The multifaceted role of propolis particles in aqueous dispersions and in oil-in-water emulsions

by

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Abstract

Consumer preferences towards formulated products that utilise natural active species over synthetic, has been generating significant industrial activity. As a result, a range of natural species possessing a multitude of functionalities has been investigated. One such natural component is propolis which is made by honeybees for healing and antiseptic purposes and has been associated with antibacterial, antifungal and antioxidant functionalities, among others. However, propolis has limited aqueous solubility which restricts its use in industrial formulations. On the other hand, it has been also established that common carrier solvents used in literature to dissolve propolis (e.g. ethanol and DMSO) can 'misrepresent' the active's functionality by themselves exhibiting a range of underlying effects (e.g. antibacterial).

The present study investigated the enhancement to propolis' performance in a purely aqueous environment, by dispersing propolis extracts into nanoparticles *via* direct ultrasonication. The hypothesis put forward was that sonication will enhance the functionality by decreasing the size of propolis particles (thus increasing the overall active surface area), and by promoting the dissolution of individual active species (present in the extracts) into the carrier (aqueous) phase. The effect of sonication time on the dispersions' particle size, polydispersity, zeta potential, chemical functionality and antibacterial activity were monitored. The aqueous carrier phase was isolated to determine the potential (further) dissolution of active species following sonication. The study indeed confirmed this hypothesis and demonstrated that the aqueous propolis particle formulations exhibited antioxidant and antibacterial activity, while sonication

was shown to encourage the extraction/dissolution of functional compounds into the carrier phase.

The functionality of the propolis particles was then ‘transferred’ into an oil-in-water (O/W) emulsion setting, by using the propolis particle aqueous dispersions as the continuous phase. Propolis particles alone were not able to produce stable emulsions, but co-stabilisation in the presence of surfactant or protein species proved more advantageous. Emulsion stability was investigated, for different oil and surfactant/protein fractions as well as for different oil droplet sizes. The role of the propolis particles in the interfacial stabilisation of the emulsions was studied by dynamic interfacial tensiometry and contact angle measurements and imaged using fluorescent microscopy. It was revealed that propolis particles exhibit some affinity for the emulsion interface (Pickering-like functionality) and thus can provide stabilisation in tandem with low concentrations of a co-stabiliser, even though some displacement of the particles was observed. Emulsions exhibited adequate stability over the course of two months, dependant on the storage temperature, pH environment and co-stabiliser used. Ultimately the choice of co-stabiliser was shown to be crucial in terms of emulsion stability as well as for the preservation of the antibacterial activity provided to the systems by the presence of the propolis particles.

In recent years, Pickering particles have been a popular research topic for the stabilisation of O/W emulsions. However, beyond their capacity for stabilisation, little research has been conducted on the ability of Pickering particles to offer further functionality to an emulsion microstructure, such as to provide or enhance their antibacterial or antioxidant character. One approach to develop such multifunctional colloidal structures is to exploit the emulsion stabilisation (Pickering functionality) of

species that already exhibit specific/desired (non-Pickering) physicochemical characteristics. Within the spirit of such an approach, the present work finally assesses the ability of propolis particles to enhance the oxidative stability and antibacterial activity of O/W emulsions. Firstly, control (non-propolis) emulsions were assessed with respect to lipid oxidation, showing that although oil content and storage temperature influence the rate of hyperoxide production, oil droplet size did not cause a significant effect. Subsequently propolis emulsions were formulated and their lipid oxidation was measured by altering key parameters such as droplet size, propolis and oil content as well as pH. It was established that propolis significantly supresses lipid oxidation in emulsion systems compared to the controls tested. Propolis emulsions also exhibited efficient antibacterial activity, eliminating the bacteria population, although their rate of action was slower than when simply present in aqueous dispersions. Finally, it was concluded that incorporation of propolis particles increased the viscosity of the emulsions.

In conclusion, the present work demonstrates that propolis' activity can be enhanced in an aqueous environment *via* ultrasound sonication. Propolis particles were also shown to exhibit Pickering-like functionality in the emulsion systems as well as oxidative stability and antibacterial properties. The ultimate goal of this research is to utilise these propolis colloidal species as natural ingredients offering both stability and antibacterial activity to industrially relevant emulsion formulations.

This thesis is dedicated to my stepfather Dr Spiros Lafis for his endless help and support throughout the years.

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Acronyms and Abbreviations

βlg	β-Lactoglobulin
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AFM	Atomic force microscopy
AMPs	Antibacterial peptides
AO	Acridine orange
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BOX	Bis-(1,3- Dibutylbarbituric acid) trimethine Oxonol
C	Control
CFU	Colony forming units
CI	Creaming index
CP	Carrier phase
CUPRAC	Cupric ion reducing antioxidant capacity
DAPI	4′6′-diamidino-2-phenylindole
DCM	Dichloromethane
DI	Deionised
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl

FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HSM	High shear mixer
IFT	Interfacial tension
LB	Luria broth
LCT	Long chain triacylglycerol oil
LMW	Low molecular weight
LO	Lipid oxidation
LPS	Liposaccharide
MBC	Minimum bactericidal concentration
MCT	Medium chain triacylglycerol oil
MIC	Minimum inhibition concentration
NA	Nutrient agar
Nacas	Sodium caseinate
NMR	Nuclear magnetic resonance
O/W	Oil-in-water
ORAC	Oxygen radical absorbance capacity
P	Propolis
p-anisidine	Para-anisidine
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate buffered saline

PD	Propolis dispersion
PdI	Polydispersity index
PG	Propyl gallate
PI	Propidium iodide
SEM	Scanning electron microscopy
Son	Sonication
TBARS	Thiobarbituric acid reactive substances
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
TRE	Troxol equivalents
TSB	Tryptic soy broth
T20	Tween 20
T80	Tween 80
W/O	water-in-oil
WPI	Whey protein isolate

Chapter 1- Introduction

1.1 Project background

The inspiration behind this study is centred on the changing preferences of a typical consumer today. People's diet and lifestyle have always been influenced by a number of factors, including environment, culture and politics.[1] As issues such as climate change, food safety, waste, animal abuse and a heightened awareness of the consumer footprint on a global scale impact people's everyday lives, naturally sourced ingredients have become more popular. This has led to the public's disapproval of synthetic additives, and to an effort towards healthier, more sustainable and cognisant food choices.[1]

Numerous studies have been conducted to comprehend this new tendency; research has led to the perception of "naturalness", reported to be equally relevant to product processing as it is to the actual ingredients.[2] This perception/misconception can also be strongly correlated to consumer familiarity with an ingredient, along with other compounding factors such as their age or whether the ingredient is named/labelled using scientific nomenclature.[3] The aspect of morality has also been studied, showing an association of natural products with health benefits and environmentally friendlier choices.[4] Similarly, when looking into consumer aversion to synthetic actives, a misunderstanding has been revealed, related to the significance of amount or dosage of synthetic chemicals in foods, along with greater exposure to contaminants and hazards.[5] Therefore, it is essential for food research and food industry to find alternative processes or ingredients that could satisfy consumers' needs, while ensuring the products are safe and consistent when it comes to texture and sensory aspects.

Naturally occurring alternative additives which address these issues would thus be highly desirable. These actives usually contain secondary metabolites from plants,[6] microbes,[7] insect[8] and animal products[9]. Focusing on plant derived natural actives, there already exists a range of multi-faceted research on extracted and/or isolated compounds and their beneficial activities including antiviral, antioxidant, antifungal etc.[10],[11],[12] Some examples include natural actives for wound healing properties such as bromelain (group of enzymes from pineapple)[13] or even essential oils and extracts coming from herbs such as parsley[14], sage[15], rosemary[16] and thyme[17] for antibacterial or antioxidant functionalities.

Propolis is another bioactive with numerous beneficial properties. It is made from bees by collecting buds and exudates from plants and trees, and it is typically utilised for sealing cracks in the hive and protecting it against intruders.[18] Therefore, the greatest abundance of its functionalities comes from plants' secondary metabolites. It has the advantage of containing multiple active compounds that have been reported to act synergistically to enhance the strength of the natural ingredients.[19],[20] Propolis is being used as a dietary supplement,[21] in dental care,[22] cosmetic products,[23] and specifically within the food industry, where it has shown effectiveness in preserving meat, fish and juice products, among others.[24],[25]

However, utilisation of propolis does not come without challenges. Like many natural products, the consistency of propolis can vary based on the local flora. The solvent utilised to extract and/or carry propolis (itself) could influence the delivery of its functionalities. However, solvents that can efficiently dissolve propolis such ethanol[26], methanol[27] or dimethyl sulfoxide (DMSO)[28] can be toxic for human consumption, or can't be used due to consumers' age or religious beliefs. In addition, the use of such

solvents creates the challenge of ensuring that any such solvents/carrier phases do not impact/enhance the studied (e.g. antibacterial) functionality of the propolis species, that is then falsely assigned to the natural species alone. Also, the use of some of these harsh solvents/carrier phases can severely impact/damage the “naturalness” credentials of propolis in the eyes of the consumer or the formulator themselves. Propolis is a complex mixture consisting of a variety of different groups of compounds such as phenolics, flavonoids or fatty acids. Due to propolis physicochemical characteristics from its individual components aforementioned, its bioavailability in water is very limited. Therefore, due to propolis’ hydrophobic nature, more applicable solvents such as water are not efficient extraction or carrier phases, which in turn, decreases its bioavailability, thereby lowering the bioefficency of the active once consumed and subsequently creating challenges in product formulation.[29]

In order for actives to be delivered in products, formulations need to be developed that retain the functionalities of natural products while also considering sensory or texture issues. Formulated products can be of various types including liquid gels, films or emulsions. Emulsions are the basis of countless formulations consumers utilise today from food (milk, margarine, mayonnaise) to cosmetics (moisturising creams, lotions, shampoo) and more. However, the consumer drive for natural products also includes natural actives for emulsion preservation and functionality. For instance, lipid oxidation is a key issue concerning emulsions as it can alter their taste and texture and lead to its overall destabilisation, and therefore antioxidant additives are essential.

Physical stabilisation of emulsions is equally essential for prolonged shelf-life of a product. Pickering particles are increasingly popular for stabilisation purposes in

emulsion systems due to the efficient stability they offer. Particles that have reported to successfully stabilise emulsions are silica particles[30], plant derived proteins or cellulose[31]; some of those can be termed as natural species. Usually, their role is solely for stabilisation and actives are encapsulated to preserve the products or offer functionality in formulations. However, the ability of particles to provide both stabilisation and functionality has not been fully investigated yet especially on natural actives.[32]

1.2 Motivation

The motivation behind this thesis was two-fold. A great abundance of natural products, including propolis, are insoluble in water. Through formulation, they could be administrated or incorporated into products in cheaper, environmental friendlier and/or more consumer acceptable carrier phases. Means of enhancing the functionality of these actives, particularly in aqueous environments need to be explored so the utilisation of natural actives, can be more widely implemented. One approach could be to disperse such insoluble matter in aqueous media in the form of micrometer/ sub-micrometer particulates.

In addition, if such colloidal systems were to be introduced within emulsion microstructures, they could potentially also act as Pickering stabilisers. Thus, in addition to their inherent functionality (which is envisaged to be passed onto the emulsions themselves), they could also physically assist in supporting/ stabilising the emulsion microstructure. Investigating the emulsion forming and supporting capacity of these colloidal systems could pave the way to ultimately deliver to food researchers

and formulators as a range of natural and multi-functional species/ingredients of immense value and utility.

1.3 Aims of this research

The aim of this research was to produce propolis aqueous based formulations, while enhancing or preserving propolis' antioxidant and antibacterial activity, and to assess the dual role of propolis particles as Pickering stabilisers as well as lipid oxidation inhibitors/ antibacterial additives in O/W emulsions.

The main objectives were to:

1. Fabricate propolis particle aqueous dispersions *via* direct ultrasonication and assess their physical characteristics, focusing on those that (at least in other particulate species) have been linked to or are associated with Pickering functionality.
2. Investigate both the antioxidant and antibacterial capacities of propolis particle aqueous dispersions, and how these are impacted by key processing/formulation parameters, such as sonication time and propolis mass fraction.
3. Establish the role of propolis particles (when their aqueous dispersions are utilised as a continuous phase) in terms of facilitating the formation and promoting the stability of O/W emulsion microstructures.
4. Determine the antibacterial activity and ability to inhibit lipid oxidation that is bestowed upon O/W emulsion utilising propolis particle aqueous dispersions as their continuous phase and assess how these both are affected by a range of

formulation parameters, including oil and surfactant content, surfactant type and pH.

1.4 Thesis layout

This thesis consists of six chapters, including introduction, a review of the literature on the topics investigated, three results chapters and a conclusion and suggestions of future work.

- **Chapter 1:** The introduction of the thesis, introducing the project, outlining the project's aims and objectives, and summarising the publications and presentations conducted related to this research.
- **Chapter 2:** An overview on the literature of plant derived natural actives and their formulation, leading to an introduction and up to date literature review of propolis, the natural active that was investigated, and finally, a theoretical background specifically on emulsion systems.
- **Chapter 3**: This chapter sets the groundwork of the propolis formulation that was used in this research. Initially the antibacterial activity of common carrier solvents used in literature to dissolve propolis such as ethanol were investigated, showing underlying antibacterial effects that could potentially be mistakenly attributed to propolis. Consecutively, propolis was suspended in water and direct ultrasonication was utilised to decrease its size and enhance its functionalities. The mass fraction of propolis was altered as well as the duration of sonication to assess the effect on the propolis particles' size and the formulations' antibacterial activity. The effect of sonication as means to force dissolution of propolis active compounds in the aqueous carrier phase was also established in terms of antioxidant and antibacterial activity. The effect the

propolis particle aqueous dispersions have on the membrane of *E. coli* cells was further investigated showing that propolis targets the membrane by depolarising it, causing permeable damage resulting in swelling of the cells absorbing water from the environment and eventual death.

- **Chapter 4:** This chapter examines the incorporation of propolis particle aqueous dispersions in an O/W emulsion system as the continuous phase. Propolis' Pickering functionality and its interactions with a low molecular weight (LMW) surfactant (Tween 80), and a protein (sodium caseinate) are assessed. The chapter is presented in two parts. The first part concerns the investigation of propolis Pickering functionality and its interactions with Tween 80. Initially the wettability of the propolis' extract in water and oil was investigated as well as the propolis particle aqueous dispersion' affinity for the O/W interface. The viscosity of the dispersions was measured to investigate whether it could influence the emulsions' formulation or stability. Subsequently, emulsions were formulated with the propolis particle aqueous dispersions as the continuous phase and with different parameters altered: surfactant content, oil content, propolis content and emulsion droplet size. Emulsions' stability was assessed for a period of two months at 4°C and 25°C storage. The second part of the chapter investigated the pH effect of on the dispersion's size, zeta potential and antibacterial activity over time. Then interactions with the dispersion and sodium caseinate were assessed and emulsions containing both were formed and characterised.
- **Chapter 5:** This chapter evaluates the functionality of the propolis emulsions investigated in the previous chapter. Lipid oxidation of the emulsions is

assessed for different oil, propolis content, surfactant type and pH. Successively, the antibacterial activity of the propolis emulsions with different surfactant, oil content and oil droplet size was determined. Finally, the rheological behaviour of the emulsions was assessed *via* measuring their viscosity and investigating the effect of incorporating the propolis particles in the emulsions along with different oil droplet size and oil content.

- **Chapter 6:** This chapter is the final chapter of the thesis. It outlines the major findings and overall conclusions of the thesis as well as suggestions for future work.

1.5 Publications and oral presentations

1.5.1 Publications

Chapter 3 of the thesis has been published as follows:

1. **Chourmouziadi Laleni, N.**, Gomes, P. D. C., Gkatzionis, K., Spyropoulos, F. (2021), Propolis particles incorporated in aqueous formulations with enhanced antibacterial performance, *Food Hydrocolloids for Health*, 1, 100040.

Chapters 4 and 5 of the thesis are also currently in preparation for publication as follows:

2. **Chourmouziadi Laleni, N.**, Gkatzionis, K., Spyropoulos, F., O/W emulsions co-stabilised by propolis particles and surfactant or protein species, *Food Hydrocolloids* (*in preparation*)
3. **Chourmouziadi Laleni, N.**, Gkatzionis, K., Spyropoulos, F., Investigating the oxidative stability, antibacterial activity and microstructural characteristics

propolis Pickering particles offer onto O/W emulsions, *Journal of Food Engineering* (*in preparation*)

1.5.2 Conferences (Presenter in bold)

The research presented in this thesis has been disseminated in academic conferences as follows:

1. **Chourmouziadi Laleni, N.**, Gkatzionis, K., Spyropoulos, F. Enhancing the antibacterial activity of propolis in aqueous-based formulations. International Conference on Formulations in Food and Healthcare, University of Birmingham, Birmingham UK, 2021. (Remote)
2. **Chourmouziadi Laleni, N.**, Gkatzionis, K., Spyropoulos, F. Characterisation of aqueous propolis dispersions with respect to stability, interfacial properties and antibacterial activity when incorporated into O/W emulsions. International Conference on Emulsion and Dispersion Chemistry, World Academy of Science Engineering and Technology (WASET), Los Angeles USA, 2021. (Remote)
3. **Chourmouziadi Laleni, N.**, Gkatzionis, K., Spyropoulos, F. Propolis Nanoparticles Offering Pickering stabilisation and Antibacterial Activity onto O/W Emulsions. Food Chemistry and Technology Conference, United Scientific Group (USG), Paris France, 2021. (Remote)

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Chapter 2- Literature Review

2.1 Natural actives

A consumer driven preference for natural products over synthetics, as well as a rise in the antimicrobial resistance partly from misuse of antibiotics has boosted the industry as well as research onto the field of natural species, their properties and formulation. Living organisms require chemical compounds that they are able to synthesise themselves for their activities and function. Apart from the substances humans can produce themselves, there are essential compounds for some biochemical functions, or compounds that are produced at insufficient quantities and are needed to be taken in the form of supplements, such as vitamins or minerals. Besides nutritional aspects, humans also tend to supplement with substances for medical or cosmetic purposes[1],[2],[3]. The intake of supplements goes back to the history of humankind with locality being the main factor[4]. Hippocrates asked patients to chew willow bark, thus extracting the anti-inflammatory agent salicin or as it is known today, aspirin.[5] Initially raw materials found in the local environment were used for supplementation, but eventually crude extraction methods were developed, complicated synthetic procedures were established and synthetic actives were discovered to cover healthcare needs.

Natural derived products are secondary metabolites which by definition are not essential for an organism's growth or reproduction, but they are beneficial for its survival. An incredible number of plant, animal, microorganisms such as bacteria and algae or insect products has been studied for their potential use as actives obtained from organisms that have been traditionally utilised for thousands of years.

Popular animal products include proteins and peptides such as lactoferrin, protamine or defensins that have shown efficient antibacterial activity against a range

of both Gram- positive and negative bacteria.[6] Products from microorganisms include bacteriocins such as nisin, that is produced by Gram- positive bacteria. Niasin possesses antibacterial activity and generates lactic acid bacteria that when added to fermentation of medical plants could enhance the production of phenolic compounds and thus the antioxidant activity.[7] One insect from which products have been utilised for milenia is the honeybee. Honeybees generate a variety of products including honey, bee venom, bee pollen, propolis, royal jelly and beeswax. The most well-known bee product, honey, has been reported to offer many benefits including but not limited to its flavour, anti-inflammatory and antioxidant properties, while its antibacterial activity mainly stems from the production of hydrogen peroxide when diluted, although dilutions up to 30-50% of honey in water need to occur to get the maximum benefit.[8] Bee venom, another product of apiculture, also holds numerous amounts of beneficial activities, such as antiviral or antifungal, with its antimicrobial activity mainly coming from peptides and enzymes, although it is not widely used in food industry due to reluctance and fears of allergic reactions.[9]

Last but not least, plants produce a variety of natural products for different purposes such as antimicrobial peptides (AMPs) that are used from plants for protection against microbes[10], toxic substances for defense or repulsion against predators[11], aromatic compounds to attract pollinators from their color[12] or scent[13] or resins for insulation and wound healing[14].

2.2 Plant derived natural actives

A lot of research has been conducted on compounds derived from the different classes of natural sources (e.g. plant, animal, insect etc.), their chemical, and physical characterisation, and their functionalities as they can be viable replacements of

synthetic actives and reformulating products. However, in the context of acceptability by the public, the class of plant actives can be more popular/ palatable. These classes are vast in themselves and therefore tackling them is a task beyond the logistics and timescales of a single thesis. For these purposes, the scope of this work is focused on plant actives.

2.2.1 Functionality of plant derived natural actives

There is a variety of secondary metabolites that are used by the plants and some of actives can even exhibit multifunctional character. Synergistic action is extremely common among plant actives as instead of producing high concentrations of one toxin with a specific mode of action, they utilize a variety of different actives at lower amount with a stronger overall effect.[15] Due to the high level of biodiversity, different plants will have different profile with respect to composition and concentration of actives, however even within the same plant species there are studies to describe their profile variations.[16],[17]

Natural products owe some of their antimicrobial function from AMPs which are small peptides that can be found mainly in plants and some mammals, and they have a broad spectrum of antibacterial properties. They are hydrophobic and very diverse[18]. Examples of plant AMPs include thionins and defensins which are subcategories of AMPs and have exhibited both antibacterial and antifungal properties.[19]

Toxic substances, such as steroids, are produced by plants mostly as repellants to predators[20]. However, while some are indeed toxic for consumption or usage, they can have beneficial effects. For example bufadienolides, steroids found in *Digitalis lutea*, can inhibit an ATPase enzyme, disrupting the ion balance across the cell

membrane and eventually strengthen the heartbeat[21]. Repellants from plants can be formulated as natural anti – lice agents or pesticides.[22]

Aromatic compounds are usually employed as natural flavorings to attract pollinators[23] although some essential oils like lavender, possess antifungal properties due to the terpenes and terpenoids present and have been utilised for post-harvest storage of various fruits like peach[24].

Resins can be utilised for protection of the plant against extreme environmental conditions such as low water content or high temperature where it will act as a sealant, wound healing and protection from pathogens[14], which could also be the end goal for formulated products. The mechanism of action is believed to be twofold. One is the direct action of actives as antiseptic and antibacterial agents like tannins[25], and the second one is the physicochemical properties of the resins. The resin can act as a semipermeable membrane[14] allowing gas exchange but can retain stable moisture content (*via* humectants[26]).[27],[28]. Creams can simulate the mode of action of resins, acting as the vehicle for the transportation of actives through tissues, as well as offering sealing protection and moisturising properties.[29] Resins commonly contain volatile compounds for defence that can be extracted into a formulation or be formulated as a resin matrix.[14]

2.2.2 Chemistry of plant derived natural actives

Generally, natural products consist of a block unit, some multiples of it and various functional groups attached. In their structural configuration they could include some form of a cyclic (quite often phenolic) group. This could be due to the complexity and flexibility of their synthesis and the subsequent properties of the final compound.

In addition, multiplication of the number of cyclics in a molecule (polycyclics or polymerization) enriches the properties of synthesized compounds. For instance, flavonoids' (a class of secondary metabolites) basic structure is a biphenol with a keto group; their synthesis is presented by Nabavi *et al*.[30] Small variations in the structure (such as inclusion of hydroxide group) result in biologically important compounds such as anthocyanins[31] or flavones. For example, the same enzyme (phenylalanine ammonia-lyase, PAL) is involved in both flavonoid and rosmarinic acid pathways[32]. These stable secondary metabolites retain some of the basic physicochemical properties of the original structure with respect to polarity or stability but differ on the enzymatic pathways they promote or inhibit.[33],[34] In the case of rosmarinic acid, its biosynthetic cycle involves phenylalanine and tyrosine, and eventually produces moieties with different linkages[35].

In literature, natural products are commonly divided by their chemical characteristics in accordance with the functional groups they possess.[36] Some major classes of secondary metabolites with antimicrobial properties, that were categorized by Cowan[36], are phenolic compounds, terpenoids, alkaloids among others; some examples are shown in Figure 2.1.

Phenolic acids are molecules where at least one of the hydroxyl groups has been oxidized to the corresponding acid (Figure 2.1A). With phenolic acids as the basic structure, more complicated molecules can occur such as:

1. Flavonoids, where the basic structure (Flavan) contains two benzene rings bridged with three carbon atoms that can form heterocyclic configuration[37] (Figure 2.1B). The hydroxyl group often forms glycoside linkage to a saccharide (eg quercetin) which provide further diversity of the final active.

2. Tannins, that are generally large molecules built from gallic acid containing numerous hydroxy groups as well as phenolic rings (Figure 2.1C).
3. Lignans, which have a chemical structure consisting of a phenol group and three carbon atom chain (Figure 2.1D).
4. Stilbenes, that are relatively simple molecules consisting of two phenolic rings bridged with three carbon atoms and enriched by functional groups on the phenols (Figure 2.1E).

Terpenes are simple hydrocarbons containing 5 carbon atoms (Figure 2.1F). Terpenoids are the actives formed when the original terpene is multiplied and enriched with functional groups.

Alkaloids contain at least one nitrogen atom and usually oxygen. They occasionally have phosphorus or halogens in their structure and are generally weak acids (Figure 2.1G).

This method of sorting the plant actives allows for understanding of the mechanisms involved in synthesis and functionality of the compounds and can give an insight on the handling of the compounds with respect to extraction or formulating methods. Concentration of the actives, their consistency and stability, are important factors to consider and could be addressed in terms of their structure.

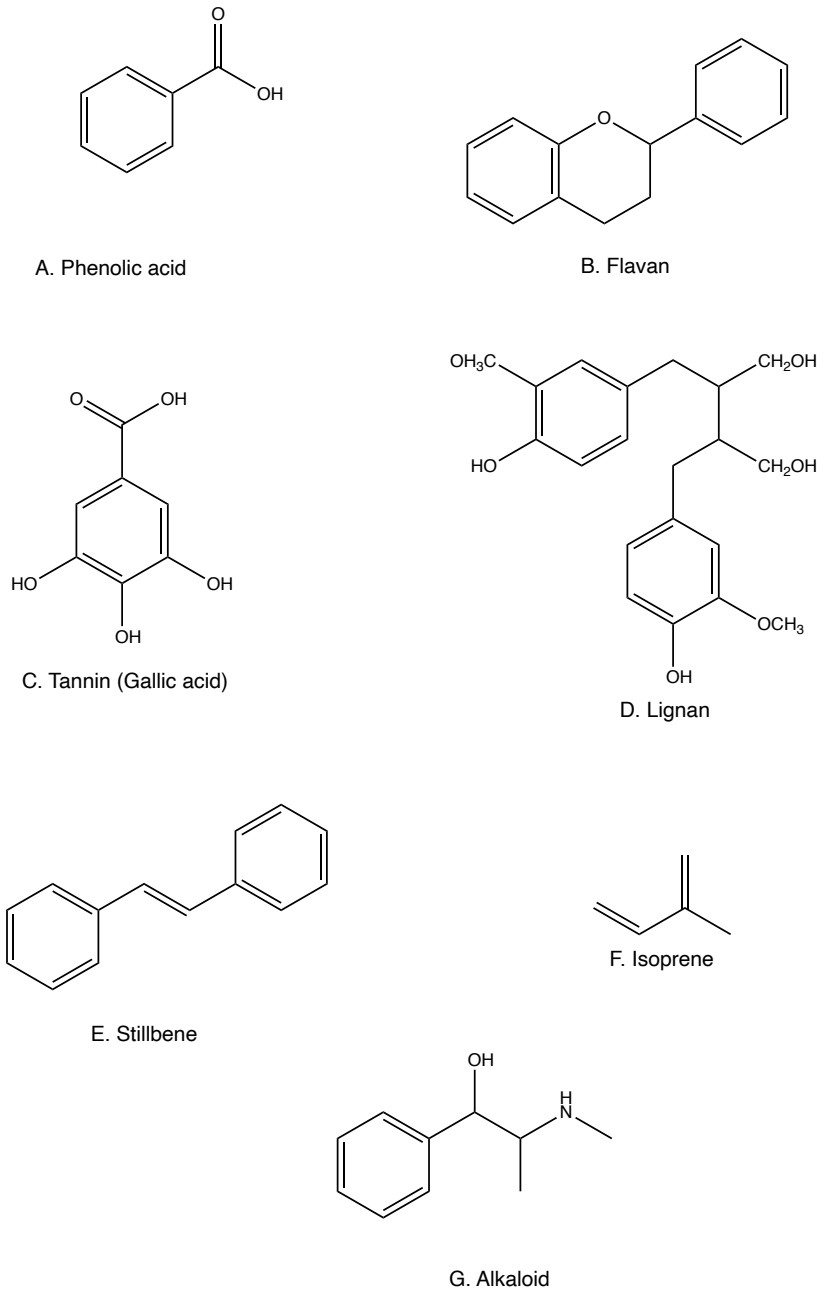

Figure 2. 1: General chemical structures of A. Phenolic acids (gallic acid[38]), B. Flavan[39], C. Tannin[40], D. Lignin[41], E. Stillbene[42], F. Isoprene[43], G. Alkaloid[44]

2.2.3 Advantages and disadvantages of plant derived natural actives

2.2.3.1 Advantages

Natural products consist of actives that can be difficult or even impossible to synthesize in the laboratory. Even though numerous synthetic actives have been

discovered and used with high success rates, new consumer drives towards natural along with a rise in antibiotic resistance, has guided research back to nature sources for novel actives[45]. Plant actives perform functions and follow biochemical pathways that are not fully understood due to the complexity of their matrix, and therefore can be hard to recreate in a laboratory setting.[46] Chemical reactions occur simultaneously and consecutively, so synergistic and complementary actions often occur. It is the complexity of those processes and their multifunction character that make the utilisation of natural products a viable addition and assistance to conventional medicines[47],[48]. It is anticipated that in the future precision and personalised medicine will gain more attention facilitated by advances in genomic technology[49]. This field will open new opportunities for natural products, a trend that is certain to continue as more medical applications are realized[50],[51].

2.2.3.2 Disadvantages and other considerations

The main disadvantages of natural products are their unpredictability and inconsistency which makes their quality control and chemical standardisation very challenging. Seasonal variations and local diversity have significant impact on the biochemical composition of the matrix. For example, the antioxidant capacity of rosemary is affected by cold weather[52] due to irreversible formation of caffeic acid from rosmarinic acid while the composition of propolis is also affected by the local flora[53].

In contrast to single chemical species, natural actives exist in complex mixtures with other compounds. The passage of such active through the digestive track can cause unpredictable structural changes, alter their functionality, and cause adverse effects on the digestive pathway of the product[54]. For instance, some flavonoids

undergo metabolic changes while digested[55] and it is therefore essential that any flavonoid formulation bypass the digestive system[56],[57]. Accordingly, many flavonoid formulations available are for external use in the form of creams, lotions etc.[58],[59] Another example is curcumin which can be quickly metabolised (reduced) as it passes through the digestive trac. In addition, it bioaccumulates in the intestine or the liver thus making it unavailable to other tissues[60].

2.3 Propolis

2.3.1 Introduction to propolis

Propolis is a resinous mixture produced by honeybees by collecting substances from plants, buds, and exudates and supplement it with salivary and enzymatic excretions.[61] Bees use it to strengthen and seal the structure of the hive, and to cover and insolate dead organisms who have entered the hive to stop contamination.[62] It comes from the Greek word “προ-“ and “πόλις” which means protection or defence of the city, therefore the hive. It has been reported to possess multiple beneficial functionalities such as antibacterial[63], antioxidant[64], antidiabetic[65], anticancerogenic[66], anti-inflammatory[67] and antifungal[68]. These properties arise from all the secondary metabolites it contains from the plant sources available near the hive.[69] Therefore, its consistency may vary from different geographical regions and time of collection.

Efforts have been made to categorise the types of propolis on its source due to some compound markers that are more abundant in propolis from different areas. For instance, Kim *et al*.[70] used analytical techniques to measure and detect propolis compounds from Argentina, Brazil, China, and Korea, finding key compositional

differences between the different countries with respect on the chemical composition of propolis. Another wide distinction identified in literature based on propolis botanical origin is between Poplar or Baccharis type. Poplar type originates from populus buds, and it is characterised by the high phenolic compounds level.[71] Baccharis type is mainly found in Brazil and some of its characteristics include specific functional actives such as anterpilin C, low phenolic and flavonoid profiles, but a great abundance of volatile compounds.[70] However, since propolis can be collected from anywhere, and the variety of flora is infinite, it would be extremely challenging to create a detailed categorical system based on its chemical composition.

2.3.2 Propolis extraction

2.3.2.1 Extraction solvents

When propolis is collected from the hive, it also contains dirty matter. Therefore, it needs to be “cleaned” or extracted prior to use. Multiple investigations and reviews have been conducted, to discuss and identify the most efficient extraction solvent for the best inclusion and then delivery of propolis functional compounds. Generally, there seems to be an agreement that aqueous ethanol (around 70-80%) has been one of the most often used extraction solvents, and was chosen in the present study.[72],[73], [74] Efforts have been made to extract propolis in environmentally friendlier solvents such as water[75] or oil[76] but without equal success.[77] However, recently, Stagkos-Georgiadis *et al.*[78] attempted to extract propolis in a greener aqueous based cyclodextrin solution which did retain propolis antifungal activity, showing promise in discovering environmentally friendly alternative extraction solvents.

2.3.2.2 Extraction techniques

Apart from the choice of extraction solvent, different extraction techniques can also influence propolis' extraction. The most conventional method is maceration, which involves leaving propolis for a specific amount of time (range from 1 hour to 10 days) and at a specific temperature (room temperature up to 70°C).[79] To speed up the extraction process, different techniques, or the use apparatus that can enhance extractions such as microwave assisted, Soxhlet and supercritical, extraction, have been investigated, a review of those has been provided by Bankova et al.[79]

In Soxhlet extraction procedures, propolis resides in a separate vessel above the solvent. As the solvent is heated it evaporates and enters the sample *via* a porous thimble. As the heated solvent passes through propolis it extracts actives and carries them to a condenser (situated above) where the enriched mixture is cooled, liquifies and returns to the original vessel *via* a siphon. The procedure can be repeated multiple times until complete extraction. Soxhlet extraction of propolis can be faster than maceration (for example 2-8 hours) and effective[80], however propolis is exposed to very high temperatures, which could result in oxidation of compounds with important functionalities[79].

Another method widely used is ultrasound assisted extraction. Oroian *et al*.[81] tested different parameters to obtain the optimum extraction conditions such as sonication amplitude, ethanol concentration, temperature and time, with the most optimum conditions being 100% amplitude, 70% aqueous ethanol, 58°C for half an hour. Sonication is considered extremely beneficial at speeding up the extraction, with lower amounts of energy needed, although not always shown to achieve better results compared to maceration.[82],[83]

A major example of how an extraction technique can influence the final result was shown by an investigation conducted by Mello *et al*.[84]. They used nanofiltration, a process that allows selective permeation of solute substances through a semi permeable membrane, where water managed to retain a much higher percentage of phenolic and flavonoid compounds than ethanol.

In the present study, maceration was used as an extraction method with occasional shaking for 7 days. That was chosen in order to not stress the system and result in potential oxidation of beneficial compounds that could influence the activity of the final formulation The 7 days duration was chosen as it was in the larger side of duration reported in the literature, in order to ensure complete extraction. [85], [86]

2.3.2.3 Carrier phases

Once propolis is extracted, it either remains in the extraction solvent for further testing or formulation, it could be dried and resuspended, or incorporated as dry matter in different systems, depending on the purpose of the study and the application. Since propolis cannot be extracted or dissolved in water, alternative solvents have been employed in literature with purpose to utilise and effectively test propolis' multiple functionalities, some of them are shown in Table 2. However, the difference in the solvents used could be another reason behind the variability of the antibacterial activities. For instance, Regueira *et al*.[87] used dimethyl sulfoxide (DMSO) as propolis' carrier phase, while Popova *et al*.[88] used dichloromethane (DCM). Even though in both papers the *E. coli* species and assay method used were the same, the antibacterial properties were different (MIC values of maximum 512 μm vs 1340 μm respectively), as it can be seen in Table 2.1 (although other reasons besides choice of carrier phases could be propolis' origin). In addition, some solvents that are used as

carrier phases to dissolve propolis such as ethanol or DMSO[89] (as shown in Table 2.1) could also have underlying antibacterial properties which creates challenges to assign the resulting activities directly and exclusively to propolis. Therefore, there needs to be an aqueous based system with enhanced propolis' activities in order to both deliver its functionalities to the best level possible and be certain that these activities originate from the propolis alone.

2.3.3 Functional compounds present in propolis

Propolis is an extremely complicated mixture of organic compounds. Over 300 compounds have been identified, their identities and concentrations differing vastly depending on time and place of collection.[90] However, it has been widely accepted that the beneficial activities propolis offers mostly stem from phenol and flavonoid compounds.[91] There are examples of both types of substances with antibacterial and antioxidant activities that can be commonly found in propolis and therefore in analytical studies are used as standards such as: gallic, p- coumaric acid (phenols), chrysin, pinocembrin, galangin, quercetin (flavonoids).[92],[74],[93] Additionally, Greek propolis, which was used in the present study, showed to possess terpenes which also when isolated, exhibited antimicrobial activities.[72] The advantage of propolis is that all the different elements work together and either synergistically or additively result in enhanced actions. For example, Amoros *et al.*[94] initially analysed propolis, detected and isolated some major flavonoid and phenolic compounds and tested them for antiviral activity. After establishing their activity alone, they tested a selection in pairs to assess synergistic effects, demonstrating that all of the pairs exhibited better antiviral activity than individually.[94] They suggest that synergistic effects can occur when compounds with different modes of action are combined, which highlights the

reason propolis is so beneficial.[94] With a more direct relevance in this study, Kharsany *et al*.[95] tested the synergistic and additive effect of the flavonoids pinocembrin, galangin and chrysin contained in propolis on their antibacterial activity, establishing that 1:1 ration combination resulted in 22% synergy while triple 1:1:1 in 66% synergy in inhibiting the bacterial growth, also concluding that propolis as a whole is more active than its individual components.

2.3.4 Antibacterial activity of propolis

As it has been aforementioned, the functional properties of propolis is due to the flavonoids and phenols. Even though propolis composition can be so variable, its antibacterial activity can be consistent. There are some general patterns such as, propolis acts much stronger against Gram- positive bacteria like *S. aureus*, *B. subtilis*, *L. monocytogenes* and *P. larvae*[96] than Gram- negative such as *E. coli* and *P. aeruginosa*.[97] That is believed to occur due to the differences in the structure of their membrane. Gram- positive bacteria are surrounded by thick layers of peptidoglycan while Gram- negative, even though they have thinner layers of peptidoglycan, have an additional outer membrane containing liposaccharide (LPS).[98] It is has been reported that it is due to LPS as well as the possible degradation of propolis by the release of hydrolytic enzymes by the Gram- negative bacteria, that makes propolis antibacterial action less pronounced.[99]

In the present study, *E. coli* was chosen for investigation as it is a Gram-negative model microorganism[100] and there is a greater research interest for Gram-negative bacteria due to the higher complexity of their membrane.[101]

Przybyłek and Karpiński[102] reviewed the antibacterial mechanism of propolis, suggesting that it involves decreasing the cell mobility, influencing the cell membrane by disrupting its potential and enhancing permeability, decrease of production of ATP (adenosine triphosphate) and enhance the activity of the immune system.

Propolis has shown an efficient preservative role when incorporated in foods such as juice[103], crayfish meat coating[104] and fruit[105]. It has also shown promising results concerning cosmetic applications[106],[107] and dentistry[108]. However, propolis performance is not consistent as its composition depends on time and place of collection, extraction solvent, carrier phase and the method to determine the antibacterial and antioxidant activity.

2.3.4.1 Methods to measure antibacterial activity

There are numerous methods in literature that have been employed to determine the antibacterial activity of an active; some more direct (treat the cells and see the effect on their growth and population) or indirect (stain the cells with fluorescent dyes). The most popular direct methods are: minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) assays, time-kill curves and zone of inhibition or diffusion tests.

MIC is the minimum concentration needed to inhibit visible bacterial growth and MBC the minimum concentration required for bacterial death. Determining the MIC value is relatively simple and fast and it is a good standard for comparison with antibacterial agents. The downside is that there is no information on the rate of action of the antibacterial agent under different concentrations. Two ways to determine the MIC are the broth and agar dilution methods. Both Goldstein *et al.*[109] and Waites *et*

al.[110] directly compared the two methods reporting a small variability in MIC values with a higher MIC appearing in solid media. The agar dilution method has the limitation that it cannot show the MBC. The type of agar medium may also affect the results. In the present study, nutrient agar was used as a non-selective agar to ensure no contamination. To better demonstrate the variability of propolis' antibacterial activity depending on the extraction solvents, carrier phases, and the method chosen, MIC and MBC values against *E. coli* were summarised and compared in as it can be seen from Table 2.1.

Table 2. 1*: MIC and MBC values of propolis extracts on different E. coli strains, showing the extraction and carrier phases, and the type of assay used.*

Bacteria	**Extraction solvent**	**Carrier phase**	**Antibacterial Assay**	**MIC value**	**MBC value**	**Reference**
E. coli	70% Ethanol	70% Ethanol	Agar dilution	14 µg/ml	-	[111]
E. coli	Methanol	TSB	Broth Microdilution	512 µg/ml	No MBC	[112]
E. coli 06	54% Ethanol	DMSO	Broth Microdilution	128-512 µg/ml	-	[87]
E. coli ATCC 11229	70% Ethanol	70% Ethanol	Broth Microdilution	340 µg/ml	-	[113]
	96% Ethanol	96% Ethanol	Broth Microdilution	290 µg/ml		
E. coli ATCC 11230	96% Ethanol	10% DMSO	Broth Macrodilution	16-128 µg/ml	-	[114]
E. coli ATCC 11775	Ethanol	DMSO	Broth Microdilution	1840 µg/ml (no activity from most samples)	-	[115]
E. coli ATCC 25922	DCM	DCM	Broth Microdilution	1340 µg/ml	-	[88]

E. coli ATCC 25922	54% Ethanol	DMSO	Broth Microdilution	161.3-512 µg/ml	-	[87]
E. coli ATCC 25922	70% Ethanol	10% Ethanol 90% TSB	Broth Microdilution	>25000 µg/ml	-	[116]
E. coli ATCC 25922	70% Ethanol	70% Ethanol	Broth Microdilution	>4096 µg/ml	>4.096 mg/ml	[117]
E. coli ATCC 25922	70% Ethanol	Ethanol	Agar Dilution	670 µg/ml	2.33 mg/mL	[118]
E. coli ATCC 25922	70% Ethanol	Ethanol	Agar dilution	1875-7500 µg/ml	-	[119]
E. coli ATCC 25922	70% Ethanol	Ethanol	Broth Microdilution	10-1980 µg/ml	-	[120]
E. coli ATCC 25922	96% Ethanol	Ethanol	Agar dilution	14300-30200 µg/ml	-	[121]
E. coli ATCC 25922	96% Ethanol	Ethanol/ DMSO	Agar dilution	>1600 µg/ml	-	[122]
E. coli ATCC 29998	80% Ethanol	DMSO	Broth Microdilution	3190 µg/ml	-	[123]
E. coli ATCC 8739	70% Ethanol	10% Ethanol	Broth Macrodilution	500 µg/ml		[124]
E. coli CECT 423	96% Ethanol	Ethanol	Agar dilution	>2000 µg/ml	-	[125]
E. coli CECT 434	60% Ethanol	60% Ethanol	Broth dilution	>1200 µg/ml	-	[126]
E. coli JMI09	96% Ethanol	Ethanol	Agar dilution	Inhibited growth by 50% 450 µg/ml	-	[91]
E. coli O157 :H7 ZMJ128	70% Ethanol	70% Ethanol	Broth Microdilution	680 µg/ml	-	[113]
	96% Ethanol	96% Ethanol	Broth Microdilution	580 µg/ml		
E. coli NCTC 09001	70% Ethanol	80% Ethanol	Zone of inhibition 1-3mm	5000 µg/ml	-	[127]

The time-kill curve assay is another method used to determine the antibacterial activity of an active. In this method, microbes are inoculated while treated with an active of interest, are sampled at specific time points and their population determined regularly to understand the effect of the active.[128] This can occur in bacteria that are both in their stationary[129] or in exponential[8] growth stage. The advantage of this method is that it can show the rate of inhibition of growth or killing. In the present study, the method utilised was a time kill curve assay, with the bacteria cells on their stationary phase, as at this stage, they are in their strongest state, having adjusted to their environment.[130] This method was chosen to obtain information on variations of the rate of killing between the different formulations.

Other ways to assess antibacterial action are diffusion and bioautographic methods with the information they provide being qualitative rather than quantitative and are described in more detail by Balouiri *et al*.[131].

Antibacterial activity can also be determined *via* the assistance of fluorescence dyes that bind to specific structural aspects of the cell and give information on its viability. The most common methods to stain cells is *via* 3,6-bis[dimethylamino]acridinium chloride (acridine orange; AO) and 4′6′-diamidino-2-phenylindole (DAPI) dyes; both are considered DNA specific and therefore as long as a cell has its DNA structure intact, the cell would be detected.[132]

Apart from enumerating, fluorescent microscopy can offer information on the structural state of the cell. For instance, propidium iodide (PI), has the ability to bind to

nucleic acids only if the membrane of the cell is damaged.[133] Other examples acting in similar ways include ethidium bromide, ethidium monoazide etc.[132]

Flow cytometry, although initially mostly used in mammalian cells, has also succeeded in examining the much smaller bacteria cells.[134] It can carry out single cell analysis by a combination of light-scattering and fluorescent measurements. Therefore, it can give fruitful information on the state of the cells such as total cell count, viability, detection of physiological changes and more.[135] In the present study it was utilised to assess the viability of the cells and whether they were considered as live injured or dead.

2.3.5 Antioxidant activity of propolis

The antioxidant activity of propolis extracts has also been extensively investigated. In a similar path with its composition and antimicrobial activity, the antioxidant activity seems to be extremely variable even though it is also recognisably stemming from its phenolic and flavonoid compounds. There are multiple different ways to assess antioxidant activity; the present study focused on spectrometric ways. Different assays rely on distinctive antioxidant mechanisms and thus indicate a characteristic aspect of propolis antioxidant activity. A few of those are discussed below.

2.3.5.1 Methods of measuring antioxidant activity

One method used to assess propolis antioxidant activity is *via* the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. This method tests the ability of a compound to donate a hydrogen atom to neutralise the stable DPPH radical. The radical has a bright violet colour which decreases as it gets oxidised, and measures of its decolourisation

determines the active's antioxidant activity.[136] Propolis has shown to neutralise the DPPH radical in literature; Zhang *et al*.[137] tested 22 propolis samples from Brazil for total phenolic, flavonoid content, DPPH scavenging activity and for specific polyphenolic compounds *via* HPLC. Their findings included that the level of this antiradical activity was varied from all the different samples tested and that this activity was correlated with the amount of total phenolics in the sample.

Similarly with DPPH, the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay also involves the ability of an active to donate a radical to neutralise the ABTS radical, and the level of decolourisation is measured and related with the active's antioxidant activity.[138] Unlike with the DPPH assay, the ABTS assay involves a radical that is positively charged. Floegel *et al*.[139], compared the two methods on a range of phenolic and flavonoid containing foods and beverages, reporting that the ABTS assay exhibited higher antioxidant activities than the DPPH assay and a higher correlation with the oxygen radical absorbance energy capacity assay (explained below), although it was highlighted that there is a discrepancy among literature in terms of different antioxidant methods correlating. Propolis has shown the ability to scavenge the ABTS radical in numerous different investigations, again with some discrepancy.[140],[141],[142]

The ORAC (Oxygen radical absorbance capacity) at its core is also a radical oxidation reaction. However, this assay is not as straightforward. It involves a UV-vis active compound which as it is being oxidised by peroxyl radicals it decolorises. Once an antioxidant compound is added, the aim is to protect the fluorescent compound by scavenging the peroxyl radicals.[143] Therefore the less decolouration occurs, the higher the antioxidant activity of the sample. Castro *et al*.[144] investigated propolis'

antioxidant activity with the ORAC assay for six different samples of Chilean propolis, reporting an efficient antioxidant action although different depending on location of collection.

Two other commonly assays used to determine propolis antibacterial activity are the FRAP (Ferric reducing antioxidant power) and CUPRAC (Cupric ion reducing antioxidant capacity). These assays are similar as they both involve redox reactions (ability of a substance to donate a pair of electrons) of iron (iii)[145] and copper (ii)[146] respectively. Propolis has been reported to exhibit antioxidant activity *via* both assays.[147],[148]

The Folin-Ciocalteu method is widely used to determine the total phenol content, again based on a redox reaction.[149] However, the method is not always very selective towards phenolic compounds and it is believed to provide a good indication of the whole antioxidant capacity of an active.[150] In the present study the method was used primarily to characterise the amount of phenolics in formulations, although a note on determining the antioxidant capacity was also taken into consideration. Similarly with all the other assays mentioned, it has been used to detect the total phenolic content but with discrepancies depending on the place of collection.[151],[152]

In the present study, the DPPH assay was used to determine the antioxidant activity as it is an accurate, fast, inexpensive, and well reported method.[153], [154] The Folin-Ciocalteu method was employed as it could not only give an indication of the total phenolic compound content, but also provide information on the antioxidant capacity of the formulations.[155]

Apart from spectrophotometric assays, there are multiple other ways to measure the antioxidant capacity of an active such as electrochemical techniques and chromatographic analysis of the sample, a more detailed description is provided by Munteanu and Apetrei[149].

