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João Filipe Dade Robalo António

DEVELOPMENT OF AN ACTIVE WHEY PROTEIN FILM USING PORTUGUESE GREEN TEA (*CAMELLIA SINENSIS* L.) EXTRACT TO ENHANCE LATIN-STYLE FRESH CHEESE SHELF LIFE

Dissertação de Mestrado em Segurança Alimentar, orientada pelo Professor Doutor Fernando Ramos e coorientada pela Doutora Ana Sanches Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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“Nothing happens quite by chance. It's a question of accretion of information and experience.”

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A todos o meu sincero
obrigado!

Resumo

O queijo fresco é um queijo não curado, ligeiramente ácido e levemente salgado, feito a partir de leite cru ou pasteurizado, muito consumido na Europa, nomeadamente em Portugal. No entanto o queijo fresco tem um tempo de prateleira reduzido devido a características como, pH elevado (>5), elevada atividade de água e elevado teor de lípidos que favorecem o crescimento de microrganismos e a oxidação lipídica.

O processo de fabrico do queijo leva, à produção de soro de leite, o principal subproduto da indústria queijeira. Infelizmente, o soro do queijo se não for eliminado corretamente pode tornar-se um grande fator de poluição ambiental devido à sua alta carência química e bioquímica de oxigénio. A nível global os produtores de queijo deparam-se com os problemas da perecibilidade do queijo fresco e com a gestão da eliminação do soro do leite. Embora anteriormente o soro do queijo fosse considerado um desperdício, atualmente é reconhecido como um subproduto valioso, uma vez que retém 55% dos nutrientes do leite. Além disso, a proteína do soro do leite compreende vários compostos bioativos que podem ser usados pela indústria nutracêutica.

Atualmente, há um interesse crescente nas "embalagens verdes" devido ao impacto ambiental negativo dos materiais de embalagem convencionais. Os avanços tecnológicos permitiram o desenvolvimento de tecnologias de separação por membrana, que permitem que o soro seja processado em concentrados de proteína de soro que podem ser utilizados para produzir películas ativas biodegradáveis com agentes antioxidantes e/ou antimicrobianos incorporados, com o objetivo de retardar a oxidação lipídica e prevenir a contaminação pós-processo.

O chá verde (*Camellia sinensis* L.) não só é a segunda bebida mais consumida mundialmente, mas é também rico em compostos bioativos como os polifenóis, tais como flavonóides e ácidos fenólicos; e portanto, é bem conhecido pelas suas propriedades antioxidantes e antimicrobianas. Portugal é um dos poucos produtores de chá (*C. sinensis* L.) na Europa. A "Gorreana" é uma das marcas mais conhecidas de chá português, e o seu chá é produzido na Ilha de São Miguel, nos Açores. Recentemente, uma empresa, a "Chá Camélia", passou a produzir chá verde numa quinta do Concelho de Vila do Conde. No entanto, este chá nunca foi caracterizado antes.

Assim, os objetivos principais deste estudo foram comparar diferentes chás verdes ("Gorreana", "Chá Camélia" e "Happy flora") quanto à sua capacidade antioxidante, incluindo o chá verde "Chá Camélia" da produção de 2020, bem como desenvolver uma embalagem ativa biodegradável e comestível usando proteína de soro de leite e extratos de chá verde para prolongar o tempo de prateleira do queijo fresco. No âmbito deste estudo, os métodos de extração sólido-líquido convencional e Soxhlet foram comparados relativamente à obtenção de extratos concentrados de *C. sinensis* L. A capacidade antioxidante de infusões de chá verde e de extratos de diferentes amostras de chá verde foi avaliada, através do sistema de inibição do radical DPPH e do ensaio do branqueamento do β-caroteno. Além disso, o conteúdo de compostos fenólicos totais e flavonóides também foi determinado. Posteriormente, foram produzidas embalagens à base de proteína de soro de leite chá verde ou extratos de chá verde incorporado. Adicionalmente, por meio de ensaios de migração, a capacidade antioxidante dos filmes foi avaliada pelos métodos mencionados anteriormente.

Os nossos resultados demonstraram que os chás verdes portugueses avaliados têm capacidade antioxidante superior (AAC=746,7) relativamente ao chá verde asiático avaliado (AAC=650). Além disso, o chá verde produzido a partir das folhas da nova plantação portuguesa de chá "Chá camélia" apresentou o maior potencial de retenção da capacidade antioxidante (97,3%). Verificou-se também que o método de extração sólido-líquido convencional permite obter extratos com maior atividade antioxidante (AAC=1500), no entanto as extrações obtidas por Soxhlet apresentaram maior rendimento (43%). Adicionalmente, a embalagem ativa com o extrato de chá verde português incorporado exibiu uma alta capacidade antioxidante (AAC≈595,4). Conclui-se também que os filmes ativos inibiram efetivamente o crescimento de microrganismos, principalmente *E. coli* (1,5 x 10 UFC/g), quando comparados com o filme controlo (2,2 x 10^2 UFC/g). Este estudo sugere que a nova embalagem à base de proteína de soro de leite incorporado com extrato de chá verde português pode prolongar a vida útil do queijo fresco.

Palavras-chave: Queijo fresco; Chá verde; proteína de soro de leite; embalagem comestível; embalagem ativa; capacidade antioxidante.

Abstract

Fresh cheese is a slightly acid and mildly salted non-ripened cheese, made from raw or pasteurized milk, widely consumed in Europe, particularly in Portugal. However, its high pH (>5), water activity, and lipid content favor the growth of microorganisms and lipid oxidation, leading to a short shelf-life.

Cheese manufacturing leads to the production of whey, the main by-product of the cheese industry. Unfortunately, cheese whey can become a major pollution factor if not managed correctly due to its high chemical and biochemical oxygen demand. Therefore, cheesemakers worldwide face both the perishability of fresh cheese and the management of cheese whey. Even though formerly, cheese whey was considered a waste, it has been acknowledged as a valuable by-product since it retains 55% of milk nutrients in recent years. Additionally, cheese whey protein comprises several bioactive compounds that can be used by the nutraceuticals industry.

Nowadays, there is a growing interest in "green packaging" due to the negative environmental impact of conventional packaging materials. Advances in technology have enabled the development of membrane separation technologies, which allow the whey to be processed into whey protein concentrates that can be used to make biodegradable active films with antioxidant/antimicrobial agents to delay lipid oxidation and prevent post-process contamination. Green tea (*Camellia sinensis* L.), the second most consumed beverage globally, is rich in bioactive compounds such as polyphenols, namely flavonoids and phenolic acids; therefore, it is well known for its antioxidant and antimicrobial properties. Portugal is one of the few producers of tea (*C. sinensis* L.) in Europe. "Gorreana" is one of the most well-known brands of Portuguese tea, and it is produced in São Miguel Island, in the Azores. Lately, a company, "Chá Camélia," started to produce green tea on a farm nearby Vila do Conde. However, this tea has never been characterized before.

Thus, the main objectives of this study were to compare different green teas ("Gorreana," "Chá Camélia," and "Happy flora") regarding their antioxidant capacity, including Chá Camelia green tea from 2020 production, as well as develop a biodegradable and edible active film using whey protein and green tea extracts to extend the fresh cheese shelf-life. In the frame of this study, solid-liquid and Soxhlet extractions were compared to obtain *C. sinensis* L. concentrated extracts. The antioxidant capacity of green teas and green teas extracts was assessed through DPPH free radical scavenging activity and ***β***-carotene bleaching assay.

Moreover, total phenolics and total flavonoids content were also determined. Furthermore, whey films incorporated with green tea and green tea extracts were produced. Additionally, through migration assays, the antioxidant capacity of the films was evaluated using the previously mentioned methods.

Our results demonstrated that Portuguese green teas have a higher antioxidant capacity (AAC=746.7) than Asian green tea (AAC=650). Furthermore, green tea produced from the leaves of the new Portuguese "Chá camélia" tea plantation had the highest potential to retain the antioxidant capacity (97.3%). Additionally, solid-liquid extraction led to extracts with higher antioxidant activity (AAC=1500) but Soxhlet extractions present higher yield (43%). Furthermore, the active film incorporated with Portuguese green tea extract exhibited high antioxidant capacity (AAC≈595.4). The active films effectively inhibited the growth of microorganisms, especially *E. coli* (1.5 x 10 CFU/g), compared to the control film (2.2 x 10^2 CFU/g). This study suggests that the new whey protein film incorporated with Portuguese green tea extract can extend fresh cheese shelf life.

Keywords: Fresh cheese; Green tea; whey protein; edible film; active packaging; antioxidant capacity.

List of Abbreviations

AAC – Antioxidant Activity Coefficient

B.C – Before Christ

BOD – Biochemical oxygen demand

C. sinensis – *Camellia sinensis* L.

CFU – Colony-forming unit

COD – Chemical oxygen demand

CVD – Cardiovascular disease

DPPH• – 2,2-Difenil-1-picrylhidrazyl

E. coli – *Escherichia coli*

EC – European Commission

ECE – Epicatechin Equivalents

EGCG – Epigallocatechin Gallate

EU – European Union

FAO – Food and Agriculture Organization

FCR – Folin-Ciocalteu reagent

FRAP – Ferric Reducing Antioxidant Power

g – Gram

GAE – Gallic Acid Equivalents

GRAS – Generally recognized as safe

HAT – Hydrogen Atom Transfer

INE – Instituto Nacional de Estatística (National Estatistics Institute)

IP – Inhibition Percentage

kg – Kilogram

L – Liter

L. monocytogenes – *Listeria monocytogenes*

LDL-C – Low-density lipoprotein cholesterol

mg – Milligram

mL – Milliliter

mm – Millimeter

nm – Nanometer

°C – Graus Celsius

ORAC – Oxygen Radical Absorbance Capacity

PGI – Protected geographical indication

POD – Protected designation of origin

ppm – Parts per million

r^2 – Coefficient of determination

ROS – Radical oxygen species

rpm – Rotações por minuto

SET – Single-electron transfer

SE-β-LG – Seleno-β-lactoglobulin

TBARS – Thiobarbituric Acid Reactive Substances

TBC – Total bacteria count

TE – Trolox Equivalents

TEAC – Trolox Equivalent Antioxidant Activity

TFC – Total Flavonoids Content

TOSCA – Total radical scavenging capacity assay

TRAP – Total Radical-trapping Antioxidant Parameter

USA – United States of America

UV – Ultraviolet

WHO – World Health Organization

WPC – Whey protein concentrate

WPI – Whey protein isolate

α-LA – Alpha-lactalbumin

µg – Microgram

µL – Microlitre

µM – Micromolar

β-LG – Beta-Lactoglobulin

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I. Introduction

Food waste, environmental impacts, increasing population, demand, and the consequent pressure on natural resources are societal challenges that suggest the need to change our food system into a more sustainable one. When applied to the cheese industry, circular economy includes utilizing by-products such as whey (the primary pollutant of the cheese industry) (Jurgilevich *et al*., 2016). Whey protein can be used as a biopolymeric matrix in active edible film formulations, in which additives can be incorporated (Campos *et al*., 2011).

