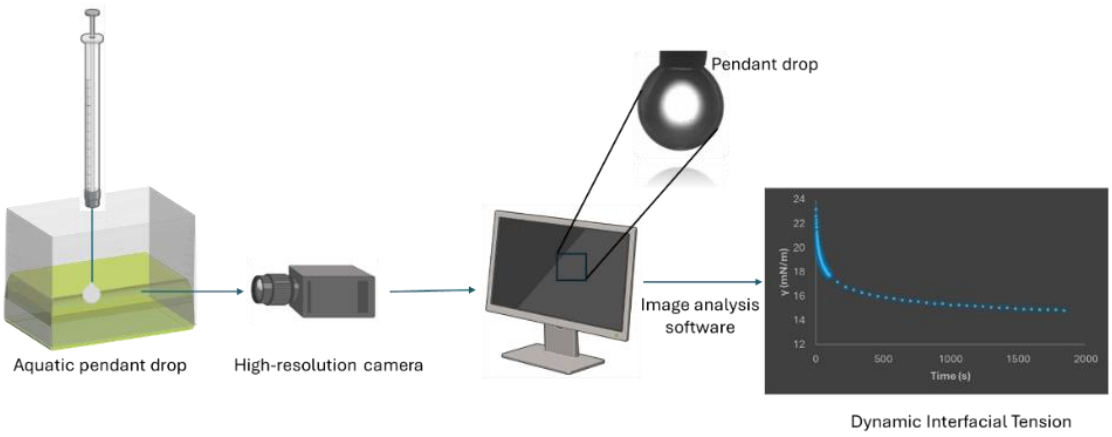


Figure 3.3b: Schematic representation of the pendant drop tensiometry technique.

4. Emulsification methods

In order to form an emulsion, external energy must be applied to aid the colloidal dispersion formation and stabilization. The main processes for obtaining conventional emulsions can also be used to produce PEs. The most commonly used emulsification techniques are high-shear homogenization (HSH), high-pressure homogenization (HPH) and ultrasonication (US). More recently, more methods have been developed and applied for the preparation of PEs, like micro fluidization emulsification and membrane emulsification (Ekanem et al., 2022; Tello et al., 2023). All these methods involve breaking down a liquid in small droplets dispersed in another liquid. They differ from one another, and their use varies depending on the desired properties of the emulsion, the nature of the involved liquids and the desired application.

During the application of ultrasonication, acoustic cavitation, created by the ultrasonic homogenizer leads to the generation and collapse of air bubbles in the medium. The physical properties of those bubbles can enable the disruption of oil droplets, facilitating the formation of stable O/W emulsions with small droplets size. At the beginning of the ultrasonication process, large emulsion droplets are formed, which then break down into smaller ones due to the continuous acoustic cavitation. Ultrasound waves with frequency ranging from 20 to 100 kHz possess the ability to interact with matter through energy transfer and thus can be leveraged for emulsification while higher frequencies of MHz range is more likely to be used for the de-emulsification process (Pang et al., 2021; Taha et al., 2020).

Micro fluidization is a technique that combines high-pressure, high-velocity, cavitation, and intense shear rate, producing highly stable and homogenous emulsions. Among its advantages, such as short processing time, low temperature, and little to zero nutritional loss, it has gained a lot of space in different industrial applications, such as the improvement of bioavailability and solubility of poorly water-soluble drugs, the development of stable emulsions and creams with fine particle sizes in cosmetics and the creation of stable emulsions for beverages, sauces, and dressings for the food industry (Ozturk & Turasan, 2021). The principle of the microfluidic method is that the disperse and continuous phase flow in different channels and finally collide to shape oil or water droplets forced by high-pressure through the micro-channels. Since micro fluidization does not use high shear forces, so it does not destroy the agglomerates of the emulsifier, thereby forming a thick film around the droplets to stabilize the emulsion (Chen et al., 2020b).

The membrane emulsification method refers to pressing pure dispersed phase or coarse emulsion into a microporous membrane and controlling the injection rate and shearing conditions to prepare a PE. There are two categories of membrane emulsification techniques, namely direct and premixed membrane emulsification. The droplet size of the resulting PE is directly linked to the pore size of the membrane, the viscosity of the continuous phase and the dispersed phase and the size of the surface tension. Compared to other emulsification methods, this one is environmentally friendly, it requires less energy to prepare an emulsion of the same particle

size, and the particle size of the emulsion is uniform. However, the process is time consuming, and suitable only for low viscosity systems (Chen et al., 2020b).

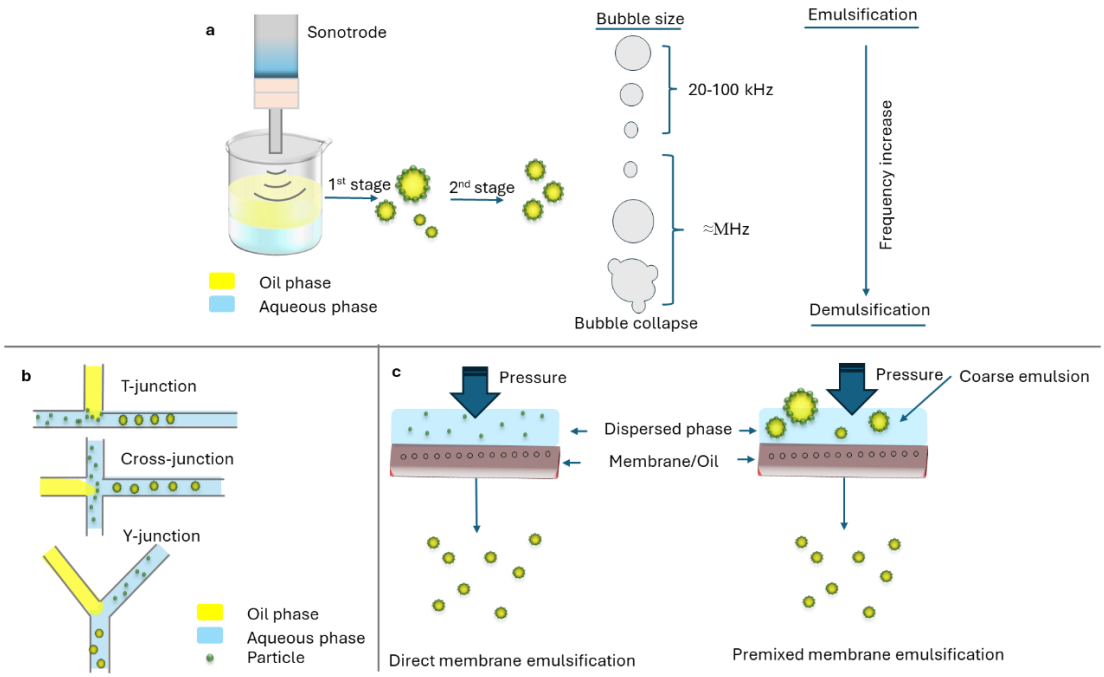


Figure 4a: Schematic representation of (a) Ultrasonication emulsification method, (b) micro fluidization emulsification method and (c) membrane emulsification method.

The HPH method is a continuous emulsification process that falls under the general category of high-energy emulsification methods. The first step is the preparation of a coarse emulsion usually by high-shear force leading to large droplets easy to collapse. This coarse emulsion is then fed to the apparatus and forced by high pressure through small channels thus creating smaller and more uniform droplets. (Levy et al., 2021). It is an effective way to break the oil and water phases to generate tiny emulsion droplets. Studies have shown that the droplets formed by HPH are smaller compared to those formed by HSH (Zhou et al., 2022). During HPH, the pressure is applied uniformly and transmitted to the pre-packed product by the high-pressure pump resulting not only in small but also uniform droplet size assuring the increased stability of the final product. The highest the pressure and the more the recirculation, the smaller the resulting droplets. Nevertheless, when a certain threshold is surpassed, a phenomenon called overprocessing happens that leads to the collapse of the emulsion (Rayees et al., 2024). Finally, it is equally important to be aware of the temperature increase because of the applied high-pressure since there are ingredients, like temperature sensitive proteins, so an ice bath or some

other cooling system may be necessary to avoid the degradation of the emulsion components (Yong et al., 2021).

In order to perform HSH a rotor-stator is needed. It is composed of an instrument with blades that rotates around its own axis, known as the internal rotor. It homogenizes the samples by means of mechanical tearing and with the aid of shear forces. The differential speed generates high levels of hydraulic cutting, promotes rapid homogenization and produces small droplets within the PE. In general, the greater the shear the smaller the emulsion droplets (Vashisth et al., 2021). However, the high shear caused by excess energy can lead to coalescence of the droplets. The droplet size is directly related to the geometry of the mixing head and the number of times it passes through the mixing zone. A rotor-stator homogenizer ensures the emulsification of different types of liquids. However, it has some disadvantages, such as the fact that only a single sample at a time can be homogenized, the point here being that one is a batch process and other (like HPH) are continuous. In addition, despite helping to overcome the energy barrier, the high mechanical shear used in homogenization causes the breakdown of particle aggregates (Kaur et al., 2023).

Overall, the choice of the right homogenization technique is of great importance to achieve the desired results. By making the right choice and by leveraging new techniques and methods, novel advanced products can be produced, problems like decreased solubility and bioavailability can be solved, and new possibilities and applications can be examined (Mesa et al., 2020; Sherman et al., 2024).

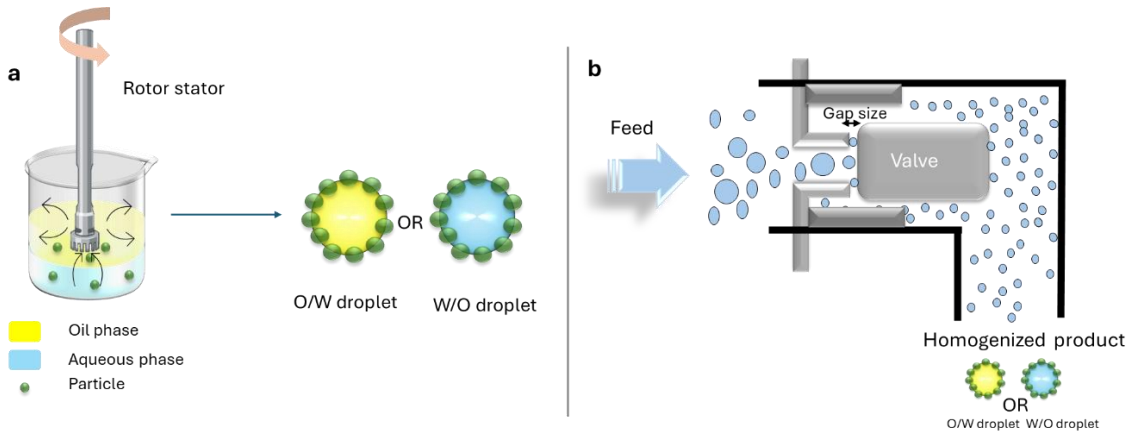


Figure 4b: Schematic representation of (a) high-shear homogenization and (b) high-pressure homogenization

5. Emulsions' structural characterization

Emulsions can often be complex systems with numerous ingredients and complicated structures. Their structural characterization is essential for comprehending their properties, and assessing their stability, and

performance. Various techniques, including microscopy, dynamic and static light scattering, ζ-potential analysis, rheology, and interfacial tension measurements, can be combined and provide insights into the size, distribution, morphology, and stability of the emulsion droplets. These characterizations are crucial for optimizing formulations, ensuring quality, and developing novel products designed for specific applications. By employing appropriate characterization methods, industries can enhance the functionality, nutritional value and overall performance of the emulsions, leading to improved products that suit consumers' demands and needs, while at the same time are of high quality. In this study, alongside the ζ-potential, DIT, FFTEM and DLS measurements of the studied particles, Static Light Scattering (SLS), Laser Diffraction (LD), optical microscopy, Confocal Laser Scanning Microscopy (CLSM) and Cryogenic Scanning Electron Microscopy (Cryo-SEM), were employed to structurally characterized the produced PEs.

5.1 Laser Diffraction (LD) / Static Light Scattering (SLS)

One of the most widely techniques used for particle or droplet size determination is LD (also known as SLS). LD measures the size of droplets or particles by analyzing the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample or emulsion. It can typically cover a particle size range of 10 nm to 4 mm. The diffraction angle is inversely proportional to the size of the droplet (Figure 5.1a), and the light diffraction pattern gives the droplet size distribution using Mie theory, and assuming that the droplets have a spherical shape (Sijs et al., 2021). Gustav Mie wrote a paper on light scattering by dielectric absorbing spherical particles in 1908. He was interested in explaining the colorful effects connected with colloidal gold solutions. Nowadays, the interest in Mie's theory is much broader and interests range from physics' problems involving interstellar dust, and near-field optics to engineering subjects like optical particle characterization. Mie theory is still being applied in many areas because scattering particles or objects are often homogeneous isotropic spheres or can be approximated in such a way that Mie's theory is applicable (Wrigglesworth & Johnston, 2021; Wriedt, 2012).

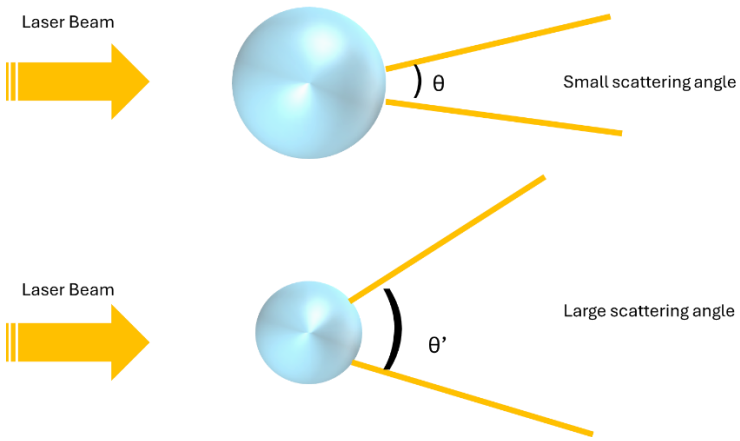


Figure 5.1a: Light scattering for particles/droplets of different size

An LD apparatus usually consists of a light/beam source that generates a coherent and monochromatic beam of light (Figure 5.1b). More commonly, helium-neon (He-Ne) lasers and solid-state lasers are used, and the laser's wavelength affects the resolution and accuracy of particle size measurements. Following, the sample dispenser ensures that the sample is well-dispersed to prevent agglomeration and achieve accurate measurements. An optical bench that includes mirrors, lenses, and beam splitters to direct and focus the laser beam through the sample provides a stable platform where the laser, sample, and detectors are aligned. A detector then measures the intensity of light scattered by the particles at various angles and the data acquisition system measures the intensity of the scattered light at various angles (Bittelli et al., 2022).

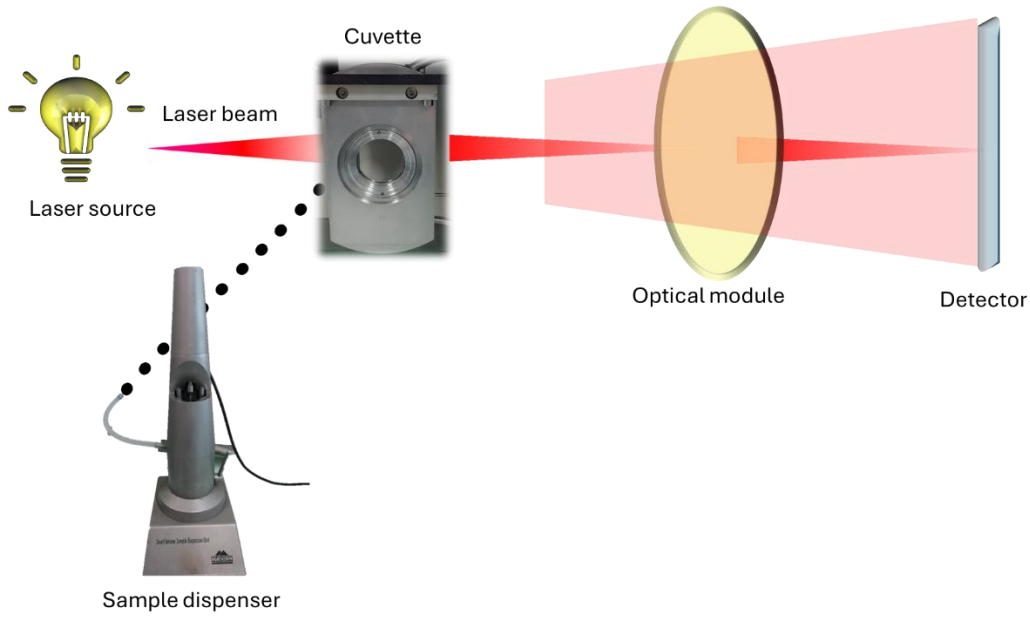


Figure 5.1b: Schematic representation of the parts of a typical LD apparatus

The LD technique offers a wide dynamic range, high sample throughput, easy operation, accuracy, and reproducibility. It can also measure samples of a broad size range, and it can handle both wet and dry samples. Furthermore, it provides quick, real-time measurements while it has the possibility of preserving the sample for further analysis. On the opposite hand, the primary equipment is rather expensive, and needs proper sample preparation as poor dispersion can lead to inaccurate results. Finally, LD assumes that all particles are ideally spherical, which can also lead to inaccurate results for irregularly shaped particles since it does not provide information on particle shape (Low et al., 2020; Polakowski et al., 2021). Overall, it is a very well-established technique, with no serious limitations and it can be used in a high number and wide range of applications to provide structural insights.

5.2 Microscopy techniques

Microscopy techniques are valuable tools in scientific research and industry for visualizing and analyzing the structure, morphology, and properties of materials at micro- and nanoscales. These techniques enable the study of a wide range of samples, from biological specimens to other materials for different applications. Some of these techniques are, Optical Microscopy, CLSM, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Atomic Force Microscopy (AFM), Fluorescence Microscopy and more. The variety of techniques that give different information on a wide variety of samples, allows for the

exploration of diverse systems, limiting the restrictions that might be encountered upon research (Clarke & Eberhardt, 2002).

5.2.1 Optical Brightfield Microscopy

Optical microscopy uses visible light and a series of lenses to magnify small objects to a level where they can be visually observed and analyzed. It is widely used in various fields, including biology, material science, and physics, among others. It is subcategorized in different types that include dark field, which enhances contrast in unstained samples by illuminating the sample with light that will not be collected by the objective lens (Gao et al., 2021). Phase contrast microscopy translates small changes in the phase of light into changes in brightness, which are then depicted as differences in image contrast. This is achieved by slowing down or advancing the background light by a quarter wavelength using a phase plate before the image plane (Mann et al., 2005). There is also fluorescence microscopy that uses high-intensity illumination to excite fluorophores in the examined sample that emit light at different wavelengths.

The most common of all is brightfield microscopy, where light that passes through the sample is used by lenses to magnify small objects, allowing the visualization and understanding of the structure and function of cells, materials, and devices at the micro- and nanoscale. The sample appears dark against a bright background, as the light is differentially absorbed or refracted by the specimen, and by modulating the distance of the specimen the lenses and the light source, as well as by adjusting the contrast and focus, a clear image can be obtained (Gutiérrez-Medina, 2022). The classic brightfield microscope is an indispensable tool for many laboratories, especially of biological interest and it can also be used for stained samples. The technique is commonly used in biology, medicine, material science, and education to study a wide range of samples, including cells, tissues, minerals, and polymers (Drey et al., 2013; Pirbodaghi et al., 2015).

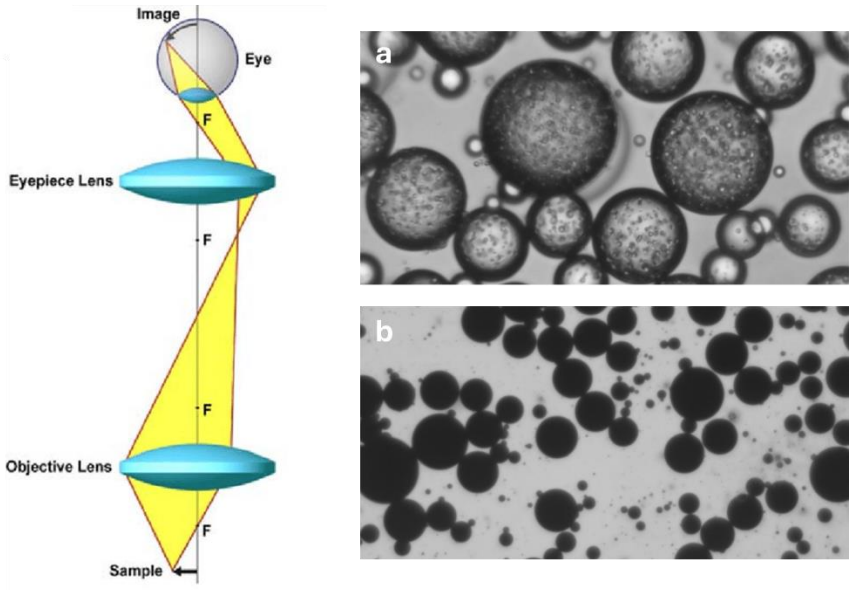


Figure 5.2.13: Typical arrangement of a classic optical microscope (Lee et al., 2011) and 2 images of emulsions (a) emulsion droplets of an O/W PE and (b) emulsion droplets of a W/O/W PE. (Both images were obtained through experiments conducted as part of the current PhD research)

Optical microscopes have been more advanced over the years providing images with higher zooming abilities and higher resolution. This allows for using optical microscopy for the structural characterization of emulsions. Microscopy is more commonly used to assess the shape of emulsion droplets and their size (Li et al., 2020; Mota da Silva et al., 2021). Furthermore, the droplets' arrangement can be observed, the effect of bridging as well as the homogeneity and stability of the emulsion (Hu et al., 2017). Especially in PEs, brightfield microscopy can be used to observe the interaction of particles that form the droplets, since modern equipment offers very good depicting abilities (Kupikowska-Stobba et al., 2024). Optical microscopy is often used in combination with other characterization techniques, such as LD for droplet size analysis, to provide a comprehensive understanding of the emulsion system. Some of the limitations of brightfield microscopy are the fact that it cannot be used easily for the analysis of three-dimensional samples because the contrast can still be low and not allow for an in-depth study.

5.2.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM is a powerful imaging technique that provides high-resolution images of structures within a specimen. It is widely used in biological, medical, and material science due to its ability to produce detailed images with reduced background noise and improved depth selectivity enabling the construction of 3D images

from 2D graphs. For this technique to be used, the sample must either carry a fluorophore or it must be stained by a fluorescent dye (Sanderson, 2022).

CLSM uses a focused beam that passes through a specific small section of the sample in a point-by-point manner. Afterwards, it is guided in a pinhole devise placed in front of the detector to eliminate all other sources of light, eliminating out-of-focus light, thus creating high resolution optical sections. By moving the focal plane through the sample in horizontal "cuts", several optical sections are obtained that can be reconstructed in a 3D image. The laser beam is scanned across the sample by a set of galvanometer mirrors or acousto-optic deflectors. Another advantage of this method is that multiple lasers can be used to excite various fluorophores in the sample. When the fluorophores that are present in the sample (native or added) are excited by the laser, they emit light, which passes through the pinhole reaching the detector. Finally, filters are used to separate the emitted light based on wavelength, enabling multi-color imaging (Elliott, 2020).

CLSM systems are sophisticated, expensive and require significant investment. The operation and maintenance of CLSM requires specialized training and expertise. Fluorophores can degrade under intense laser illumination, leading to the phenomenon called bleaching, limiting the duration of live imaging experiments. Finally, samples require careful preparation to ensure proper staining and preservation of samples. Despite its high cost and complexity, the benefits of CLSM make it a valuable technique for advancing scientific knowledge and innovation.

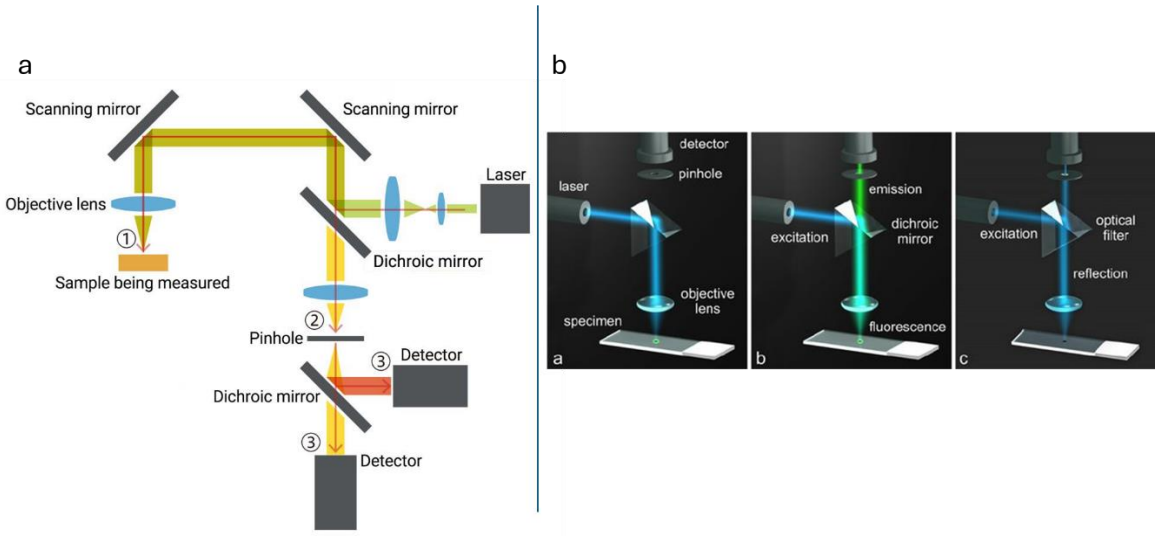


Figure 5.2.2: (a) Basic principle of a CLS microscope (Laser Scanning Microscope | Hamamatsu Photonics, n.d.) and (b) 3 different laser wavelengths appearing in different colors (Prasse et al., 2013)

The applications of CLSM vary, from medical and biological research to pharmacology and material science (Reigoto et al., 2021; Stožer et al., 2021). Especially in material science it can be employed for analyzing the structure and composition of polymers as well as to visualize nanoparticles and nanostructures like colloidal dispersions, assess their distribution and interactions (Sharif et al., 2020). It can also be used to observe interfaces and detect specific molecules such as proteins or polysaccharides (Xue et al., 2020). In the case of PEs, their emulsifiers, aqueous and oil phases are often stained to get a visual of how they are structured inside the emulsion, what is the encapsulation efficiency as well as to follow the release of encapsulated substances (Bi et al., 2020; Kubota et al., 2020; Sharkawy et al., 2021).

5.2.3 Cryogenic Scanning Electron Microscopy (Cryo-SEM)

Cryo-SEM is an advanced, sophisticated microscopy technique that combines the principles of SEM with cryogenic preparation methods. It is used to observe specimens containing water or fats at a frozen state. It is particularly useful for analyzing liquids, biological materials, and "wet" food products since otherwise artifacts would be introduced due to evaporation, chemical fixation or thermal damage during conventional SEM analysis. The principle of Cryo-SEM involves rapid sample freezing to preserve its native structure and composition. The frozen sample is then fractured, sublimated, and coated with a thin layer of conductive material (usually gold or platinum) before the final observation.

The main components of a Cryo-SEM are the same in all devices and follow the same principles. First, there is a preparation chambre where freezing, fracturing, and coating are performed under vacuum conditions to avoid contamination. Afterwards, there is a system for transferring the frozen sample from the preparation chamber to the SEM while maintaining cryogenic temperatures and finally the main instrument where the sample is imaged. It includes an electron gun, lenses, detectors, and a vacuum system. Inside the SEM there is also a stage that keeps the sample at cryogenic temperatures during imaging.

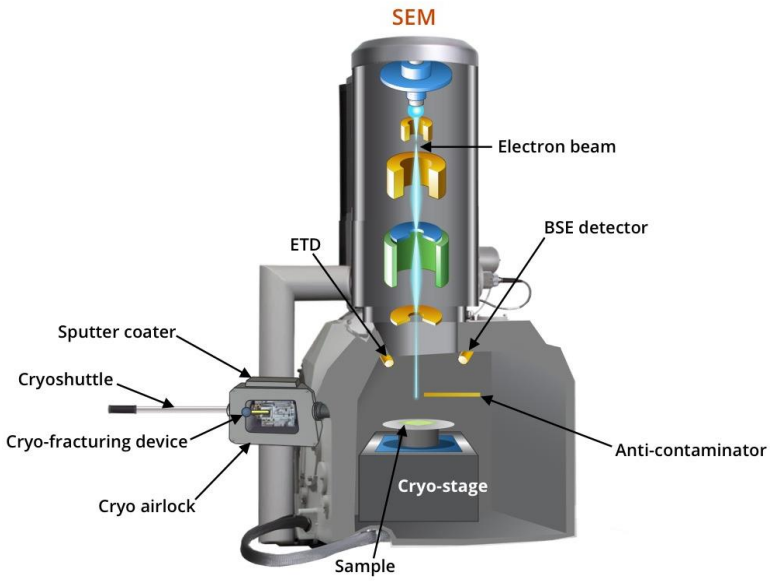


Figure 5.2.34: Illustration of the parts of a typical Cryo-SEM device (Cryo-SEM Design CRYO)

The first challenge of this method is met during its very first step of rapid freezing. It is important to ensure that the liquid sample, quite often with a high vapor pressure system, is compatible with high vacuum in the electron microscope, as all of them must operate under high vacuum typically 10^{-5}-10^{-11} Torr. The sample is rapidly frozen using liquid nitrogen or other cryogens with techniques, such as plunge freezing, high-pressure freezing, or slam freezing. Vitrification requires very high cooling rates of at least 105 K/s for water and of about 7000 K/s for organic solvents. The frozen sample is then fractured in a vacuum chamber to expose internal structures. Following, the sample undergoes controlled sublimation to remove a thin layer of ice to enhance surface contrast and reveal more structural details. The image quality is also preserved by coating the sample with a conductive material to avoid charging by the beam. The prepared sample is finally transferred to the SEM chamber, where it is kept at cryogenic temperatures. An electron beam scans the sample, and secondary electrons emitted from the sample surface are detected to form an image (Koifman & Talmon, 2021; Liang et al., 2021).

Cryo-SEM can be used in a wide range of applications including biological systems, material science and pharmaceuticals. More specifically, in biological systems it can be used to monitor cells, tissues, and organelles in their native, hydrated state. Imaging bacteria, viruses, and biofilms without the need for dehydration is also possible (Meibom et al., 2023). Furthermore, studying plant tissues, including leaves and roots, in their natural hydrated state gives insights on their morphology as close as possible to their native state (Wightman, 2022). The examination of the morphology and phase separation of hydrated polymer gels as well

as the visualization of nanoparticles in liquid dispersions is efficient and detailed through Cryo-SEM (Stewart, 2017; K. Yu et al., 2023). The food industry has also taken advantage of this method's refined results by investigating the microstructure of food products, such as emulsions and gels. For example, it is possible to observe the ice morphology in frozen foods that affects the overall quality and also observe and measure nonencapsulated food ingredients (Pérez-Bermúdez et al., 2023; Sarabandi et al., 2020).

Overall, Cryo-SEM is a powerful technique with significant advantages for depicting hydrated and biological samples in their native state (Wightman, 2022). It combines the high-resolution abilities of SEM with cryogenic sample preparation, providing detailed and artifact-free images. Despite its high costs and complexity, it is an invaluable technique for biology, material science, and pharmaceuticals, where preserving the natural state of samples is essential for accurate analysis and understanding of complex structures.

6. Emulsions' functional and bioactive properties assessment

Emulsions are complex systems composed of different components that carry certain activities/properties. An emulsion, in order to be fully characterized, needs to be assessed for its functionality and not only its structural properties. Emulsion's ingredients can endow the system with desirable characteristics, such as antioxidant and antimicrobial abilities, biological properties, conductivity, porosity and many more. These properties can come either from potential encapsulated molecules or, as in the case of PEs, from the materials used for their stabilization. Assessing the functional properties of emulsions is crucial for ensuring their stability, performance, and suitability for specific applications.

6.1 Antioxidant activity

Oxidative stress is defined as an increase in cellular free radicals' levels, which create an imbalance toward shifting the cellular environment toward an oxidant state. Free radicals in the forms of reactive oxygen species (ROS) are continuously produced in human cells. However, cells are also equipped with an antioxidant system that can neutralize the adverse effect of free radicals. In order to increase the antioxidant potential of a food, products are fortified with bioactive, antioxidant ingredients, usually extracted from natural sources. The presence of antioxidants in foods is highly important as they protect the body from oxidative stress, inhibit the oxidation of lipids, and help maintain the color, flavor, and texture of the food matrix. This enhances the overall quality and shelf-life of food products. Foods rich in antioxidants are considered to be of high nutritional value. Antioxidants are often introduced in the final products encapsulated in an emulsion. This condition has led to the need for methods that could accurately estimate the overall antioxidant capacity (Siddeeg et al., 2021).

The methods used for the assessment of antioxidant capacity are usually divided in two categories namely, hydrogen atom transfer (HAT) method, which donates a hydrogen from a stable molecule thus allowing

the antioxidant to scavenge the ROS, and single electron transfer (SET), which depends on the potential of the antioxidant to reduce certain molecules and compounds by receiving an electron (Figure 6.1a). Typical examples of HAT-based tests are the Oxygen Radical Absorption Capacity (ORAC), Total Peroxyl Radical Trapping Antioxidant Parameter (TRAP) and Total Oxyradical Scavenging Capacity (TOSC) assays. On the other hand, the SET tests, include the Folin–Ciocalteu method (FC), the ferric reduction of antioxidant power (FRAP), and the tests of reducing the cupric antioxidant capacity (CUPRAC) (Munteanu & Apetrei, 2021).