2.4 Incorporation of natural actives in formulations

Due to the bioactives' nature or the needs of the consumers, natural actives have to undergo through formulation to be incorporated into products. There is a variety of purposes for an active to be incorporated in formulations such as: protection of the active from degradation caused by environmental conditions, enhancement of its bioavailability due to the active's potential hydrophobic nature, controlling of the release for more efficient action, preservation and increase in nutrition of the product itself and for texture and sensory aspects. Most of those techniques include encapsulation of the actives in carriers.

As some natural actives can be quite sensitive to environmental stresses, for instance flavonoids can easily be oxidised when exposed to light[156], they often are formulated by being encapsulated in protective matrices to prevent or delay degradation. For instance, Dai *et al*.[157] incorporated curcumin in zein-lecithin nanoparticles which improved curcumin's thermal and light stability. Hu *et al*.[158] encapsulated different flavonoids in gum Arabic and whey protein concentrate microcapsules, which increased the retention rate massively than free flavonoids (63.39% vs 19.29%) post 3 months storage.

Enhancing the bioavailability and the solubility of actives is a crucial ability of formulations as a lot of plant actives are hydrophobic and possibly not retainable when

administrated. Encapsulating actives in materials to increase their solubility as well as decreasing the size of the carriers seem to be common pathways followed in literature. For instance, Celebioglu *et al*.[159] encapsulated thymol, a monoterpene phenol, in cyclodextrin complex nanofibers which enhanced both thymol's water solubility and thermal stability. Similarly Cecchi *et al.*[160] encapsulated an olive extract, due to its high phenolic compounds consistency, in microemulsions aimed for oral dosage, achieving enhancement in the active's solubility by four times, and increased significantly the intestinal permeability.

Controlled release of an active brings many advantages on its administration such as improved bioavailability, reduced doses and increased efficacy,[161] (ie *via* temperature, shear or pH changes, depending on the mechanism of release[162]). For instance, Taofiq *et al*.[163], encapsulated a range of phenolic compounds such as p-coumaric acid, cinnamic acid and ergosterol in semisolid base creams for cosmetic applications, reporting that by delaying the release, their bioactivity was prolonged.

An extremely common phenomenon observed is incorporating actives into formulations and comparing them with synthetic equivalents to assess their action and whether they can stand as efficient replacements (ie. for preservative roles), often with promising results. Naveena *et al*.[164] carried out a study testing the antioxidant activity of pomegranate juice and pomegranate rind powder extract in cooked chicken patties, and compared them to the artificial BHT (butylated hydroxytoluene) preservative. The study revealed that both the pomegranate juice and the rind powder extract achieved inhibiting oxidation more efficiently and for longer times than BHT. In similar fashion, Coruh *et al.*[165] compared the antioxidant activity of aqueous and methanolic extracts of the aerial part of the *Rumex crispus* L. with synthetic additives BHA (butylated

hydroxyanisole) and BHT, reporting that even though the methanolic extract's activity was lower, the difference was not statistically significant, highlighting the extracts' potential as both antioxidant and antibacterial additives.

Finally, as Premathilaka *et al*.[166] recently highlighted in a review, natural actives, for instance flavonoids, when incorporated in oral delivery formulations, can cause adverse sensory effects, which can be masked if they are encapsulated. Sun *et al*.[167] encapsulated tartary buckwheat flavonoids in polymeric whey protein to then be incorporated in yoghurt, due to flavonoids' dark colour and bitter taste. They concluded that the encapsulated flavonoids' sensory changes were masked effectively and even improved the physicochemical characteristics of yoghurt.

2.4.1 Incorporation of propolis in formulations

Because of propolis' well reported beneficial activities, research has been conducted concerning different methods it can be formulated. Some of the main reasons it needs to go through formulation is its high hydrophobicity, unpleasant sensory properties, and sensitivity of its functionable compounds to oxidation from environmental conditions such as light or heat.[168]

Attempts to cover propolis' undesirable sensory properties have been conducted. For example, Goncalves *et al*.[169] investigated whether the sensorial assessment of propolis based creams would improve with the addition of tocopheryl acetate, for cosmetic applications, showing encouraging results. Similarly Jansen-Alves *et al*.[170] encapsulated propolis in pea protein for applications in fortified cake, resulting in similar sensory aspects with the control.

To decipher propolis small aqueous bioavailability, offer protection and simultaneously control its release, attempts have been carried out to encapsulate propolis in different nanocarriers such as liposomes[171], solid lipid nanoparticles[172] or with inorganic nanoparticles such as gold[173] or silver[174], all exhibiting promising results. A complete review can be found by Mendez- Pfeiffer *et al*.[175].

Otherwise, propolis has also been added in formulations to enhance the formulations' properties. For example, it has been incorporated in films to increase the functionality of packaging and edible coatings, for its antimicrobial and antioxidant activities.[176] Recently Chrila *et al*.[177] incorporated propolis in an emulsion system and then in cotton textiles for medical applications. El-Guendouz et al.[178], added propolis in water based emulsions in order to increase their oxidative stability.

Overall, natural actives including propolis, are usually incorporated in multiphase and multicomponent microstructures ranging from dispersions to films or emulsions, depending on the purpose of the formulation, the application, or the needs of the consumers. Emulsions, beyond the natural active space, are convenient systems that through their microstructure can offer multiple benefits, such as sensory or rheological control, including the coexistence of two phases that can tolerate both hydrophilic and hydrophobic components. Therefore, the present study is focused on emulsion systems.

In the present study, issues such as the hydrophobicity of propolis were tackled through formulation by reducing its particle size and increasing its active surface area. Furthermore, the ability of propolis to possess Pickering functionality in an emulsion system was also examined, which to the best of the author's knowledge has not been reported yet.

2.5 Emulsions

Colloidal dispersions are formulations where one phase (dispersed phase) is dispersed into another (continuous phase) while keeping their physical characteristics, ie. without being dissolved. Depending on the physical state (solid, liquid, gas) the states are, the formulations have different properties and can be used for diverse applications.

An emulsion is a type of formulation that consists of two or more immiscible liquids, typically water and oil, one being dispersed in the other as droplets. The area between the oil and water phases is the interface. Depending on which liquid is in the continuous or dispersed phase, emulsions can be categorised as oil-in-water (O/W) or water-in-oil (W/O). Another way they can be classified is depending on the size of the dispersed droplets as macro- (1-100 μm), micro- (20-500 μm) and nano- (10-100 nm) emulsions.[179] Complex emulsion based systems can exist as well, such as double emulsions[180] (oil-in-water-in-oil or vice versa) and emulsion gels (where both emulsion droplets and gelling agents are present)[181].

Emulsions are widely used for encapsulating actives or flavours, protecting active compounds from degradation, enhancing their bioactivity, controlling their release or altering the texture of a product.[182] They are the basis behind numerous products in industries such as foods (dressings, dips sauces)[183], pharmaceuticals (as carriers of actives in drugs)[184], cosmetics (body care, hair care, personal care etc)[185], agrochemicals (pesticides or herbicides)[186] and more.

The formulation of emulsions consequently results in an increase of the contact area between water and oil, i.e. the interfacial area. However, the interfacial tension

between the two phases is too high deeming the emulsion as thermodynamically unstable and results in a tendency to minimise the interfacial area until eventually the two phases become completely separate. A more detailed explanation of the thermodynamic instability of emulsions has been provided by McClements[187].

2.5.1 Emulsifiers

In order to enhance the stability of emulsions, emulsifiers are used. Emulsifiers are surface active species that during emulsification have the ability to adsorb in the water/oil interface and create a layer around the oil droplets increasing the stability of the emulsions. They can be classified as low molecular weight (LMW) surfactants, phospholipids, amphiphilic biopolymers (proteins or polysaccharides) and certain types of particular matter.[187] The present study focuses on LMW surfactants, proteins and particles.

2.5.1.1 LMW surfactants

LMW surfactants are species with a molecular structure that possesses hydrophilic heads and hydrophobic tails. During emulsification, they place themselves in the oil/water interface and orientate themselves so that their hydrophobic parts to side with the oil and hydrophilic ones with the water phases respectively, interrupting the unfavourable interaction of water and oil, thus decreasing the interfacial tension.[188] They could be non-ionic, charged, or zwitterionic and therefore they can also (if charged) stabilise emulsions electrostatically.[189] Due to their low molecular weight, they have the advantage of being able to move rapidly and adsorb at the emulsion interface. Depending on the purpose of the formulation, if it needs to be oil or water continuous, the appropriate surfactant needs to be selected. This is based on its

hydrophilic-lipophilic balance (HLB) which determines their solubility in water or oil, and therefore which phase will end up being the continuous phase.[190]

2.5.1.2 Proteins

Proteins are large molecules and can act as emulsifying agents, by adsorbing at the interface and protecting sterically and electrostatically the oil droplets from destabilisation mechanisms. Compared to LMW surfactants, proteins create a thicker layer with higher viscoelastic properties around the droplets, as it is depicted in Figure 2.2A. However, due to their larger size, their mobility is lower so the rate of diffusion, adsorption and subsequent orientation at the droplets' interface is decreased, as it is demonstrated in Figure 2.2B; Tween 80 (T80) has dramatically decreased the interfacial tension compared to whey protein isolate (WPI). Also, proteins are not able to reduce the interfacial tension as efficiently as LMW surfactants (Figure 2.2B), and usually end up with larger oil droplet sizes (Figure 2.2C). Proteins can be highly affected by parameters such as pH and temperature.[191] For example, if the pH is close to their isoelectric point (where the net charge of the protein is zero), due to the absence of charge required to keep the protein unfolded and able to surround the droplets, they flocculate and cannot act as efficient emulsifying agents.[192]

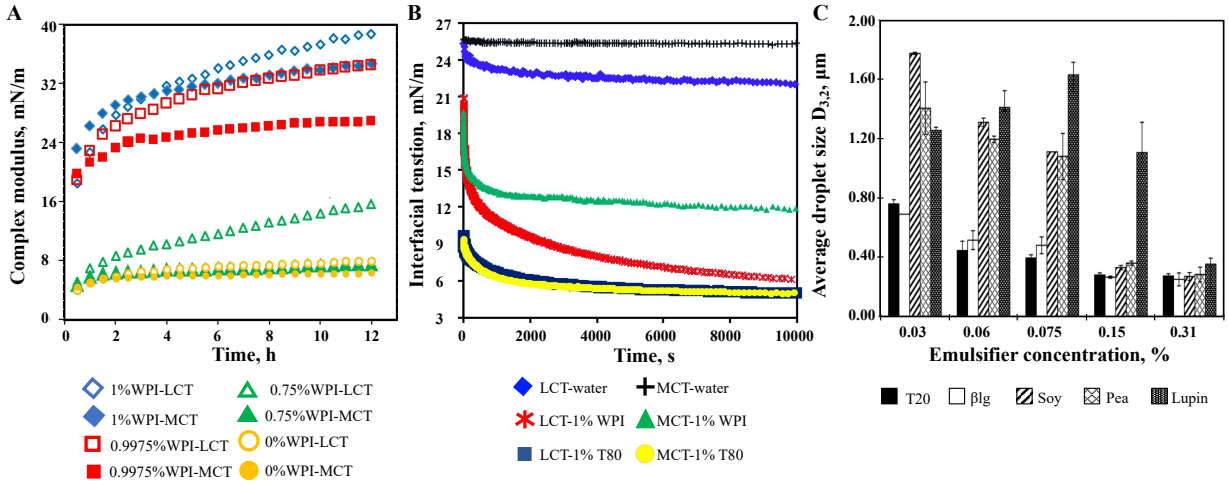

Figure 2. 2: A. Viscoelastic properties of different concentrations of whey protein isolate (WPI) and Tween 80 (T80) against a long chain triacylglycerol oil (LCT) and a medium chain triacylglycerol oil (MCT) and B. Interfacial tension, produced by high shear mixer followed by homogenization, adapted from Gomes et al.[193]. C. Average oil droplet size with increasing emulsifier concentration: Tween 20 (T20), β-Lactoglobulin (βlg), soy, pea and lupin protein, produced by high shear mixer, followed by a microfluidizer, adapted from Benjamin et al.[194]

2.5.1.3 Protein/ surfactant mixed systems

Due to the ability of LMW surfactants to adsorb rapidly at the emulsion interface, and the proteins' advantage to form stronger viscoelastic layers, efforts have been made to combine them together for more stable emulsions. Su and Zhong[195], demonstrated that using sodium caseinate in tandem with Tween 20 produced smaller oil droplet sizes (by a high shear mixer) than using sodium caseinate alone. However, Dixit *et al*.[196] highlighted that there can also be a competitive behaviour between two stabilisers, reporting that Tween 20 shows a tendency to displace the protein from the interface, due to the surfactant's hydrophobic parts interacting with the protein's, preventing the protein from reaching the interface and resulting in its denaturation, something that has been reported by other authors as well.[197],[198]

2.5.1.4 Pickering Particles

Another way to stabilise emulsions is by using colloidal particles, termed Pickering particles. Pickering particles started being investigated in the early 20th century[199],[200], Since then a lot of research has been conducted on the capacity of colloidal species such as inorganic, food grade or polymer based particles, to stabilise emulsions.[201] These particles can be adsorbed at the O/W emulsion interface, create a packed layer around the droplets and thus protecting them from destabilisation sterically, though it is notable to mention that they tend to be less mobile than low molecular weight surfactants, in reaching the interface.[202] The degree of protection is determined by the density and viscoelasticity of the layer.[203] Pickering emulsions tend to be thermodynamically favourable than emulsifier stabilised emulsions due to the higher energy required to displace the particles from the interface.[204] This energy is related to the assumption that the particles are spherical, to the particles' size being small enough for the gravity effect to be negligible, and to the particles' wettability which determines their position at the interface and what emulsion system they will promote formulating (in some respect it is similar to the emulsifiers' HLB value). Depending on the value of the contact angle θ, the particles will have a preference of being wetted more by either the continuous or dispersed phase. Figure 2.3 illustrates the three different circumstances that could take place. If the angle θ is acute or obtuse, the particles will have a hydrophilic or hydrophobic character and form the appropriate emulsion (O/W or W/O respectively).[202] For example, if hydrophilic (Figure 2.3A) the particle prefers to be wetted predominantly by the aqueous phase, which therefore will end up being the continuous phase of the emulsion.

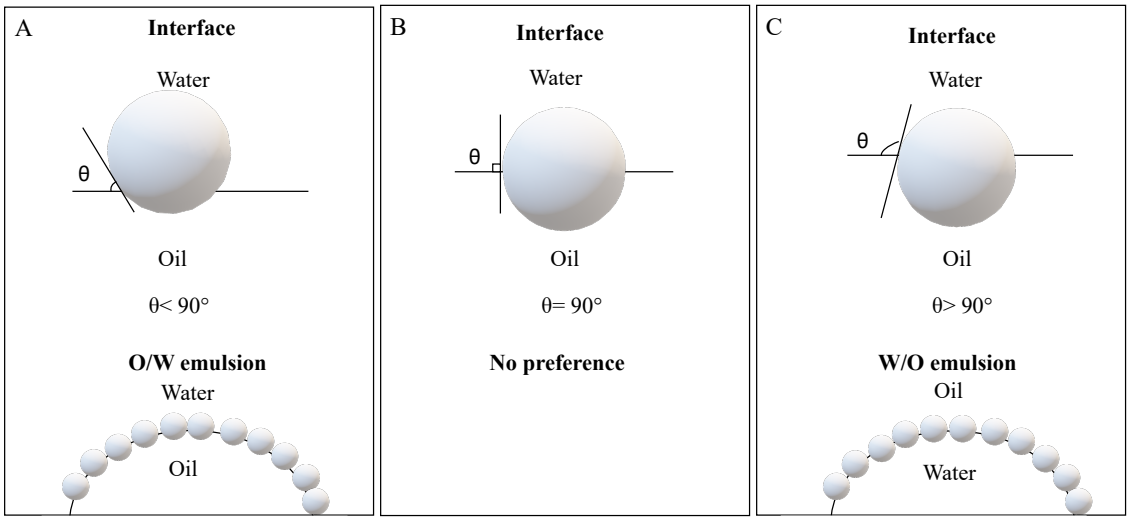

Figure 2. 3 Preferential position of Pickering particles determined by their A. hydrophilic, B. amphiphilic and C. hydrophobic character (adapted from Binks[205]).

The energy, *E*, required to remove them from the droplet surface is given by equation (2.1)[206]

$$E = \pi r^2 \gamma (1 - |cos\theta|)^2 \qquad (2.1)$$

where *r* is the particle radius, *γ* is the interfacial tension between the water and oil phases and *θ* is the contact angle of the particle at the interface (Figure 2.3).

It can be seen by the equation (2.1) that the desorption energy is immensely dependant on the particle size. Therefore, smaller particles can more efficiently stabilise emulsions. It is believed that for better coverage, the particles need to be an order of magnitude smaller than the droplets they aim to surround and stabilise.[203] For instance, for a 5 μm emulsion droplet, Pickering particles of at least 500 nm should be employed.

Another factor that can also highly influence the desorption energy is the contact angle *θ.* If *θ* equals 90°, that results in the strongest adsorption of particles at the interface and these particles can stabilise both O/W and W/O emulsions (Figure 2.3B). For instance, Duffus *et al*.[207] investigated a range of colloidal particles with different contact angles on their ability to stabilise both O/W and WO emulsions, reporting that ethyl cellulose particles with a water contact angle of 90° but an oil contact angle of 40° could not produce stable O/W emulsions but successfully fabricated W/O emulsions with an excellent stability for 21 days of storage.

Research has been carried out on particles from multiple plant sources to examine their potential ability as Pickering stabilisers. Different kinds of particles have provided Pickering stabilisation from proteins such as soy[208], zein[209] or quinoa[210] as well as complexation of these species with polyphenolic anthocyanins with purpose to increase the physical and oxidative stability and potentially their antibacterial activity of emulsions.[211],[212] Other widely used particles are polyssacharides such as cellulose [213] or starch[214]. The usage of plant-based proteins and polyssacharides is cheaper and more environmentally friendly than animal sourced proteins, as well as being more popular among consumers.[215] Plant derived fat and wax particles, termed solid lipid nanoparticles, have also been extensively studied both for their capacity to stabilise emulsions[216] as well as their ability to encapsulate actives themselves, providing the advantage of a multi-delivery emulsion system[217].

2.5.1.5 Emulsifiers/ particles mixed systems

One advantage of a combined system where both emulsifiers and Pickering particles are used to stabilise emulsions is that surfactants can influence the wettability of the particles, and therefore potentially enhance the stability of the final emulsions.

[218],[219] For example Hu *et al*.[220] demonstrated that adding a highly hydrophobic cationic alkyl ammonium surfactant resulted in electrostatic as well as hydrophobic/philic interactions with hydrophobic anionic cellulose nanocrystals, as well as better emulsion stability.

Another important advantage of mixed systems is that they can produce emulsions with smaller oil droplet sizes and enhanced long term stability than they would if the individual components were used alone. It is believed that this is achieved by the mechanism of stabilisation of the two species; the surfactants are able to travel fast to the interface, decrease the interfacial tension, enhance the droplet break up and delay coalescence while the particles reach the interface slower and are able to then provide long term stability.[202]

However, similarly to the surfactant/protein systems, competitive adsorption has been reported in case of Pickering particles and surfactants as well. Song *et al.*[221] concluded that even though using surfactants in tandem with Pickering particles created smaller oil droplet size emulsions than using solely the Pickering particles, a competition for the interface was observed. This has been also observed by other authors[222],[223], for instance Pichot *et al*.[224] reported the eventual removal of silica particles from the interface as the concentration of surfactants increased.

2.5.2 Methods of emulsification

There are multiple ways to formulate emulsions, depending on a variety of factors such as the volume of the sample, the type of the emulsion or additives, the required droplet size, viscosity of the continuous and disperse phases and more. These could be categorised as high, intermediate, or low energy techniques and a full

description of each is provided by McClements[187]. Another distinguish that can be made among the different emulsification methods is droplet break up (such as high shear mixers, direct ultrasonication, homogenisers, microfluidisers etc.) *versus* drop by drop formation methods (ie. membrane emulsification, microfluidic devices). Although drop by drop methods have numerous advantages such as low polydispersity and accurate tuning of the wished droplet size, in case of Pickering emulsions, the particles are often unable to diffuse and reach the interface, something that happens spontaneously in high energy break up methods.[225] Therefore, the present work focuses on high energy, droplet break up methods which are discussed below.

High shear mixers (HSM) are widely used in food, cosmetical and pharmaceutical industries.[226] HSM consist of a mixing head rotor with a specific diameter that gets submerged in the sample while its speed can be controlled and the different phases are mixed by the rapid movement of the rotor head. An important advantage HSM can offer is that they can work on both small and large scales. However, the final droplet distribution is usually wider compared to other emulsification techniques and they are often used as the primary homogenisation step to be followed by a different technique and achieve smaller droplet sizes.[226]

Direct ultrasonication is another high energy method employed to achieve emulsification. Sonication introduces high power ultra sound that create small vacuum bubbles which grow absorbing energy and collapse violently.[227] Such method can promote dissolution by breaking intermolecular forces [228] and can be also used for extraction purposes[83]. It can achieve small droplet sizes and it is beneficial compared to other formulation techniques as less energy is needed to achieve a small droplet

size[187]. It is ideal for samples with small volume, however it can be difficult to scale up[229].

The energy input of the breakup method can influence the size of the oil droplets in an emulsion system. Droplets in the microscale region could be achieved relatively easily via a high shear mixer whereas below the micrometer level, is more challenging and requires even higher input.[230] Siddiqui[231] tested a range of different methods while controlling the energy dissipation rates. They showed that for similar energy dissipation rates, the high shear mixer method consistently produced the largest oil droplets, while sonication could produce similar oil droplet sizes with other break up methods such as high-pressure homogenizers but with 1000 times less energy dissipation, highlighting its efficiency.[231]

2.5.3 Emulsion physical stability

Depending on the ingredients used, storage conditions or other factors, emulsions can be prone to destabilisation mechanisms. Phase separation is the major cause of denaturation of emulsions which in turn destabilises the system. The mechanisms responsible are Oswald ripening, coalescence, flocculation, gravitational separation (creaming and sedimentation), and phase inversion, all usually leading to complete phase separation.[232] They are summarised in Figure 2.4.

Coalescence is an irreversible process that can take place when the electrostatic or steric forces that keep the droplets apart are overpowered over the attractive van der Waals forces between the droplets, and the layer covering the droplets isn't strong or viscoelastic enough for the droplet structure to stay intact.[233] This is achieved by thinning of the film surrounding the emulsion droplets and then

rapture. The result is that the droplets eventually will merge, and the overall oil droplet size will increase. In the present study increase in droplet size over time was taken as a sign of instability due to coalescence.

Flocculation, like coalescence, takes place when the short-range van der Waals forces are stronger than the long-range repulsive forces.[187] The droplets floc together reversibly (with no interfacial rupture). There can be two types of flocculation; depletion which is caused by an osmotic pressure gradient triggered by the exclusion of a non-adsorbed or depleting agent (ie a surfactant) from two droplets in close proximity, and bridging which is caused by the connecting of droplets at the interface, forming a bridge due to incomplete coverage or electrostatic interactions. Depending on the adequacy of the repulsive long-range forces, this could lead to gravitational separation and eventually to phase separation.

Gravitational separation in emulsions can occur when the density of the continuous phase of the emulsion is different from the dispersed, and although it usually is a reversible process, it could also result in complete phase separation.[234] Creaming takes place when the continuous phase is denser than the dispersed and usually occurs in O/W emulsions (as oil is less dense than water) whereas sedimentation usually takes place in W/O emulsions. The oil droplet size also plays an important role in the rate of gravitational separation as for instance larger droplets will cream faster than smaller droplets. Especially in O/W emulsions, industries use thickeners in the continuous phase in order to avoid creaming.[235]

Ostwald's ripening can take place in a system where the oil droplet sizes are variable, therefore in a polydisperse system. It is an irreversible process and it includes transport of matter from smaller oil droplets to larger ones.[236] It occurs due to the

solubility of material in the dispersed phase increasing as the oil droplet size decreases.[236]

Phase inversion is a process where the dispersed and continuous phases switch. It can either be traditional, meaning changing parameters that could affect a surfactant's HLB value such as additives or environmental factors such as temperature, or pH and could be reversed[237], or catastrophic, which can be caused by increasing the volume of the dispersed phase.

Depending on the type of emulsifier used, different destabilisation mechanisms can be observed. For instance, Pickering particles primarily stabilise emulsions sterically and do not always reduce the interfacial tension (depending on the material)[238]. Therefore, the resulting droplet size is usually larger compared to surfactant stabilised emulsions and can be more prone to creaming[239]. On the other hand, due to the rigid layers around the droplets, and can almost fully arrest coalescence, phase inversion and Ostwald ripening phenomena.[240]

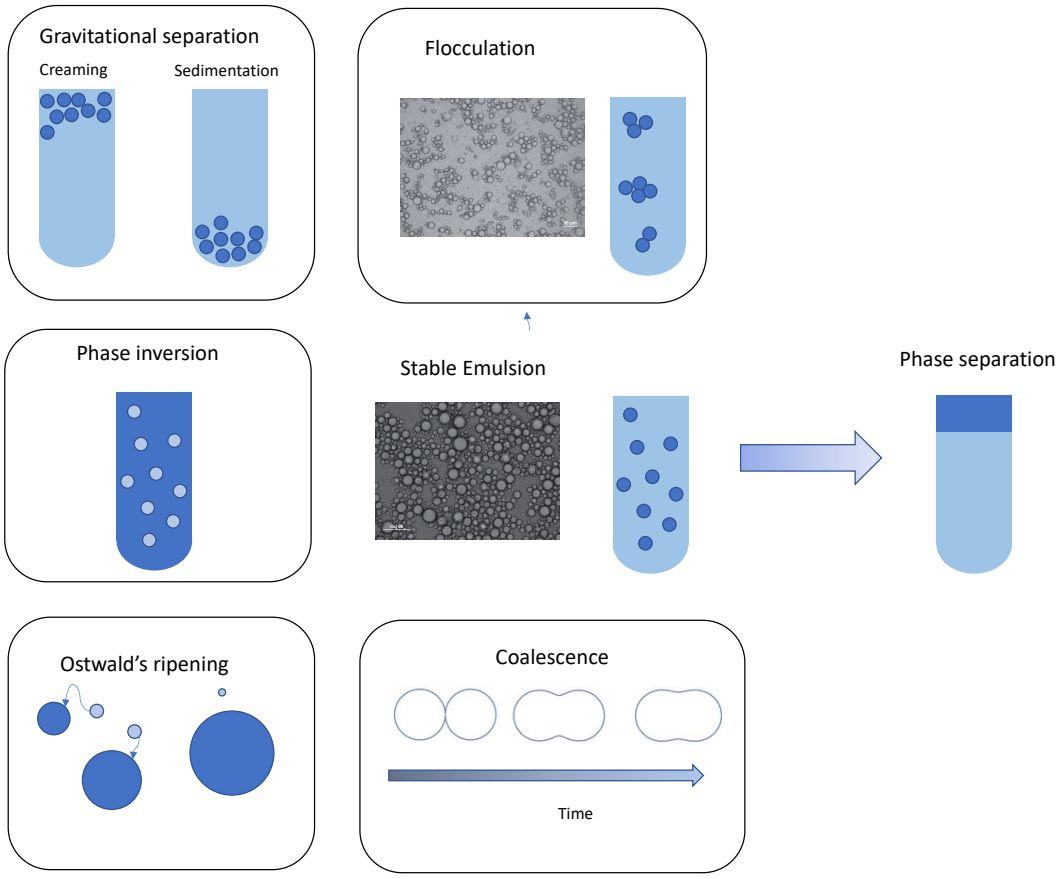

Figure 2. 4 Physical destabilisation that can occur in emulsions, adapted from McClements[232] and Dahiya et al.[241]

2.5.4 Emulsion oxidative stability

Lipid oxidation is a natural process that affects the quality of perishable goods particularly in the food industry. It contributes to the reduction of self - life as it is responsible for the deterioration of texture, smell and taste.[187] Free radicals generated by light, microbiological activity or chemical processes are responsible for lipid oxidation. The reaction involves the 3-step free radical mechanism: initiation, propagation, and termination. A brief description of the progress of lipid oxidation is given on Table 2.2.

Table 2. 2: Brief description of the lipid oxidation process, adapted from Schaich[242]

STEP	REACTION	Description
Initiation	$R_1H \longrightarrow R_1^{\bullet} + H^{\bullet}$	The initiation reaction is the first step where alkyl radicals are formed. This is caused by initiators such as free metals acting as catalysts, light, heat and more.
Propagation	$R_1^{\bullet} + O_2 \longrightarrow R_1OO^{\bullet}$ $R_1OO^{\bullet} + R_2H \longrightarrow R_1OOH + R_2^{\bullet}$	The propagation reaction is where the free- radical chain reaction commences. Once the alkyl radical is formed, it can react with oxygen to form peroxyl radicals and then lipid hyperoxides.
Termination	$R_1^{\bullet} + R_2^{\bullet} \longrightarrow R_1R_2$ $R_1OO^{\bullet} + R_2^{\bullet} \longrightarrow R_1OOR_2$	The termination reaction involves two reactive radicals coming together, forming new stable molecules.

There are plenty of factors than can encourage lipid oxidation, some of those being temperature, pH, interfacial area, lipid content and surfactant type, and are investigated and discussed in more detail in Chapter 5. An important element that influences lipid oxidation in emulsion systems is transition metal ions, the most common cause of oxidative instability.[243] They can enhance the oxidative process by reacting with the lipids on the O/W interface and producing highly reactive peroxyl and alkoxyl radicals that can then initiate the radical chain reaction.[243] The most important prooxidant metal is considered to be iron, more specifically ferrous which is even more reactive than ferric due to its higher solubility.[244] Therefore, the microstructure of the emulsion has a crucial role on lipid oxidation, for instance the thicker the protective layer around the droplets, the harder it would be for prooxidant metals to reach the oil phase and initiate lipid oxidation. In literature, surfactants that are primarily used to create thick interfacial layers, such as sodium caseinate[245] or whey protein isolate[246], and physically protect the lipid phase from oxidation, are also capable in preventing oxidation chemically for instance by chelating the prooxidant metals or scavenging free radicals.

2.5.4.1 Methods of monitoring lipid oxidation

A variety of different methods have been reported in literature to monitor lipid peroxidation for both primary and secondary oxidative products; a detailed review can be found by Abeyrathne *et al*.[247]. In the present study, the formation of primary and secondary oxidation products was investigated through spectrophotometric assays; a review of those is briefly described below.

2.5.4.1.1 Primary oxidation products

Primary oxidation products are considered to be the hydroperoxides produced from peroxyl radicals[248] showed in the propagation step in Table 1. There are three main techniques to detect primary peroxidation products: the ferric cyanate method (determining the peroxide value), the iodine value and the diene conjugation measurement[249]. In the present study the ferric thiocyanate assay was used where, detection of hydroperoxides spectrophotochemically involves the measurement of the presence of a ferric thiocyanate complex which is formed *via* the reaction of ferrous iron with hydroperoxides.

2.5.4.1.2 Secondary oxidation products

Due to the nature of the radical chain reactions, they can form a far larger amount of secondary products compared to primary oxidation. Depending on the compounds of interest the appropriate method can be used. A couple of methods widely used to determine secondary lipid oxidation products are the para-anisidine (p-anisidine) method and the thiobarbituric acid reactive substances assay (TBARS).[249] The method selected in the present study was the TBARS which measures the

appearance of a complex of malondialdehyde (one of the most common secondary oxidation products).[250]

2.5.4.2 Antioxidant additives

In order, to avoid or at least delay lipid oxidation the food industry utilises methods such as sealed packaging or the inclusion of antioxidant agents. Antioxidant agents can be natural (eg. Ascorbic acid[251]) or artificial (eg. BHA or BHT[252]). Artificial agents are often compared with natural additives in literature in order to establish alternative ways to oxidatively stabilise emulsions, with natural ingredients but with equal preserving qualities.[253],[254],[255]

2.5.5 Emulsion antibacterial stability

Antibacterial preservation of emulsions is crucial as due to their composition (water, oil or fats) they can promote growth of microorganisms.[256] There are some popular options of specific antibacterials used such as acetic acid, benzoic acid and or sorbic acid, all of them chosen based on the pH of the emulsion, the thermal processing and the compatibility of these additives with the other ingredients used[187].

Emulsions and especially nanoemulsions are believed to have an innate antibacterial activity stemming from the energy stored which was input into the system while it was formulated, to destabilise the cells' membrane, leading to cell lysis. [257] Kadri *et al*.[258] summarised reports on the antibacterial activity of nanoemulsions, correlating them with the emulsion droplet size, as well as testing nanoemulsions on a range of different bacteria. They concluded that experimentally the nanoemulsions did not exhibit an antibacterial activity against all the bacteria tested, although at the same

time they highlighted that there is a big discrepancy in literature concerning this topic and more research needs to be conducted.

2.5.6 Emulsion microstructure characterisation and behaviour

2.5.6.1 Characterisation of the emulsion interface

Characterisation of the interface is extremely important as it can give insights into differences between stabilisation elements (i.e., surfactants or particles) and their role in the emulsion microstructure. Two parameters are important to investigate, one being the ability of emulsifiers to decrease the interfacial tension and the other one being the appearance or thickness of the interfacial layer.

Interfacial tension can be measured with a variety of different techniques such as Wilhelmy plate, capillary rise or sessile drop method some of those being static and some dynamic, a review of those is provided by Zhou *et al.*[259]. In the present study the pendant drop method was used to measure the dynamic interfacial tension. It is one of the most frequently used techniques, it can be employed if there is sufficient density difference between the two phases and it can measure both surface and interfacial tension as well as dilatational rheology.[260], [261]

Dilatational or interfacial rheology can provide information of the viscoelastic properties of an interface and therefore could be related with the short-term stability of an emulsion. It can also be measured *via* the pendant drop method once a low amplitude is applied, and the response of the interfacial layer is determined. For example, Santini *et al*.[262] showed a correlation between increasing concentration of surfactant with better viscoelastic properties and increase emulsion stability. However, further work is needed to establish a relation with long-term stability of emulsions,

something that can be achieved if combined with other techniques such as shear interfacial rheology.[263]

Different microscopy techniques can also be adopted to characterise the interface such as fluorescence microscopy. Fluorescent dyes can be used to tag the compounds of interest and observe their placement in emulsion systems, as it is shown in Figure 2.5A, where rhodamine B, a fluorescent dye, was used to stain silica particles.[264] Some substances or particles are naturally fluorescent so can be observed without the aid of dyes.[265] Polarised light microscopy has also been utilised if the species of interest are in a crystal from (Figure 2.5B).[266] Scanning electron microscopy (SEM) can be used to observe emulsion systems in smaller scales, as the morphology of the layer can be studied (Figures 2.5C and D).[224]

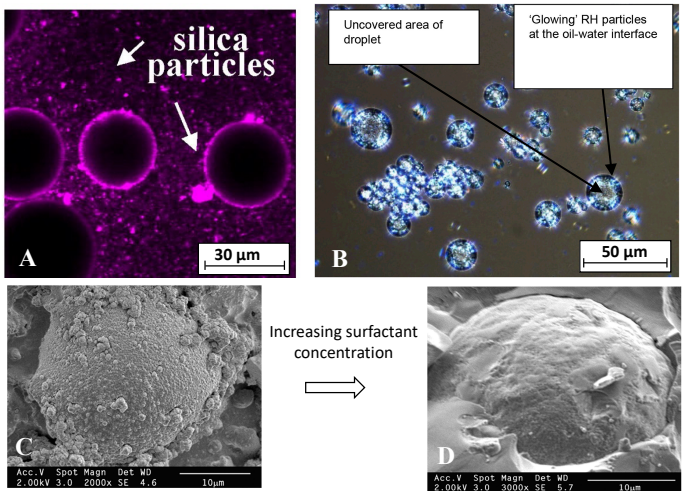

Figure 2. 5 A. Fluorescent microscopy of emulsions stabilised by silica particles dyed with Rhodamine B (adapted from Juarez et al.[264]), B. Polarised light microscopy of emulsions stabilised by rutin hydrate (adapted from Noon et al.[266]), C. emulsions stabilised with 1% silica particles and 0.4% Tween 60 and D. with 1% silica particles and 1.5% Tween 60 (adapted from Pichot et al.[224]).

2.5.6.2 Size measurement of the emulsion droplets

One important method to characterise emulsions and assess their stability over time is by measuring the oil droplet size. There are various techniques to achieve that, such as laser diffraction or dynamic light scattering (DLS).

The DLS technique takes into consideration the Brownian motion the dispersed phase tends to move in. As a laser beam passes through the sample, it is attenuated by the dispersant droplets according to their size and Brownian motion (as free diffusion), is taken into account in calculating their hydrodynamic diameter.[267] To describe the width of the distribution, the polydispersity index is also calculated. It is dimensionless and ranges from 0 to 1. The closer the value is to 0, the less polydisperse the sample is.

In laser diffraction, a laser beam passes through the sample, determining the dispersant's size by measuring the angular variation in the intensity of the laser, and the particle size distribution can be obtained.[267] The average size, $D_{(p,q)}$, from the distribution is then given by equation (2.2):

$$D(p,q) = \frac{\sum_1^n D_i^p \times v_i}{\sum_1^n D_i^q \times v_i} \qquad (2.2)$$

where v_i is the number of droplets with diameter D_i, and p and q are the powers selected to obtain the appropriate average.

Depending on the needs of the experiment, suitable values for the powers p and q need to be selected. If p is 1 and q is 0, then the number average will be calculated

If p is 2 and q is 1, then the length average is obtained. For p equal to 3 and q equal to 2 the surface and p equal to 4 and q equal to 3 the volume-based means. The more one increases the powers, the more the calculation favours larger droplet sizes. Surface- and volume-based means are the ones most commonly used.

From the laser diffraction method, the span of the distribution can also be determined. The value of the span gives an indication about how monomodal the size distribution is, which can be related with the stability of the emulsions. It can be determined *via* the equation (2.3):

$$Span = \frac{D_{90} - D_{10}}{D_{50}} \quad (2.3)$$

where D_{90} signifies that the size given is contained in up to 90% f the size distribution, D_{50} up to 50% and D_{10} up to 10%.

Both dynamic light scattering, and laser diffraction require information on the optical properties of the sample, since both methods are based on the ability of particles to scatter light once illuminated with a laser beam. In the case of dynamic light scattering only the refractive index of the dispersant is needed, as it is an intensity-based technique from a single angle measurement.[268] In laser diffraction, the refractive index of both the dispersant and the dispersed material are necessary, as it is a volume-based method and uses Mie theory to convert the light scattering data to volume, needing both parameters.[269] In both cases, if the refractive index of the samples is unknown it could lead to unreliable size determination.

Particle size can also be determined *via* imaging. Light microscopy has been widely used to assess the droplet size of the emulsions as well as determine any destabilisation mechanisms such as coalescence or flocculation. However, it is appropriate for larger oil droplet sizes, down until 1 μm.[187]

The technique used is dictated by the expected dispersed phase size as well as other parameters such as volume and concentration of the sample. DLS is used for from 1 nm to a few micrometers[270], while laser diffraction is preferred for samples 0.1-2000 μm.[270] It is important to emphasize that the two different techniques may give different results for the same sample as it was demonstrated by Staven *et al.*[271], therefore the methods are not directly comparable.

2.5.6.3 Rheology of emulsions

Rheology can be defined as the study of flow and deformation of a material.[272] Rheological measurements involve applying a force on the sample of interest and measure its flow or deformation. Viscosity is the internal friction of a fluid or a measurement of its resistance to flow.[273] It can be influenced by a variety of different parameters such as the composition of the material, the shear rate of the external force or the temperature.[274] With direct relevance to emulsions, it is an important tool to assess their rheological characteristics in terms of its texture and stability.[275] For instance, assessment of the viscosity of the continuous phase is important as it can determine whether gravitational separation will occur. Different parameters to study emulsions such as emulsion droplet size[276], viscosity of continuous or dispersed phase or incorporation of particles or actives can also alter the rheological properties of an emulsion.

There are multiple ways to measure the viscosity of a sample such as falling ball viscometers glass capillary viscometers etc.[275] In the present study, the viscosity was measured *via* a rotational test by inserting the sample in an appropriate geometry and commence rotation at a pre-set rotation rate which will steadily increase.

Viscosity measurements can be reported as a single value at a specific shear rate or the whole viscosity curve can be given. If the curve increases the sample presents a shear thickening behaviour whereas if it decreases, it is called shear thinning. If it stays steady throughout the measurement, then it possesses a Newtonian behaviour.

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Chapter 3- Propolis particles incorporated in aqueous formulations with enhanced antibacterial performance

Abstract

In recent years, the use of natural bioactives in food, pharmaceutical and cosmetic industries has emerged as a global formulation development trend. Although natural bioactives exhibit promising properties, they are also associated with chemical instability or poor aqueous solubility. One such bioactive with beneficial functionalities but limited industrial applicability within industry is propolis. The purpose of this study was to investigate means of enabling enhancement to the antibacterial activity of propolis-based aqueous formulations by increasing its surface area when suspended in water and encourage dissolution of active compounds in the carrier phase *via* ultrasonication. Dry propolis was firstly extracted from crude material and the effect of common carrier phases used for dissolution of propolis for antibacterial assays was investigated. Consequently, the extract was formulated into propolis sub-micron aqueous dispersions *via* direct ultrasonication. Processing time was varied, and all formed particles were characterised immediately after production in terms of size, polydispersity and zeta potential, and then again after a month-long storage period. Depending on the propolis content and the duration of sonication, particle sizes of 200 nm to around 1 μm were produced. When tested against *E. coli* cells, 15% propolis dispersions caused a bactericidal effect, which was sonication time and time of exposure dependent. Particles formed at the shortest sonication period (4 min) resulted in higher cell injury while those processed the longest (10 min) caused greater cell death and with AFM imaging, cell membrane alterations were confirmed. Chemically, for whole dispersions and carrier phases alone, free radical scavenging

activity and total phenol content were slightly enhanced at longer sonication times. Overall, the present work suggests that formulating propolis extract sub-micron aqueous dispersions *via* sonication enhances their antibacterial performance *via* a synergistic effect involving both their carrier and dispersed phases.

3.1 Introduction

In recent years there has been a movement to reduce the use of synthetic actives in food, pharmaceuticals and cosmetics. This largely stems from the consumers' conceptualisation of the term "natural", which ultimately leads to preferences towards products containing natural bioactives, as they are perceived to possess positive attributes such as being healthier or more environmentally friendly than synthetic equivalents.[1],[2],[3] Studies demonstrate the public's apprehension on being exposed to synthetic chemicals, concerns that may stem from misunderstandings related to the significance of the quantities used, despite the fact that these are closely regulated.[4] Consequently, more and more research is being directed towards natural bioactives shown to possess a wide range of functional performances that could be utilised within the context of formulated products. Some examples include curcumin which has been reported to possess antioxidant and anti-inflammatory properties[5], aloe vera which has been associated with antitumor/anticancer, anti-diabetic and anti-inflammatory properties [6] and is widely used among cosmetic products [7], and essential oils from oregano or lavender [8] that have been reported to exhibit antibacterial, antifungal and antiviral properties.[9]

Another versatile bioactive with multiple reported benefits is propolis or bee glue; a resinous mixture produced by *Apis mellifera* honeybees.[10] It consists of 50%

resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and other substances such as organic debris.[11] Propolis extracts have been reported to exhibit antibacterial activity against both Gram- positive (*S. aureus*[12], *S epidermidis* [13], *L. monocytogenes* [14], *S. typhimurium* [15]) and Gram- negative (*E. coli* [16], *P. aeruginosa* [17] and *K. pneumoniae* [18]) bacteria; with a less pronounced antibacterial action on Gram- negative [19]. In current literature, propolis extracts have also been associated with antifungal, antioxidant and anti-inflammatory properties.[20] In terms of applications, propolis has been used as a dietary supplement [21] ,in dental care[22], in dermatological creams and lotions [23] and in food industries it has been effective in preserving meat, fish and juice products.[24],[25]

Although the consistency of propolis varies depending on the local flora, season and climate, it is widely accepted that phenols and flavonoids are the main compounds responsible for its antibacterial activity.[26],[27] By being such a complex mixture, propolis has the advantage of providing a synergistic functional response that is stronger compared to that of its individual components alone.[28],[29] However, it is the level of heterogeneity that can cause issues such as variability in chemical constitution and functional performance (e.g. antibacterial activity), and its plethora of organic compounds that greatly diminishes its water solubility.[30],[31] Moreover, crude propolis needs to be purified prior to use as it contains contaminants and debris that are not functional nor safe.[32] This usually involves an extraction process, which itself can be highly variable depending on the solvent and method used. While efforts have been made to extract propolis' bioactive compounds in water [33] or oil [34], aqueous ethanol (a mixture of ethanol and water) tends to be the most effective solvent [35],[36].

Propolis can remain diluted in the extraction solvent for further investigation, or the solvent can be evaporated to recover the extracts as dry matter. The solid propolis extracts then can be incorporated within a formulation medium that amongst others should easily allow the realisation of the desired propolis functionalities in the final application. Solvents that effectively dissolve propolis extracts and tend to be used in literature (such as ethanol, methanol or dimethyl sulfoxide (DMSO)) [37],[17],[38], have limited industrial applications and can themselves give rise to an antibacterial response that is beyond the sole capacity of propolis; for instance some *E. coli* strains can be susceptible to DMSO.[39],[40]

To avoid such issues, water can be used as the formulation medium instead. However, literature on aqueous-based propolis systems and their antibacterial activity is much more scarce, since, although ideal for application purposes, water is not an efficient extraction solvent nor carrier/formulation medium for propolis.[25],[41] Alternative approaches to enhance the bioavailability of poorly water-soluble functional compounds have been reported in literature. *Basniwal et al.*[42] produced aqueous dispersions of curcumin nanoparticles that were shown to exhibit a similar or even stronger response against different cancer cell lines, when compared to curcumin dissolved in DMSO. Elsewhere, attempts to increase the bioavailability of quercetin in water have studied its inclusion within solid dispersions fabricated using a range of carrier compounds such as monoolein, polyvinylpyrrolidone and hydroxypropyl methyl cellulose, and utilising homogenization, solvent evaporation or freeze drying methods.[43],[44],[45] Finally, and with direct relevance to the presence study, Elbaz *et al.*[46] developed chitosan-based nano-in-microparticles carriers to facilitate the oral

delivery of propolis, enhance its aqueous solubility and bioavailability, enable its controlled release and enhance its anticancer activity.

The current work adopts the formulation engineering ethos of such studies and aims to investigate approaches that enable enhancement to the antibacterial activity of propolis-based aqueous systems. Initially, the study reveals that the use of ethanol or DMSO as carrier phases for the dissolution of propolis extracts, produces antibacterial responses beyond the capacity of the bioactive itself. Therefore, water was exclusively used as the carrier phase of crude propolis dry extracts which were formulated into sub-micron propolis aqueous dispersions *via* direct ultrasonication. Although sonication has been shown to offer advantages when employed as part of the propolis extraction process [47],[48] or to facilitate encapsulation (e.g. in β-cyclodextrin)[49], its use to promote functionality of the sole active directly within the final formulation/carrier medium, to the best of our knowledge, has not been previously explored. Here, sonication time was varied and all formed propolis particles were characterized in terms of size, polydispersity and zeta potential both immediately after production as well as over a month-long storage period. Sonication time and time of exposure were considered for their impact on the antibacterial activity (tested on *E. coli* cells) of all propolis dispersions and AFM imaging was selectively utilized to confirm potential damage to the cell membrane. All dispersions were tested for their free radical scavenging activity and total phenol content as means to gain further insight into their antioxidant activity and the causes of their antibacterial performance. Finally, the contribution to the overall bactericidal functionality arising by the carrier phase of the propolis aqueous dispersions alone, was investigated. Overall, the present work demonstrates that formulating propolis extracts into sub-micron propolis aqueous

dispersions can indeed enhance their antibacterial performance *via* a synergistic effect involving both their carrier and dispersed phases.

3.2 Materials and Methods

3.2.1 Propolis Samples and Materials

Crude propolis was collected from Fthiotida region, Greece during spring 2019, purchased from ANEL (Thessaloniki, Greece) and was stored in the dark at -20°C. Ethanol (Absolute,99.8%, analytical reagent grade), Nutrient agar (NA), Dimethyl Sulfoxide (DMSO) and Phosphate Buffered Saline (PBS) were purchased from Fisher Scientific (Loughborough, UK). Luria Bertani broth (LB), Tween 80, Propidium Iodide (PI), Bis-(1,3- Dibutylbarbituric acid) trimethine Oxonol (BOX), Folin-Ciocalteu reagent and gallic acid were purchased from Sigma-Aldrich Ltd (Gillingham, UK). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Trolox were purchased from EMD Millipore Corp. (Darmstadt, Germany). Sodium Carbonate was purchased from LP Chemicals Ltd (Winsford, UK).

3.2.2 Microbial culture

E. coli K12 (MG1655) cells were maintained on Luria Bertani agar at 4°C. In order to obtain cells in the stationary phase, a colony was transferred into 50 ml of Luria Broth, incubated at 37°C for 18 hours while shaking at 150 rpm. The concentration of overnight cultures was fixed at approximately 1.5 x 10^7 CFU/ml. Cells were centrifuged at 4600 *g* for 3 minutes (Eppendorf Centrifuge 5430, Germany) and washed thrice with PBS.