Furthermore, consumers' growing concern about food safety has pushed the food industry to turn to natural additives (Ramos *et al.,* 2012). Composed essentially by polyphenols, mainly catechins (including epicatechin; epigallocatechin; epigallocatechin-gallate (EGCG) and epicatechin-gallate), and caffeine (2.5-4% dry weight), green tea extracts may be potential natural antioxidant agents (Colombo *et al*., 2020; Kim *et al.,* 2014; Rubab *et al.,* 2020). Moreover, green tea extracts and EGCG have been shown to have the capacity to suppress foodborne pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes, Salmonella typhimurium, Clostridium perfringens, Pseudomonas aeruginosa, Pseudomonas fragi, Helicobacter pylori, and Brochothrix thermosphacta* and therefore may be potential natural antimicrobial agents as well (Nikoo *et al*., 2018). This actively demonstrates that green tea extracts may act as antioxidant and antimicrobial agents when incorporated into biopolymeric matrices such as whey. Castro *et al.* (2019) and Andrade *et al.* (2019) have developed whey protein-based films incorporated with green tea extracts and applied them to salmon and salami, respectively, to inhibit lipid oxidation. Portugal has, up to date, three plantations, "Gorreana", "Chá Camélia" and "Chá Porto Formoso", that produce outstanding green teas that can be used to obtain green tea extracts.

Even though whey protein-based films incorporated with green tea extracts have been applied to other food products, they have never been applied to Latin-style fresh cheeses, a very perishable dairy product very consumed in Portugal. In fact, according to the literature, no active packaging was developed for this type of cheese.

In this context, the present research study aims to characterize the antioxidant capacity of Portuguese and Asian green teas, optimize the preparation of green tea extract and develop an active edible film based on whey protein and green tea extracts. This package incorporated with Portuguese green tea extracts is expected to prolong Latin-style fresh cheese shelf-life.

II. State of the Art

2.1 Cheese: history and classification

Cheese is a fermented dairy product whose origins are unknown. Nevertheless, there is written and iconographic evidence of cheese making from mid-third-millennium BC. Furthermore, archaeological discoveries provided evidence of cheese making from the sixth millennium BC. Therefore, cheese is rather prehistoric, and its true origins lie in the Neolithic, many millennials BC (Salque *et al*., 2013).

Throughout history, several types of cheese have been developed and, currently, there are approximately 1400 different cheese varieties (Associação Portuguesa de Nutrição, 2018). According to the Codex Alimentarius, cheese is defined as "the ripened or unripened soft, semi-hard, hard, or extra-hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by: (a) coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from the coagulation, while respecting the principle that cheesemaking results in a concentration of milk protein (in particular, the casein portion), and that consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made; and/or (b) processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a)" (FAO/WHO, 2011).

Worldwide there are multiple cheese varieties, and consequently, multiple cheese classification models exist (Almena-Aliste & Mietton, 2014). Cheeses can be classified according to ripening (unripened, ripened, and ripened by the action of molds); composition (with or without the addition of foodstuffs other than cheese), fat content (low-fat, medium-fat, fat, and very fat); and paste type and moisture (fresh paste, soft paste, semi-soft paste, semi-hard paste, hard and extra hard-paste, blue cheeses, pasta filata cheeses, fruit & flavored cheeses, and processed cheeses) (Associação Portuguesa de Nutrição, 2018; Modesto & Barbosa, 2007).

2.2 From milk to cheese

Over the centuries, the basic principles of cheese production have not undergone significant changes. Currently, the final product is produced through a set of steps from milking to packaging, shipping, and transport. In the particular case of fresh cheese, the stages of ripening and maturation do not occur (Associação Portuguesa de Nutrição, 2018).

Cheese production is a somewhat complex process. Briefly, cheese making involves ensuring the safety of the main raw material, i.e., milk. Afterward, the milk is heated until it reaches approximately 74°C (Pasteurization), after which it is necessary to cool down to proceed to the next stage. Some traditional cheeses do not go through the pasteurization step. Then, one of the essential operations, coagulation, takes place (Associação Portuguesa de Nutrição, 2018). At this stage, the milk will pass from a liquid state to a gel by the enzymatic way, yielding the curd (a combination of casein, protein, and milk fat) (Modesto & Barbosa, 2007; Salque *et al.*, 2013). Subsequently, the curd is cut. A lighter cut releases less whey, and it is applied in the production of soft cheeses, while a deeper cut releases more whey, and it is applied in the production of semi-hard and hard cheeses (Modesto & Barbosa, 2007). In the draining stage, the curd is placed on a table with a groove, through which the whey is drawn. After being separated from the whey, the curd is shaped, pressed, and salted, resulting in fresh cheese. However, following the draining stage, most cheeses (soft, semi-hard and hard) go through a ripening stage.

2.3 Fresh cheese

Fresh cheese is a very consumed dairy product in the European Union (Hinrichs, 2001). Classified as an unripened cheese, fresh cheese is obtained by coagulation and draining of milk by lactic fermentation, with or without the addition of rennet. In addition, it is also considered a fresh paste cheese, since it is a product that contains a lot of moisture (Associação Portuguesa de Nutrição, 2018; Modesto & Barbosa, 2007).

Within the fresh cheeses category, Latin-style fresh cheese is a fresh cheese produced by enzymatic coagulation of milk with rennet, without adding starter cultures, traditionally made in the Iberian Peninsula, widely consumed by the Portuguese population. With a mutually fresh and slightly acidic flavor, it is characterized by its soft texture and high moisture content, culminating in a slightly salty, nutritious, and healthy cheese (Coelho *et al.*, 2014; C. C. G. Silva *et al.*, 2015).

Furthermore, fresh cheese is one of the essential ingredients in the Mediterranean diet. This diet recommends the daily consumption of two to three portions of dairy products, with one portion being equivalent to, approximately, a small fresh cheese (50g) (Associação Portuguesa de Nutrição, 2018).

2.3.1 Nutritional composition and health benefits

Globally there are hundreds of cheese varieties, with multiple nutritional compositions that can vary according to several factors, such as the manufacturing process, the milk used for production, bacterial cultures, and ripening conditions (Associação Portuguesa de Nutrição, 2018). However, despite the differences, they are essentially made up of fat, water, proteins, vitamins, and minerals (Walther *et al*., 2008). Cheese fat is mainly made up of saturated fatty acids, which may represent a risk factor for cardiovascular disease (CVD), as they tend to increase low-density lipoprotein cholesterol (LDL-C) (Chen *et al*., 2017). Despite what was previously mentioned, there is no evidence of a correlation between the consumption of fresh cheese and increased risk of hypertension or stroke (Associação Portuguesa de Nutrição, 2018). Nevertheless, the World Health Organization (WHO) stated that "to avoid unhealthy weight gain, total fat should not exceed 30% of total energy intake" (World Health Organization, 2019). However, the average fresh cheese fat content is lower (8g/100g) when compared to soft (22g/100g), semi-hard (27g/100g), hard (31g/100g), and extra hard cheese (33g/100g) (Walther *et al*., 2008). Furthermore, Asensio-Grau *et al*. documented that fresh-cow cheese presented lower fat content when compared to fresh-goat, mild, and aged cheeses. Moreover, according to the same authors, although fresh-cow cheese has the lowest fat content, fresh-goat cheese triglycerides are more digestible than fresh-cow cheese triglycerides, increasing its nutritional value(Asensio-Grau *et al*., 2019).

Nevertheless, the fresh cheese lactose content is higher when compared to other cheese types, as it still contains traces of whey. Thus, unlike most cheese varieties, it can cause harmful effects in lactose-intolerant individuals; however, there are lactose-free options on the market (Associação Portuguesa de Nutrição, 2018; Walther *et al*., 2008).

Cheese is also an essential source of calcium (Ca), zinc (Zn), phosphorus (P), and magnesium (Mg), with the levels of Ca and P being much higher than those in milk. Since the Ca/P ratio makes calcium bioavailable, the consumption of cheese, associated with a good source of vitamin D, has a potential preventive effect against osteoporosis (López-Expósito *et al*., 2012). In addition to minerals, cheese is essentially rich in vitamins A, B2, B3, B6, B12, and folates (López-Expósito *et al*., 2012; Walther *et al*., 2008).

Besides being an essential source of easily digestible proteins with high biological value, cheese also provides a wide variety of essential amino acids in higher amounts than those recommended for children and adults (Associação Portuguesa de Nutrição, 2018; Walther *et al*., 2008). Nevertheless, many of the nutraceutical properties of cheese are due to bioactive peptides that perform various biological activities (López-Expósito *et al*., 2012).

According to FAO/WHO, probiotics may be defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill *et al*., 2014). Because of their characteristics, such as high moisture content, lack of cultures that may compete with potential probiotic bacteria, and nearly neutral pH, Latin-style fresh cheeses, such as the ones used in this study, may serve as an ideal food carrier for the delivery of probiotic cultures into the intestine (C. C. G. Silva *et al*., 2015).

Overall, fresh cheese is a vital dairy product for a healthy diet due to its nutritional characteristics (Table I). It provides essential macronutrients, micronutrients, minerals, and vitamins that are important for humans during the different stages of growth and development (Lordan *et al*., 2018). Therefore, fresh cheese may be considered the most recommended type for a healthy and balanced diet.

Table I Nutritional composition of sheep fresh cheese (per 100g) according to the Food Composition Table available on the PortFIR website (http://portfir.insa.pt/#).

Sheep fresh cheese	
Energy [kcal]	286
Energy [kJ]	1190
Lipids [g]	22.9
Saturated fatty acids [g]	13.2
Monounsaturated fatty acids [g]	5.3
Polyunsaturated fatty acids [g]	0.7
Linoleic acid [g]	0.6
Trans fatty acids [g]	0.8
Carbohydrates [g]	0.1
Sugars [g]	0.1
Lactose [g]	0.1
Salt [g]	2.5
Protein [g]	19
Water [g]	52.1
Organic acids [g]	1
Cholesterol [mg]	89
Total Vitamin A (retinol equivalents) [µg]	234
Carotene [µg]	95
Vitamin D [µg]	0.3

Sheep fresh cheese	
a-tocopherol [mg]	0.48
Thiamine [mg]	0.05
Riboflavin [mg]	0.45
Niacin [mg]	0.45
Niacin equivalents [mg]	5.35
Tryptophan/60 [mg]	4.9
Vitamin B6 [mg]	0.1
Vitamin B12 [µg]	1.45
Folates [µg]	27
Gray [g]	3.51
Sodium (Na) [mg]	1000
Potassium (K) [mg]	120
Calcium (Ca) [mg]	580
Phosphorus (P) [mg]	420
Magnesium (Mg) [mg]	39
Iron (Fe) [mg]	0.7
Zinc (Zn) [mg]	2.4

2.3.2 Fresh cheese spoilage

Due to its high pH (above 5.0), water activity, and lipid content, fresh cheeses are very susceptible to the oxidation of lipids and microbial contamination, which affect their quality and safety (Andrade *et al*., 2019; Coelho *et al*., 2014; Costa *et al*., 2018; C. C. G. Silva *et al*., 2015).