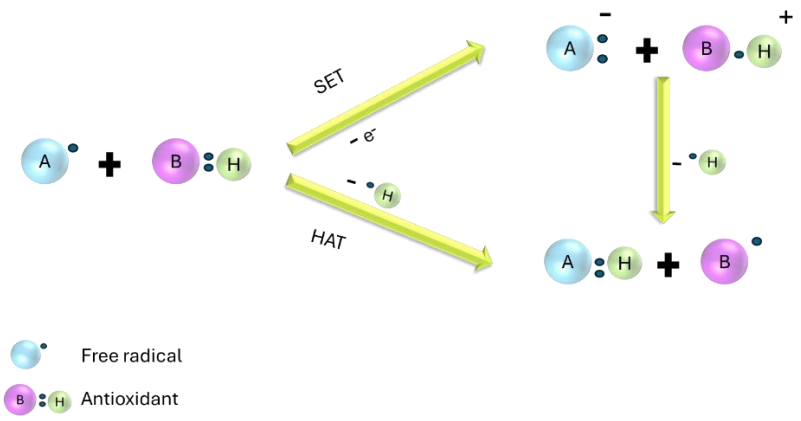


Figure 6.1a: Illustration of the SET and HAT methods mechanisms for antioxidant assessment

Additionally, there are spin trapping techniques that can be used for identifying the effects of various oxidative stresses in biological samples by providing a direct identification of free radicals. One of the most sophisticated and accurate methods of spin trapping is Electron Paramagnetic Resonance (EPR) spectroscopy. This technique involves the reaction of these radicals with a spin trap to generate stable adducts whose signal is detected by EPR (Chatzidaki et al., 2022; Marchi et al., 2022).

Some of the most used methods of antioxidant activity assessment are the mixed SET/HAT tests. These mixed tests are generally based on the elimination of a stable chromophore, like 2,2'-azinobis-3-ethylbenzthyazolin-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazil (DPPH), where HAT, SET, and proton-coupled electron transfer (PCET) mechanisms play different roles depending on the corresponding reaction conditions (such as pH and solvent). Mixed mode tests (HAT/SET) mainly include the ABTS/Trolox equivalent antioxidant capacity (TEAC) test, the DPPH colorimetric assay, and the N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) radical neutralization test (Siddeeg et al., 2021).

DPPH is a stable free radical present in its monomer form in a solid state as well as in solution. It can be dissolved in different organic solvents, but not in water. The neutralization of DPPH is what the test

measures and it is based on donating electrons from the antioxidant in order to neutralize the DPPH free radical. The reaction is accompanied by changing the DPPH color measured at ≈515 nm. The decrease in absorbance because of the discoloration acts as an indicator of antioxidant activity (Figure 6.1b). The results are often expressed as % Inhibition given by the following equation, where A_0 represents the absorbance of the control sample, and A_1 represents the absorbance observed after a reaction time period (Liu et al., 2023).

$$\%Inhibition = \left[\frac{A0-A1}{A0}\right] * 100 \quad (9)$$

The DPPH method has certain limitations as any other method. First, poor solubility of DPPH in water creates the need for fine tuning in different solvents. Furthermore, it has been shown that the reaction of DPPH and the antioxidant activity are highly dependent on accessibility and steric properties since different materials tend to get "on the way" of the antioxidant. This makes complex systems like foods more difficult in their assessment by this method. Additionally, the overlapping spectra of compounds that absorb in the same wavelength range as DPPH can be a significant drawback. For instance, anthocyanins exhibit absorption at 500–550 nm as does DPPH, which could introduce interference into the data and affect their interpretation (Christodoulou et al., 2022). Nevertheless, DPPH is one of the most widely applied methods in food and pharmaceutical applications. It gives a better response mostly for phenolic compounds followed by compounds with limited polarity. The reaction rate of DPPH with antioxidants depends on the varying ratios of mixed SET and HAT mechanisms (Gulcin & Alwasel, 2023).

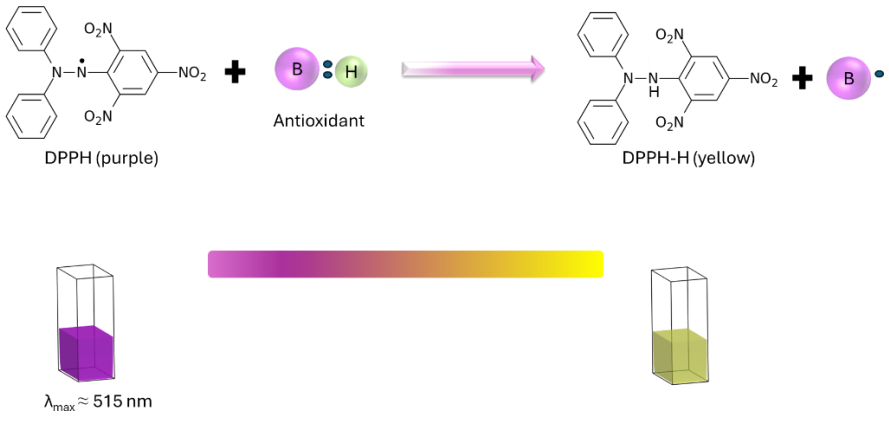


Figure 6.1b: Reaction mechanism of DPPH with an antioxidant

6.2 Antimicrobial activity of fortified or empty emulsions

The antimicrobial activity refers to the potential of compounds to kill or inhibit the growth of microorganisms. This property is crucial for several fields, such as medicine, agriculture and food preservation. The effectiveness of an antimicrobial agent depends on several factors, including its chemical nature, concentration, mode of action, and the type of targeted microorganisms. Emulsions of different types can also exhibit this ability that is usually bestowed upon them by their ingredients, by the molecules they carry or by their combination (Figure 6.2). Emulsions with antimicrobial abilities are increasingly being explored for their potential applications in food preservation, pharmaceuticals, and cosmetics. These emulsions can prevent or reduce microbial growth, extending the shelf life of products and ensuring safety.

Some of the natural antimicrobial agents that can be found in emulsions usually as encapsulated molecules are essential oils and organic acids, like lactic and citric acid, can disrupt cell membranes, inhibit enzyme activity, and affect microbial DNA (Angane et al., 2022; Coban, 2020). A group of antimicrobial compounds called bacteriocins, like nisin, produced by LAB, form pores in bacterial cell membranes, leading to cell death can also be used in emulsions as parts of their aqueous phase or encapsulated in reverse micelles (Mani-López et al., 2022). Lately, the antimicrobial potential of the natural biopolymer chitosan is widely studied with promising results. These polymers can also be used as emulsifiers, leading to the formation of functional carriers. (Ke et al., 2021). Many more antimicrobial agents can be found in nature in sources, like herbs, spices, and plant extracts, and they can be delivered through encapsulation in emulsions (Shwaiki et al., 2021, Wu et al., 2022).

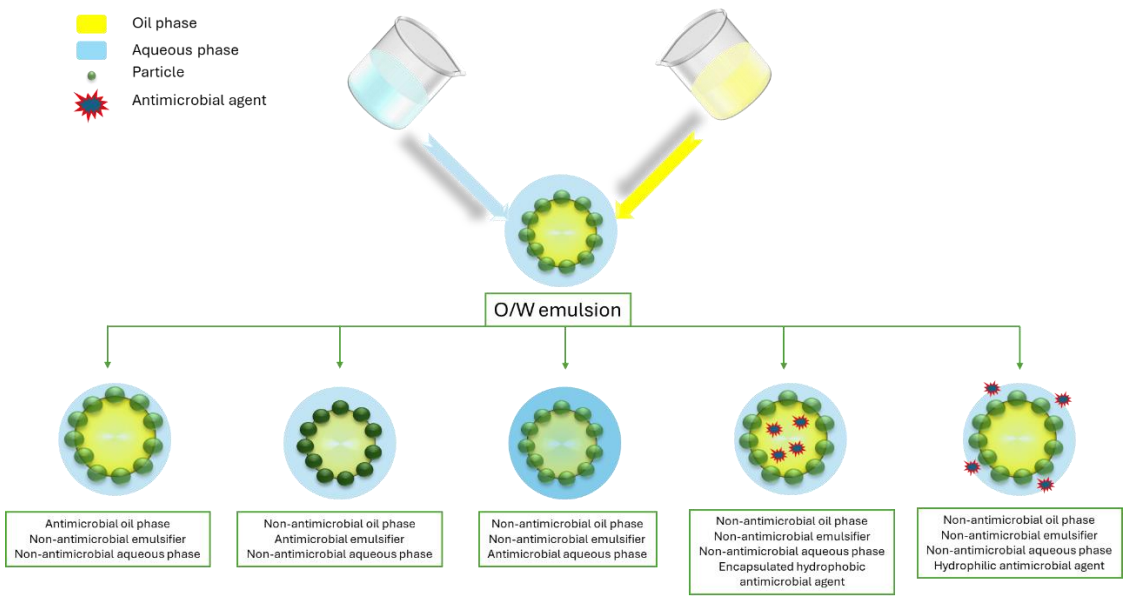


Figure 6.2: Different antimicrobial emulsions

There is a plethora of methods for the determination of a substrate's antimicrobial potential that are widely used. First, there are agar diffusion methods including the Disk Diffusion (Kirby-Bauer Test) and Well Diffusion assays which operate on similar principles (Chatzidaki et al., 2019). In these methods, the antimicrobial agent is placed on an agar plate inoculated with the test microorganism. The agent's insertion is either on a disk or in wells formed in the agar. The halo around the disk or well indicates antimicrobial activity. Both techniques are useful for the assessment of liquid samples. Following, there are broth dilution methods divided in Minimum Inhibitory Concentration (MIC), where the lowest concentration of the antimicrobial agent that inhibits visible growth of the microorganism in broth culture is estimated, and Minimum Bactericidal Concentration (MBC), where the lowest concentration of the antimicrobial agent that kills 99.9% of the target is measured. Another commonly used method is flow cytometry that uses fluorescent dyes to differentiate living and dead cells after treatment with the antimicrobial agent (Paparella et al., 2008). Finally, there are Time-Kill assays that monitor the reduction in viable microorganism's counts over time after exposure to the antimicrobial agent. For the kill-assays, usually microorganisms are exposed to the antimicrobial agent at various concentrations. Samples are taken at different time points and plated on agar and viable cells are enumerated to assess the antimicrobial activity (Balouiri et al., 2016; Hossain, 2024).

Antimicrobial tests are essential for evaluating the effectiveness of substances in inhibiting or killing microorganisms. The number of existing methods for the determination of antimicrobial ability, gives a very

wide range of monitoring different parameters and mechanisms (Jamali et al., 2021). These tests are crucial in the food industry, pharmaceuticals, clinical microbiology, and environmental monitoring, ensuring safety, efficacy, and quality.

6.3 Probiotic bacteria

6.3.1 Health Benefits

The general concept of microbial cells as particles has also been transferred in food industry related applications exploiting their probiotic activities. Probiotic microorganisms are living microorganisms that when administered in adequate amounts, offer the host some benefit (FAO: *Food Safety and Quality: Probiotics*, n.d.). Although the list of probiotics in literature keeps growing, the question that arises is how to determine the actual probiotic potential of microorganisms. Potential probiotics are chosen after conducting *in vitro* or *in vivo* assays that evaluate basic characteristics like resistance to stomach acidity or intestine bile, or their effects on complex host functions, such as immune development, metabolic processes, or gut-brain interactions. Although human clinical trials are essential for validating health benefits, only a limited number of strains with positive study results have successfully convinced regulatory authorities to approve these health claims (Papadimitriou et al., 2015).

Probiotic microorganisms have demonstrated several beneficial functionalities with respect to human health (Figure 6.3.1(a)). Their most recognized action is the improvement of gut health either by maintaining the balance of gut bacteria, or by repopulating the gut with beneficial bacteria after a possible disturbance (dysbiosis) of the gut's microbiome (Bodke & Jogdand, 2022). Additionally, they can stimulate the immune system, increasing its ability to fight off infections and diseases. They have been shown to relieve symptoms, such as irritable bowel syndrome (IBS) and respiratory tract infections (Maftei et al., 2024). Furthermore, they have been linked to improved skin health, particularly in infants and children reducing symptoms of atopic dermatitis (Amalia et al., 2020). Some research suggests that probiotic supplementation can contribute to weight loss (Álvarez-Arraño & Martín-Peláez, 2021), while others also claim that the consumption of probiotics can improve inflammatory markers and glycemic control and insulin (Ding et al., 2021; Lacerda et al., 2022) It has also been suggested that the administration of probiotics improve markers of cardiovascular diseases, including hypertension (Hendijani & Akbari, 2018). Finally, the past decade gave more space for the study of probiotics in relation to diseases affecting other parts of the human body. Liver disease has become a focus of probiotic research, driven in part by observations of alterations in gut microbiota linked to the development of the disease. Probiotics' activity mechanisms have been also studied for battling even more serious conditions, such as colon cancer or HIV. This focus is due to the significant impact of colonic bacteria on gut metabolism, the immune system, and colonic cell division as well as their potential to influence gut barrier function and mucosal

immunity. Although limited, there are few clinical studies related to the effect of probiotics on the gut-brain axis, mostly investigating mood and/or psychological distress (Zoumpopoulou et al., 2017).

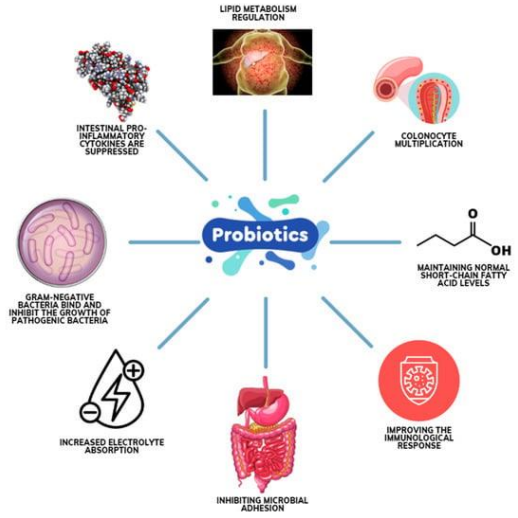


Figure 6.3.1(a): Mechanisms of action of probiotics (Maftei et al., 2024)

The most studied probiotic strains so far belong to the following genera: *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus, Leuconostoc*, and *Bacillus*. In the present thesis three lactic acid bacteria strains (Table 6.3.1), namely, *S. thermophilus* ACA-DC 26, *Lb. bulgaricus* ACA-DC 87, and *Lb. fermentum* ACA-DC 179, were used and they are going to be presented. Since the probiotic potential is a strain dependent trait, below are presented the data obtained, for each of the three aforementioned strains, after extensive research.

In general, *S. thermophilus* is a gram-positive, lactic acid bacterium that plays a crucial role in the production of fermented milk products, such as yogurt and cheese. It has a coccus structure and thrives at temperatures typically above 40°C, while it can survive in the GIT (Uriot et al., 2017). More specifically, *S. thermophilus* ACA-DC 26 was isolated from artisanal sheep milk yogurt. It exhibits antimicrobial activity against the oral pathogen *Streptococcus* mutans LMG 14558^{T}, as well as anti-inflammatory action since it induces IL-10 production in THP-1 cells–. It is not hemolytic and susceptible to eight common antibiotics, namely, gentamycin, kanamycin, tetracycline. streptomycin ampicillin, vancomycin, erythromycin and chloramphenicol. (Zoumpopoulou et al., 2018).

Another very well-known starter culture to produce yogurt and cheese is *L. bulgaricus*, a gram-positive, rod-shaped, non-motile, and non-sporulating bacterium (Crow & Curry, 2002). In particular, *L. bulgaricus* ACA-DC 87 isolated from traditional Greek yogurt (Tsakalidou et al., 1994) was studied for its probiotic properties. It exhibited antihypertensive ACE-I activity when grown in milk, with the activity being linked to the type of

milk, be it goat, sheep or cow milk. The same study reported that *L. bulgaricus* ACA-DC 87 also demonstrates strong proteolytic activity, compared to other LAB strains. This strain is resistant to the antibiotic kanamycin, while susceptible to all others tested (Georgalaki et al., 2017). Furthermore, in order to shed more light on the technological traits of this strain, the complete genome sequence has been performed and analyzed (Alexandraki et al., 2017).

L. fermentum, in general, is a gram-positive, rod-shaped bacterium that belongs to the genus *Limosilactobacillus*. It is most commonly found in fermented animal and plant materials, including sourdough and cocoa. Specifically, when it comes to *L. fermentum* ACA-DC 79, it was found that it can produce bacteriocins against oral pathogens, more specifically, against *Streptococcus oralis* (Zoumpopoulou et al., 2013) and against *Listeria monocytogenes* and *Salmonella enteritidis* in raw meat (Maragkoudakis et al., 2009). In addition, it shows anti-inflammatory activity i*n vitro* and *in vivo*. Furthermore, when it was used in a *Salmonella*-infected mouse model, its administration revealed an *in vivo* anti-*Salmonella* activity (Zoumpopoulou et al., 2008). Finally, *L. fermentum* ACA-DC 179 exhibited antiviral activity efficiently protecting human and animal intestinal epithelial and immune cells from enteric virus infection (Maragkoudakis et al., 2010).

In general, several techniques can be used to determine the anti-inflammatory activity of probiotic bacteria strains. These methods can be categorized based on *in vitro* (laboratory-based), ex vivo (using tissue samples), and *in vivo* (animal or human studies) approaches. *In vitro* techniques, also used for the present dissertation, include cell culture models, like human intestine epithelial cells, where the effect of the probiotics on them is observed and cytokine assays like ELISA tests. In that case, the levels of pro-inflammatory and anti-inflammatory cytokines (e.g., TNF-α, IL-6, IL-10) in cell culture supernatants after treatment with probiotics are determined.

Table 6.3.1: Bacteria strains used in this dissertation and their origin

Strain	**Origin**
Streptococcus thermophilus ACA-DC 26	Artisanal sheep milk yogurt
Lactobacillus delbrueckii subsp. *bulgaricus* ACA-DC 87	Yogurt
Lactobacillus fermentum ACA-DC 179	Kasseri cheese

The assessment of probiotic potential involves evaluation of functional properties as well as safety of the examined microorganisms, such as their ability to survive in the GIT, inhibit the growth of pathogens, and produce beneficial substances (Aziz et al., 2023; Casarotti et al., 2017; Zoumpopoulou et al., 2018). One of the

methods that can be employed to determine probiotic potential is the Enzyme-Linked Immunosorbent Assay (ELISA) test. ELISA measures the ability of the probiotic strain to stimulate the immune system by quantifying the level of specific antibodies produced in response to the strain. ELISA can specifically detect target microbial and thus probiotic strains using antibodies, reducing cross-reactivity with non-target microorganisms. It measures the level of specific antibodies produced in response to the probiotic strain, indicating its ability to stimulate the immune system. ELISA kits usually follow the same basics steps starting with the coating where the antigen or the antibody is immobilized on a microplate surface. The tested sample is added, allowing the target antigen to bind to the immobilized antibody (in a sandwich ELISA) or the antibody in the sample to bind to the immobilized antigen (in a direct or indirect ELISA). Following, a secondary antibody, conjugated with an enzyme is added, which then binds to the antigen-antibody complex. Finally, a substrate for the enzyme is added, producing a measurable color change, fluorescence, or chemiluminescence and the intensity of the signal is measured using a plate reader, and the concentration of the target is determined with the use of a standard curve (Plested et al., 2003).

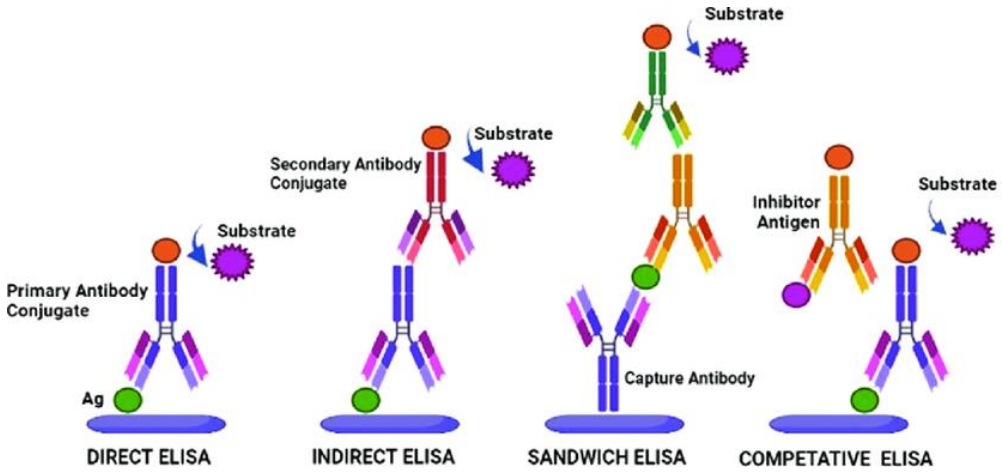


Figure 6.3.1 (b): Different ELISA types (Khan et al., 2023)

In some cases, untargeted metabolomics analysis is performed in order to avoid any possible biases coming from the ELISA test. During this procedure the complete set of unidentified metabolites within a biological sample are analyzed. The metabolome is defined as the complete set of small-molecule chemicals within a biological sample, including endogenous (produced by the organism) and exogenous metabolites (from the environment, diet, etc.). Unlike targeted metabolomics, which focuses on pre-selected metabolites, untargeted metabolomics aims to detect and quantify as many metabolites as possible in a sample. To do so, after the extraction of metabolites from the tested cells, chromatography and mass spectroscopy (MS) are usually performed. Initially, for the separation of metabolites techniques, such as liquid chromatography (LC)

or gas chromatography (GC) are performed. These methods help to reduce the complexity of the sample before analysis. After, MS detects and quantifies metabolites based on their mass-to-charge ratio (m/z). Advanced MS techniques can also provide structural information about metabolites. Finally, the data processing involves peak detection, alignment, normalization, and annotation using databases and software tools, while for the statistical analysis multivariate methods are used to interpret the data, identify patterns, and distinguish between different sample groups based on their metabolic profiles (Broeckling et al., 2023; Schrimpe-Rutledge et al., 2016).

Overall, the assessment of probiotic activity can be challenging and complex. Nevertheless, it provides valuable insights on the activity and functionality of the tested subject. Evaluating the probiotic potential of microorganisms is a comprehensive process that ensures their beneficial impact on health efficacy and safety. This evaluation is essential for regulatory approval, consumer confidence and safety, and the advancement of probiotic research and applications in various health domains.

6.3.2 Probiotics in emulsion applications

The most common way, probiotics are connected to emulsions is by encapsulation, meaning that the emulsions are used as vehicles for the delivery of the microorganisms and as a means of their protection. Reports showed that encapsulated probiotics exhibit enhanced durability in harsh environments thus increasing their potential post administration (Haji et al., 2022). In another case, inulin was exploited to develop a stable dressing emulsion able to sustain the viability of the probiotic microorganism *Lactobacillus paracasei* subsp. *paracasei* DC412. The encapsulation protected the cells when exposed to GIT enzymes (Mantzouridou et al., 2012). Spherical microgels formed by gellan gum were found resistant to oral and gastric digestion and were able to encapsulate and deliver *L. rhamnosus* protecting the microorganism from the environmental conditions (Picone et al., 2017).

In limited cases, probiotic microorganisms have been used for the stabilization of PEs. After extensive literature review only two other research articles were found that examined the stabilization of PEs by probiotic bacteria. In the first and more recent study, 19 food-grade microorganisms were tested as PE stabilizers. Thermally inactivated yeast, cocci, *Bacillus* spp. and lactobacilli cells were tested in terms of morphology, surface charge, interfacial tension, and contact angle. The reported physical stability of microorganism-stabilized PEs was given in the following order: lactobacilli > *Bacillus* spp. > cocci > yeast. The variations were attributed to the morphology and cell wall composition while it was also observed that by increasing the microorganism concentration, the physical stability of PEs also increased (Nejadmansouri et al., 2023). In the second study, thermally inactivated LAB strains (*Lactobacillus acidophilus and Streptococcus thermophilus*) and yeast (*Saccharomyces cerevisiae*) cells were used as Pickering colloidal particles. The study reported that oil droplets were stable against

coalescence and bulk phase separation for over four months. The microbial cells acted as particle stabilizers by residing at the oil–water interface (Firoozmand & Rousseau, 2016).

Saccharomyces cerevisiae, *Lactobacillus acidophilus*, and *Streptococcus thermophilus* were used untreated and produced stable emulsions (Firoozmand & Rousseau, 2016). In another case, *Lactobacillus acidophilus* was treated by octenyl succinic anhydride (OSA) and the treatment increased PEs stability (Jiang et al., 2019). Finally, a very recent study explored the potential of food-grade microorganisms as PE stabilizers. More specifically, thermally inactivated yeast, cocci, *Bacillus* spp. and lactobacilli cells were tested. The resulting emulsions were of various droplet sizes and stability times with the differentiations being attributed to the bacteria morphology and cell wall composition (Nejadmansouri et al., 2023). Nevertheless, none of these studies examine whether the microorganisms retain their probiotic potential when adsorbed on an interface to support their claim.

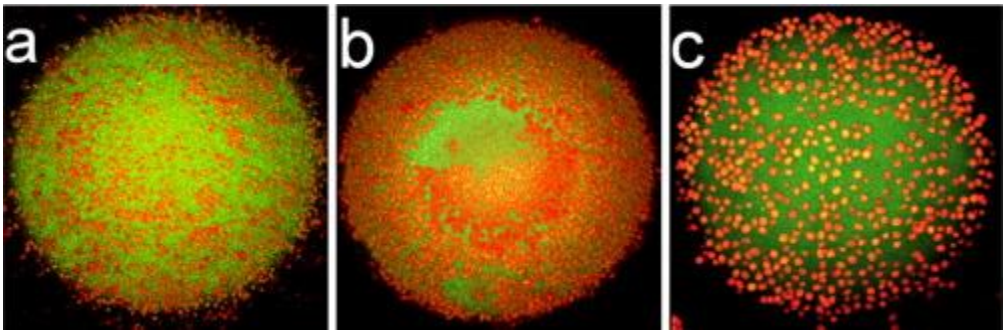


Figure 6.3.2: Adsorbed cells on oil droplets: a) S. thermophilus;b) L. acidophilus;c) S. cerevisiae (Firoozmand & Rousseau, 2016)

Aim of the study

The modern food industry is facing an increasing demand for healthier and more sustainable food options. As the human population grows, the production of processed foods from animal sources is increasing. Unfortunately, this type of diet often contains saturated fats and results in diet-related diseases, such as obesity and diabetes. It has also led to the outbreak of infectious diseases and antibiotic resistance. Moreover, the increased demand for animal origin products, such as milk, eggs, and meat, has a huge effect on the environment and raises many ethical issues concerning animal welfare. Additionally, food safety has become an urgent matter for discussion due to the increasing globalization of food supply chains, which are associated with potential health risks from unsafe or contaminated food.

Functional foods are specially designed or modified to promote the well-being and health of consumers beyond basic nutrition. The demand for these foods has increased due to modern lifestyles that necessitate quick, convenient food options like store-bought meals. One of the ways functionality is achieved is by the introduction of emulsions in food matrices. When emulsions are used, it is usually in order to achieve properties, such as the extension of shelf-life, the replacement of ingredients like conservatives or antibiotics and endowing products with desirable characteristics. The interest has lately shifted from conventional to Pickering emulsions (PEs).

Therefore, the aim of this thesis was to develop novel, sustainable, and functional PEs that could also serve as carriers for bioactive molecules. For this purpose, PPI, SPI, PC, FC, and BFCs were used as emulsifiers. In all prepared emulsions 2 model bioactives, namely α-tocopherol or squalene, known for their antioxidant properties, were encapsulated.

PPI's properties were further enhanced through particle formation with pH-shifting combined with HPH, which resulted in finely dispersed smaller particles that were able to stabilize emulsions of smaller droplet size and increased stability. In order to further functionalize the emulsions, PC was added carrying a triple role as co-emulsifier, natural coloring and antioxidant agent. HPH was also applied for the emulsion formation, following the preparation of a coarse emulsion through HSH.

Additionally, functional PEs were formulated using FC particles. The resulting emulsions aimed to the use of FC both as an emulsifier and an antimicrobial agent.

Finally, FC was combined with probiotic LAB strains to form particles capable of creating stable emulsions. The particles were formed by exploiting the electrostatic interaction of the oppositely charged bacteria and FC. Their ability to form emulsions was also studied by examining the DIT between water and oil in their presence. The biggest challenge of this project was to support the claim that FC and the bacteria retained their properties while they were conjugated and adsorbed at the water-oil interface.

All previously described systems were used as carriers for two very important natural bioactive components, namely α-tocopherol and squalene. They are both oil soluble substances and can be incorporated in the core of oil droplets. Herein, they were studied for their antioxidant activity, and it was found that they exhibit this potential when encapsulated in emulsion droplets. All emulsions were structurally analyzed, and further chemical analyses were conducted to target the unique functional properties of each emulsion. The several experimental methods performed, as well as the results obtained are presented in Chapters 2-4 as they have already been published. Overall, the work of the current thesis is analytically described in 3 publications and 1 submitted manuscript.

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Chapter 2

Pea and Soy Protein Stabilized Emulsions: Formulation, Structure, and Stability Studies

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Pea and Soy Protein Stabilized Emulsions: Formulation, Structure, and Stability Studies

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Abstract: During the last decades, there has been a huge consumer concern about animal proteins that has led to their replacement with plant proteins. Most of those proteins exhibit emulsifying properties; thus, the food industry begins their extensive use in various food matrices. In the present study, pea and soy protein isolates (PPI and SPI) were tested as potential candidates for stabilizing food emulsions to encapsulate α-tocopherol and squalene. More specifically, PPI and SPI particles were formulated using the pH modification method. Following, emulsions were prepared using high-shear homogenization and were observed at both a microscopic and macroscopic level. Furthermore, the adsorption of the proteins was measured using the bicinchoninic acid protein assay. The emulsions' droplet size as well as their antioxidant capacity were also evaluated. It was found that the droplet diameter of the SPI-based emulsions was 60.0 μm, while the PPI ones had a relatively smaller diameter of approximately 57.9 μm. In the presence of the bioactives, both emulsions showed scavenging activity of the 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\cdot+}$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, with the ones loaded with α-tocopherol having the greatest antioxidant capacity. Overall, the proposed systems are very good candidates in different food matrices, with applications ranging from vegan milks and soups to meat alternative products.

Keywords: pea protein isolate; soy protein isolate; food emulsions; particle-stabilized emulsions

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1. Introduction

The modern food industry is facing an increasing demand for healthier and more sustainable food options. As the human population grows, the production of processed foods from animal sources is increasing. Unfortunately, this type of diet often contains saturated fats and results in diet-related diseases, such as obesity and diabetes [1,2], or has led to the outbreak of infectious diseases and antibiotic resistance [3]. Moreover, the increased demand for animal products, such as milk, eggs, and meat, has a huge effect on the environment and raises many ethical issues concerning animal welfare [4]. According to the suggestions of the EAT-Lancet commission, there should be a significant decrease on the consumption of animal-derived products by 2030 [5]. For the specific case of dairy products, allergies as well as lactose intolerance have led consumers to search for healthier alternatives. The commercial milk-alternative products based on coconut, soy, almond, and other sources are poorly accepted by the consumers due to the differences in quality, organoleptic characteristics, and functional attributes, such as stability, creaming, undercooking, etc. [6]. Most of the plant proteins currently used in the food industry are derived from wheat or soybeans. Nevertheless, due to the allergenicity of both ingredients and consumer demand for alternative options, there is an increasing interest in the use of proteins from peas, faba beans, rice, and other plants. Even though soybean may face some

challenges since it is listed as a food allergen [7], it remains one of the most promising plant proteins in terms of stability and functionality. More specifically, soybean proteins have shown important functional properties such as emulsifying, wetting, and/or gelling [8]. Pea protein has also been found to show important properties when used in food matrices [9]. These important properties have made soybean and pea proteins acceptable as alternatives to animal proteins in the food industry, especially in products such as plant-based milks or even meat-analog products [10,11].

Among the technological strategies used to design and develop these novel products are those based on structural modifications of food systems. This could be accomplished by changes in the pH, temperature, solubility, and other parameters to improve the texture and mouthfeel of the end product. Emulsions are systems widely used for the development of food products. However, in the past decades, one specific type of emulsion has received increasing interest, namely particle-stabilized emulsions. Ramsden and Pickering were the first to study the interfacial phenomena taking place in these emulsions, and independently observed that solid particles were able to stabilize the interface between two immiscible liquids [12,13]. Particle stabilized emulsions have the great benefit of being surfactant-free, and functional particles with high nutritional value, such as proteins, lipids, polysaccharides, etc., can be used as stabilizers [14]. The peculiarity of these types of emulsions is that the particles used are partially wettable, both by the water and the oil phases, and they are adsorbed at the oil-water interphase, forming a steric barrier, that prevents oil and water from coming into contact, thus stabilizing the emulsion droplets [15,16]. In this respect, protein-based emulsions have been proposed as versatile systems with tunable characteristics [17,18] that could act as good candidates for food applications.