3.2.3 Preparation and characterisation of propolis samples

3.2.3.1 Propolis extraction

The method selected for propolis extraction was based on a literature review on standard methods for propolis research by Bankova *et al*.[50] with a few adjustments. Frozen propolis samples (Figure 3.1A were ground (using a commercial food grade grinder, MosaicAL) until a fine particle powder was obtained with particles in a submillimetre range as shown in Figures 3.1B and C. Extraction was carried out by mixing 30 g of propolis with 300 ml of 70% ethanol in water (i.e. a mass to volume ratio of 1:10 propolis to solvent) and stored at 25°C in dark for one week with occasional shaking.[51] The resulting tincture was centrifuged (Sigma 3K30, UK) at 8800 *g* and 4°C, and filtered through no 4 Whatman filter paper, in order to remove waxes and other non-soluble compounds.[52] This process was repeated for three consecutive days or until no visible precipitation was present, while the tincture was stored at 4°C. The solvent was evaporated under reduced pressure (approximately -90 kPa gauge) at 40°C (Buchi Rotavapor R-215, Switzerland) and the dry propolis extracts (Figure 3.1D) were obtained at approximately 20% yield. 25, 10, 5, 1 and 0.1 mg/ml of propolis dry extracts were diluted in 20% ethanol and 80% water for further testing.

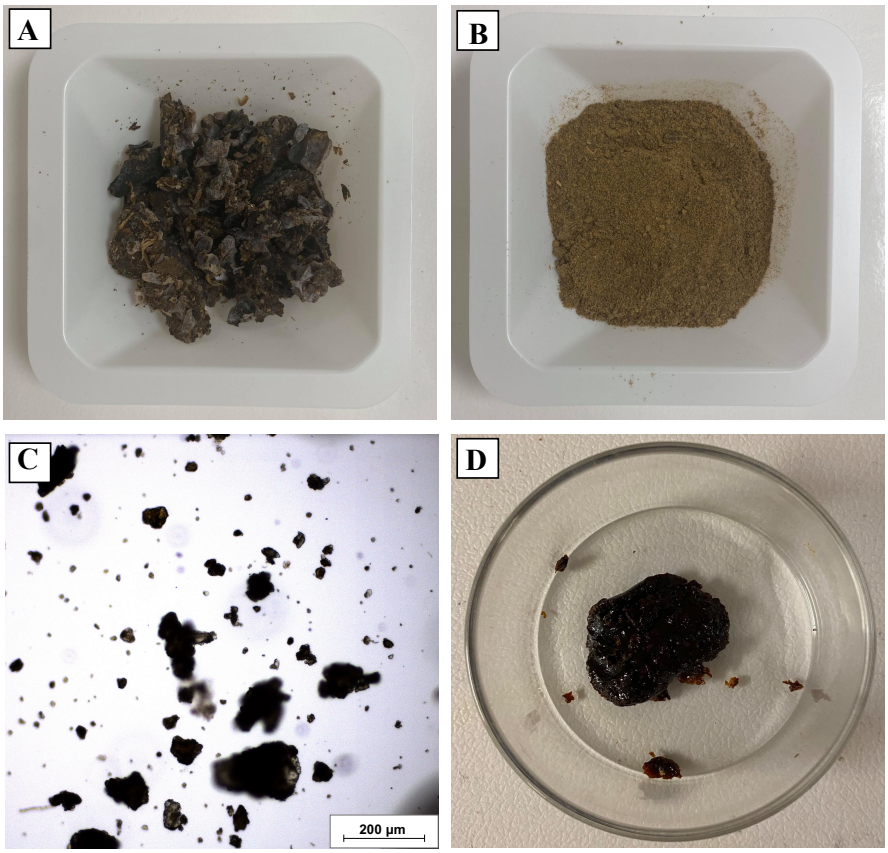

Figure 3.1:A) Frozen crude propolis, B) grinded propolis C) light microscopy image (10× magnification) of grinded propolis particles and D) propolis post extraction.

3.2.3.2 Preparation of propolis aqueous dispersions

An aqueous dispersion was made of 1.88% propolis dry extract (approximately 1-2 g), 1% Tween 80 and water. Tween 80 was selected as a non-ionic, small weight surfactant, typically used in foods as an emulsifier or dispersing agent. [53] The mixture was sonicated using a high intensity ultrasonic vibracell processor with a 13 mm probe (Sonics & Materials, Inc., CT, USA) for 2, 4, 6, 8 and10 min at 20 kHz, 750W and 95% amplitude. Pulse mode of 4 sec on 2 sec off was used to avoid heat buildup. Sonication lasting for half an hour up to 100 min, with a temperature range of 50-60 °C is reported in the literature with often increased efficiency for functional compounds of propolis.[54],

[55], [56] In the present study sonication lasted for up to 10 min in order to prevent any active compound degradation, with results not showing any decrease of propolis activity following longer sonication times.

An initial dispersion was made of 7% propolis dry extract (approximately 4-6 g), 1% Tween 80 and water and processed as described above. Part of some water content of the dispersion was evaporated under reduced pressure (approximately -95 kPa gauge) at 40°C, which resulted in dispersions with a higher propolis content of 15%. Dispersions of 4 and 10 minutes were diluted to 11.24%, 7.5%, 5.6% and 3.75% for further testing. A 15% propolis, non-sonicated, aqueous sample was also prepared for reference testing.

It should be stressed that the exact content of propolis ultimately retained within the formed aqueous dispersions (post sonication) used in this study differs from the amount of propolis extract initially added (prior to sonication). The discrepancy arises due to extract losses during the sonication stage where a fraction of the propolis was not possible to be dispersed; either precipitated at the bottom and/or adhered onto the walls of the vessel. A series of preliminary experiments have indicated this loss to be more or less consistent across the different propolis contents (and sonication times) used; amounting to ~1/3 of the initially introduced propolis extract mass. The authors have elected to retain the initial propolis content in the discussion that follows only because this represents a more unambiguous means to distinguish between different samples.

3.2.3.3 Isolation of the aqueous carrier phase of the propolis dispersions

The aqueous carrier medium was isolated from the propolis dispersion by ultrafiltration (Vivaspin 100kDa, Uppsala, Sweden) and was further tested separately. The filtration tubes were filled with 1.2 ml propolis dispersions and centrifuged for 30 min at 3900 *g* (Eppendorf Centrifuge 5810, Germany).

3.2.3.4 Size, polydispersity index and zeta potential measurements

A zetasizer Nano (Malvern Panalytical Ltd., U.K.) instrument was used to measure the particle size distribution (intensity, %) and determine the mean particle size and polydispersity index (PdI) using dynamic light scattering (DLS). Zeta potential was measured by electrophoretic mobility at room temperature. Samples were diluted 10 fold prior to measurement using ultrapure water (Milli-Q).

3.2.3.5 Total phenol content

Total phenolic content was determined using the colorimetric Folin-Ciocalteu method. The method was adapted from Kubliene *et al*.[35] The propolis dispersions and carrier phases were diluted 1:10 with distilled water and 77 µl were mixed with 77 µl of Folin- Ciocalteu reagent, 385 µl 20% sodium carbonate and 960 µl distilled water in order to make up a total of 1.5 ml. After 1 hour incubation in the dark at room temperature absorbance was taken at 760 nm using the Orion AquaMate 8000 UV–vis spectrophotometer (Thermo-Scientific, UK). Total phenol content was calculated as gallic acid equivalents (GAE) in micrograms per ml using a calibration curve ($y=0.007x-0.045$, $R^2=0.9997$), from a concentration range of 15.63- 250 µg/ml (Appendix 1, Figure A.1.1).

3.2.3.6 Free radical scavenging activity

The method was adapted from Jansen- Alves *et al*.[57] All dispersions and carrier phases were diluted 1:100 with distilled water and 100 µl were mixed with 100 µl 0.78 mg/ml DPPH reagent and 2.3 ml ethanol. Absorbance was taken after half an hour at room temperature in the dark at 517 nm using the Orion AquaMate 8000 UV–vis spectrophotometer (Thermo-Scientific, UK). Results were expressed as Trolox equivalents (TRE) in micrograms per ml using a calibration curve (y=-0.0041x+0.9624, R^2=0.9988), from a concentration range of 12.5-200 µg/ml (Appendix 1, Figure A.1.3).

3.2.4 Assessment of biological properties of samples

3.2.4.1 Antibacterial assay

The antibacterial activity was determined by using a dilution assay in a 96-well microplate format. Samples were mixed with the microbes in a 1:1 ratio, incubated at 37°C while at constant shaking at 150 rpm and plated at appropriate time points from 0 up to 24 hours.

3.2.4.2 Plate counting (CFU)

Viability of bacteria after treatment was assessed by serial 10-fold dilutions in PBS. 10 µl were measured and plated in Nutrient Agar plates and incubated overnight at 37°C. Colony Forming Units (CFU) were counted optically at the appropriate dilution (3-30 colonies) to determine cell viability. The lower limit of detection was 30 CFU /ml.

3.2.4.3 Flow cytometry

The viability of the cells and the physiology of their membrane was analysed using an Attune NxT, Acoustic Focusing Cytometer (ThermoScientific, Singapore). Samples were diluted with filtered PBS and stained with Propidium Iodide (PI) and Bis-

(1,3- Dibutylbarbituric acid) trimethine Oxonol (BOX) at final concentrations 5 µg/ml and 10 µg/ml respectively. PI binds to the nucleic acid but cannot permeate a healthy cytoplasmic membrane.[58] BOX is a lipophilic and anionic dye which binds with depolarised (damaged) cytomplasmic membranes. [59] In order for a cell to be significantly damaged and be characterised as "dead", detection of both dyes is needed. If there is only BOX dye detection that gives an indication of injury to the cell membrane. Samples were vortexed and incubated for five minutes in the dark. Cells were irradiated using a blue laser at 488 nm and the emitted fluorescence was detected through a 400 nm band- pass filter for both dyes. The trigger was set for the green fluorescence (550 nm) channel and data were acquired on forward versus side scatter. Untreated bacteria cells in PBS were tested as controls. The results consisted of percentages of live, injured, and dead cells that add up to 100%.

3.2.4.4 Atomic Force Microscopy

Atomic force microscopy (AFM) images of control and treated bacterial samples with the 10 min sonicated dispersions (15%), were acquired with a JPK NanoWizard II atomic force microscope (Oxford Instruments, UK) in dry condition on a 25 mm^2 p-type silicon wafer (Sigma-Aldrich, UK). The silicon wafer was initially cleaned with a CO_2 snow jet while being held on a hot surface at 300°C, followed by placing a suspension of 5 µl of *E. coli* (approximately 10^5 CFU/ml) in filtered PBS. Samples were allowed to air dry for 3-4 hours. Then they were rinsed with deionised water to avoid crystallisation of PBS. The images were acquired in tapping mode using NCHV-A cantilever (Bruker, UK) (T: 8 nm; L: 117 µm; W: 33 µm; f_0: 320 kHz; k: 40 Nm^{-1}).

3.2.5 Statistical analysis

Experiments were conducted at least in duplicate unless otherwise stated. Results are expressed as averages and error bars represented the standard deviation. Statistical analysis was performed using IBM SPSS Statistics software. Kaplan- Meier survival plots were used to compare the antibacterial action of the various formulations. They can be useful to easily detect the antibacterial advantage of a one formulation over another, as well as the magnitude of this action and provide an indication of the rate of killing.[60] The survival plots were constructed also using the IBM SPSS Statistics software.

Results were compared with 1-way ANOVA (Tukey's test) and 2-way ANOVA (Sidak's multiple comparisons test). Differences were considered significant at $p<0.05$.

3.3 Results and discussion

3.3.1 The impact of the carrier phase used to dissolve propolis extracts on the overall antibacterial performance of the systems.

Different quantities of propolis extracts ranging from 0.1 to 25 mg were dissolved in 1 ml of a carrier phase consisted of 20% aqueous ethanol solution and tested against *E. coli* cells in their stationary phase. PBS was used as the continuous phase for *E. coli* cells as it does not provide any nutrients for growth and is an appropriate medium for bacteria preservation.[61] Data were collected immediately after the active-containing formulations were added to the cells and following 24 hours of incubation.

Results show that at immediate contact, the highest concentrations of propolis (10 and 25 mg/ml) eliminated the population completely, while a dose dependent

bactericidal effect was prominent for the concentrations ranging from 0.1 to 5 mg/ml (Figure 3.2). However, after 24 hours of incubation, there was no population present for any of the samples, including the control (20% ethanol). Therefore, the antibacterial effect appeared to be dominated by the carrier phase.

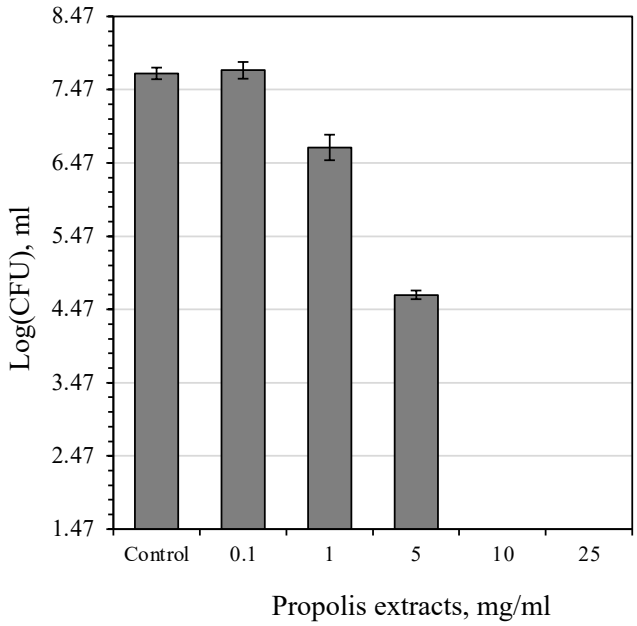

Figure 3.2 Antibacterial activity of propolis extracts dissolved in 20% ethanol. Data for population of stationary phase E. coli MG1655 are presented as a function of concentration of propolis extracts added (0.1, 1, 5, 10 and 25 mg/ml) after immediate contact. The control is 20% ethanol. Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

Flow cytometry was utilised to investigate the effects of the propolis extracts and carrier phase (20% ethanol in water) on the *E. coli* membrane potential and cellular integrity. Measurements were taken after immediate exposure (Figure 3.3A) and after 24 hours (Figure 3.3B). All propolis extracts concentrations caused depolarisation of the cell membrane (injury) and affected cell integrity (death), even after immediate exposure, further confirming the CFU measurements (Figure 3.2). The 20% aqueous

ethanol mixture alone (control) caused a significant amount of injury to the cell membrane (43%), at immediate contact. As the concentration of propolis applied increases, the amount of injury is maintained, while the total amount of cell death is increasing, showing signs of propolis' bactericidal activity. After 24 hours, the control and 0.1 mg/ml of propolis extracts exhibited similar antibacterial effects of low injured cells and a higher percentage of dead cells, whereas the rest of the samples mainly contained either alive or dead cells with low injury percentages. That could be due to the time effect which at high propolis extract quantities, results in eventual death of the previously injured cells, whereas for the control and 0.1 mg/ml propolis concentration, the rate of action is slower.

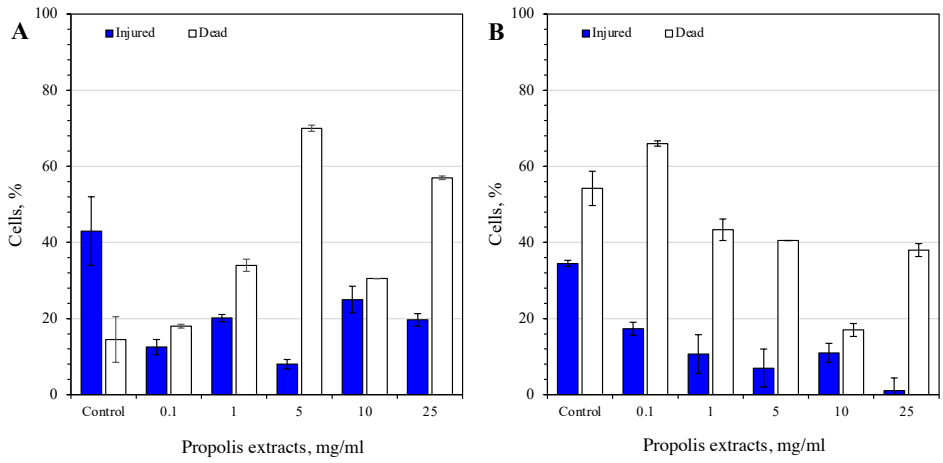

Figure 3.3 Effect of membrane integrity of E. coli (MG1655) cells A) after immediate exposure and B) after 24 hours with propolis extracts diluted in ethanol 20%. Data for percentages of injury and death are presented as a function of propolis extracts concentration (0.1, 1, 5, 10, and 25 mg/ ml). Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

It is apparent that ethanol, even at a relatively low percentage of 20%, provides an underlying antibacterial response. Ethanol, like other alcohols, is anticipated to cause damage to the cell membrane, denaturation of the proteins, followed by

inhibition of the cell metabolism and eventually causing lysis.[62] To further establish the antibacterial contribution arising from the carrier phase alone, a range of ethanol content (below the previously examined 20%) aqueous solutions were tested, while also investigating another popular carrier phase used in literature to dissolve propolis for antibacterial assays, DMSO (Figure 3.4). The lowest percentage of ethanol tested (5%) achieved two logs of *E. coli* CFU /ml reduction, showing the sensitivity of this method to antibacterial agents. After 24 hours of incubation, 100% DMSO completely eliminated the population and 20% DMSO achieved approximately one log CFU /ml reduction. DMSO has the ability to cross cell membranes [63], causing morphological changes in the cytoplasm [64]. However it should be noted that DMSO has been reported to protect *E. coli* cells by scavenging ROS (reactive oxygen species) caused by antibacterial agents ampicillin, kanamycin, oxolinic acid and ciprofloxacin [65].

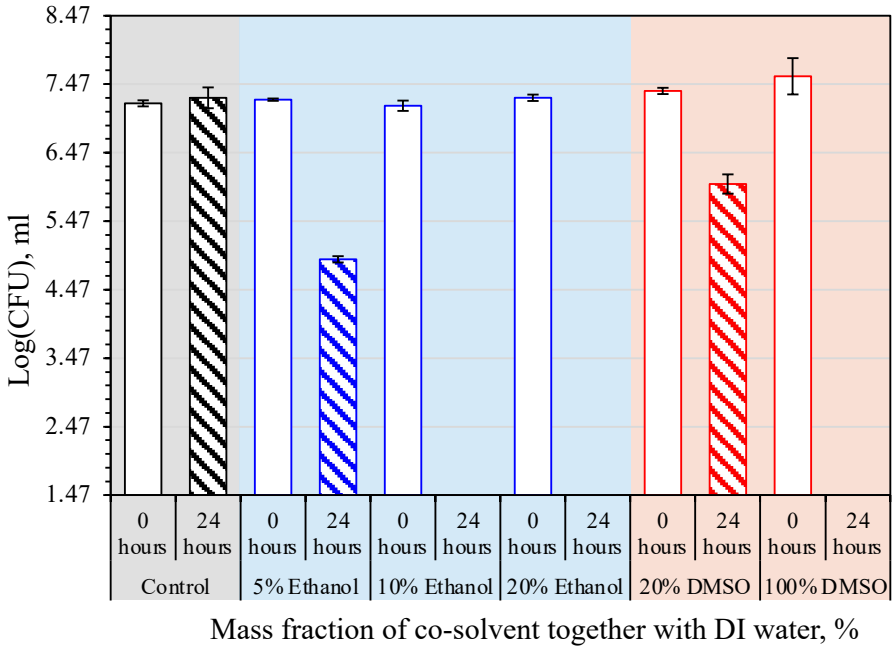

Figure 3.4: Antibacterial activity of different fractions of ethanol and DMSO mixed with deionised water. Data for population of stationary phase E. coli MG1655 are presented as a function of different percentages of ethanol and DMSO in water after immediate exposure and 24 hours. The control is

deionised water. Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

Although both carrier phases are widely used in literature for dissolving propolis extracts for MIC assays [13],[24],[66],[67], they are usually added in overall more dilute concentrations [68],[17],[69], to cells suspended in broth [70],[38],[67] which provides nutrients for the bacteria population to grow [66],[71],[38] rather than at stationary phase in PBS. Furthermore, during a 37°C incubation, part of the ethanol (particularly at such low concentrations) can evaporate, minimising/masking its antibacterial contribution. In the current method used, the actives to be tested are added in higher quantities (1:1) making their effect more prominent even at low ethanol concentrations (i.e. 5%). MIC assays show the minimum concentration needed to inhibit the growth of a microorganism [72], without being able to distinguish between static and cidal status. The current method was chosen as there was greater interest in the rate of killing and how that can be influenced under different formulation conditions while understanding the effects on the cell membranes of fully grown stationary cells. In addition to choosing a carrier phase that is relevant to industrial applications, the results presented here clearly demonstrate the importance of such selection in terms of anticipated antibacterial performance by the bioactive of interest, to disentangle any effects arising from the bioactive alone, thus reducing environmental interferences/contributions.

3.3.2 The effect of sonication on the formation of propolis extract dispersions within an aqueous carrier phase

In order to produce formulations with less toxic, environmentally friendly and more industrially applicable solvents, as well as to study the propolis' antibacterial performance without external interferences, water was selected as the carrier phase

for all further investigation. As propolis is insoluble in water, the hypothesis that its bioavailability and activity would be enhanced by formulating it as a colloidal aqueous dispersion, was tested.

Initially, aqueous dispersions of 1.88% of propolis extracts were formed in the presence of 1% Tween 80. The effect of the duration of the sonication step and the subsequent storage period on the particle size and polydispersity index (PdI) of the formed propolis particles were investigated (Table 3.1). The average particle size decreases with increasing sonication time, reaching a minimum at 6 min (206±1 nm), with possible aggregation after; this was also reflected in the PdI values, as it can be seen in the supplementary material provided (Appendix A1, Figure A1.1). What is more, all particle sizes remain constant with storage time.

Table 3.1: Average size and polydispersity index (PdI) of propolis particles dispersed in aqueous media. Data for aqueous dispersions containing a 1.88% propolis extract mass fraction are presented as a function of sonication time (2, 4, 6, 8 and 10 min) and storage period (7, 14, 21 and 30 days at 25°C in the dark). Data shown are averages (n=3; replicates) and errors represent ± one standard deviation (s.d.).

Sonication time (min)	Storage period (days)	Size (nm)	PdI
2	7	492±9	0.63±0.07
	14	565±55	0.54±0.01
	21	606±20	0.60±0.06
	30	727±42	0.54±0.03
4	7	333±1	0.24±0.01
	14	328±6	0.18±0.02
	21	393±8	0.31±0.01
	30	427±17	0.34±0.04
6	7	206±1	0.25±0.01
	14	209±2	0.23±0.01
	21	215±3	0.24±0.01
	30	218±2	0.23±0.01
8	7	239±1	0.36±0.01
	14	248±1	0.22±0.01
	21	251±2	0.23±0.01

	30	256±1	0.24±0.01
10	7	245±7	0.42±0.02
	14	230±3	0.32±0.02
	21	231±2	0.29±0.01
	30	227±4	0.27±0.01

Aqueous dispersions containing a higher propolis mass fraction of 7% were also prepared. These were further concentrated post sonication (by evaporating the appropriate amount of water) to form systems with 15% propolis content. The effect of the duration of sonication (> 2 min), water evaporation and period of storage on the formed propolis dispersions was investigated by measuring the average particle size, polydispersity index (PdI) and zeta potential; results are presented in Table 3.2. The average particle size between 1.88% and 7% propolis mass fraction was not statistically significant, but significantly increased with 15% propolis mass fraction ($p<0.05$).

One- way ANOVA of the average size measurements of the initial 7% propolis dispersions revealed no statistically significant differences between the sonication times. However, considering the PdI data, there is a significant increase in polydispersity after 6 min of sonication ($p<0.05$), compared with 4, 8 and 10 min sonication. This can be explained by observing the particle size distributions (Figure 3.5A). The 6 min distribution is multimodal, justifying the rise in average size.

Time of sonication, along with other parameters including sonication power or the diameter of the probe can affect the particle size.[73] This could lead to a reduction of size and particle agglomeration [74], which can be observed particularly in the 6 min distribution. It is challenging to compare these results with literature as sonication concerning propolis or other bioactives, appears to be used mostly as means of

extraction [75],[76],[77],[78] or encapsulation [49],[79], rather than creating dispersions. Kalogeropoulos *et al.* [49] formulated propolis extract suspensions in aqueous *β*-cyclodextrin solutions, but size measurements were not reported in this study.

Concentrating the propolis content in the dispersions *via* water evaporation (from 7% to 15%) reveals a consistent and statistically significant increase ($p<0.05$ for all sonication times). Particles move in a Brownian motion and tend to collide and flocculate [80], which can be further enhanced at smaller sizes due to the higher surface area created [81]. Although solvent evaporation is used as means to concentrate formulations [82],[83], aggregation can be a result [83] due to an increased likelihood of particles colliding. Therefore, the significant rise in particle size (Table 3.2) is expected and the same phenomena can equally explain the observed increase in polydispersity; also seen in Figure 3.5.

Comparing the particle sizes between 0, 7 and 30 days, in some cases a significant increase is observed for both 7% and 15% propolis. However, the standard deviations between measurements are too large indicating deterioration of samples.

Zeta potential was also measured as it is a good indicator of dispersion stability (while the pH of the formulation was not altered). Zeta potential values were all highly negative, in agreement with other propolis formulated systems,[84],[85] and remained mostly stable over 30 days of storage, with sample deterioration being accounted for some variability. In the 7% propolis systems, the zeta potential was not statistically significant between 8 and 10 min, due to the possibility of the sonication time not causing any effect after that. In the 15% propolis fraction systems (post evaporation) there was no statistically significant difference between 6 and 10 min, although the 8 min sonication was close. This could be occurring due to flocculation of the particles.

Two- way ANOVA showed a statistically significant difference of zeta potential values between 7% and 15% propolis fractions ($p<0.05$), with increasing zeta potential (towards less negative values) as the fraction of propolis was raised from 7% to 15%. This could be a result of particle agglomeration (post water evaporation; see Table 3.2); the subsequent reduction to the particles' surface area would be associated with a decrease in the 'overall' repulsion. This increase in zeta potential with increasing propolis amount is a subject of some disagreement in literature. Ramli *et al.* [86] studied the zeta potential with an increasing propolis (encapsulated in liposomes) concentration, reporting the same trend as here, although the authors claim this was due to interactions between the phospholipids and propolis. However, other studies [46, 87] have reported the opposite effect.

Table 3.2: Average size, polydispersity index (PdI), and zeta potential of propolis particles dispersed in aqueous media. Data for aqueous dispersions containing a 7% and 15% (concentrated) propolis mass fraction are presented as a function of sonication time (4, 6, 8 and 10 min) and storage period (0,7 and 30 days at 25°C in the dark). Data shown are averages (n=3; repeats) and errors represent ± one standard deviation (s.d.).

Sonication time (min)	Storage Period (days)	7% propolis dispersions			15% propolis dispersions		
		Size (nm)	PdI	Zeta Potential (mV)	Size (nm)	PdI	Zeta Potential (mV)
4	0	357±51	0.51 ±0.07	-26.85±2.62	928±162	0.71±0.01	-20.27±2.93
	7	250±26	0.19±0.03	-22.0±1.13	456±68	0.41±0.03	-28.76±7.44
	30	2799±2494	0.30±0.06	-24.99±4.96	462±3	0.39±0.05	-21.76±2.89
6	0	466±84	0.78±0.08	-41.24±4.22	1031±187	0.86±0.12	-30.12±2.32
	7	321±48	0.37±0.08	-38.08±4.13	3531±312	0.40±0.09	-29.11±3.46
	30	350±156	0.38±0.23	-38.84±1.13	3134±102	0.49±0.05	-31.07±1.07
8	0	330±47	0.39±0.03	-36.59±3.29	694±76	0.63±0.11	-33.09±2.69
	7	289±24	0.36±0.05	-36.11±1.35	2810±177	0.47±0.05	-30.28±1.49
	30	368±131	0.42±0.21	-38.11±0.69	2965±43	0.62±0.13	-30.26±4.44
10	0	347±24	0.48±0.07	-35.80±3.32	666±87	0.72±0.12	-26.84±3.51
	7	249±19	0.27±0.03	-37.87±2.20	455±50	0.61±0.05	-32.35±1.76
	30	3224±2279	0.60±0.23	-29.22±7.19	1116±745	0.62±0.21	-22.45±6.77

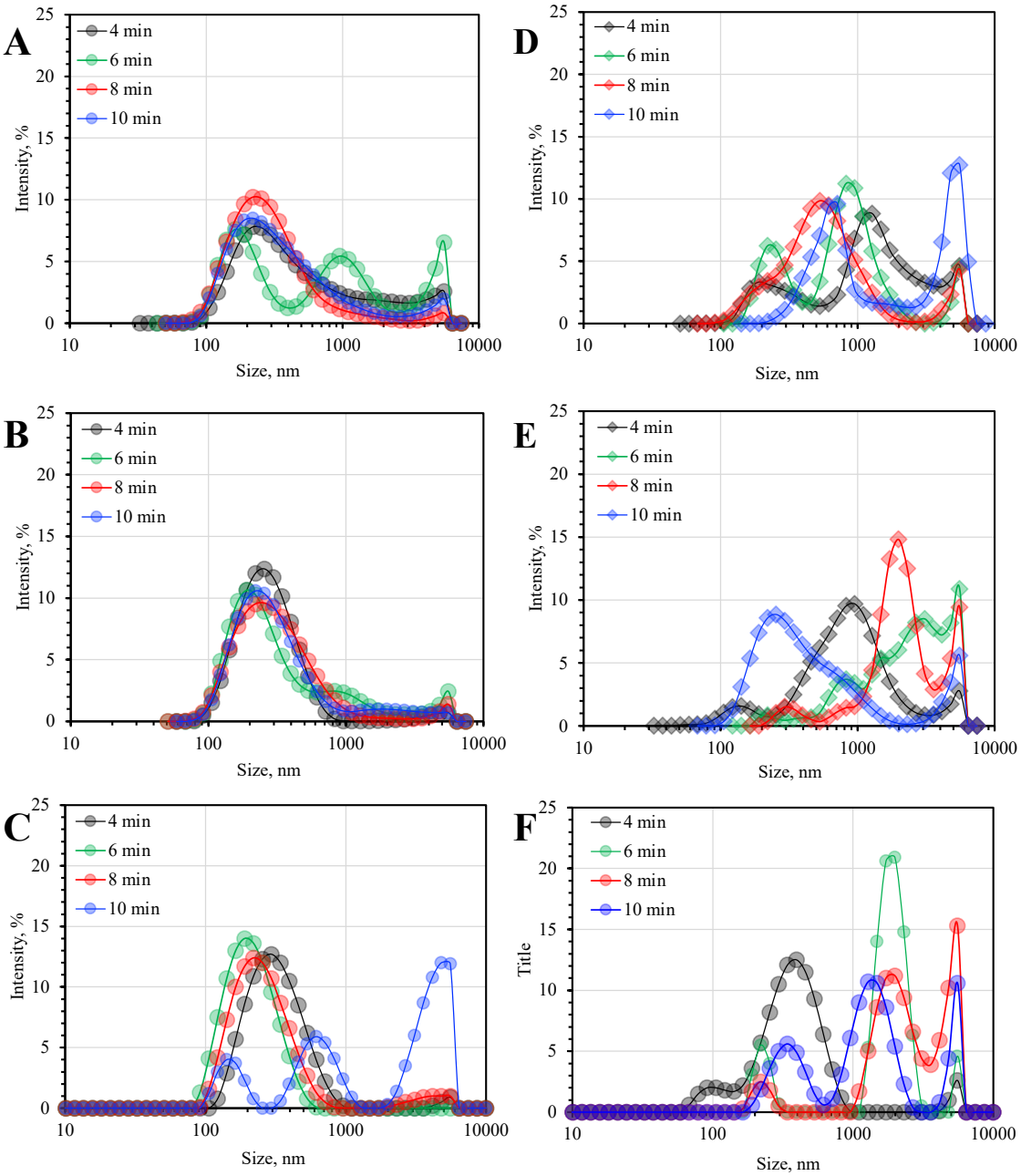

Figure 3.5 Average particle size distributions of propolis particles dispersed in aqueous media. Data for dispersions of 7% (A, B and C) and 15% (D, E and F) (concentrated) propolis mass fractions are presented for different sonication times (4, 6, 8 and 10 min) and storage periods; at 0 (A and D), 7 (B and E) and 30 (C and F) days, at 25°C in the dark. Data shown are averages (n=3; repeats), except results shown for 30 days which represent averages n=3, replicates due to deterioration of samples.

3.3.3 The effect of sonication on the content of 'relevant' chemical species in propolis extract dispersions within an aqueous carrier phase

Although the duration of sonication did not have a significant effect on the average particle size, further investigation was performed on its effect on the chemical composition of the formed propolis dispersions. The dispersions were characterised in terms of propolis extracts' functional compounds; total phenol content (reducing capacity), which can indicate if there are phenolic compounds present that could contribute to propolis' antibacterial activity (Figure 3.6A) and free radical scavenging activity, which is associated with propolis' antioxidant activity (Figure 3.6B).[88]

The data suggest that there were no statistically significant differences between different sonication times and the amount of phenolic compounds present. However, there is an increasing trend in the total phenol content of dispersions and sonication time from 4 min to 8 min (from ~1226 to ~3199 μg/ml GAE) or above (Figure 3.6A). This could be due to the increased forced dissolution of phenolic compounds in the aqueous carrier phase.

The total phenol content values although in some cases comparable [88], [89], were generally smaller than reported literature values [90], [91], [76], probably due to the fact that less quantity was used in the formulation from the total amount extracted or less was available for testing since the highest abundance when sampling, was not in solution. Other systems have shown that especially phenolic compounds are bound to the biological cell walls and sonication has assisted their release into solution resulting in the detection of larger phenolic amounts.[92],[93] However, in terms of propolis, the variability of the results is frequent, making it challenging to compare between different

samples, as the amount detected is not only related with the local flora or time of collection, but also with the degree of digestion by β-glycosidase from bees' saliva, and the percent of beeswax mixed with propolis.[38]

The free radical scavenging activity method can provide an indication of the antioxidant capacity using the stable free DPPH radical by the ability of an active to donate a hydrogen atom to the nitrogen radical. In terms of free radical scavenging activity, there was a statistically significant difference ($p<0.05$) between the 10 min sonicated dispersions and all the others tested (Figure 3.6B), indicating that the time of sonication influences the antioxidant capacity of the samples. The stability was adequate after 30 days, revealing that the storage period of samples did not affect their antioxidant activity.

Similarly with the total phenol content, the free radical scavenging activity was comparable with the literature [52],[38], although still lower probably due to less compounds being available in solution or due to the lower amount of propolis being used in the formulation.

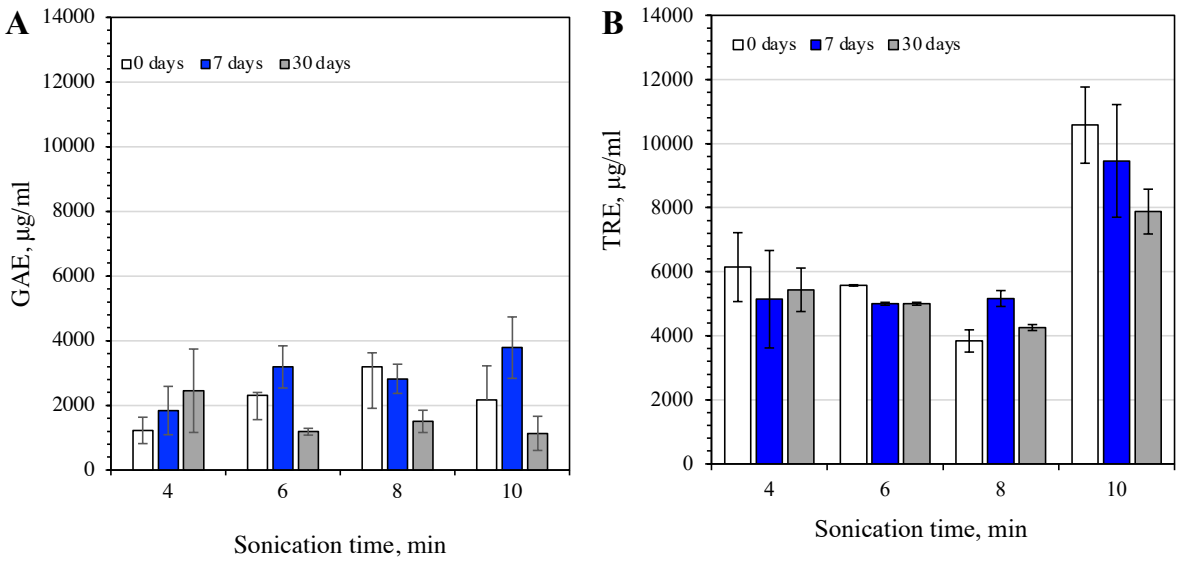

Figure 3.6: A) Total phenol content (in gallic acid equivalents, GAE) and B) Free radical scavenging activity (in trolox equivalents) of propolis particles dispersed in aqueous media. Data for 15% (concentrated) propolis mass fractions are presented as a function of sonication time (4, 6, 8 and 10 min) and storage period (0, 7 and 30 days at 25°C in the dark). Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

To distinguish between chemical compounds associated with the propolis particles and those in the carrier phase, the aqueous phase of the formed dispersions was isolated and examined further for its total phenol content and free radical scavenging activity Figure 3.7.

The total phenol content of the carrier phase (Figure 3.7A) showed a similar pattern to that of the full systems (Figure 3.6A); i.e. total phenol content increased for systems sonicated for 6 min and over, although once more the trend was not statistically significant. The phenol content detected in the aqueous carrier phase is lower than that in the full systems (i.e., ~2164 and ~850 μg/ml GAE for 10 min of sonication, respectively), an average ratio of 0.34±0.13 across all systems. This is expected as plenty of phenolic compounds, including flavonoids, found in propolis show a higher affinity for the solid phase,[94] were probably detected early on without further assistance of ultrasound energy, which could explain the non statistical significance of the results. However, there is evidence of sonication-assisted dissolution of functional compounds to the carrier phase. Campos *et al.* [95], compared the amount of phenolic compounds extracted in different fractions of water and ethanol, reporting that the percentage concentration reached a maximum when 1:1 ratio of ethanol and water was used, clarifying that some phenolic compounds can be partially water soluble.

When examining the full systems (Figure 3.6), there was higher variability of the results than their respective carrier phases. This high variability could be associated with the alteration of the physical properties of the dispersions that were evident from the size measurements of the propolis particles (Table 2), rather than from chemical degradation of functional compounds.

Sonication time although not statistically significant, in terms of free radical scavenging activity of the carrier phase (Figure 3.7B), presented a clear trend of increasing antioxidant activity with increasing sonication time (~880 and ~1587 μg/ml TRE after 4 and 10 min of sonication, respectively). The average ratio of compounds exhibiting free radical scavenging activity in the carrier phase over those in the whole dispersion was 0.19±0.06, showing that similarly with the total phenol content, free radical scavenging activity was primarily associated with the solid phase. There has been evidence in the literature that antioxidant activity could be correlated with the total phenol content which also agrees with our results.[38]

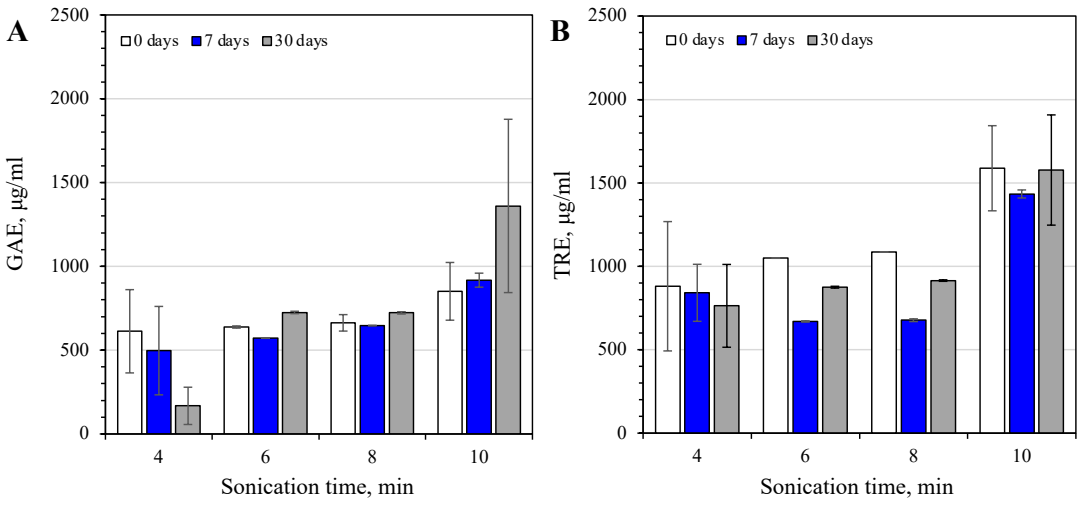

Figure 3.7: A) Total phenol content (in gallic acid equivalents, GAE) and B) Free radical scavenging activity (in trolox equivalents) of propolis aqueous carrier phases post isolation from dispersions. Data

for propolis carrier phases are presented as a function of sonication time (4, 6, 8 and 10 min) and storage time (0, 7 and 30 days at 25°C in the dark). Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

Sonication has shown to encourage dissolution, especially as part of an extraction set-up. Oroian *et al.* [54] investigated sonication as means of propolis extraction by altering parameters such as, ethanol and water concentration in the extraction media, temperature, amplitude and time, revealing that time did not have a significant effect on propolis extraction. That could be because the minimum amount of time tested (15 min) was already sufficient enough for complete extraction. In this study the maximum amount of sonication investigated was 10 min to avoid aggregation of particles or chemical deterioration of the sample. In different systems the duration of sonication has played a significant role in the antioxidant capacity and phenolic compounds dissociated in the carrier phase. Bhat and Goh [96] showed that a sonication time of 30 min increased significantly the total phenol compounds and free radical scavenging activity of strawberry juice. The present work suggests that sonication times between 8 and 10 min are enough to maximise the chemical species in the propolis extract aqueous dispersions, both in terms of total phenol content as well as free radical scavenging activity.

3.3.4 The effect of sonication on the antibacterial performance of propolis extracts within an aqueous carrier phase

The antibacterial response 1.88% and 7% propolis extract aqueous dispersions were tested against *E. coli* cells (Figure 3.8; showing the antibacterial response from the 10 min sonicated 1.88% and 7% propolis aqueous dispersion). The 1.88% propolis mass fraction did not reveal any meaningful effect. There have been reports in

literature where propolis extracts were not effective against *E. coli*. [97],[67],[98]. This is typically ascribed to an inadequate (low) propolis concentration or differences in chemical consistency depending on the location and the time of collection [99]. The 7% propolis mass fraction eliminated the population after 24 hours.

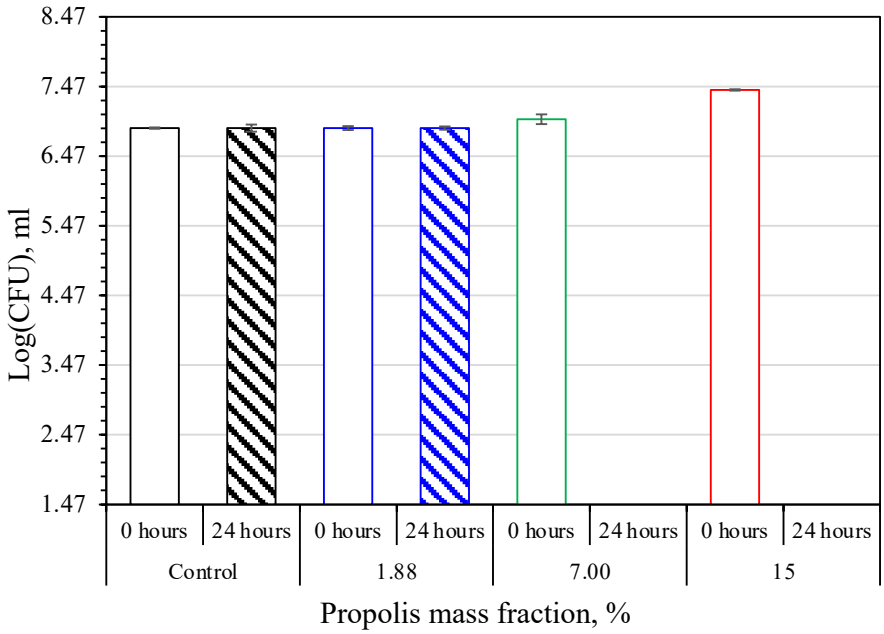

Figure 3.8: Antibacterial activity of propolis particles dispersed in aqueous media. Data for population of stationary phase E. coli MG1655 are presented as a function of propolis mass fraction (1.88%, 7% and 15%) and time (0 and 24 hours). The control is deionised water. Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

The antibacterial effect (over a 24-hour period) of the aqueous formulations with a higher propolis content (15%) was subsequently determined by exposing *E. coli* cells in their stationary phase to propolis dispersions formed at varying sonication times (Figure 3.9A). The non-sonicated sample exhibited a similar behaviour with the control, highlighting the major enhancement of antibacterial activity of the samples post sonication. The 10 min sonicated dispersions acted rapidly, eliminating the bacterial population within one hour. Both the 6 min and 8 min sonicated systems responded

similarly, eliminating *E. coli* population after 2 hours of exposure, while finally the 4 min sonicated dispersions managed the same after 8 hours of exposure

In addition, the data were fitted to a Kaplan-Meier survival plot model to estimate the percentage of survival of *E. coli* cells post exposure at each time point within the 24-hour testing period (Figure 3.9B). Unlike the particle size or chemical characterisation data, two- way ANOVA revealed a statistically significant ($p<0.05$) effect of sonication time on the antibacterial performance of the propolis extract aqueous dispersions.

There was no statistical difference between fresh samples and those stored for 30 days, with both data sets showing a practically equivalent antibacterial response. As such, this is hypothesised to predominantly arise by the preservation of compounds possessing antibacterial activity in the propolis aqueous systems and not to be affected by the deterioration of their physical characteristics (reported earlier in terms of particle size).

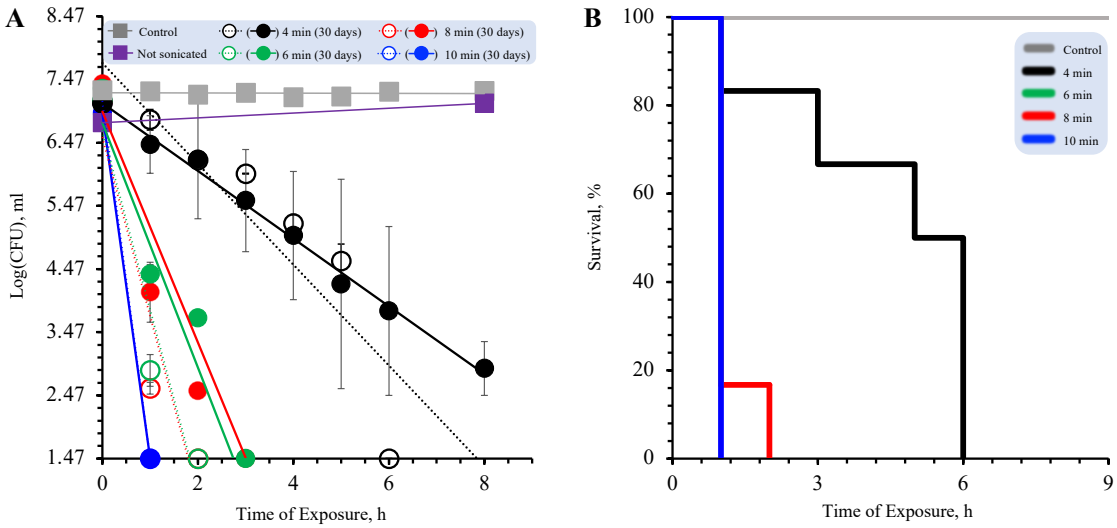

Figure 3.9: Antibacterial activity of propolis particles dispersed in aqueous media. Data for the population of stationary phase E. coli MG1655 are presented as a function of 15% (concentrated) propolis mass

fractions' exposure time, sonication time (4, 6, 8 and 10 min and not sonicated) and storage time (0 and 30 days at 25°C in the dark). The control is deionized water. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d). B. Kaplan-Meier survival plot of stationary phase E. coli MG1655 cells are presented as a function of 15% (concentrated) propolis mass fractions' exposure time and sonication time (4, 6, 8 and 10 min).

Propolis particles have previously shown to exhibit antibacterial activity. Dobrowolski *et al.* [100] suspended propolis granules and tablets in water with 1% acacia and tested them against *E. coli* exhibiting an inhibition zone of 13.8-15 cm after 30 minutes at a concentration of 10 mg/ml. Similarly, Abdullah *et al.* [101] suspended 20 mg/ml propolis particles in water which also exhibited antibacterial activity against *E. coli, B. subtilis, S aureus and P. aeruginosa* after 24 hours, even though the concentrations used in the present study were greater in comparison. Pobiega *et al.* [77] investigated the antibacterial effect of propolis extracts, extracted *via* different sonication times in 70% ethanol in water, showing that higher sonication times were related with stronger inhibition effects of the extracts against the organisms tested, agreeing with our results.

The antibacterial activity of the dispersions' carrier phases alone was also tested (Figure 3.10). However, the resulting antibacterial response was weaker compared to the full systems, but a similar pattern of action was revealed (Figure 10A); the obtained data were also fitted to the Kaplan-Meier survival plot model (Figure 10B). 2-way ANOVA showed once more that the differences in the antibacterial activities of the carrier phases of propolis dispersions sonicated for varying times were statistically significant ($p<0.05$). The carrier phases of propolis dispersions that had undergone 10 min sonication had the highest bactericidal effect, followed by those processed for 8

and 6 min (which had a similar effect), and lastly by 4 min where the effect was bacteriostatic.