The oxidation of lipids, which results in the development of volatile chemicals associated with unwanted off-flavors or rancidity and shortens shelf life, is one of the most common deterioration pathways (Nikoo *et al*., 2018). Lipid oxidation can alter the type and concentration of certain chemical compounds in cheese, altering their nutritional value, causing the loss of liposoluble vitamins and organoleptic properties, resulting in unpleasant tastes and/or aromas, shortening the shelf life of the food. Some chemical compounds generated by lipids' oxidation have been associated with aging, mutagenesis, carcinogenesis, neurological disorders, atherosclerosis (such as Parkinson's disease and Alzheimer's disease), and chronic inflammatory diseases; therefore, they may pose a risk to human health (Andrade *et al*., 2019).

In addition, fresh cheeses are particularly vulnerable to post-process microbial contamination. During handling and storage, fresh cheese is vulnerable to the growth of nonpathogenic and pathogenic microorganisms. The growth of bacteria, yeast, and molds may not only reduce their quality but also affect their safety since the colonization by pathogenic

bacteria, such as *Escherichia coli, Salmonella, Staphylococcus aureus,* and *Listeria monocytogenes* may endanger consumers by causing foodborne diseases (Costa *et al*., 2018; Nikoo *et al*., 2018; C. C. G. Silva *et al*., 2015).

Therefore, due to the previously mentioned characteristics, fresh cheese is very perishable and has a reduced shelf life (Modesto & Barbosa, 2007). Thus, it must be sold in closed packages and transported and stored at temperatures below 5°C in order to avoid contamination. In addition, it is essential to emphasize that its primary raw material is milk. Therefore, it must be produced from healthy animals, and the transport between the milking place and the cheese production site must be as fast as possible, respecting food safety standards (Rocha, 2019).

Nevertheless, despite their perishability, fresh cheeses are very nutritious and therefore recommended for a healthy diet.

2.4 Socioeconomic value of cheese in Portugal

Although cheeses have similar forms of manufacture, the differences are related to the territory where they are made, and Portuguese cheeses are no exception. Portugal is a country that produces cheese of different varieties, whether from cow; goat; sheep, or mixtures, of excellent nutritional and organoleptic quality, and there are several kinds of cheese, from fresh to ripened, with Protected Designations (POD) of Origin and Protected Geographical Indication (PGI), such as "Travia da Beira Baixa POD" or "Queijo Serra da Estrela POD" (Rocha, 2019; Portuguese Nutrition Association, 2018). In this line, cheese is considered one of the pillars of Portuguese culture.

According to the National Institute of Statistics (INE) data, in Portugal, total cheese production increased around 8% between 2016 and 2019. The volume of cow's cheese was the highest, followed by sheep, mixed, and goat cheese (INE 2021). In addition, cheese is one of the most consumed dairy products, with fresh cheese comprising 15% of the entire Portuguese market (Associação Portuguesa de Nutrição, 2018).

In the economic sector, cheese plays an important role, being the EU the leader of the market in terms of production (32%) and exports of cheese (43%), followed by the USA (Tecnoalimentar, 2015).

2.5 Cheese whey

Whey is a liquid by-product of the cheese industry, obtained following casein coagulation. Its color can vary between greenish-yellow and shades of blue according to the quality and type of milk used in cheese making (Pintado *et al*., 2001; Smithers, 2008).

In Western countries, there is higher production of bovine milk; therefore, higher production of bovine cheese, resulting in higher whey production from bovine milk. In fact, in Portugal, in the year 2020, according to the National Statistical Institute (INE, 2021), cow's milk accounted for about 95% of the total milk production. Furthermore, cow's cheese accounted for about 74% of the total cheese production in the same year. However, depending on the region of the world, other female mammals' milk, such as goat, sheep, and camel milk, is also used in cheese making (Smithers, 2008).

Overall, whey retains about 55% of the nutrients found in milk, being particularly rich in lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried extract) (Siso, 1996). However, whey's composition can vary according to several factors such as the female mammal's species and breed, the feed given to those mammals, the season, the lactation stage, and also the processing techniques applied during the removal of casein from milk (Gurrola *et al*., 2017; Kaur *et al*., 2020).

The type of whey also determines whey's composition. Depending on the technique applied during casein coagulation, two types of whey, sweet whey (pH 6-7) and acid whey (pH <5), can be obtained (Siso, 1996). Usually, cheesemakers apply enzymatic coagulation, in which rennet of animal origin (or functionally similar coagulants, such as *Cyanara cardunculus* L.) is used to coagulate the casein, resulting in sweet whey as a by-product (Modesto & Barbosa, 2007; Panesar *et al*., 2007). However, in some cheeses manufacturing, such as acid-coagulated fresh cheeses, acidic coagulation is performed through fermentation or adding organic or mineral acids, resulting in acid whey as a by-product. Sweet whey and acid whey are essentially made up of water, lactose (approximately 70-72% of total solids), minerals (approximately 12-15% of total solids), and whey protein (approximately 8-10% of total solids). The mineral content, acidity, and whey protein fraction composition are the main differences between the two types of whey (Panesar *et al*., 2007). It is important to remark that due to its high salt content and acidic flavor, acid whey has a restricted utility (Carvalho *et al*., 2013).

Regarded as a highly nutritious by-product, whey owes much of its biological value to its protein fraction (whey protein), a heterogeneous mixture of several biologically active

proteins with high nutritional and therapeutic properties (Lappa *et al*., 2019; Ramiro Chacón Gurrola *et al*., 2017).

2.5.1 Whey protein – biological & nutritional value

Overall, whey protein is a combination of different individual whey proteins, specifically, ***β***-lactoglobulin (***β***-LG), α-lactalbumin (α-LA), and glycomacropeptide. It is worth noting that glycomacropeptide is an exclusive component of sweet whey since its production requires enzymatic coagulation of casein. In addition, whey protein fraction also includes residual protein/peptide components, such as lactoperoxidase, lysozyme, lactoferrin, serum albumin, immunoglobulins, and growth factors (Smithers, 2008).

β-LG and α-LA together comprise 70% of total whey proteins. β-LG is the major whey protein found in bovine (2.3 to 4.9 g/L) ovine (2.7 to 5.0 g/L) and caprine milk (1.8 to 2.8 g/L) (Hernández-Ledesma *et al*., 2011). It has fascinating, documented properties against cancer. For instance, it is a promising protein nanomaterial, due to its gelling properties, that can be used in cancer therapy (Lohcharoenkal *et al*., 2014). Furthermore, it can stimulate glutathione synthesis, a substance known for its intestinal-level anticancer activity (Gurrola *et al*., 2017). Moreover, when associated with inorganic selenium, it forms a se-β-LG complex with antitumor activity (Lappa *et al*., 2019). However, in the scope of this thesis, it is important to remark that ***β***-Lg is an ingredient of choice in the formulation of novel foods due to its high functional and nutritional value (Hernández-Ledesma *et al*., 2011). α-LA is the second major whey protein found in bovine (0.8 to 1.2 g/L), ovine (1.2 to 2.6 g/L) and caprine (0.6-1.1 g/L) milk (Hernández-Ledesma *et al*., 2011). This metalloprotein has the potential to be used in multiple medical and nutritional applications, for instance: (1) it can be used as a therapeutic agent against a wide variety of diseases and conditions such as mood disorders, seizures, sarcopenia, type II diabetes, and cancer; (2) It has potential to be used as a supplement to modulate neurological function, to promote gastrointestinal health, to aid infant development and to protect muscle mass in old adults (Gurrola *et al*., 2017; Layman *et al*., 2018). Furthermore, its physical characteristics, such as clean flavor profile, heat stability, and high-water solubility, and biological properties, make α-LA an attractive protein to be used in developing novel functional food products (Layman *et al*., 2018).

The whey protein comprises other components also noteworthy such as lactoperoxidase and lactoferrin. Lactoperoxidase is an enzyme that, in the presence of hydrogen peroxide, catalyzes the chemical reaction of thiocyanate, resulting in a compound with antibacterial properties (Food and Agriculture Organization of the United Nations &

World Health Organization, 2005). Lactoferrin (or lactotransferrin) is an iron-binding globular multifunctional protein with reported antimicrobial effects against a broad spectrum of harmful bacteria and fungi (Hernández-Ledesma *et al*., 2011).

In addition, whey proteins are precursors of bioactive peptides (mainly produced by enzymatic hydrolysis) that have a significant role in human health by regulating both the nervous system (opioid activity) and cardiovascular system (antithrombotics, antihypertensives, and antioxidants), increasing resistance to infectious diseases (antimicrobials and immunomodulators) and facilitating the transport of minerals (Gurrola *et al*., 2017; Hernández-Ledesma *et al*., 2011).

To summarize, whey proteins possess important biological and nutritional properties that can potentially improve human health and prevent health conditions and diseases. This actively demonstrates that whey's transformation into value-added products may positively impact both the nutraceutical and food industries. However, the use of whey in natura is limited, so in order to be applied to food formulations or other value-added products, such as edible films/coatings, whey needs to be processed.

2.5.2 Whey environmental impact

Large quantities of whey are produced in cheesemaking. In fact, during the manufacture of 1 kg of cheese, around 8 to 9 L of whey are generated. Furthermore, worldwide cheese whey production is estimated to be over 10^8 tons per year. Actually, in 2016 2×10^8 tons of whey were generated (annual growth rate of 3%) (Chalermthai *et al*., 2019; Lappa *et al*., 2019). However, cheese is the main pollutant of the dairy industry (Lappa *et al*., 2019).

Essentially, two parameters characterize the environmental impact of whey, the biochemical oxygen demand (BOD) and chemical oxygen demand (COD). BOD is defined as the amount of dissolved oxygen required by aerobic microorganisms for the decomposition of organic matter present in a given volume of water to occur, at a certain temperature, during a specific period. COD is an indicative measure of the total amount of oxygen that can be consumed during the reactions, used as an indicator of the amount of organic matter in the water. In this context, whey has both a high BOD (>35,000 ppm) and COD (>60,000 ppm) (Kareb & Aïder, 2019). Therefore, due to its high organic content and the vast amount generated, cheese whey cannot be directly discharged to the environment, or otherwise, eutrophication processes can take place, resulting in excessive growth of aquatic plants and microbes (Ahmad *et al*., 2019).