These systems also have the ability to entrap bioactives with different solubilities, giving the final product health benefits. Such valuable bioactive compounds are α-tocopherol, widely known as vitamin E, and squalene. α-tocopherol is a plant-derived, lipid-soluble essential micronutrient and has been used in various fields, including pharmaceutics, cosmetics, and foods [19–22]. α-tocopherol has been found to play an important role in the maintenance of good health as well as the prevention and/or treatment of some diseases and disorders. The recommended daily intake is 15 mg (22.4 IU, International Unit) for adults [23]. Multiple functions of α-tocopherol have been reported, including antioxidant activity by scavenging free radicals, membrane stabilization by forming complexes with destabilizing molecules, regulation of enzyme activity, and prevention of diseases, including neurological disorders, cardiovascular diseases, and age-related skin damage [24–26]. Among these functions, the role of α-tocopherol as an antioxidant against free radicals has been unequivocally demonstrated and it appears that this is the most important function of α-tocopherol. α-tocopherol also inhibits air oxidation of foods and oils, expanding their shelf- life [27,28]. Squalene is a natural triterpene, and a lipid that acts as a precursor to the biosynthesis of sterols, including cholesterol. It is widely found in nature, and especially in vegetable oils, such as olive oil. Squalene has several beneficial properties. It is a natural antioxidant, serves in skin hydration, and has been used as an emollient in vaccines [29–31]. As a compound of olive oil, it has also been studied for anticancer and cardio-protective properties, while it decreases the cholesterol level [32–34]. In countries around the Mediterranean Sea, a high squalene consumption was observed (200–400 mg per day) through the intensive use of olive oil. Since squalene is not approved by the FDA for treating any conditions, there is no official dose. Users and supplement manufacturers have established unofficial doses based on trial and error. The average intake of squalene is estimated to be around 30 mg per day [35]. However, when olive oil plays a more prominent role in the diet, such as the Mediterranean diet, levels of squalene can reach anywhere from 200 to 400 mg per day. Shark liver oil supplements commonly contain between 120 to 500 mg of squalene per dose. Studies have indicated that squalene supplements are tolerable up to 27 g with mild side effects [36].

The aim of the present study is to examine the formation of particles from pea or soy protein isolates that would stabilize oil-in-water emulsions for food applications. In these

emulsions, α-tocopherol or squalene were encapsulated to enhance the system's antioxidant capacity while increasing the health benefits of the system. Overall, the proposed systems could be good candidates for novel food applications.

2. Materials and Methods

2.1. Materials

Pea protein isolate (NUTRALYS® F85F) (≥83% protein content) was purchased from Roquette, France. Soy protein isolate (≥90% protein content) was purchased from Ingretia Global Trading LLC (Miami, FL, USA). Acetic acid, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), α-tocopherol and potassium persulfate were purchased from Sigma-Aldrich (Chemie Gmbh Munich, Germany). The bicinchoninic acid assay kit (BCA) and Squalene (98%) were purchased from Thermo Fisher Scientific, (Waltham, MA, USA). Mygliol 812N (MCT) was purchased from IOI Oleochemical (Penang, Malaysia). Extra virgin olive oil (EVOO) and sunflower oil (SFO) were purchased from a local supermarket.

2.2. Experimental Design

2.2.1. Preparation of Particles

Both kinds of particles were prepared at four different concentrations, namely 0.1, 0.2, 0.3, and 0.5% w/v. This was in order to evaluate the ability of the particles to formulate emulsions and choose the most suitable to proceed with the rest of the experiments.

Pea Protein Isolate (PPI) Particles

The preparation of PPI particles was based on the previous study by Liang et al., (2014) [37], with some modifications. More specifically, native PPI was measured in glass vials in proper quantities to achieve the concentrations previously mentioned. Afterwards, the required volume of deionized water was added and then the dispersions were stirred for 30 min using a magnetic stirrer. Subsequently, as recommended by Liang et al., the pH was adjusted to 3.0, using HCl 1 M. The dispersions remained under magnetic stirring for 2 h and were finally stored overnight at 4 °C to allow the complete hydration of the proteins [37].

Soy Protein Isolate (SPI) Particles

Particles were prepared from native SPI using a variation of the method used by Jiang et al., (2009) [38]. In this method, proper quantities of SPI were dispersed in the required volume of deionized water, and the dispersions were placed under magnetic stirring for 30 min. Afterwards, the dispersions' pH was shifted to 3.0 with HCl 1 M, and the dispersions remained under magnetic stirring for 2 h. Following that, pH was adjusted to 7.0 to induce the refolding of the proteins, and the magnetic stirring was carried on for one more hour [38].

Both the PPI and SPI particles were stored in the fridge to avoid microbiological growth.

2.2.2. Determination of Particles' Dimensions and ζ-Potential by Dynamic Light Scattering (DLS)

A Zetasizer Nano ZS (ZEN3600) analyzer (Malvern Instruments Ltd., Malvern, UK) equipped with a He-Ne laser (633 nm) and non-invasive backscatter (NIBS) optics was used for initial particle size measurements of the SPI and PPI dispersions. The polydispersity index (PDI) of the particles was also determined. All samples were diluted to 0.01 mg/L, and the pH was readjusted, if necessary, because dilution with distilled water can affect the pH. For the ζ-potential measurements, no dilution was needed. For comparative purposes, the size of soluble proteins of PPI and dispersions prior to pH modification. For this, PPI and SPI were dispersed in water for 30 min. The dispersions were centrifuged to remove any insoluble parts (8000× g, 15 min), and the supernatant was diluted 500 times. Results were processed with the Malvern Zetasizer Nano software, version 6.32 (Malvern

Instruments Ltd., Malvern, UK), which fits a spherical model of diffusing particles with low polydispersity. Measurements were carried out in triplicate at 25 °C [39].

2.2.3. Morphology Observation of Particles by Freeze-Fracture Transmission Electron Microscopy (FFTEM)

Freeze-fracture (FF) replica preparation was performed by putting a drop of the sample onto a gold planchette, then freezing the sample by quickly plunging the holder into liquid propane that was held at the temperature of liquid nitrogen. The freezing step must be fast in order to vitrify the sample and avoid structure disruption due to crystallization. Frozen samples were then introduced in the freeze-fracture enclosure of a BAF 060 Leica Microsystems apparatus held at a temperature of −50 °C and a pressure of 10^{-8} mBar. The samples were fractured using a metal knife held at a temperature of −200 °C. The fractured surface was immediately shadowed by the successive deposition of platinum at an angle of 45° and carbon at an angle of 90°. Outside the BAF060, the gold planchettes were immersed in the sample solution to detach the replicas from the samples. These replicas were then immersed in several consecutive baths of ethanol and NaOH (1M) in order to destroy the left sample, and finally in pure water for a few more hours. The replicas were finally collected on 400 mesh copper grids and dried before transmission electron microscopy (TEM) imaging. TEM was performed with a HITACHI H 600 microscope operating at 75 kV [40].

2.3. Analytical Measurements

For the present work, two O/W emulsions were prepared and stabilized by SPI or PPI particles. For the preparation, the particles' dispersions (0.1, 0.2, 0.3, and 0.5% w/v), deionized water, acted as the aqueous phase, and EVOO as the oil phase. Both empty and loaded emulsions were prepared, with the loaded ones containing α-tocopherol or squalene as the encapsulated substance (1% w/w). α-tocopherol and squalene were dissolved in EVOO by magnetic stirring. To ensure the best dispersion in EVOO, the mixture was kept under stirring at 800 rpm for 20 min. EVOO containing the bioactives was then incorporated in the systems in the way presented below in Section 2.3.1. Furthermore, different water:oil ratios were examined, namely 80:20, 70:30, and 60:40 w/v, to assess the highest quantity of oil that can be encapsulated by each emulsion.

2.3.1. Preparation of Emulsions

The preparation method was based on the previously published work by Mwangi et. al., (2016) [41]. More specifically, the aqueous phase was measured in a glass vial followed by the stepwise addition of the oil, with or without the 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, a 4eflon bearing, an immersion depth of 150 mm, and at 10,000 rpm. Following the complete addition of the oil, the homogenization was carried on for 3 more minutes at 20,000 rpm [41]. Afterwards, emulsions were allowed to reach a steady state for a few hours before any further experiments or observations were made. Due to the drop sizes and the density mismatch between oil (density equal to 0.916 g/cm^3) and water, the systems were then separated into two layers, namely a serum and cream layer. The cream layer (emulsion) was used for all further analysis as well as for the stability assessment. Creaming is a natural phenomenon that is reversible under stirring. Coalescence is an irreversible phenomenon that leads to a macroscopic oil layer that is visible on the top of the emulsion. In the following sections, we will distinguish the two phenomena and discuss stability against creaming or coalescence.

2.3.2. Droplet Dimension Determination by Static Light Scattering (SLS)

A Mastersizer 2000 granulometer (Malvern Instruments Ltd., Malvern, UK) and the Mie theory were used to measure the emulsions' droplet size. Samples were added to

the "small volume sample dispersion unit" after proper dilution. The size distribution was characterized in terms of the surface-averaged diameter D and polydispersity P. The polydispersity index, also called uniformity (U), is defined as the volume-average difference between the diameter and the median diameter, normalized by the median diameter. The median diameter corresponds to the midpoint of the size distribution, meaning the diameter for which half of the dispersed phase is distributed in drops smaller than the median diameter and the other half is distributed in drops larger than the median diameter. These values result from Equations (1) and (2), where N_i is the total number of droplets with diameter D_i and D_{50} is the median diameter, i.e., the diameter for which the cumulative undersized volume fraction is equal to 50% [42,43].

$$D = \frac{\sum_i N_i D_i^3}{\sum_i N_i D_i^2} \quad (1)$$

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

2.3.3. Droplet Morphology Observation by Cryogenic Scanning Electron Microscopy (Cryo-SEM) and Microscopic Observation

A Leica DM IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted research microscope was used for the emulsion droplet observation. The microscope was equipped with ×4, ×10, ×20, ×40, and ×63 lenses. Samples were diluted using distilled water at the appropriate pH for each sample, specifically pH 7 for SPI samples and pH 3 for PPI samples. Samples were then placed on a microscope slide without a cover, and they were observed in a bright field.

Cryo-SEM observations were carried out with a ZEISS GEMINI 300 field emission scanning electron microscope operating at 1.5 kV. This SEM is equipped with a cryo stage PP3010T from Quorum Technologies, England. A small amount of emulsion was first set on the specimen holder and then frozen in a slushy nitrogen freezing station. Rapid freezing reduces ice crystal damage and results in improved specimen preservation. The sample was transferred into the cryo preparation chamber, held at −140 °C, and fractured with a cold blade. Then it was etched at −90 °C for 3 min or directly coated with platinum. Finally, the sample was inserted onto a highly stable SEM cold stage for observation [44].

It is worth noting that FFTEM and cryo-SEM differ in two major aspects. In FFTEM, after freeze fracturing, a replica of the sample is observed by TEM, while in cryo-SEM, after freeze fracturing, it is the sample itself that is observed by SEM and not by TEM.

2.4. Assessment of the Emulsions' Stability against Creaming

2.4.1. Determination of the Creaming Index Percentage (CI%)

To assess the stability of the emulsions against creaming, storage tests in two conditions were performed. In one case, emulsions (empty and loaded) were prepared in 12 mL flat bottom glass tubes (15 mm in diameter and 50 mm in height) with screw cups and were kept at ambient temperature. In the second case, the vials were kept in the fridge at 4 °C. The systems were separated into two layers (cream/emulsion and serum), within a few minutes after they were prepared. The formation and proportion of the cream layer (emulsion) during storage were observed. The data were collected every 5 days. Their stability against creaming was quantified by the creaming index ($CI\%$), which represents the cream layer height (H_c) expressed as a percentage of the total height of the emulsion height (H_E) in the tube and is calculated by Formula (3) [45]. The procedure was carried out three times.

$$CI = \left(\frac{Hc}{Ht}\right) \times 100(\%) \quad (3)$$

2.4.2. Determination of the Percentage of Adsorbed Proteins (AP%)

The bicinchoninic acid (BCA) method was also employed in order to determine the percentage of adsorbed proteins (AP%) as a means of explanation for each emulsion's stability time. The BCA assay method is based on the fact that the sodium salt of bicinchoninic acid reacts with the cuprous ion generated by the biuret reaction under alkaline conditions. The bicinchoninic acid cuprous complex forms a deep blue color that is read at 562 nm, and the detection range is 0.2–50 μg [46]. For this procedure, 1 mL of the pH-treated SPI and PPI particle dispersions, as well as 1 mL of the serum phase of the emulsion, were centrifuged (10,000× *g*, 5 min). The protein content of the supernatants was measured by the bicinchoninic acid (BCA) assay [47]. The protein fraction (*AP*%) was calculated by Equation (4), in order to assess the amount of proteins that are adsorbed on the oil-water interface.

$$AP = \left[\frac{C_0 - Cs}{C_0}\right] \times 100(\%) \quad (4)$$

In the formula, C_0 was the protein concentration in the initial dispersions, and *Cs* was the protein concentration in the aqueous phase [48]. The absorbance measurements were performed with a Safire2 (Tecan, Männedorf, Switzerland) plate reader. The procedure was carried out three times.

2.5. Assessment of the Emulsions' Antioxidant Capacity by DPPH Free Radical or ABTS $^+$ Cation Scavenging

The emulsions' antioxidant capacity was measured by a colorimetric DPPH assay. The procedure followed was the one previously described by Bi et al., (2021) [49] with slight modifications. Briefly, 2 mL of a DPPH solution in ethyl acetate (0.108 mM) was measured in a plastic tube, and afterwards, 100 μL of sample was added. The mixture was vigorously shaken and kept in the dark for 30 min. Following that, the absorption was measured at 515 nm. Ethyl acetate was used as a blank and a mixture of 2 mL DPPH and 100 μL of ethyl acetated was used as the control sample. The % inhibition of the free radical was calculated with Formula (5), where A0 is the control sample's absorption and A1 is the absorption after the 30 min reaction time [49].

The ABTS assay was also employed as a means to verify the scavenging ability of the bioactives against free radicals. The total antioxidant activity is measured by the ABTS + radical cation decolorization assay involving a preformed ABTS ÷ radical cation. ABTS ÷ is prepared by reacting ABTS (7 mM) with potassium persulfate (140 mM) in a ratio of 1:0.5, and the mixture is allowed to stand in the dark at room temperature for 16 h before use. The radical cation is stable in this form for more than 2 days in storage in the dark at room temperature. Prior to assay, the solution is diluted in water to give an absorbance at 734 nm of 0.70 + 0.02. The ratio of radical to sample was 1:20. Formula (5) was also used in this case in order to deduce the samples' antioxidant activity. The absorbance was measured for the ABTS at 734 nm before the addition of the samples. After the addition of the samples, the absorbance was measured again after 30 min [50].

$$\%Inhibition = \left[\frac{A0 - A1}{A0}\right] \times 100(\%) \quad (5)$$

3. Results and Discussion

In the present study, two O/W emulsions stabilized by PPI or SPI particles were prepared, and α-tocopherol or squalene were encapsulated in the oil droplets. The PPI and SPI particles were produced from native PPI and SPI, respectively, via the pH shifting method. The emulsions were prepared by high-speed homogenization. The particles shape was assessed by FFTEM, and their size was measured with the use of DLS, while the emulsions were macroscopically observed to assess their stability against creaming with the aid of the creaming index and against coalescence. Their morphology was evaluated microscopically with an optical microscope, and their size was measured with the use of a

Mastersizer granulometer. The antioxidant capacity of both empty and loaded emulsions was evaluated by a DPPH colorimetric assay.

3.1. Particles' Preparation, Dimensional Determination, and Morphology Observation

The preparation of both SPI and PPI particles has been studied before with time and pH conditions to optimize the particles' preparation methods [51–54]. The proper modification of the particles is of great importance, as the stability, type (O/W or W/O), and morphology of particle emulsions are highly dependent on the properties of solid particles [55]. Liang & Tang (2014) found that at pH 3.0, most of the proteins in PPI were in the nanoparticle form [37]. For SPI particles, Jiang et al. suggested that the best method to obtain nanoparticles is the one described at Section 2.2, after treating SPI at several pH values and different treatment times, having found that pH 3.0 is the optimum for SPI [38]. In order to quantify the amount of protein converted to particles, the following test was performed: After the preparation of particles at 0.3% w/w, a measured volume was placed in a centrifuge tube. The content of the tube was centrifuged at 8500$\times$ g for 20 min in order to collect the particles. Afterwards, the supernatant, which contained the dissolved proteins that had not converted to particles, was collected in a pre-weighted container. The water was removed by freeze-drying, and the container was weighed again. The difference in weight was converted into a percentage, and the results for PPI were that 77% of the protein is converted, compared to 67% of the SPI proteins.

Dynamic light scattering was applied in order to measure the size of the particles after their acidic treatment. All dispersions exhibited a wide and heterogeneous particle size distribution profile, with sizes ranging from 128–255 nm for the PPI particles and from 26–422 nm for the SPI particles. The fact that the particles are not monodispersed is also mirrored in the PDI values. These results are in accordance with those of previous studies [37,56]. PPI and SPI dispersions were also measured by DLS prior to pH treatment for comparative purposes. Both protein isolates had two major size groups. For PPI the 2 size populations were at 1041 nm (79.5% Intensity) and at 109.4 nm (20.5% Intensity), while for SPI at 1259 nm (70.1% Intensity) and 190.5 nm (29.9% Intensity) [57]. Furthermore, DLS was used to determine the ζ-potential of the particles. It was found that for SPI the ζ-potential was -31.37 ± 0.67 mV, and for PPI it was $+32.77 \pm 0.32$ mV. ζ-potential for both those particles has been measured before. The results presented are in accordance with those previously published [53,58].

FFTEM images were also obtained and proved that the particles are of spherical shape, thus the DLS's hypothesis of spherical objects can be applied without misrepresenting the obtained results. The main goal of the FFTEM was indeed to assess particle morphology. We chose not to exploit this technique to deduce particle sizes because many artefacts could alter this determination (dilution, filtration, the position of the fracture that may not capture the equatorial position, etc.). Nevertheless, this method was preferred to TEM, which would have required a drying process that could have altered the particle morphology. Instead, the sizes were determined by DLS. In Figure 1, FFTEM images of PPI particles (A) and SPI (B) are presented. The images provided are of the main size population of each protein. As can be seen in Table 1, most of the PPI particles' diameter was around 128 nm, while for SPI particles it was mainly about 26 nm. Images of protein particles have been obtained before with the freeze-fracture technique and are in accordance with the ones obtained for this project [59].

Table 1. Dynamic light scattering results of the particles' hydrodynamic diameters and PDI. Each value in the table is represented as the mean ± SD (n = 3).

	Peak 1 (nm)	Peak 2 (nm)	Peak 3 (nm)	PDI
PPI particles	128 ± 57	293 ± 50	555 ± 50	0.494 ± 0.049
SPI particles	26 ± 3	270 ± 43	422 ± 32	0.404 ± 0.099

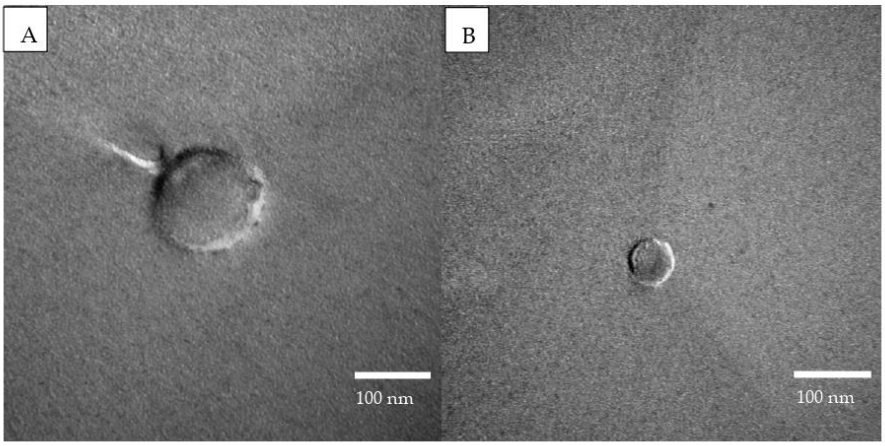

Figure 1. FFTEM images at 75.0 kV of (**A**) PPI particles (150,000× magnification) and (**B**) SPI particles (200,000× magnification).

3.2. Emulsions' Preparation, Droplets Dimensional Determination, and Morphology Observation

In order to form emulsions with the best possible characteristics, such as stability against coalescence, droplet dimensions, and antioxidant ability, several factors have to be considered. Such parameters are the concentration of particles, the oil type, and its volume fraction, as well as the homogenization method. These parameters are the most important factors for emulsion stability and droplet size [60]. For this purpose, several combinations of particle concentrations and water:oil ratios were tested. Furthermore, different homogenization speeds and homogenization times were applied. One more parameter that was tested was the type of oil that was used. Both particles (PPI and SPI) were used at four different concentrations, namely 0.1, 0.2, 0.3, and 0.5% w/v. All concentrations were tested in 80:20, 70:30, and 60:40 water: oil ratios. The homogenization times that were tested were 1, 2, and 3 min. Homogenization speeds ranged from 5000 to 20,000 rpm. For the oil phase EVOO, sunflower oil and MCT oil were examined. With these combinations, the one with 0.3% w/v particles and 70:30 water: oil ratio was considered the best in terms of stability and incorporation capability, as all of the oil was able to be encapsulated, and the droplet size that was acquired was the smallest. EVOO was chosen as the oil phase since sunflower oil and MCT oil could not be fully incorporated into the emulsion at the 70:30 water:oil ratio. In pursuit of an explanation, control emulsions of oil and water (with no particles) were prepared under the same conditions as the emulsions. It was observed that EVOO formed emulsions that remained stable for several days, while the emulsions formed by sunflower oil or MCT collapsed after 2 days. This could be due to the fact that EVOO is a mixture of triglycerides of several fatty acids and small quantities of other components that display surface-active abilities [61–64]. This means that EVOO may also exhibit emulsifying/stabilizing properties or components able to promote particle adsorption; thus, it was chosen as the most promising candidate for the present study. Nevertheless, the emulsions were observed at times larger than the control destabilization time to ensure that they were also stabilized by the Pickering effect. Hence, all systems that were used to encapsulate the bioactive compounds and that were further analyzed had a 0.3% w/v particle concentration and a 70:30 water:EVOO ratio with both protein isolates.

3.2.1. Droplet Size Determination by Static Light Scattering (SLS)

SLS uses the technique of laser diffraction to measure the particle size and particle size distribution of materials. It does so by measuring the intensity of light scattered as a laser

beam passes through a dispersed sample. This data is then analyzed to calculate the size of the particles that created the scattering pattern.

In Table 2, the droplet size of empty and loaded emulsions is presented. All samples show a uniformity ranging from 0.3 to 0.45, showing that the emulsions are quite polydisperse; nevertheless, the distributions remain monomodal. In addition, a representation of the PPI empty sample's droplet diameter distribution is given as an example in Figure 2. This came as no surprise since the particles that stabilized them were very heterogeneous in terms of size and therefore led to droplets of various sizes. Several studies have examined the fact that particles' size affects the emulsions' droplet diameter in a proportional manner, meaning that bigger particles form larger droplets [65–67] in the case where particles are in excess in the continuous phase. The two kinds of emulsions seem to have similar droplet sizes, since the difference in diameter is not significant when taking into account the polydispersity of the emulsions. This is also supported by the fact that the particles that stabilize these emulsions do not differ much, in terms of size, as shown in Table 1. Another useful conclusion that could be drawn from the measurements is that the encapsulation of both α-tocopherol and squalene does not have an effect on the droplet size. Previous studies have also reported that no size differences were observed after the encapsulation of bioactive compounds [68]. This indicates that the two molecules are likely encapsulated in the core of the drops and do not perturb the interface.

Table 2. Determination of the emulsions' droplet diameter and uniformity by static light scattering. Each value in the table is represented as mean ± SD (n = 3). A *t*-test was employed for the statistical analysis. Significant differences between values in the same column are indicated by different letters a, and b ($p < 0.1$).

Sample	d (μm)	Uniformity
PPI empty	57.9 ^{a} ± 0.1	0.30 ^{a} ± 0.01
PPI α- tocopherol	57.0 ^{a} ± 0.1	0.31 ^{a} ± 0.01
PPI squalene	62.2 ^{b} ± 0.5	0.31 ^{a} ± 0.01
SPI empty	60.0 ^{a} ± 6.9	0.45 ^{a} ± 0.03
SPI α- tocopherol	66.4 ^{a} ± 1.5	0.38 ^{b} ± 0.01
SPI squalene	67.7 ^{a} ± 5.0	0.41 ^{a} ± 0.02

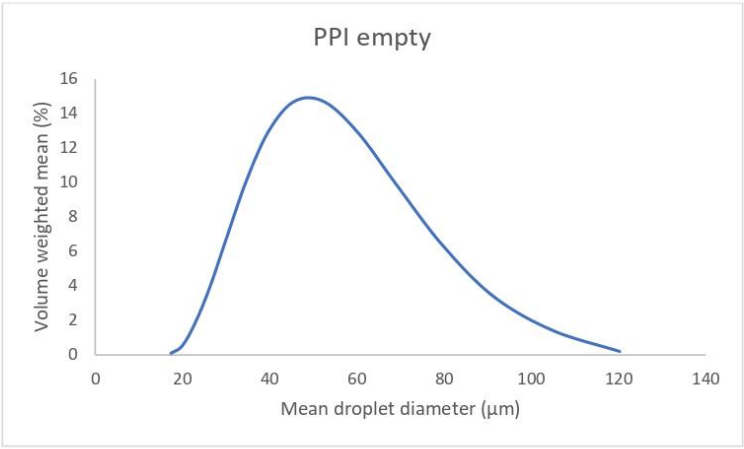

Figure 2. Mean droplet diameter of the PPI empty emulsion obtained by static light scattering.

3.2.2. Droplet Morphology Observations by Cryogenic Scanning Electron Microscopy (Cryo-SEM) and Microscopic Observation

The emulsions' droplets were observed by cryo-SEM and an optical microscope. The images obtained from the microscope showed spherical droplets with various sizes, which supports the large value of the uniformity obtained from the SLS measurements. Such images have been observed in previous works that have focused on the preparation of O/W emulsions stabilized by pea and soy protein isolates [58,69–71]. The texture of the droplets can also be commented on, since in some images, droplets appear to have a smooth surface (Figure 3A), while in others, there seems to be a rough layer (Figure 3B) around them of probably larger particles or insoluble proteins. 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.

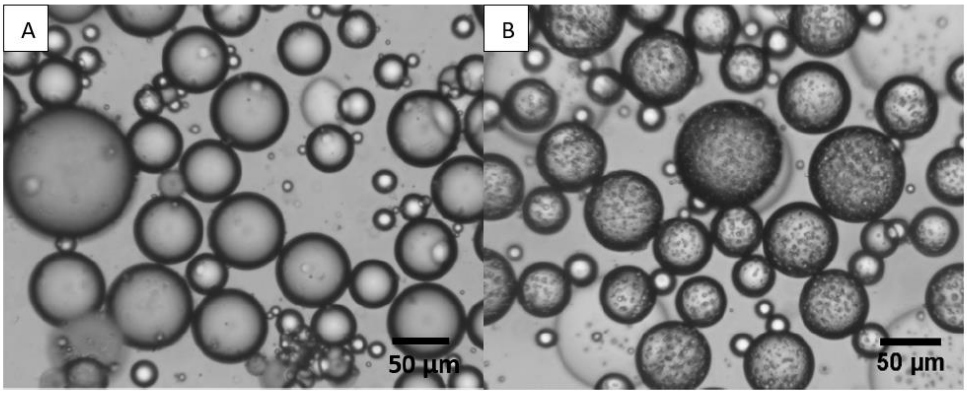

Figure 3. A × 10 magnification of PPI empty emulsions of (**A**) droplets with a smooth surface, and (**B**) droplets with a rough surface. The two images were taken from the same emulsion.

On the other hand, cryo-SEM analysis made it quite difficult to provide a distinct outline of individual particles at the interface of droplets. It was also challenging to make the observations, especially at high magnification, because samples were thermally sensitive and the radiation from the electron beam led to melting. Such a phenomenon can be seen in Figure 4A, where cracks are visible. It sometimes seems as though there are two levels of roughness, but it is hard to say because zooming is not possible due to the sensitivity of the samples (Figure 4B). Nevertheless, some concrete results can be collected, such as the round shape of the particles as well as the droplets. In addition, a clear image of a droplet covered by SPI particles is presented in Figure 4C. Furthermore, the data and images that were collected are supported by figures published in other studies [72,73].

3.3. Stability Assessment against Creaming and Coalescence of the Emulsions

The stability of the emulsions against creaming was assessed by monitoring the emulsions' CI% during storage. Two different storage conditions were examined, namely 4 °C and 25 °C. All the emulsions, freshly prepared, underwent a fast creaming process. The emulsions stability was assessed by their respective creaming stability in terms of percentage creaming index (CI%) upon storage. The emulsions' CI% was measured every three days up to the point of collapse. The %CI of the SPI empty as well as the loaded emulsions is 66.7% at day 0 (Figure 5a), while for the PPI it is 53.3% (Figure 5e). If the emulsion occupies 53.3% of the volume while the volume fraction of oil is 31.9% (density of EVOO = 0.916 g.cm^{-3}), this means that droplets are packed with a capacity of 0.60 in the PPI-stabilized emulsion, a value close to the random close packing, or, in other words,

drops of cream without strong interactions. The compacity is a bit lower (0.48%) in the SPI-stabilized emulsions, likely due to some weak drop attractions. For the SPI samples that were stored at ambient temperature, the CI% started to decrease gradually, and at 10 days, oil was visible on top of the emulsions and reached 13.3% (Figure 5b) meaning a destabilization through coalescence. On the other hand, PPI emulsions stored at the same conditions were more stable (Figure 5e,f) against both creaming and coalescence. At 10 days, a small quantity of oil was visible on top of the empty emulsion and the one loaded with squalene. CI% remained the same for the α-tocopherol containing emulsion, while it reached 50.1% for the other two PPI emulsions (Figure 5f). Microbial growth was also observed on some of the emulsions after the tenth day of storage at 25 °C. Microbial growth was not observed for samples containing α-tocopherol and squalene that were stored at ambient temperature and for all samples that were stored at 4 °C. It has been previously reported that microbial growth started for SPI emulsions after 6 days of storage at ambient temperature and after 9 or 10 days for PPI emulsions [74,75]. Furthermore, squalene as well as α-tocopherol have been found to act against microorganisms, which explains the resistance of the samples containing them against microbial growth [76,77].

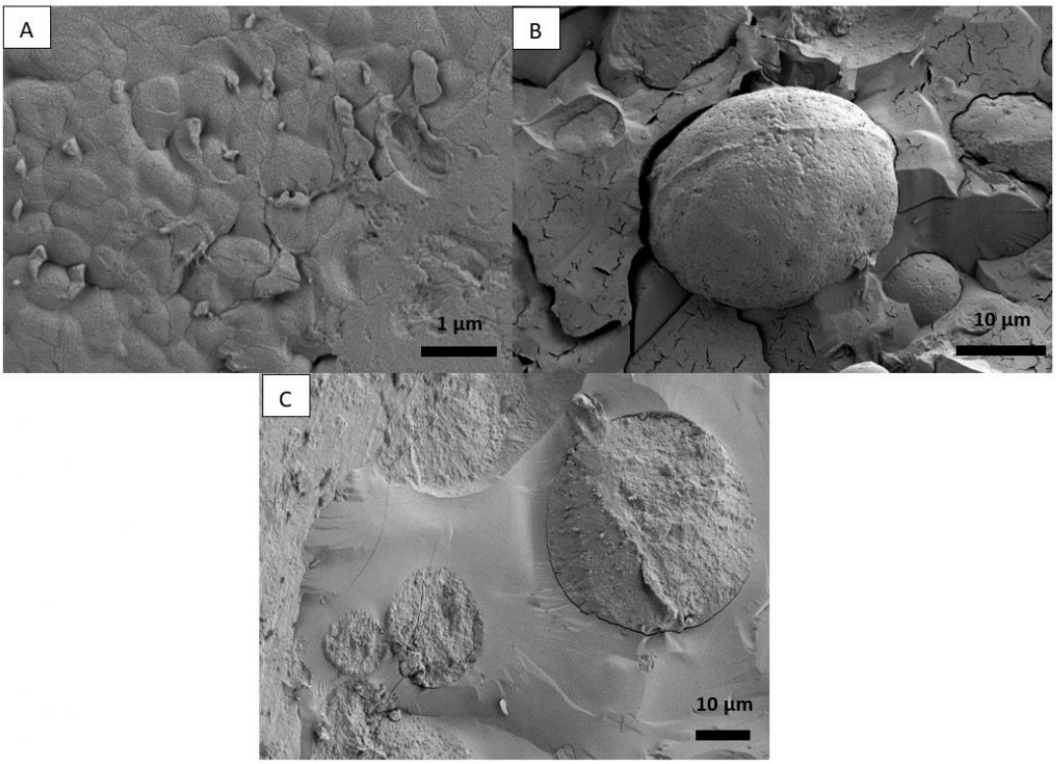

Figure 4. Cryo-SEM image of (**A**) PPI emulsion, (**B**) PPI emulsion droplet covered by PPI particles, and (**C**) SPI emulsion droplets covered by SPI particles.