Both whole systems and carrier phases showed statistically significant difference ($p<0.05$) in antibacterial activity with increasing sonication time. It is hypothesized that this enhancement is probably not anticipated from the solid phase but rather from an increased forced dissolution of functional compounds to the aqueous phase. In terms of the resulting antibacterial activity, both the colloidal solid propolis component and the aqueous carrier phase contribute, exhibiting a synergistic antibacterial action. Propolis particles need foremostly to come in contact with the cells in order to act [102], whereas functional compounds in the carrier phase are free to exhibit their antibacterial performance.

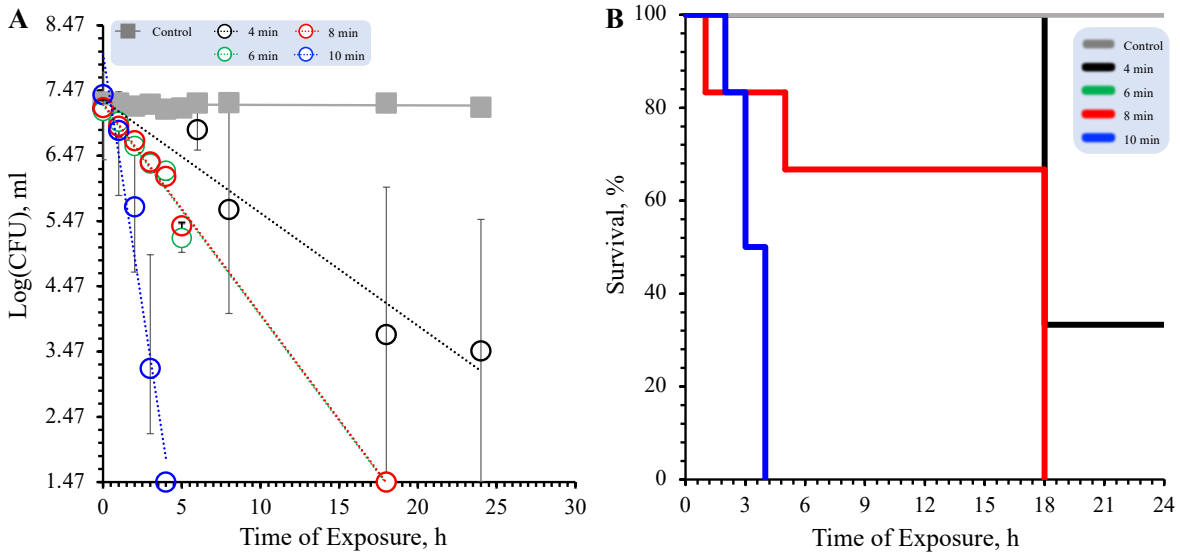

Figure 3.10A: Antibacterial activity of aqueous carrier phases post isolation from dispersions. Data for the population of stationary phase E. coli MG1655 are presented as a function of 15% (concentrated) propolis carrier phases' exposure time and sonication time (4, 6, 8 and 10 min). The control is deionised water. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d).

B. Kaplan-Meier survival plot of stationary phase E. coli MG1655 cells are presented as a function of 15% (concentrated) propolis carrier phases' exposure time and sonication time (4, 6, 8 and 10 min).

3.3.5 Effect of concentration of propolis extracts within an aqueous carrier phase on the membrane integrity of *E. coli* cells

Propolis dispersions were sonicated for 4 and 10 min and further diluted in water to different propolis mass fractions; these were taken to represent the two extremes in terms of processing intensity and were tested against *E. coli* cells in their stationary phase (Figure 3.11). The 4 min sonicated dispersions eliminated the bacteria population at the highest propolis mass fraction of 15%, and caused approximately one log CFU reduction at 11.25%. The 10 min sonicated dispersions eliminated the *E. coli* population at most propolis mass fractions apart from the lowest (3.75%) tested. This additionally confirms that the 10 min sonicated dispersions had a stronger antibacterial activity than the 4 min sonicated dispersions. In order to further understand the difference in the biological activity of the samples, flow cytometry was utilised to assess the level of injury and death of the cells, at immediate contact with the dispersions and at the same propolis mass fractions.

According to the flow cytometry data (Figure 3.12), the propolis extract aqueous dispersions that had undergone 4 min sonication predominantly caused injury (rather than death) to the *E. coli* cells. In this case there is a gradual (almost linear) rise to the injured cell population that follows the increase in the propolis extract mass fraction in the systems (Figure 3.12A). Cell death was both minimal and relatively independent of the propolis extract content (Figure 3.12B). However, the mode by which the propolis extract aqueous dispersions subjected to 10 min impacted cell integrity was much more severe. Here, although at the lowest propolis content, injury and death is brought

upon more or less equally across the cell population (Figure 3.12A), further increases to the propolis mass fraction resulted in an almost linear enhancement to cell fatality. What is more, for the higher propolis fractions tested, cell injury upon immediate exposure is significantly reduced while cell mortality dominates (Figure 3.12B). The fact that even the 15% propolis extract aqueous dispersions sonicated for 4 min did not significantly affect cell membrane permeability, provides further evidence that the propolis particles themselves are not the sole culprits in terms of antibacterial activity and that chemical species present in their aqueous carrier phases (dissolution of which is much more pronounced at higher sonication times) also play an important role. To the best of the authors' knowledge, this is the first time flow cytometry has been utilised to study the physiology of *E. coli* cells after exposure to propolis extracts either dissolved in ethanol (Figure 3.3) or formulated in an aqueous dispersion (Figure 3.12). Flow cytometry associated with propolis is commonly used to examine eukaryotic cells. [103],[104],[105]

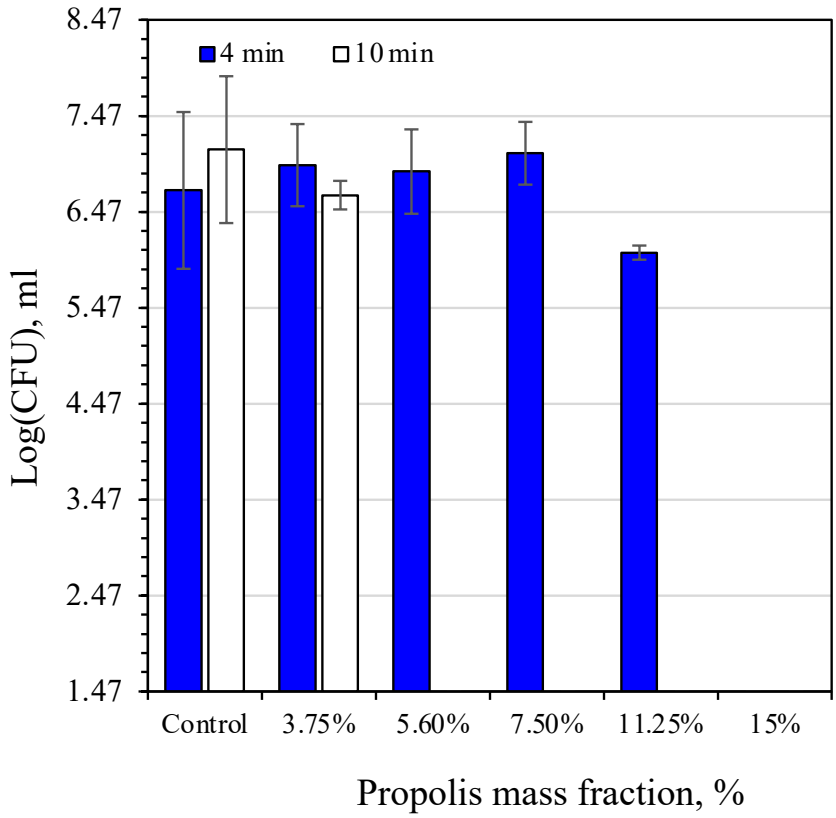

Figure 3.11: Antibacterial activity of propolis particles dispersed in aqueous media. Data for population of stationary phase E. coli MG1655 are presented as a function of 15% propolis mass fraction sonicated for 4 and 10 min and further diluted to 11.25%, 7.5%, 5.60% and 3.75%; after 24 hours of exposure. The control is deionised water. Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

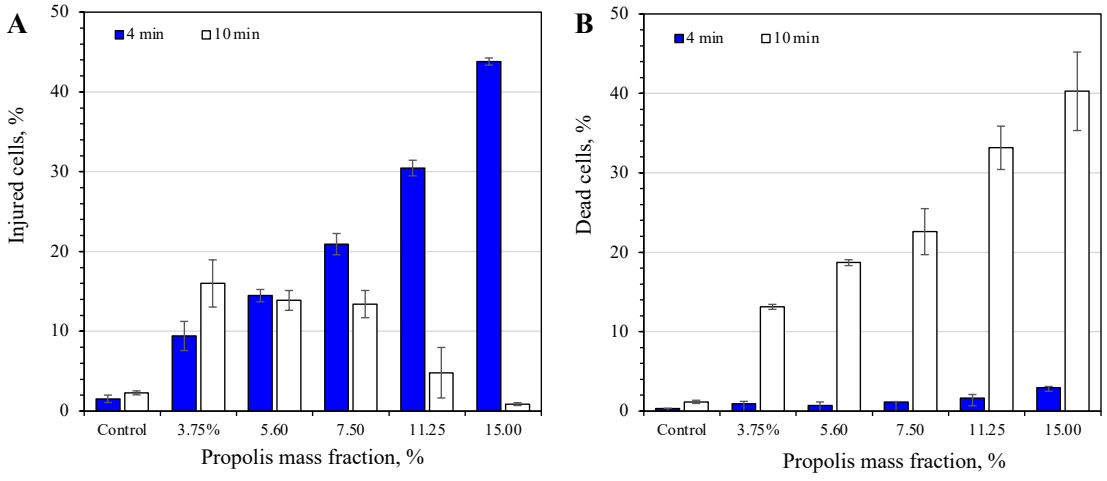

Figure 3.12: Effect of membrane integrity of E. coli MG1655 cells after immediate exposure with propolis particles dispersed in aqueous media. Data for percentages of A) injury and B) death are presented as a function of propolis mass fraction added (15%, 11.25%, 7.5%, 5,6% and 3.75%) and sonication time (4 and 10 min). Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

AFM imaging and topography were employed to detect the *E. coli* cell membrane disruption caused by the 15% propolis extract aqueous dispersions sonicated for 10 min (Figure 3.13). Cells A and B are examples of untreated bacteria with dimensions of around 250 nm height and approximately 1 μm width; these are similar to the previously reported dimensions for the same *E. coli* strain using AFM. [106] Following 1 hour of exposure to the propolis extract aqueous dispersion there was evidence of membrane alteration, with a shift in cell dimensions to around 3 μm in height and approximately 150 nm in width; evidence of membrane disruption can also be seen. This results in a higher cell volume and therefore indicating swelling possibly due to water absorbance by the compromised membrane. This way of propolis antibacterial action has been observed in literature with different strains. [107] Campos *et al.* [95] imaged *E. coli* and *S. aureus* cells after being treated with propolis extracts diluted in different concentrations of ethanol in water. The cells had ruptures in the cell membrane which caused leakage of intracellular contents along with swelling due to possible water absorption, agreeing with our results. The concentration of propolis extracts used at 137.5 mg/ml was higher compared to ours. Similarly, when *S. aureus* was exposed to magnetite propolis nanoparticles in Brain Heart Infusion broth, disruptions on the cell wall were observed, at a lower concentration of propolis extracts compared to ours. [108] To the best of our knowledge, is the first time cell swelling has been observed post propolis particles exposure, in an aqueous carrier phase.

Therefore, there are similar mechanisms of action for propolis extracts both in a solid form (particles) and diluted in a solvent. The antibacterial performance of propolis has been mainly ascribed to phenolic and flavonoid compounds. Molecules such as galangin, pinocembrin and chrysin, often found in propolis extracts, have been shown to act synergistically and compromise the outer membrane of cells. [109] Terpenoids, species that are more specific to Greek propolis samples, have also been associated with targeting the cell membrane. [110],[111],[112]

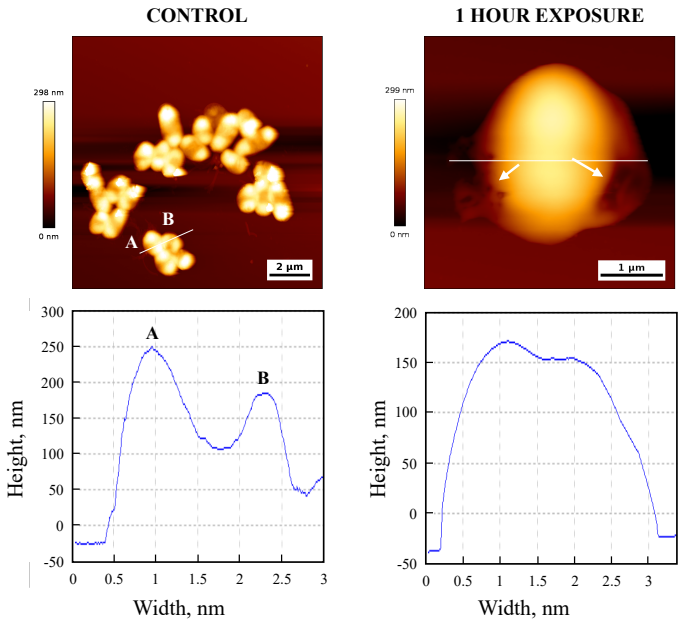

Figure 3.13: AFM images and topography of E. coli MG1655 cells. White lines represent the cross section where the height and width measurements were taken. Data for control (deionized water) and treated (15% propolis mass fraction sonicated for 10 min) cells are presented after 1 hour exposure. Cells within the white circles were used for measurement while the white arrows indicate potential membrane damage.

3.4 Conclusions

In conclusion, the use of sonication to formulate aqueous dispersions of propolis extracts has shown promise. DMSO and ethanol were inadequate carrier solvents for

investigation of antibacterial performance *via* the antibacterial assay used. Water based propolis formulations sustained propolis' antibacterial and antioxidant activity, diminishing any underlying effects. This study has demonstrated that utilising direct ultrasonication to formulate propolis aqueous dispersions, enables taking advantage of the ability to reduce the particle size and force dissociation of compounds in the aqueous carrier phase, resulting an enhanced bioactivity of propolis in an aqueous environment. Dispersions exhibited adequate physical and chemical stability and excellent antibacterial activity over the course of a month. Concentrating the dispersions resulted in agglomeration of particles, increasing the polydispersity. Assays gave an indication of the activity and antioxidant capacity of the formulations exhibiting similar results among different sonication times, with a trend of increasing total phenol content and free radical scavenging activity with increasing sonication time. Investigating the dissociation ability of direct ultrasonication *via* separating the aqueous carrier phase showed a high affinity of phenolic compounds and antioxidant capacity to the solid phase. Exploration into the antibacterial activity of the two phases showed synergism, revealing a stronger effect than when the isolated carrier phase was tested separately. The bactericidal effect was sonication and exposure time dependant. Combination of flow cytometry and AFM gave an insight into the formulations dose dependent antibacterial activity and its mechanism of action targeting the cell membrane.

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Chapter 4- O/W emulsions co-stabilised by propolis particles and surfactant or protein species

Abstract

Pickering stabilisation has been explored in depth as an advantageous way to stabilise emulsions. Recent trends towards the use of natural over synthetic actives, have also been extended to assess the capacity of such alternative colloidal particles to act as Pickering stabilisers in emulsion systems. In the present study, propolis particle aqueous dispersions, which have been shown to possess enhanced antibacterial and antioxidant activities in an aqueous environment, were used as the continuous phases of oil-in-water (O/W) emulsions. Propolis particles alone were not able to produce stable emulsions, but co-stabilisation in the presence of surfactant or protein species proved more advantageous. Emulsion stability was investigated, for different oil and co-stabiliser fractions, as well as for different oil droplet sizes. The role of the propolis particles in the interfacial stabilisation of the emulsions was studied by dynamic interfacial tensiometry and contact angle measurements and imaged using fluorescent microscopy. It was revealed that propolis particles exhibit some affinity for the emulsion interface (Pickering-like functionality) and thus can provide stabilisation in tandem with low concentrations of a co-stabiliser, even though some displacement of the particles was observed. Emulsions exhibited adequate stability over the course of two months, dependant on the storage temperature, pH environment and co-stabiliser used. Ultimately, the choice of co-stabiliser was shown to be crucial in terms of emulsion stability as well as for the preservation of the antibacterial activity provided to the systems by the presence of the propolis particles.

4.1 Introduction

Consumer preference towards products that utilise natural over synthetic active species, has been generating significant industrial interest. As a result, a range of natural actives possessing a multitude of functionalities (including antibacterial or antioxidant) has been investigated. However, natural actives can be prone to oxidation, can alter the end product's sensory qualities and if being lipophilic, can be less bioavailable. Formulating/introducing these in emulsion systems, could provide protection, enhancement of bioavailability and even mask any change in the organoleptic properties of the foods.[1]

One such bioactive is propolis, a complex resinous mixture produced by bees, with a variety of functionalities such as antibacterial, antioxidant and antifungal.[2] With active compounds originating from plants and exudates, the consistency of propolis' functionality relies on the local flora and season.[3] Propolis consists of over 300 compounds, with flavonoids and polyphenols mostly being responsible for its multiple beneficial functionalities.[4] However, in order for propolis to be incorporated in formulations, one has to account for the phenolic and flavonoid compounds' lipophilic character, and their unpleasant sensory features.[5] In literature, propolis has been added in formulations (including emulsions) for the purpose of increasing its bioavailability[6], masking odour or flavour[7], increasing nutritional value[8] or even as a building block to develop solid lipid nanoparticles for encapsulation and delivery purposes[9]. Nonetheless, available research on propolis emulsions seems to focus on the encapsulated capacity achieved,[10],[11],[12] and there is much less emphasis on the microstructural support that propolis can offer to emulsions. Within propolis, phenolic compounds like quercetin[13], and flavonoids such as rutin[14] have been previously

shown to provide Pickering stabilisation, and thus propolis colloidal particles themselves could play a role in terms of maintaining/supporting an emulsion microstructure.

Pickering stabilisation in emulsions has been reported to be advantageous due to the capacity of colloidal particles to form a strong interfacial layer, offering high resistance to destabilisation mechanisms such as coalescence, Ostwald ripening or coagulation.[15] The performance of Pickering stabilised emulsions could be further improved in the presence of surfactants (co-stabilisers).[16] When incorporated in the same system, the surfactants can lower the interfacial tension and encourage droplet breakage, while the particles arrange themselves around the droplets to provide (stronger) long-term, stability.[17] Such co-stabilisation approach has also been shown to be beneficial in terms of preventing lipid peroxidation[18],[19] and enhancing the antibacterial activity[20],[21] of emulsions, albeit these have been scarcely shown for particulates from natural sources in food applications[22].

The present work aims to investigate the microstructural role that propolis colloidal particles can offer to O/W emulsions, whether this can be further augmented or assisted by co-stabilisation strategies and whether the adoption of such microstructural responsibility is to the detriment of propolis' inherent antibacterial capacity. Propolis aqueous dispersions, with the antibacterial capacity that has been previously established by the same authors[23], were used herein as the continuous phases for the production of a range of O/W emulsion systems. Initially, the interfacial tension lowering capacity and the wettability of propolis particles were assessed in order to establish the magnitude of their affinity for the oil/water interface. O/W emulsions co-stabilised by propolis particles and surfactant or protein species were

produced, and their microstructure and stability were assessed as a function of propolis, co-stabiliser and oil content and oil droplet size. The effect of the type of co-stabiliser was investigated by comparing a non-ionic, low molecular weight surfactant with a positively charged protein at the propolis dispersion's native pH; Tween 80 and sodium caseinate, respectively. Tween 80 and sodium caseinate were chosen as both have been used extensively in food, pharmaceutical and cosmetic applications, and their stabilisation mechanisms in an oil- water have been well established in literature.[24],[25],[26],[27]. All emulsions were assessed in terms of thermal stability over the course of two months and imaged using light and confocal fluorescence microscopy. The current study concludes that propolis particles can indeed provide microstructural support in emulsions with the aid of a co-stabiliser, the choice of which however is proven to be crucial for this dual functionality to be successfully exhibited.

4.2 Materials and methods

4.2.1 Propolis Samples and Materials

Crude propolis was collected from Fthiotida region, Greece during spring 2019, purchased from ANEL (Thessaloniki, Greece) and was stored in the dark at -20°C. Ethanol and methanol (Absolute,99.8%, analytical reagent grade), Nutrient agar (NA), and Phosphate Buffered Saline (PBS) were purchased from Fisher Scientific (Loughborough, UK). Luria Bertani broth (LB), Tween 80, Sodium caseinate, Sodium hydroxide (1M) and Hydrochloric acid (1M) were purchased from Sigma-Aldrich Ltd (Gillingham, UK). Sunflower oil was purchased from a local supermarket.

4.2.2 Microbial culture

E. coli K12 (MG1655) cells were maintained on Luria Bertani agar at 4°C. In order to obtain cells in the stationary phase, a colony was transferred into 50 ml of Luria Broth, incubated at 37°C for 18 hours while shaking at 150 rpm. The concentration of overnight cultures was fixed at approximately 1.5 x 10^7 CFU/ml. Cells were centrifuged at 4600 *g* for 3 minutes (Eppendorf Centrifuge 5430, Germany) and washed thrice with PBS.

4.2.3 Preparation and characterisation of propolis and control samples

4.2.3.1 Preparation of propolis particle aqueous dispersions and isolated carrier phases

Propolis extraction and preparation of the dispersions and the isolation of the carrier phase is described in previous publication[23]. In the present study propolis particle aqueous dispersions at 15% propolis mass fraction were prepared without surfactant, with 2% Tween 80 and with 2% sodium caseinate and the carrier phases were isolated.

4.2.3.2 Dynamic Interfacial tension

The interfacial tension between sunflower oil and ultra-pure water, 1 and 2% Tween 80, 1% Sodium caseinate at pH 4.6 and 7, propolis particle aqueous dispersions (at 1.87, 3.75, 11.25 and 15% propolis mass fraction) and their isolated carrier phases produced with Tween 80 or sodium caseinate and propolis particles aqueous dispersions and carrier phases made without surfactant were measured. 15% propolis dispersions with increasing Tween 80 % was measured but the interfacial tension dropped to the minimum interfacial tension value that can be detected by the

software (results are presented in Appendix 2, Figure 2.1) A profile analysis tensiometer (PAT-1M, Sinterface Technologies, Berlin, Germany) at 20°C, where the measurement involves of fitting the image of the drop profile by the Gauss- Laplace equation, and the temperature as well as the densities of the two liquids need to be imported. The equipment was fitted with a straight stainless-steel capillary (3 mm outer diameter) to create a pendant drop (15 mm^2 cross-sectional area) of the aqueous phase in a sunflower oil containing quartz cuvette. Dynamic interfacial tension data were recorded for 3000 seconds, although some samples did not reach equilibrium. Both oil- and water-based phase density values were determined to carry out the data analysis by the tensiometer software, utilising a force tensiometer (K100, Kruss GmbH, Hamburg, Germany) and a solid measuring probe (2.33 g/cm^3). All water-based density measurements were identical to water, giving a value of 0.997 g/cm^3, and the density of sunflower oil was 0.916 g/cm^3.

4.2.3.3 Particle size, polydispersity index and zeta potential measurements

A zetasizer Nano (Malvern Panalytical Ltd., Malvern, UK) instrument was used to measure the particle size distribution (intensity, %) and determine the mean particle size and polydispersity index (PdI) using dynamic light scattering (DLS), of the propolis particle dispersions formulated without the aid of surfactant. Zeta potential of the propolis aqueous dispersions, 1% sodium caseinate and a mixture of both was determined by electrophoretic mobility at room temperature. Samples were diluted 10-fold prior to measurement using ultrapure water (Milli-Q). To investigate the effect of pH on the zeta potential, a pH meter (Mettler Toledo, Leicester, UK) was used along with HCL (1 M) and NaOH (1 M) as titrants.

4.2.3.4 Contact angle measurements

Contact angle *θ* measurements were taken using a goniometer EasyDrop from Kruss, Germany. The sessile drop method was used to calculate the contact angle. Propolis extracts (solid material) were dissolved in methanol and layered on a glass slide. When the glass slide was covered, the methanol was allowed to evaporate overnight while being placed in a half-opened petri dish to avoid contamination. Ultra pure (Milli Q) water and sunflower oil were loaded in a syringe (Hamilton 1750 TLLX with stop, 500 µl) equipped with a needle diameter 0.5 mm. A drop of aqueous or oil phase (16 mm^2) was placed on the propolis covered slide and the contact angle was measured by the Drop Shape Analysis software (Kruss, Germany), using Young's equation. A schematic representation is showed in Figure 4.1.

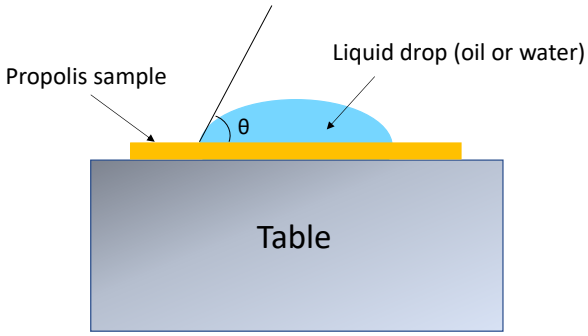

Figure 4.1A.: Schematic representation of contact angle *θ* measured with the goniometer.

4.2.3.5 Viscosity measurements

Viscosity measurements of the propolis aqueous dispersions at 15, 11.25, 7.5 and 3.75% propolis mass fraction, and water, were performed using a Kinexus Pro Rheometer (Netzsch GMBM, Selb, Germany), with a parallel 50 mm sandblasted plates using a shear rate profile from 1 to 100 s^{-1} at 20°C. To assess the shear viscosity profiles of the samples, the viscosity curves were fitted to a power-law model[28] presented in equation (4.1):

$$\eta = K\gamma^{n-1} \quad (4.1)$$

where η is the viscosity, K is the consistency constant and n is the power law index. If n is equal to 1, then the sample is exhibits Newtonian behaviour. If it is less than 1 it is shear thinning and if it is greater than 1, shear thickening.

4.2.3.6 Formulation of propolis and control emulsions

Oil-in-water (O/W) emulsions were prepared including either 15% or 25% sunflower oil phase and 84% or 74% aqueous phase (containing the aqueous particle propolis dispersions or just water in case of the controls) and 1% Tween 80 or sodium caseinate. Two processing methods were employed; using a high shear mixer (HSM) (Silverson L5M, Silverson Machines Ltd, UK) with an emulsion screen of 25 mm diameter for 2.5 minutes at 7500 rpm, and a high intensity ultrasonic vibracell processor with a 13 mm probe (Sonics & Materials, Inc., CT, USA) for 30 seconds with having a pause for two seconds every four seconds. 100-150 ml beakers were used. Firstly, 1% Tween 80 or sodium caseinate were dispersed in water and left to stir for

10 min. Then, the oil phase was included, and the samples were placed under the Silverson rotor or sonication probe. The resulting emulsions were either analysed directly postproduction or stored at 4°C and 25°C until further analysis.

4.2.3.7 Oil droplet size analysis

Propolis and control emulsions' average droplet size was obtained using a Malvern Mastersizer MS 2000 (Malvern Panalytical Ltd., Malvern, UK), using a Hydro SM manual volume sample dispersion unit. The refractive index values required by the software to determine the droplet size were 1.33 and 1.467 for water and sunflower oil respectively. Mastersizer utilises laser diffraction which is a volume-based technique; *via* detecting the light scattered and using Mie theory the particle size diameters can be obtained. The sample was dispersed in distilled water at 1300 rpm until an obscuration value of 4-5% was achieved. Surface (Sauter, $D_{3,2}$) and volume (de Brouckere, $D_{4,3}$) weighted mean diameters were obtained along with the span value. Emulsions were tested upon production and at appropriate time points, at 4°C and 25°C storage.

4.2.3.8 Light microscopy

Control and propolis emulsions were examined using an optical microscope (dM 2500 LED, Leica, at right after production and at 60 days while being stored at 4°C and 25°C, at 40× magnification. Results are presented in Appendix A2, Figures A2.3, A2.4, A2.6, and A2.8.

4.2.3.9 Confocal fluorescence laser scanning microscopy

Propolis emulsion samples were imaged using a Leica DM2500 confocal microscope (Leica, CH). Objective lenses of 40× and 63× magnification were used with

immersion oil. A laser at 408, 488, 532 and 634 nm wavelength was used at 100% intensity to excite the samples and emissions at the range of 200-800 nm wavelength were detected for imaging.

4.2.3.10 Creaming index (CI)

The creaming index of the emulsions was measured after emulsions were stored at 4°C and 25°C for 24 hours. A cylindrical container of dimensions 30x68 mm was used. An identical empty container was weighted, and then filled up with water to match the serum layer, and then the weight was taken. Then it was filled up to match the creaming layer and the weight was recorded again. The creaming index was determined according to equation (4.2)[29]:

$$Creaming\ Index = \frac{Serum\ weight}{Total\ Emulsion\ weight} \quad (4.2)$$

Results are presented in Appendix A2, Figures A2.2, A2.5, A2.7 and A2.10.

4.2.4 Assessment of biological properties of samples

4.2.4.1 Antibacterial assay

The antibacterial activity was determined by using a dilution assay in a 96-well microplate format. The pH of deionised (DI) water and propolis particle aqueous dispersions at 15% propolis mass fractions was adjusted to 2.5, 3.6 (dispersion's native pH), 5 and 7 using HCL (1 M) and NaOH (1 M) as titrants. The samples were then mixed with the microbes in a 1:1 ratio, incubated at 37°C while at constant shaking at 150 rpm and plated at appropriate time points from 0 up to 24 hours.

4.2.4.2 Plate counting (CFU)

Viability of bacteria after treatment was assessed by serial 10-fold dilutions in PBS. 10 μl were measured and plated in Nutrient Agar plates and incubated overnight at 37°C. Colony Forming Units (CFU) were counted at the appropriate dilution (3-30 colonies) to determine cell viability. The lower limit of detection was 30 CFU /ml.

4.2.5 Statistical analysis

Experiments were conducted at least in duplicate. Results are expressed as averages and error bars represented one standard deviation. Statistical analysis was performed using IBM SPSS Statistics software. Results were compared either pairwise, with independent T- test or One- way Anova with Tukey's test. Differences were considered significant at $p<0.05$.

4.3 Results

4.3.1 Investigation of emulsions co-stabilised by propolis particles and Tween 80

4.3.1.1 The capacity of propolis particles to impact the oil/water interfacial tension

As propolis is a complex system consisting of a variety of organic compounds, there is potential capacity to present interfacial activity when it comes to contact with oil. What is more, the sub-micron dimensions of the formulated propolis particles might facilitate its capability to act as a Pickering stabilizer of an oil/water interface. Interfacial affinity was assessed *via* interfacial tension measurements between the propolis particle aqueous dispersions or their isolated aqueous carrier phases, and sunflower oil (Figure 4.1A). The interfacial tensions (IFT) between deionized (DI) water, a 2%

Tween 80 aqueous solution (the latter estimated to correspond to the amount of Tween 80 in the dispersions post water evaporation) or 1% Tween 80 (to account for free Tween 80 in the propolis particle aqueous dispersions) and sunflower oil were also measured as reference.

As expected, the DI water/ sunflower oil system presented the highest interfacial tension with sunflower oil, with a final IFT value of 13.29 mN/m. Although this value tends to be lower than literature [30], [31] it is comparable with refined sunflower oil IFT values [32]. The pendant drop method is an optical method that utilizes a droplet of one phase submerged in another immiscible phase at mechanical equilibrium while being recorded by a camera and by determining the droplet profile and from by means of a series of different algorithms, the interfacial tension is calculated.[33] Experimentally, it is common to utilize the dispersed phase of an emulsion to be studied as the droplet and place the continuous phase in the cuvette, in order to simulate the actual emulsion system.[34],[35],[36] However, in the present study, the opposite occurred, due to the nature of the continuous phase, as it includes particles making the phase opaque and prevents the camera to detect the droplet. Therefore, the surface area of the water is much smaller compared to oil, which is the opposite to what takes place within an O/W emulsion, making it much easier for the surface-active substances existing in commercial oil to reach the small water interface and lower the interfacial tension than in an opposite system.

The presence of 1% or 2% Tween 80 resulted in a much faster rate of IFT reduction and lower equilibrium. IFT values of 4.79 mN/m and 4.38 mN/m, respectively, were in agreement with literature [37]. The similarity of these values suggests that the interface created during measurements was saturated.

The equilibrium IFT values between sunflower oil and either the propolis particle aqueous dispersions or their isolated aqueous carrier phases were 1.36 mN/m and 9.89 mN/m, respectively. Both values were much lower compared to the sunflower oil/DI water equilibrium IFT, showing a higher surface action at the oil/water interface. The propolis particle aqueous dispersions, which were produced in the presence of Tween 80, gave a lower equilibrium IFT value than the oil/water interface in the presence of 2% Tween 80 alone. This suggests that the propolis particles and Tween 80 components decrease the oil/ water interfacial tension additively, and certainly to a greater extent than when present as individual entities.

However, since the propolis particle aqueous dispersions are produced in the presence of Tween 80, it is challenging to determine the exact amount of “unbound” Tween 80 in the system (present in the carrier phase), versus the proportion of the surfactant that is strongly associated with the propolis particles and thus cannot freely adsorb at an oil/water interface. In order to better understand the distinction between free and bound surfactant in the system, propolis particle aqueous dispersions were produced in the absence of Tween 80. The average particle size of the propolis particle aqueous dispersions with Tween 80 was 666 nm[23], which was statistically smaller ($p<0.05$) compared to the average particle size of the Tween 80-free propolis particle aqueous dispersions, with an average particle size of 2202 nm. The equilibrium IFT values between sunflower oil and either these Tween 80-free propolis particle aqueous dispersions or their isolated aqueous carrier phases were subsequently measured (Figure 4.1A). The equilibrium IFT of the carrier phase of the Tween 80-free propolis particle aqueous dispersions was 9.74 mN/m. What is more, this value was not statistically different ($p>0.05$) from the equilibrium IFT of the propolis particle aqueous

dispersions produced in the presence of Tween 80 (9.89 mN/m). This clearly suggests that the amount of free Tween 80 in the latter systems is limited, to the extent that the reduction to the interfacial tension observed in either case (in comparison to that of a vegetable oil/ deionized water system) largely comes from the presence of dissolved species in the carrier phase, originating from the propolis extracts. However, the equilibrium IFT value of the Tween 80-free propolis particle aqueous dispersions (particulate matter and carrier phase together) was even lower than 7.00 mN/m. Therefore, there is indication that the propolis particles themselves do provide further IFT reduction to what is achievable by the action of the carrier phase alone, although the significantly larger propolis particle size of the Tween 80-free propolis particle aqueous dispersions could also have played a significant role in not lowering the interfacial tension further[38] as smaller particles can be assembled into a more compact arrangement at the interface.[39]. Still, the IFT reduction exhibited by the propolis particle aqueous dispersions produced in the presence of Tween 80 (1.36 mN/m) is statistically more significant ($p<0.05$), providing further evidence of the oil/water interfacial tension and without signs (at least over the experimental timescales) of the competitive desorption of the latter by the surfactant.

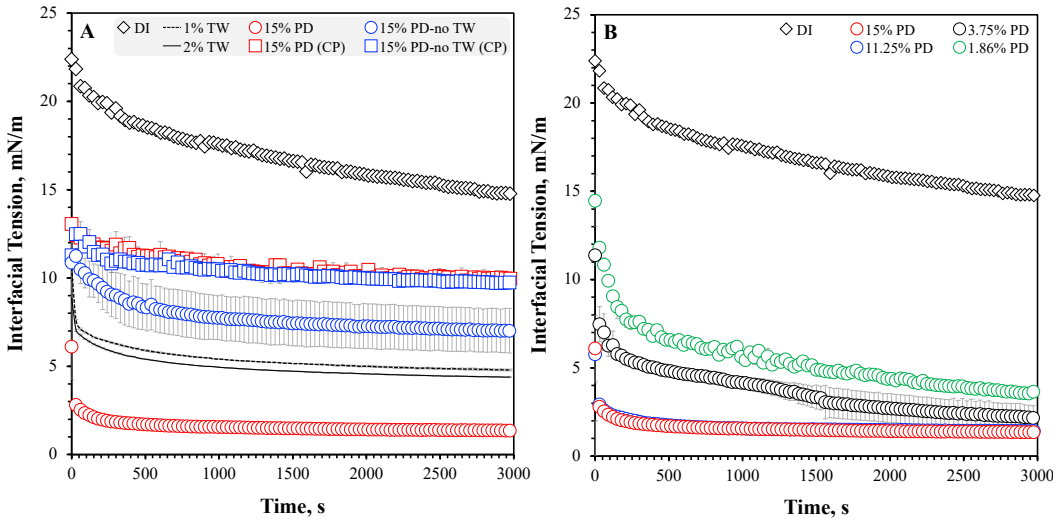

Figure 4.2A.: Dynamic interfacial tension between sunflower oil and deionised water (DI), 1% and 2% Tween 80 (1% TW and 2% TW, respectively) in DI, 15% propolis particle aqueous dispersions produced in the presence of Tween 80 (15% PD) and their aqueous carrier phases (15% PD (CP)), and 15% propolis particle aqueous dispersions produced in the absence of Tween 80 (15% PD- no TW) and their aqueous carrier phases (15% PD-no TW (CP)). B. Dynamic interfacial tension between sunflower oil and DI, 15% propolis particle aqueous dispersions produced in the presence of Tween 80 (15% PD) and dilutions of the former (using DI) producing dispersions of 11.25% (11.25% PD), 3.75% (3.75% PD) and 1.87% (1.87% PD) propolis mass fractions. All data were acquired at 20°C and are averages of three measurements, with error bars corresponding to ± one standard deviation (s.d.).

To the best of the authors' knowledge, this work reports for the first time on oil/water interfacial tension reduction in the presence of propolis extract particles. Propolis is a complex mixture [4] and it is therefore challenging to identify in detail which of these species would contribute to the interfacial behavior observed. Within propolis, the capacity of flavonoids to act as stabilisers of oil/water interfaces has been previously studied. Luo *et al.* [14] investigated *n*-tetradecane O/W emulsions, showing that insoluble flavonoids (rutin, naringin and tiliroside) acted as Pickering particles; the authors proposed that flavonoids would interact with the continuous water phase *via* their hydroxyl groups and show an affinity for the oil phase *via* their ring structures.

Similarly, phenolic compounds within propolis, have been shown to possess surface activity. Di Mattia *et al.* [40] investigated the surface and water/oil interfacial properties of three phenolic compounds which can also be found in propolis (quercetin, gallic acid and catechin) and the oxidative stability they could offer to olive oil-in-water (O/W) emulsions. All phenolic compounds decreased the interfacial tension, with quercetin and catechin showing a concentration dependent behaviour, whereas gallic acid remained constant probably due to a saturation effect. Zembyla *et al.* [13] investigated Pickering emulsions containing polyphenol crystals quercetin and curcumin, which also showed a strong affinity with the oil interface.

In an attempt to further elucidate the role of propolis particles in lowering the oil/water interfacial tension, the effect of the propolis extract mass fraction in the dispersion was investigated. A 15% (propolis extract, in terms of mass) propolis particle aqueous dispersion, in the presence of 2% Tween 80, was initially created and subsequently diluted using deionised water to produce dispersions of 11.25%, 3.75% and 1.87% propolis mass fractions; the interfacial tensions of the parent and diluted systems against vegetable oil were all measured (Figure 4.1B). The obtained IFT data show that the 15% and 11.25% propolis mass fractions practically had the same overall dynamic behaviour at the oil/water interface, giving equilibrium IFT values of 1.36 mN/m and 1.44 mN/m, respectively. The more diluted systems of 3.75% and 1.85% propolis mass fractions had equilibrium IFT values of 2.18 mN/m and 3.54 mN/m, respectively. It was apparent that even the most diluted system (1.87% propolis mass fraction) gave an equilibrium interfacial tension that was very close to the more concentrated systems, indicating that the system (even at its most diluted state) results to a more or less saturated oil/water interface. The rate of IFT reduction does differ

from one system to the other however, it was believed to be a concentration/content driven effect. at the interface differs showing dispersion's fraction dependence. Regardless of the comparable equilibrium IFT values between the systems of different propolis mass fractions, the latter distinction with regards to IFT decrease kinetics can become crucial during emulsification, as expedited interfacial adsorption would, amongst others, result in smaller droplet sizes that are also more stable (during processing).[16]

4.3.1.2 Wettability of propolis extracts

Having established that the propolis particles and the propolis existing in the carrier phase have an affinity for the oil/ water interface, it was important to investigate which phase it would promote as continuous or dispersed in an emulsion system. This was examined by determining propolis extracts' wettability *via* measuring its contact angle with deionised water (θ_w) and sunflower oil (θ_o). If the contact angle of water is greater than oil, this indicated that the propolis extracts are more hydrophilic than hydrophobic, promoting a water continuous emulsion and *vice versa*. The results along with pictures of the droplets are shown in Figure 4.2.

The contact angle θ_w was significantly larger than θ_o, showing that propolis was hydrophilic and would favour the stability of O/W emulsion systems. Within the propolis extracts, components such as rutin hydrate[41] showed similar results of high hydrophilicity and low hydrophobicity (θ_o value of approximately 75° and θ_w of 35°). In terms of propolis, to the best of the authors' knowledge its wetting properties have not been previously reported. Contact angle of water with propolis has been investigated as part of a formulation. However, the results are mixed as it highly depends on nature of the surface onto which it has been incorporated. For example, Kim *et al.* [42] showed

that increasing the propolis percentage in their polyurethance composite nanofibre formulations, decreased the water contact angle with increasing propolis fraction (from 114.5° to 92.4°), while Cho *et al.* [43] showed that quercetin, a flavonoid often found in propolis, decreased the water contact angle when it was incorporated with titanium dioxide (from 54.6° to approximately 47°).

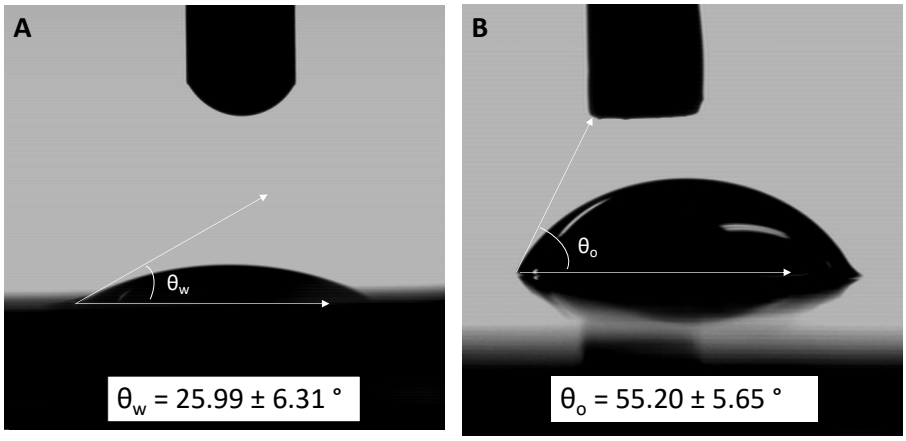

Figure 4.3: Contact angles for A. water and B. sunflower oil for a propolis surface. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

4.3.1.3 Effect of the propolis content on the viscosity of the dispersions

The viscosity of the propolis particle aqueous dispersions with different propolis content was investigated to establish if it would influence the emulsification or stability of O/W emulsions, where the dispersions would be used as the continuous phase. Viscosity of the continuous phase can influence the overall stability of the emulsions and therefore it often deliberately is increased in applications; at the same time if too high it could enhance coalescence or prevent droplet breakup during emulsification.[44] The viscosity of water was also measured, and the results are presented in Figure 4.3A.

Figure 4.3A shows that high propolis samples (≥ 7.5%) exhibited slight shear thinning behaviour. This was further confirmed from the power law indices. Therefore, the viscosity of the dispersions was dependent on the propolis content, but not by far. It is notable to mention that sometimes Newtonian fluids may appear as shear thinning due to surface tension effects that can cause a torque which can influence their viscosity.[45]

For comparison purposes, a single shear rate of 100 s^{-1} was selected and the viscosities were plotted against the propolis fraction used (Figure 4.3B). A similar trend is followed of 15% exhibiting the highest viscosity of 1.32 mPa.s, following 11.25% of 1.09 mPa.s, 7.5% of 1.06 mPa.s, 3.75% of 1.04 mPa.s and deionised water of 0.91 mPa.s. There was a statistically significant difference ($p<0.05$) between the 15% propolis dispersion and deionised water, further establishing the difference in viscosity due to the incorporation of propolis.

To the best of the authors' knowledge these propolis aqueous systems' viscosities have not been examined in previous literature. However, dispersions' shear thinning behaviour until a specific fraction of the dispersed phase, have been well reported. For instance, Silva et al. [46] reported the promoting increase in viscosity of aqueous tragacanth gum dispersions *via* the addition of sugars. Similarly, Hsu *et al.* [47] reported that ceria dispersions exhibited a shear thinning behaviour down until 2% dispersed volume fraction, where it resembled Newtonian behaviour. In the present study although there seemed to be a slight shear thinning behaviour in the concentrated propolis dispersions and a larger viscosity at shear rate 100 s^{-1}, it is not expected to cause a significant difference in the emulsions compared to water.

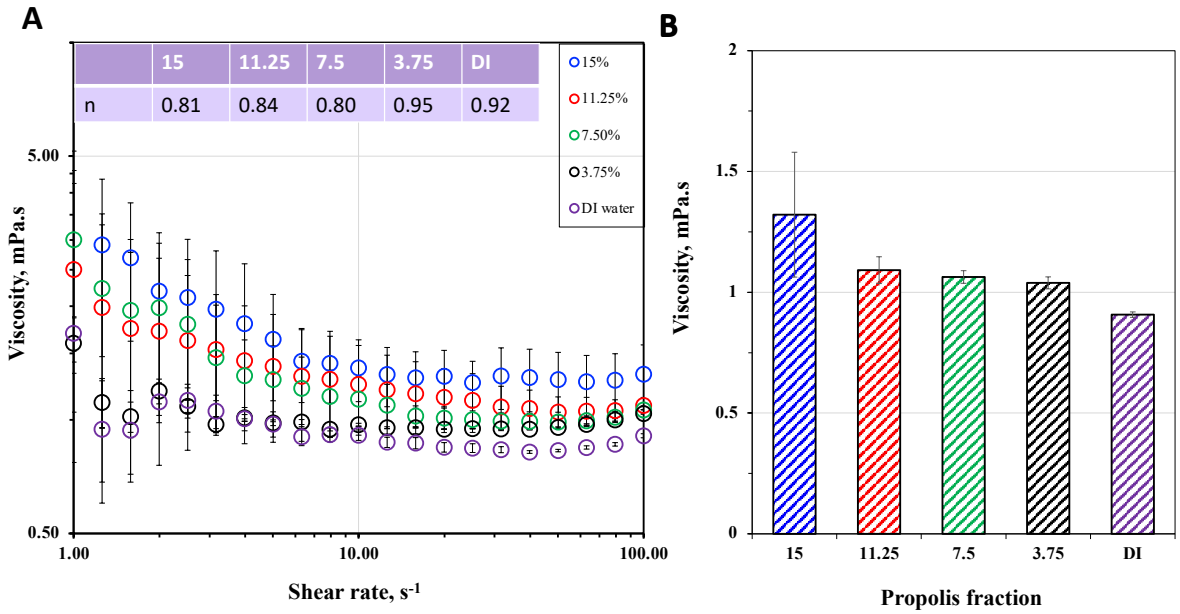

Figure 4.4: Viscometry and B. Viscosity at shear rate 100 s^{-1} of the dispersions with different propolis content of 3.75, 7.5, 11.25 and 15%. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

4.3.1.4 Effect of the surfactant content on the stability and microstructure of propolis emulsions.

After establishing that the viscosity of the propolis aqueous dispersions would not significantly influence the emulsification process or the emulsion stability, they were utilised to formulate O/W emulsions. The emulsions contained 15% oil and 0.1, 0.5 and 1% Tween 80 as the emulsifier and were formulated *via* a high shear mixer device. The 15% propolis particle aqueous dispersions were used as the continuous phase, while DI water was used to produce control emulsions for comparison purposes. The propolis emulsions that contained 0.1% Tween 80 phase separated immediately after mixing, showing that the particles covered in Tween, even though they associate with the oil/water interface, are not solely able to protect the droplets against coalescence

and more surfactant is needed to stabilise emulsions. The 0.5 and 1% Tween 80, and their controls, were stored at 4°C and 25°C, over a 60-day period. The average span of the size distributions along with the $D_{3,2}$ and $D_{4,3}$ average oil droplet diameters, were measured and compared with the control emulsions. Tables 4.1 and 4.2 show the evolution of the mean droplet size and span values for emulsions made in the presence of 0.5 and 1% Tween 80, respectively.

From Table 4.1 it can be seen that during 60 days storage, at 25°C the span, $D_{4,3}$ and $D_{3,2}$ droplet average sizes stayed consistent with no statistical difference, indicating good stability. Considering the span average values at 4°C storage, there was a statistical increase after 60 days which was only similar with the 30 days measurement. The $D_{4,3}$ average values exhibited a statistical difference between 0 and 30 and 0 and 60 days, showing an increase in oil droplet sizes and possibly explaining the increase in span. However, the $D_{3,2}$ means presented no statistically significant difference for the whole period of storage at both temperatures. Overall, the emulsions were stable with a significant increase of oil droplet size after 60 days at 4°C.