Nowadays, standard treatments for cheese whey include anaerobic digestion or landfill disposal; however, the current treatments focus on reducing BOD and COD rather than producing value-added products such as biochemicals, bioenergy, or other novel products (Lappa *et al*., 2019). Nevertheless, it is essential to remark that processing whey into value-added products implies high costs in research and development. Therefore, in order to justify the investment, it is essential to evaluate the economic and technical potential of each factory in order to obtain adjusted, high added-value products that benefit cheese factories while minimizing the negative environmental impact of cheese whey (Mirabella *et al*., 2014; Prazeres *et al*., 2012). The whey disposal constitutes a significant loss of an abundant, affordable, renewable, nutrient-rich effluent with remarkable biological properties (Lappa *et al*., 2019; Prazeres *et al*., 2012). In addition, by processing whey into value-added products, a circular economy is adopted, which is a more sustainable and efficient economic model.

2.5.3 Circular Economy

Food production and consumption patterns are currently unsustainable. Food destined for human consumption is wasted at several points in the food supply chain. Furthermore, current food economy inefficiencies mean we waste productivity, energy, and natural resources, as well as pay the expenses of wasting food. This actively demonstrates that we need to change to a more self-sustainable food system (Jurgilevich *et al*., 2016).

A Circular Economy is a regenerative system in which material and energy loops are slowed, closed, and narrowed to reduce resource input and waste, emissions, and energy leakage (Geissdoerfer *et al*., 2017). Reduced waste generation, food reuse, utilization of by-products (e.g., cheese whey) and food waste, nutrient recycling, and dietary modifications toward more diverse and efficient food patterns are all examples of the circular economy within the food system (Jurgilevich *et al*., 2016).

A circular economy, in this sense, is a key economic model for a paradigm change, boosting productivity and competitiveness while promoting resource efficiency and reducing environmental impacts (FCT, 2019).

Applying the circular economy model to the cheese industry allows adding value to cheese by-products; however, in order to be processed into value-added products, cheese whey needs to be further processed.

2.5.4 Processing Whey into Value-Added Products

Despite its high nutritional value, whey is a very perishable product, as its composition and temperature promote bacterial growth, resulting in protein degradation and lactic acid formation. Therefore, whey's use in natura is limited (Brandelli *et al.*, 2015; Bylund, 2015).

Advances in separation technologies, such as chromatography and membrane filtration, have underpinned affordable business processes for the fractionation of whey into highly purified lactose and protein products (Fig.1). After processing, end-users may take advantage of the functional properties of individual whey components and apply them into a wide variety of fields: (1) animal feed; (2) human consumption; (3) baby food; (4) diet food; (5) sports nutrition; (6) clinical nutrition; (7) sausages; (8) soups; (9) bakery; (10) salad dressings; (11) ice cream; (12) whey spread/ cheese; (13) cheeses; (14) beverages; (15) confectionery; (16) pharmaceutical (e.g., probiotics); (17) yeast products; and (18) industrial products (e.g., bioplastics, fuels) (Bylund, 2015; Yadav *et al.*, 2015).

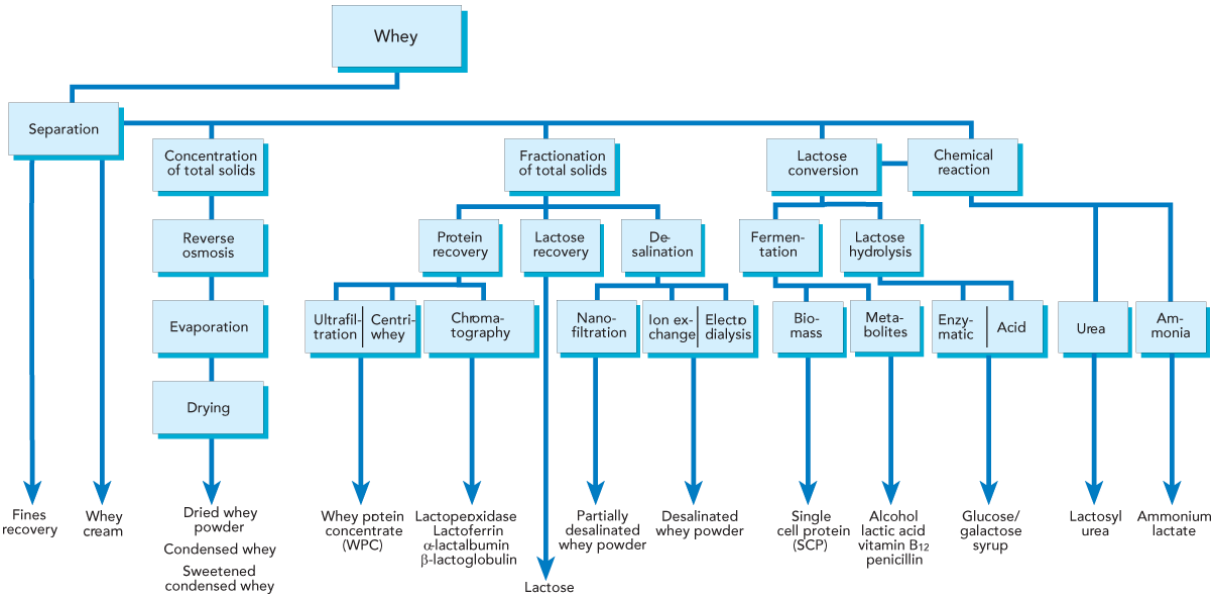

Figure 1 Whey Processing alternatives; Source: (Bylund, 2015)

Whey powder is used in most application fields, and the majority of whey is processed into it (Foegeding *et al.*, 2011). However, processing cheese whey into powder requires a high cost drying process that most cheese manufacturers cannot afford. Therefore, processing whey into whey protein concentrates (WPCs) is a more economical solution to add value to cheese whey since it requires less expensive processing technologies, such as ultrafiltration, that do not require any heat-induced phase change (evaporation) (Kaur *et al.*, 2020).

2.5.5 Whey protein concentrates and isolates

Food packaging makes it easier to store, handle, transport, and preserve food, and it is crucial for avoiding food waste. Aside from these advantages, food packaging is causing increasing environmental concern due to its large production volume, typically short usage period, and waste management and littering issues (Geueke *et al*., 2018). Most food packaging is made of petroleum-based non-biodegradable polymers, and their disposal is becoming a major environmental concern. The partial replacement of these materials with biodegradable polymers derived from renewable sources (e.g., biopolymers) can help lessen the environmental impact of packaging materials (de Camargo Andrade-Molina *et al*., 2013). Therefore, among all the cheese whey application fields, packaging materials are a vital area of exploration.

Through different processing technologies, whey can be processed into whey protein concentrates (WPC) and whey protein isolate (WPI) powders that can be used as film-forming biopolymers in edible packaging (Costa *et al*., 2018). Both of these fractions have characteristics that make them acceptable for use in edible packagings, such as good film-forming capacity, biocompatibility and generally recognized as safe status; lack of pathogens and toxic metabolites, high amino acid and protein content; low calorie and fat contents; ready availability; and low cost (Kandasamy *et al*., 2021; Costa *et al*., 2018) WPC, with a protein content that ranges from 35 to 80%, is obtained by drying the retentates from ultrafiltration, while WPI, with a protein content of approximately 90%, results from extensive diafiltration with ultrafiltration membranes followed by evaporation and spray drying (Bylund, 2015; Tetra Pak, 2021). WPCs have a low fat and cholesterol content but are high in bioactive components, while WPIs are high in protein but low in bioactive compounds (Kandasamy *et al*., 2021). Because these are salt-free, they are acceptable for human consumption, including dietetic and infant foods (Kotoupas *et al*., 2007). In addition, the amino acid profile of protein concentrates is excellent, with significant quantities of accessible lysine and cysteine (Bylund, 2015). Processing whey into WPC or WPI generates a permeate as by-product that cannot be disposed into the environment. However, the ultrafiltration permeates high biodegradability (BOD_5/COD≈0.5-0.8) allows biological treatments (Pescuma *et al*., 2015; Prazeres *et al*., 2012).

Nevertheless, due to their high protein content and functional capabilities, WPC and WPI can be a fascinating and economic biopolymeric matrix to utilize in the development of whey protein-based edible films with excellent properties. The stretchability of WPC and WPI films is similar, but WPI leads to more resistant films in terms of mechanical properties.

However, the WPC-based films are usually smooth, flexible, and have better moisture barriers than WPI-based films. Therefore, WPC-based films seem to be more attractive since they have better functional performance and are also less expensive than WPI based films (Banerjee & Chen, 1995).

2.6 Packaging

2.6.1 Conventional packaging materials: Absolute or Obsolete?

Global issues that affect the food industry are arising and worsening. The world's population is expected to reach around 10 billion by 2050, which means 2.1 billion more mouths to feed (Lal, 2021). Furthermore, according to FAO (FAO, 2011), one-third of global food production is lost or wasted. Moreover, Earth is increasingly contaminated by petroleum-based plastics. 6.3 billion metric tons of plastic waste were produced until 2015, of which only 9% was recycled (Agudelo-Cuartas *et al*., 2020). These pressing issues, together with the changes in retailing practices and consumers way of life, act as driving forces for the development of innovative, sustainable, renewable, and improved packaging concepts that extend the shelf-life of perishable products, in order to reduce food waste, while being aware of the feeding issues related to the increasing world population (Dainelli *et al*., 2008).

Currently, packaging may be the most effective way to preserve food products since it not only protects and preserves, allowing commercialization and distribution, but also provides essential information about the products (Díaz-Montes & Castro-Muñoz, 2021). However, conventional packaging materials are responsible for a large portion of municipal waste. For instance, according to "Eurostat" (Eurostat, 2021), in 2018, municipal waste was generated in European Union (EU) per person amounted to 492kg per capita. Each EU citizen generated around 174.1kg of packaging waste in the same year, of which 66.3% was recycled. Therefore, packaging waste accounted for 35% of the total municipal waste generated, with Plastic (19.0%) being the primary packaging waste material after paper and cardboard (40.9%).

In addition, even though most conventional packaging materials (paper, glass, metal) are either biodegradable or recyclable, plastic, one of the most used, is neither (Díaz-Montes & Castro-Muñoz, 2021). In this context, through the Directive on single-use plastics (Directive (EU) 2019/904), different measures are being applied by the EU, such as (1) market restrictions of multiple plastic products, including food containers made of expanded polystyrene; (2) consumption reduction of single-use plastics for which there are no alternatives; (3) separate collection and design requirements for plastic bottles; (4) monitorization of single-use plastics

consumption; (5) effective and informative compulsory marking, extended producer responsibility ("polluter pays" principle); and (6) awareness-raising. With this Directive, the EU aims to promote a transition to a circular economy while preventing and reducing the impact on the environment of certain plastic products. However, only some certain plastic products are considered single-use plastic products for this Directive. For instance, food containers are only considered single-use plastic products when used to contain food intended for immediate consumption, food typically consumed from the receptacle, and food ready to be consumed without further preparation, such as cooking, boiling, or heating.