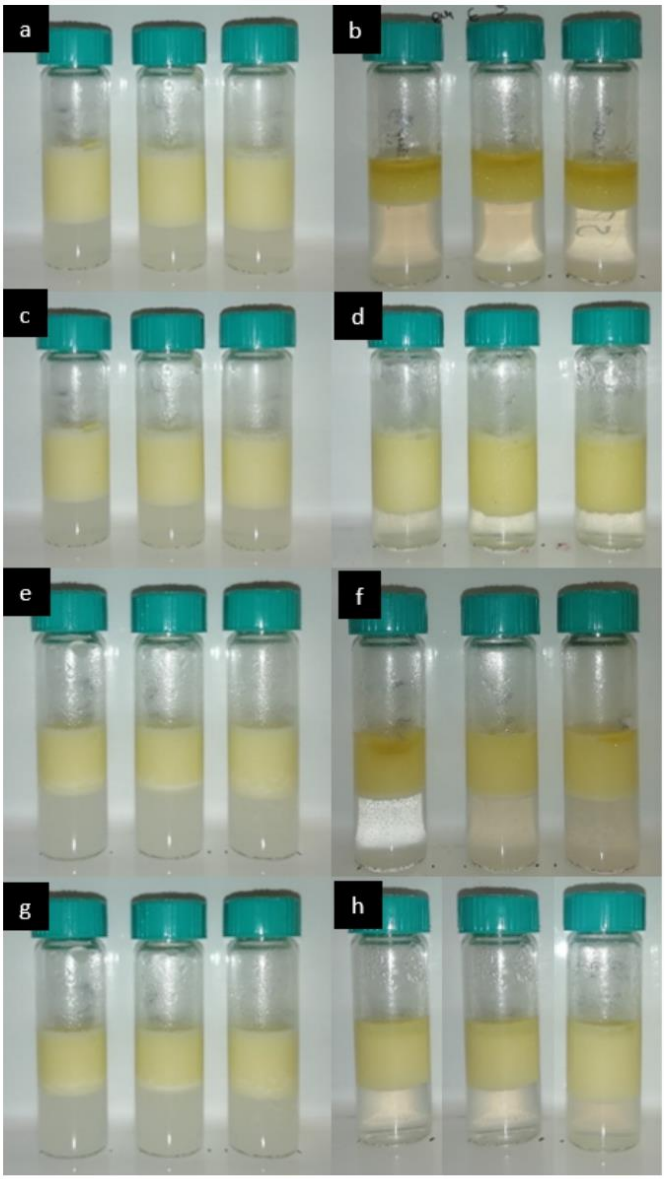

Figure 5. (**a**) SPI-stabilized emulsions at day 0, 25 °C, from left to right: empty, with α-tocopherol, with squalene, (**b**) SPI-stabilized emulsions at day 10, 25 °C, from left to right: empty, with α-tocopherol, with squalene, (**c**) SPI-stabilized emulsions at day 0, 4 °C, and (**d**) SPI-stabilized emulsions at day 10, 4 °C, from left to right: empty, with a-tocopherol, with squalene, (**e**) PPI-stabilized emulsions at day 0, 25 °C, from left to right: empty, with α-tocopherol, with squalene, (**f**) PPI-stabilized emulsions at day 10, 25 °C, from left to right: empty, with α-tocopherol, with squalene, (**g**) PPI-stabilized emulsions at day 0, 4 °C, and (**h**) PPI-stabilized emulsions at day 10, 4 °C, from left to right: empty, with α-tocopherol, with squalene.

Storage at 4 °C increased the stability of both types of emulsion. 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 [78]. The CI% does not change for any of the samples for a period of more than 60 days (Figure 5), and no oil release is observed for any of the emulsions.

The AP% is also a strong indication of an emulsion's stability against coalescence and creaming, since a higher amount of protein stabilizes the emulsion further, but there is a certain threshold of protein concentration that can be adsorbed at the oil-water interface. In this case, 70% of the initial protein content was adsorbed at the oil-water interface of PPI emulsions, while the adsorption decreased to 45% for the SPI emulsions. This fact explains, in a certain degree, the increased stability of the PPI emulsions against those stabilized by SPI. The higher amount of non-adsorbed SPI may also explain the lower compacity of the creamed emulsion as the non-adsorbed protein can induce attractive interactions through depletion. Previous studies have reported that the AP% for PPI stabilized emulsions, with PPI concentrations up to 1% *w*/*w*, reached 84.33% [79]. On the other hand, lower AP% values that reach 19.9%, for concentrations under 1% *w*/*w* have been reported for SPI [80]. The main explanation behind these results lies in the isolates' composition and surface hydrophobicity. The consistency of both SPI and PPI has been previously studied, and it has been found that 80% of the SPI proteins are 7S (b-conglycinin) and 11S (glycinin), which are highly soluble, while convicillin, vicilin, and a- and b- subunits of legumin constitute the most part of the PPI protein fraction [81,82]. The highest hydrophobicity of pea proteins explains their better adsorption at the oil-water interface. PPI is also the only isolate that was found to contain fat, increasing its affinity towards the oil phase of the emulsion [9]. It has also been reported that the surface hydrophobicity of SPI proteins decreases when the SPI treatment happens under 90 °C, affecting its emulsifying capacity [83].

3.4. Assessment of the Emulsions' Antioxidant Capacity by DPPH Free Radical Scavenging

The antioxidant capacity of O/W emulsions was measured spectrophotometrically, with the DPPH and ABTS cation scavenging techniques over time to observe the reaction between the free radicals and the emulsions. Table 3 includes the % scavenging of the DPPH and ABTS cation free radicals 30 min after the addition of the emulsion in the DPPH solution. The 30-min time frame gives the reaction enough time to reach a plateau, so that the scavenging potential measured in each sample would be the maximum.

Table 3. % scavenging ability of empty PPI and SPI emulsions and their respective loaded ones with α-tocopherol or squalene. Each value in the table is represented as the mean ± SD (n = 3). A t-test was employed for the statistical analysis. Significant differences between values in the same row are indicated by different letters a, b, and c ($p < 0.1$).

% DPPH Scavenging		
PPI empty	PPI α-tocopherol	PPI squalene
$37.3^{a} \pm 0.7$	$86.1^{c} \pm 0.1$	$62.0^{b} \pm 0.1$
SPI empty	SPI α-tocopherol	SPI squalene
$39.5^{a} \pm 0.1$	$83.6^{c} \pm 0.1$	$60.6^{b} \pm 0.1$
% ABTS Scavenging		
PPI empty	PPI α-tocopherol	PPI squalene
$26.1^{a} \pm 0.9$	$77.8^{c} \pm 0.3$	$52.4^{b} \pm 0.1$
SPI empty	SPI α-tocopherol	SPI squalene
$25.5^{a} \pm 0.1$	$68.5^{c} \pm 0.2$	$58.7^{b} \pm 0.3$

The results obtained show that all emulsions have a scavenging effect against both radicals, suggesting that they can be characterized as antioxidants at least for this kind of antioxidant mechanism. Both for the SPI and the PPI emulsions, the empty ones exhibit the lowest antioxidant effect. The difference in the antioxidant effect between the two empty emulsions is not significant, indicating that whatever antioxidant effect appears comes from the EVOO and the protein contribution is minimal, if not nonexistent. EVOO's antioxidant capacity is well-known and widely studied, while for SPI and PPI, antioxidant capacity has been reported when they are previously hydrolyzed [84,85]. Moving on to the loaded emulsions, it is clear in both systems that the ones containing α-tocopherol exhibit higher antioxidant activity than those that contain squalene. Previously published studies that have examined the roles of several molecules, including squalene and α-tocopherol, in lipid environments have shown that α-tocopherol is the dominant antioxidant compound. More specifically, Naziri et al., 2015 studied the contribution of squalene and tocopherols on the oxidative stability of cold-pressed pumpkin seed oil and showed that the antioxidant activity of squalene commenced 3 weeks into the study, while α-tocopherol was the molecule that acted as an antioxidant during that time [86]. Furthermore, studies that examined the degradation rates of squalene, α-tocopherol and phenolics, reported that α-tocopherol had the highest degradation rate because it acted protectively over squalene's and phenols' oxidation. Those studies also suggest, as an explanation of squalene's reduced antioxidant capacity, the competitive oxidation of different lipids present in olive oil [87]. Moreover, antioxidant activity, in general, mainly depends on the number and positions of phenolic hydroxyl groups. This fact stands in favor of α-tocopherol's superior antioxidant ability [88–90]. On the other hand, α-tocopherol has been proven to be a rather effective antioxidant, especially in lipid-based systems, since it can act synergistically with other molecules that exhibit antioxidant activity [91,92]. One more factor that can affect the system's antioxidant ability is the placement of the antioxidants in the oil droplet. Although both compounds are oil soluble, α-tocopherol has the hydroxy-phenol group that could orient the molecule closer to the water-oil interface. One more factor that affects the antioxidant ability of a molecule is the length of the alkyl chain. The antioxidant capacity increases with the increase of the alkyl chain up to a point, and then it starts to decrease. It has been found that the optimum antioxidant activity is achieved with chain lengths from 2 to 13 carbon atoms. From that point of view, the antioxidant activity declines [93]. If we do not take into account the carbon atoms of α-tocopherol's phenol group, which are not in an open chain, then we can see that squalene has a significantly higher number of carbon atoms in an open chain that α-tocopherol. Between the SPI and PPI systems, loaded with either α-tocopherol or squalene, no remarkable difference can be reported.

4. Conclusions

Pea and soy protein isolates were used to create particles via the pH shifting method to form two O/W emulsions. The particles formed were of spherical shape and polydispersed, leading to emulsions with a quite high value of uniformity in terms of droplet size while maintaining a monomodal drop size distribution. These emulsions were then used for the encapsulation of two bioactive compounds, namely α-tocopherol and squalene. Both kinds of emulsions had EVOO as the oil phase. The water:oil ratio that was finally examined was 70:30 w/w. Both particles successfully formed emulsions at this ratio. The droplets of the two kinds of emulsions were of similar size and spherical shape. The encapsulation of the bioactives, at least at the concentration used in the present study, did not appear to have a great effect on the size. The stability of the emulsions was tested under two conditions. More specifically, empty and loaded emulsions, were stored at ambient temperature and at 4 °C, and it was found that while at room temperature PPI emulsions were more stable than SPI emulsions, when stored at 4 °C, no big differences in stability were detected. Empty and loaded emulsions exhibited antioxidant activity, with a noticeable increase in antioxidant activity when loaded at 1% of α-tocopherol or squalene. SPI and PPI emulsions loaded

with α-tocopherol exhibited the highest scavenging ability of all other samples against the DPPH free radical and the ABTS cation.

Overall, PPI and SPI were successful in forming particles that stabilized emulsions that were used for the encapsulation of α-tocopherol or squalene. The additives enhance the systems' antioxidant capacity. The use of EVOO as the oil phase provides the system with added nutritional value. The use of proteins as stabilizers, as well as the presence of olive oil and the two bioactive compounds, lead us to believe that these systems are very good candidates as delivery systems of nutraceuticals in foods and especially plant-based products.

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Chapter 3

Formulation and characterization of edible pea protein stabilized emulsions: the role of phycocyanin as a co-stabilizer

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Formulation and characterization of edible pea protein stabilized emulsions: the role of phycocyanin as a co-emulsifier.

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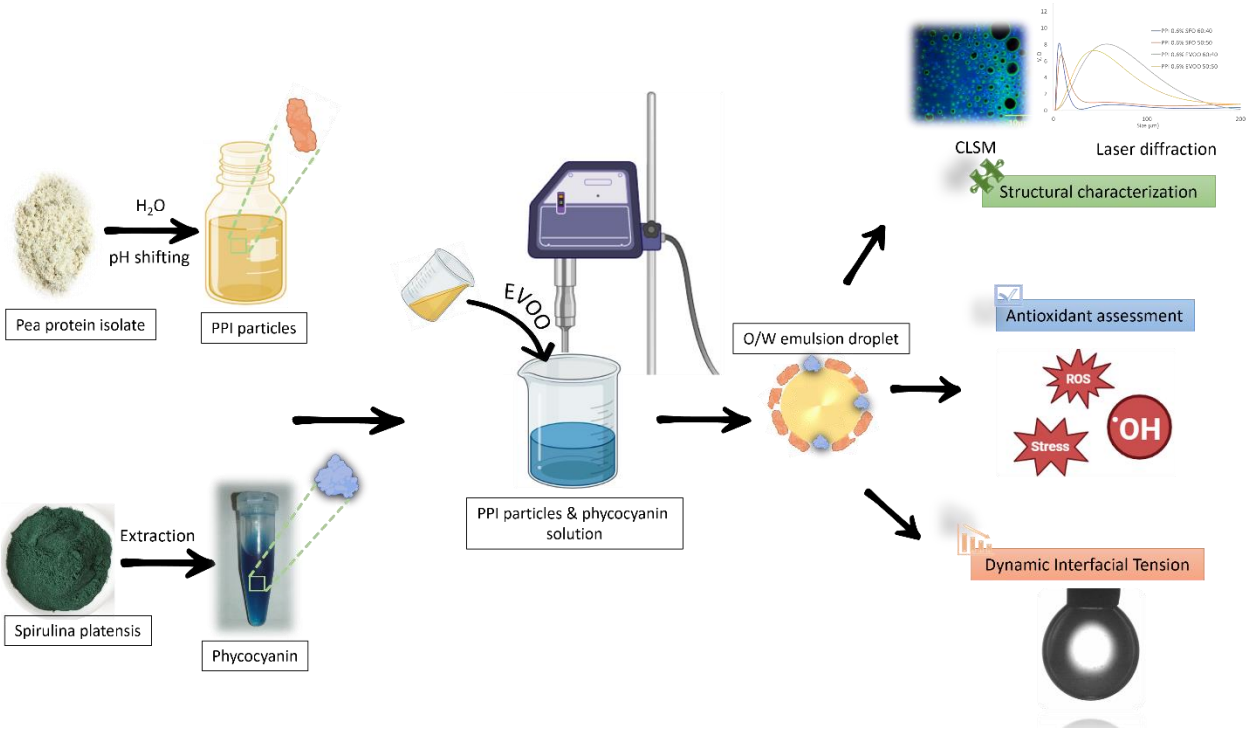

Abstract

Food sustainability and functionality are of great importance and the increasingly burdened lifestyle makes them an issue urgent to be addressed. Herein, pea protein isolate (PPI) and phycocyanin (PC) from *Spirulina platensis* were used as emulsifiers of edible oil-in-water (O/W) emulsions. PPI particles formed by combining the pH-shifting method and high-pressure treatment, while PC was dissolved untreated in the occurred particles' solution. The obtained PPI particles- PC solution was analyzed in terms of size with Dynamic Light Scattering (DLS), and for their Dynamic Interfacial Tension (DIT) with the pendant drop technique. Emulsions were produced using high-pressure and were analyzed in terms of size using Laser Diffraction (LD), and microscopically with Confocal Laser Scanning Microscopy (CLSM). The effect of Extra Virgin Olive Oil (EVOO) and Sunflower Oil (SFO) was examined for emulsions' formulation and stability. It was found that PPI particles were at 200 nm with small PDI values, while the ζ-potential of proteins varied from negative for PC, positive for PPI and close to neutral for their combination. The droplet size of the emulsions varied from 4.4 μm to 111.6 μm, affected by the protein concentration, oil volume fraction as well as the oil type. To depict the oil phase, adsorbed proteins and particles on the CLSM was employed. Overall, the systems proposed are novel with high encapsulation potency due the high oil volume fraction. They are stable for more than 20 days, and stained blue with PC that can substitute other chemical coloring agents that are used in foods.

Keywords: Pea Protein Isolate; Pickering emulsion; Spirulina; Food coloring; Interfacial tension

1. Introduction

Emulsions are colloidal dispersions of two immiscible liquids, where one is dispersed in the other. Systems like these are not stable unless some surface-active ingredient is present, namely an emulsifier. Emulsifiers are added in order to reduce the interfacial tension between the two liquids or form a steric barrier at their interface. Many processed foods and beverages are, or contain some kind of emulsion including nanoemulsions, or Pickering emulsions (Cai et al., 2023; Hong et al., 2023; Tavassoli et al., 2023).

When emulsions are used, it is usually to achieve properties such as the extension of shelf-life (Travičić et al., 2023), the replacement of ingredients like conservatives or antibiotics (Galani et al., 2024; Mitsou et al., 2016) and endowing products with desirable characteristics (Xu et al., 2023). Consumers nowadays, are well informed and alert on those matters, so the scientific community works very closely with the food industry, to produce products that meet certain criteria. Furthermore, the interest is lately focused on the production of "functional foods". The term "functional foods" refer to foods that promote the health and well-being of the consumers beyond their nutritional value (*FAO Terminology Portal | Food and Agriculture Organization of the United Nations*, n.d.; *Novel Food | EFSA*, 2024). Thus, foods are attempted to be fortified with ingredients such as proteins, minerals, fibers, probiotics etc. (Tur & Bibiloni, 2016). For that reason, much attention is lately paid on Pickering and particle stabilized emulsions. The distinction between the two is mainly a matter of definition, since Pickering emulsions are solely stabilized by particles that adsorb at the water-oil interface, but when other molecules affect the stabilization process (e.g. fatty acids of the edible oils used), they are mainly referred to as particle stabilized emulsions (Cui et al., 2023).

Among the multitude of particles used for stabilizing emulsions, there are proteins of different origins, like animal, plant or even algae. Pea protein isolate (PPI) derives from the edible pulse pea and is used more and more in food applications because of its multifunctional properties. To start with, PPI is an excellent substitute for soy protein isolate (SPI) which was the main plant protein used for foods but is has recently raised concerns of being a potential allergen (J. Wang et al., 2023). Furthermore, PPI exhibits certain health benefits, like consisting of more than 89% pure protein while it is also gluten-free, leading to much needed products with no gluten (Lu et al., 2020). PPI has also been found to have antihypertensive properties, and it can regulate the intestinal bacteria populations (Ge et al., 2020). Besides its health benefits PPI can be a practical solution for the food industry's

challenges, with its excellent foaming, emulsifying and gelling abilities (Liu et al., 2020). PPI solubility and structure can be easily modified by simply changing its pH, to increase its stabilizing and emulsifying abilities (Zhi et al., 2022). It has been found that PPI can also form strong gel networks and improve the properties of other gels when added in smaller quantities (Moreno et al., 2020). PPI has a balanced amino acid profile with all essential amino acids being present (Daba & Morris, 2022).

Phycocyanins (PCs), on the other hand, are a group of proteins obtained from blue-green algae or cyanobacteria. PCs global market value steadily increases and it is estimated that it will reach 245.5 million US dollars by 2027 (*Phycocyanin Market to Be Worth $279.6 Million by 2030*, n.d.). PC is a high-value protein that contains a chromophore that gives it its distinctive blue color. In the need for substitution of chemicals in foods, PC has been lately used as a dietary supplement and natural dye, while its use as food colorant is regulated by the FDA (*CFR - Code of Federal Regulations Title 21*, n.d.) and complies also with the regulations of the EU (Bratinova, 2015; REGULATION EU No 1333/2008). PC is well known for its antioxidant activity against free radicals (Fratelli et al., 2021). It has also been found that PC can act as an antidiabetic and anti-inflammatory agent (Prabakaran et al., 2020; Ziyaei et al., 2023). The above mentioned benefits of PC, have led to its consideration and use as a nutraceutical in several formulations (Ashaolu et al., 2021). In literature, the main way of use or delivery for PCs is their encapsulation in different kinds of emulsions (Li, Li, et al., 2022; Yu et al., 2022). However, an increasing number of recent studies have been published utilizing PC as either an emulsifier or co-emulsifier. Usually, it is used alongside other molecules such as silica or gelatin and at different pH values in order to improve its surface activity, and stability overtime under different storage conditions (Tello et al., 2023; H. Wang et al., 2022). Ongoing research efforts aim to improve PC's properties, to increase its use in the food industry and exploit to the fullest its beneficial characteristics (Eriksen, 2008; Yuan et al., 2022).

In the present study, novel functional systems stabilized by PPI and PC, two naturally derived proteins, are presented. To our knowledge, it is the first time such a combination is being tested. The proposed O/W systems exhibit excellent stability and great oil incorporating capacity, while at the same time the use of natural, eco-friendly, and sustainable ingredients make them ideal candidates for "greener" applications in the food and other sectors. The use of PC is an alternative to more conventional proteins used as stabilizes and endows the presented systems with its biological activities apart from its obvious natural blue color.

2. Materials and Methods

2.1 Materials

Pea Protein Isolate (PPI) (NUTRALYS® F85F) (≥83% protein content) was purchased from Naturalys (L'Isle sur la Sorgue, France), Hydrochloric acid (HCl), nile red, dilution membrane (MW cut-off 14000 Da), and phosphate buffer tablets were from Sigma-Aldrich (Chemie Gmbh Munich, Germany), *Spirulina platensis* was kindly provided by Hellenic Spirulina Net L.P., ammonium sulfate was purchased from Alfa Aesar (Kandel, Germany), Extra Virgin Olive Oil (EVOO), and Sunflower Oil (SFO) were bought from a local super market in Athens, Greece. Ultra-pure water was used for the dynamic interfacial tension measurements.

2.2 Preparation of particles

Two kinds of particles were used for the stabilization of O/W emulsions, namely PPI or a combination of PPI and PC. When solely PPI was employed, concentrations of 0.6% and 1% w/v were used. PPI and PC's total concentrations were also 0.6 or 1% w/v, with the mixing concentration ratio of the two being 1:1.

Pea Protein Isolate (PPI)

The preparation of PPI particles was made following a protocol previously used by our team with some modifications (Galani et al., 2023). Briefly, PPI was dispersed in deionized water at the two aforementioned concentrations, namely 0.6 or 1% w/v, and the mixture was left under stirring at 800 rpm for 30 minutes. Subsequently, the pH was adjusted to 3.0, using HCl 1 M and the dispersions remained under magnetic stirring for 2 h. Finally, they were stored overnight at 4°C to ensure complete hydration of the proteins. Afterwards, the mixture passed through a high-pressure homogenizer Panda PLUS1000 (GEA, Niro Soavi, Parma, Italy). After 10 recirculation passings at 400 bar the final solution was immediately put in an ice bath to achieve rapid cooling, thereby preventing denaturation of the proteins.

Extraction of phycocyanin (PC) from Spirulina platensis

For the extraction of PC, 5 g of powdered *Spirulina platensis* were dissolved in 50 mL phosphate buffer 50 mM and the mixture was kept under stirring in the dark for 3 h. Following, it was centrifuged at 8500 x g for 20 min at 4°C and the supernatant was collected. In the supernatant, ammonium sulfate was added to achieve 30 % saturation and the mixture was left under stirring overnight at 4°C. Afterwards, it was centrifuged at 8500 x g for 20 min at 4°C and the supernatant was collected and saturated again to 70% with ammonium sulfate. After 3 h of stirring in the dark at 4°C the centrifugation was repeated and this time the precipitate was collected and dissolved in deionized water. The solution was then placed in a dialysis membrane with a molecular cutoff at 12000-14000 KDa and was kept in beakers with deionized water which was changed regularly for 2 days. The solution was then freeze dried and the PC powder was kept in the freezer (Veličković et al., 2023).

PC's concentration in the extract was calculated spectrophotometrically. More specifically, after proper dilution with distilled water, the extracts' absorption was measured at 620 and 650 nm. The concentration was calculated using the formula indicated below in equation (1), where PC concentration is in mg/mL, A620 is the sample's absorption at 620 nm, and A650 is the sample's absorption at 650 nm (Yang et al., 2017).

$$\text{PC concentration} = \frac{[A_{620} - 0.474\text{A}_{650}]}{5.34} \quad 1)$$

At the same time, the extract's purity was determined with the formula presented in equation (2). The extract's absorption was measured at 280 nm after proper dilution. The purity was determined using the ratio indicated below, where A620 is the maximum absorbance of PC, and A280 is the absorbance of total proteins (Schwarz et al., 2001).

$$\text{EP} = \frac{A_{620}}{A_{280}} \quad 2)$$

Combination of PPI and PC

For using both PPI and PC as emulsifiers they were mixed in a concentration ratio of 1:1, to achieve a total protein concentration of 0.6 or 1% w/v leading to a mixture of particles (PPI) and colloidal dispersion of proteins (PC). To do that, freeze dried PC was added to a PPI solution after it had already passed from the high-pressure homogenizer. The mixture of the two proteins was left under stirring in the dark for about 2 h and then in the fridge overnight to achieve the best dissolution of PC in the PPI solution.

2.3 Determination of particles' dimension and ζ-potential by Dynamic Light Scattering

For the size, PDI, and ζ-potential determination of the particles, a Zetasizer Nano ZS (ZEN3600) analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a He-Ne laser (633 nm) and Non-Invasive Backscatter (NIBS) optics was used. Results were processed with the Malvern Zetasizer Nano software, version 8.02 (Malvern Instruments Ltd., Malvern, UK) which fits a spherical model of diffusing particles with low polydispersity. For the size and PDI measurements a glass 1 cm cuvette with a round aperture was used, while for the ζ-potential measurements the Folded Capillary Zeta Cell DTS 1070 were used (Demisli et al., 2020).

2.4 Preparation of emulsions

For the emulsions' preparation, the aqueous phase consisted of either PPI (0.6 or 1% w/v) or PPI and PC (0.6 or 1% w/v total protein concentration), while the oil phase consisted of either EVOO or SFO. The aqueous-to-oil phase ratio was 60:40 or 50:50. Initially, a coarse emulsion was prepared by high-shear 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 20,000 rpm for 1 min. The oil was added gradually in the dispersion

during the homogenization. Afterwards, the coarse emulsions passed through a high-pressure homogenizer Panda PLUS1000 (GEA, Niro Soavi, Parma, Italy) 2 times at 200 bar. Due to the droplet sizes and to the different densities of oil (0.916 g/cm^3) and water, some emulsions separated into two distinct layers, namely serum and emulsion layer. The emulsion was used for all further analysis and for stability assessment. Creaming, is a natural phenomenon that can be reversed by applying simple stirring, in contrast to coalescence which is irreversible and leads to a macroscopic oil layer visible on the top of the emulsion (McCarthy et al., 2016).

2.5 Droplet size distribution determination by Laser Diffraction (LD)

The droplet size distribution was determined using LD with a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) and the Mie theory was used to determine the emulsions' droplet size. Samples were added in the instrument via a Hydro MU liquid sampler (Malvern Instrument, Malvern, Worcestershire, UK) and after proper dilution (1:20 in an SDS solution 1.3% w/v). The SDS solution in a concentration 10 times the Critical Micelle Concentration was used in order to protect the initial droplet sizes against coalescence or other phenomena (Charisis & Kalogianni, 2023). The size distribution was expressed in terms of %Volume of the dispersed phase as a function of droplet size. In addition, the volume mean diameter D[4,3] was determined for all the systems.

2.6 Emulsions' microscopic observations by Confocal Scanning Electron Microscopy (CLSM)

For the microscopical analysis of the samples with CLSM, emulsions were stained with rhodamine, or nile red. Rhodamine was used for the staining of PPI, since phycocyanin is fluorescent on its own. Finally, nile red was used for coloring EVOO inside the droplets. Microstructure images of the emulsions' droplets were acquired using an EVO 50XVP confocal laser scanning microscope (Carl Zeiss, CZ Miscoscopy GmbHm, Jena, Germany). Ar/K and He/Ne dual-channel laser mode was used (Huang et al., 2004).

2.7 Assessment of the emulsions' stability against creaming using the Creaming Index percentage (%CI)

Stability tests were conducted on the emulsions stabilized both by PPI and by the combination of PPI and PC. The emulsions were prepared in 12 mL glass tubes with screw caps, and stored under two different conditions: room temperature and refrigerated at 4°C. After preparation, some of the emulsions initially separated into cream and serum layers. The cream layer's changes during storage were monitored and data were recorded every 5 days. The stability of the emulsion was evaluated using the creaming index (CI%), which is the cream layer height (Hc) expressed as a percentage of the total sample height (Ht) within the tube. The CI% was calculated using a specific formula given in equation (3), and the procedure was repeated three times (Firebaugh & Daubert, 2005).

$$CI\ (\%) = \left(\frac{H_c}{H_t}\right) * 100 \quad (3)$$

2.8 Dynamic Interfacial Tension measurements

The pendant-drop method was employed to examine the proteins' effect on the dynamic interfacial tension of the water oil interface. The dynamic interfacial tension between oil and water was measured with a pendant-drop tensiometer (CAM 200, KSV, Biolin Scientific, Stockholm, Sweden). For the experiments two oils were used, namely SFO treated with ultra-pure water, to remove as much as possible any surface-active ingredients, and EVOO. For SFO's treatment, SFO was mixed with ultra-pure water in a 1:1 ratio. The mixture was vigorously shaken and kept overnight at ambient temperature. Afterwards it was centrifuged at 5000 x g for 30 minutes and the aqueous phase was removed, while the oil phase was rewashed. The obtained data were analyzed using an axisymmetric 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. For the experiment a pendant drop of the aqueous phase was created within the oil phase, contained in a quartz (Hellma Analytics, Müllheim, Germany) cell (Charisis & Kalogianni, 2023).

2.9 Antioxidant assessment by DPPH colorimetric assay

The antioxidant capacity of the emulsions was assessed using a colorimetric DPPH assay, which was carried out following a previously described procedure with some adjustments (Bi et al., 2021). Briefly, 2 mL of a DPPH solution dissolved in isooctane at a concentration of 0.108 mM was measured using VIS spectroscopy. Subsequently, 500 μL of the sample being tested was added to this solution. The resulting mixture was vigorously agitated and then its absorbance was measured with time scan for a duration of 10 minutes until it reached a plateau, and the absorbance was measured at a wavelength of 515 nm. Isooctane was employed as a blank, while a mixture comprising 2 mL of DPPH and 500 μL of isooctane served as the control sample. The percentage of inhibition of the free radical was calculated using equation 4, where A0 represents the absorbance of the control sample, and A1 denotes the absorbance observed after 2.5 min.

$$\%Inhibition = \left[\frac{A_0 - A_1}{A_0}\right] * 100 \quad (4)$$

3. Results and discussion

In this study we investigated the ability to formulate edible O/W emulsions using PPI particles. These particles were formed via high pressure homogenization to ensure the finest possible dispersion. High-shear, alongside high-pressure homogenization was used for the emulsions' preparation. Furthermore, we introduced PC as a co-emulsifier, natural coloring agent and antioxidant compound. PC was added to the particles after homogenization, and the resulting mixture was employed as the

aqueous phase. The particles were characterized in terms of ζ-potential, particle size, and polydispersity index (PDI) using Dynamic Light Scattering (DLS). The stability of the emulsions was assessed through macroscopic observations employing the Creaming Index (CI%). The droplet size of the produced emulsions was also measured using a Mastersizer granulometer. Furthermore, we investigated the microscopic structure of the emulsions by Confocal Electron Scanning Microscopy (CLSM), by staining the oil phase and the proteins. Furthermore, we evaluated the antioxidant capacity of the emulsions through a DPPH colorimetric assay. Finally, the dynamic interfacial tension of the emulsions was assessed using the pendant drop technique.

3.1 Particles' preparation, dimensional and ζ-potential characterization

Preparing PPI particles can present several challenges, like the protein's relatively low solubility in water and its tendency to exhibit size variations. Additionally, the formation of aggregates can occur, which can act as destabilizing factor when formulating emulsions. Another very important aspect of preparing PPI particles is the pH of the aqueous medium chosen for dispersing the protein, since each isolate's consistency in legumin, vicilin and globulins is different, bestowing each with different properties. Finally, particles wettability should be tuned in a way that will allow them to interact with both the aqueous and oil phase, thus forming the steric barrier between the two (Burger & Zhang, 2019).

We investigated how particles formed at 3 different pH values, namely 3.0, 5.0 and 9.0, would affect the emulsions' stability. We observed that at pH 9.0 creaming happened instantly after preparation and that the emulsion would collapse in only a few hours. Similar results were observed at pH 5.0 where many visible aggregates were formed, while at pH 3.0 the emulsions formed were stable and the creaming process did not take place immediately. Studies have shown that PPI's solubility increases when the pH is away from its isoelectric point and that at pH 9.0 it exhibits its greatest solubility (Tanger et al., 2022). On the other hand, pH 5.0 was very close to the isoelectric point (IP ≈ 5.9), with the solubility being low, explaining the presence of aggregates. At pH 3.0, the solubility of the proteins was increased but not to the extent that they could not interact with the oil phase thus, giving the most stable emulsions. Many published studies have used PPI at pH 3.0 to form stable, edible emulsions (Liang & Tang, 2014; Sridharan et al., 2020).

In a previous study published by our group PPI particles were prepared solely through the pH shifting method without any further treatment (Galani et al., 2023). Although those particles formed stable emulsions, they were quite polydispersed in terms of size, tended to aggregate quickly, and resulted in emulsions that underwent fast-creaming. Consequently, it was decided to also use physical modification through high-pressure homogenization. For this purpose, several pressure values and passing cycles through the homogenizer were tested. It was found that the procedure of 400 bar with 10 recirculation passings gave the smallest particles' size ranging from 200 to 310 nm with a PDI of 0.19 to 0.31, meaning that a very narrow distribution was achieved (Table 1). High pressure treatment has been found to be very beneficial when using proteins as emulsifiers since it can reduce the solubility by exposing sulfhydryl groups after protein unfolding and improves its functional properties

(Queirós et al., 2018). These structural changes have been shown to improve the proteins' thermal stability and emulsifying properties (Chao et al., 2018).

The addition of PC presented some difficulties due to its very sensitive nature. At first, it was attempted to be added to the PPI dispersion prior to the high-pressure treatment but the pressure and temperature resulted in PC's decolorization. Thus, it was decided to be added to the PPI particles solution after they passed through the high-shear homogenization. The sensitivity of PC in high temperatures depends on the pH of the dispersion medium since in different pH values shift tertiary structure alongside its properties. In fact, it has been reported the stability of PC is at a maximum level at pH 6 (Chaiklahan et al., 2012). After the addition of PC, the mixture was left under stirring for 2 h and then overnight at 4°C to achieve the maximum PC dissolution at pH 3.0. The resulting dispersion of proteins (PC) and particles (PPI) was used for the preparation of emulsions.