Propolis emulsions for both storage temperatures were compared with controls (formulated with distilled water as the continuous phase). The average span of the size distributions of the propolis emulsion was statistically larger compared with the control at 60 days of storage for both 4°C and 25°C. The $D_{4,3}$ average droplet size of the propolis emulsions remained statistically smaller than the control over the 60-day storage period at 25°C. However, post 60 days storage at 4°C the oil droplet sizes ($D_{4,3}$) were statistically similar with the control. The $D_{3,2}$ average value of the oil droplet size of propolis emulsions remained statistically smaller than the control, for both at 4°C and 25°C during 60 days storage period. Therefore, having propolis particles in

the continuous phase instead of only distilled water significantly decreased the oil droplet size ($p<0.05$).

Examining Table 4.2, where results for a higher percentage of Tween 80 were reported, it was determined that the span of the distributions stayed consistent over 60 days. The $D_{4,3}$ average droplet size at 0 days was statistically smaller after 1 day and after 60 days of storage whereas the $D_{3,2}$ average at 0 days was statistically different from all other measurements. That indicates an increase of average oil droplet size with the progress of time, causing an increase in the span of the distributions. At 4°C storage temperature, the distribution span was statistically different between 60 days and all the other time points apart from 30 days. The $D_{4,3}$ and $D_{3,2}$ averages exhibited a similar behaviour with the span, with the size being significant larger at 60 days compared to all other days tested.

Table 4.1: Average volume ($D_{4,3}$) and surface ($D_{3,2}$) weighted mean oil droplet sizes (and associate span values) of 15% O/W emulsions produced with either aqueous propolis particle dispersions (15% Propolis mass fraction) or water (Control) as their continuous (aqueous) phase, in the presence of 0.5% Tween 80, *via* HSM. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Storage Temperature, (°C)	Storage Period (days)	Propolis			Control		
		$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span
25	0	7.97±2.09	3.86±0.61	2.49±0.03	17.11±0.03	9.79±0.03	1.58±0.00
	1	8.25±1.57	3.90±0.43	2.63±0.02			
	7	9.52±1.60	4.12±0.37	2.62±0.05			
	14	9.73±2.42	4.42±0.64	2.48±0.09			
	30	12.20±2.75	5.13±0.88	2.51±0.27			
	60	9.37±2.33	4.09±0.15	2.47±0.40	15.67±1.22	8.93±0.73	1.68±0.07
4	0	7.97±2.09	3.86±0.61	2.49±0.03	17.11±0.03	9.79±0.03	1.58±0.00
	1	9.53±1.90	4.07±0.63	2.76±0.22			

	7	9.10±2.33	3.89±0.36	2.59±0.11			
	14	9.68±2.65	4.19±0.76	2.05±0.61			
	30	14.87±2.15	5.00±0.39	2.68±0.19			
	60	14.24±1.56	3.98±0.05	3.55±0.16	15.32±0.19	8.57±0.17	1.73±0.02

Table 4.2: Average volume ($D_{4,3}$) and surface ($D_{3,2}$) weighted mean oil droplet sizes (and associate span values) of 15% O/W emulsions produced with either aqueous propolis particle dispersions (15% Propolis mass fraction) or pure water (Control) as their continuous (aqueous) phase, in the presence of 1% Tween 80, *via* HSM. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Storage Temperature, (°C)	Storage Period (days)	Propolis			Control		
		$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span
25	0	6.75±0.39	2.76±0.41	3.04±0.15	15.05±1.64	9.08±1.67	1.52±0.21
	1	12.11±1.23	4.16±0.48	2.67±0.08			
	7	11.10±1.04	4.73±0.01	2.33±0.11			
	14	11.68±1.56	3.97±0.45	2.95±0.30			
	30	11.11±1.93	4.28±0.57	2.69±0.09			
	60	13.69±0.45	4.57±0.37	3.25±0.53	16.70±1.56	7.84±0.51	1.79±0.13
4	0	6.75±0.39	2.76±0.41	3.04±0.15	15.05±1.64	9.08±1.67	1.52±0.21
	1	9.18±0.38	3.76±0.18	2.48±0.07			
	7	10.71±1.35	3.68±0.90	2.44±0.09			
	14	8.34±0.55	3.43±0.18	2.79±0.10			
	30	11.37±3.18	3.33±0.79	2.94±0.20			
	60	16.09±3.79	3.76±0.43	3.69±0.59	14.44±0.20	8.00±0.21	1.67±0.04

Subsequently, the propolis emulsions were compared with their respective controls to identify the effect of propolis and higher percentage of Tween 80 on the formulation. The average span value of propolis emulsions was statistically larger than the control and both $D_{4,3}$ and $D_{3,2}$ averages were statistically smaller upon production. After 60 days of storage at 4°C, the span of the propolis emulsion progressed to be

similar with the control emulsion, while at 25°C, the distribution span remained statistically larger. The $D_{4,3}$ and $D_{3,2}$ averages exhibited similar behaviours for both storage temperatures; the $D_{4,3}$ averages showed no statistical difference between the propolis emulsions and the controls while the $D_{3,2}$ averages remained smaller for the whole period of 60 days.

Examining the entire size distributions for all produced emulsions (Figure 4.4), in all cases the control emulsions presented sharper singular peaks compared to their respective propolis emulsions, as it was reflected from the statistically smaller span values shown in Tables 4.1 and 4.2. Increasing the fraction of Tween 80 in the emulsion from 0.5% to 1%, did not influence the emulsion droplet size, indicating a saturation of coverage of the O/W interface. The average span value was statistically smaller at the 0.5% than 1% Tween content immediately after production with the progress of time they became statistically similar, as it is reflected in Figure 4.4. The difference in average size between propolis and control emulsions can also be observed. Post 60 days storage the propolis emulsion peaks shift closer to those of the controls with some evidence of potential coalescence in Figures 4.4B and C.

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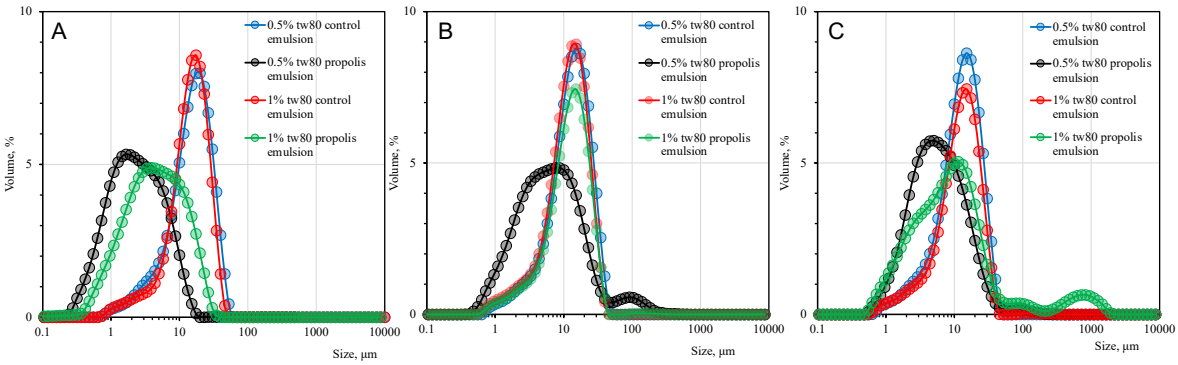

Figure 4.5: $D_{4,3}$ average droplet size distributions of 15% propolis and control O/W emulsions. Data for emulsions at 0 days (A), 60 days at 4°C (B) and 25°C (C) storage are presented. Data shown are averages (n=3; repeats).

The emulsions were also observed using light microscopy; images were taken and presented in Figures A2.3 and A2.4. As it can be seen all emulsions were flocculated regardless of the amount of surfactant or the existence of propolis in the continuous phase. Tween 80 is a non-ionic surfactant with a hydrophilic head group and a hydrophobic tail group, by arranging their hydrophobic and hydrophilic parts on the respective phases.[48] However, Tween stabilised interfaces possess lower viscoelastic properties, resulting in emulsions that are more prone to flocculation compared to interfaces stabilised by other emulsifiers like proteins.[49] In this case, flocculation was probably related with the coarse size of the emulsion droplets or the processing conditions. All the emulsions also presented a creaming layer which likewise was the same across all systems (Figure A2.2). The flocculation along with the creaming may provide an insight to the mechanism of destabilisation as both can be indications of coalescence which was present in some propolis emulsions (Figure 4.5B and C).

So far, it has been established that propolis particles have an affinity for the O/W interface and have a preference for the aqueous phase. To determine if this is the case, fluorescence microscopy was employed and results for propolis emulsions containing 0.5% Tween are presented in Figure 4.5. Propolis particles themselves (no dye was used to carry out these experiments) fluoresced after excitation at 532 nm, surrounding the oil droplets from the continuous phase and exhibiting Pickering-like functionality which to the best of our knowledge is not included in previous literature.

Immediately after production there are more droplets surrounded by propolis particles (Figure 4.5A) than after 60 days where there is lower fluorescence emission around the oil droplets This observation agrees with the earlier results showing that with the passage of time, there was a shift in the oil droplet size distributions of the propolis emulsions towards the control emulsions. This shift was probably caused from less particles existing at the interface. It can additionally be seen from Figures 4.5B and C that there are less particles in the continuous phase, which could be due to precipitation of propolis present at the bottom of the vessel post 60 days storage.

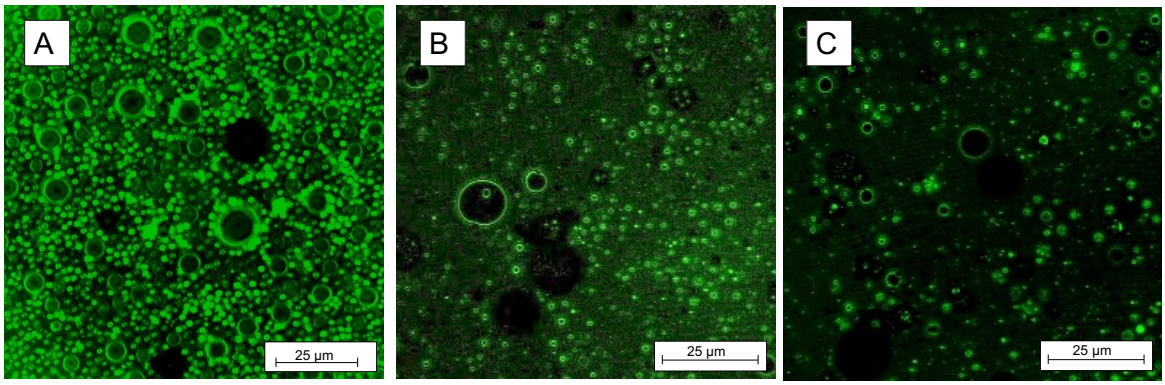

Figure 4.6: Confocal fluorescence images of propolis emulsions containing 0.5% Tween 80 and 15% oil A. upon production (63× magnification) ,B. post 60 days storage at 4°C and C. post 60 days storage at 25°C (40× magnification) excited with a 532 nm wavelength.

Overall, the propolis emulsions containing 0.5% Tween 80 showed a slightly better stability especially at 25°C storage that 1% Tween (as shown in the Appendix, Figure A2.10). Mixed systems of Pickering particles and surfactants have been reported in literature to produce more stable emulsions.[50],[51],[52] However, cases have been reported of displacement of particles from the interface by the surfactant molecules. For example Pichot *et al.* [53] studied the interfacial tension and contact

angle of hydrophilic silica particles in the presence of lecithin, Tween 60 and sodium caseinate. The authors report that as the concentration of the surfactant is increased, it becomes the dominant factor over the silica particles for the wetting ability of the continuous phase due to displacement of the particles from the interface. This competition could be the reason behind the droplet size increase with increasing Tween for the propolis emulsions. In the opposite system of W/O emulsions stabilised by hydrophobic silica particles and sorbitan monoolate, Drelich *et al.* [54] detected a change in the water droplets freezing transition which was believed to occur due to a displacement of the silica particles by the surfactant.

It is safe to hypothesise that the same phenomenon occurs in the present study. Following production, the droplet sizes were smaller than the controls, most probably due to a synergistic effect between the propolis and the surfactant in lowering the interfacial tension (Figure 4.1). It has been highlighted that at particle/low surfactant concentration the stability of an emulsion can increase due to less antagonistic effects taking place.[55] As the surfactant concentration increases, it showed a higher instability which could be due to the increased displacement of the particles from the interface. It is also important to note that the flocculation of oil droplets observed in both control and propolis emulsions, which can enhance the creaming taking place, could promote coalescence. In addition, there was a difference in storage conditions with 4°C being a more unfavourable environment. It is believed that increase of temperature decreases the interfacial tension as the intermolecular forces at the interface weaken.[56]

4.3.1.5 Use of different processing to alter the oil droplet size and its effect on the stability and microstructure of propolis emulsions.

Different processing conditions were employed (high shear mixer vs direct ultrasonication), to produce emulsions with different oil droplet sizes. The impact of the type of processing and oil droplet size that this generated was investigated on the capacity of propolis particles to exhibit a Pickering functionality. Table 4.3 presents the span, $D_{4,3}$ and $D_{3,2}$ values of 15% propolis and control emulsions. All emulsions were formulated with 15% oil and 1% Tween 80 *via* sonication.

All systems showed adequate stability at 25°C storage temperature. The span of the distributions did not alter significantly with the passage of time. This was reflected by the distributions in Figure 4.7A and C, and the $D_{3,2}$ average. The $D_{4,3}$ average values showed an increase after two months storage while at 4°C storage temperature, samples presented a better stability as all measurable values were statistically similar throughout the whole period of storage.

Comparing the propolis samples with controls, upon production there was a statistically significant difference between the span values of the two systems, showing a greater span with the propolis emulsions. The $D_{3,2}$ average droplet sizes upon production were statistically smaller compared to the controls, however with the passage of time they became larger. The $D_{4,3}$ averages of the propolis emulsions were statistically larger than the controls during 25°C for 60 days of storage, while the 4°C remained statistically similar. Therefore, there was an increase in the average droplet size of the propolis emulsions compared to the controls for both storage temperatures. This was more pronounced at 25°C storage as can be seen from the distribution in Figure 4.6.

Table 4.3: Average volume ($D_{4,3}$) and surface ($D_{3,2}$) weighted mean oil droplet sizes (and associate span values) of 15% O/W emulsions produced with either aqueous propolis particle dispersions (15% Propolis mass fraction) or pure water (Control) as their continuous (aqueous) phase, in the presence of 1% Tween 80, *via* sonication. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Storage Temperature, (°C)	Storage Period (days)	Propolis			Control		
		$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span
25	0	1.42±0.01	0.71±0.00	3.44±0.03	1.43±0.20	0.74±0.01	2.28±0.19
	1	1.77±0.44	0.76±0.02	3.50±0.39			
	7	1.46±0.02	0.70±0.18	2.95±0.02			
	14	1.62±0.36	0.56±0.14	3.80±0.72			
	30	1.46±0.08	0.83±0.07	2.68±0.16			
	60	3.94±0.89	0.87±0.04	3.64±0.51	1.10±0.09	0.67±0.09	1.94±0.17
4	0	1.42±0.01	0.71±0.00	3.44±0.03	1.43±0.20	0.74±0.01	2.28±0.19
	1	1.51±0.06	0.75±0.00	3.33±0.12			
	7	1.94±0.64	0.75±0.02	3.23±0.20			
	14	2.34±1.03	0.76±0.17	3.25±1.09			
	30	1.78±0.54	0.83±0.11	3.05±0.43			
	60	1.94±0.80	0.81±0.02	3.13±0.27	1.30±0.05	0.73±0.01	2.04±0.04

Emulsions with different oil droplets sizes showed similar behaviours in terms of their stability, suggesting that the increased interfacial area did not significantly influence the system (as shown in the Appendix, Figure A2.11). The span of the distributions was larger than their respective controls and although initially the oil droplet sizes were consistently smaller, there was an increasing trend in oil droplet size, with the passage of time especially at 25°C storage. One indication of the better stability of the emulsions produced by sonication, was that no creaming took place compared with the HSM produced emulsions, due to increased capacity of the sonicated emulsions (with smaller droplet sizes) to withstand flocculation and

coalescence phenomena[57]. Looking at Figure 4.6, the high shear mixer emulsions present signs of coalescence post 60 days storage, which is less pronounced with the sonicated emulsions.

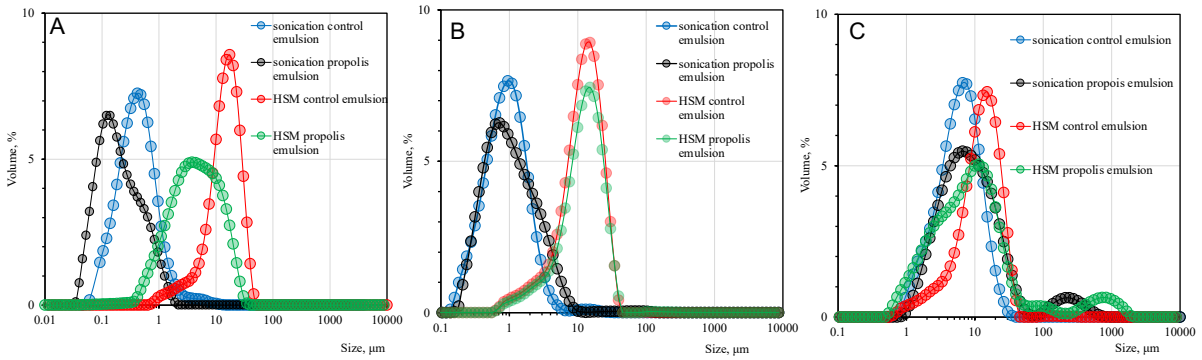

Figure 4.7: $D_{4,3}$ average droplet size distributions of propolis and control O/W emulsions. Data for emulsions at 0 days (A), 60 days at 4°C (B) and 25°C (C)storage, produced by sonication and HSM are presented. Data shown are averages (n=3; repeats).

The presence of propolis in emulsions produced *via* sonication did not decrease the average droplet size even though smaller droplet populations were generated as demonstrated in the size distribution data (Figure 4.6). That could be due to the system being saturated and not being able to reduce the droplet size even further. In both systems, there was increase in average droplet size with the passage of time; the average oil droplet size of the sonicated emulsion systems became greater than the control. However, this was much more pronounced at 25°C and more or less insignificant at 4°C.

4.3.1.6 Influence of the propolis content on the stability and microstructure of propolis emulsions

Concentrated propolis dispersions showed an affinity for the interface, and when used with Tween 80, produced emulsions with smaller oil droplets than just the

surfactant itself. To investigate the role of propolis in this phenomenon, emulsions with less propolis mass fraction of 3.75% were formulated and compared with the concentrated propolis emulsions of 15%. All emulsions contained 15% oil and 1% Tween 80 and were formulated *via* HSM.

Similarly to when less surfactant was used, all measurable values (Table 4.4) remained statistically consistent throughout storage and for both storage temperatures, with all emulsions exhibiting sufficient stability, as it can also be seen from Figure 4.7B where there are still particles surrounding the oil droplets after 60 days storage at 25°C. This provides evidence that fewer propolis particles in the system increased the stability due to less competition between the particles and the surfactant. In addition, the 3.75% propolis emulsions showed flocculation throughout storage, and creaming which occurred shortly after production, as it can be seen in Figures A2.5 and A2.6.

Table 4.4: Average volume ($D_{4,3}$) and surface ($D_{3,2}$) weighted mean oil droplet sizes (and associate span values) of 15% O/W emulsions produced with either aqueous propolis particle dispersions (3.75% propolis mass fraction) or pure water (Control) as their continuous (aqueous) phase, in the presence of 1% Tween 80 *via* HSM. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Storage Temperature, (°C)	Storage Period (days)	Propolis			Control		
		$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span
25	0	13.12±1.27	5.80±1.13	2.06±0.25	15.05±1.64	9.08±1.67	1.52±0.21
	1	10.99±0.78	5.11±0.43	2.14±0.15			
	7	13.78±1.39	6.13±0.73	1.96±0.12			
	14	10.82±2.30	5.51±1.86	2.20±0.44			
	30	14.07±1.28	6.23±1.28	2.11±0.34			
	60	12.38±1.54	5.36±0.37	2.14±0.03	16.70±1.56	7.84±0.51	1.79±0.13
4	0	13.12±1.27	5.80±1.13	2.06±0.25	15.05±1.64	9.08±1.67	1.52±0.21
	1	11.51±0.98	5.45±0.44	2.08±0.08			

	7	12.94±0.29	5.64±0.17	2.04±0.04			
	14	11.87±0.62	5.57±0.30	2.03±0.06			
	30	13.18±2.22	5.75±0.54	2.09±0.02			
	60	10.78±0.94	5.96±0.48	2.23±0.10	14.44±0.20	8.00±0.21	1.67±0.04

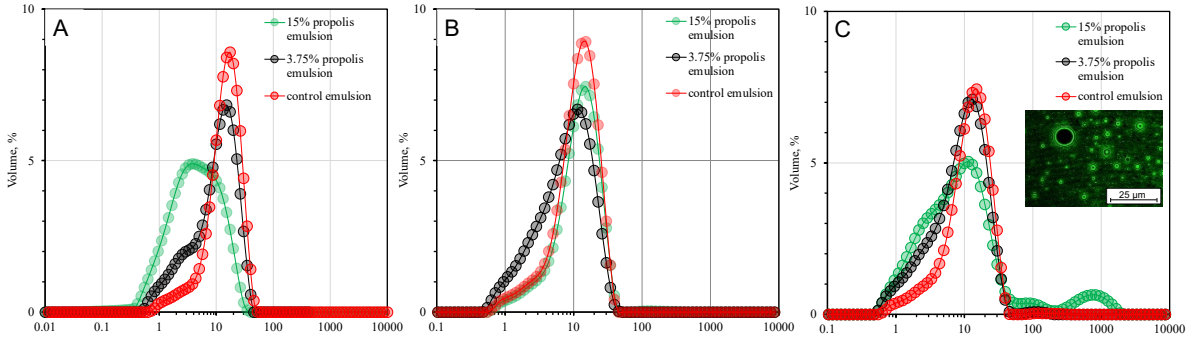

Figure 4.8: $D_{4,3}$ average droplet size distributions of 3.75% and 15% propolis and control O/W emulsions. Data for emulsions at 0 days (A), 60 days at 4°C (B) and 25°C storage, produced by HSM are presented. Data shown are averages (n=3; repeats). Inset Figure: Confocal fluorescence images of 3.75% propolis emulsions containing 15% oil and 1% Tween 80 produced via HSM (40× magnification) excited with a 532 nm wavelength post 60 days storage at 25°C.

Emulsions containing the highest propolis mass fraction of 15% upon production produced significantly smaller oil droplet sizes than the 3.75% propolis mass fraction emulsion (as shown in the Appendix, Figure A2.12), unlike the widths in the size distributions which showed the opposite pattern, as it is depicted in Figure 4.7A. Comparing the span values between the 15% and 3.75% propolis emulsions showed that, at a 3.75% propolis fraction it remained smaller for both storage temperatures, up until 60 days where it resembled the 15% propolis emulsions. As the size of the oil droplets of the 15% propolis dispersions increased, it became similar with the 3.75% propolis emulsions. This was more pronounced at 25°C over 4°C. Overall, it can be seen that even though the 15% propolis containing emulsions produced smaller oil

droplets than the 3.75%, there is a level of instability at 15% which causes the size to shift to values similar with the 3.75% after prolonged storage.

The 3.75% propolis emulsions were imaged with fluorescence microscopy under different excitation wavelengths to examine the difference in the emissions. It can be seen from Figure 4.8 that the different laser excitations influence different part of the propolis emulsions. Figure 4.8A focuses on the more hydrophilic compounds contained in propolis and as the wavelength increases, the focus shifts to more hydrophobic compounds which are believed to be located at the interface of the O/W emulsions. Therefore, by changing excitation wavelengths, different positions of the emulsion microstructure (continuous phase vs interface) are in focus. Since propolis is a complex mixture it is difficult to determine which compounds are responsible for these images. However, Luo *et al.*[14], when comparing emulsion systems made with rutin and tiliroside, the rutin stabilised emulsions produced a similar image to Figure 4.9A while tiliroside stabilised emulsions were similar to Figure 4.9D. This difference was attributed by the author to the differences in water solubility (a partition coefficient ($\log_{10}$P) of -0.27 for rutin and 2.71 for tiliroside respectively).

The fact that the 3.75% propolis emulsions are similar with the controls both in terms of average oil droplet size and shape of the size distribution curves means that the contribution of propolis particles to the emulsion microstructure created is concentration specific, as it was also seen by measuring the interfacial tension of the dispersions containing different propolis mass fractions. In terms of the 15% propolis emulsions, there was strong evidence of particle desorption which was not as evident in the 3.75% which Is believed to occur due to less competition between particles and surfactants.

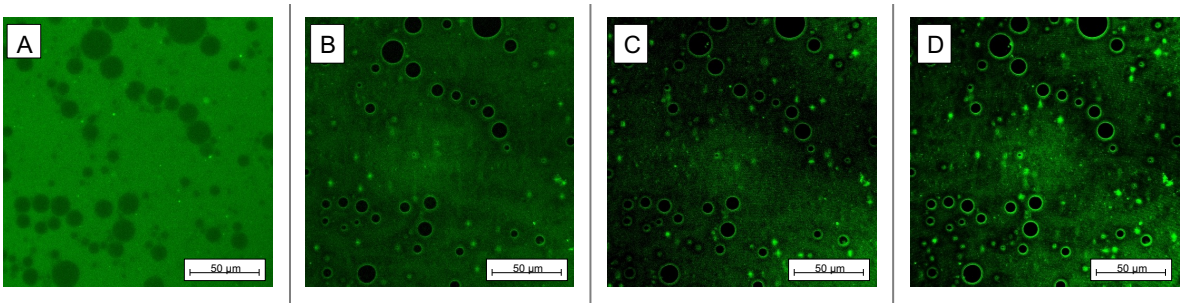

Figure 4.9: Confocal fluorescence images of propolis emulsions containing 3.75% propolis mass fraction, 15% oil and 1% Tween 80 immediately postproduction (40× magnification) excited with a A. 408 nm, B. 488 nm C. 532 nm and D. 634 nm wavelength.

4.3.1.7 Effect of oil content on the stability and microstructure of propolis emulsions.

So far, the effect of propolis particles in emulsion systems has been investigated by altering key parameters such as surfactant content, surface area of oil droplets (size) and propolis mass fraction. Essentially, all these variations have a direct effect on the O/W interface either by increasing its size, or the amount of available components that could contribute to its coverage. One final factor examined was to increase the amount of the dispersed phase itself. Therefore, propolis emulsions with 25% sunflower oil and 1% Tween 80 were produced using a high shear mixer and were compared with 15% oil propolis emulsions as well as the controls in terms of mean size, span value and stability; the data obtained are shown in Table 4.5.

The 25% oil fraction of the propolis emulsions had excellent stability under both storage temperatures as the volume and weighted mean sizes were not statistically different over the passage of time. The span of the distribution at 4°C post 60 days storage was statistically larger than the rest. This is also illustrated by the fluorescence images of the emulsions, where it can be seen that the oil droplets are surrounded by particles even after two months storage (Figure 4.9).

Both control and propolis emulsions were flocculated for all days of storage as it is depicted in Figure A2.8. There was a cream layer present in the propolis and control emulsions that was much larger from all the 15% oil containing emulsions as expected since there was a higher oil content included in the emulsion (Figure A2.7).

Propolis emulsions produced smaller oil droplet sizes than the controls ($p<0.05$) which remained smaller at 25°C for over 60 days of storage. At 4°C the oil droplet sizes of the propolis emulsions measured after 60 days ($D_{4,3}$) were statistically similar with the controls indicating an increase in oil droplet size. All the span values of the distributions remained statistically larger than their respective controls, as it is evident in Figure 4.10.

Table 4.5: Average volume ($D_{4,3}$) and surface ($D_{3,2}$) weighted mean oil droplet sizes (and associate span values) of 25% O/W emulsions produced with either aqueous propolis particle dispersions (15% Propolis mass fraction) or pure water (Control) as their continuous (aqueous) phase, in the presence of 1% Tween 80 *via* HSM. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Storage Temperature, (°C)	Storage Period (days)	Propolis			Control		
		$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span
25	0	8.67±1.97	4.09±0.60	2.43±0.10	15.36±0.02	9.13±0.02	1.50±0.00
	1	9.23±1.89	4.24±0.59	2.52±0.16			
	7	9.90±1.42	4.37±0.47	2.52±0.15			
	14	10.55±2.29	4.66±0.62	2.40±0.13			
	30	11.17±0.49	5.10±0.04	2.37±0.14			
	60	9.37±2.33	4.16±0.08	2.47±0.40	16.44±0.20	10.16±0.17	1.42±0.02
4	0	8.67±1.97	4.09±0.60	2.43±0.10	15.36±0.02	9.13±0.02	1.50±0.00
	1	10.12±1.77	4.50±0.80	2.54±0.36			
	7	9.91±2.22	4.39±0.77	2.43±0.24			
	14	10.40±2.39	4.57±0.81	2.40±0.21			
	30	14.32±0.56	5.20±0.20	2.46±0.21			

	60	14.24±1.56	3.98±0.05	3.55±0.16	15.65±0.18	9.83±0.13	1.42±0.03

Emulsions made with 15% and 25% oil were compared to determine the effect of the propolis containing continuous phase on different fractions of the dispersed phase and consequently different interfacial areas (as shown in the Appendix, Figure A2.13). Upon production 15% propolis emulsions contained smaller droplet sizes ($p<0.05$) following production ($D_{3,2}$) than the 25% propolis emulsions as expected, since the same content of surfactant, propolis or processing was inputted in both systems.[58] Interestingly the distributions of the 25% propolis emulsions had a smaller span value compared to the 15% and remained smaller, as it is shown in Figure 4.10A. It is also observed from Figure 4.10 that the 25% oil propolis distributions remained constant whereas the 15% oil propolis distributions shifted with the passage of time approaching the controls. This level of stability was similar for the sonicated emulsions as well. This provides evidence of less displacement occurring with larger interfaces, which could be the reason the 25% oil propolis emulsion as well as the sonicated ones were more stable than the 15% oil HSM propolis emulsion especially at 25°C storage. All values for both 15% and 25% oil containing propolis emulsions were statistically similar at 4°C storage.

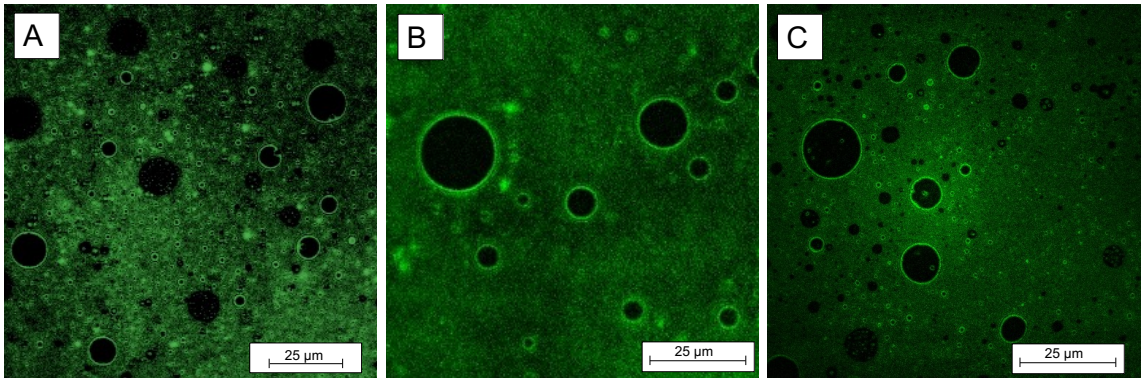

Figure 4.10: Confocal fluorescence images of propolis emulsions containing 1% Tween 80 and 25% oil A. upon production (40× magnification ,B. post 60 days storage at 4°C (40× magnification and C. post 60 days storage at 25°C (63× magnification) excited with a 532 nm wavelength .

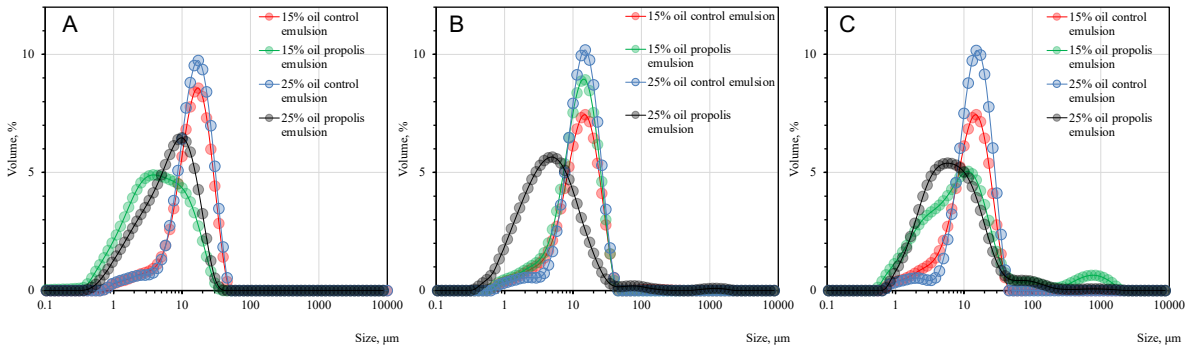

Figure 4.11: $D_{4,3}$ average droplet size distributions of 15% and 25% oil propolis and control O/W emulsions. Data for emulsions at 0 days (A), 60 days at 4°C (B) and 25°C storage, produced by HSM are presented. Data shown are averages (n=3; repeats).

4.3.2 Investigation of emulsions co-stabilised by propolis particles and sodium caseinate

4.3.2.1 Investigation of the pH environment on the properties of propolis aqueous dispersions and their electrostatic interactions with sodium caseinate

Propolis particle aqueous dispersions were prepared *via* direct ultrasonication. The dispersions were made with Tween 80 which is a small weight, non-ionic surfactant. Tween 80 has previously been used in the formation of dispersion systems.[59]. For instance, Xiong et al.[60] dispersed titanium carbide with the aid of Tween 80, reporting that the zeta potential of the powder decreased, and its electrostatic repulsion improved with the aid of Tween 80 due to better dispersability of the sample. However, the opposite was observed by Correa *et al*.[61] who reported

fewer electrostatic repulsions when a non-ionic surfactant was added, attributing it to the non-ionic surfactants shielding the electric charge.

Consequently, the pH environment of the resulting dispersion was adjusted to 2.5, 5 and 7 to investigate its effect on the size, PdI, zeta potential and stability of the dispersions. Figure 4.11 shows an inversely proportional relationship between pH, size and zeta potential. Increasing the pH resulted in a decrease in the zeta potential. As fabricated, propolis particle aqueous dispersions had a native pH value of 3.65, slightly more acidic than literature [62], and a zeta potential value of -26.84 mv[23]. The negative zeta potential is believed to be due to free fatty acids contained in propolis.[61] Increasing the cations in the dispersion would result in an increase in the zeta potential and *vice versa*. By decreasing the pH and thus increasing the zeta potential, the electrostatic repulsion between particles is reduced, encouraging particle aggregation, and therefore explaining the immense increase in particle size seen at pH 2.5. Similarly at pH 7, the zeta potential increased to -43.80 mv encouraging electrostatic repulsion and therefore resulting in smaller average particle size.

Size, PdI and zeta potential were tested over the course of 30 days while stored at 25°C to determine the effect of pH on the stability of the propolis particle aqueous dispersions. Figure 4.12 shows that at the lowest pH value of 2.5, the size significantly increased post 30 days storage. The instability due to the acidic pH is reflected in the PdI data in Figure 4.12B which also increased over time due to sample deterioration. At greater pH values (5 and 7), the size stability was improved. This was hypothesized to be due to the increased electrostatic repulsion between particles.

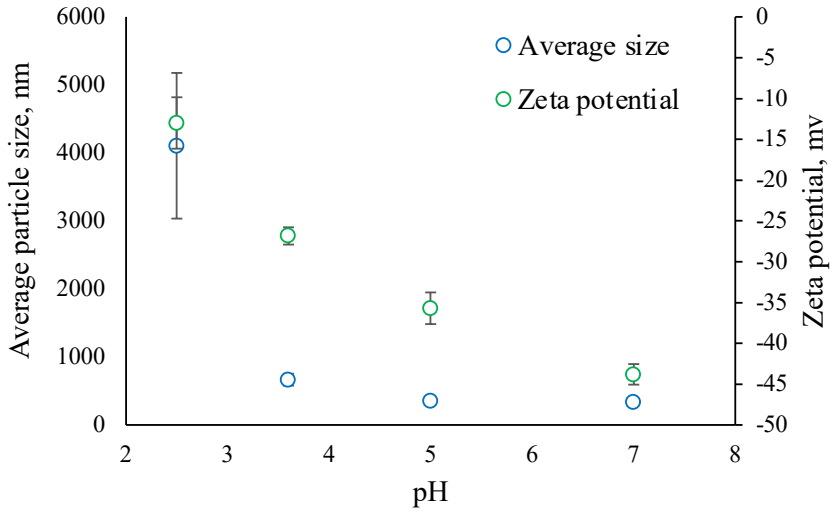

Figure 4.12: Average particle size and zeta potential as a function of pH (2.5, 3.65, 5 and 7) for propolis particles. Data points are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.). Where error bars are not shown, it is because they are too small to be seen.

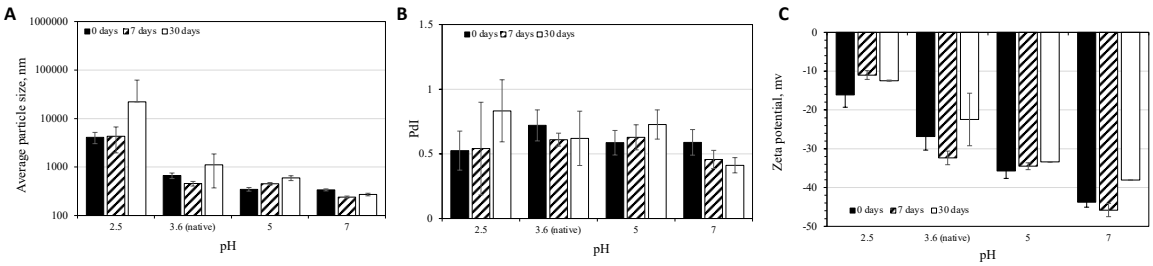

Figure 4.13: A. Average particle size, B. PdI and C. zeta potential as a function of pH for (2.5, 3.65, 5 and 7) and storage period (0, 7 and 30 days). Data points are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

4.3.2.2 Effect of the pH environment on the antibacterial activity of propolis dispersions

The influence of pH was further investigated on the antibacterial activity of the propolis particle aqueous dispersions as, so far, the antibacterial activity of propolis particles aqueous dispersions has only been reported at pH value 3.65 (native)[23]. Propolis dispersions at different concentrations and at varied pH values of 2.5, 5 and

7 were tested against *E. coli* and compared with propolis native pH of 3.65. Results are shown in Figure 4.12.

As it can be seen from Figure 4.13, decreasing the pH environment increased the antibacterial activity of the propolis dispersions drastically. At pH 2.5 even at 50% dilution (Figure 4.13C) the bacteria population was eliminated after an hour. However, increasing the pH decreased the bactericidal rate. At pH 5, there was an approximate 2 log reduction after 18 and 24 hours exposure (Figure 4.13A) which at propolis lower concentrations, was not apparent (Figure 4.13B and C), whereas at pH 7 there was no antibacterial activity present post 24 hours for all propolis concentrations.

The reduced antibacterial activity of the propolis particle aqueous dispersions at higher pH values of 5 and 7 are believed to be due to acidity playing an important role in propolis antibacterial activity. In addition, by changing the pH environment, the compounds responsible could be affected, lowering the efficacy of the natural product. When Ivančajić *et al*. probed the antibacterial activity of propolis against *E. coli* in different solvents with respect to pH, the highest inhibition was determined to be present at the lowest pH value of 6, with propolis being diluted in ethanol, diethyl ether, toluol and chloroform.[63] Similarly Lu *et al*. [64] tested the effect of pH on the antibacterial activity of propolis in 80% ethanol against *S. aureus* also showing the best antibacterial activity at the lowest pH. It is challenging to locate the exact reason behind this activity as propolis is such a complex mixture. Within propolis, Cushnie *et al.* [65] proposed that when galangin (a compound present in propolis) was incubated with sodium carbonate (a base), it formed a salt, resulting in a reduction to its antibacterial activity. This observation could be applied to the propolis system since the dispersion was acidic and sodium hydroxide solution was added to increase the pH. Additionally, the pH can

influence the dissociation of phenolic acids present. Wen *et al*.[66], investigated the antibacterial activity of different phenolic acids including caffeic acid, p-coumaric acid and ferulic acid (which are also present in propolis), establishing that at as the pH value increases, dissociation of the phenolic acids is encouraged and that can diminish their antibacterial activity.

When the water solution with different pH values was tested as a reference (Figure 4.13D), there was no antibacterial activity observed, meaning that in this study just by altering the pH itself does not solely explain the difference presented in the antibacterial activity of the propolis dispersions. In literature water solutions of different pH values has shown antibacterial activity when tested against a range of bacteria. Rahman *et al* [67] investigated its antibacterial activity against *E. coli* as well as numerous other Gram- negative and Gram- positive microorganisms, showing that at pH 2.5 and 4.5 there was evidence against *S. aureus, S. pseudintermedius, and S. intermedius* but not on all the others tested including *E. coli*, agreeing with our results. *E. coli* cells' resistance to the antibacterial activity of acidic water could be due to its capability to sustain pH homeostasis by adjusting its amino acid levels resulting in a reduction of the environmental effect.[68]

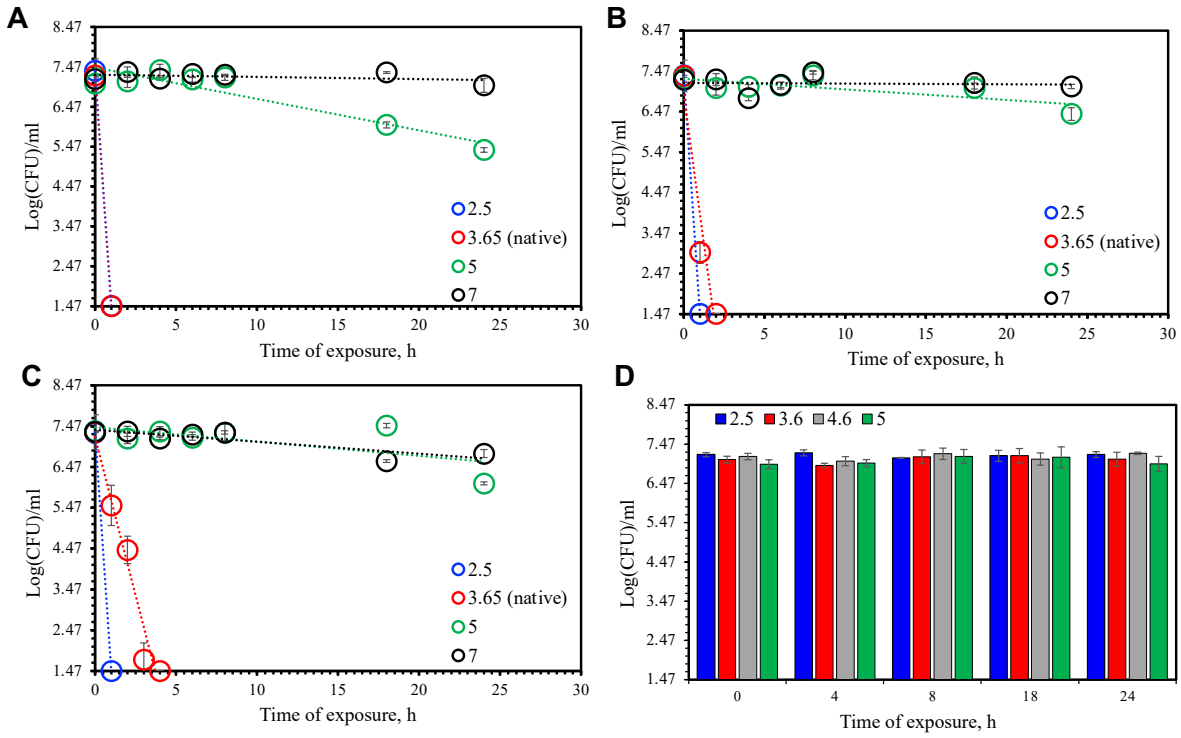

Figure 4.14: Antibacterial activity of aqueous propolis dispersions. Data for the survived population of stationary phase E. coli *MG1655 are presented as a function of propolis mass fractions A. 15%, B.11.25%, C. 7.5% propolis dispersions between immediate exposure and 24 h and pH (2.5, 3.65, 5 and 7). D. Antibacterial activity of water at pH 2.5, 3.65, 5 and 7. Data for the survived population of stationary phase* E. coli *MG1655 are presented as a function of pH (2.5, 3.65, 5 and 7). Data shown are averages (*n = *3; biological replicates) and error bars represent ± one standard deviation (s.d.). The control was deionised water (results not shown).*

4.3.2.3 Effect of propolis dispersions and sodium caseinate on the interfacial tension between water and oil

Proteins have been shown to provide excellent long-term stability in emulsions; by unfolding around the oil droplets and adsorbing on the interface, providing an efficient interfacial barrier with higher viscoelastic properties, indicating better stability.[49] In addition, there is an interest in exploring the prevalence of Pickering

behaviour observed in the case of Tween 80 for proteins, a class of different emulsifiers.

In the present study, sodium caseinate was chosen for investigation.

Sodium caseinate like other dairy proteins, is pH sensitive and the acidity of its environment influences its efficacy as a stabiliser.[69] However, to the best of the author's knowledge the electrostatic interactions between propolis and sodium caseinate haven't been examined. Within previously reported literature, propolis has been investigated in the presence of sodium caseinate through formation of complexes with other components[70],[71], for example Zhang *et al*.[72] enhanced the bioavailability of propolis by encapsulating it in zein/caseinate/alginate nanoparticles. In a similar system, Zembyla *et al*.[73], investigated water-in-oil (W/O) emulsion systems stabilised by phenolic compounds quercetin and curcumin, with whey protein isolate showing enhanced stabilisation over three weeks of storage (than the phenolic compounds alone). They attributed this enhanced stabilisation to the ability of the phenolic compounds and whey protein isolate to form complexes with the protein due to the oppositely charged species as well as hydrogen bonding. Similarly, testing emulsions under different pH values of the dispersed aqueous phase showed that at a pH value of 3, the emulsions were more stable than at pH 7 possibly due to stronger complexation as well as less chemical degradation of the phenolic compounds at neutral pH.

An attempt was made to form propolis particles in the presence of sodium caseinate. However, this resulted in the propolis particles aggregating, forming a continuous solid matrix which adhered to the walls of the vessel, even post sonication. According to the literature that complex coacervation (which results in phase

separation) can occur between oppositely charged proteins and polysaccharides.[74] In the present study, sodium caseinate would take the role of the polymer while propolis can contain polysaccharides in its complex matrix.[75] Guo *et al*.[76] investigated the complex coacervation between whey protein isolate and pectin, with emphasis that phenolic compounds such as tannic acid (also present in propolis[77]) could be used to stabilise the complexation further by functioning as crosslinking agents. Therefore, complex coacervation could explain the favourable interaction between the two species observed.

To investigate this interaction further, the interfacial tension of the dispersion with sodium caseinate at its native pH was measured and compared with a propolis dispersion made without emulsifier. This experiment was conducted to determine if sodium caseinate was successfully incorporated and what was the effect. Measurements of the carrier phases of the two dispersions were also investigated as well as 1% sodium caseinate, without propolis at pH 3.65 and 7 (Figure 4.14).

Figure 4.13 shows that sodium caseinate at pH 7 had a slow adsorption at the interface compared to all the other samples tested and resulted in the lowest IFT value of 2.13 mN/m. Sodium caseinate incorporated at pH 3.65 exhibited a faster adsorption than at pH 7 at the interface and a final interfacial tension of 4.98 mN/m, a significantly higher value than at neutral pH. At a pH of 3.65 the absolute value of the zeta potential of sodium caseinate was smaller than at pH 7 (Figure 4.15), meaning the electrostatic repulsion was reduced, encouraging the protein to form big aggregates, and therefore preventing it from covering the interface as efficiently.

Notably, the carrier phase produced without the aid of sodium caseinate or any other emulsifier resulted in the highest IFT value, higher than the one made with

sodium caseinate (9.61 mN/m vs 7.15 mN/m). The lower IFT value of the carrier phase with sodium caseinate provides evidence of the presence of the protein in the carrier phase responsible for decreasing the interfacial tension further. However, when comparing the whole systems, both dispersions exhibited very similar interfacial tension behaviours (6.34 mN/m vs 6.89 mN/m), revealing that the affinity of the interface was possibly dominated by the particles, explaining the little influence of the carrier phase with sodium caseinate. As it is seen in Figure 4.15, sodium caseinate and propolis particles at pH 3.65, are oppositely charged and therefore would interact electrostatically. These interactions could limit or potentially inhibit the adsorption of sodium caseinate at the interface. However, similarly with the emulsions co-stabilised by Tween 80 and propolis particles, the interfacial tension of all systems was smaller compared to DI water.