In addition to the environmental impacts, the concept of conventional packaging itself may become outdated. The primary safety criteria for traditional packaging is that interactions between food and packaging should be minimal; however, this principle may become obsolete (Yildirim *et al*., 2018). Active packaging is a new system of packaging designed to intentionally interact with food in a positive way, which can help the resolution of multiple issues related to food packaging. Active food contact materials and articles are defined according to Regulation (EC) No 450/2009 as "materials and articles that are intended to extend the shelf-life or maintain or improve the condition of packaged food". Within this group, edible films and coatings can help achieve a progressive and sustained reduction in the consumption of disposable plastics and therefore mitigate their environmental impact.

Furthermore, due to their capacity to extend the shelf-life of packaged food, they may help prevent food waste and future hunger. Moreover, the edibility and biodegradability of edible films may appeal to consumers, whose awareness regarding food safety and environmental issues is increasing (Ramos *et al*., 2012). For these reasons, edible films and coatings may be the innovative, sustainable, renewable, and improved way to preserve perishable food products that the food industry needs.

2.6.2 Edible films as bio-packages

An edible film or coating is any material formed through the combination of biopolymers and additives with a thickness of less than 0.3mm (Díaz-Montes & Castro-Muñoz, 2021). Overall, there are no differences between edible films and coatings regarding their material composition. However, the incorporation technique applicable to each one is different. In edible films, the design is previously shaped, using molds, and it is only adhered to food after drying, while in edible coatings, the material is formed directly on the food's surface, usually by immersion or aspersion (Fig.2) (Díaz-Montes & Castro-Muñoz, 2021; Oliveira *et al*., 2017; Ramos *et al*., 2012). In addition, edible coatings are designed to be consumed along with the final product and therefore are not usually disposed of separately from the coated food.

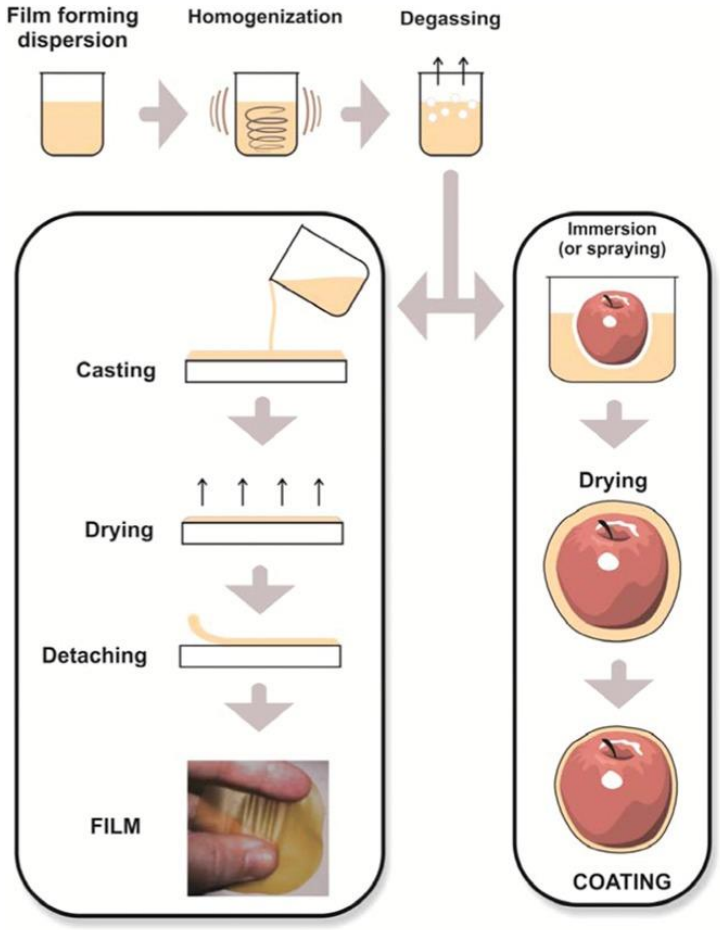

Figure 2 Schematic representation of the development and incorporation technique of films and coatings; Source: (Otoni et al., 2017)

In addition to increasing food shelf-life of packaged products and reducing bacterial growth and fungal contamination, edible films can present protection against UV light, act as barriers against mechanical damage, transport gases, water vapor, organic vapors, and solutes and act as carriers of healthy microorganisms (e.g., probiotics) (Díaz-Montes & Castro-Muñoz, 2021). Furthermore, besides enhancing food quality and safety, these active materials can also

reduce the demand for synthetic packaging materials and, therefore, reduce packaging waste while adding value to natural polymer materials. Moreover, compared to conventional packaging materials, edible films and coatings have the advantage of being comestible and biodegradable (Khwaldia *et al*., 2004; Ramos *et al*., 2012).

Economically, edible films and coatings might lower food prices by reducing post-harvest losses and using more affordable packaging materials. Nevertheless, the relation cost-benefit must be addressed, and therefore in order to reduce costs and increase benefits, it is recommended to use as biopolymeric matrices hydrocolloids (e.g., whey proteins) obtained from by-products of the food industry (e.g., whey) (Campos *et al*., 2011).

Active edible films and coatings are edible polymeric materials incorporated with biologically active agents, such as antioxidants or antimicrobials, able to reduce the risk of foodborne bacteria growth, fungal contamination or able to inhibit oxidation, allowing to packaged foods shelf-life and quality (Cagri *et al*., 2004; Díaz-Montes & Castro-Muñoz, 2021; Ramos *et al*., 2012). It is important to remark the difference between food antioxidant and food antimicrobial. Any agent capable of delaying, retarding, or preventing the development of rancidity in food or other flavor deterioration due to oxidation is referred to as a food antioxidant. While any agent capable of preventing the growth of both deteriorating and pathogenic microorganisms in food is referred to as a food antimicrobial (Ganiari *et al*., 2017; Ramos *et al*., 2012).

2.6.3 Whey protein-based edible films

Even though edible films can be obtained from polysaccharides, lipids, proteins, or a combination of these compounds, proteins-based films are more attractive due to their nutritional value (Galus & Kadzińska, 2016). Additionally, proteins-based films have a more attractive barrier and mechanical properties than polysaccharides-based ones (Khwaldia *et al*., 2004).

Within proteins-based films, whey protein-based ones have exhibited not only good mechanical properties, compared to standard synthetic polymer films, but also good barrier properties against aromas, lipids, and oxygen. However, their hydrophilic nature limits their capacity to act as barriers against water vapor (Castro *et al*., 2019; Oliveira *et al*., 2017). Plasticizers (e.g., glycerol - E 422) can be added in order to surpass this limitation. This way, whey protein-based films obtain better moisture resistance and improve their resilience (extensibility and flexibility)(Castro *et al*., 2019). Additionally, whey proteins can be obtained

from renewable resources and degrade faster than other polymeric materials (Ramos *et al*., 2012). Nevertheless, despite all the positive impacts, these active materials should meet many functional, legislative, and marketing requirements.

2.6.4 Functional, legislative, and marketing requirements for edible Packaging

Several specific requirements must be accomplished to produce safe and effective edible films without compromising food safety, good manufacturing practices, good hygiene practices, consumer information, or traceability requirements set out in Regulations (EC.) No 178/2002 (EC), No 1935/2004 (EC), No 852/2004 (EC), Regulation (EC) No 2023/2006 and their amendments of the European Parliament and of the Council and other relevant legislation related not only to food safety and hygiene but also labelling.

In order to be available on the European market, edible films should follow the applicable food contact legislation and therefore comply with Regulation (EC.) No 1935/2004 on materials and articles intended to come into contact with food. Overall, under article 3, food contact materials (including edible films) must not display any human health risk and be as inert as possible. Specifically, following article 4, edible films may cause alterations in the composition or organoleptic characteristics of food if those alterations comply with community provisions or national provisions (in the absence of community provisions). Furthermore, deliberately incorporated active substances must be authorized and used in compliance with Community provisions applicable to food.

Moreover, edible films must not change the composition or organoleptic characteristics of food in a way that misleads the consumer (e.g., masking the spoilage of food). Again, given information about food conditions must not mislead consumers. Finally, active edible films must be labelled in order to be identified as active materials. Under article 15, food business operators must be informed on how to use edible films safely and appropriately in order to comply with relevant legislation clauses.

In addition to complying with current legislation, an optimal edible film should meet some requirements: (1) it must not contain any toxic, non-digestible, or allergic compounds (for the consumer); (2) it should only contain food-grade components; (3) structure stability should maintain the integrity and prevent mechanical damage throughout the food supply chain; (4) In order to maintain optimal moisture content, water migration should be controlled; (5) it should be semipermeable in order to maintain the intern equilibrium of anaerobic and aerobic gases, thus preventing undesired physicochemical reactions that lead to

senescence or agedness; (6) it should maintain flavor as well as nutritional and organoleptic characteristics and, therefore, avoid avoiding the loss or absorption of the key components (7) it should prevent degradation, pesticide infestation; bacterial reproduction and fungal contamination; (8) it should maintain or enhance food products organoleptic features and (9) it should be easily manufactured and affordable (Dinika *et al*., 2020; Otoni *et al*., 2017).

Finally, edible films, as packaging materials, have the first contact with consumers and should meet their demands. When developing an edible film, marketing factors such as the price of the final product, handling instructions, and consumer's cultural background should also be addressed to appeal to consumer's acceptance (Ramos *et al*., 2012).

2.6.5 Edible films and coatings in fresh cheese preservation

Polyethylene, polypropylene, and polyamide are standard materials used for cheese packaging; however, legislation concerning the migration of materials into cheese can restrict their utilization. In addition, their characteristics, such as non-biodegradability and non-edibility, can lead to environmental issues. These significant problems motivated the research community to search for new packaging solutions for cheese (Costa *et al*., 2018).

Films or coatings applied on cheese surfaces have long been regarded as a key treatment for maintaining quality standards and microbiological safety (Mileriene *et al*., 2021). A wide variety of edible films and coatings have been developed for multiple types of cheese (Costa *et al*., 2018). However, according to the bibliography, the number of edible films or coatings developed for unripened cheeses, such as fresh cheese, is scarce. For instance, by searching the Web of Science Core Collection (https://www.webofscience.com/ - Accessed 19.08.2021) for the keywords "cheese" and "edible film," we obtained 186 results while using the keywords "fresh cheese" and "edible film" we obtained only 22 results. Nevertheless, at least six edible packages, including films and coatings, have been developed to date (Table 2).