Measurements of ζ-potential were made both for PPI and PC alone as well as for their combination. The results presented in Figure 1 show the ζ-potential values of the proteins. It is evident that the ζ-potential of PPI is negative while for PC it is positive. Similar values have been previously obtained by other studies (Burger & Zhang, 2019; Li, Zhang, et al., 2022). Concentration does not seem to affect the values obtained neither for PC nor for PPI. It has been previously shown that if the size and solution buffer do not change, the ζ-potential remains the same for different protein/particle concentrations (Jailani et al., 2008). In general, the more positive or negative the ζ-potential values are for a particle or protein, the highest is their stability, hence the stability of the produced systems (Schneider et al., 2011). Since the combination of the two proteins leads to a solution that has a ζ-potential very close to zero, it could be deduced that this mixture is not a very stable one. Furthermore, the fact that after their mixing occurs one charged population gives the indications that the two proteins interact. It is stated in literature that particles with ζ-potential above +30 mV or below -30 mV are highly dispersed hence more stable against forming aggregates leading to the destabilization of the emulsions they are part of (Barhoum et al., 2018).

Table 1: PPI particles size and PDI in different concentrations.

PPI (% w/v)	Size (nm)		PDI
	Peak 1	Peak 2	
0.3	200 ± 2		0.19 ± 0.07
0.5	216 ± 1		0.20 ± 0.04
0.6	270 ± 3		0.26 ± 0.02
1	310 ± 2	150 ± 1	0.31 ± 0.03

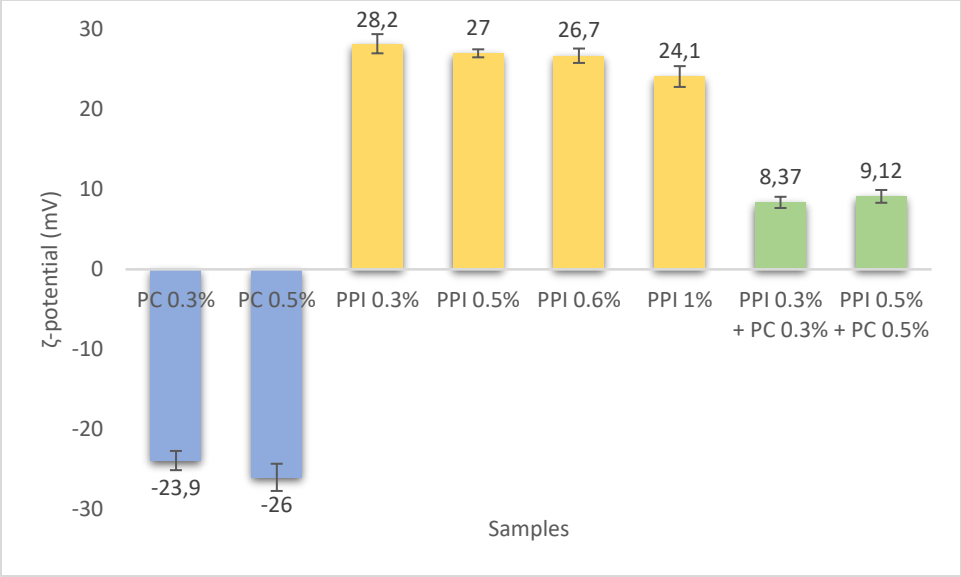

Figure 5: ζ-potential measurements on PC, PPI and their combination by Dynamic Light Scattering (DLS).

3.2 Emulsions' preparation and Droplets' dimension determination by Laser Diffraction

3.2.1 Emulsions' preparation

While preparing the following systems, the main goal was to produce stable emulsions with an extended shelf-life, while at the same time they present inherent antioxidant properties and act as a natural coloring agent. The preparation method was optimized, by testing several pressures and recirculation times through the high-pressure homogenizer. High-pressure treatment was introduced here to optimize the preparation method presented in our previous work that resulted in less stable emulsions and in order to increase the oil content (Galani et al., 2023). To achieve the optimal system, many protein concentrations and water-to-oil ratios were tested alongside the two oils chosen, namely, EVOO and SFO. To be precise 0.3, 0.6 and 1% w/v of PPI were tested at 70:30, 60:40 and 50:50 water-to-oil ratios. All experiments were carried out at 200 bar pressure and 2 recirculation passings. In Figure 2, macroscopic images of the emulsions 24 h after their preparation are presented. Results showed that at 0.3 % w/v PPI, emulsions underwent fast creaming at all water-to- oil ratios, while at 0.6% w/v PPI and with the increase of the oil volume fraction, the stability and creaming of the emulsions, as well as their droplet size homogeneity were improved. At 0.6% w/v PPI serum was visible shortly after homogenization for emulsions with water-to-oil ratios 70:30 and 60:40, but no creaming occurred at 50:50 water-to-oil ratio. When preparing emulsions with 1% w/v PPI, creaming

only occurred at 60:40 and 70:30 water-to-oil ratios but in a smaller degree than with the other two concentrations. No creaming was detected at a water-to-oil ratio of 50:50.

After reviewing the results, we decided to focus our further analysis on systems that have 0.6% or 1% w/v protein (either PPI alone or the combination of PPI and PC). Additionally, we considered using either EVOO or SFO for the oil phase and chose water-to-oil ratios of 60:40 or 50:50. In Figure 2 images of the produced emulsions stabilized either by PPI or PPI and PC, with EVOO or SFO as the oil phase and 50:50 or 60:40 water-to-oil ratios are presented. The red circles indicate the serum layer, in the cases that it was formed. In those chosen emulsions it is evident that with the increase of the protein concentration and oil volume fraction, there is a decrease in creaming. At 0.6 % w/v PPI a thin serum layer is present only at 60:40 water-to-oil ratio for both EVOO (Figure 2a right) and SFO (Figure 2e right) emulsions. When 1% w/v PPI is used no serum is detected in both oil volume fractions (Figure 2b, 2f). When PC was introduced in the systems, the results were slightly different. A serum layer was present in all emulsions except the one prepared with 0.5% w/v PPI and 0.5% w/v PC at 50:50 water-to-oil ratio (Figure 2h left). Nevertheless, even in this case when the oil volume fraction is increased, the phenomenon of creaming is significantly reduced. Furthermore, in samples that present creaming, when SFO is used (Figure 2g left) as the oil phase instead of EVOO (Figure 2c left), the serum layer is thinner. The protein content also affected the serum layer as shown in Figure 1h right, stabilized by 1% w/v total protein content, where the serum layer is less than in Figure 2d right that the emulsion is stabilized by 0.6% w/v total protein content.

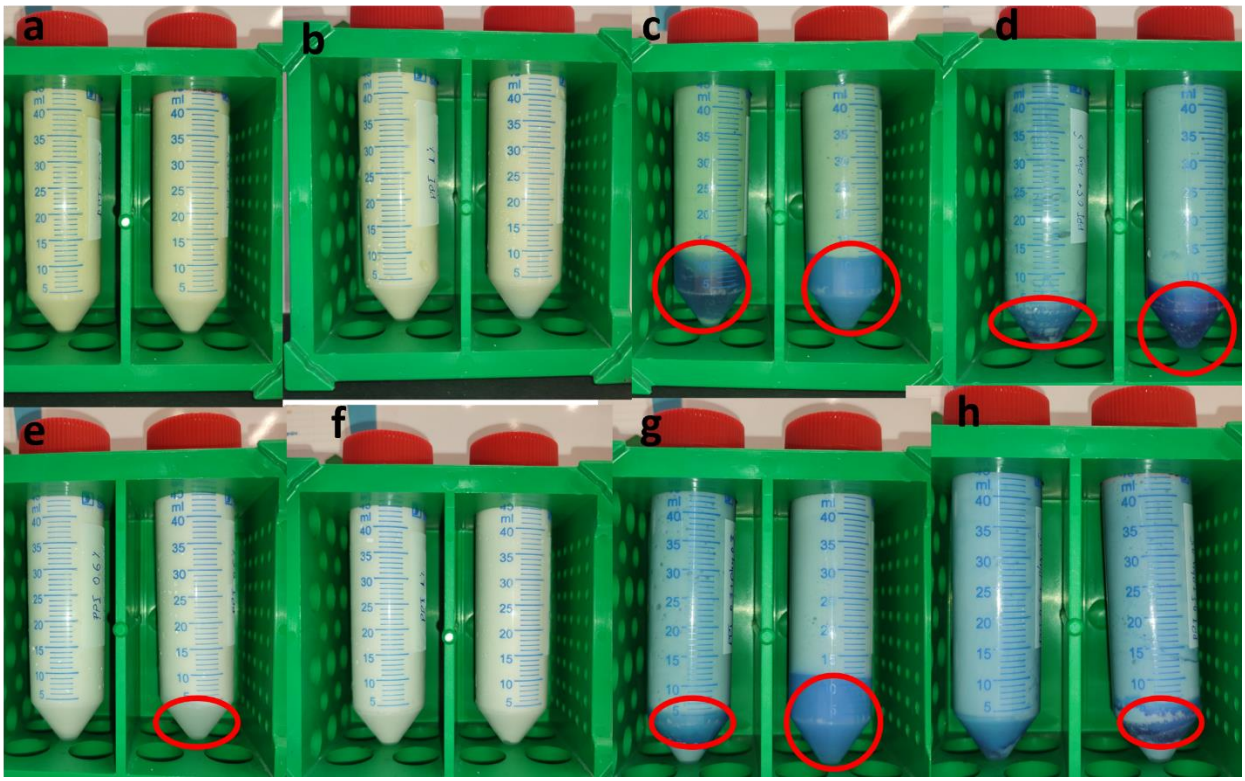

Figure 6:Emulsions stabilized by PPI or by PPI and PC 24 h after preparation. From the left to the right in the following images (a) emulsions stabilized by 0.6% w/v PPI at 50:50 and 60:40 water: EVOO ratio, (b) emulsions stabilized by 1% PPI at 50:50 and 60:40 water: EVOO ratio, (c) emulsions stabilized by 0.3% w/v PPI and 0.3% w/v PC at 50:50 and 60:40 water: EVOO ratio, (d) emulsions stabilized by 0.5% w/v PPI and 0.5% w/v PC at 50:50 and 60:40 water: EVOO ratio, (e)) emulsions stabilized by 0.6% w/v PPI at 50:50 and 60:40 water: SFO ratio, (f) emulsions stabilized by 1% PPI at 50:50 and 60:40 water: SFO ratio, (g) emulsions stabilized by 0.3% w/v PPI and 0.3% w/v PC at 50:50 and 60:40 water: SFO ratio, (h) emulsions stabilized by 0.5% w/v PPI and 0.5% w/v PC at 50:50 and 60:40 water: SFO ratio.

3.2.2 Droplets size distribution by Lazer Diffraction (LD)

The LD technique was used to measure the droplet size distribution of the emulsions described above. In Figure 3, the droplet size distributions of emulsions stabilized by PPI or a combination of PPI and PC with total protein content 0.6% or 1% w/v, with EVOO or SFO as the oil phase and 60:40 or 50:50 water: oil ratios are presented. Among all samples a wide range of droplet sizes is detected, from 4.4 to 111.6 μm depending on protein concentration and kind, type of oil, and oil volume fraction.

When comparing samples that have been stabilized solely by PPI, it is evident that the ones were SFO is used as oil phase, have smaller droplets than those where EVOO was used. Furthermore,

1% w/v of PPI leads also in smaller droplet sizes than 0.6% w/v PPI. Combining these results with the macroscopic image of the emulsions it is evident that EVOO leads to bigger droplets than SFO based systems. This could, probably, be attributed to the much more complex composition of EVOO in comparison to the simpler one of SFO. More specifically, EVOO, a virgin oil, has an acidity which is at least an order of magnitude higher compared to the refined SFO. EVOO acidity is mostly due to the presence of fatty acids (mainly oleic acid) which have been shown to adsorb at the water-oil interface displacing the proteins which confer stability to these systems whereas oleic acid is not an emulsifier itself (Kalogianni et al., 2017). When it comes to protein concentration, studies have showed that with the increase of the protein concentration there is a decrease in droplet size, while at the same time it prevents them from flocculating (M. Chen et al., 2019). Finally, when comparing Figure 3a to Figure 3b, it is evident that PPI concentration overcomes any destabilizing effect of EVOO since the size distribution of samples containing EVOO are significantly more narrow and smaller at 1% w/v than their equivalents with 0.6% w/v PPI shown in Figure 3a.

The introduction of PC in the systems changed the obtained results, leading to almost completely different systems. First, the comparison of the droplet sizes shows larger droplets when PC is used alongside PPI than when solely PPI is used for the stabilization, both in the case of 0.6 and 1% w/v total protein content. This leads to the conclusion that PC cannot fully replace PPI as an emulsifier. This fact combined with the macroscopic image of the emulsions containing PC that have the serum layer that is colored blue, shows that not all the PC is adsorbed at the oil-water interface. Furthermore, these results are complimented by the ζ-potential measurements that suggested less stable colloidal particles when PPI and PC were combined. Naturally, less stable particles led to more unstable emulsions. Nevertheless, the observations made for EVOO, and protein concentration are also applied here, since samples with EVOO have larger droplet sizes (Figure 3c), and the size distribution lowers and becomes narrower when total protein content increases (Figure 3d). The explanation behind these observations probably lies on PC's properties, like solubility and structural stability which are affected by pH, temperature etc. In order to achieve the best of those characteristics, PC is often treated with deamidation and succinylation protocols, as well as by ultra high-pressure treatment (Li, Zhang, et al., 2022; Zheng et al., 2020). Since in this case PC was used in the pure form isolated from *Spirulina platensis,* to retain its color and biological abilities, its emulsifying properties were not amplified.

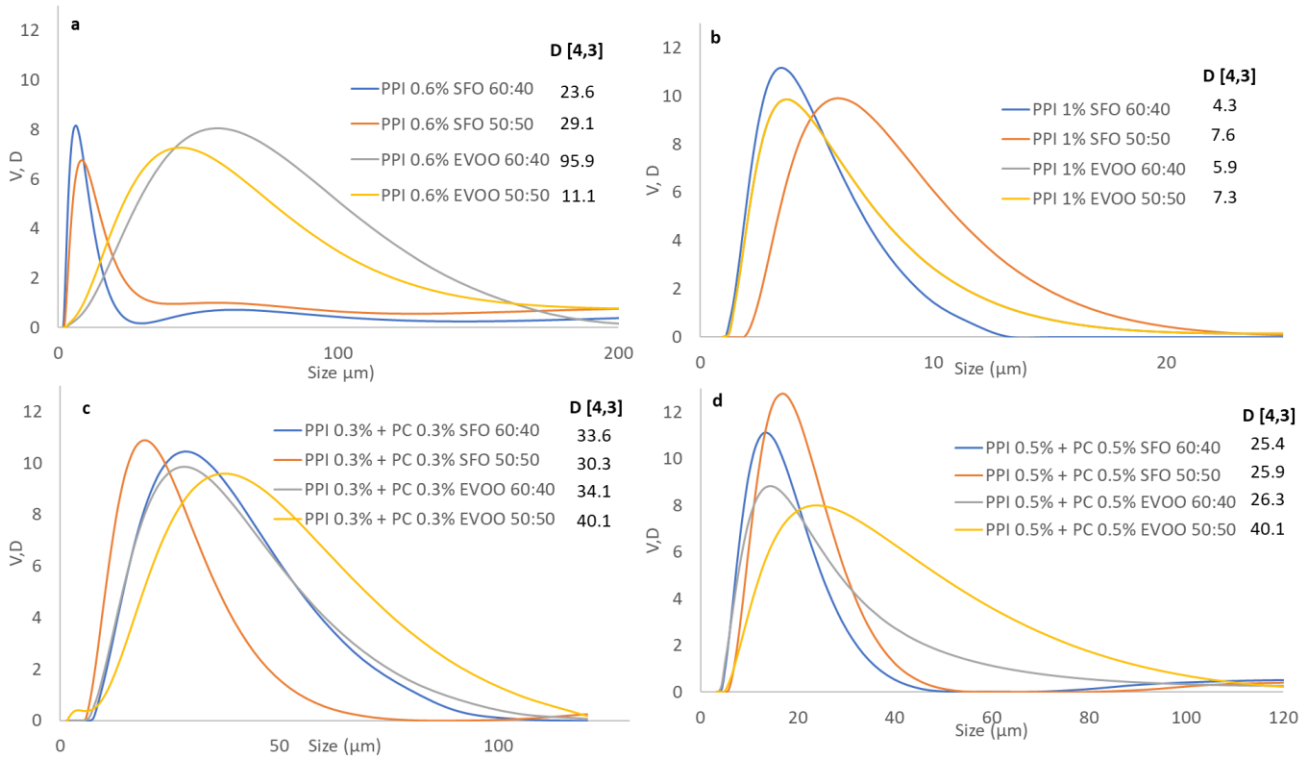

Figure 7: % Droplet size distribution of emulsions where V is the oil volume fraction in the oil phase and D is the droplet diameter. The systems where stabilized by either(a) PPI 0.6% w/v, with SFO or EVOO as the oil phase and at 60:40 or 50:50 water oil ratio, (b) stabilized by PPI 1% w/v, with SFO or EVOO as the oil phase and at 60:40 or 50:50 water oil ratio, (c) PPI 0.3% w/v and PC 0.3% w/v, with SFO or EVOO as the oil phase and at 60:40 or 50:50 water oil ratio, and (d) PPI 0.5% w/v and PC 0.5% w/v, with SFO or EVOO as the oil phase and at 60:40 or 50:50 water oil ratio.

3.3 Emulsions' microscopic observations by Confocal Scanning Electron Microscopy (CLSM)

CLSM was employed to visualize the individual components of the emulsions and especially PPI and PC in the aqueous phase, as well as adsorbed on the oil-water interface. In addition the oil droplets were depicted. The images obtained showed spherical droplets with different sizes, in the size range aquired also by the LD measurements. In Figure 4b the PPI "ring" around the oil droplets is depicted showing the strong adsorption of PPI on the interface forming a thick interfacial layer. Furthermore, disolved PPI or PPI aggregates can also be observed dispersed in the aqueous phase. Such images have been obtained by other studies as well, showing PPI around oil droplets acting as an emulsion stabilizer (Yi et al., 2021).

In addition to the PPI, PC can also be observed since it is a self-fluorescent protein and several conclusions can be drawn (Figure 4c). First of all, in this case, PPI is detected at the water-oil interface, while PC is present in the aqueous phase both in partuculate form as well as most probably as a

colloidal dispersion. PC can be detected as a bright blue ring around the smaller droplet populations when zooming in, and at the same time it can be seen in the background dissolved in the aqueous phase. This fact is also mirrored in the macroscopic images of the emulsions containing PC, where the serum left under the emulsion is of blue color. Furthermore, the phenomenon of creaming is more intense in the PC containing systems, suggesting lower adsorption and stabilization by proteins in the case of mixed systems. Nevertheless, in the few previous studies that have used PC as a emulsifier, it has been depicted as a "ring" around the oil droplets (Bai et al., 2023; X.-H. Chen & Tang, 2021). Also, in this case the antagonism between PC and PPI in adsorbing at the water-oil interface, is not easy to be depicted with CLSM and better insight is gained by the intefacial tension measurements as well as the pH of the aqueous medium should be taken into consideration for the explanation of the obtained images.

The oil droplets were also stained with Nile red and observed with CLSM and the results are shown in Figure 4a in red color. Red color is seen only inside the spherical droplets meaning that all of the oil resides in the oil droplets and also confirms the O/W type of the emulsion. This corraborates with the macroscopic observations that were made, that showed no evidence of oil on top of the emulsions meaning that all of the oil resided in the droplets. Previous images have reported through CLSM the formulation of O/W emulsions stabilized by PPI (Vall-llosera et al., 2021; Zhan et al., 2022).

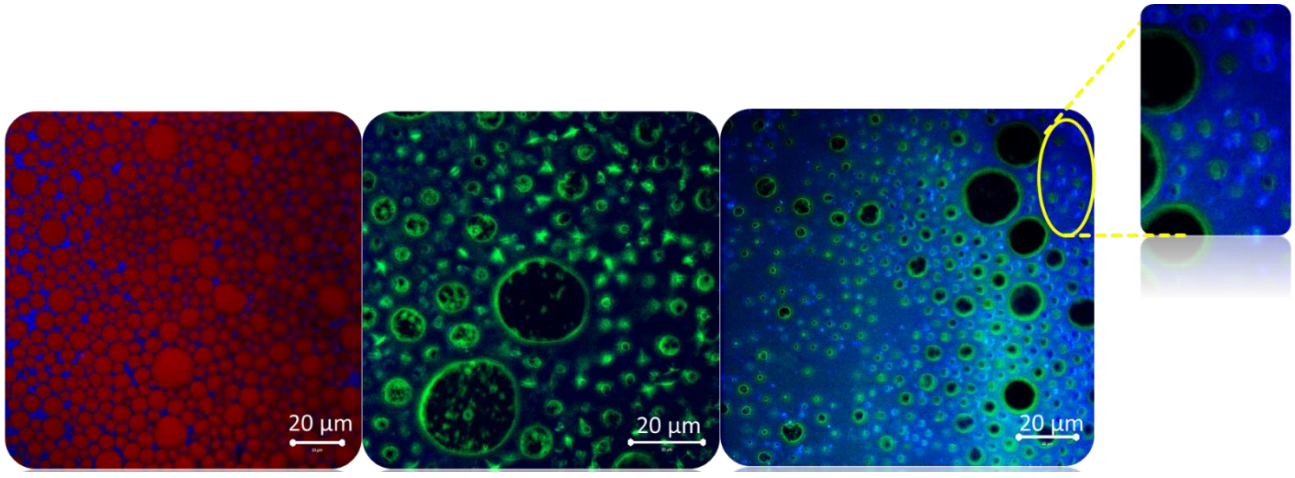

Figure 8: Confocal images of emulsions stabilized by PPI and PC. (a) oil droplets dispersed in the aqueous phase stained with nile red, (b) stained with rhodamine, and (b) PPI stained with rhodamine (green) and self-fluorescent PC (blue).

3.4 Assessment of the emulsions' stability against creaming using the Creaming Index percentage (%CI)

The emulsions' stability against creaming was assessed by monitoring the %CI overtime, when systems were stored at 25°C or 4°C. PPI when used on its own and the combination of PPI and PC were compared for their stabilizing abilities as well as EVOO and SFO were compared to each other when acting as the continuous phase respectively. The emulsions exhibited different stability depending on protein kind, oil phase and water-to-oil ratio.

In Sup Figure 1 results concerning emulsions stored at ambient temperature are presented. First, it was found that when 1% w/v PPI was used, the % CI was equal to 1, meaning that no creaming was observed at both 50:50 and 60:40 water-to-oil ratio. Furthermore, the two oils did not affect the creaming outcome in this case. All these emulsions remained stable for over 2 months when stored at ambient temperature and 3 months when stored at 4°C. After 10 weeks, emulsions stored at room temperature had a % CI of 5.2 and after 12 weeks, oil was visible on top. Emulsions stored in the fridge had a % CI of 4.8 at 12 weeks but with no oil being visible on top of the emulsion. When the PPI concentration used for the stabilization was 0.6% w/v, slight differences where observed. Both in the case of SFO and EVOO as the oil phase, no creaming occurred at 50:50 water-to-oil ratio and it remained so for up to 8 weeks, when the %CI was 2.1 and finally reaching 6.7 at 12 weeks. On the other hand, at 60:40 water-to-oil ratio the %CI was 4.8 for the emulsion with EVOO as the oil phase and 4.6 for the one with SFO, with the difference between the two not being significant. The % CI remained stable for 3 weeks, after which it started to increase to 5.6 at 6 weeks for the emulsions with SFO and 10.2 for the EVOO-containing emulsions. The quickest destabilization or creaming of the emulsions with EVOO as the oil phase can also be explained by their droplet size and droplet size distribution presented in Figure 3, where larger droplets and distributions are reported for systems containing EVOO. The results presented here, are also supported by previously published works, which have confirmed that the stability of emulsions is increased through the treatment of PPI using high pressure, as well as through the homogenization of emulsions using high pressure along with high shear (Feng et al., 2022; Zhao et al., 2022).

The presence of PC in the systems significantly changed their behavior, as well as their stability. The most obvious observation made was the fact that almost every emulsion underwent creaming upon preparation in comparison to when solely PPI was used as an emulsifier. The only emulsion where no creaming happened shortly after homogenization, was the one with 1% w/v total protein content (0.5% w/v PPI & 0.5% w/v PC), and with SFO as the oil phase at 50:50 water-to- oil ratio. On the contrary, when EVOO was employed as the oil phase at the same water-to- oil ratio, with 1% w/v total protein content, the resulting emulsion had a % CI of 7.9. This further highlights the destabilizing effect of EVOO's components, which is directly reflected in the droplet size as depicted in Figure 3. The % CI was higher for the emulsion stabilized by 1% w/v total protein, and 60:40 water-to-oil ratio, when EVOO was used as the continuous phase rather than when SFO was used. To be more precise, the % CI in the EVOO based emulsion was 16.4, while for the SFO based emulsions the % CI was 12.3. When the total protein concentration used for the emulsions', stabilization was 0.6% w/v, creaming was observed in all emulsions. Again, in this case the % CI was higher for EVOO based emulsions and for the 60:40 water: oil ratio. Finally, an observation made that is visible in Fig 2, was that the serum layer for the emulsions where EVOO was used, was a darker shade of blue than when SFO was used, indicating a higher concentration of PC. This, alongside the % CI of the emulsions, and their droplet size distribution, corroborate with each other. As for the stability of the emulsions containing PC, we saw that when they were stored at 25°C, PC started to lose its color after 2 weeks of storage, but the % CI did not alter for 10 weeks. Studies have shown that the stability of PC starts to decrease after 36 h at ambient temperature and one of the first sign

of this is the alteration of its color (Wu et al., 2016). On the contrary when stored at 4°C their stability time was the same as when solely PPI was used for the stabilization. They remained stable for over 3 months, with no change in their % CI. The stability results in general agree with the predictions made after the ζ-potential measurements presented in Figure 2. PC is not usually applied as an emulsifier, and this is a fact that was confirmed through extended literature review. In those articles that do use PC as a stabilizer, it is not used on its own but it is often treated with other compound such as urea, to improve its structural and emulsifying properties (Bai et al., 2023; X.-H. Chen & Tang, 2021; Zhong et al., 2024). Nevertheless, PC has been successfully used as a co-emulsifier, and previously published works support the findings presented above (Bai et al., 2023; Sun et al., n.d.).

3.5 Dynamic Interfacial Tension measurements

Upon the absorption of proteins at a water-oil interface, the dynamic interfacial tension (DIT) alters, and more specifically it decreases for stabilizing phenomena. Those changes of the DIT between EVOO or SFO and water when PPI or PPI and PC were present, were monitored through the pendant drop tensiometry technique, and the results obtained are presented in Figure 6.

The results obtained, when the adsorption of proteins was examined at a purified SFO-water interface, were a bit clearer. To be more specific in Figure 5a, the results of the dynamic adsorption of PPI or the combination of PPI and PC on an SFO-water interface are presented. The DIT values and reduction rate of SFO in the absence of proteins is lower than that of EVOO due to its simpler composition and the results obtained corroborate with previously reported ones (Kirtil et al., 2021). The distinction among the different DIT changes between the different proteins and different protein concentrations and is more distinct that it is in the case of the EVOO-water interface of Figure 5b. The reduction rate of the DIT follows again the pattern of a concentration dependent action, a fact supported by previously published research (A. Bos & van Vliet, 2001). The action of PPI 0.3% w/v is followed by PPI 0.5% and 0.6% w/v. The adsorption rate of PPI 0.6% w/v is the same as the one of PC 0.3% w/v. Also, the same dynamic adsorption rate was measured for the combined PC and PPI both at 0.6% and 1% w/v total protein concentration. The decrease of DIT over the increase of concentration, reaches a quasi-equilibrium constant value since the surface becomes saturated with the proteins. Finally, the highest DIT reduction rate was observed for PPI 1% w/v and PC 0.5% w/v. The adsorption motif of PC when it was measured on its own differs from the one observed when it was combined with PPI. This could be due to the different nature of the two proteins, their different stabilizing potencies and their different polarities leading to antagonistic effects. The DIT of PC has primarily been studied in conjunction with another molecule. However, in the few reviews that have addressed this, it has been demonstrated that the adsorption of PC at a water-oil interface depends on its concentration and, naturally, the pH of the aqueous medium. Research has showed that at pH 3, which is used in the present study because it favors PPI, reduces the surface activity of PC and a higher pH would be needed (Bertsch et al., 2023; Hardiningtyas et al., 2018).

In Figure 5b, the results of the dynamic process of absorption of the proteins at the water: EVOO interface as well as the DIT between EVOO and water in the absence of proteins are presented. It is evident that even when no proteins were present, the DIT between EVOO and water decreases overtime. The decrease can be attributed to EVOO's composition, EVOO issues from physical processes without any refining, this results to a higher acidity (up to an order of magnitude) due to the presence of free fatty acids with respect to refined oils, and also mono and diacylglycerols and phenolic compounds which adsorb at the interface (Cong et al., 2020; Dopierala et al., 2011; Kalogianni et al., 2017). The reduction rate as well as the total reduction of the DIT between EVOO and water is much less than when proteins are present indicating the strongest adsorption of the proteins on the interface with comparison to EVOOs biosurfactants. What can be easily observed is the fact that DIT when proteins are present, decreases in a dose dependent manner. This shows that the critical micelle concentration has not been reached at least up to a concentration of 0.6 % for the PPI and a concentration of 0.3 % for the PC. The results obtained indicate that both proteins are present at the interface when they are in a mixture. Furthermore, the combination of PPI and PC (0.6 or 1% w/v total protein) does not differ significantly from their equivalent PPI concentrations in terms of DIT decrease. In the case of EVOO the results of the different protein systems are similar probably proteins, particles and surfactants adsorb at the interface with the latter filling the gaps between particles and or proteins. It should be noted however that some of the adsorbed molecules in EVOO such as free fatty acids adsorb strongly inducing a decrease of the stability of protein emulsions (Kalogianni et al., 2017).

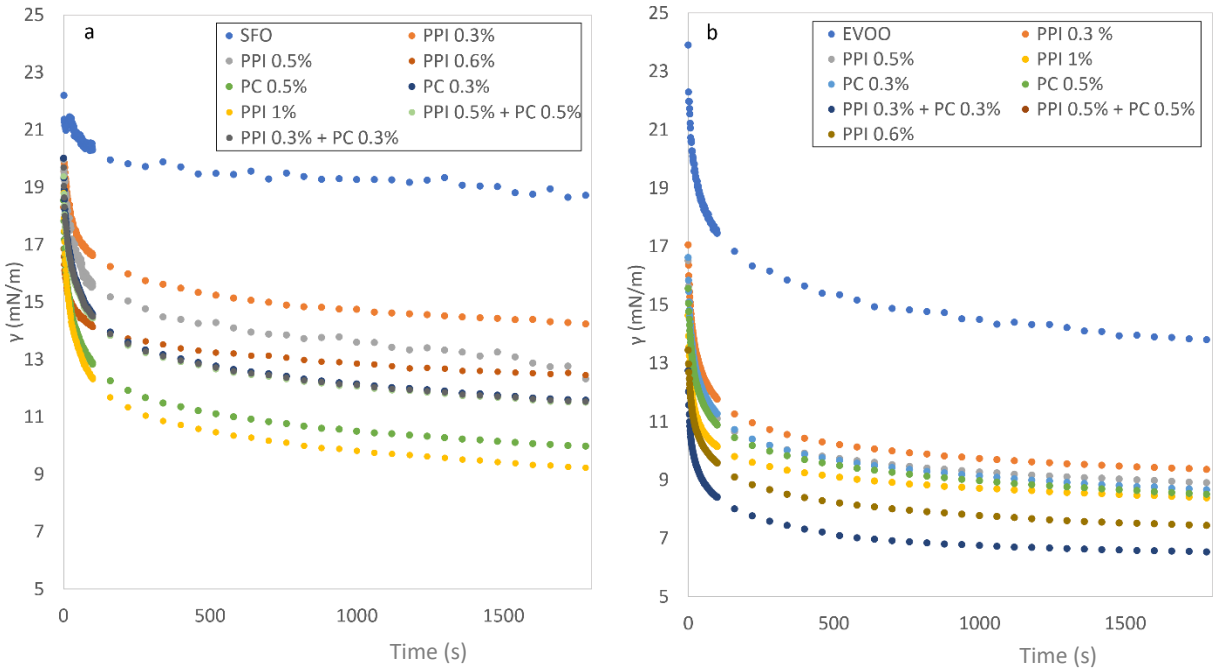

Figure 9: Dynamic interfacial tension (γ) as a function of time of PPI or the combination of PPI and PC at (a) a water: SFO (treated with ultra-pure water) interface and (b) a water: EVOO interface.

3.6 Antioxidant assessment by DPPH colorimetric assay

PC among its many beneficial abilities, possess also antioxidant properties and is able to scavenge and neutralize free radicals. There have been many studies presenting results on this matter and confirm PC's antioxidant effect (Romay et al., 1998). The question in hand here was if PC retains its activity in the emulsion. Even though the pH at which the experiments were conducted (pH 3) was not the optimum (pH 5.5-6.0) for PC to be more active at the water-oil interface it did not affect its antioxidant ability (Agrawal et al., 2021). The results showed that in the presence of PC, the system's antioxidant ability was increased two times than in its absence (Figure 6). Previously published research has confirmed PC's antioxidant ability in different matrices (Agrawal et al., 2021; Fratelli et al., 2021). Furthermore, as observed by the confocal images (Figure 4c), PC is only partly adsorbed at the water-oil interface, meaning that it is also free in the emulsion to react with DPPH. This confirms the functionality enhancement of the emulsions in the presence of PC since they remain stable, they are colored blue and possess increased antioxidant ability.