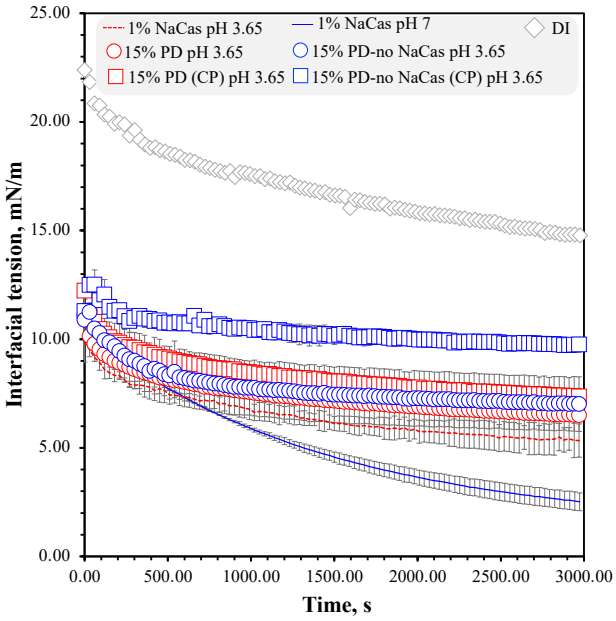

Figure 4.15: Dynamic interfacial tension between sunflower oil and deionised water (DI), 1% and sodium caseinate at pH values of 3.65 (NaCas pH 3.65) and 7 (NaCas pH 7) in DI, 15% propolis particle aqueous dispersions produced at pH 3.65 in the presence of sodium caseinate (15% PD pH 3.65) and

their aqueous carrier phases (15% PD (CP) pH 3.65) and 15% propolis particle aqueous dispersions produced at pH 3.65 in the absence of Tween 80 (15% PD- no NaCas pH 3.65) and their aqueous carrier phases (15% PD-no NaCas (CP) pH 3.65). All data were acquired at 20°C and are averages of three measurements, with error bars corresponding to ± one standard deviation (s.d.).

4.3.2.4 Effect of pH on the zeta potential of propolis particles and sodium caseinate dispersions

Zeta potential titration curves of 1% sodium caseinate, the propolis particle aqueous dispersions, as well as a mixture of the two were carried out. The propolis particle aqueous dispersions were already produced with the aid of Tween 80. Figure 4.15 shows the dependency of the zeta potential on the pH of sodium caseinate and propolis particle aqueous dispersions. The zeta potential of sodium caseinate alone was positive at low pH and as this was increased, it decreased steeply until it reached a plateau, in agreement with literature [78]; the isoelectric point was observed at around pH 4.6. The zeta potential of the propolis particle aqueous dispersions alone was negative even at low pH and decreased gradually as the pH increased. When sodium caseinate was mixed with the propolis particle aqueous dispersion, the titration curve exhibited a similar behaviour to that of sodium caseinate alone. The propolis particles to sodium caseinate resulted in the isoelectric point of the system shifting to approximately 4, implying formation of a complex due to electrostatic interactions.[79]

Figure 4.15 (insert) also depicts the dependency of the pH on the formation of aggregates between propolis and sodium caseinate. As the pH increases from 2.5 to 4, the formation of aggregates is encouraged since the zeta potential approaches zero. Beyond pH 4, the zeta potential decreases, and the electrostatic interactions are enhanced, encouraging the dispersibility of the sample.

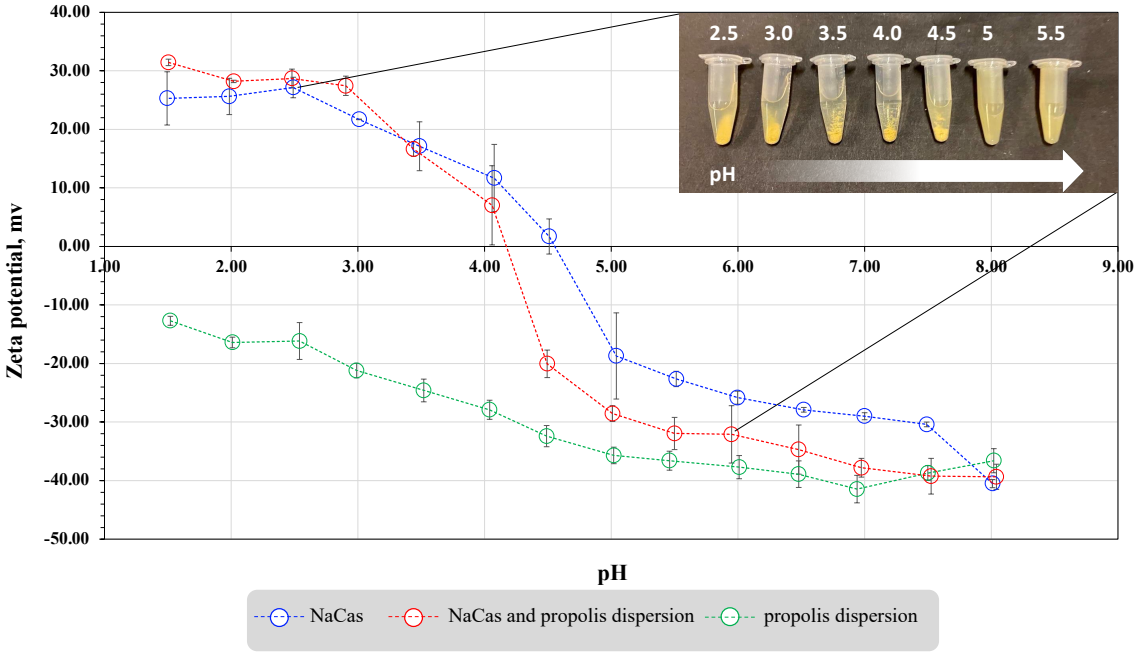

Figure 4.16: Zeta potential as a function of pH for sodium caseinate in water (NaCas), propolis dispersions and sodium caseinate with propolis dispersions mixed (NaCas and propolis dispersion). Data points are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.). Inset figure: Images of the propolis particle aqueous dispersions with sodium caseinate at pH 2.5-5.5 highlighting the aggregation occurring at the mixed system's isoelectric point.

4.3.2.5 Effect of propolis dispersions and sodium caseinate on the stabilisation of O/W emulsions

Aqueous emulsions containing propolis dispersions and sodium caseinate were formulated *via* high shear mixing. As it was shown from Figure 4.15, sodium caseinate's isoelectric point was at pH of 4.6, thus, the pH of the continuous phase of the emulsions was adjusted to 4.6 as this is expected to be the most unstable conditions of sodium caseinate to act as an emulsifier, and propolis effect towards the microstructure of the emulsions will be more pronounced. The emulsions' droplet size was measured and both surface weighted (Sauter, $D_{3,2}$) and volume weighted (de

Brouckere, $D_{4,3}$) mean diameters were reported over a storage period of 60 days while at either 4°C or 25°C (Table 4.6) (Figure also shown in the Appendix, A2.14).

Table 4. 6: Average volume ($D_{4,3}$) and surface ($D_{3,2}$) weighted mean oil droplet sizes (and associate span values) of 15% O/W emulsions produced with either aqueous 15% propolis particle dispersions (Propolis) or pure water (Control) as their continuous (aqueous) phase, in the presence of 1% sodium caseinate (pH 4.6), made *via* HSM. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

Storage Temperature (°C)	Storage Period (days)	Propolis			Control		
		$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	Span
25	0	16.98±2.16	9.48±0.86	1.75±0.31	26.87±4.79	19.45±4.34	1.25±0.11
	1	16.40±1.20	9.09±1.06	1.84±0.46	26.47±1.45	18.91±1.60	1.39±0.12
	14	59.18±5.69	28.73±1.04	2.52±0.16	25.51±2.07	20.31±1.19	1.48±0.18
	30	63.99±3.41	28.77±0.44	2.81±0.21	28.37±0.65	20.44±1.27	1.28±0.07
	60	101.35±20.13	25.99±2.49	3.00±0.79	104.29±2.11	33.03±2.43	2.40±0.02
4	0	16.98±2.16	9.48±0.86	1.75±0.31	26.87±4.79	19.45±4.34	1.25±0.11
	1	15.60±1.27	8.58±1.31	1.57±0.17	26.47±1.45	18.91±1.60	1.36±0.05
	14	16.76±2.96	9.37±1.71	1.69±0.16	27.95±3.02	21.73±3.16	1.27±0.13
	30	13.93±1.00	9.65±0.67	1.40±0.04	38.39±5.66	22.18±2.19	1.48±0.18
	60	33.53±1.83	18.20±1.16	1.60±0.14	45.04±23.16	16.89±1.52	1.06±0.11

Initially emulsions without propolis, possessed an average oil droplet size of 26.87 μm ($D_{4,3}$) and a span value of 1.25. According to all measurable values, emulsions remained stable over a course of a month at 25°C, with a significant increase in oil droplet size after two months (104.29 μm). That increase is most likely due to the absence of electrostatic interactions of the sodium caseinate protein at its isoelectric point, whereas it was discussed previously, the protein forms aggregates and cannot unfold as efficiently around the dispersed phase, leading to flocculation of the oil droplets.[80] Since the protein is not providing a strong barrier, the droplets are more

prone to flocculation or coalescence. At 4°C storage, the stability of emulsions improved with no significant differences among the average sizes and with smaller oil droplets post 60 days storage compared to 25°C (45.04 μm), although still some deterioration at 60 days was evident.

Upon incorporation of the propolis particles, the average oil droplet size ($D_{4,3}$) decreased significantly ($p<0.05$) from 26.87 to 16.98 μm, whereas the width of the distribution was similar and quite narrow (Figure 4.16A). The stability of the emulsions at 25°C was adequate with evidence of coalescence or flocculation after 14 days of storage and beyond which the measurable values were statistically larger compared to the control ($p<0.05$). This was reflected by their distributions (Figure 4.16B) where the peak shifted to larger sizes. Since all systems presented creaming (Figure A2.9) it is further postulated that the mechanism of destabilisation was by flocculation which could lead to coalescence. At 4°C the increase in oil droplet size was much slower, and the propolis emulsion oil droplets remained statistically smaller for 30 days of storage ($D_{4,3}$) or even 60 ($D_{3,2}$) depending on which average is examined. However, the span of the distribution was statistically larger after 60 days of storage. Figures 16C and D depict the evolution of the size distribution of the propolis systems with time for 4°C and 25°C respectively showing in both cases broadening of the distributions with the passing of time.

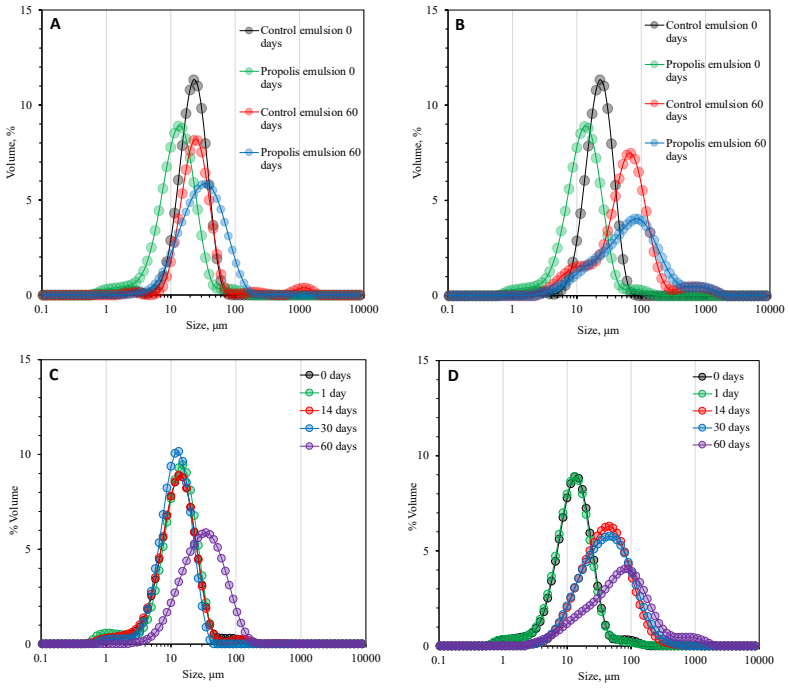

*Figure 4.17: $D_{4,3}$ average droplet size distributions of O/W emulsions containing 15% propolis particles in the aqueous phase or water (control) with 1% sodium caseinate at pH 4.6. Results are presented for different storage periods (0, 1, 14, 30 and 60 days) and different storage temperatures; at 4°C (A, C) and 25°C (B, D). C and D include only the propolis emulsions. Data shown are averages (*n = *3; repeats).*

Due to the possible aggregation of propolis particles, or complexation with sodium caseinate that was observed in previous sections, the surface weighted distributions were plotted as well, as they favour smaller sizes, and any aggregation present would be better observed. Figure 4.17 depicts the surface weighted distributions of the control and propolis emulsions for the different storage temperatures, over a period of 60 days. Unlike in the volume distributions (Figures 4.16A and B) for the control samples, there were two smaller peaks at around 0.88 µm and 2.50 µm, (Figure 4.17A and B). The one at 2.50 µm remains throughout the whole storage period for both temperatures, apart from 60 days at 25°C. Propolis emulsions distributions also contained one peak immediately after production at around 1 µm which was present for a month at 4°C (Figure 4.17C) but was diminished after two

weeks at 25°C (Figure 4.17D). These smaller peaks could correspond to sodium caseinate aggregates or sodium caseinate flocculated with propolis for the controls and propolis emulsions respectively (as they were not present when emulsions were produced with Tween 80). It is important to note that the refractive index included for these measurements was that of sunflower oil (1.467). Sodium caseinate has a different refractive index of 1.57.[81] Therefore, the exact size of the aggregates might differ slightly. Aggregates stopped being detectable over time, more prevalently at 25°C for the propolis emulsions. This was thought to be due to their size increasing over time and precipitating, therefore not being part of the sampling to carry out the measurements.

Another element not apparent from the volume weighted distributions, was the bimodal distribution post 60 days storage at 25°C explaining the increase in the ($D_{3,2}$) average oil droplet size (Figure 4.17B), showing signs of instability of the sodium caseinate emulsions at its isoelectric point as expected. The width of the distributions for both 4°C and 25°C increased with the passage of time, again more rapidly at 25°C.

Overall, emulsions stored at 4°C showed better stability than at room temperature. When the temperature is low, it can slow down coalescence due to a higher viscosity if the continuous phase and an increase of the surface tension of the oil droplets [82] which could be the difference in storage conditions.

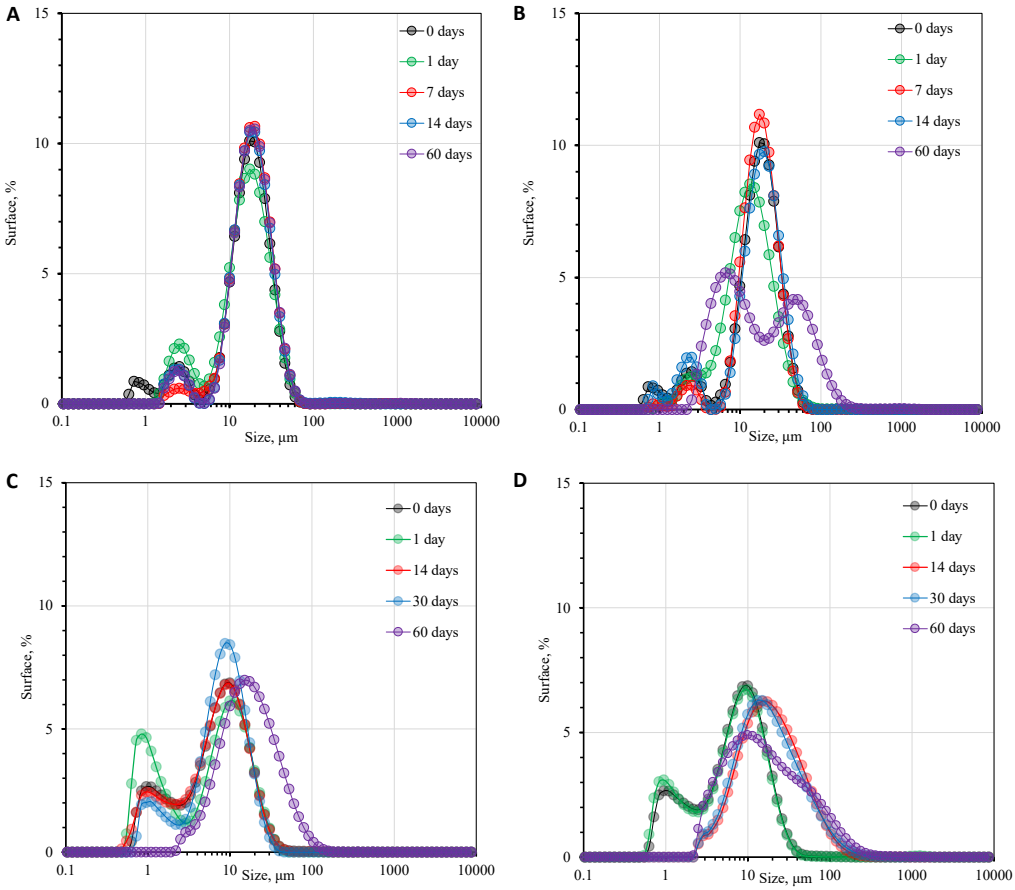

Figure 4.18: $D_{3,2}$ average oil droplet size distributions of O/W emulsions containing water (control) (A and B) or propolis particles in the aqueous phase (C and D) with sodium caseinate at pH 4.6. Distributions are presented for different storage periods (0, 1, 14, 30 and 60 days) and different storage temperatures; at 4°C (A, C) and 25°C (B, D). Data shown are averages (n = 3; repeats).

Interestingly, although propolis emulsions initially produced a smaller average oil droplet size than the control, with the passage of time, they became more unstable, which is surprising since the electrostatic interactions as depicted in Figure 4.15 were stronger compared to purely sodium caseinate (-20.05 vs 1.71). Confocal fluorescence microscopy was employed to give an indication behind this instability, and images of the emulsions are shown in Figure 4.18. At 25°C (Figure 4.18B) the propolis particles have aggregated and are not equally distributed throughout the sample like for the systems stored at 4°C (Figure 4.18A). That could be due to the complexation of the

propolis particles with sodium caseinate which was apparent from Figure 4.15. With time the particles aggregate with the protein and interact less with the oil droplets eventually causing flocculation or coalescence, something that was also evident from the interfacial tension measurements (Figure 4.14). Generally it is known that particle aggregation can often lead to destabilisation of emulsions, dominated by the interactions between dispersed particles.[83]

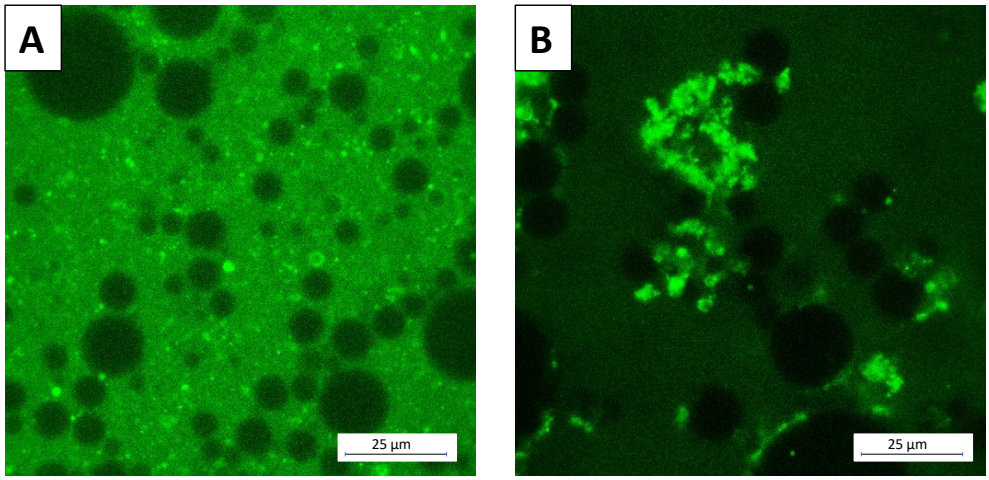

Figure 4.19: Fluorescence confocal images (62 × magnification) of O/W emulsions with propolis dispersions as the continuous phase in the presence of sodium caseinate post 60 days of storage at A. 4°C and B 25°C, excited with an 408 nm wavelength.

4.4 Conclusions

Propolis colloidal particles have been shown to exhibit a level of Pickering functionality in O/W emulsion systems in the presence of a co-stabiliser. Assessment of the propolis dispersions used as the aqueous phase of these emulsions revealed that the colloidal species can lower the oil/water interfacial tension, while their (aqueous) carrier phase is practically deprived of any appreciable surface active content. In addition, the wetting properties of propolis extracts suggest that O/W

emulsion formation would be facilitated. Emulsions co-stabilised by propolis particles and surfactants possessed smaller droplets than their respective controls (surfactant alone), however at higher surfactant or propolis content there was evidence of displacement of the particles from the interface leading to destabilisation of the emulsions. Emulsions co-stabilised by propolis particles and proteins, were found to be prone to changes to their pH environment. An enhancement to the stability of these emulsions exhibited at pH values above the protein's isoelectric point, was unfortunately offset by a reduction to the antibacterial activity of the propolis particles. Overall, propolis particles can indeed provide microstructural support in tandem with antibacterial activity in emulsions with the aid of a co-stabiliser, the choice of which however is crucial for this dual functionality to be successfully exhibited. Further research in this area could pave the way to ultimately deliver to food researchers and formulators a range of natural and multi-functional species/ingredients of immense value and utility.

4.5 References

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Chapter 5- Investigating the oxidative stability, antibacterial activity and microstructural characteristics propolis Pickering-like particles offer into O/W emulsions

Abstract

In recent years Pickering particles have been a popular research topic for the stabilisation of O/W emulsions. However, beyond their capacity for stabilisation, little research has been conducted on the ability of Pickering particles to offer further functionality to an emulsion microstructure, such as to provide or enhance their antibacterial or antioxidant character. One approach to develop such multifunctional colloidal structures is to exploit the emulsion stabilisation (Pickering) functionality of species that already exhibit specific/desired (non-Pickering) physicochemical characteristics. Within the spirit of such approach, the present study builds up on previous research on the antioxidant and antibacterial activity, and Pickering potential of propolis particles and aims to assess the ability of these colloidal species to enhance the oxidative stability and antibacterial activity of oil-in-water (O/W) emulsions. Firstly, control (non-propolis) emulsions were assessed with respect to lipid oxidation, showing that although the oil content and storage temperature influence the rate of hyperoxide production, oil droplet size did not cause a significant effect. Subsequently propolis emulsions were formulated and their lipid oxidation was measured by altering key parameters such as: droplet size, propolis and oil content, as well as pH. It was established that propolis significantly supressed lipid oxidation in emulsion systems compared to the controls tested. Propolis emulsions also exhibited efficient antibacterial activity, eliminating the bacteria population, although their rate of action was slower than when simply present in aqueous dispersions. Finally, it was concluded that incorporation of propolis particles increased the viscosity of the emulsions. Overall, propolis particles showed significant promise as multifunctional colloidal species in O/W emulsions, and the present study offers clear understanding to researchers and

formulators in the area in terms of maximising the potential of these structures upon usage.

5.1 Introduction

In recent years there has been a renewed consumer drive to adopt healthier lifestyles.[1] This includes a variety of nutritional and/or dietary changes such as moving away from synthetic additives[2], animal products[3] or replacing saturated fats with healthier unsaturated options[4].

In turn this trend has presented food formulators with a number of challenges, For example, even though healthier for human consumption, replacing saturated with unsaturated fats, can make products more prone to lipid oxidation.[5] Lipid oxidation is a free radical chain reaction that can alter lipid containing products' taste, appearance and even produce toxic compounds. Hence, several synthetic additives such as butylhydroxyanisole (BHA), or propyl gallate (PG)[6], are added in products to inhibit lipid oxidation and prolong the self-life of products.

In a similar manner, especially water-based products, are subject to bacterial spoilage, which can also in turn lead to instability and undesirable sensory effects, and cause damage to health if consumed. Common antibacterial additives used to tackle bacteria spoilage are nitrates, benzoates and sorbates.[7] Specifically in emulsion systems, a microstructural approach can also result to an antibacterial effect being exhibited. Due to their specific architecture, nanoemulsions have been shown to possess antibacterial functionality against a range of both Gram- positive and Gram-negative bacteria, and even against prokaryotic cells by disrupting their membrane.[8]

Despite this, the majority of research in the emulsions area focuses on the use of natural actives as alternatives (to the more conventional synthetic species) in order to assess their preserving properties. For instance Noon *et al.*[9], examined the inhibition of lipid peroxidation arising from a range of natural actives such as quercetin, rutin and curcumin which were added in the continuous phase of O/W emulsions, exhibiting efficient oxidative stability. Djokhdem et al.[10] incorporated essential oil from the *Juniperus phoenicea* in an O/W emulsion, which protected soft cheese against *E. coli* and *S. aureus* in 4°C storage.

In terms of the physical stabilisation of emulsions, the use of Pickering particles has been gaining significant momentum even in the area of foods.[11] Pickering particles are colloidal species that place themselves in the oil/water interface providing a strong barrier against emulsion instability phenomena. They have the advantage of being irreversibly adsorbed at the interface, and therefore being highly resistant towards coalescence.[12] At their colloidal state, natural species have also been reported to exhibit the potential to act like Pickering stabilisers. For instance, Hu *et al*.[13] reported that gliadin, a water and oil insoluble protein from cereal, formed Pickering emulsions with stability that could be manipulated *via* changes to pH conditions. Chitin, the second most commonly found natural polysaccharide after cellulose[14], is another such example. Tzoumaki *et al*.[15] demonstrated that chitin crystal nanoparticles could stabilise O/W emulsions over the course of a month, also providing a gel-like inter-droplet network which was assessed *via* rheology.

Interestingly, enough number of natural species reported to possess Pickering functionality, have been shown (in separate studies by different authors) to also offer antibacterial and antioxidant stabilisation. For instance, Luo *et al*.[16] reported that the

flavonoids rutin, naringin and tiliroside can provide stable Pickering emulsions, and Duffus *et al*.[17] even confirmed this (in the case of rutin hydrate) in double emulsions[18]. However, the antibacterial and antioxidant activities of these flavonoids have also been established elsewhere in literature.[19],[20],[21] Similarly, phenolic compounds such as curcumin and quercetin have also been separately reported to provide Pickering stabilisation[22] as well as possessing antibacterial and antioxidant functionalities[23]. Elsewhere phenolic compounds such as gallic and tannic acid when used in tandem with whey protein isolate, produced stable emulsions of smaller average droplet sizes and enhanced oxidative stability.[24] It is worth noting that both acids have also been reported to exhibit antibacterial activity, albeit by different authors.[25],[26],[27]

Therefore, there is a significant potential for such natural colloidal species to exhibit/demonstrate a dual functionality in emulsions, where they not only offer enhanced microstructural performance, but also 'fortify' these two-phase assemblies in terms of antibacterial and antioxidant stabilisation. Propolis is such one compound, a product made by honeybees by collecting different substances from local plants and exudates, and it is used to strengthen the structure of the hive.[28] It has multiple beneficial properties such as antibacterial, antioxidant, antiviral etc.[29] It is a very complex mixture, where its antioxidant and antibacterial activities among others have been attributed to phenols and flavonoids[30] such as rutin, gallic acid or naringin[31]. Propolis so far has shown both antibacterial and antioxidant activities when encapsulated in emulsion systems. [32],[33],[34] Work by the present authors, has shown that propolis colloidal particles exhibit a significant antibacterial activity when present in simple aqueous dispersions (Chapter 3), and also have an affinity for the O/W

interface, can lower the oil/water interfacial tension, and overall possess a Pickering-like functionality in emulsions (Chapter 4) when assisted by a co-stabiliser (Tween 80).

The present study aims to demonstrate that these features can be expressed in tandem, and therefore provide clear evidence that propolis particles can indeed be utilised as multifunctional natural colloidal species in O/W emulsions. More specifically, this work focuses on the oxidative and antibacterial stability that propolis particles can offer in O/W emulsions, while also assesses their effect on the rheological behaviour of these systems. The oxidative stability of propolis emulsions was investigated with reference to key formulation parameters such as oil content, oil droplet size and storage temperature, and compared to that of control (non-propolis) emulsions to then select the appropriate experimental conditions for further investigation. Subsequently, upon confirming that control emulsions did not have an effect on bacterial populations, the antibacterial activity of the propolis emulsions was studied in systems with varied oil and co-stabiliser content, as well as different oil droplet sizes. Finally, the viscosity of the propolis emulsions was characterised and compared with that of the control systems. Overall, the present work demonstrates that propolis particles can indeed offer both Pickering stabilisation and oxidative and antibacterial stability to O/W emulsions. The present work additionally offers valuable and formulation-specific insight to researches and formulators in the area in terms of maximising the potential of these structures upon usage.

5.2 Materials and Methods

5.2.1 Materials and propolis samples

Crude propolis was collected from Fthiotida region, Greece during spring 2019, purchased from ANEL (Thessaloniki, Greece) and was stored in the dark at -20°C. Ethanol (Absolute,99.8%, analytical reagent grade), Nutrient agar (NA), and Phosphate Buffered Saline (PBS) were purchased from Fisher Scientific (Loughborough, UK). Luria Bertani broth (LB), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), iron (III) chloride, 1,1,3,3-tetraethoxypropane (TEP), Tween 80, Sodium caseinate, Sodium hydroxide (1 M), Hydrochloric acid (1M), were purchased from Sigma-Aldrich Ltd (Gillingham, UK). Iron (II) chloride tetrahydrate was purchased from Across Organics (New Jersey, USA). Methanol was purchased from Merck (Darmstadt, Germany). Butanol was purchased from SLS (Nottingham, UK). Ammonium thiocyanate was purchased from Honeywell (Spain). Sunflower oil was purchased from a local supermarket.

5.2.2 Microbial culture

E. coli K12 (MG1655) cells were stored on Luria Bertani agar at 4°C. To acquire cells in their stationary phase, a single *E. coli* colony was transferred into 50 ml of LB, incubated at 37°C for 18 hours while shaking at 150 rpm. The population of cultures grown overnight was fixed at approximately 1.5 x 10^7 CFU/ml. The microbes were centrifuged for 3 minutes at 4600 *g* (Eppendorf Centrifuge 5430, Germany) and washed three times with PBS.

5.2.3 Preparation and characterisation of propolis samples

5.2.3.1 Preparation of propolis particle aqueous dispersions

Propolis extraction and preparation of the dispersions is described in previous publication.[35] In the present study propolis particle aqueous dispersions at 15% propolis mass fraction were prepared with approximately 2% Tween 80.

5.2.3.2 Formulation of propolis and control emulsions

Oil-in-water (O/W) emulsions were prepared containing either 15% or 25% sunflower oil phase and 84% or 74% aqueous phase (including the aqueous particle propolis dispersions or just water in case of the controls). 0.5 or 1% Tween 80 and 1% Sodium Caseinate (NaCas). The pH of the emulsions was adjusted *via* titration of either 1 M of HCl or NaOH with a pH meter (Mettler Toledo, Leicester, UK). Two processing methods were employed; using a high shear mixer (HSM) (Silverson L5M, Silverson Machines Ltd, UK) with an emulsion screen of 25 mm diameter for 2.5 minutes at 7500 rpm, and an ultrasonic vibracell processor with a 13 mm probe (Sonics & Materials, Inc., CT, USA) for 30 seconds with having a pause for two seconds every four seconds.

5.2.3.3 Oil droplet size analysis

Propolis and control emulsions' average droplet size was obtained using a Malvern Mastersizer MS 2000 (Malvern Panalytical, Malvern, UK), with a Hydro SM manual volume sample dispersion unit. The refractive index values required by the software to determine the droplet size were 1.33 and 1.467 for water and sunflower oil respectively. The sample was dispersed in distilled water at 1300 rpm until an obscuration value of 4-5% was achieved. The surface (Sauter, $D_{3,2}$) weighted mean diameter was obtained.

5.2.3.4 Confocal fluorescence microscopy

Propolis emulsion containing 15% propolis mass fraction, 15% oil content and 1% Tween 80, produced *via* HSM was imaged using a Leica DM2500 confocal microscope (Leica, CH). Objective lense of 63× magnification was used with immersion oil. A laser at 532 nm wavelength was used at 100% intensity to excite the samples and emissions at the range of 200-800 nm wavelength were detected for imaging.

5.2.3.5 Peroxide value assay

The peroxide value assay detects primary oxidation products, usually hyperoxides as a result of auto-oxidation[36], and was adapted from Papotti *et al*.[37] Emulsions were stored at 40°C to speed up lipid oxidation. 20 µl of emulsions was mixed with 2.975 ml of a 2:1 methanol/butanol solution (v/v), 25 µl of 0.2 M HCL, 15 µl of 3.094 M ammonium thiocyanate solution in water and 15 µl of 72 mM iron(ii) chloride solution in water. The mixture was left for 20 min and the absorbance was measured at 510 nm using a UV-vis spectrophotometer (Jenway Genova Bio life science, Cole-Parmer, UK). A calibration curve was constructed (y=0.1554x-0.0455, R^2=0.9993) using iron (iii) chloride as a standard, at a concentration range of 0.94-15 µM. Results were expressed as hyperoxide concentration (µM). To eliminate the effect of the surface area on lipid oxidation, the hyperoxide concentrations were divided over the total surface area of the droplets. The total surface area was calculated according to equations (5.1)-(5.3).[38]

$$A_s = N_d \times SD_{3,2} = \frac{6\varphi}{D_{3,2}} \quad (5.1)$$

$$SD_{3,2} = 4\pi(\frac{D_{3,2}}{2})^2 \quad (5.2)$$

$$N_d = \frac{\varphi}{\frac{3}{4}\pi(\frac{D_{3,2}}{2})^3} = \frac{6\varphi}{\pi(D_{3,2})^3} \quad (5.3)$$

where A_s is the total surface area, N_d is the number of oil droplets per 100 g of emulsion, $SD_{3,2}$ is the surface of one droplet, φ is the oil fraction and $D_{3,2}$ is the mean oil droplet size

5.2.3.6 TBARS assay

The TBARS reactive substances assay is used to detect the secondary lipid oxidation product, malonaldehyde, which usually comes from polyunsaturated fatty acids.[39] The method was adapted from Papotti *et al*.[37] 100 µl of emulsions was mixed with 1.9ml of distilled water and 4ml of TBA solution. Samples were vortexed and heated in boiling water for 30 min. Subsequently. they were let to cool for 20 min and were centrifuged (Eppendorf Centrifuge 5810, Germany) for 15 min at 1000 rpm. The absorbance was measured at 532 nm using a UV-vis spectrophotometer (Jenway Genova Bio life science, Cole-Parmer, UK). A calibration curve was constructed ($y=0.4433x+0.0546$, $R^2=0.9955$) with TEP as a standard, at a concentration range of 0.12-3.91 mM. Results were expressed as TBARS (mM).

5.2.3.7 Viscosity measurements

Viscosity measurements of the propolis and control emulsions, were performed using Kinexus Pro Rheometer (Netzsch GMBM, Selb, Germany), with a parallel sandblasted plates, 60 mm diameter (1 mm gap), serrated plates, 60 mm diameter (1 mm gap), cup and bob, 25 mm bob diameter (62.5 mm gap) and double gap, 25 mm diameter (59.5 mm gap) using a shear rate profile from 1 to 100 s^{-1} at 20°C.

5.2.4 Assessment of biological properties of samples

5.2.4.1 Antibacterial assay

The antibacterial activity of the formulations was determined by using a dilution assay in a 96-well microplate format. The samples were diluted by half and quarterly with sterilised deionised (DI) water and then mixed with *E. coli* cells in a 1:1 ratio. They were incubated at 37°C while at constant shaking at 150 rpm and plated at appropriate time points from 0 up to 24 hours. DI water was used as a control.

5.2.4.2 Plate counting (CFU)

Viability of bacteria after treatment was assessed by serial 10-fold dilutions in PBS. 10 µl were measured and plated in NA plates and incubated overnight at 37°C. Colony Forming Units (CFU) were counted at the appropriate dilution (3-30 colonies) to determine cell viability. The lower limit of detection was 30 CFU /ml.

5.2.5 Statistical analysis

Experiments were conducted in triplicate. Results are expressed as averages and error bars represent the standard deviation. Statistical analysis was performed using IBM SPSS Statistics software. Results were compared with one way ANOVA (Tukey's test). Antibacterial results were fitted to Kaplan-Meier survival plots and compared with a Log-rank test. Differences were considered significant at $p<0.05$. Lipid oxidation curves were fitted *via* MATLAB's curve fitting tool.

5.3 Results

5.3.1 Oxidative stability of propolis emulsions

5.3.1.1 Screening of different parameters to investigate the oxidative stability of control emulsions.

Prior to testing the incorporation of propolis particle aqueous dispersions in the emulsion systems and measure the production of lipid oxidation occured, a study testing different parameters that could influence the formation of hyperoxides, such as different oil content and droplet size, was carried out. It has been suggested previously that increasing the oil content would result in higher lipid oxidation due to the higher concentration of lipids [9], as well as due to the increase in the proximity of the oil droplets resulting in a greater probability of pro-oxidant species to initiate and propagate lipid oxidation[40]. It is also believed than the interface between water and oil which is increased with smaller oil droplet size, is one of the main components influencing oxidative stability as the water phase can contain prooxidants such as metals or radicals, initiating the lipid oxidation.[41] Emulsions consisting of 15%, 25% and 40% sunflower oil were formulated *via* HSM (high shear mixer) or sonication (son), to achieve different droplet sizes, were tested (some data were taken from Chapter 4). The average oil droplet size ($D_{3,2}$) was measured, and the total surface area of the oil droplets was calculated and presented in Table 5.1.

Table 5.1: Mean droplet size ($D_{3,2}$), surface area, final concentration of hyperoxides after 10 days storage at 40°C and rate of lipid oxidation of oil-in-water emulsions stabilised with 1% Tween 80. Emulsions were formulated with 15%, 25% and 40% sunflower oil and processed *via* sonication or HSM. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Processing method	Oil fraction (%)	Size (μm)	Total surface area of droplets (μm^2)	Hyperoxide Concentration @ day 14 (μM)	Rate of Lipid Oxidation (μM/days)
Son	15	0.74±0.01	121.48±2.34	5.82±0.19	0.56±0.18
	25	0.80±0.04	188.26±9.54	8.13±0.29	0.62±0.07
	40	1.31±0.18	186.49±24.20	9.97±0.24	0.77±0.22
HSM	15	9.08±1.67	10.22±1.68	6.67±0.61	0.56±0.15
	25	9.13±0.02	16.43±0.03	8.82±0.38	0.76±0.11
	40	13.28±1.40	18.26±1.88	7.54±0.44	0.69±0.45

Table 5.1 establishes that there is a significant difference ($p<0.05$) in oil droplet size arising from the processing, independently of oil content. Within the same processing, different oil contents were compared showing that the oil droplet size of the 40% oil content emulsions was significantly larger than the 15 and 25% oil content emulsions ($p<0.05$). The total surface area of the droplets was also statistically significant depending on the processing as expected. Through the same processing, in both cases the total surface area of the 15% oil containing emulsion was statistically smaller ($p<0.05$) than from the 25% and 40%.

The emulsions were subsequently stored at 40°C and samples were taken every two days over the period of 10 days to measure the concentration of hyperoxides. The data obtained are presented in Figure 5.1A. As the lipid oxidation can take place on the interface between the oil and the water, the total surface area could influence the result. In order to diminish the influence, the hyperoxide concentrations were divided by the total surface area of the oil droplets (Table 5.1) and results are shown in Figure 5.1B. Attempt to monitor the concentration of secondary oxidation products of control emulsions using the TBARS method[42] was also carried out but preliminary results showed that the production was very slow even at 60°C post

two months storage (data not shown) so the experimental efforts were focused on primary oxidation products alone.

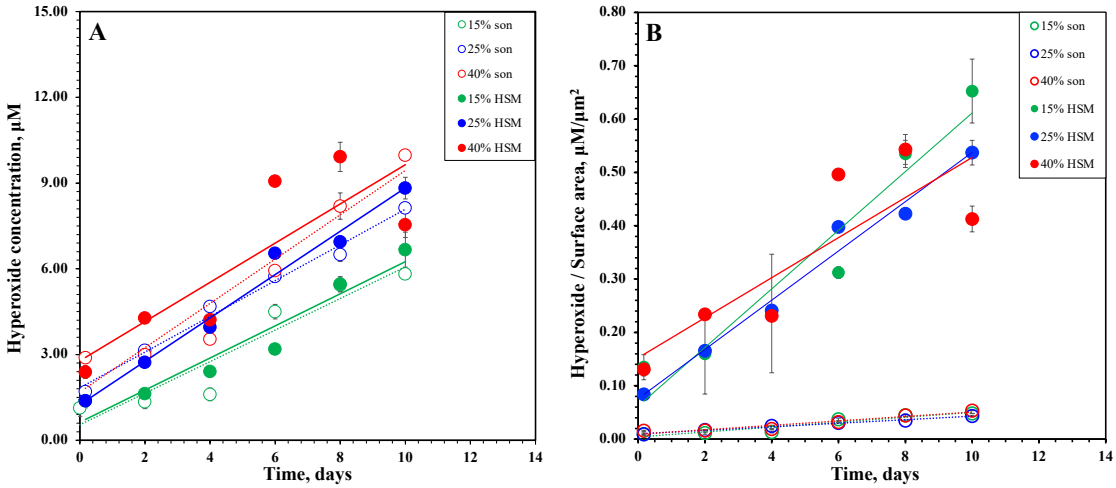

Figure 5.1: A. Effect of processing (HSM and sonication) and oil content (15%, 25% and 40%) of emulsions formulated with sonication (son) and HSM, and 1% Tween 80 on the production hydrogen peroxides generated for a maximum 10-day period at 40 °C and B. Normalised hydrogen peroxide production to total oil droplets surface area. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Figure 5.1A shows the results of the production of hydrogen peroxides depending on the oil content of the emulsions or the oil droplet size arising from different processing. The 15% oil containing emulsions produced less hyperoxide concentration compared to the other emulsions ($p<0.05$) independent of the processing. This was also reflected by rates of lipid oxidation, where the 15% oil emulsions had a slower rate than the rest (Table 5.1).

Concerning the HSM emulsions, at day 10, the 25 and 40% oil ones exhibited a statistically similar concentration of hyperoxides, whereas in the sonicated emulsions all were statistically different from each other, increasing with increasing oil content.

Comparing within the same oil content, only the 40% oil emulsions presented a significant difference with the sonicated emulsions having a statistical higher hyperoxide production ($p<0.05$).

Figure 5.1B shows that for the same oil content, the hyperoxide concentration is dramatically less in the sonicated emulsions once it is calculated per total surface area of the oil droplets. That could be due to an enhanced production of hyperoxides in the case of the HSM emulsions, due to creaming accelerating the lipid oxidation. *Gohtani et al.*[43] made a similar observation and claimed that this could be due to the larger oil droplets being closer-packed at the surface of the emulsion once they cream, accelerating the chain reaction, noting that their emulsions were stored in a static state, similarly with the present study.

Additionally, the reaction was tested under 60°C storage. In this case, emulsions with the highest oil content of 25 and 40% were chosen for further investigation where the oxidation is expected to be increased. They were formulated with 1% Tween 80 *via* HSM or sonication and compared over the period of 10 days. Table 5.2 presents the hyperoxide concentration at day 10, and the rate of lipid oxidation. Figure 5.2A presents the concentration of hyperoxides measured for all the days tested and Figure 5.2B the normalised hydrogen peroxides to total droplet surface area.

Table 5.2: Final concentration of hyperoxides after 10 days storage at 60℃ and rate of lipid oxidation of oil-in-water emulsions stabilised with 1% Tween 80. Emulsions were formulated with 25% or 40% sunflower oil and processed *via* sonication (son) and HSM. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Processing method	Oil fraction (%)	Hyperoxide Concentration @ day 14 (μM)	Rate of Lipid Oxidation (μM/days)
son	25	20.03±0.25	2.09±0.40
	40	30.94±1.36	2.93±0.72
HSM	25	26.56±0.64	2.61±0.36
	40	28.05±0.83	2.84±0.82

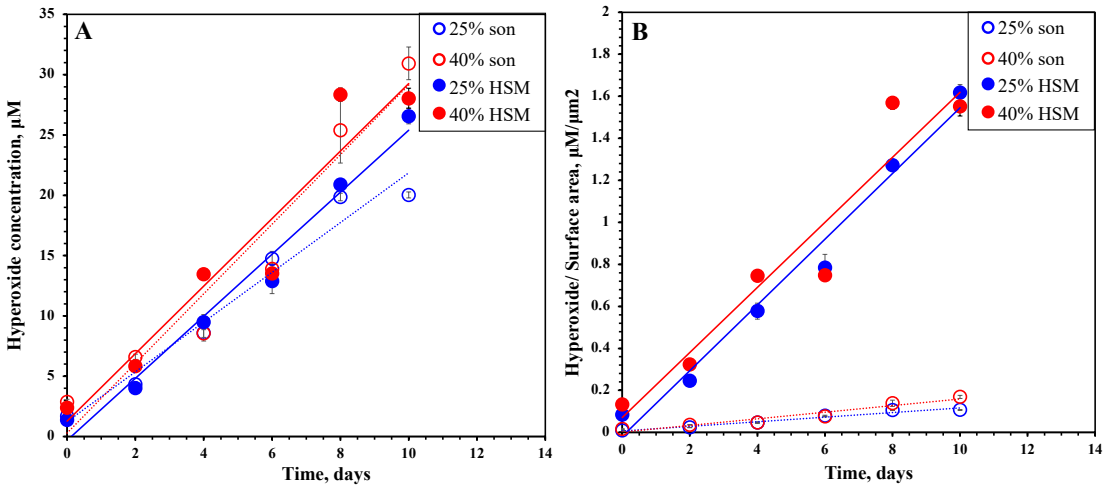

Figure 5. 2: A.Effect of processing (HSM and sonication) and oil content (25% and 40%) of emulsions formulated with sonication and HSM, and 1% Tween 80 on the production hydrogen peroxides generated for a maximum 10-day period at 60 °C and B. Normalised hydrogen peroxide production to total oil droplets surface area. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Figure 5.2A shows that the lipid peroxidation production among the different emulsion systems remained proportional with time at 60 °C storage. For both sonicated and HSM emulsions the concentration of peroxides produced from the 25% oil content was statistically smaller than the 40% oil content (Table 5.2). However, the rate of lipid oxidation was similar across all samples (Table 5.2).

Interestingly, the 25% oil content sonicated emulsions produced less hydrogen peroxides at day 10 than the HSM ones. That is again believed to occur due to

creaming. Additionally, in literature, mixed results have been reported on the effect of oil droplet size on the amount of lipid peroxidation occurring in emulsion systems. [42], [44], [45], [46] Figure 2B depicted similar results with Figure 1B; a dramatic increase in the lipid oxidation per total surface area in the HSM emulsions, that could also stem from the creaming of the oil droplets.

The concentration of hydrogen peroxides produced at 60°C storage was statistically larger in all emulsions tested ($p<0.05$), compared to those stored at 40°C, a difference also reflected by the rate of lipid oxidation. This was expected as this method is temperature sensitive and higher temperatures are often used at to accelerate the production of hydrogen peroxides.[47] Since sufficient difference was detected at 40°C storage and there was no need to further stress the system at higher temperatures, this temperature was chosen for further investigation.

5.3.1.2 Investigating the effect of processing and oil content on the oxidative stability of propolis emulsions

Following the screening of different parameters to investigate the occurrence of lipid peroxidation in control emulsions, propolis emulsions were fabricated with propolis aqueous dispersions as the continuous phase. Propolis particles have shown to possess a Pickering-like functionality by surrounding the oil droplets when being included in the processing (Chapter 4), as it is also depicted in Figure 3 (propolis emulsion with 15% oil and 1% Tween 80, formulated *via* HSM). With direct relevance in offering oxidative stability to emulsions, propolis or its constituents has been identified to inhibit the formation of both primary and secondary products in O/W emulsions[37],[48], in one instant acting even better than some synthetic antioxidants such as BHA or BHT.[49]

Initially, different ways of processing were compared to achieve different oil droplet sizes (HSM and sonication). Emulsions also contained 1% Tween 80. The oil droplet sizes of the emulsions, the total surface area, the hyperoxide concentration post 14 days storage at 40°C and the rate of lipid oxidation production are presented in Table 5.3.

Table 5.3: Mean droplet size ($D_{3,2}$), surface area, final concentration of hyperoxides and rate of lipid oxidation after 14 days storage at 40℃ of oil-in-water emulsions stabilised with 1% Tween 80. Emulsions were formulated with 15% sunflower oil and processed *via* son and HSM. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Sample	Processing method	Size (μm)	Total surface area of droplets (μm^2)	Hyperoxide Concentration @ day 14 (μM)	Rate of Lipid Oxidation (μM/days)
Propolis	son	0.71±0.00	127.12±0.51	1.41±0.13	0.05±0.03
	HSM	2.76±0.41	33.29±4.90	0.99±0.09	0.01±0.02
Control	son	0.74±0.01	121.48±2.34	7.84±0.09	0.56±0.18
	HSM	9.08±1.67	10.22±1.68	6.93±0.11	0.56±0.15

The 15% oil containing propolis emulsions formulated with HSM possessed an average droplet size of 2.76 μm which was significantly larger ($p<0.05$) than the sonicated propolis emulsion with an average droplet size of 0.71 μm ($D_{3,2}$). (Chapter 4) The same trend was followed with the total surface area. The concentration of hyperoxides leading up to two weeks as well as the estimation per total surface area are presented in Figure 3.