Table 2. Edible films/coatings developed to enhance unripened cheeses preservation

Unripened-Cheese Application	Biopolymeric Matrix	Additives	Coating Technique	Results	Reference
Fresh soft rennet-curd cheese	Furcellaran + whey protein isolate	Yerba mate | White tea extracts	Edible film - Wrapping	(1) Cheese shelf life extended; (2) Addition of YM or WT impacted the thermal stability of FUR/WPI films; (3) Addition of YM caused a reduction in water solubility, water content, and water vapor transition rate; (4) small negative impact on organoleptic quality.	(Pluta-Kubica *et al*., 2020)
Quark	Furcellaran + whey protein isolate	Green tea | Pu-erh extracts	Edible film - Wrapping	(1) PE addition strengthened the film structure; (2) FUR/WPI films with PE and GT had similar antioxidant activity (3) tested films were found to be inappropriate as packaging of an acid-curd cheese; (4) Most of the films bore a negative influence on quality properties.	(Pluta-Kubica *et al*., 2021)
Manaba fresh white cheese	Cassava starch	*Lactobacillus acidophilus*	Coating - dipping	(1) Encapsulated *L. acidophilus* showed higher viability than free cells during storage at 25 °C. (2) cassava starch film containing encapsulated *L. acidophilus* may be a potential tool to control mesophilic aerobic bacteria during storage of Manaba cheese at refrigeration conditions.	(Santacruz & Castro, 2018)
Ricotta cheese	Chitosan + whey protein	NA	Coating - dipping	(1) Coating of Ricotta surface with a CWP edible coating, under modified atmosphere, reduced the growth of microbials and extended the shelf-life of the cheese; (2)The coating inhibited the development of undesirable acidity, better maintained the texture, and did not seem to modify organoleptic characteristics.	(di Pierro *et al*., 2011)

Eastern European curd cheese	Liquid whey protein concentrate	Chinese cinnamon bark - *Cinnamomum cassia* - CO2 extract	Coating - dipping	(1) The applied coating significantly affected the appearance and color of the curd cheese; (2) coating did not affect moisture, color, or texture, of vacuum-packed curd cheese and had a strong antimicrobial effect, especially against Yeasts and molds; (3) coating had no significant effect on pH, lactic acid, protein, or fat contents of curd cheese and vacuum-packed curd cheese; (4) no negative effect of applied antimicrobial coating on the flavor of curd cheese.	(Mileriene *et al*., 2021)
Colombian fresh cheese	Chitosan	Bacteriocins - *Pediococcus pentosaceus* 147	Coating - dipping	(1) chitosan coatings with cell-free supernatant of *P. pentosaceus* 147 can inhibit the growth of *L. monocytogenes* during the storage of cheese contaminated after production.	(Jutinico-Shubach *et al*., 2020)

As previously mentioned, whey protein can be used as a material in the development of biodegradable packaging. Whey protein has already been applied to edible coatings, and film formulations developed for unripened cheeses (di Pierro *et al*., 2011; Mileriene *et al*., 2021; Pluta-Kubica *et al*., 2020, 2021). In addition, no scientific findings are yet available on edible film application and effect on Latin-style fresh cheeses; therefore, it may be interesting to evaluate the effect of whey protein-based edible films on the preservation of this type of fresh cheeses.

A whey protein-based edible film to be effective, besides a polymeric matrix, requires an active agent. One of the latest active packaging trends is using natural plant extracts as antioxidant and antimicrobial agents (Mileriene *et al*., 2021; Mosavinezhad *et al*., 2020). Within natural plants extracts, green tea (*Camellia sinensis* L.) extract, due to its physicochemical composition, is a promising antioxidant and antimicrobial agent to be incorporated in an edible packaging intended to extend fresh-cheese shelf life.

Although multiple polymetric matrices and active compounds have been used to develop active packages for fresh cheese, the effect of whey protein films incorporated with green tea extract on Latin-style fresh cheeses preservation has never been studied.

2.7 Green tea

Tea is a popular beverage obtained by immersing the leaves, flowers, or stems of the *C. sinensis* L. plant in hot water. Even though it was first consumed in Asia, its consumption has spread throughout the world, and currently, worldwide, each person consumes, on average, approximately 120 ml of tea per day (Prasanth *et al*., 2019). It is important to remark that only hot water infusions of the leaves, flowers, or stems of the *C. sinensis* L. can be classified as tea. Other plant infusions (e.g., chamomile (*Matricaria recutita L.*), rooibos (*Aspalathus linearis*)) should be termed "herbal teas" or tisanes (da Silva Pinto, 2013).

Depending on the manufacturing process, tea can be classified as black tea (fully fermented), oolong tea (semifermented), yellow tea (partly fermented), white tea (slightly fermented), and green tea (non-fermented) (J. Ning *et al*., 2016). Within the green tea, category exist several varieties of green tea: (1) Aracha – pre-processed green tea; (2) Bancha (tea with little caffeine); (3) Fukamishi sencha (green tea with prolonged vaporization); (4) Genmaicha (green tea with puffed rice); (5) Guricha or Tamaryokucha. (traditional green tea); (6) Gyokuro (green tea with a prolonged period of growing in the shade); (7) Houjicha (roasted green tea); (8) Kabusecha (green tea grown in the shade); (9) Kamairicha (panfired green tea); (10) Kukicha – green tea with stems; (11) Matcha – fine powder green tea; (12) Mizudashi sencha (green tea for cold infusions); (13) Sencha (steamed green tea); and (14) Shincha (very first harvest green tea) (Chá camélia, 2021).

Due to a manufacturing process that involves drying techniques such as steaming or roasting, green teas, compared to other teas, are particularly rich in antioxidants since those techniques will lead to the preservation of the active substances (Xing *et al*., 2019). However, green teas chemical compositions may vary according to their horticultural conditions and manufacturing process. Furthermore, there are a wide variety of green teas, as previously mentioned, and consequently, their characteristics vary between them. Moreover, the edaphoclimatic conditions in which the tea plant is grown impact the green tea final composition (Sanaeifar *et al*., 2020).

2.7.1 History of tea: from China to Portugal

According to historical records, tea cultivation and consumption originated in China around two millennia BC (Prasanth *et al*., 2019). However, several local legends and tales confound the story, and it is impossible to date the genesis of the tea culture precisely. Due to its medicinal nature, this drink quickly became popular across different layers of Chinese

society, which stimulated the interest of neighboring nations, namely Japan. The introduction of tea in Japan occurred around the 6th century due to cultural interactions between Japanese and Chinese monastics; however, it was only in the 9th century that the first tea plants were cultivated (Silva, 2014).

The development of commercial interactions between China and Japan in the middle of the 16th century inevitably led Portuguese navigators to interact with the local culture and, consequently, with tea. Religion played an essential role in introducing tea in Portugal, with Dominican and Jesuit missionaries being the first to record tea consumption in the Portuguese language (Silva, 2014).

Considered an exotic drink with a high price, the notoriety of tea in Portugal began to increase due to the demand and consumption by the upper classes of society. In fact, Catherine of Braganza - D. Catarina de Bragança – (1638-1707) is often credited with the introduction of the habit of drinking tea (five o'clock tea) into Britain, around the 17th century, since she gave an aura of nobility to this habit by establishing it at the royal court, which led to the spread of this costume into aristocratic circles and wealthier classes. However, in Portugal, unlike in Britain, the popularity of this product, throughout the 18th century, fell short compared to tobacco and coffee (Silva, 2014).

2.7.2 Tea Production in the Azores

Although there have been some attempts to grow tea plants in Portuguese territory, according to historical records, the Azores were the first to produce tea at an industrial level successfully, having reached its peak in the early 20th century (Silva, 2014).

Although there are several versions of the introduction of tea cultivation in the Azores, it is estimated that it took place at the beginning of the 19th century. However, it was only in the middle of that century, due to the action of the Promoting Society of *Micaelense* Agriculture (PSMA), driven by the economic crisis that the archipelago was going through, that industrial tea cultivation began in the island of São Miguel. This entity promoted the culture of tea on the island by hiring, after negotiations with the Macau government, two tea-producing specialists, Lau-a-Pan (master) and Lau-a-Teng (assistant), who not only instructed São Miguel's inhabitants but also conducted several experiments and trials (Silva, 2014).

With the help of the Chinese technicians, the production of Azorean tea grew exponentially, and the marketing of tea on the islands began in 1884. At the beginning of the 20th century, the development of the tea industry allowed the appearance of new factories

and the improvement of existing factories. However, the increase in taxes on Azorean tea and the State protectionism offered to tea produced in the Portuguese colonies in the mid-twentieth century led to the closure of all tea factories in São Miguel, except the Gorreana factory (Silva, 2014).

The Gorreana factory, founded by Ermelinda Gago da Câmara and her son José Honorato, prevailed over five family generations despite all the difficulties. Gorreana currently has the largest tea plantation in Europe, with thirty-two hectares and an annual production of approximately thirty-three tonnes of tea. Additionally, it is internationally recognized not only for the quality and uniqueness of its teas (black, green, and oolong) but also for the production of 100% organic, chemical-free teas, since its use is not justified once the island's climate does not allow the development of tea plant pests. They produce and market several varieties of green tea, namely Encosta de Bruma, Pérola, and Hysson. The latter variety was selected as a sample in this study (Chá Gorreana, 2021)

Porto Formoso tea factory, located on the north coast of the island of São Miguel, labored between the twenties and the eighties and was one of the factories that closed, however in 2001 this factory reopened, and currently produces black tea of the following varieties: Orange Pekoe, Pekoe, Broken Leaf and Azores Home Blend (Chá Porto Formoso, 2021).

2.7.3 Tea production in mainland Portugal

From the middle of the 19th century to the beginning of the 20th century, there were several attempts to introduce the tea plant in mainland Portugal; however, due to the complexity of the tea plant culture (*Camellia sinensis* L.) and economic and social reasons, they all ended up failing (Silva, 2014).

Aiming to produce a high-quality organic green tea, in 2011, a couple of tea lovers, Dirk Niepoort and Nina Gruntkowski, embraced the challenge of introducing tea culture in mainland Portugal, having started by planting 200 tea plants in their garden in Porto. The success of this plantation led to these being transferred, in 2014, to the final land, in Fornelo, near Vila do Conde, where the "Camélia tea" project was born. Subsequently, with the advice of the Morimoto family, a tea-producing couple from southern Japan, 12,000 *Camellia sinensis* L. were planted and cared for biologically and sustainably. After two years of experimental production, in 2019, the 1st green tea harvest was carried out. After manual harvesting and a mostly artisanal transformation process, the tea used in this study was obtained. In addition

to currently being the only producers of artisanal, organic, Asian-style green tea in mainland Portugal, they also promote the development of tea culture. Additionally, they also distribute teas from family producers in Japan (Chá Camélia, 2021).