Emulsions with no PC in their aqueous phase also exhibit scavenging ability against DPPH. Since the other two main ingredients of the emulsion are PPI and EVOO there is where to search the potent antioxidant. EVOO contains ingredients like polyphenols well-known and studied as antioxidants. Squalene as well as tocopherols also add to its antioxidant profile (Lanza & Ninfali, 2020). The fact that oil is fully incorporated in the emulsion droplets and not free (Figure 4a) can

explain the decreased scavenging activity against DPPH compared to when its action is combined with PC. Finally, there are some studies reporting an antioxidant activity of PPI against free radicals and oxidative stress which is characterized as superior to that of other plant proteins (Chang et al., 2021). Figure 4b also suggests that PPI is mostly adsorbed at the water-oil interface, thus making it less available for the free radical neutralization. Overall, the proposed systems of this study exhibit potent antioxidant ability which increases even more in the presence of PC. Finally, the synergistic effect of all of the emulsions' components when they are all present cannot be ruled out.

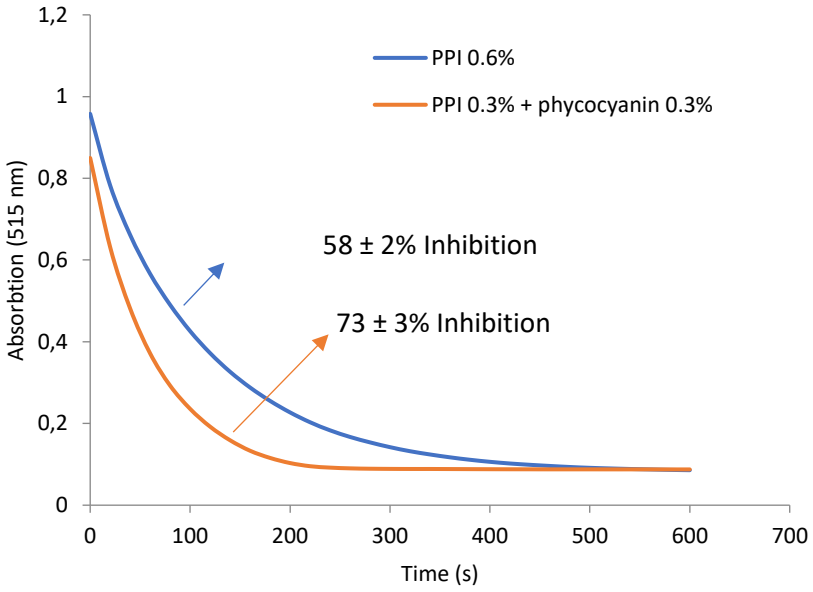

Figure 10: Time scan measurements of DPPH free radical's absorbance decrease in the presence of the emulsions.

4. Conclusions

The present study investigated the formulation of edible oil-in-water (O/W) emulsions using Pea Protein Isolate (PPI) particles and phycocyanin (PC). The PPI particles were formed through high-pressure homogenization to achieve a fine dispersion. Additionally, PC was introduced as a co-emulsifier, while retaining its functional properties as natural color agent, and antioxidant. The study aimed to optimize the emulsion preparation method and assess the stability, droplet size, and microscopic structure. The impact of PPI particles formed at different pH values (3.0, 5.0, and 9.0) on emulsion stability was explored as well as different concentrations (0.3, 0.5, 0.6 and 1 % w/v). It was found that emulsions at pH 3.0 were stable. For the formulation of particles optimal conditions

included also high-pressure treatment at 400 bars with 10 recirculation passings, resulting in smaller particles with a narrow size distribution. The addition of PC faced challenges due to its sensitivity to high temperatures. The study revealed that PC's addition after homogenization and careful pH adjustment enhanced its stability in the system. Moreover, different water-to-oil ratios (60:40 and 50:50), and two oil types (Extra Virgin Olive Oil and Sunflower Oil) were explored. Higher protein concentration and oil volume fraction were found to reduce creaming. Emulsion stability, droplet size, and distribution were influenced by protein concentration, oil type, and water-to-oil ratios. CLSM revealed the distribution of PPI and PC around oil droplets, indicating their role in emulsion stabilization. Droplet size and droplet size distribution were measured by LD and showed variations based on protein concentration and oil type. Emulsion stability over time was assessed through %CI measurements, revealing differences in stability for various protein concentrations, oil types, and water-to-oil ratios. DIT measurements using pendant drop tensiometry demonstrated the proteins' adsorption at the water/oil interface, with a concentration-dependent reduction in interfacial tension. PC's adsorption behavior differed from PPI, and their combination exhibited distinct dynamics. The findings contribute to the understanding of protein-stabilized emulsions with potential applications in food industry, such as carriers of lipophilic bioactive molecules or as coloring agents. The encapsulation and delivery of model molecules α-tocopherol and squalene has been previously described by our group in particle-stabilized emulsions. Taking into consideration the enhanced stability and oil volume fraction of these emulsions compared to the previously described ones the encapsulation of these model components or other oil soluble substances will be improved. Furthermore, the addition of phycocyanin acting as an emulsifier, a coloring agent and an antioxidant, carries even more benefits to these proposed systems. This novel carrier brings alongside multiple advantages, formulated from the combination of functional ingredients of natural origin without chemical treatment.

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Chapter 4

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

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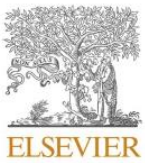

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Fungi-derived chitosan as an emulsion stabilizer for the encapsulation of bioactives

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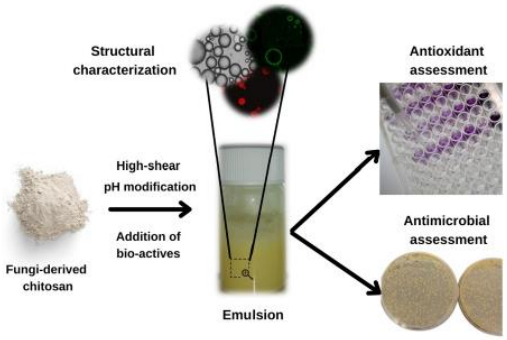
GRAPHICAL ABSTRACT



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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 μm. Furthermore, α-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

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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 α-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.

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, wall 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-β-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 Sections 2.7 and 3.6. 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 α-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

FC particles were prepared in an aqueous solution at four different concentrations, namely 0.1%, 0.2%, 0.3% and 0.5% w/v. 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 0.1 M. Since chitosan is a polycationic polymer it is fully soluble in acidic conditions. Although, in order for it to be a good candidate as a stabilizer for Pickering emulsions it needs to be partially wettable both by the oil and aqueous phase. Thus, with the pH increase it is deprotonated in a certain degree with the solubility being slightly decreased, giving it the ability to interact better with the oil phase. Finally, the particles were sonicated for 20 min (Analytika Ultrasonic Cleaner Machine, Thessaloniki, Greece), 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 − 150 °C and a pressure of 10^{-8} mBar. Subsequently, a metal knife, cooled to − 200 °C, was employed to fracture the samples. The freshly fractured surface was immediately coated with successive depositions of platinum at a 45° angle and carbon at a 90° 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 cups. 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.

$$CI \quad (\%) = \left(\frac{Hc}{Ht}\right) * 100 \tag{1}$$

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 α-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.

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,

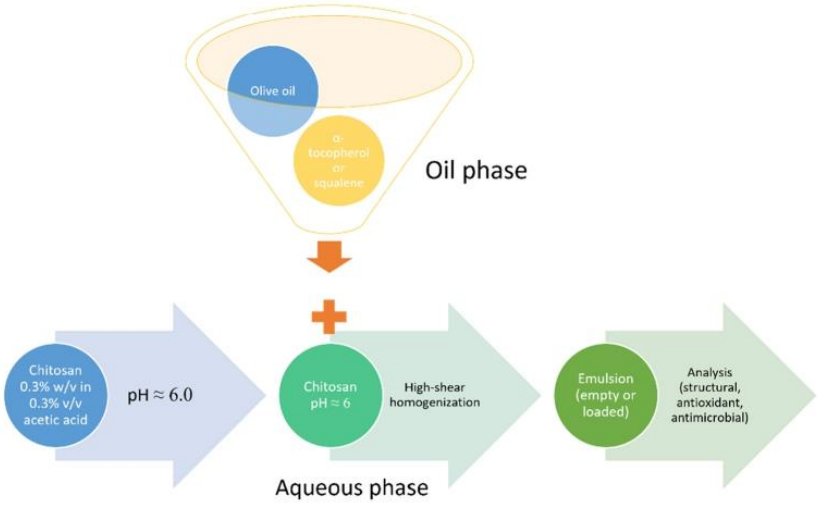

Fig. 1. Schematic representation of the emulsions' preparation method.

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 Ni representing the total number of droplets having a diameter Di and D50 signifying the median diameter, which is the diameter at which the cumulative undersized volume fraction equals 50% [32,33].

$$D = \frac{\sum_i N_i D_i^4}{\sum_i N_i D_i^3}(2) and \quad P = \frac{1}{D_{50}} \frac{\sum_i N_i D_i^3 |\quad D_{50} - D_i|}{\sum_i N_i D_i^3} \tag{2}$$

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 °C, where they were fractured using a cold blade. Ultimately, the processed samples was positioned onto an 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 μL 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 μL 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].

$$\%Inhibition = \left[\frac{A0 - A1}{A0}\right] * 100 \tag{3}$$

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 α-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

In this study, we assessed the capability of fungi-derived chitosan to form particles and subsequently stabilize Oil-in-Water (O/W) emulsions. These particles were produced from chitosan derived from mushrooms using the pH shifting method. The emulsions were prepared by high-shear homogenization. The particles were characterized in terms of their size and shape using Dynamic Light Scattering (DLS) and Freeze Fracture Transmission Electron Microscopy (FFTEM). Macroscopic observations employing the creaming index evaluation were conducted. Additionally, the droplet morphology and size were measured using a Mastersizer granulometer. Furthermore, we investigated the capacity of FC-based emulsions to efficiently encapsulate lipophilic bioactive compounds, such as squalene and α-tocopherol, using the aforementioned techniques. We also conducted stability studies to assess their performance over time. Furthermore, we evaluated the antioxidant capacity of both empty and loaded emulsions through a DPPH colorimetric assay. Finally, the antimicrobial ability of the emulsions was assessed by using *E. coli* alongside *S. aureus* as target strains starting from two different initial populations.

3.1. Particles preparation, dimensional determination and morphology observation

Crafting 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.

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%

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

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

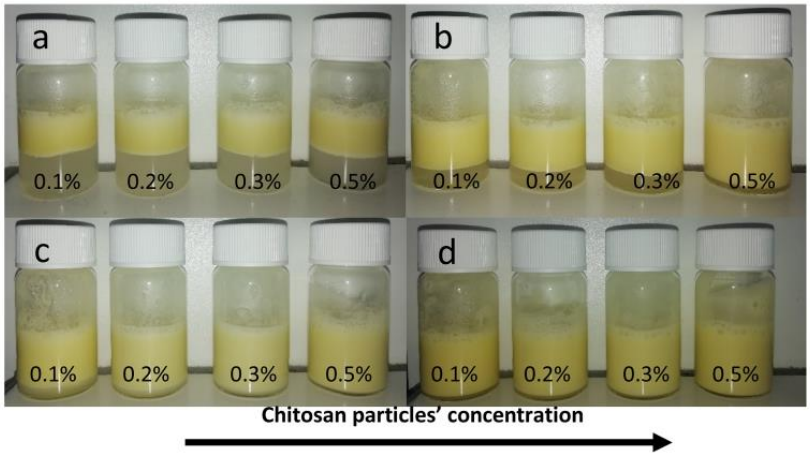

Fig. 2. Emulsions prepared with chitosan particles at different concentrations (%w/w) presented from lowest to highest concentrations (left to right) in 4 different water:oil ratios (a) 80:20 water:oil ratio, (b) 70:30 water:oil ratio, (c) 60:40 water:oil ratio and (d) 50:50 water:oil ratio.

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 μm). 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 α-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 that 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.

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 that 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 of 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 that 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 α-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 α-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.

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 α-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].

Table 2
Determination of the emulsions' droplet diameter and polydispersity P by Static Light Scattering. Each value in the Table is represented as mean ± SD (n = 3).

Sample	d (μm)	P
Chitosan empty	75.2 ± 4.2	0.63 ± 0.02
Chitosan α- tocopherol	61.6 ± 4.6	0.57 ± 0.03
Chitosan squalene	71.8 ± 2.7	0.46 ± 0.01

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 α-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 α-tocopherol appeared to be more drastic that squalene. In that case empty emulsions' inhibition against DPPH ranged from 25 to 39% while for α-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 α-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

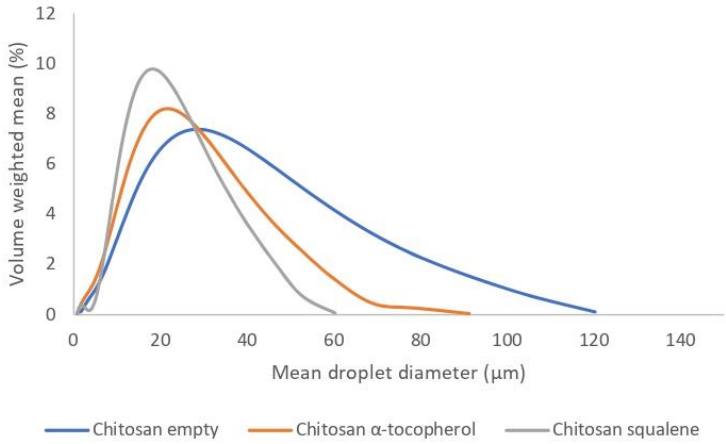

Fig. 3. % Volume weighted mean diameter of chitosan emulsions droplets for empty emulsions, loaded with α-tocopherol or loaded with squalene emulsions.

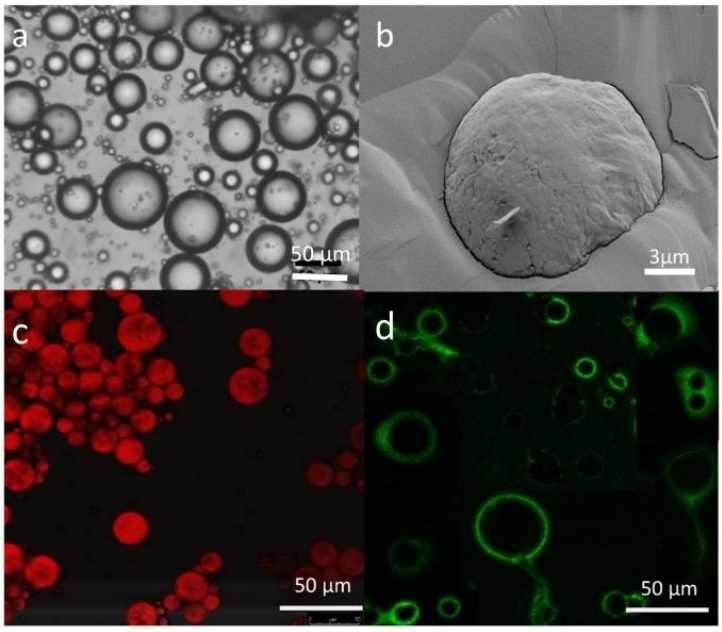

Fig. 4. (a) brightfield image x10 magnification of chitosan empty emulsion with optical microscope (b) Cryo-SEM image of chitosan emulsion, (c) oil droplet confocal image x20 magnifichation of chitosan empty emulsion and (d) chitosan "ring" confocal image x20 magnification of chitosan empty emulsion.

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

% DPPH Scavenging			
Chitosan 0.3% w/w	Chitosan empty	Chitosan α-tocopherol	Chitosan squalene
5.3 ± 0.1	37.3 ± 0.7	86.1 ± 0.1	620. ± 0.1

both oil soluble the hydroxyphenyl group of α-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 α-tocopherol is dispersed in an oil medium with squalene, then α-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.

3.5. Assessment of the emulsions' antimicrobial activity

Chitosan's antimicrobial activity has been previously assessed. The

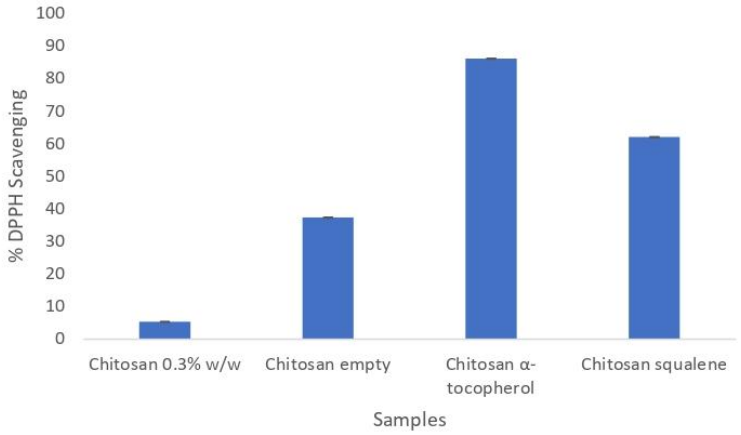

Fig. 5. % Scavenging ability of empty chitosan emulsions and their respective loaded ones with α-tocopherol or squalene.

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 α-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 α-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 °C 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).

	E. coli (3 log cfu/mL)				*E. coli* (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	1.3	2.3	1.8	2.6	3	5.5	0	4.9
Loaded with α-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
	S. aureus (3 log cfu/mL)				*S. aureus* (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 α-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 μm), depending on each chitosans 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 was 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 ± 1.2 μm, while crab-derived chitosan gave emulsions with larger droplets of 157.3 ± 2.6 μm. 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 crustacean-derived chitosan forms was measured with DLS. Results showed very large particles of different size populations (240.2 nm ± 10.2, 830 nm ± 3.7) accompanied by a rather high PDI value of 0.98 ± 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 (γ) between oil and water in the presence of the two chitosans. It is evident that both polymers rapidly decrease γ values with fungal chitosan having a small lead. The final values recorded after allowing the systems to reach an equilibrium were 9.78 ± 0.01 for crab derived chitosan and 8.98 ± 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 support the results of the present study [82].

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 α-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 α-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.

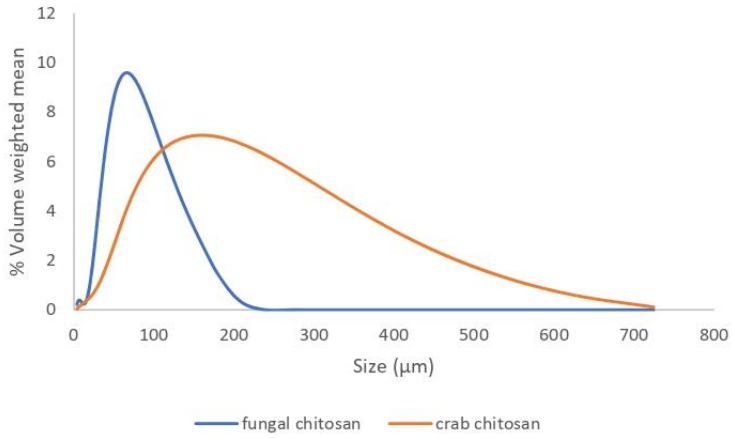

Fig. 6. % Volume weighted mean droplet diameter of emulsions stabilized by mushroom or crab chitosan.

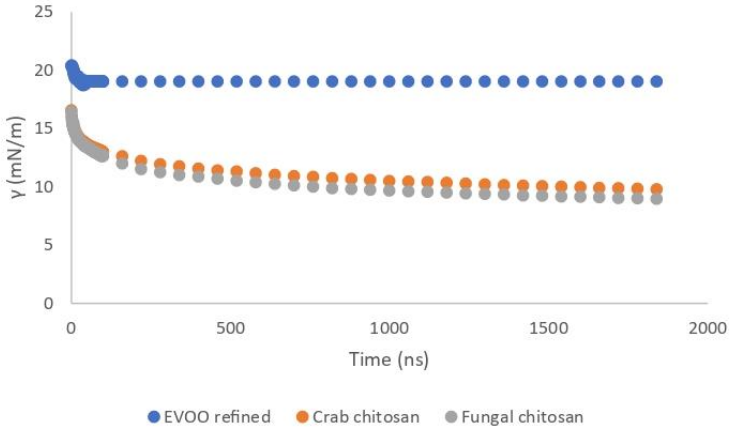

Fig. 7. Dynamic interfacial tension γ of fungal and crab derived chitosan with refined olive oil as a control.

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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Chapter 5

Multifunctional Pickering emulsions stabilized by probiotic bacteria-fungal chitosan conjugates

Galani E., C. Fragkou, G. Zoumpopoulou, A. Topali, Theodora Katsila, E. Tsakalidou, V. Papadimitriou, M.D. Chaztidaki, **"Multifunctional Pickering emulsions stabilized by probiotic bacteria-fungal chitosan conjugates"** (to be submitted to *Nature Communications*, 2024)

Multifunctional Pickering emulsions stabilized by probiotic bacteria-fungal chitosan conjugates

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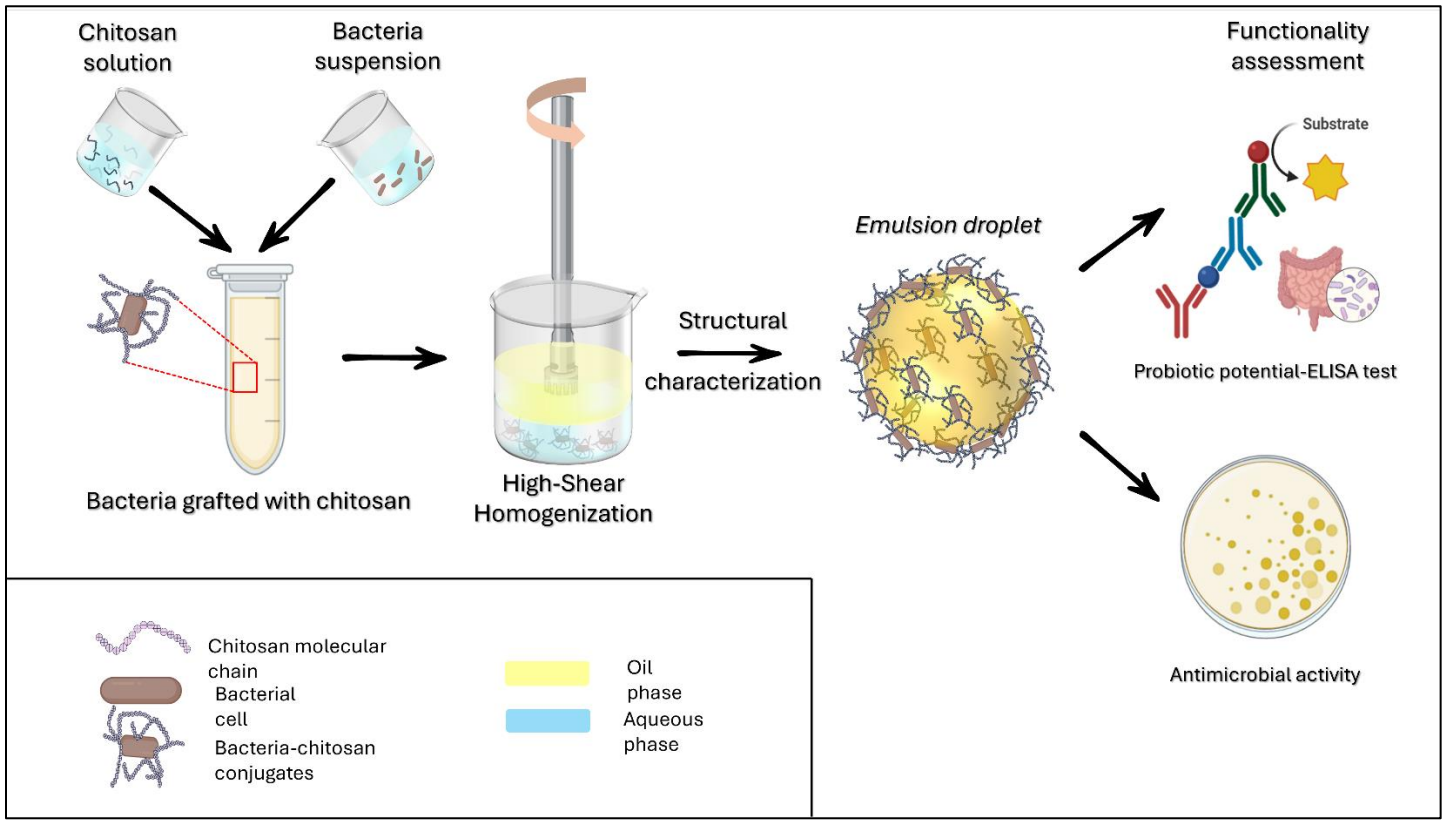
Keywords: Pickering emulsions, probiotic bacteria, fungal chitosan, encapsulation, functionality

Graphical abstract



Abstract

This study introduces a novel Pickering emulsion (PE) stabilized by probiotic bacteria-fungal chitosan conjugates (BFCs). These BFCs, formed via electrostatic interactions between fungal chitosan (FC) and probiotic bacteria, enabled the formulation of PEs with 50% wt. extra virgin olive oil (EVOO) through high-shear homogenization. Bacterial viability assays showed minimal reduction, and ζ-potential measurements confirmed FC-bacteria interactions. Laser Diffraction (LD) revealed droplet sizes of 50-110 μm. Confocal microscopy confirmed BFCs surrounding oil droplets. Functionality tests showed antimicrobial activity against *E. coli* and *S. aureus*. ELISA tests and untargeted metabolomics demonstrated that BFCs and BFC-stabilized emulsions exhibited anti-inflammatory properties by reducing pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. These findings highlight the potential of BFC-stabilized PEs in delivering bioactive compounds with enhanced antimicrobial and anti-inflammatory activities.

Introduction

Pickering emulsions (PEs) are stabilized solely by particles that can be adsorbed at a water-oil interface[1]. They present certain advantages over conventional emulsions stabilized by surfactants including increased stability[2], absence of potentially harmful chemical surfactants[3], tailorable properties by selecting appropriate particles[4], stimuli responsiveness[5] and biocompatibility[6]. Their superiority over other types of emulsions lies in the fact that they can carry their own functionality apart from being delivery vehicles for other components. This ability originates from the multitude of particles that can be chosen to endow a system's desirable characteristics. These particles include proteins[7], polysaccharides[8], inorganic particles (such as TiO_2[9]) and other compounds[10]. Additionally, more recent studies exploit the benefits of PEs in fabricating microreactors for chemical synthesis via flow cascade reactions[11].

Chitosan is considered as one of the most attractive polysaccharides derived from crustaceans, invertebrates, and fungi. Chitosan has several valuable diverse applications in a wide range of industries as it exhibits several applications including biomedical and pharmaceutical applications for wound healing and drug delivery, in the food industry as an antimicrobial agent and emulsifier, as well as in cosmetics applications due to its moisturizing, anti-aging, and antimicrobial properties[12]. As a natural material, chitosan creates opportunities for the development of sustainable and environmentally friendly products[13]. Moreover, as an emulsion stabilizer, it has excellent potential owing to its surface activity, its ability for electrostatic interactions with negatively charged molecules as well as its gelling and film-forming properties[14,15]. The introduction of chitosan in emulsion formulations can improve stability, texture and appearance while contributing to the extension of products' shelf-life[16,17]. Chitosan's natural origin, biocompatibility, and biodegradability make it highly appealing for food applications, aligning with consumer preferences for clean labels and sustainable ingredients. Fungal chitosan (FC) offers a vegan-friendly and sustainable alternative, free from animal-derived allergens and suitable for animal-free applications. Recent studies have highlighted its superior performance as a PE stabilizer in terms of stability and also, as an antimicrobial agent compared to conventional chitosan[18]. These attributes underscore FC's potential to enhance the stability and functionality of PEs, thereby contributing to the development of sustainable and effective formulations.

There is a limited number of published papers concerning the stabilization of PEs by microbial cells. These cells rarely need elaborated treatment before using them while they can be excellent stabilizers and remain active as part of the emulsions. *Mycobacterium neoaurum* has been reported twice as a PE stabilizer alongside modified silica particles or alone, being able to form stable emulsions[19]and act as a biocatalyst[20]. *Alcaligenes faecalis* ATCC 8750 was used for interfacial catalysis in another study that also reported excellent results[21]. An earlier study dealt with the stabilization mechanisms underscoring bacterial cell adsorption and demonstrated

that bacteria can produce the so-called biosurfactants. In this study, several bacterial strains were tested indicating the dependence of PE stability on the bacterial genus, species and strain[22].

Even fewer studies deal with the stabilization of PEs by food-grade microorganisms. When it comes specifically to probiotic bacteria, the most common way they are reported as part of PEs is by encapsulation, meaning that the emulsions are used as vehicles for the delivery of the microorganisms and a means of protection. Reports showed that the encapsulation of probiotics enhances their durability in harsh environments and thus, increase their potential after administration[23]. For stabilizing emulsions, *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, and *Streptococcus thermophilus* were used untreated and produced stable emulsions. The same study reported that the stability of the emulsions was affected by the kind of bacteria used[24]. In another case, *L. acidophilus* was treated by octenyl succinic anhydride (OSA) that increased PEs stability[25]. Finally, a recent study explored the potential of 19 food-grade microorganisms as PE stabilizers. In particular, thermally inactivated yeast, cocci, *Bacillus* spp. and lactobacilli cells were tested. The resulting emulsions were of various droplet sizes and stability times with the differentiations being attributed to the bacteria morphology and cell wall composition[26].

Taking into account the need for multifunctional carriers suitable for various applications we combined for the first time, to our knowledge, two bioactive substances, namely FC and probiotic bacteria to form a novel conjugate. After extensive literature review only one study was found combining animal-derived chitosan with *Escherichia coli* DH5α to form non-edible PEs[27]. The objective of the present study was to assess the ability of BFCs forming functional emulsions by retaining their individual functionalities and thereby, transferring them to the formulated carrier. Therefore, three different bacterial strains, known for their probiotic potential, namely *Lactobacillus delbrueckii* subsp. *bulgaricus (L. bulgaricus)* ACA-DC 87[28], *Streptococcus thermophilus (S. thermophilus)* ACA-DC 26[29] and *Limosilactobacillus fermentum (L. fermentum)* ACA-DC 179[30–32]were tested alongside FC for their stabilizing ability via surface charge measurements, Dynamic Interfacial Tension (DIT) determination and viability assessment after high shear. To further study the encapsulation properties of the carriers, two model bioactives, namely α-tocopherol and squalene were chosen to be encapsulated in the oil droplets. Even though they are both oil-soluble substances, they possess unique properties by altering the overall performance of the carriers in different ways and leading to multifunctional systems with tunable characteristics. All systems were structurally characterized microscopically as well as for their droplet size and in terms of stability. The dual functionality of the proposed systems was tested, first via a killing assay determining antimicrobial activity towards pathogenic bacteria, and secondly, by evaluating the anti-inflammatory potential of the carrier. Our results highlight the potential of creating multifunctional carriers with tunable potency (by choosing the right ingredient combination) that can find application in various industries. Furthermore, they provide valuable

information regarding the ability of FC and tested probiotics to act while they are conjugated and adsorbed on an emulsion interface.

Results and Discussion

Optimization of the emulsion system

Preparation of emulsions

Because of the study's novelty and the gap in peer-reviewed literature concerning the existence of probiotic bacteria-chitosan conjugates for stabilizing emulsions, the first step taken was to verify which stains, if not all, could form conjugates able to act as emulsifiers. The factors that were altered in the attempt to optimize the produced systems were the bacterial strain [*S. thermophilus* ACA-DC-26 (ST) or *L. bulgaricus* ACA-DC-87 (LB) or *L. fermentum* ACA-DC-179 (LF)], the FC concentrations (0.03, 0.10, 0.20, or 0.4 %, w/v), and the homogenization speed and its duration (15000 rpm/3 min or 20000 rpm/1 min). Different combinations of the aforementioned conditions led to different properties of BFCs and prepared emulsion systems[33]. Of note, in all formulation processes, extra virgin olive oil (EVOO) was used and the aqueous-to-oil phase ratio (1:1) remained the same.

In terms of the BFCs' stabilizing potential, it was observed that neither ST nor LB could incorporate oil in the case of C-ST1, C-ST2 and all LB samples (C-LB1, C-LB2, C-LB3, C-LB4 and C-LB5) as the emulsions collapsed within 1 h after preparation. BFCs C-ST3, C-ST4, and C-ST5 could provide emulsions that collapsed 4 days after preparation. On the other hand, LF conjugates resulted in the best outcomes when the formulation of emulsions was explored. EVOO was incorporated fully with all LF conjugates tested (C-LF1, C-LF2, C-LF3, C-LF4, and C-LF5), while only a small amount of oil was visible on top of the emulsions in the case of C-LF1. Emulsions remained stable for more than 20 days with no alteration of the emulsions after macroscopic observation. Thus, *L. fermentum* ACA-DC-179 was chosen for further testing.