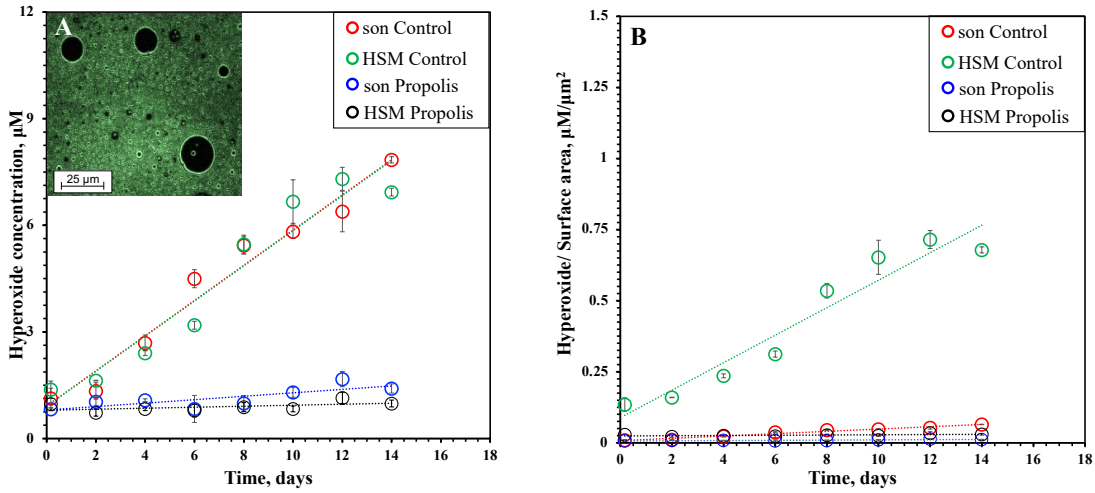

Figure 5.3: A. Effect of processing (HSM and sonication) of propolis and control emulsions formulated with sonication and HSM, 15% oil and 1% Tween 80 on the production hydrogen peroxides generated for a maximum 14-day period at 40 °C and B. Normalised hydrogen peroxide production to total oil droplets surface area. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.). Inset figure: Confocal fluorescence images of 15% propolis emulsions upon production containing 15% oil and 1% Tween 80 produced via HSM (63× magnification) excited with a 532 nm wavelength (Chapter 4).

Figure 5.3A shows the production of hyperoxides from emulsions consisting of different oil droplet sizes arising from their processing. Similar with the control emulsions, the different oil droplet size did not result in significant difference in final concentration of hyperoxides. However, unlike with the control emulsions, the storage time did not influence the production of lipid peroxidation in both propolis emulsions due to the presence of propolis supressing dramatically the lipid oxidation, as it can be seen from the much smaller rates in Table 5.3. Figure 5.3B highlights propolis ability to inhibit lipid oxidation in case of the HSM emulsions for the whole period of 14 days, even though creaming also took place (Chapter 4).

Subsequently, the oil content of the emulsions was altered and tested as it caused a significant difference in the control emulsions tested earlier. The oil droplet size of the emulsions and the controls, as well as the calculated total surface area, the final hyperoxide concentration measured at day 14 and the rate of lipid oxidation are presented in Table 5.4.

Table 5.4: Mean droplet size ($D_{3,2}$), surface area, final concentration of hyperoxides and rate of lipid oxidation after 14 days storage at 40°C of oil-in-water emulsions stabilised with 1% Tween 80. Emulsions were formulated with 25% sunflower oil and processed *via* HSM. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Sample	Oil content, %	Size (μm)	Total surface area of droplets (μm^2)	Hyperoxide Concentration @ day 14 (μM)	Rate of Lipid Oxidation (μM/days)
Propolis	15	2.76±0.41	33.29±4.90	0.99±0.09	0.01±0.02
	25	4.09±0.60	32.11±0.94	4.32±0.22	0.13±0.05
Control	15	9.08±1.67	10.22±1.68	6.93±0.11	0.56±0.15
	25	9.13±0.02	16.43±0.03	8.82±0.38	0.76±0.11

From Table 5.4 it can be seen that the 25% oil propolis emulsions have an average droplet size of 4.09 μm ($D_{3,2}$) which were significantly larger ($p<0.05$) than the 15% oil HSM propolis emulsions. (Chapter 4) The propolis emulsion containing 25% oil yielded a higher amount of hydrogen peroxide products than the 15% oil one. However, the rates of lipid oxidation were similar between the two oil contents and both propolis emulsions produced fewer lipid peroxides than their respective controls. In order to clarify the trend, concentration of hyperoxides were plotted against the appropriate sampling dates and presented in Figure 5.4A.

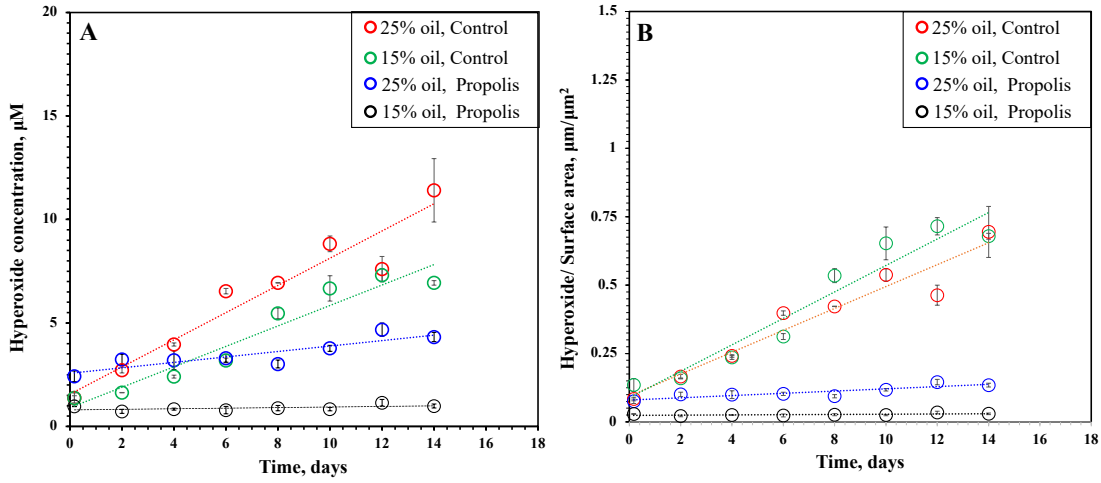

Figure 5.4: A. Effect of oil content (15% and 25%) of propolis and control emulsions formulated with HSM and 1% Tween 80 on the production hydrogen peroxides generated for a maximum 14-day period at 40 °C and B. Normalised hydrogen peroxide production to total oil droplets surface area. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Figure 5.4A shows the production of lipid peroxides from control and propolis emulsions fabricated with different oil content. Propolis emulsions followed a similar pattern with the controls, as there was a significant difference between the different oil contents. In the 25% oil propolis emulsion, even though the final concentration of hyperoxides was larger than the 15% oil content propolis one, the slope was reduced, also exhibiting efficient suppressing of lipid oxidation and showing the excellent antioxidant efficacy of propolis. This was reflected in Figure 5.4B where the difference in lipid oxidation between the 15% and 25% propolis emulsions decreased once the total surface area was considered. Additionally, normalisation of the data highlights the difference between the propolis and control emulsions containing 25% oil, emphasising the ability of propolis to offer oxidative stability.

Propolis' antioxidant activity (while it was dissolved in appropriate solvents) has been well investigated in the past, by a variety of different assays which can clarify the mechanism of its action.[37] Notably, the antioxidant activity of propolis has been reported to proceed by reducing ferric cations and chelating ferrous cations[50], which if both occur in an emulsion system, could promote the initiation or propagation steps of lipid peroxidation [51]. Propolis has also been shown to scavenge species such as hydrogen peroxides, superoxide anions, and hydroxyl radicals (intracellularly)[52], which could directly promote the initiation of lipid peroxidation. Specifically within this system, in previous publication, the propolis particle aqueous dispersions that were used as the continuous phase in the emulsions, were able to act as a hydrogen donors to the DPPH free radical and succeeded in reducing the Folin -Ciocalteu reagent[53]; an oxidation reaction which has shown to occur mainly due to propolis' phenolic compounds[54]. Additionally, as it is shown in Figure 5.3 and reported in Chapter 4, propolis exhibits Pickering-like functionality as it surrounds the oil droplets creating a thick layer. It is believed that the thicker the protective layer around the oil droplets, the more protected the interface is from prooxidant metals in the aqueous phase that can initiate lipid oxidation.[55] To the best of the authors' knowledge this is the first time propolis has suppressed lipid peroxidation in the form of particles, in emulsion systems.

5.3.1.3 Investigating the effect of propolis mass fraction, pH and type of surfactant on the oxidative stability of propolis emulsions

As it has been discussed previously, lipid peroxidation has shown to be related to the interface or the amount of lipids in the emulsions, and propolis was established to play a major role in the system's oxidative stability. The dependency of the mass fraction of propolis on the lipid oxidation was subsequently tested. Emulsions were

formulated with 0 (control), 3.75%, 7.5% and 15% propolis mass fraction, 15% oil and 1% Tween 80 *via* HSM. The emulsions' oil droplet size, as well as the calculated total surface area, the final hyperoxide concentration measured at day 14 and the rate of lipid oxidation are presented in Table 5.5.

Table 5.5: Mean droplet size ($D_{3,2}$), surface area, final concentration of hyperoxides and rate of lipid oxidation after 14 days storage at 40°C of oil-in-water emulsions stabilised with 1% Tween 80. Emulsions were formulated with 0 (control), 3.75, 7.5 and 15% propolis mass fraction,15% sunflower oil and processed *via* HSM. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Propolis content, %	Size (μm)	Total surface area of droplets (μm^2)	Hyperoxide Concentration @ day 14 (μM)	Rate of Lipid Oxidation (μM/days)
0	9.08±1.67	10.22±1.68	6.93±0.11	0.56±0.15
3.75	5.80±1.13	15.29±3.06	5.09±0.15	0.33±0.05
7.5	5.04±0.95	18.43±3.12	2.86±0.31	0.13±0.06
15	2.76±0.41	33.29±4.90	0.99±0.09	0.01±0.02

Table 5.5 shows the influence of the propolis fraction in the emulsion system on the size and total surface area of the oil droplets, the hydroxide concentration after 14 days storage and the rate of lipid oxidation. With increasing mass fraction of propolis the average oil droplet size and consequently the total surface area of the droplets is decreasing. It has been established previously (Chapter 4) that propolis can decrease the interfacial tension between oil and water, and therefore probably contributes to the smaller oil droplet size. All peroxide values at 14 days storage were statistically different ($p<0.05$) indicating that even at the lowest percentage tested, propolis could support the emulsions' oxidative stability.

The hydrogen peroxide concentration produced and the normalised data per the surface area of the oil droplets were plotted for the duration of sampling. Results are presented in Figure 5.5.

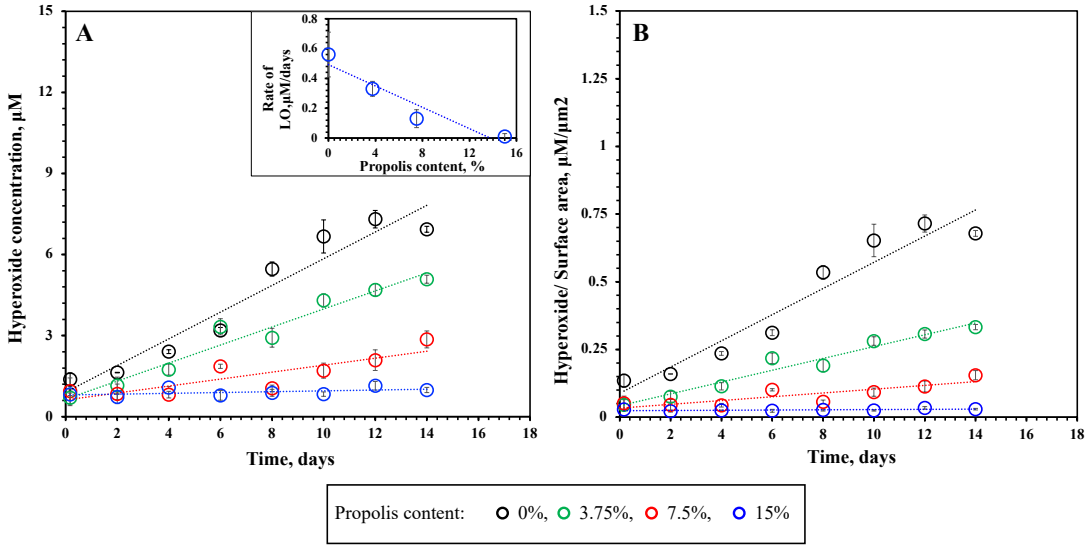

Figure 5.5: A. Effect of propolis mass fraction (0. 3.75, 7.5 and 15%) in emulsions formulated with 15% oil and 1% Tween 80 via HSM on the production hydrogen peroxides generated for a maximum 14-day period at 40 °C and B. Normalised hydrogen peroxide production to total oil droplets surface area. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.). Inset graph: Effect of propolis mass fraction (0. 3.75, 7.5 and 15%) on the rate of lipid oxidation (LO). Error bars represent ± one standard deviation (s.d.).

It is evident from both Figures 5.5A and B that there was a linear dependency between propolis mass fraction and hyperoxide production. This concentration dependency between propolis and lipid peroxidation has been reported in literature. El- Guendouz *et al.*[56] studied propolis' ability to decrease lipid peroxidation at the highest concentrations tested of 0.02% and 0.04%, agreeing with the present study, even though in their system a lower propolis amount was used, dissolved in ethanol, and added to the oil phase. Coban[57] tested different concentrations of propolis

containing emulsions (0.3% and 0.6%) used for chitosan coatings, on the lipid peroxidation of crayfish, also showing the concentration dependency on both primary and secondary lipid peroxidation products.

Lipid oxidation can be influenced by a variety of different factors such as surface area of the droplets, oil content, concentration of antioxidants and storage temperature that have been examined and discussed in previous in sections. Other factors influencing lipid peroxidation could be the pH of the emulsion or the type of emulsifier. An acidic pH environment in an emulsion system could reduce the rate of oxidation by having a greater abundance of hydrogen ions which can terminate lipid radicals and reduce prooxidant metals such as ferric ions. An alkaline pH environment could increase the amount of monoacylglycerols, diacylglycerols and free fatty acids, which can then increase the rate of lipid oxidation *via* production of associated colloids.[58] Different types of surfactants could inhibit lipid oxidation by creating ticker layers around the oil droplets and therefore protecting them from the aqueous continuous phase, interacting with metal ions preventing them to encourage lipid oxidation or by scavenging free radicals that are participate in propagating the oxidation reactions.[59]

To investigate those parameters, control and propolis emulsions were formulated consisting of 15% oil, 1% Tween 80 or sodium caseinate (NaCas) and pH values of 4.6 and 7. Emulsions with propolis and NaCas at neutral pH were also investigated but the emulsions phase separated after 2 days of storage at 40 °C so the experiment was not included at the lipid oxidation study. The emulsions' oil droplet size, as well as the calculated total surface area, the final hyperoxide concentration measured at day 14 and the rate of lipid oxidation are presented in Table 5.6.

Table 5. 6: Mean droplet size ($D_{3,2}$), surface area, final concentration of hyperoxides and rate of lipid oxidation after 14 days storage at 40°C of oil-in-water emulsions stabilised with 1% Tween 80 or sodium caseinate (NaCas). The continuous phase consisted of either distilled water or propolis aqueous particle dispersions 15% propolis mass fraction) at pH 3.65, 4.6 and 7. Emulsions possessed 15% sunflower oil and processed *via* HSM. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Sample	Surfactant	pH	Size (μm)	Total surface area of droplets (μm^2)	Hyperoxide Concentration @ day 14 (μM)	Rate of Lipid Oxidation (μM/days)
Control	Tween 80	7	9.08±1.67	10.22±1.68	6.93±0.11	0.56±0.15
Propolis	Tween 80	7	3.35±0.73	28.44±7.34	5.33±0.04	0.25±0.08
Propolis	Tween 80	3.6 (native)	2.76±0.41	33.29±4.90	0.99±0.09	0.01±0.02
Control	NaCas	7	9.73±0.91	9.33±0.90	4.08±0.11	0.21±0.04
Control	NaCas	4.6	19.44±4.35	4.91±1.25	0.64±0.06	0.01±0.07

Table 5.6 shows the effect of surfactant and pH conditions on the average oil droplet size, hyperoxide concentration post 14 days storage at 40°C and the rate of hyperoxide production. The pH of the continuous phase did not influence the average oil droplet size of the emulsions containing Tween 80. However, at neutral pH the NaCas emulsions produced a much smaller oil droplet size than at pH value of 4.6. When NaCas exists in an environment with pH close to its isoelectric point (4.6) it forms aggregates and cannot unfold around the oil droplets, lower the interfacial tension and stabilise them as efficiently, explaining the differences in the oil droplet sizes observed.[60]

The 15% propolis emulsions at native pH of 3.6 exhibited similar behaviour with the 4.6 pH NaCas ones; the lipid oxidation was completely supressed during the 14 days of storage. Therefore, emulsions containing both were not examined further.

Interestingly both propolis and NaCas emulsions at neutral pH had similar oxidation rates, even though the final hyperoxide concentration of NaCas emulsion was slightly smaller than the propolis emulsion. Acidic pH also resulted in decrease of production of hyperoxides.

Figure 5.6A shows the primary oxidation products from emulsions consisting of 15% oil, 1% Tween 80 or sodium caseinate (NaCas) and pH of 4.6 and 7 of propolis and control emulsions. Figure 5.6B presents the normalised data per the surface area of the oil droplets.

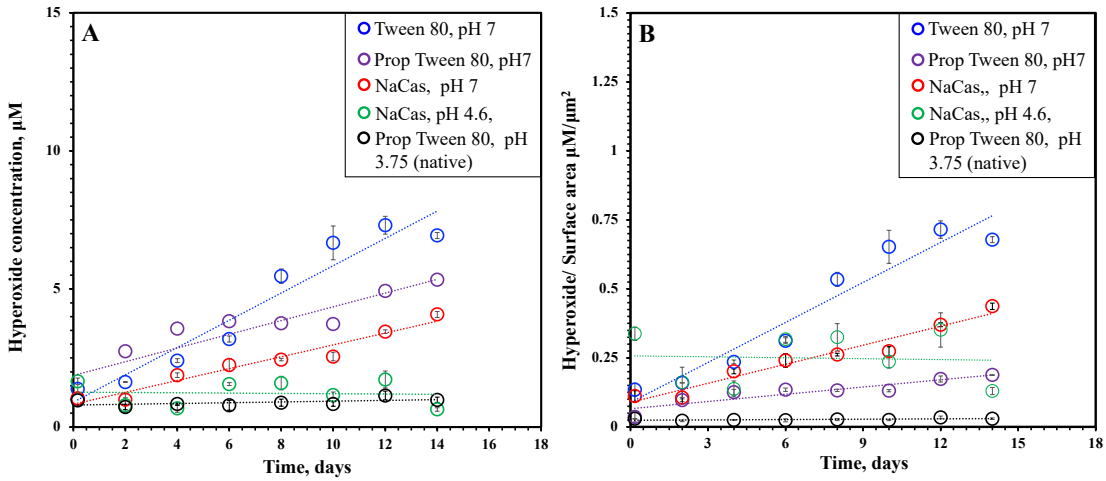

Figure 5.6: Effect of pH (4.6 vs 7) and surfactant type (NaCas vs Tween 80 at 1% concentration) of emulsions formulated via HSM on the amount hydrogen peroxides generated for a maximum14-day period at 40 °C storage temperature. Data shown are averages (n=3; replicates) and error bars represent ± one standard deviation (s.d.).

Sodium caseinate tends to perform better as a stabiliser in neutral environments,[61] thus emulsions containing NaCas at neutral pH were also tested for their ability to prevent lipid peroxidation. The NaCas containing emulsions showed more efficient antioxidant activity than the Tween 80 stabilised control ones. Ries *et al.*[62] suggested that NaCas' ability to prevent lipid peroxidation was due to its efficacy

in binding trace metal cations as well as scavenging free radicals. Additionally, Kargar *et al*.[38] highlighted NaCas' ability to form a thick layer around the oil droplets, inhibiting prooxidant metals to reach the interface. However, when comparing the propolis emulsions with the NaCas ones, one can conclude that in the case of an emulsifier that does not seriously impact the oxidative stability of the system (Tween 80), the presence of propolis can offer a very significant suppression of lipid oxidation.

In all cases, emulsions with acidic pH performed better than emulsions with neutral pH. It has been concluded that more alkaline pH and presence of oxygen or transition metal ions can cause auto-oxidation in phenolic compounds from plants, with an increase in pro-oxidant activity, which partially explains the decrease in antioxidant efficiency of the neutral propolis emulsions shown in Figure 5.6A.[63] This has been reported in emulsion systems containing both low molecular weight surfactants[64] and dairy proteins[65]. However, it is notable to mention that at neutral pH, propolis emulsions performed better than control emulsions, showing that propolis can offer some oxidative stability even at higher pH values. Once the data were normalised per the total droplet surface area (Figure 5.6B), propolis oxidative properties were more prominent in both acidic and neutral environments as they offered equivalent or more suppression of lipid oxidation than all the NaCas containing emulsions tested.

5.3.2 Antibacterial activity of propolis emulsions

5.3.2.1 Assessing the antibacterial effect of control O/W emulsions

Control emulsions were tested initially for antibacterial activity against *E. coli* to assess if they alone can influence the bacteria population. It is believed that the antibacterial effect of emulsions, especially at a nanometre level, can stem from the

energy input while the system is formulated with high energy methods such as ultrasonication (which was used in this study),[66] and as the oil droplets' fuse with the lipid containing microorganisms is favoured, the energy stored during the emulsion formation could cause destabilisation of the lipid membrane leading to cell lysis and eventually death.[67] In literature there has been a disagreement in terms of the antibacterial activity of emulsions especially concerning nanoemulsions. For example, Majeet *et al*[68] fabricated nanoemulsions (151 nm average oil droplet size) containing clover (with eugenol as the compound of interest) and canola oils and stabilised by Tween 80 and starch at exhibited antibacterial action against *E. coli HB2151.* On the other hand Terjung *et al*.[69] 's emulsions (80 nm average oil droplet size) containing Miglyol 812N and Tween 80 encapsulating carvacol and eugenol did not exhibit any activity against *E. coli C 600*. Therefore, in the present study, it was essential to determine whether the antibacterial response detected would stem from the presence of propolis in the emulsions or from the emulsion microstructure itself and/or the emulsification method used.

Emulsions were formulated, containing different oil contents (15% vs 25%), surfactant contents (0.5% vs 1% Tween 80) and size arising from different processing (HSM vs sonication) and their antibacterial activity was compared. The emulsions formulated *via* HSM possessed an average droplet size of around 9 μm ($D_{3,2}$) while the sonicated one had average oil droplet size of 0.74 μm ($D_{3,2}$) (Chapter 4). *E. coli* cells at their stationary state were treated with the emulsions and samples were taken as soon as they came in contact and after 24 hours. Results are presented in Figure 5.7.

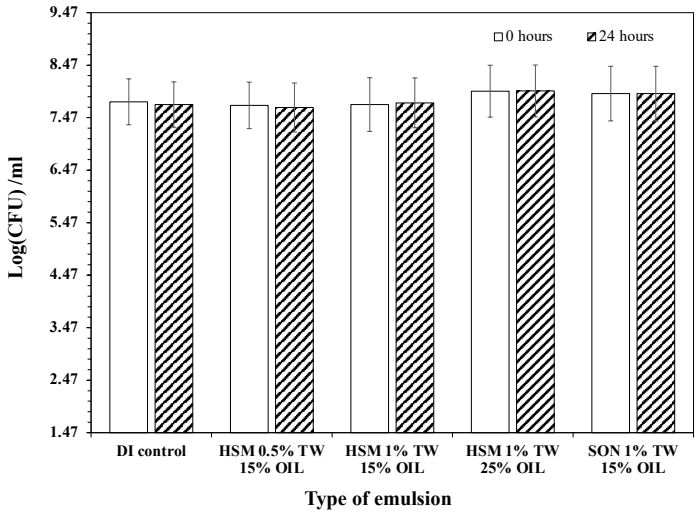

Figure 5.7: Antibacterial activity of O/W emulsions formulated with different parameters. Data for population of stationary phase E. coli MG1655 are presented as a function of different percentages of surfactant (0.5 and 1% Tween 80), oil content (15 and 25%) and processing conditions (HSM and sonication) after immediate exposure and 24 hours. Deionised water (DI) was also tested as a control. Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.).

Figure 5.7 suggests that independently of the variable parameters tested, there was no decrease of the *E. coli* population when they were treated with the non-propolis O/W emulsions, and there was also no statistical difference with deionised water (control). Kadri *et al*.[8] investigated the antibacterial activity of both coarse (15-35 μm) and nanoemulsions (170-650 nm) containing sunflower oil and Tween 80 and illustrated that both emulsions did not have an effect on the population of *E. coli K-12 MG1655* and on their membrane and cellular integrity, further agreeing with the present study. Therefore, any advantage in terms of antibacterial activity that could arise from the incorporation of propolis in emulsion would solely originate from propolis itself.

5.3.2.2 Investigating the propolis mass fraction dependency on the rate of bactericidal action of E. coli

Propolis extracts' antibacterial activity has already been investigated extensively.[70] Literature includes a variety of formulations that have incorporated propolis to include an additional antibacterial functionality, from hydrogels[71], cotton fabrics[72] or even in the form of nanoparticles encapsulated by alginate[73]. The propolis particle aqueous dispersions that were included as continuous phase in the emulsion systems so far, had shown enhanced antibacterial activity of propolis in an aqueous environment.[53] The minimum bactericidal concentration was at 5.6% propolis mass fraction and the dispersion acted by targeting the cellural membrane causing cell swelling and possibly lysis[53]. However, the rate of eliminating the bacteria population from different propolis mass fractions post formulation in the aqueous particle propolis dispersions has not been yet investigated. For comparison purposes, Kaplan-Meier survival plots of the antibacterial activity of the dispersions were estimated and results were compared using the Mantel-Cox log rank comparison test. Figure 5.8 compares the rate of bactericidal action caused by the highest propolis mass fraction of 15% and by its dilutions of 11.25% and 7.5% respectively.

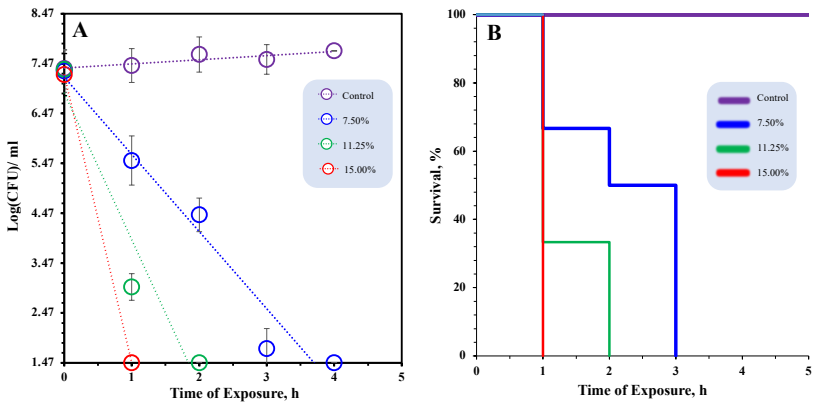

Figure 5.8: A. Antibacterial activity of propolis particles dispersed in aqueous media. Data for the population of stationary phase E. coli MG1655 are presented as a function of propolis mass fractions (15%. 11.25% and 7.5%) and exposure time. The control is deionized water. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d). B. Kaplan-Meier survival plot of stationary phase E. coli MG1655 cells are presented as a function of propolis mass fractions (15%. 11.25% and 7.5%) and exposure time.

It can be seen from Figure 5.8A that the rate of decrease in the *E. coli* population by the propolis particle aqueous dispersions is clearly affected by the propolis mass fraction. Mantel Cox log rank test showed that overall, there was a statistical difference between the survival plots ($p<0.05$) However, when compared pairwise, 15% and 7.5% propolis aqueous dispersions caused significantly different bactericidal rates ($p<0.05$) while 11.25% propolis mass fraction was not different from either. Therefore, since the the 15% and the 7.5% propolis mass fractions gave statistically different bactericidal rates, they were chosen for further analysis when testing propolis emulsions.

5.3.2.3 Investigating the surfactant and oil content, and processing on the antibacterial activity of propolis emulsions

Antibacterial surfaces/interfaces have been researched in literature, especially concerning materials that cells need to encounter *via* covalent forces to act.[74] The antibacterial effect of propolis particle aqueous dispersions has been examined, where it was suggested that propolis particles could eliminate the *E. coli* population via contact.[53] It has also been previously suggested (Chapter 4) that the propolis aqueous dispersions have an affinity for the oil/water interface. Overall, it was proposed that increasing the area available for the propolis particles to place themselves at the oil/ water interface decreased the competition with Tween 80 and enhanced the stability of the emulsions. In the current study, the antibacterial effect of propolis emulsions

containing the aqueous dispersions as the continuous phase is examined while different key parameters such as surfactant and oil content as well as oil droplet size were altered.

Propolis containing emulsions have shown to exhibit antibacterial activity against different pathogens such as *S. aureus*, *E. coli* or spores either incorporated in polyethylene glycol 400[75] or even diluted in olive oil or ethanol[76]. However, to the best of the authors' knowledge it is the first time propolis antibacterial activity is being investigated in the form of particles, while existing in an emulsion system.

The effect of incorporation of propolis particle aqueous dispersion in an emulsion system was investigated on the antibacterial activity of the dispersions, *via* comparing propolis emulsions with the propolis dispersions as well as altering the oil volume fraction, oil droplet size and surfactant concentration. Propolis emulsions and their half dilution with deionised water (15% and 7.5% propolis mass fractions) were tested. Emulsions were formulated with 15% oil, 1% Tween 80 and *via* HSM.

Raw data (Figures 5.9A and B) and Kaplan-Meier survival plots for propolis emulsions containing 15% oil and different surfactant percentages; 0.5 and 1% Tween 80, formulated by HSM (Figures 5.9C and D) are presented. The average droplet size of the propolis emulsions was at around 3 μm ($D_{3,2}$) (Chapter 4).

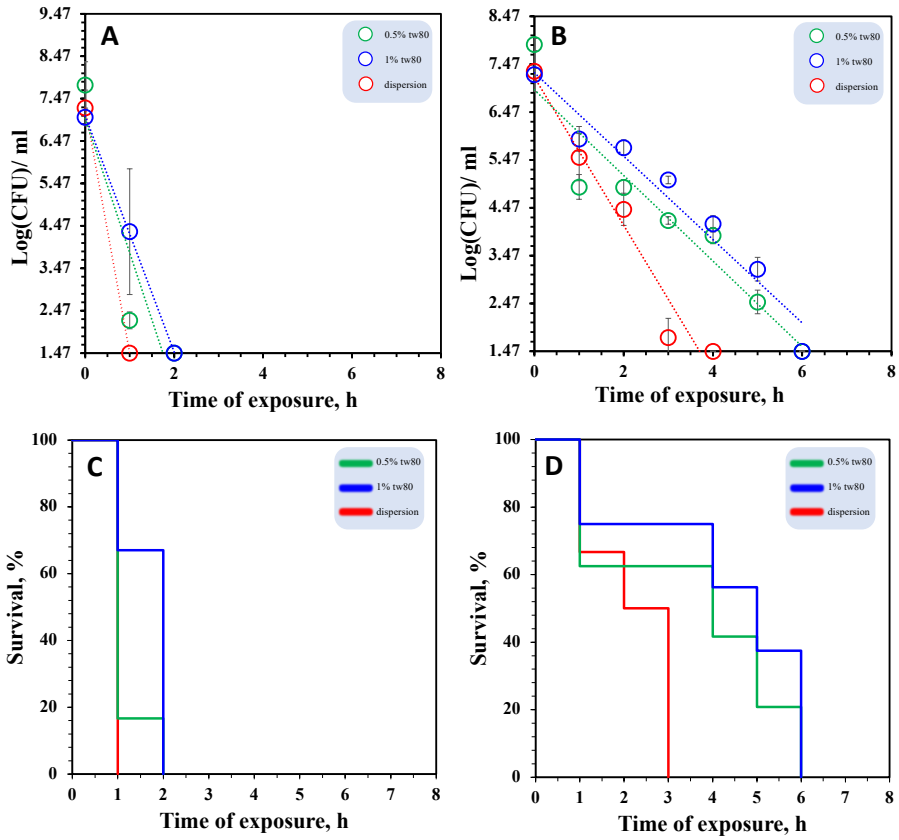

Figure 5.9: Antibacterial activity of A. propolis emulsions and B. propolis emulsions diluted by half, formulated via HSM containing 15% oil. Data for population of stationary phase E. coli MG1655 are presented as a function of different percentages of surfactant (0.5 and 1% Tween 80). Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.). Kaplan-Meier survival plots of C. propolis emulsions and D. propolis emulsions diluted by half.

From Figure 5.9 it is established that the dispersions caused the fastest bactericidal rate. Overall, for both concentrated and diluted systems there was a statistical difference of the survival plots ($p<0.05$), however pairwise comparison resulted in the dispersion being statistically different with the 1% Tween 80 propolis emulsion while 0.5% was similar to both. Since propolis can be diluted in oil to some extent[77], a portion could be transferred in the oil phase during emulsification, influencing the bactericidal rate. Additionally, when less surfactant is included in the

emulsions, the oil droplets could possibly have more propolis particles surrounding them, assisting in the diffusion of particles to the cells.[78]

Moreover, the effect of oil content on the antibacterial activity of the propolis emulsions was tested. Emulsions were formulated with 15 and 25% oil content with average oil droplet size of 2.76 μm and 4.09 μm ($D_{3,2}$) respectively which were statistically different ($p<0.05$) (Chapter 4), and 1% Tween 80 *via* HSM. Kaplan-Meier survival plots are presented in Figure 5.10C and D, and the raw data are included in Figures 5.10A and B.

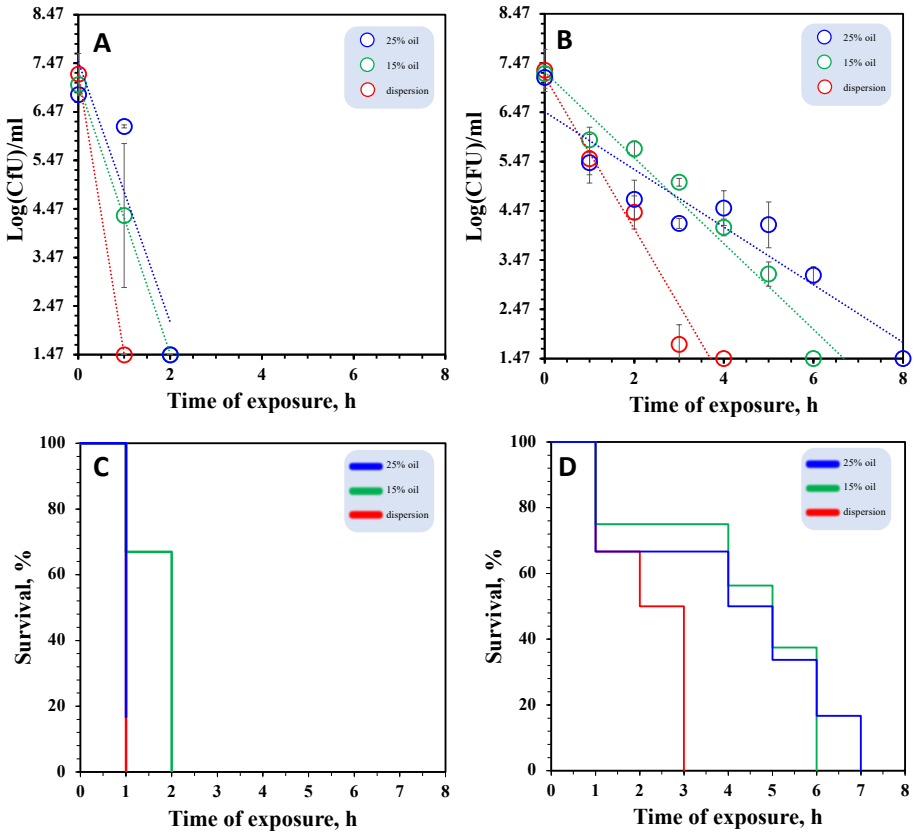

Figure 5.10: Antibacterial activity of A. propolis emulsions and B. propolis emulsions diluted by half, formulated via HSM containing 1% Tween 80. Data for population of stationary phase E. coli MG1655 are presented as a function of different percentages of surfactant (15 and 25%). Data shown are

averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.). Kaplan-Meier survival plots of C. propolis emulsions and D. propolis emulsions diluted by half.

Figure 5.10 shows that the highest bactericidal rate came from the propolis dispersions. In the concentrated systems (Figure 5.10C) the 25% oil containing emulsions were statistically similar to the dispersions and resulted in a higher antibacterial action than 15% oil containing emulsions. That could be due to a better fuse of the particles in the oil/ water interface with the cells, similarly with when less surfactant was used. Post dilution (Figure 5.10D), all the systems were statistically different ($p<0.05$). The 15% oil treatment acted slightly faster than the 25% but both were slower than the dispersion. This is believed to be a consequence of oil being added to the propolis particle aqueous dispersions to formulate the emulsions, and therefore resulting in a less propolis per volume treated on the cells with increasing oil content.

Lastly, the effect of the droplet size was tested on the antibacterial activity of the propolis formulations. Propolis emulsions were formulated containing 15% oil and 1% Tween 80 *via* HSM and sonication. The difference in processing resulted in emulsions of statistically different oil droplet sizes ($p<0.05$); 2.76 μm and 0.71 μm ($D_{3,2}$) for the HSM and sonication methods respectively. (Chapter 4) Kaplan-Meier survival plots are presented in Figures 5.11C and D and the raw data are included in Figures 5.11A and B.

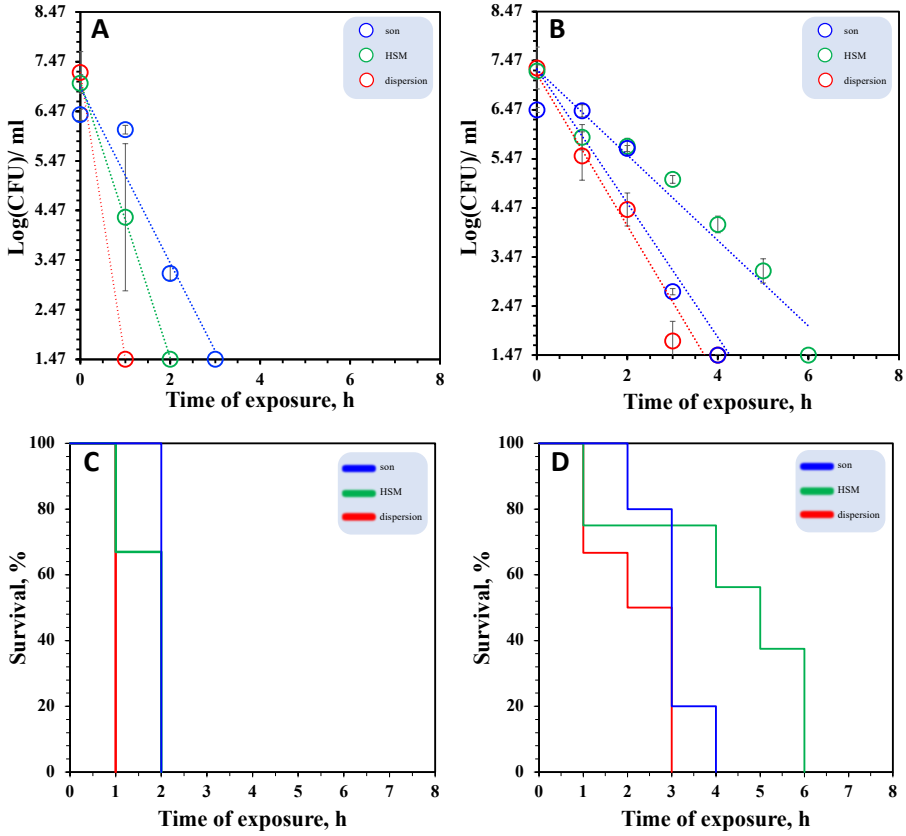

Figure 5.11: Antibacterial activity of A. propolis emulsions and B. propolis emulsions diluted by half, containing 15% oil and 1% Tween 80. Data for population of stationary phase E. coli MG1655 are presented as a function of different processing (sonication and HSM). Data shown are averages (n=3; biological replicates) and error bars represent ± one standard deviation (s.d.). Kaplan-Meier survival plots of C. propolis emulsions and D. propolis emulsions diluted by half.

Figure 5.11C shows that the dispersion exhibited the fastest bactericidal rate followed by the HSM emulsion and then the sonicated emulsion (with only an hour difference in eliminating the *E. coli* population). Log-rank test revealed that all the bactericidal rates were significantly different ($p<0.05$). Post dilution (Figure 5.11D) the systems' bactericidal action exhibited a different pattern, with dispersion and sonicated emulsion having similar bactericidal rates and with the dispersion being significantly different from the HSM emulsion ($p<0.05$), even though the rate was again delayed by an hour only (Figure 5.11A). It is believed that in the concentrated treatments, the much

smaller oil droplets could also emerge as inhibitors for particle diffusion towards the cell[79]. As the system is diluted, there are smaller oil droplets acting as obstacles in the case of sonicated emulsions, which could be the reason behind the increased bactericidal rate compared with the HSM emulsions.

5.3.3 Rheological behavior of propolis and control emulsions

Propolis and control emulsions were formulated to assess whether propolis will have a pronounced effect on the emulsions' rheological behaviour as a function of emulsion droplet size or emulsion dispersed phase content. It has already been established that altering the propolis mass fraction of the continuous phase alone was not significant and only the most concentrated system of 15% was more viscous than water. (Chapter 4) Therefore, in the present study, emulsions were compared according to their oil content (15% *vs* 25%) and their droplet size (HSM *vs* sonication). All emulsions contained 1% Tween 80. The results are presented in Figure 5.12.

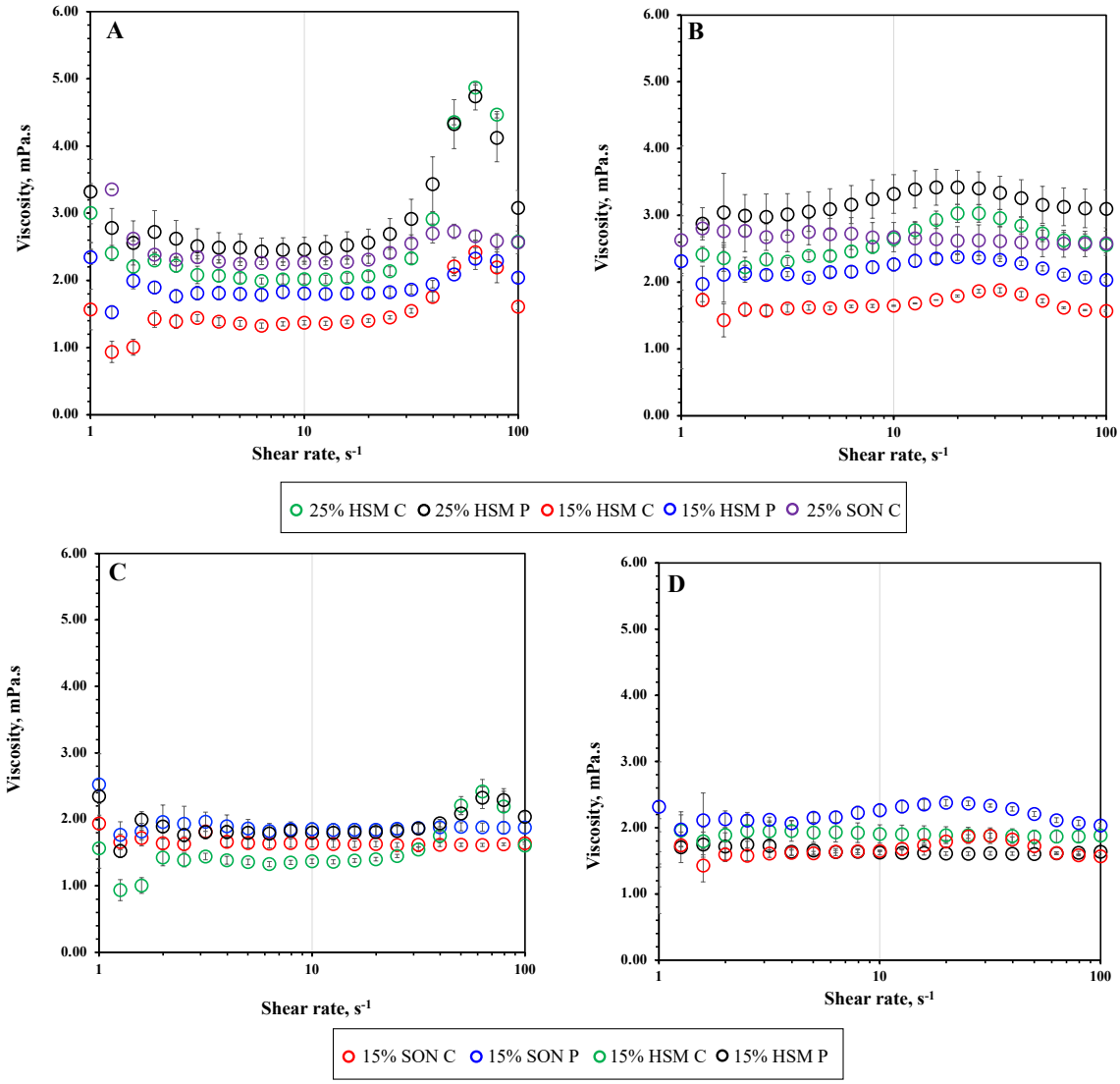

Figure 5.12: Viscosity of propolis (P) and control (C) emulsions containing 15% or 25% oil content (A, B) and fabricated via HSM or sonication (SON) (C, D) at increasing (A, C) and decreasing (B, D) shear rate. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

From Figures 5.12A and B it can be seen that the viscosity of the emulsions follows a specific trend; the control emulsions with 15% oil had the lowest viscosity, followed by the propolis ones with same oil content, then the controls with 25% oil and finally the propolis emulsions with 25% oil. The viscosity seems to be predominantly influenced by the fraction of oil, as it was also reported by Farah *et al*.[80]. In addition,

it is believed that Pickering emulsions have higher viscosities than surfactant stabilised emulsions due to the thick layer of nanoparticles around the oil droplets[81], something also observed in the present study.

Interestingly even though the HSM formulated emulsions initially exhibited a Newtonian behaviour, since the oil content is not very high, there was a shear thickening behaviour at higher shear rates from approximately 25 s^{-1} followed by a shear thinning one at around 75 s^{-1}. This effect was more pronounced as the shear rate increased (Figure 5.12A) than decreased (Figure 5.12B), and was stronger on the control rather than the propolis emulsions. According to Onaizi that could be due to flocculation of the oil droplets[82], which possibly subsided as the shear persisted. A 25% oil sonicated emulsion was also tested, exhibiting a purely Newtonian behaviour. When the HSM emulsions were tested, a creaming layer was pronounced once the measurements were completed, something not observed for the sonicated emulsions and could therefore be the reason behind the shear thickening and thinning behaviour observed as the shear rate increased. To test this further, the viscosity of the 25% oil control emulsions was measured under shear for different geometries (Figure 5.13) showing that the emulsions tested *via* parallel plates, where the creaming effect would be evident, showed shear thickening and thinning behaviour, whereas geometries with smaller exposed surface area (cup and bob, double gap) followed a Newtonian behaviour. Therefore, it can be concluded that creaming is the reason behind the effect observed in Figure 5.12, and it is less pronounced when the shear rate is decreasing, as the creaming layer has already been formed.

The sonicated emulsions exhibited a small decrease in viscosity at low shear rate and a Newtonian behaviour onwards (Figures 5.12C and D). This behaviour was also

observed by Onaizi[82], who tested the viscosity of nanoemulsions of low oil content (5%- 20%) under increasing shear. The initial decrease was attributed to the disentanglement of the oil droplets at low shear rate and once the droplets get aligned with the flow there is no effect on the flowability. They also noted that once the system surpassed the 100 s^{-1} shear rate, there was an increase in viscosity due to possibly formation of flocks, but that was not tested in our case, although it was observed for the HSM emulsions.

There was a clear increase in viscosity with the addition of particles, but the oil droplet size did not alter the viscosity of the emulsions. In literature, a finer oil droplet size could result in an increase in viscosity due to the higher interactions between the small oil droplets causing flocculation[83]. This was observed for the propolis emulsions although, probably due to the small oil content the flow behaviour was predominately affected by the addition of solid propolis particles. Overall, the presence of propolis has a more prominent effect on the emulsions' rheological properties as a fraction of emulsion droplet size or emulsion dispersed phase content. El-Guendouz *et al* [56], tested the rheology of W/O emulsions containing liquid propolis extracts, also observing an increase in viscosity with the addition of propolis compared to control emulsions, even though their system is different to the emulsions examined in the present study.