2.7.4 Chemical composition and health benefits

Green tea has been known for centuries for its medicinal properties. When it was first discovered, it was only used as a medicine (R. E. E. Silva, 2014). Nowadays, green tea is consumed as a non-alcoholic beverage, mainly for relaxation purposes, rather than being classified as a medicine. Even though green tea is used mainly as a relaxation drink, its consumption over the years has been rising due to documents that report not only its health benefits, such as blood pressure reduction, weight control, reduced risk of cardiovascular diseases, diabetes and osteoporosis, but also its health-boosting properties, such as antioxidant, antibacterial, anticancer and anti-radiation properties (Ly *et al*., 2020; Malar *et al*., 2020; Sanaeifar *et al*., 2020; Wang *et al*., 2020; Xing *et al*., 2019).

Green tea contains, predominantly, caffeine, a known central nervous system stimulant, vitamins, carbohydrates, amino acids, and polyphenols, such as flavonoids, anthocyanins, and phenolic acids (Fakae *et al*., 2020; Prasanth *et al*., 2019; Xing *et al*., 2019). Polyphenols are phytochemicals that have drawn increasing scientific attention due to their potential therapeutic effects against a wide variety of diseases (de Araújo *et al*., 2021). Notably, due to the green tea manufacturing process, polyphenol oxidase activity is inactivated, preventing any oxidation reaction from occurring. Therefore, when compared to other types of teas, green tea contains much higher amounts of phenolic compounds since it maintains not only their original structure but also preserves its overall composition (da Silva Pinto, 2013; Xing *et al*., 2019).

Polyphenols can be divided into three different groups: flavonoids, allied phenolic, and polyphenolic compounds. In particular, flavonoids play an essential role in the color and taste of fruits and vegetables and are essential for synthesizing vitamins and enzymes and inhibiting lipid peroxidation effects. Flavonoids can be classified as flavonones, isoflavones, flavonols, anthocyanins, and flavanols, depending on their aromatic rings' hydroxylation pattern and methylation (de Araújo *et al*., 2021). Among the different groups of flavonoids, flavanols, also known as catechins or flavan-3-ols, are particularly interesting since they are found in high concentrations in green tea, making it the primary dietary source of these phenolic compounds (Panche *et al*., 2016; Prasanth *et al*., 2019; Sanaeifar *et al*., 2020). Specifically, green tea major catechins are (-)- epicatechin-3-gallate (ECG), epigallocatechin 3-gallate (EGCG), (-)-

epigallocatechin (EGC), and (-)-epicatechin (EC) (Mosavinezhad *et al*., 2020). Following the European Food Safety Authority report, in 100ml of green tea there are 70.2 of mg EGCG; 29.2mg of EGC; 17.9mg of EGC and 8.3mg of EC (Younes *et al*.,2018).

Multiple studies have demonstrated that green tea catechins possess a wide variety of health-promoting activities, such as antioxidant, antibacterial, hypolipidemic, antitumor, hypotensive, hypoglycemic, and antiviral effects (Fakae *et al*., 2020; Upadhyay *et al*., 2020). However, EGCG, besides being the most abundant, as previously mentioned, is also the most pharmacological active phenolic compound; therefore, it is very likely that the therapeutic effects of green tea documented in animal and clinical studies as well as in cell culture studies are due to the high concentrations of this flavanol (Kim *et al*., 2014; Rubab *et al*., 2020). Moreover, EGCG has exhibited health-promoting effects in diabetes, Parkinson's disease, Alzheimer's, stroke, and obesity (Mosavinezhad *et al*., 2020).

2.7.5 Green tea extract as an antioxidant and antimicrobial Agent

Synthetic food additives, such as propyl gallate, sulfites, or nitrites, have been utilized to address the issues caused by oxidative processes and the growth and development of pathogenic microorganisms in foods, as well as to maintain the nutritional and organoleptic features that customers desire. However, growing consumer concerns about the safety of synthetic antioxidants and antimicrobials have led to a reduction in their use and replacement with safe, natural alternatives (Nikoo *et al*., 2018). Green tea has antibacterial and antioxidant properties, owing to its high content on polyphenols and other substances such as minerals and amino acids in its composition, which actively demonstrate that tea extracts are suited for usage as a natural additive in edible packaging (López de Lacey *et al*., 2014).

Green tea phenolic compounds can stop the radical chain reactions that occur during the oxidation of triglycerides in food systems by acting as free radical scavengers, mitigating food spoilage. Furthermore, it has been demonstrated, in vitro, that these polyphenols can inhibit the growth of bacteria that cause foodborne illness, such as *Shigella flexneri, Salmonella typhimurium, Staphylococcus aureus, Vibrio cholerae, Escherichia coli O157:H7,* and including *Listeria monocytogenes* (Siripatrawan & Noipha, 2012). In addition, it has been proven that phenolic compounds can improve various functional aspects of milk and dairy products, such as foaming, thermal, microbiological, and oxidative stability (Giroux *et al*., 2013).

The attractive properties of green tea have led to an increasing trend in the utilization of green tea extract (GTE) as a food antioxidant and/or antimicrobial agent. Multiple studies

have incorporated green tea extracts into a wide variety of edible polymeric or copolymeric matrices such as gelatin (*Gelidium corneum*) (Hong *et al*., 2009), furcellaran-gelatin (Kulawik *et al*., 2019), alginate (Falcó *et al*., 2019; Kristam *et al*., 2016), whey protein (Castro *et al*., 2019), furcellaran-whey Protein(Pluta-Kubica *et al*., 2021), chitosan (Montaño-Sánchez *et al*., 2020; Sabaghi *et al*., 2015), tapioca starch/decolorized hsian-tsao leaf gum (Chiu & Lai, 2010) and agar (López de Lacey *et al*., 2014) in order to extend the shelf life of multiple products such as: (1) chicken nuggets (Kristam *et al*., 2016); (2) pork Loins (Hong *et al*., 2009); (3) pork chops (Montaño-Sánchez *et al*., 2020); (4) pork slices (Chiu & Lai, 2010); (5) strawberries and raspberries (Falcó *et al*., 2019); (6) salmon (Castro *et al*., 2019); (7) salmon sushi (Kulawik *et al*., 2019), (8) hake (López de Lacey *et al*., 2014); (9) walnut kernels (Sabaghi *et al*., 2015) and (10) acid-curd cheese (Pluta-Kubica *et al*., 2021).

Therefore, green tea is a promising bioactive agent to be incorporated into a whey-protein-based film that can retard the oxidation reactions and control microbial spoilage in Latin-style fresh cheese. A very interesting whey-protein-based film incorporated with green tea extract was developed by Castro *et al.* (Castro *et al*., 2019). It would be interesting to evaluate the effect of a similar edible film upon Latin-style fresh cheese preservation.

2.8 Analytical Methodologies to evaluate the potential of natural extracts to be used as bioactives in active packaging

A suitable selection of the antioxidant compound to be incorporated in the packaging material is crucial. Therefore, it is necessary to evaluate the antioxidant capacity of the bioactive compound. However, multiple analytical methodologies can be used to evaluate the antioxidant capacity, and therefore it is vital to select which techniques are more suitable for the research.

The mechanisms of action by which the applied chemicals block chain-breaking processes can be used to classify the existing methods for quantifying antioxidant activity. They are split into two categories: hydrogen-atom transfer (HAT) reactions and single electron transfer (SET) reactions (compound reduction reactions through electron transfer from an antioxidant) (Chaves *et al*., 2020).

The ability of a putative antioxidant to transfer one electron to decrease any chemical, including metals, carbonyls, and radicals, is detected using SET-based approaches. As the oxidant is reduced, the SET is represented as a change in coloration. The following assays are part of the SET-based methods: (1) Folin–Ciocalteu reagent assay for total phenolics; (2)

Trolox equivalence antioxidant capacity assay (TEAC); (3) Ferric ion reducing antioxidant power assay (FRAP); (4) Assay for total antioxidant potential with a Cu^{2+}-complex as the oxidant; (5) 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH). HAT-based approaches assess an antioxidant's potential to quench free radicals through hydrogen donation. HAT reactions are solvent- and pH-independent, and they are usually relatively quick, taking just seconds to minutes to complete. In HAT assays, the inclusion of reducing agents, such as metals, is a problem that might result in erroneously high apparent reactivity. The following are some examples of HAT reaction-based methods: (1) Chemiluminescent assay; (2)Total radical trapping antioxidant parameter (TRAP); (3) Inhibition of induced LDL oxidation; (4) Total radical scavenging capacity assay (TOSCA); (5) Oxygen radical absorbance capacity (ORAC); (6) β-Carotene bleaching assays (Gulcin, 2020).

The following ideal requirements should be addressed by a standardized assay for antioxidant activity of a food component: (1) It assesses chemistry that occurs in possible applications; (2) it uses a biologically relevant radical source; (3) it should be straightforward; (4) it employs a method with a defined endpoint and chemical mechanism; and (5) it uses readily available chemicals and equipment; (6) It has good reproducibility within-run and between-day; (7) It should be adaptable for the assay of both hydrophilic and lipophilic antioxidants, as well as the usage of various radical sources; and (8) It should be adaptable to high-throughput analysis for routine quality control tests. Furthermore, antioxidant activity should not be determined solely through the use of a single antioxidant test model. In order to test the antioxidant activity of the samples of interest, several *in vitro* antioxidant procedures should be performed. Another crucial factor to remember is that antioxidant tests differ in many ways. As a result, thoroughly comparing one procedure to another is challenging (Gulcin, 2020).

2.8.1 *In vitro* methods to determine antioxidant capacity

2.8.1.1 β-carotene bleaching assay

The β-carotene bleaching test allows us to evaluate antioxidant effectiveness against lipid peroxidation (Nickavar & Esbati, 2012). Specifically, when linoleic acid is in the presence of reactive oxygen species (ROS) or oxygen, a peroxyl radical is formed (LOO•). This radical will form a stable β-carotene radical by reacting with β-carotene (Fig. 3), reducing the amount of β-carotene.

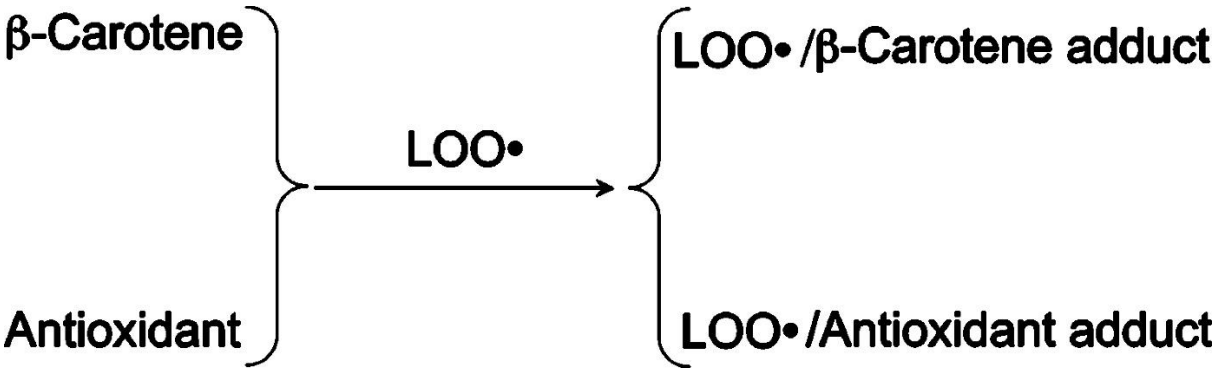

Figure 3 Competitive reaction between the antioxidant and β-carotene with the peroxyl radical (Moon & Shibamoto, 2009).