Strain viability before and after high-shear treatment

The viability of the three strains used for the preparation of the emulsions was tested against high-shear treatment and results are presented in Figure 1. The experiment took place for unconjugated LF, LB and ST because in preliminary tests FC crated a "masking" effect, leading to false enumeration[34]. Thus, all three strains were resuspended in water and then, they were treated or not with high shear

At first, the initial population of the strains before any treatment was determined. LF was found to have the highest initial cell count (9.15 ± log cfu/mL) followed by LB and ST with initial populations 8.45± and 8.22± log cfu/mL, respectively. Next, strains' suspensions were mixed with EVOO (1:1 ratio) using high

shear at both conditions, namely, 15000 rpm for 3 min and 20000 rpm for 1 min. As shown in Figure 1, ST and LB showed a significant decrease in their initial cell count (>1 ± log cfu/mL), while LF exhibited high viability and minimal mortality in both test conditions (<0.5 ± log cfu/mL).

Considering the viability results obtained by the high-shear treatment tests, the two homogenization conditions did not differ in terms of the final cell count, suggesting that any choice could be made (i.e. to procced with either 15,000 rpm/3 min or 20,000rpm/1min). Nevertheless, a homogenization speed of 15,000 rpm for 3 min was employed for all subsequent experiments to minimize any potential stress on the strains. Finally, the decision to continue the study with LF was supported by viability results as they showed that LF exhibited the least mortality among the three strains as even after high-shear treatment, the cell count did not drop below 8.9± log cfu/mL.

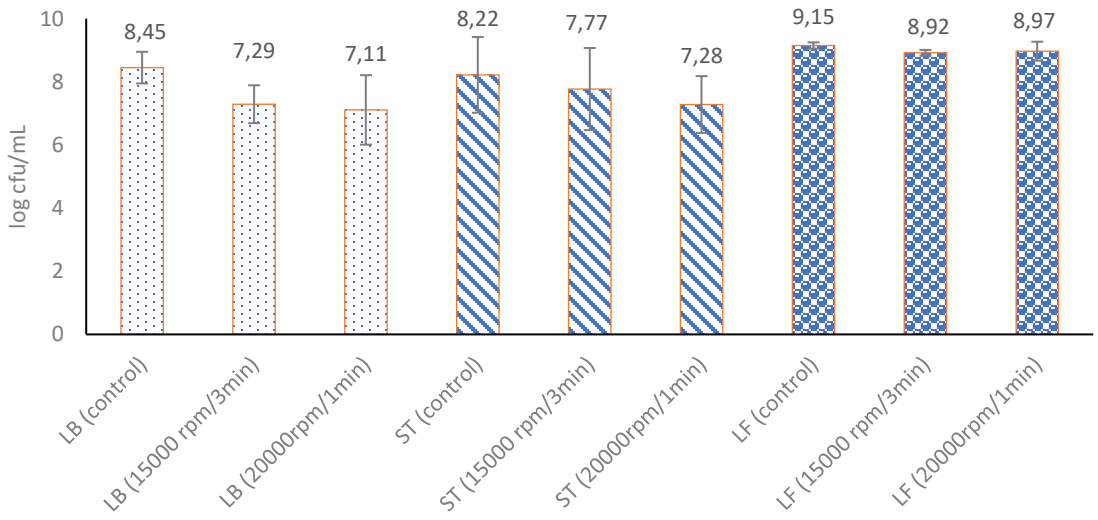


Figure 11: Enumeration of LF, ST, and LB before any treatment and viability assessment of them when mixed with EVOO in 1:1 ratio and subjected to high-shear treatment at either 15000 rpm for 3 min or at 20000 rpm for 1 min

Characterization of conjugates

ζ-potential

For the LF conjugates, the ζ-potential values were determined. The ζ-potential measurement allowed us to determine the interactions between LF and FC by assessing the electrostatic surface charge of their combination. Additionally, when it comes to Pickering, particles' surface charge indicates the final stability of the emulsions, as a higher surface charge means an increased repulsion between the droplets decreasing coalescence.

From the findings presented in Figure 2, it is evident that the only negative value comes from the bacteria (-8.1 mV). This finding is consistent with results from previous studies and it can be attributed to the Gram-positive nature of the strains considered, with their cell wall being rich in teichoic acids, i.e. co-polymers of glycerol- or ribitol phosphate and carbohydrates linked via phosphodiester bonds[26]. Furthermore, FC shows a positive surface charge that increases in a FC dose-dependent manner with values ranging from 59.6 to 75.6 mV. These results were anticipated, as FC is a polycationic polysaccharide, and its surface properties have been well-studied[35]. These values indicate that the oppositely charged bacterial cells and FC are likely to electrostatically interact with one another. This interaction is reflected in the ζ-potential values recorded when the bacteria (9 log cfu/mL) were resuspended in the various FC concentrations (Figure 2).

The BFCs formed had positive surface charges that increased from 14.4 mV to 40.7 mV as the concentration of FC increased(Figure 2). The conjugates' ζ-potential values were lower than those of FC and significantly higher than the one obtained for unconjugated LF resuspended in distilled water[36]. For a colloidal dispersion to be considered stable, its ζ-potential should be either more positive than +30 mV or more negative than -30 mV[37]. Therefore, it can be assumed that the conjugates formed with 0.2, 0.3, and 0.4% w/v FC were the most stable ones, leading to the formulation of more stable emulsions. The results from ζ-potential measurements indicate that the structure of the formulated conjugates comprises of bacteria covered by FC. TEM images obtained in a previous study proposed the same structure for chitosan-*E.coli* conjugates[27].

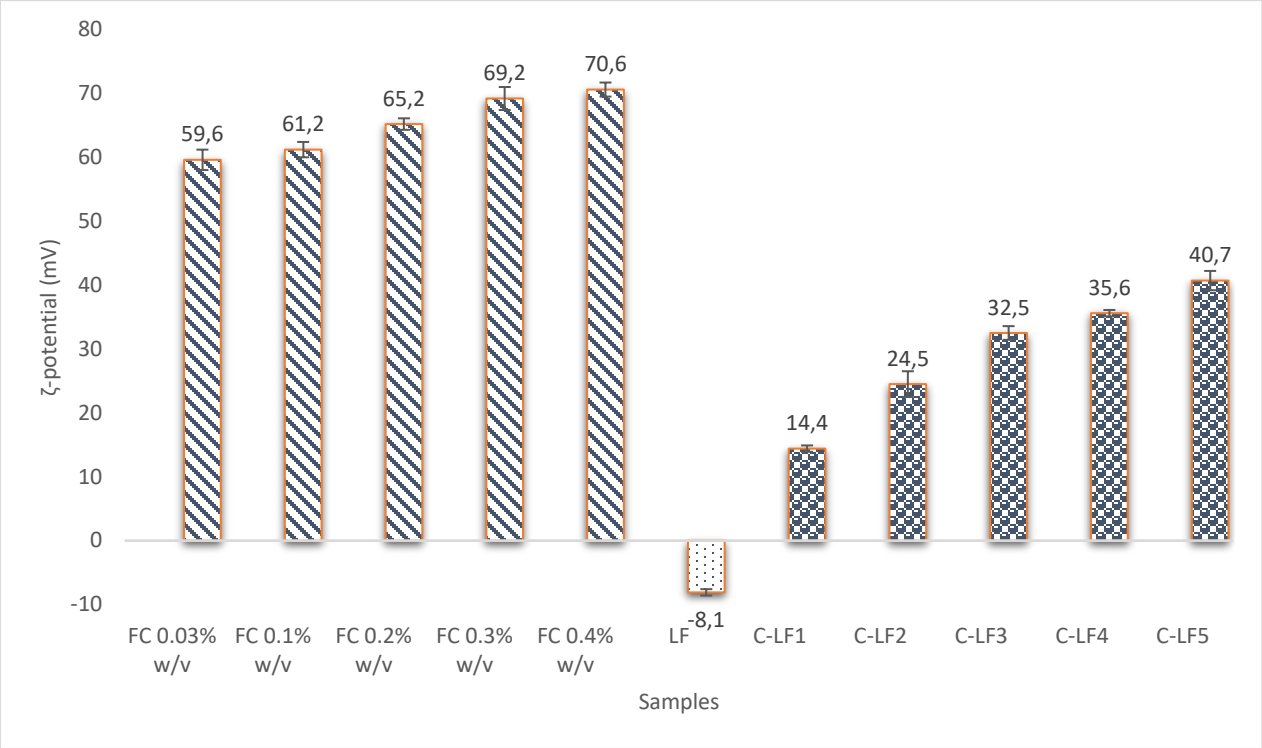


Figure 12: The ζ-potential values of FC solutions at concentrations of 0.03, 0.1, 0.2, 0.3, and 0.4% w/v of L. fermentum ACA-DC-179 (9 log cfu/mL) and their combinations

Dynamic Interfacial Tension (DIT)

DIT tendency shows the adsorption rate of particles on an interface. High DIT decrease indicates enhanced interfacial adsorption and increased stability[38]. Conjugates C-LF1, C-LF2, C-LF3, C-LF4, and C-LF5 were studied and compared to one another in terms of their ability to decrease DIT. As a control sample, Ultra-pure water in SFO was tested. Results presented in Figure 3 showed that DIT remains stable through time in the absence of surface-active ingredients for the control sample. The results of the conjugates' behavior at the water-SFO interface show a dose-guided decrease pattern when FC concentration increases in the conjugates. The DIT decrease recorded (Figure 3) suggests that the conjugates could, indeed, act as suitable stabilizers for Pickering emulsions. The course of DIT that BFCs follow is also mirrored in the ζ-potential values obtained. As mentioned above, the more positive the ζ-potential value the more stable the particles in question are. In the same way, the lowest the DIT, the highest the adsorption of the examined particles at the water-oil interface. Conjugates C-LF-1, C-LF2, and C-LF3 exhibited similar decrease among them in terms of DIT. On the other hand, the two most potent conjugates were C-LF4 and C-LF5, but their respective activities did not differ significantly. In conclusion, we have two major groups of conjugates when it comes to DIT decrease potency.

The less potent group consists of C-LF1, C-LF2 and C-LF3, while the most potent group includes C-LF4 and C-LF5. Finally, conjugates C-LF2 and C-LF5 were chosen as two extreme conditions to be examined for their anti-inflammatory potential and antimicrobial activity

Both bacteria and chitosan can present some surface-active properties but under specific conditions. Chitosan itself has a very weak surface activity because it lacks hydrophobic segments according to the provided sources and needs to be modified [39,18]. These facts can lead to the conclusion that the decrease of the DIT is a result of the conjugates' themselves and not of their individual components. Although to our knowledge no other study so far has used the pendant drop technique to determine the effect of such conjugates at the emulsions' interface, there are peer-reviewed literature data on the θ contact angle of such particles. It was found that in the presence of chitosan bacteria cell surface hydrophobicity increased drastically providing particles that could act as emulsion stabilizers supporting our obtained data[40].

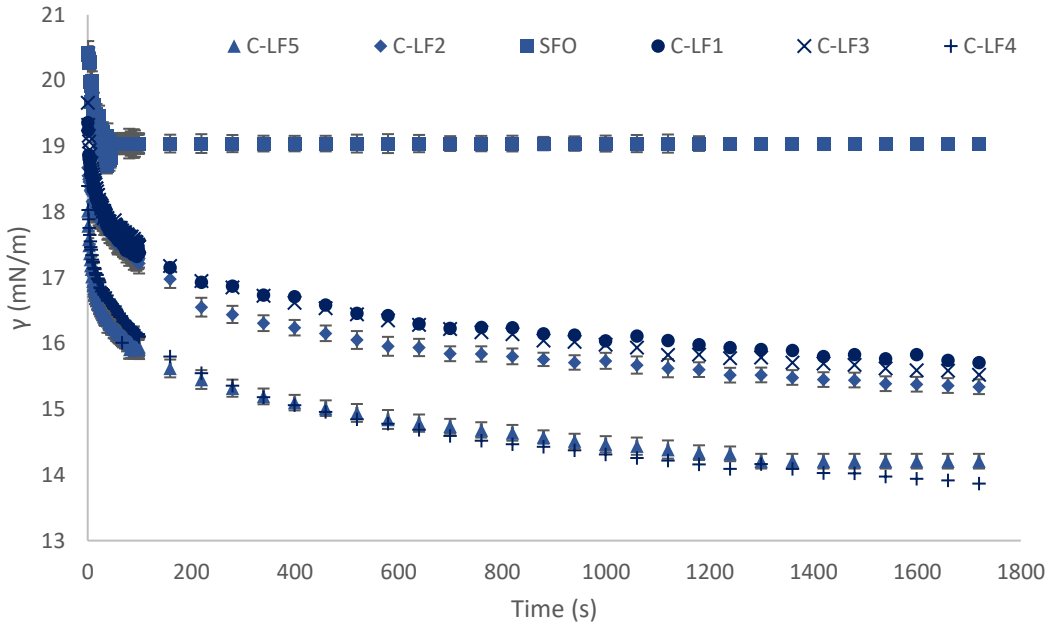


Figure 13: Dynamic Interfacial Tension of L. fermentum ACA-DC-179 and FC conjugates in the presence of clean SFO. The interfacial tension between EVOO and ultra-pure water is also presented

Structural characterization of emulsions

Droplet size determination by Laser Diffraction (LD)

LD was used to determine the droplet size and droplet size distribution of emulsions stabilized by BFCs of LF resuspended in FC solutions of various concentrations (Figure 4).

The droplet sizes recorded ranged from 50.6 μm to 110.2 μm. It was evident that LF1 and LF2 gave the largest droplets of 110.2 μm and 75.2 μm, respectively. These two emulsions also appeared to have a second very small sub-population with droplet sizes of about 10 μm. Furthermore, the droplet size distributions were quite wide, especially for the emulsions stabilized by LF1. With the increase of FC concentration in the BFCs, there was also a decrease in the emulsions' droplet size. The emulsions stabilized by LF3, LF4 and LF5 had the smallest droplet sizes, namely 51.9 μm, 51.6 μm and 50.6 μm, respectively, with a narrow size distribution.

To our knowledge, only two previous studies have explored the stabilization of emulsions using bacterial cells and chitosan, with one focusing on food-grade formulations. Although emulsions were successfully prepared, droplet sizes exceeded 0.5 mm[27,41]. The present study, however, demonstrates the competence to achieve smaller droplet sizes, thus potentially advancing emulsion stabilization approaches.

The results of the droplet sizes were also in accordance with the ones of DIT and ζ-potential measurements[42]. In fact, BFCs with the highest ζ-potential values were presumed to be the most stable. Concurrently, DIT measurements indicated enhanced adsorption at the water-oil interface, resulting in smaller droplet sizes and suggesting more stable emulsions.

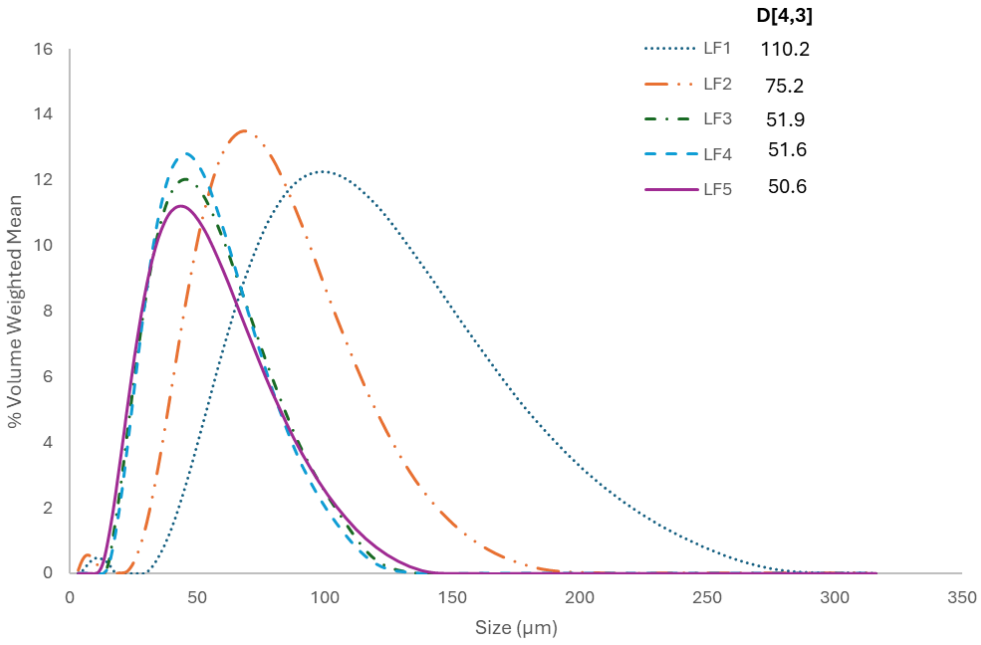


Figure 14: % volume weighted mean diameter of emulsion *droplets* stabilized by BFC conjugates. D [4,3] values are also presented

Confocal Laser Scanning Microscopy (CLSM) and Optical Microscopy (OM)

CLSM was chosen to visualize the BFC interfaces as well as the incorporated oil. FITC was employed to stain FC used for the preparation of the BFCs, and nile red for EVOO's staining. Samples were analysed after being properly diluted (1:10 in distilled water). For the OM observations no staining or dilution was necessary.

In Figures 5a and 5b the BFCs are depicted. In brief, Figure 5a depicts one single droplet covered by BFCs. Furthermore, the BFC rings are visible around the droplets in Figure 5b and there is no green color detected in the aqueous phase suggesting that BFCs are mostly adsorbed at the water-oil interface. In Figure 5c, red spherical drops correspond to EVOO stained with nile red. The fact that the red colour appears only inside the droplets and not in the background can safely lead to the conclusion that all of the oil was incorporated into the droplets and the emulsions were of O/W type as predicted. Additionally, CLSM images of the BFCs suspended in water were obtained (Figure 5d). This image shows bacteria cells covered by chitosan, which were previously stained with FITC dye. Finally, in Figure 5e, a brightfield image of the emulsion is presented. A similar chitosan-bacteria cell network was also depicted in the work of Wongkongkatep et al., showing a bright ring around the droplets [27].

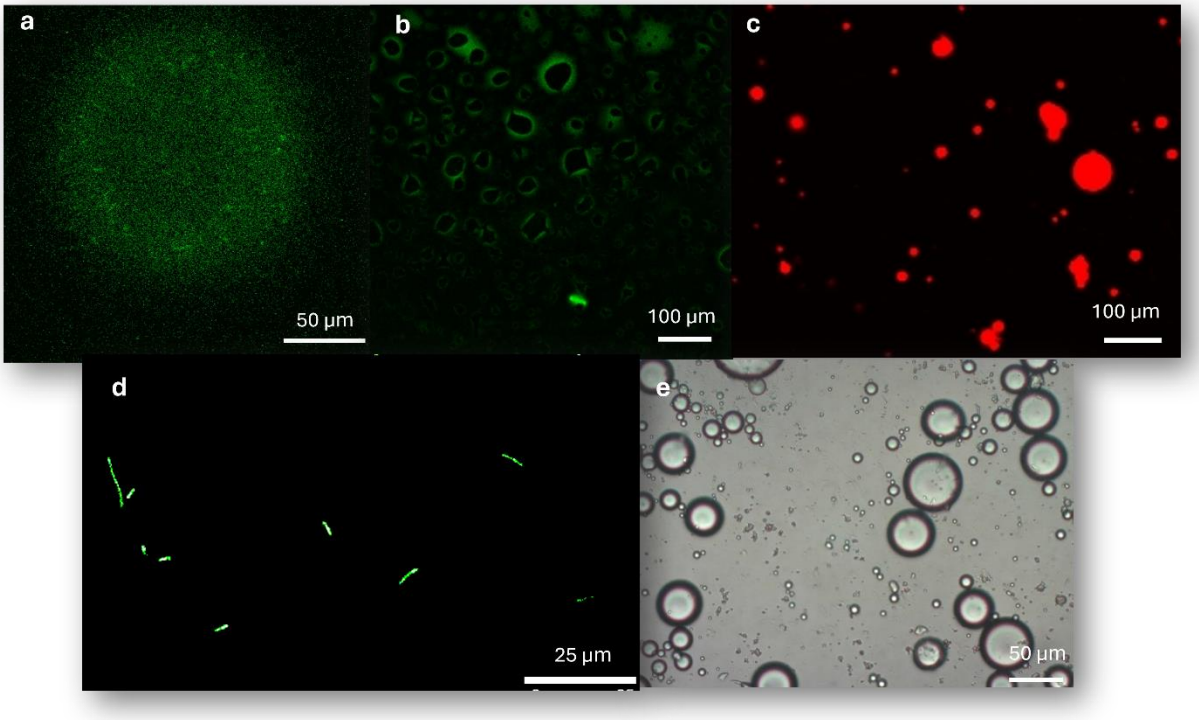


Figure 15: Confocal images of (a) a x60 empty emulsion droplet at z=10 µm and BFCs stained with FITC, (b) x20 empty emulsion droplets at z=60 µm and BFCs stained with FITC (c) x20 empty emulsion droplets at z=60 with the oil phase being stained with nile red, (d) x40 BFCs stained with FITC, and (e) x20 brightfield image of empty emulsion

Stability of the emulsions against creaming and coalescence

The assessment of the emulsions' stability against creaming was carried out through the observation of the % Creaming Index (% CI). Creaming is a naturally occurring, reversible phenomenon attributed to the density mismatch between EVOO and the aqueous phase as well as to the non-Brownian nature of the droplets that are larger than 1 µm. When creaming occurs, a serum layer is formed under the emulsion[43]. The progression of this layer was followed for all LF emulsions (Figure 2) when they were stored at either ambient temperature or 4°C. The evolution of the creaming in the sample containing 0.03% w/v FC (LF1) was also observed despite the presence of the small amount of oil being visible on top.

All LF emulsions presented creaming within 1 h after preparation and the increase of FC concentration did not seem to affect creaming so all emulsions on day 0 had a CI of 89%. The sample with the lowest FC concentration in the BFCs (LF1) had also a small amount of oil on top not fully incorporated in the system. Nevertheless, it remained stable at ambient temperature for 10 days with no measureable changes in the % CI until the emulsion's sudden collapse. System LF2 was the next one to progressively collapse. Its CI was 85.6 % on day 15 reaching finally 81.2%on day 22, when it collapsed and the oil was visible on top. All other three

emulsions (LF3, LF4 & LF5) were stable for more than 35 days with no changes in their % CI. After 90 days, their consistency appeared to change and on day 96 the oil was visible on top of them. When samples were refrigerated at 4^{o}C no changes were observed in any of the samples, except LF1. The latter collapsed after 18 days of storage while the other four (LF2, LF3, LF4 & LF5) remained stable for more than three months. The quicker destabilization of LF1 and LF2 emulsions is a direct reflection of the lower ζ-potential and DIT they presented. Finally, the BFCs with the highest ζ-potential values and with the highest decrease in DIT, indeed formed more stable emulsions.

Although no other studies so far have specifically examined the stability of such systems against creaming, the results presented here are consistent with peer-reviewed literature data on stability against creaming of other Pickering emulsions using particles, such as solely chitosan, or solely bacterial cells[7,19].

Functionality of emulsions

Antioxidant activity

The DPPH colorimetric assay was used to compare the overall antioxidant activity of LF emulsions stabilized by BFC conjugates being either empty or with encapsulated α-tocopherol or squalene. The antioxidant activity of the samples was measured spectrophotometrically after 30 min of reaction between the free radical and the samples (Figure 6). It was evident that all samples exhibited antioxidant activity, which increased in the presence of the bioactives. The antioxidant activity of the empty emulsions can be attributed to the presence of EVOO[44]. The inhibition of the DPPH reaches 82.8% when squalene is present and 91.4% when α-tocopherol is the encapsulated molecule. Similar results were obtained by previous studies of our group where the two bioactives were incorporated in emulsions stabilized by plant proteins or fungal chitosan[7,18]. Furthermore, other studies corroborate these results, presenting a more effective action of α-tocopherol compared to squalene against oxidation[45]. Although research on the antioxidant activity of α-tocopherol is much more extended than that of squalene, the main argument in favor of α-tocopherol's superior antioxidant activity lies in its unique structure and reactivity[46]. The structure of the two molecules plays a key role in the particular environment described in this work. Their chemical structure indicates their location inside the droplet and since squalene is a triterpenoid oil it can be deduced that it is perfectly compatible with EVOO and fully dissolved in it. On the other hand, even though α-tocopherol is a fat-soluble vitamin, it contains a polar group comprising hydroxyl groups and the chromanol ring. This polar group has been proven to be oriented towards the surface of the membrane making it easier to interact with the exterior environment, which in this case is the environment of the free radical[47].

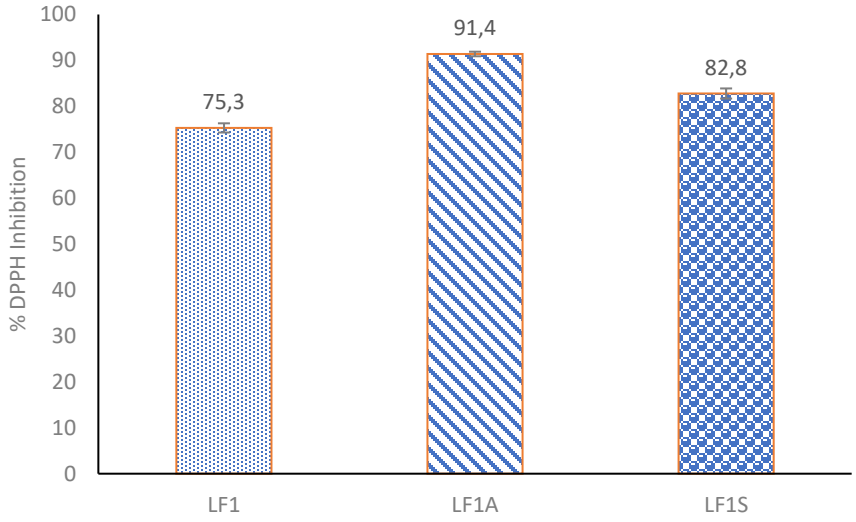


Figure 16: % Inhibition of the DPPH free radical by empty emulsions (LF1), emulsions loaded with α-tocopherol (LF1A), and emulsions loaded with squalene (LF1S) after 30 min of reaction time

Antimicrobial activity

The antimicrobial efficacy of LF2 and LF5 emulsions (empty and loaded with α-tocopherol or squalene) against *E. coli* and *S. aureus* was evaluated. Our previous research demonstrated that the emulsions stabilized by FC particles possess excellent antimicrobial properties[18]. The challenge in this study was to determine if the antimicrobial action of FC would be maintained when conjugated with microbial cells as well as how the rest of the ingredients (LF, α-tocopherol, squalene) would add to it. The emulsions were mixed at a 1:1 ratio with *E. coli* 3 log cfu/mL and 6 log cfu/mL, respectively (Figures 7a and 7b), and *S. aureus* 3 log cfu/mL and 6 log cfu/mL (Figures 7c and 7d). It was evident that all emulsions were distinctively more effective against *S. aureus* than against *E. coli* [48].

S. aureus showed much higher susceptibility to the emulsions. As shown in Figures 7c and 7d, all LF emulsions significantly affected the microorganism. In Figure 7c, it is shown that the initial population of *S. aureus* (3 log cfu/mL) was reduced by all samples after 4h of incubation reaching counts of 1.03-2.0 log cfu/mL. After 24h of incubation, *S. aureus* was present only in three samples, namely, LF2 loaded with squalene (LF2S), LF5 and LF5 loaded with α-tocopherol (LF5A), (1log-1.3log/cfu/mL). In Figure 7d, when the initial population of *S. aureus* was 6log cfu/mL, results highlighted again the better antimicrobial ability of samples LF2, LF2 loaded with α-tocopherol (LF2A), and LF2S that at 4h have reduced the population reaching 1.2log-2.9log cfu/mL. By 24h, no counts were enumerated for all three samples. The reduction of the population was also significant for LF5, LF5A, and LF5 loaded with squalene (LF5S) samples reducing the microbial population at

2.5log-3.1log cfu/mL after 24h of incubation. The differences observed and presented above, in the efficacy of each sample against the pathogen lies on the samples' ingredients. It is clear in Figure 7c and Figure 7d that LF2 samples appear to be more effective than LF5 samples. The question rising in this case is how samples with higher FC content are less potent. The answer can be found in the collective action. FC is not the only antimicrobial agent present. The probiotic strain *L. fermentum* ACA-DC 179 used in the present study has been shown to have antimicrobial activity against Gram + *Streptococcus* and *Listeria* strains[31,32]. When FC and LF are conjugated with higher FC concentrations, the ζ-potential results (Figure 2) showed increased interaction and stability of the conjugates. Thus, not only is FC strongly attached to the conjugates and not "free" to interact with the targets, but also LF is highly covered by FC not being able to express its antimicrobial potential.

The most potent emulsions against *E. coli* (3 log cfu/mL) were LF2 with α-tocopherol (LF2A) and LF2 with squalene (LF2S), both causing a 3 log cfu/mL reduction after 4 h and hence, completely vanishing *E. coli* counts after 24h of incubation (Figure 7a). *E. coli* seemed to remain unaffected by LF2 at 4h, but at 24h no counts for *E. coli* were enumerated. Emulsions LF5, LF5A and LF5S showed a small, but not significant, effect on *E. coli* after 24h. The results with *E. coli* initial population of 6 log cfu/mL, followed the same pattern, with a decrease of 1.4-1.3 log cfu/ml, after 4h of incubation with LF2A and LF2S, respectively, while LF2 had no effect. The results imply that besides FC, α-tocopherol and squalene also added to the antimicrobial activity of the whole emulsions. The findings are supported by previously published results on the antimicrobial ability of chitosan, α-tocopherol, and squalene[49–51]. As in the case of *S. aureus*, herein, variation among samples can be observed and attributed to their ingredients. The clearest difference in this case is that in the presence of the encapsulated molecules (α-tocopherol and squalene) the reduction of microbial cell counts is greater pointing towards the antimicrobial efficacy of these compounds[52,53].

Alongside the aforementioned 1:1 mixing ratio, a mixing ratio of 1:10 (emulsion:bacteria suspension) was also tested. On the contrary to the 1:1 mixing ratio, the 1:10 ratio showed no effectiveness against pathogen targets. On the contrary, in our previously published work[18], emulsions stabilized solely by FC (0.3% w/v) where able to act on both microorganisms at both mixing ratios. This difference in results of the same mixing ratio (1:10) between the two studies can be attributed to the physical state of FC. Herein, the partial binding of FC for the formation of conjugates took place leading to less "available" FC to act against the pathogens.

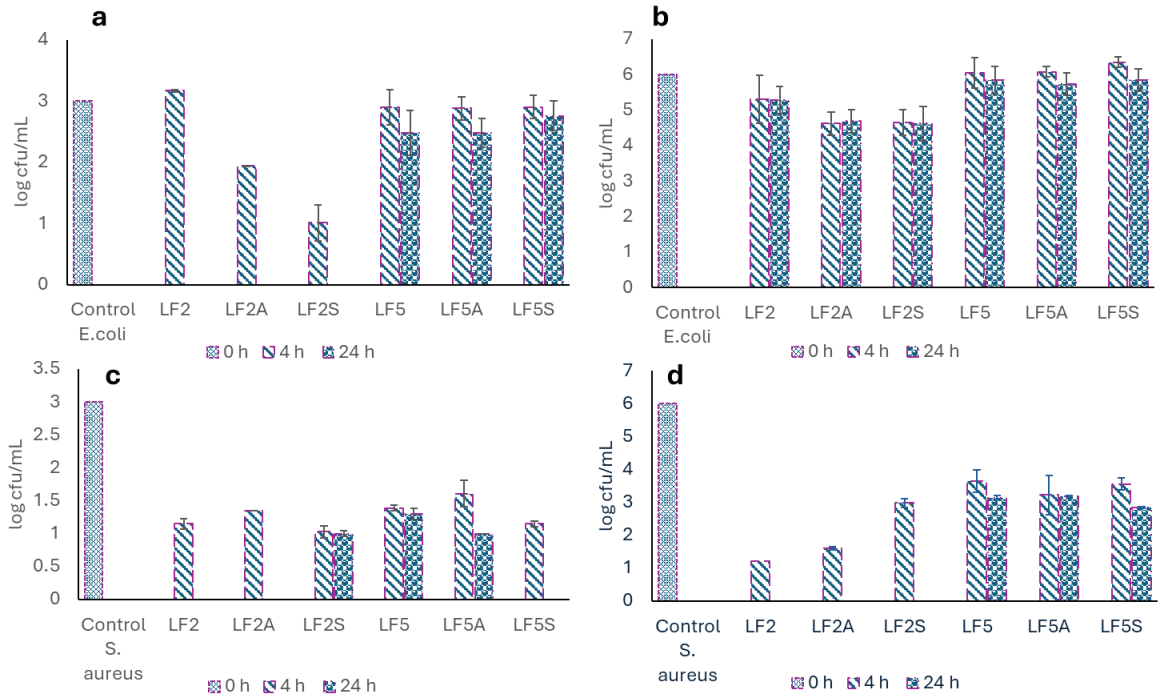


Figure 17: Microbial counts of E. coli and S. aureus after 4 and 24 h incubation at 37°C with empty emulsions (LF2, LF5), emulsions loaded with a-tocopherol (LF2A, LF5A) and emulsions loaded with squalene (LF2S, LF5S). Bacterial suspensions and emulsions were mixed at a 1:1 ratio (emulsion: bacterial suspension)

Anti-inflammatory activity of emulsions

The strain *L. fermentum* ACA-DC 179 has been previously proven to have anti-inflammatory activities i*n vitro* and *in vivo*. To name but a few, when it was used in a *Salmonella*-infected mouse model, its administration revealed an *in vivo* anti-*Salmonella* activity[30]. Additionally, it exhibited antiviral activity efficiently protecting human and animal intestinal epithelial and immune cells from enteric virus infection[54]. In the present study, we also tested whether the strain would retain its anti-inflammatory activity while conjugated (covered by FC) and at the same time, when adsorbed at a water-oil interface. To do so, we used the LPS-induced RAW 264.7 cell model as macrophages have a key role in the recognition of threats from specific receptors upon the multifaceted process of the inflammatory response and next, we determined the levels of the major pro-inflammatory cytokines, being the first ones to be produced by the activated macrophages during innate immunity[55]: interleukin 6 (IL-6), interleukin 1-beta (IL1-β), and tumour necrosis factor-alpha (TNF-α) by ELISA upon stimulation by lipopolysaccharide (LPS). Figure 10 depicts the levels of IL-1 β, IL-6 and TNF-α in LPS-induced RAW 264.7 cells, following treatment with various suspensions and emulsions including *L. fermentum* ACA-DC-179: 1) LF in distilled water; (2) C-LF2; (3) C-LF5; (4) LF2 empty emulsion; (5) LF5 empty emulsion; (6) LF2A loaded with α-tocopherol; (7) LF5A loaded with α-tocopherol; (8) LF2S loaded with squalene; and (9) LF5S loaded with squalene. Findings suggest that BFCs and BFC stabilized emulsions reduce

the levels of the pro-inflammatory cytokines and hence, exhibit anti-inflammatory activity (when compared to the control group and depending on the test-conditions). LF5S loaded with squalene decreases IL-6 levels (Fig 10a), while LF2A loaded with α-tocopherol decreases IL-1β levels (Fig 10b). TNF-α levels were decreased by LF2 (and LF2 empty) as well as LF5 (Fig 10c). We suggest a combined action of the strain, FC and α-tocopherol[56–58], while the anti-inflammatory activity of squalene has been already reported and related to its antioxidant capacity[59].