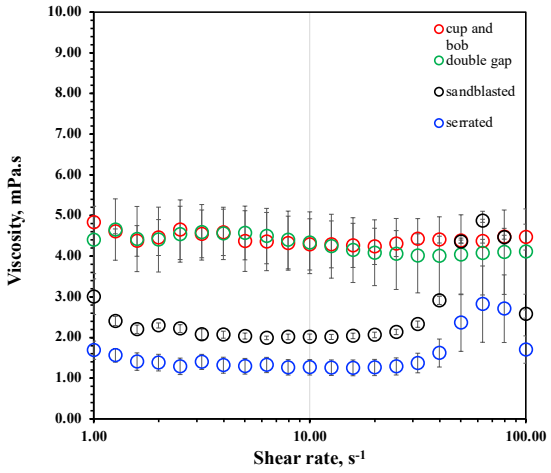

Figure 5.13: Viscosity of control emulsions containing 25% oil content and fabricated via HSM at increasing shear rate, measured via serrated and sandblasted parallel plates, cup and bob and double gap geometries. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

5.4 Conclusions

In conclusion, the propolis particles can adopt a microstructural role (Pickering functionality and increased viscosity) while also offering both oxidative and antibacterial stability to O/W emulsions. Screening key parameters that could influence lipid oxidation on control systems showed that temperature as well as oil content influence the production of hyperoxides considerably, whereas oil droplet size did not significantly influence the rate of lipid oxidation. Compared to the (non-propolis) controls, lipid oxidation in propolis O/W emulsions was diminished at a high content of propolis particles and low pH, while it decreased at a lower propolis fraction, or at neutral pH conditions. This was attributed to both propolis antioxidant activity as well as the protective layer that propolis particles form around the oil droplets, reducing their contact with the aqueous phase where the lipid oxidation-enhancing prooxidant metal ions are present. The antibacterial activity of both control and propolis emulsions was

tested against *E. coli* cells. Control emulsions showed no antibacterial activity, while propolis emulsions successfully eliminated the bacteria population, a performance that was slightly enhanced with decreasing co-stabiliser (surfactant) concentrations and oil droplet sizes and increased oil content. The presence of propolis particles increased the viscosity of the emulsions, which was additionally influenced by droplet size and oil content, with both these attributed to the thick layer of particles around the oil droplets. Overall, propolis particles showed significant promise as multifunctional colloidal species in O/W emulsions, and the present study demonstrates how this (multi)functionality is impacted by variations to key emulsion formulation characteristics.

5.5 References

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Chapter 6- Conclusions and Further Work

6.1 Conclusions

This research aimed to produce propolis aqueous based formulations, while enhancing or preserving propolis' antioxidant and antibacterial activity, and to assess the dual role of propolis particles as Pickering stabilisers as well as lipid oxidation inhibitors/ antibacterial additives in O/W emulsions. As it has been discussed beforehand, Pickering particles are most commonly studied for their interfacial behaviour and associated emulsion stability performance, while other potential functionalities (exhibited in tandem with the former) are only scarcely investigated and usually overlooked. Particularly with regards to natural species, there is significant potential to identify colloidal (natural) structures that exhibit/demonstrate a dual functionality in emulsions, where they not only offer enhanced microstructural performance (Pickering stabilisation), but also 'fortify' these two-phase assemblies in terms of antibacterial and antioxidant stabilisation. The current study has explored the potential for propolis particles to act exactly in this capacity and specifically in the case of O/W emulsions. The main conclusions arising from the findings of the present work are here discussed in more detail.

6.1.1 Commonly used propolis carrier phases can lead to misinterpretation of the active's antibacterial performance

In most of the current literature, an aqueous ethanol mixture is used as the extraction solvent and often the carrier phase for propolis. However, such solvents can themselves exhibit an antibacterial and/or antioxidant response, that is then conflated with that originating form propolis alone. The impact of the carrier phase used to dissolve and test propolis extracts was investigated for ethanol and DMSO solvents,

by direct colony counting and assessing the membrane integrity of the cells *via* flow cytometry. The present work concludes that both ethanol and DMSO even at low concentrations of 5-10%, can influence the cell population, highlighting the need for alternative formulations/carrier phases for water-insoluble actives.

6.1.2 Increasing propolis extract content influences the physical characteristics and stability of the corresponding propolis particle aqueous dispersions formed *via* sonication

The pathway chosen to enhance propolis' activity in an aqueous environment was to increase the surface area of the insoluble material, thus decreasing its size, *via* direct ultrasonication. Tween 80 was chosen to aid the break-up of the propolis extracts into micrometre/sub-micrometre particles. The propolis content and sonication time was initially examined, establishing that less propolis content as well as higher sonication time result in smaller particle size. However, as the concentration of propolis was not sufficient for antibacterial testing, water was reduced from the formulation by evaporation thus strengthening the antibacterial character further. This resulted in an increase of particle size, probably due to a higher chance of the particles colliding and aggregating. The zeta potential was highly negative suggesting good stability, although after a month of storage, there was a general increase in average particle size, as well as an increase in the polydispersity of the dispersions indicating physical deterioration of the samples.

6.1.3 Direct ultrasonication influences the dissolution of species from the propolis extracts into the aqueous carrier phase and enhances the overall antibacterial activity of the propolis particle aqueous dispersions

Ultrasonication showed to enhance extraction, thus improving further the formulation by forcing some dissolution of compounds from the insoluble matter to aqueous carrier phase. The dissolution effect arisen from sonication was examined on the total phenol content, the antioxidant, and antibacterial characters, with sonication time being a key parameter. Both total phenol content and antioxidant activity were mainly stemming from the insoluble propolis although some was detected in the carrier phase. However, sonication time did not influence the activities significantly. Concerning the antibacterial activity, sonication time played a significant role in the antibacterial action of both whole dispersions and carrier phases, with stronger bactericidal effect caused by increased sonication time. Both insoluble matter and carrier phase exhibited bactericidal effects, showing synergism in the whole dispersion. The effect of the propolis particle aqueous dispersions on the *E. coli* cells was examined under flow cytometry and AFM. Flow cytometry showed that lower sonication times cause more injury while higher sonication times result in more cell death. Observing the cells *via* AFM showed that post treatment, there was cell swelling possibly due to fractures triggering absorption of water, caused by propolis which can result in lysis and eventual cell death.

6.1.4 pH conditions influence the physical stability and antibacterial activity of propolis particle aqueous dispersions

Increase of the pH resulted in an increase of the zeta potential of the propolis particle aqueous dispersions, therefore increasing the electrostatic interactions

between the propolis particles and thus increasing the stability of the formulation. The antibacterial activity of the dispersions for different pH values was tested against *E. coli*. It was observed that as the pH increased the antibacterial activity of the propolis particle aqueous dispersions decreased. This was believed to be due to the dissociation of the phenolic compounds with increased pH.

6.1.5 Propolis particles exhibit Pickering(-like) functionality in O/W emulsions

Consequently, the propolis particle aqueous dispersions were examined for their affinity to an O/W interface. Propolis particle aqueous dispersions and carrier phases with and without surfactant were produced and tested. Results showed that no free surfactant existed in the carrier phase. Also, carrier phase reduced the interfacial tension more than water, but similar with the whole dispersion (without Tween). Adding Tween 80 to the dispersion, resulted in the highest reduction of the interfacial tension, even more than solely the surfactant itself. This was believed to be due to a synergism between Tween 80 and propolis in lowering the interfacial tension as well as due to the much smaller particle sizes of the dispersions produced with the surfactant. The wetting properties of propolis indicated that it would promote the formation of an O/W emulsion. The viscosity of the dispersions was significantly higher than water but not enough, thus not expecting to influence the emulsification process.

The propolis aqueous particle dispersions were incorporated in O/W emulsions as the continuous phase and compared with control emulsions. Key parameters were altered such as surfactant, propolis and oil content, and oil droplet size. It was concluded that even though propolis emulsions produced a significant smaller oil droplet size than the controls, and did surround the oil droplets, there was a competition between the particles and surfactant resulting in higher oil droplet sizes with the

passage of time. This was enhanced with smaller surface area, more surfactant or more propolis content.

Furthermore, sodium caseinate was tested instead of Tween 80 towards the stability of propolis emulsions. Initially when propolis and sodium caseinate were mixed at propolis' native pH of 3.65, induced propolis aggregation at the walls of the vessel. Since sodium caseinate as a protein is pH sensitive, zeta potential titration curves of sodium caseinate, propolis particle aqueous dispersions and both mixed were conducted, showing that propolis particles and sodium caseinate interact electrostatically. Subsequently, the interfacial tension of the propolis and sodium caseinate dispersion was measured (at dispersions' native pH) revealing that the electrostatic interactions of the particles with sodium caseinate prevented the protein from reaching the interface. Finally, emulsions at pH 4.6 were formulated containing sodium caseinate and propolis (at sodium caseinate' s isoelectric point). It was revealed that even though initially the propolis emulsions contained smaller oil droplet sizes, the emulsions over time were less stable than the controls. That is believed to be due to the particles aggregating with sodium caseinate and interacting less with the interface.

6.1.6 Propolis particles exhibit significant promise as multifunctional colloidal species in O/W emulsions

Once propolis Pickering functionality was established, its antioxidative and antibacterial effect on the emulsions was investigated as well as its effect on the rheological characteristics of the emulsions. Propolis inhibited lipid oxidation in emulsion systems, and the rate of inhibition was dependent on the propolis and oil content and pH of the emulsion. The antibacterial activity of the propolis emulsions

was tested and compared against the propolis particle aqueous dispersions. Although the dispersions showed the most potent antibacterial activity, the emulsions showed efficient short term antibacterial activity which was dependant on the oil and surfactant contents as well as oil droplet size. Finally, it was shown that when propolis particles are incorporated into emulsions, they increase the viscosity of the formulations. Other parameters that made a difference were the oil content and oil droplet size. Generally, emulsions exhibited a Newtonian behaviour apart from the made with a high shear mixer that resulted in creaming, where there was a shear thickening and thinning behaviour with increasing shear rate.

6.2 Further work

Based on the findings of the present thesis, further work can be conducted on different elements of the research, either arising from the specific systems examined such as chemical characterisation of the propolis extracts or altering their surface charge to extending and applying the enhancement of this specific active (propolis) in aqueous formulations to other natural actives. Some suggestions of further work are being discussed below.

6.2.1 Chemical characterisation of the propolis extracts, propolis particles and aqueous carrier phases

A huge challenge concerning propolis extracts (as it is as well with many other natural products), arising from its extremely variable consistency is its standardisation.[1] For this purpose, it can be extremely beneficial to carry out chemical analysis prior to antioxidant or antibacterial studies. More knowledge will be gained by identifying the exact molecular make-up of propolis and deduce which species are responsible for the properties of the parent matrix. With relevance to the present study, it would be beneficial to identify the compounds in propolis and then compare them post sonication, to the ones present in the solid and liquid phases. Therefore, it would be known which compounds were forced to dissolve in water and still exhibited antibacterial and antioxidant activities. Furthermore, the effect of the duration of sonication could also be examined. Possible techniques to measure and identify propolis compounds are *via* HPLC analysis paired with Mass Spectrometry[2] and NMR[3].

6.2.2 Investigation of the surfactant type and charge of the propolis particles and their antibacterial and antioxidant activity.

Bacteria cells tend to be negatively charged and it is expected that they would be more attracted to positively charged surfaces.[4] As propolis showed to also be negatively charged, this is not a beneficial interaction for the cells to reach the particle's surface. Similarly, charge plays an important role in lipid oxidation, as since a large amount of lipid oxidation occurs in the interface, charged surfactants for instance could repel or attract pro-oxidant metal cations.[5] In literature, there is plenty of research aimed to increase the positive charge of antibacterial surfaces and consequently their antibacterial activity.[6],[7] To determine if there is an effect of the charge surface on the antibacterial activity of the dispersions, a range of ionic surfactants with different charges could be used, such as positively charged amine surfactants or negatively charged sodium dodecyl sulfate.[8]

6.2.3 Investigation of the surfactant type and charge of the propolis particles and their antibacterial and antioxidant activity.

Two surfactant types were tried out in order to stabilise O/W emulsions; Tween 80 as a low molecular weight surfactant and sodium caseinate as a non-synthetic dairy protein. To align with the ethos of the thesis in testing natural additives to offer beneficial functionalities in formulations, more natural derived proteins that have assisted in emulsion stabilisation, such as quinoa[9] or pea protein[10], could be tested to investigate whether they would be good emulsion stabilisation alternatives.

6.2.3 Investigation of the surfactant type and charge of the propolis particles and their antibacterial and antioxidant activity.

Two surfactant types were tried out in order to stabilise O/W emulsions; Tween 80 as a low molecular weight surfactant and sodium caseinate as a non-synthetic dairy protein. To align with the ethos of the thesis in testing natural additives to offer beneficial functionalities in formulations, more natural derived proteins that have assisted in emulsion stabilisation, such as quinoa[9] or pea protein[10], could be tested to investigate whether they would be emulsion stabilisation alternatives.

6.2.4 Assessment of the specific contribution towards oxidative stability arising from propolis particles present at the emulsion interface versus those residing in the continuous phase

Through this study, it has been established that the propolis particles surround the oil droplets, thus providing both steric and chemical protection against lipid oxidation that could protect the (oil) droplet core from prooxidant metals. To investigate this effect further, the influence of the location of propolis particles in the emulsion system could be determined. This can be conducted by comparing emulsions with the propolis particle aqueous dispersions added post emulsification (while keeping all the parameters such as propolis, oil and surfactant content consistent). The oil droplet size and stability of emulsion systems could be assessed as, if propolis particles are not able to diffuse to the interface easily from emulsification, it is not expected to reduce the oil droplet size and compete as much with Tween 80 for the interface. Subsequently the effect on lipid oxidation could be investigated.

6.2.5 Extending the approach taken to (multi-)functionalise propolis into other natural compounds

Ultrasonication caused a clear enhancement of propolis antibacterial and antioxidant activity of propolis in water both by increasing the surface area and by forcing dissolution of actives in the aqueous carrier phase. This method of enhancing the activity of an insoluble natural active in water could potentially be applied for multiple other extracts with similar solubility issues as propolis.

The double role propolis particles can occupy in an emulsion in the present study has been investigated and reported. The potential of Pickering particles to act more than stabilisers could be investigated for different natural actives that can act as Pickering particles. Good examples include the flavonoids (naringin, rutin and tiliroside)[11] and phenolic compounds (quercetin and curcumin)[12] that have already successfully stabilised Pickering emulsions. More examples could include cocoa particles, which have also stabilised successfully O/W emulsion systems[13] as well as possessing antioxidant and antibacterial properties[14].

6.2.6 Studying the potential multifunctionality of propolis particles within other microstructures

Since propolis particles were shown to exhibit Pickering-like functionality in O/W emulsions, their performance in a more complex double emulsion system could be also investigated. To the best of the authors' knowledge, there was only one paper in literature that attempted to incorporate propolis in a double emulsion system where propolis was encapsulated in the internal phase which consisted of 70% ethanol and 30% water (O/W/O emulsion). Rutin which can be found in propolis, has shown ability

to stabilise double emulsions[15]. Therefore, there is potential that the propolis particle aqueous dispersions could also exhibit Pickering functionality when incorporated in double emulsion systems.

Furthermore, the Pickering ability of the propolis particle aqueous dispersions could be tested in foam formulations. There are examples in literature where propolis has been part of a foam formulation, for instance as coating of a polyurethane-nanolignin composite for wound healing purposes[16], as part of a polyurethane foam for antibacterial preservation of the formulation[17] and as part of a foam for shampoo based application in order to combat dandruff causing bacteria[18]. However, its surface tension as well as its ability to stabilise foams has not been investigated yet. Provided that propolis particles aqueous dispersions are surface active, they could potentially show an affinity to the liquid- air interface, providing Pickering-like stabilisation.

6.3 References

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Chapter 7- Appendices

A1.1: UV-vis calibration and raw absorption data for the determination of total phenol content investigated in Chapter 3.

A)

Gallic acid concentration (µg/ml)	Absorbance
15.625	0.153±0.002
31.250	0.251±0.008
62.500	0.500±0.043
125.000	0.914±0.020
250.000	1.790±0.068

B)

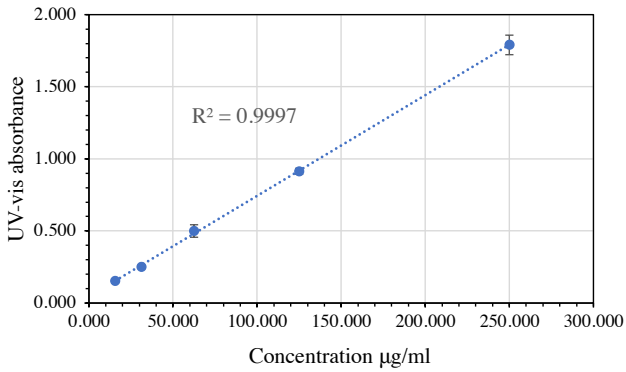

Figure A1. 1: A) Average molecular absorbance vs concentration of gallic acid and B) calibration curve of the absorbance vs concentration of gallic acid. Data shown are averages (n=3; repeats).

A1.2: UV-vis calibration and raw absorption data for the determination of DPPH assay investigated in Chapter 3.

A)

Trolox concentration (µg/ml)	Absorbance
200.00	0.14±0.07
100.00	0.56±0.06
50.00	0.77±0.09
25.00	0.85±0.05
12.50	0.91±0.04

B)

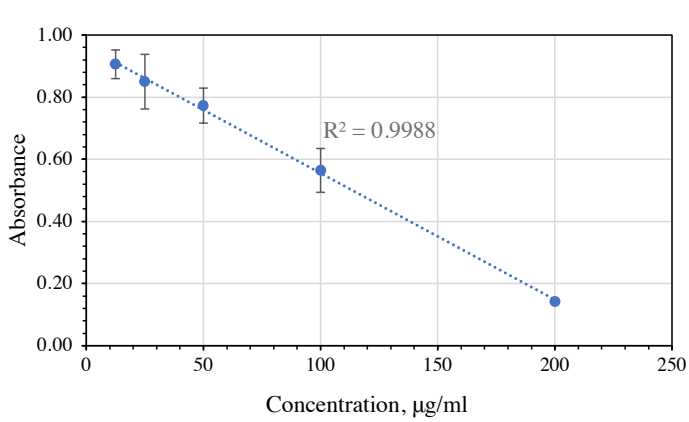

Figure A1. 2: A) Average molecular absorbance vs concentration of trolox and B) calibration curve of the absorbance vs concentration of trolox. Data shown are averages (n=3; repeats).

A1.3: Size distribution of 1.88% propolis aqueous dispersions investigated in Chapter 3

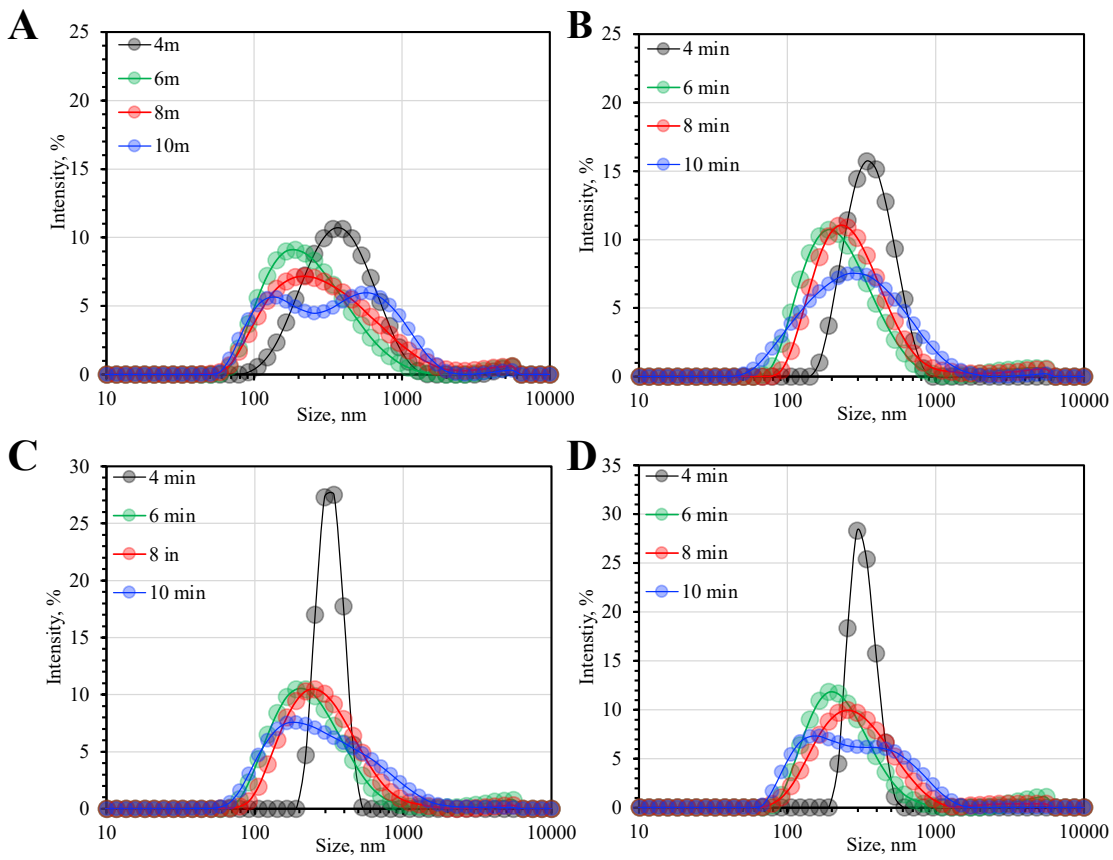

Figure A1. 3: Average particle size distributions of propolis particles dispersed in an aqueous media. Data for dispersions of 1.88% propolis mass fractions are presented for different sonication times (4, 6, 8 and 10 min) and storage periods; A. 7 days, B. 14 days, C. 21 days, D. 30 days at 25°C in the dark. Data shown are averages (n=3; replicates).

A2: Interfacial tension of 15% propolis dispersion with increasing amount of tween 80.

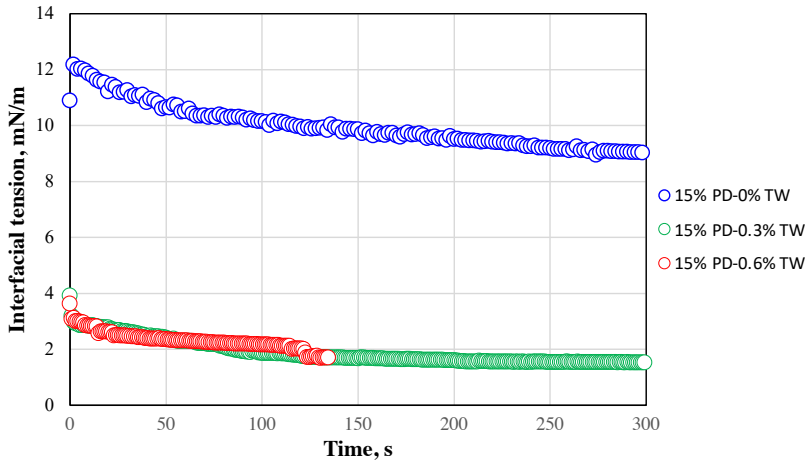

Figure A2.1: Dynamic interfacial tension between sunflower oil and 15% propolis dispersion (15% PD)), 0.3% and 0.6% Tween 80 (0.3% TW and 0.6% TW, respectively).

The 0.6% sample's measurement was stopped automatically by the software because the interfacial tension stopped being detected.

A2.2: Creaming index and light microscopy of propolis and control emulsions investigated in Chapter 4

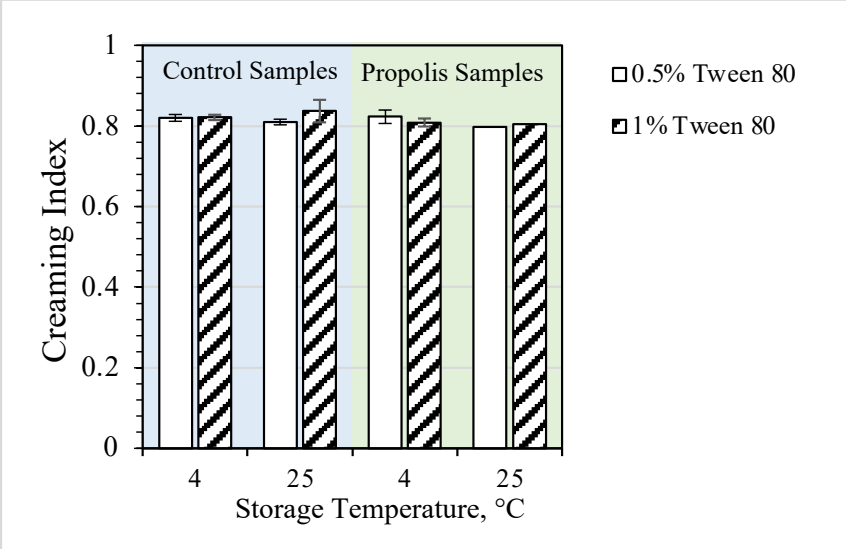

Figure A2.2: Creaming indices for control and 15% propolis emulsions, formulated with 0.5 and 1% Tween 80 and 15% oil at 4 and 25 °C storage temperatures. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

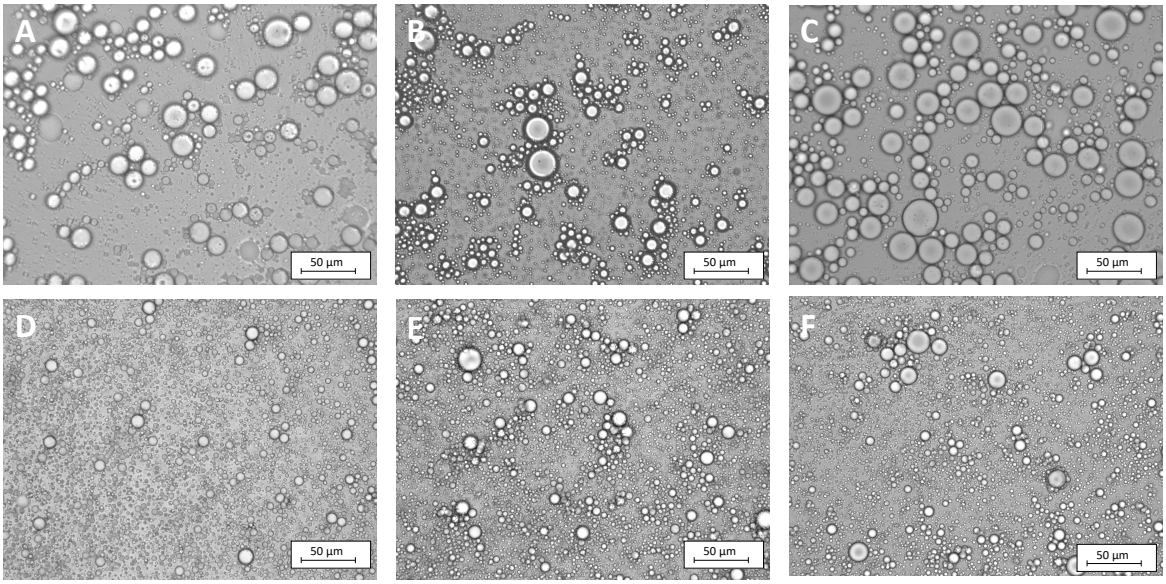

Figure A2.3: Light microscopy images of (A, B, C) control and (D, E, F) 15% propolis emulsions formulated with 15% oil and 0.5% Tween 80 via HSM. Images of the emulsions were taken at: A-D) upon production, B-E) post 60 days storage at 4 °C and C-F) post 60 days storage at 25 °C (40× magnification).

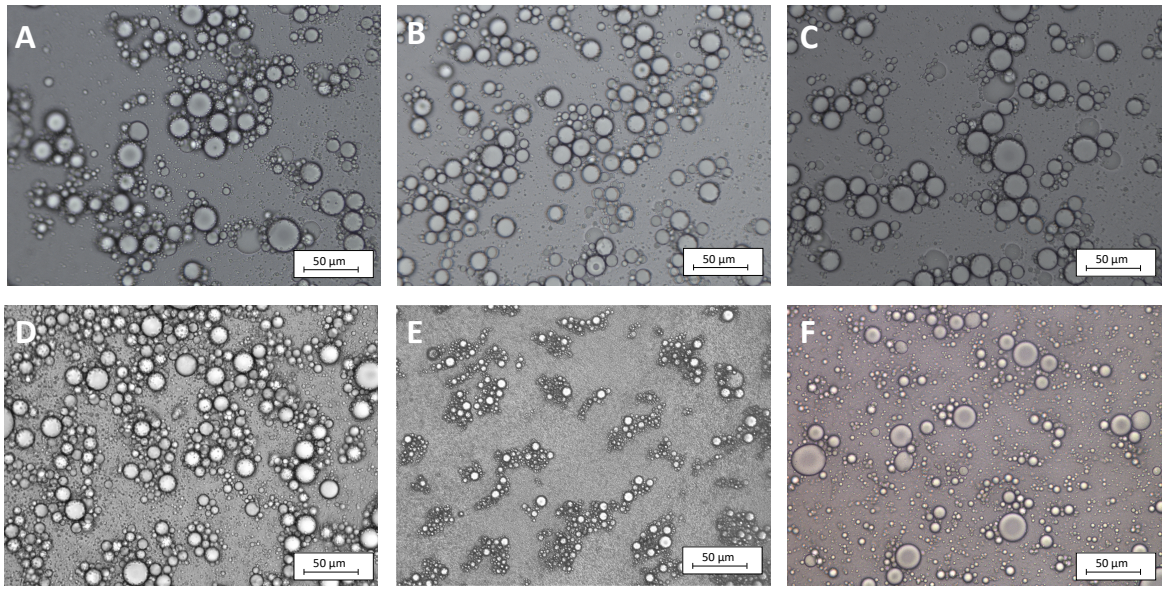

Figure A2.4: Light microscopy images of (A, B, C) control and (D, E, F) propolis emulsions formulated with 15% oil and 1% Tween 80 via HSM. Images of the emulsions were taken at: A-D) upon production, B-E) post 60 days storage at 4 °C and C-F) post 60 days storage at 25 °C (40× magnification).

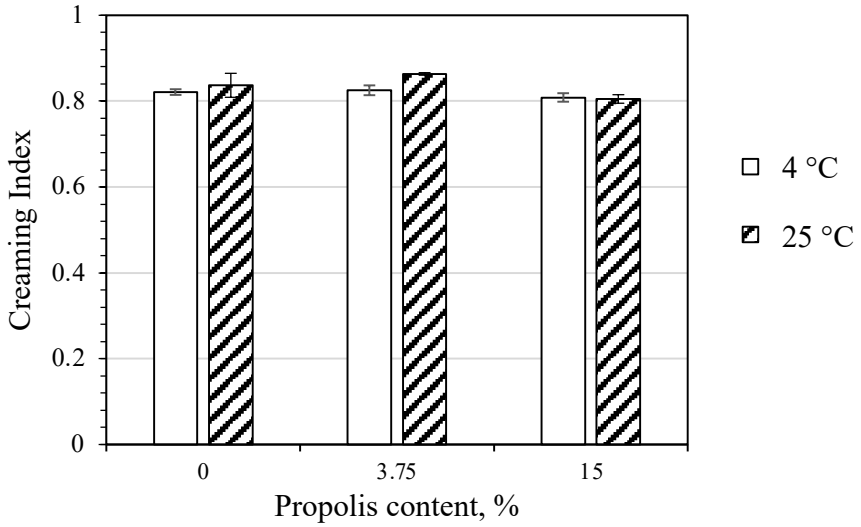

Figure A2.5: Creaming indices for control and propolis emulsions, formulated with 0, 3.75 and 15% propolis mass fraction, 1% Tween 80 and 15% oil at 4 and 25 °C storage temperatures. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

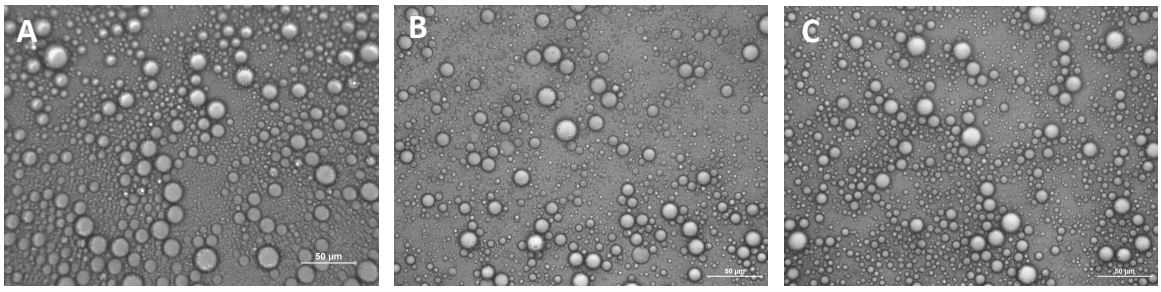

Figure A2.6: Light microscopy images of propolis emulsions formulated with 3.75% propolis, 15% oil and 1% Tween 80 via HSM. Images of the emulsions were taken at: A) upon production, B) post 60 days storage at 4 °C and C) post 60 days storage at 25 °C (40× magnification).

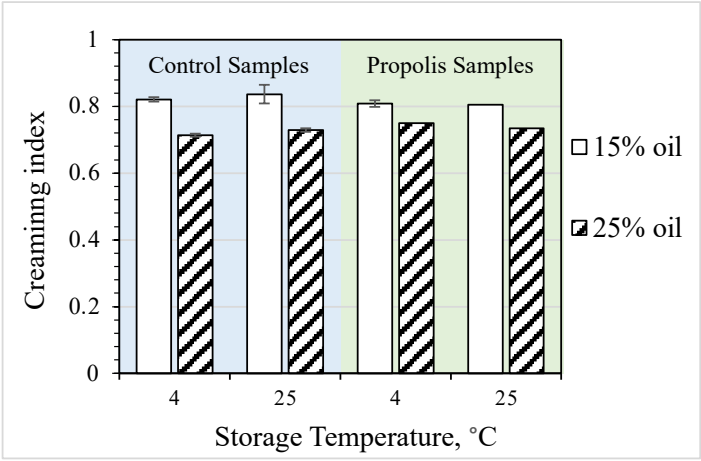

Figure A2.7: Creaming indices for control and propolis emulsions, formulated with 15% and 25% oil and 1% Tween 80 at 4 and 25 °C storage temperatures. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

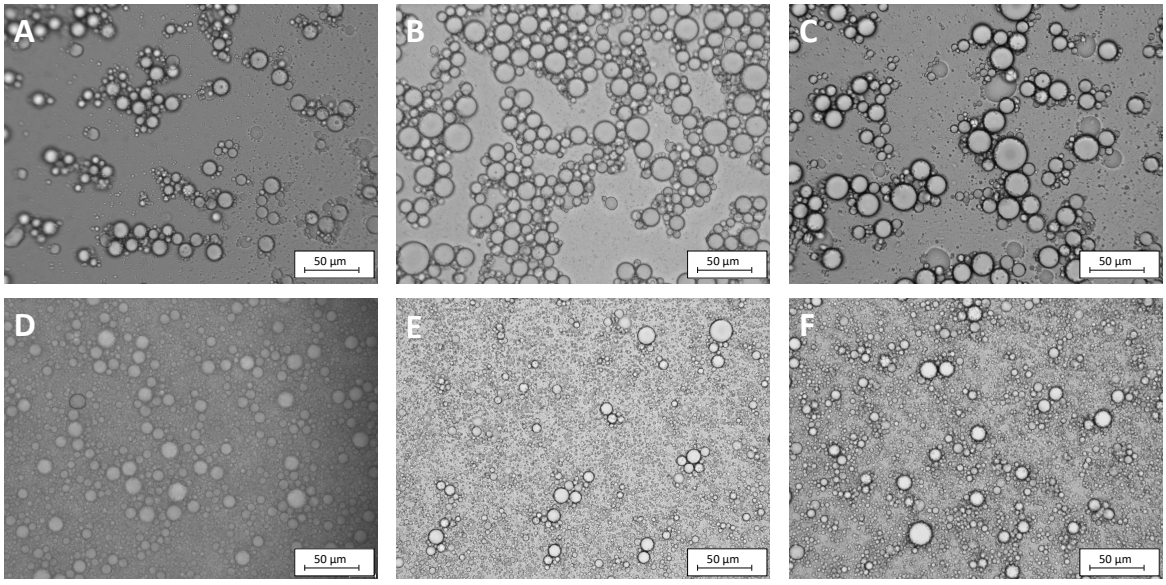

Figure A2.8: Light microscopy images of (A, B, C) control and (D, E, F) propolis emulsions formulated with 25% oil and 1% Tween 80 via HSM. Images of the emulsions were taken at: A-D) upon production, B-E) post 60 days storage at 4 °C and C-F) post 60 days storage at 25 °C (40× magnification).

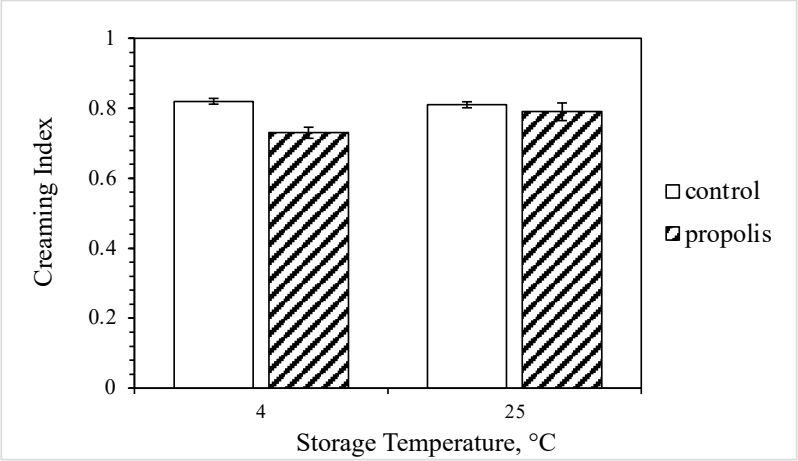

Figure A2.9: Creaming indices for control and propolis emulsions, formulated with 15% oil, 1% sodium caseinate at pH 4.6 stored at 4 and 25 °C storage temperatures. Data shown are averages (n=3; repeats) and error bars represent ± one standard deviation (s.d.).

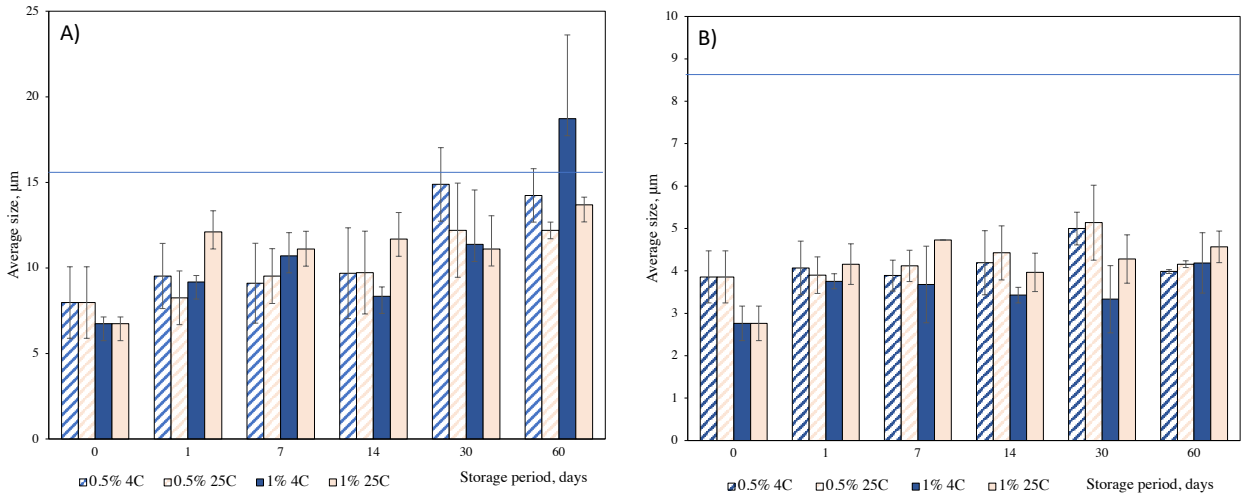

Figure A2.10: A) Average volume ($D_{4,3}$) and B) surface ($D_{3,2}$) weighted mean oil droplet sizes of 15% O/W emulsions produced with propolis particle dispersions (15% Propolis mass fraction) as their continuous (aqueous) phase, in the presence of 0.5% and 1% Tween 80, via HSM. Lines on the figures represent an approximation of the control (emulsions with water as their continuous phase). Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (n = 3; replicates) and errors represent ± one standard deviation (s.d.).

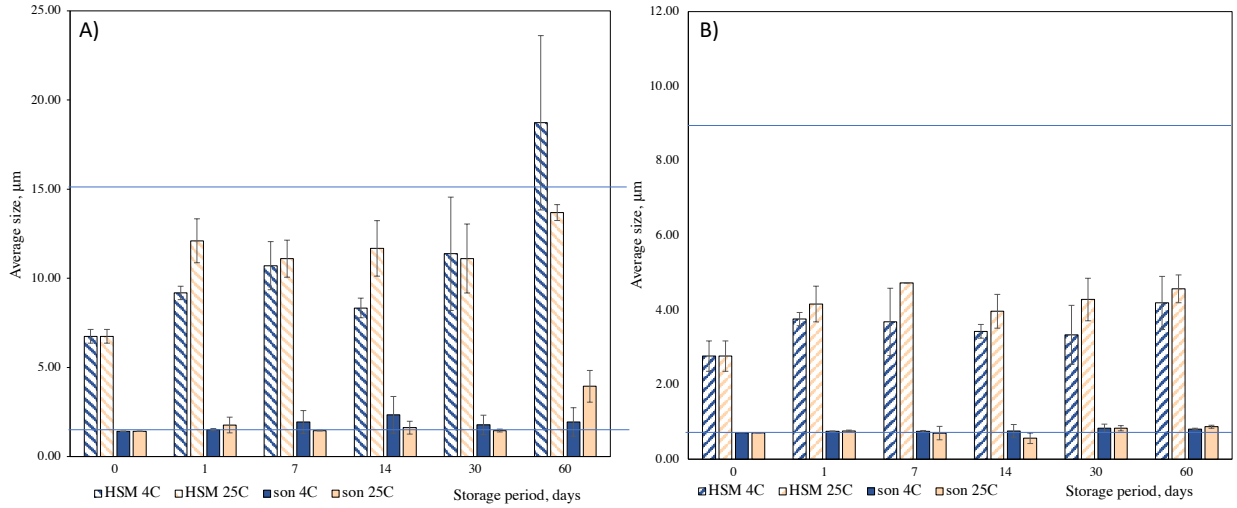

*Figure A2.11: A) Average volume ($D_{4,3}$) and B) surface ($D_{3,2}$) weighted mean oil droplet sizes of 15% O/W emulsions produced with propolis particle dispersions (15% Propolis mass fraction) as their continuous (aqueous) phase, in the presence of 1% Tween 80, made via HSM and sonication (son). Lines on the figures represent an approximation of the control (emulsions with water as their continuous phase). Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (*n = *3; replicates) and errors represent ± one standard deviation (s.d.).*

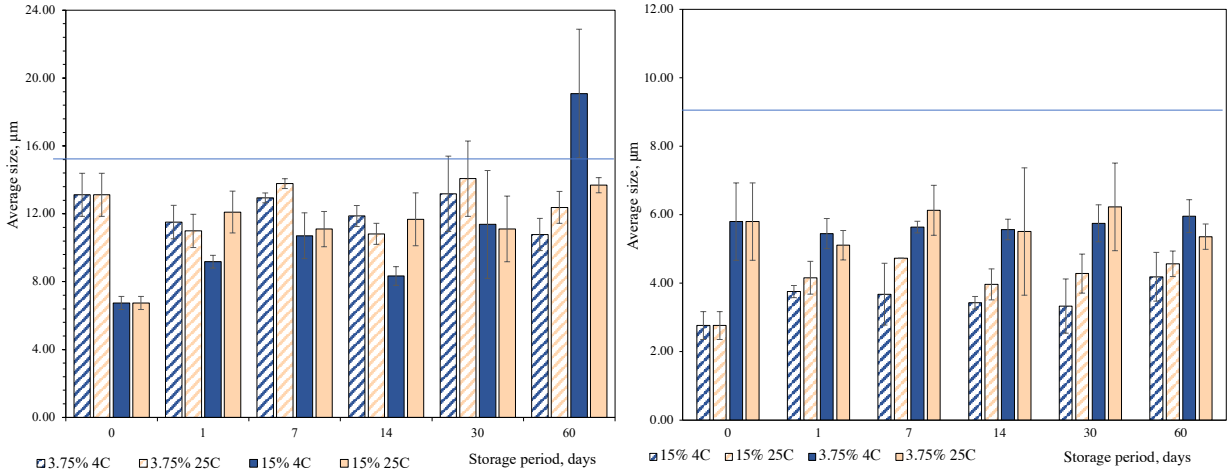

Figure A2.12: A) Average volume ($D_{4,3}$) and B) surface ($D_{3,2}$) weighted mean oil droplet sizes of 15% O/W emulsions produced with propolis particle dispersions (3.75% and 15% propolis mass fraction) as their continuous (aqueous) phase, in the presence of 1% Tween 80, made via HSM. Lines on the figures represent an approximation of the control (emulsions with water as their continuous phase).

*Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (*n = *3; replicates) and errors represent ± one standard deviation (s.d.).*

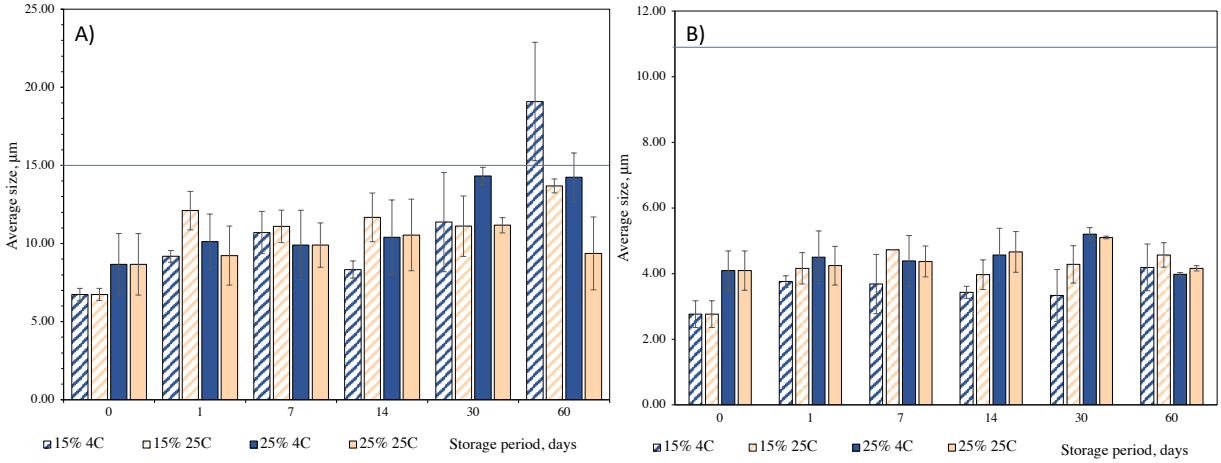

*Figure A2.13: A) Average volume ($D_{4,3}$) and B) surface ($D_{3,2}$) weighted mean oil droplet sizes of 15% and 25% O/W emulsions produced with propolis particle dispersions (15% propolis mass fraction) as their continuous (aqueous) phase, in the presence of 1% Tween 80, made via HSM. Lines on the figures represent an approximation of the control (emulsions with water as their continuous phase). Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (*n = *3; replicates) and errors represent ± one standard deviation (s.d.).*

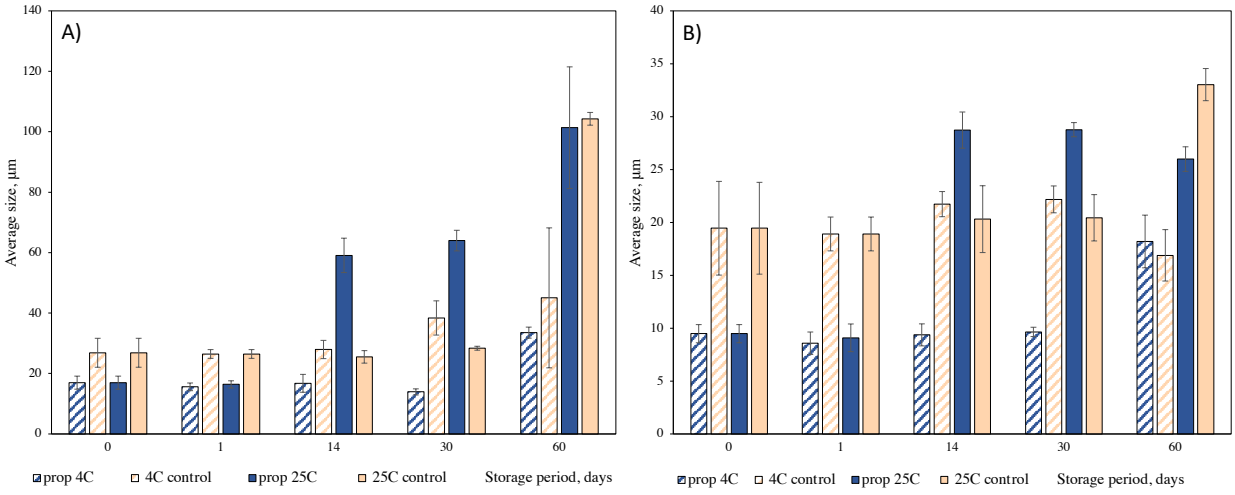

*Figure A2.14: A) Average volume ($D_{4,3}$) and B) surface ($D_{3,2}$) weighted mean oil droplet sizes of 15% O/W emulsions produced with propolis particle dispersions (15% propolis mass fraction) as their continuous (aqueous) phase or water (control), in the presence of 1% sodium caseinate, made via HSM. Measurements were performed immediately after production and over a storage period of 60 days, at 4 and 25°C. Data shown are average values (*n = *3; replicates) and errors represent ± one standard deviation (s.d.).*