Metabotypes reflect alterations in cellular processes and pathways and thus, highlight the mechanisms underlying anti-inflammatory responses. For this, we performed pilot untargeted cell culture supernatant metabolomics by liquid chromatography-mass spectrometry (LC-MS). Overall, distinct metabotypes were revealed across the different treatment groups, implying metabolic shifts associated with the anti-inflammatory responses obtained. In all cases of anti-inflammatory activity, a marked increase in metabolites associated with antioxidant activity (such as glutathione) is noted. Additionally, a decrease in pro-inflammatory lipid mediators, including various eicosanoids, was observed. Metabolites related to energy metabolism, such as pyruvate and lactate, showed a trend towards normalization in those anti-inflammatory activity cases compared to the control group, indicating a possible restoration of metabolic homeostasis disrupted by LPS-induced inflammation. Phospholipids and sphingolipids, including phosphatidylcholines and sphingomyelins were also modulated, highlighting their involvement in inflammatory signaling. Branched-chain amino acids, such as leucine, isoleucine, and valine, which play a role in immune cell function and metabolism, were also impacted.

The reduction in IL-6, IL1-β, and TNF-α levels aligns with previous studies that have demonstrated the anti-inflammatory potential of Lactobacillus strains and in particular, *L. fermentum*[60]. The pilot metabolomic data support these findings, showing that the most effective formulations not only reduced pro-inflammatory cytokine production but also modulated key metabolic pathways involved in inflammation and oxidative stress. The increase in glutathione and other antioxidant-related metabolites aligns with the known role of α-tocopherol in enhancing cellular antioxidant defenses. The observed decrease in eicosanoids, which are potent lipid mediators of inflammation, further underscores the anti-inflammatory potential of these formulations. Interestingly, squalene is a precursor in cholesterol biosynthesis and has been reported to possess anti-inflammatory properties, likely through its ability to modulate lipid metabolism and membrane structure. Of note, this is a pilot study of limited sample size and biological replicates and thus, future studies should aim to elucidate the precise molecular mechanisms underpinning these observations, particularly focusing on the interactions between probiotics and bioactive compounds at the cellular level.

Overall, the results of this study show that LF not only maintains its anti-inflammatory activity when conjugated with FC and emulsified but also acts in parallel and cumulatively with other anti-inflammatory agents, namely, FC, α-tocopherol, and squalene. It can be safely assumed that LF can be part of a functional

emulsion with other biologically active compounds. Finally, through mechanisms that have not yet being defined, the presence of the encapsulated molecules and their combination with FC and LF create emulsions of different activity. Even though we were unable to find similar results that corroborate studies that have used ELISA tests and metabolomics to provide insights on probiotics' anti-inflammatory activity [61,62].

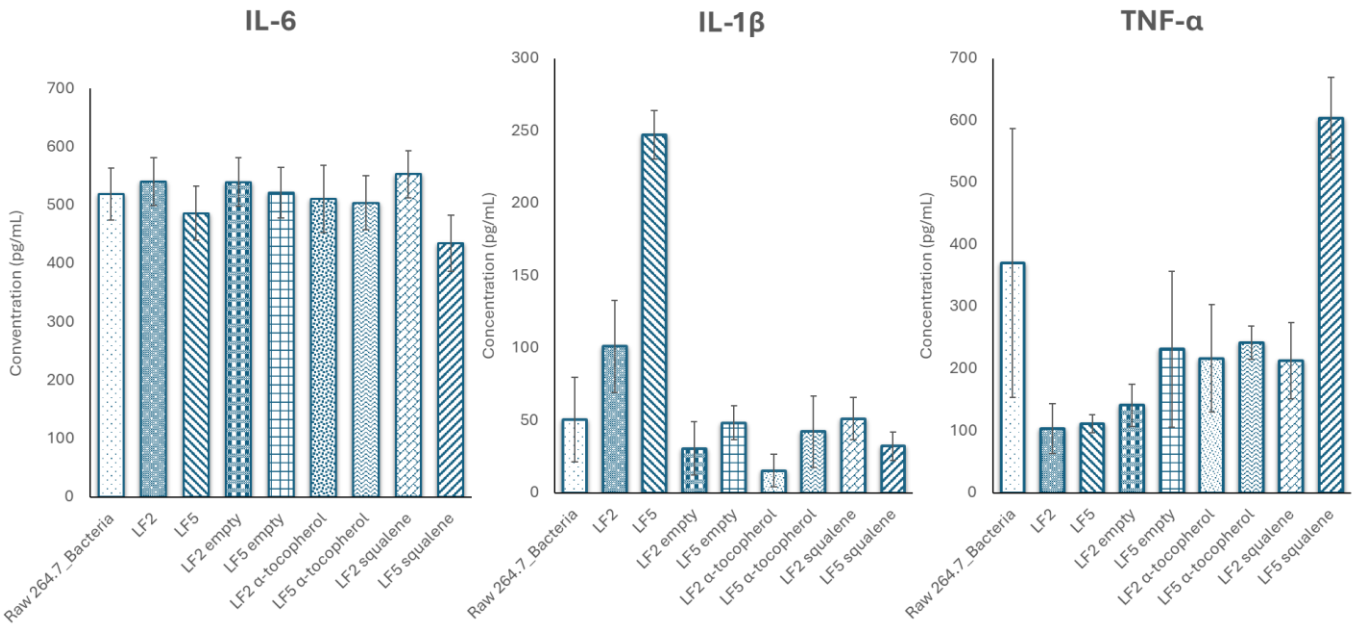


Figure 18: (a) IL-6 levels in LPS-induced RAW 264.7 cells upon pre-treatment of conjugated chitosan-*L. fermentum*, (b) IL-1β levels in LPS-induced RAW 264.7 cells upon pre-treatment of conjugated chitosan-*L. fermentum*, and (c) TNF-a levels in LPS-induced RAW 264.7 cells upon pre-treatment of conjugated chitosan-*L. fermentum*

Materials and Methods

Materials

Fungi-derived chitosan was sourced from Qingdao Chibio Biotech, (Qingdao, Shandong, China). 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) was obtained from a local supermarket in Athens, Greece. 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)

Preparation of bacteria-fungal chitosan (BFC) conjugates

Three lactic acid bacteria strains, namely *L. bulgaricus* ACA-DC 87 (LB), *L. fermentum* ACA-DC 179 (LF) and *S. thermophilus* ACA-DC 26 (ST), belonging to the ACA-DC Collection at the Agricultural University of Athens, Greece, were included in this study. All strains were stored at −80°C in the respective growth media, supplemented with 20% (v/v) glycerol. For biomass collection, strains were subcultured twice (1% v/v inoculum) in MRS (lactobacilli) or M17 (*S. thermophilus*) broth and were incubated at 37°C for 24 h. After incubation, cells were collected by centrifugation (centrifugation conditions) and washed twice with distilled sterile water[29]. The biomass was finally suspended in FC solutions (0.03, 0.1, 0.2, 0.3 & 0.4% w/v) to produce BFC conjugates (Table 1).

Table 2: Name-coded BFCs of each bacterial strain and the different FC concentrations

	FC 0.03% w/v	FC 0.1% w/v	FC 0.2% w/v	FC 0.3% w/v	FC 0.4% w/v
S. thermophilus ACA-DC-26	C-ST1	C-ST2	C-ST3	C-ST4	C-ST5
L. bulgaricus ACA-DC-87	C-LB1	C-LB2	C-LB3	C-LB4	C-LB5
L. fermentum ACA-DC-179	C-LF1	C-LF2	C-LF3	C-LF4	C-LF5

Selection of the optimum system

Emulsions' preparation

For the emulsions' preparation, BFCs were used as the aqueous phase and EVOO as the oil phase. at a 1:1 ratio and high shear was employed as the homogenization method. Several homogenization speeds and durations were tested and the two chosen for further experiments were 15000 rpm for 3 min and 20000 rpm for 1 min. For the homogenization process, the oil was added gradually to the aqueous phase under high-shear treatment (X1000D Unidrive, Ingenieurbüro CAT, Ballrechten-Dottingen, Germany) with a 10 mm Diameter Generator, Teflon Bearing, Immersion Depth 150 mm[18]. For the incorporation of the bioactive molecules, α-tocopherol or squalene were added in the oil phase to reach a final concentration of 1% w/w[7].

Table 3:Name coded emulsions stabilized with the respective BFCs.

C-ST1 emulsion	**C-ST2 emulsion**	**C-ST3 emulsion**	**C-ST4 emulsion**	**C-ST5 emulsion**
ST1	ST2	ST3	ST4	ST5
C-LB1 emulsion	**C-LB2 emulsion**	**C-LB3 emulsion**	**C-LB4 emulsion**	**C-LB5 emulsion**
LB1	LB2	LB3	LB4	LB5
C-LF1 emulsion	**C-LF2 emulsion**	**C-LF3 emulsion**	**C-LF4 emulsion**	**C-LF5 emulsion**
LF1	LF2	LF3	LF4	LF5

Strains' viability before and after high-shear treatment

ST, LB and LF viability after high shear treatment was assessed. The bacteria were resuspended in distilled water and then mixed with EVOO at a 1:1 ratio and finally subjected to high shear. Water was used instead of FC solutions for the resuspension of the bacteria because of FC's masking effect, leading to false results. For the experiment two high shear treatment conditions were tested, namely 15,000 rpm for 3 min or 20,000 rpm for 1 min. As control samples, strains resuspended in water and not treated by high shear were used. All samples including the controls were placed in separate petri dishes with the appropriate culture medium for each strain. The petri dishes were incubated at 37^{o}C for 24 h and then the bacteria population was enumerated for each sample. The procedure was performed in triplicate[63].

Characterization of conjugates

ζ-potential

A Zetasizer Nano ZS (ZEN3600) analyzer (Malvern Instruments Ltd., Malvern, UK) equipped with a He-Ne laser (633 nm) and non-invasive backscatter (NIBS) optics was used for the ζ-potential determination, while the results were processed with the Malvern Zetasizer Nano software, version 6.32 (Malvern Instruments Ltd., Malvern, UK). For the measurements folded capillary cells DTS1070 were used (Malvern Instruments Ltd., Malvern, UK)[7].

Dynamic Interfacial Tension (DIT)

DIT between oil and water was measured with a pendant-drop tensiometer (CAM 200, KSV, Biolin Scientific, Stockholm, Sweden). For the experiments, SFO treated with ultra-pure water, to remove as much as possible any surface-active ingredients, was used. The obtained data were analyzed using an axisymmetric 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. For the experiment a pendant drop of the aqueous phase was created within the oil phase, contained in a quartz cell (Hellma Analytics, Müllheim, Germany)[64].

Structural characterization of emulsions

Droplet size determination by Laser Diffraction (LD)

The emulsions' droplet size distribution was determined using LD with a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were added in a Hydro MU liquid sampler (Malvern Instrument, Malvern, Worcestershire, UK) after dilution (1:20 in SDS)[64]. The size distribution was expressed in terms of Volume Weighted Mean Diameter ([D 4,3]) with the values appearing on the figures.

Confocal Laser Scanning Microscopy (CLSM) and Optical Microscopy

Two different dyes were used for emulsions' staining, namely nile red for the oil phase and FITC for the particles' staining. For the preparation of the oil phase with nile red, the dye was dissolved directly in EVOO at 0.01% w/v. For particles' staining, FITC was dissolved at 0.03 % w/v in a FC solution of 0.1% w/v, under magnetic stirring at 400 rpm for 1 h. The solution was left overnight to ensure maximum interaction between chitosan and FITC. The samples were then subjected to confocal laser scanning microscopy (Leica TCS SPE, Leica Microsystems, Heidelberg, Germany), employing a x20 lens. The LAS AF software from Leica Microsystems was utilized for image acquisition[18].

The examination of the emulsions' droplets using optical microscopy was carried out using an AXIO Scope.A1 with Axiocam ERc 5s, microscope (Carl Zeiss Microscopy GmbH, Germany). Samples were placed on microscope slides without being subjected to dilution and observed with the x20 magnification lens. The obtained images were analyzed using the ZEISS ZEN Imaging Software.

Stability of emulsions against creaming and coalescence

For stability tests, emulsions were prepared in 12 mL glass tubes with screw caps, and stored under two different conditions, namely at ambient temperature and refrigerated at 4°C. After preparation, some of the emulsions initially separated into cream and serum layers. Alterations of the cream layer during storage were

monitored and data were recorded every 5 days. The emulsion stability was evaluated using the % Creaming Index (% CI), which is the cream layer height (H_c) expressed as a percentage of the total sample height (H_t) within the tube. The % CI was calculated using equation (1), and the procedure was repeated three times[65].

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

Functionality of emulsions

Antioxidant activity by DPPH assay

The assay followed a previously established procedure with minor modifications. Initially, a 2 mL solution of DPPH dissolved in 40% v/v methanol at a concentration of 0.108 mM was measured using UV-VIS spectroscopy in a plastic tube. Then, 100 µL of the sample was added to this solution. The resulting mixture was vigorously agitated and kept in the dark for 30 min. After incubation period, the absorbance was measured at a wavelength of 515 nm. The percentage of inhibition of the free radical was determined using equation (2), where A_0 represents the absorbance of the control sample, and A_1 represents the absorbance observed after the 30-minute reaction period[66].

$$\%Inhibition = \left[\frac{A0-A1}{A0}\right] * 100 \ (2)$$

Antimicrobial activity

For the assessment of the emulsions' antimicrobial activity two bacterial strains were used as targets, namely, *E. coli* C1845 (kindly provided by Prof. Luc De Vuyst, VUB, Brussels, Belgium) and (*S. aureus* DSM 21705. Both strains were grown for 18 h in BHI broth reaching approximately 9 log cfu/mL. Afterwards, the cultures were serially diluted in buffered peptone water to prepare bacterial suspensions of two different populations for each target strain, namely 6 and 3 log cfu/mL. Following, three different types of emulsions, namely empty, loaded with α-tocopherol and loaded with squalene, were mixed with the prepared target bacterial suspensions in two ratios, 1:1 and 1:10 (emulsion:bacteria suspension). All mixtures were incubated at 37°C and after 0, 4 h and 24 h of incubation, samples were collected, serially diluted and plated on BHI agar to enumerate surviving target cells following the incubation of plates for 48 h at 37°C[67].

Anti-inflammatory activity

Enzyme-Linked Immunosorbent Assay (ELISA)

The murine macrophage cell line RAW 264.7 was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific), enriched with L-Glutamine and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Corning™) at 37◦C under a humidified atmosphere of 5% CO_2. Subcultures were performed when cell confluency reached 80-90%.

To test the anti-inflammatory potential of the suspensions and emulsions that included *L. fermentum* ACA-DC-179 the following test-conditions were determined: 1) LF in distilled water; (2) C-LF2; (3) C-LF5; (4) LF2 empty emulsion; (5) LF5 empty emulsion; (6) LF2A loaded with α-tocopherol; (7) LF5A loaded with α-tocopherol; (8) LF2S loaded with squalene; and (9) LF5S loaded with squalene. The levels of major pro-inflammatory cytokines, namely interleukin 6 (IL-6), interleukin 1-beta (IL1-β), and tumour necrosis factor-alpha (TNF-α) were quantified by ELISA upon stimulation by lipopolysaccharide (LPS). For this, cells were seeded at a density of $375x10^3$ cells/mL at 37◦C in black, flat-bottom 96-well plates (SPL Life Sciences) under a humidified atmosphere of 5% CO_2, overnight (18h). Each test-condition was added for 12h at 37◦C under a humidified atmosphere of 5% CO_2 (50 µL), followed by LPS treatment (1 mg/mL) for an additional 12h at 37◦C under a humidified atmosphere of 5% CO_2. Next, all cell culture supernatants were collected and centrifuged at 2500 rpm at 4◦C for 5 mins. Cell culture supernatants were stored at -20°C prior to analyses. Cells treated only with L. fermentum and/or LPS (1 mg/mL) served as controls. Each test-condition refers to n=3 biological replicates and n=3 technical replicates. The quantitative determination of cytokines was carried out using ELISA kits (Thermo Fisher Scientific) for IL1-β, IL-6, and TNF-α according to the manufacturer's recommendations. All data obtained were analyzed and presented with mean ± standard deviation (SD) using Prism9 and CurveFit softwares.

Untargeted metabolomics

For untargeted metabolomics by liquid chromatography-mass spectrometry, sample collection, processing, and storage for cell supernatants were performed as described previously[68,69].

The liquid chromatography separation was performed with an Accela ultra-high-performance LC (UHPLC) system. An ACQUITY UPLC BEH C18 column (1.7µm, 2.1 x 100mm) was used operating at 45°C. The injection mode was set at 2 µL, and the mobile phase flow rate was set at 0.25 mL/min. Mobile phase solvents were A (95% H_2O, 5% methanol, 0.1% formic acid) and B (100% methanol, 0.1% formic acid). The eluting gradient program in both positive and negative ion mode was the following: 0.00–0.10min (50% A, 50% B), 0.10–0.50 min (20% A, 80% B), 0.50–0.60 min (5% A, 95% B), 0.60–6.50 min (5% A, 95% B), 6.50–6.51 min (50% A, 50% B), 6.51–8.00 min (20% A, 80% B). The UHPLC system was coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an APCI source,

operating in both positive and negative modes. To monitor the instrument performance over time and the chromatographic integrity, including retention time shifts, QC samples were prepared as a mix of each sample. Data were pre-processed with Xcalibur software (version 2.1, Thermo Scientific, Waltham, MA, USA).

Data processing and analysis were performed as described previously[71,72]. ProteoWizard MSConvert was used to centroid all raw MS data (Adusumilli and Mallick, 2017) and convert them into MzML files prior to MetaboAnalyst 5.0 (Pang et al, 2021). LC-MS spectral processing was performed using the auto-optimized parameter setting and blank subtraction. All test-groups were cross-compared, first to gain insights into the metabolomes and then to identify key metabolites. Both positive and negative ion modes were employed during LC-MS analysis. Subsequent analyses included metabolites detected in more than 33% of the samples. Following the removal of uninformative features, the resulting number of metabolites was decreased drastically to ~1/4. For those metabolites surviving our criteria, empty values were annotated with a small value (1). Data centering and unit variance scaling were carried out. Univariate and multivariate statistical analysis were applied where appropriate. Student's t-test and ANOVA (One-way Analysis of Variance) test followed by post hoc analysis (Fisher's least significant difference) were used. Critical value was set at <0.05, including FDR correction. For all comparative analysis, we performed Log2fold calculation and PCA and PLS analyses. Next, we determined PLS VIP (variable importance in projection) values. Only metabolites with a log2fold ≥ 2 were selected for subsequent enrichment analysis. Enrichment analysis was performed using Metaboanalyst 5.0 employing pathway-associated metabolite sets (SMPDB). For the interrogation of metabolic pathways, the mummichog algorithm was applied. This algorithm facilitates one-step functional analysis through tandem mass spectra feature tables[70]. The top 10 most significantly associated m/z features were used as input to the mummichog algorithm v.2. KEGG (Kyoto Encyclopedia of Genes and Genomes) and REACTOME databases were selected as the pathway libraries of interest. Only those metabolic pathways containing at least 3 significant metabolites were included. Significance threshold was set at a p-value < 0.05, including FDR correction.

Conclusions

This study explored the formation and functionality of Bacteria-Fungal Chitosan Conjugates (BFCs) for stabilizing emulsions. Through extensive experimentation, we examined the interactions between various bacterial strains and Fungal Chitosan (FC) concentrations, and the impact on emulsion stability, and bacterial viability post-high-shear homogenization. The emulsions' antioxidant, antimicrobial, probiotic, and anti-inflammatory properties were also evaluated.

L. fermentum ACA-DC-179 combined with FC exhibited the highest potential for stabilizing emulsions, at FC concentrations of 0.1% to 0.4% w/v, with a stability of 90 days. This strain maintained high viability after high-shear treatment. ζ-potential measurements indicated robust electrostatic interactions between negatively charged bacterial cells and polycationic FC, particularly at higher FC concentrations, enhancing BFC formation and stability. The BFCs significantly reduced dynamic interfacial tension (DIT) at the water-oil interface, particularly at FC concentrations above 0.2% w/v, leading to smaller droplet sizes and improved emulsion stability as confirmed by Laser Diffraction (LD) and visualized through Confocal Laser Scanning Microscopy (CLSM). Emulsions stabilized with higher FC concentrations demonstrated prolonged stability against creaming, lasting up to 96 days at 4°C. Furthermore, BFCs and BFC-stabilized emulsions retained strong anti-inflammatory and antimicrobial activities, showcasing the functional synergy of FC and probiotic bacteria within complex structures. Finally, herein it was highlighted that it is possible to form BFC stabilized emulsions with tunable characteristics by encapsulating squalene and α-tocopherol. Both molecules brought along their unique properties, including antioxidant ability. The most interesting revelation was that upon their addition, emulsions differed in their antimicrobial and anti-inflammatory abilities.

These findings highlight a novel and highly effective multifunctional carrier capable of encapsulating oil-soluble bioactive ingredients with an oil volume fraction up to 50% wt. The proposed systems represent a significant advancement in the field, with versatile applications in the food, pharmaceuticals, and cosmetics industries. It was presented for the first time how emulsions with tailorable abilities can occur from probiotic bacteria-fungal chitosan conjugates. Since there are no other reported cases on such conjugates that study not only the emulsions' structural characteristics but also whether their ingredients' functional properties are retained in this complex matrix, further exploration is necessary. Future research should explore the applicability of these emulsions in various industrial sectors, potentially leading to the development of innovative and commercially viable products.

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Chapter 6
Concluding remarks

The oldest food emulsion as well as the first one we come across after birth is milk, dating the history of emulsions at the beginning of existence. Even if the science behind the preparation of an emulsion is often complex, the concept of emulsions is quite straight forward. Two unmixable liquids combined with the aid of an emulsifier. In its simplicity, this concept has offered over the years infinite opportunities for progress, goal achievements and resolution of many persisting issues related with what. Extensive research has made the formation of emulsions simple, and their characteristics have been harnessed to create products, such as with improved texture, and stability. The ongoing research and development in this field continue to expand the potential applications of emulsions, making them an indispensable tool in modern science and technology. Thus, it is of great importance to take advantage of this knowledge and stir the colloids science towards new paths that follow and address issues of modern life such as food sustainability and functionality.

Over the past years, food industry has been governed by the need for healthier foods, low-fat foods and plant-based alternatives. PEs, stabilized by particles, have only recently begun to be tapped in food systems and can address many rising food issues. This type of emulsion gives the opportunity of using natural components as emulsifiers like proteins, polysaccharides, and even microbial cells. The multitude of sources for these components offer the chance for diverse applications that lately focus on the production of plant-based products, as well as of products with increased functionality. Plant-based products are valuable alternatives not only for those who choose not to consume meat and animal origin products but also for those who are unable to do so because of allergies or intolerances. Thus, emulsions that are stabilized by plant proteins or other particles of plant origin can encapsulate bioactive ingredients and be used to fortify products like plant-based beverages. Additionally, food functionality can be increased by the carrier itself since PEs offer the opportunity to use as particles ingredients with specific characteristics, such as antimicrobial activity, pH sensitivity, and many more. Among those ingredients is chitosan, known for its antimicrobial properties, for its ability to act as a fat replacer and an agent to improve mouthfeel. Although chitosan is more commonly obtained by crustaceans there is the possibility to get it also from fungus such as mushrooms. The use of fungal chitosan in PEs can address both the issue of functionality and be used in animal-free applications. Microbial cells as PE stabilizers have been studied mainly at a primary level using model oils, such as dodecane and bacteria easy to grow and handle like *E. coli*. For food applications there are limited studies focusing on the use of probiotic bacteria as part of the emulsions' stabilization steps, although lately there is a clear tendency leading to that direction.

In this framework, during this thesis, PEs suitable for plant-based applications that also carried functional properties were successfully designed and formulated. For this purpose, PPI and SPI were used initially as a first approach. Spherical particles were formed and stabilized O/W emulsions able to encapsulate bioactive ingredients. Both the preparation of particles and of the emulsions were optimized by the introduction

of HPH. This indicated the possibility of preparing particles and emulsions of tunable particle- and droplet sizes while having also the possibility to increase the oil's encapsulation efficiency up to 50%. The encapsulation of α-tocopherol and squalene increased the antioxidant ability of the emulsions proving they are an appropriate vehicle for the efficient delivery of bioactive ingredients.

Another novelty deriving from this study is the preparation of carriers that possess functional properties without the need of adding an encapsulated ingredient. This was possible by introducing PC to PPI emulsions. PC was able to perform as a co-emulsifier, a natural coloring agent and an antioxidant. The stability of the emulsions was also increased, and no degradation of PC was observed (sedimentation of color degradation). The effect of PC and PPI were studied, and the stabilization mechanism was explained. When used together, results showed that the two proteins interacted giving a different stabilization mechanism. The same was deduced by the DIT measurements that showed that both particles act on the interface by decreasing the interfacial tension, when combined the results differ showing a slower decrease rate because of the proteins' interaction. Nevertheless, the combination of PPI and PC as well as the application of high pressure led to very stable emulsions with added functionality and improved encapsulation efficiency.

FC as an alternative plant-based form of conventional chitosan was used as an emulsifier. FC was able to produce spherical particles with tunable sizes leading to the formation of stable emulsions. Not only FC proved to be an excellent emulsifier but also a very potent antimicrobial agent. Emulsions stabilized by FC showed antimicrobial activity against both Gram + and Gram – pathogens. FC's functionality was successfully combined with the encapsulation and antioxidant capacity of the two model bioactives used throughout the thesis.

The biggest challenge of the present thesis was to further enhance the functionality of the emulsions with probiotic potential. This was achieved by three LAB strains, *L. fermentum* ACA-DC 179, *S. thermophilus* ACA-DC 26 and *L. bulgaricus* ACA-DC 87, from the ACA-DC culture collection of the Laboratory of Dairy Research at the Agricultural University of Athens. Conjugates of the aforementioned bacteria and FC were produced and to our knowledge, this is the first time this combination was tested. After extensive literature review the only similar reference was of *E. coli* interacting with chitosan from crustaceans for the formation of non-edible emulsions. The main idea behind this endeavor was the preparation of a particle, and in extension of an emulsion, that would possess both the antimicrobial properties of FC and the already demonstrated probiotic bacteria potential and at the same time act as a potent PE emulsifier. Results showed clearly the interaction between FC and the bacteria for the formation of conjugates whose stability was directly affected by FC concentration. Emulsions were successfully formulated with all three conjugate strains and the conditions chosen were able to maintain minimum fatality of the bacteria. These novel conjugates and emulsions were fully characterized. It should be highlighted that complex structures of probiotic bacteria and FC have not been

previously studied for their functionality. The great bet won in this case proved that both FC and the probiotics are not affected by conjugation and homogenization. The tests revealed the ability of the emulsions to act as antimicrobial agents and to reduce the levels of major pro-inflammatory cytokines thus proclaiming anti-inflammatory abilities. The importance of this specific result lies in the fact that no previous study had shown whether conjugated and emulsified probiotics retain their potential to act as such. Each emulsion, depending on its ingredients, affected different pro-inflammatory cytokines, a fact that favors the use for targeted applications while avoiding general immunosuppression. The results were further cross-checked by untargeted metabolomics.

This study showed not only the possibility of improving already well-known and studied formulations for food applications, but also the ability to form novel ones. Plant proteins are possible to form stable emulsions with high encapsulation efficiency and the introduction of unconventional ones like PC can enrich the system with more functional characteristics. Furthermore, FC is an excellent alternative to crustacean derived chitosan with very good emulsifying capacity and antimicrobial properties. When FC is combined with probiotic bacteria, a novel particle is formed that leads to emulsions that combine the stability and robustness of PEs with functional characteristics such as antimicrobial and anti-inflammatory potential. At the same time this carrier can exhibit antioxidant capacity when proper molecules are encapsulated. The continuous progress and new findings in colloidal science and specifically PEs can lead to novel food products that are safe and offer combined multiple benefits. More extensive research of the proposed systems could lead to new dairy-free products and meat alternatives enriched with probiotics they naturally do not contain, with antimicrobial potential to extend shelf-life and the introduction of any oil soluble bioactive substances needed in aqueous-based foods.

The current dissertation has yielded three published papers, with an additional paper currently under submission, all of which are detailed above. Moreover, during the course of this thesis, three additional papers were generated. One of these papers presented the findings of my master's thesis, a collaboration between the University of Ioannina and the National Hellenic Research Foundation, Institute of Chemical Biology. Biomimetics and Nanobiotechnology group (1). The second originated from the Operational Program Competitiveness, Entrepreneurship, and Innovation, under the RE-SEARCH–CREATE–INNOVATE call, T2EΔK_03640 (2). Finally, a review paper on "Soft nanostructures for sun protection formulations" was also produced (3).

(1) E. Galani, et al., "Antioxidant Activity of Methyl Caffeate-Enriched Olive Oils: From Extra Virgin Olive Oil to Extra Virgin Olive Oil-Based Microemulsions", https://doi.org/10.1002/ejlt.202100249

(2) E. Galani, et al., "Natural Antioxidant-Loaded Nanoemulsions for Sun Protection Enhancement". https://doi.org/10.3390/cosmetics10040102.

(3) A. Xenakis, E. Galani, et al., «Soft nanostructures for sun protection formulations https://doi.org/10.1016/j.cocis.2024.101803.

Supplementary materials

Pilot untargeted cell culture supernatant metabolomics by liquid chromatography-mass spectrometry (LC-MS)

Untargeted metabolomics allows for accounting for biases when exploring metabotypes (i.e. individual metabolomes). Herein, we explore RAW 264.7 cell supernatants to gain insights into the metabolic changes and pathways affected by the treatments (to understand the mechanisms underlying the anti-inflammatory responses and identify potential biomarkers or metabolic pathways involved in the regulation of IL-1β, IL-6, and TNF-α secretion.

Taking into account that:

- Metabotypes: Metabolomics provides a comprehensive profile of metabolites present in the cell supernatant. Changes in metabolite levels can reflect alterations in cellular processes and pathways.
- Pathway Involvement: Identify metabolic pathways that are influenced by the treatment.
- Biomarker Identification: Specific metabolites that change in response to treatments can serve as biomarkers for the anti-inflammatory effect.

When dosing RAW 264.7 macrophage cells with *L. fermentum*, chitosan, and squalene, particularly in the context of LPS stimulation, data suggest pathways related to energy metabolism, redox balance, lipid metabolism, and amino acid metabolism that are linked to inflammation and immune response.

Key metabolite classes that are suggested herein, include:

- metabolites in the arachidonic acid pathway (e.g., PGE2, LTB4)
- the tryptophan metabolism (e.g., kynurenine)
- glutathione - reduced (GSH) and oxidized (GSSG) forms – play a key role in oxidative stress and inflammation along with metabolites, such as malondialdehyde (MDA), a marker of lipid peroxidation
- Glycolysis and TCA Cycle intermediates: metabolites such as lactate, pyruvate, citrate, succinate, and fumarate can indicate shifts in cellular energy metabolism due to LPS stimulation and treatment effects
- Cholesterol and squalene: as squalene is a precursor in cholesterol biosynthesis, monitoring can provide insights into lipid metabolism alterations.
- Phospholipids and sphingolipids: alterations in membrane lipid composition, such as phosphatidylcholines (PC) and sphingomyelins (SM), are involved in inflammatory signaling.

- Branched-Chain Amino Acids (BCAAs): Leucine, isoleucine, and valine can be involved in immune cell function and metabolism.

Furthermore, considering that a selective modulation of the inflammatory response is obtained herein (when we compare IL-1β, IL-6, and TNF-α levels), untargeted pilot metabolomics may interpret and further investigate such findings. IL-1β, IL-6, and TNF- α are pro-inflammatory cytokines but can be independently regulated. For instance, IL- 1β production often involves inflammasome activation, whereas TNF-α is typically produced via NF-ϰB signaling. Herein, we may also have a case of differential sensitivity: cells might have differential sensitivity to treatments regarding IL-1β, IL-6, and TNF-α production. This could be due to variations in receptor expression, signaling intermediates, or feedback mechanisms.