However, in the presence of an antioxidant, a competitive reaction will occur between the antioxidant and β-carotene with the peroxyl radical (LOO•) (Moon & Shibamoto, 2009). This reaction occurs in an aqueous emulsion of linoleic acid prepared using a phase stabilizer, namely Tween® 40 (Amorati & Valgimigli, 2015). Under standard settings, thermal induction (50°C) will result in the oxidation of the fatty acids generating radicals and, consequently, causing the discoloration of the yellow-colored emulsion (Prieto *et al*., 2012; Ueno *et al*., 2014). However, in the presence of an antioxidant, this discoloration can be delayed due to the previously mentioned competitive reaction. These antioxidant effects can be quantified through spectrophotometry (470nm) by measuring the rate at which β-carotene absorbance decays (Moon & Shibamoto, 2009; Prieto *et al*., 2012).

Unfortunately, the reagent complexity, the recurrent use of single reaction time, the unspecified settings of heat induction, and the reaction vulnerability to a wide variety of factors (e.g., pH, presence of metals) diminish this assay reproducibility. Furthermore, it is important to remark that β-carotene is sensitive to oxygen and temperature both in the absence or presence of linoleic acid. Moreover, this assay has been criticized for its representativeness, lacking adequacy to mimic lipid oxidation in foods (Prieto *et al*., 2012). Nevertheless, besides being one of the most common methods for assessing lipid peroxidation, this assay allows rapid and straightforward screening of antioxidant effects (Gulcin, 2020).

2.8.1.2 DPPH free radical scavenging method

Blois developed the α,α-diphenyl- β-picrylhydrazyl (DPPH) free radical scavenging method in 1958 to assess antioxidant activities at room temperature, using a mechanism that measures the scavenging of DPPH radical. In other words, we can evaluate a sample's capability to inhibit lipid oxidation by determining its free radical scavenging capacity (Bondet *et al*., 1997; Kedare & Singh, 2011; Škrovánková *et al*., 2012). In a nutshell, the antioxidant reacts with the

radical DPPH• reducing it to diphenylpicrylhydrazine, leading to the discoloration of the solution (Fig. 4) (Amorati & Valgimigli, 2015). Visually the violet-colored DPPH solution turns yellow (Rahman *et al.*, 2015).

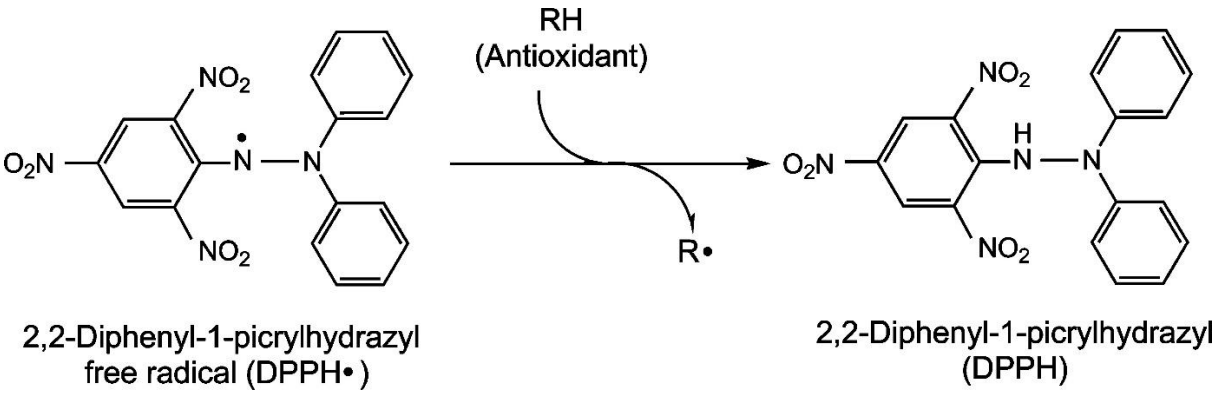

Figure 4 "Reaction between DPPH• and antioxidant to form DPPH" (Moon & Shibamoto, 2009)

However, this assay relies on the reaction time; therefore, this parameter alone does not provide sufficient data on the actual antioxidant reactiveness. Furthermore, comparisons can only occur if the data is obtained under similar conditions (Amorati & Valgimigli, 2015). Nevertheless, due to its simplicity and inexpensiveness, it is a particularly useful test in food science (Kedare & Singh, 2011). Furthermore, since the measuring occurs at room temperature, the risk of thermal degradation of the molecules is eliminated (Bondet *et al.*, 1997). Moreover, the short time required for the analysis makes this assay a rapid way to predict the antioxidant activity of foods by measuring the compound's capacity to act as hydrogen donors or free radical scavengers (Kedare & Singh, 2011; Rahman *et al.*, 2015).

2.8.1.3 Determination of total flavonoids content (TFC)

The spectrophotometric assay based on the production of aluminum complexes and their spectrophotometric determination is one of the most widely used procedures for determining total flavonoid content (Pękal & Pyrzynska, 2014). According to the basic concept of the aluminum chloride ($ALCL_3$) colorimetric method, $ALCL_3$ forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols, resulting in yellow color (Ahmed & Iqbal, 2018; Pontis *et al.*, 2014; L. Silva *et al.*, 2015).

2.8.1.4 Determination of total phenolic compounds

The Folin–Ciocalteu reagent (FCR) assay is commonly used to determine total phenolic content (TPC). This test was created to assess polyphenols in wine, but it has since then been widely used to measure phenols or antioxidants in food and extracts, such as those from herbs, spices, and fruits, as well as cereals and legumes. This colorimetric assay of phenolic

and polyphenolic antioxidants is done with Folin's phenol reagent, a combination of phosphomolybdate and phosphotungstate. The FCR assay is based on the single electron transfer (SET) from phenolic chemicals to FCR in an alkaline solution resulting in a blue-colored chromophore that may be detected spectrophotometrically at 750–765 nm. Specifically, phenolics are energetically oxidized, yielding O2, which combines with molybdate to yield colored molybdenum ions, MoO^{4+}. Although this test is supposed to determine a sample's total phenolic content, it is not specific for phenols or antioxidants in general, as any reducing agent will score well on this test and be classified as an antioxidant. Although various phenols have been used, usually, the results are reported in relation to gallic acid. However, the reaction time and sensitiveness to pH and temperature are some limitations of this assay. Nevertheless, in addition to its precision, sensitivity, and simplicity, the FRC capacity test has high linear correlations with other SET-based antioxidant assays such as DPPH (Amorati & Valgimigli, 2015; Gulcin, 2020).

III. Objectives

The main objectives of this Master thesis are: (i) to compare infusions and extracts obtained from different green teas ("Gorreana," "Chá Camélia," and "Happy flora") regarding their antioxidant capacity, and (2) to develop a biodegradable and edible active film using whey protein and green tea extract and (3) to characterize the new active films in terms of antioxidant and antimicrobial capacity in order to evaluate its potential to extend the shelf-life of Latin-style fresh cheese.

For this purpose, tea infusions from Gorreana," "Chá Camélia," and "Happy flora" will be prepared to assess their antioxidant capacity and their capacity to retain it. Furthermore, Two extraction methods (conventional solid-liquid and Soxhlet) will be compared to determine which one is more efficient. Moreover, the antioxidant capacity of "Gorreana" and "Happy flora" concentrated extracts obtained through the previous extraction methods will also be evaluated to determine which one has the highest antioxidant capacity. The antioxidant capacity of infusions and extracts obtained from different green teas will be evaluated through DPPH free radical scavenging activity assay and β-carotene bleaching method. Additionally, the total phenolics and total flavonoids content will also be determined.

In order to produce the active film, whey protein-based films will be incorporated with 2.5% of green tea extract. Furthermore, their antioxidant capacity will be evaluated through migration assays using the previously mentioned methods. Moreover, the antimicrobial efficiency of the active films will also be evaluated by wrapping the product (Latin-style fresh cheese) with active and control films for over a week and count of the viable mesophilic bacteria and psychrophilic bacteria as well as coagulase + Staphylococci and *Escherichia coli* present in the fresh cheese.

IV. Materials and Methods

4.1 Green tea samples

In this study, three Portuguese samples from *C. sinensis* L. were used: a green tea sample from the oldest tea plantation in Europe, called "Gorreana," and two samples from the first tea plantation in continental Portugal "Chá Camélia" (Fig. 5), which were produced according to traditional organic farming methods.

From the "Gorreana" plantation, located on the island of São Miguel (Azores), the "Hysson" variety was chosen. In July and August, this tea is produced after harvesting the first three leaves of *C. sinensis*.

Two samples were evaluated from the "Chá Camelia" plantation, located on the northern Portuguese coast. One was obtained from the leaves of *C. sinensis* from the 1st green tea harvest (Asian style) in continental Portugal, and the other was produced in autumn, resulting from the dehydration of flowers of the tea plant (*C. sinensis*). Furthermore, an Asian tea marketed as "Happy Flora" was selected for the study (Fig. 5).

The green tea brand "Gorreana" and the "Happy Flora" green tea were purchased locally in a commercial area in Coimbra. The company kindly supplied the samples from the "Chá Camélia" brand (green tea and *C. sinensis* flowers).

Figure 5 Green tea samples. From left to right: Gorreana, Happy flora, Chá Camélia (leaves), Chá Camélia (Flowers)

4.2 Preparation of green tea infusions

The tea infusions were prepared as traditionally as possible (Fig. 6). 200ml of tap water was heated to 75°C, and, immediately afterward, 2g of *C. sinensis* leaves/flowers, weighed previously, were soaked for 3 minutes, with the aid of a tea filter, in the absence of a sachet. At this stage, for each variety, four infusions were prepared. Initially, the 1st infusions were prepared, using dry leaves of different varieties of green tea, which were designated as G (Gorreana), H (Happy flora), CA (Chá Camélia-leaves) and CB (Chá Camélia- Flowers). Then, the second, third, and fourth infusions were prepared sequentially from the reuse of the same leaves, these being designated by *1, *2, and *3 respectively (* - corresponds to G/H/CB/CA) (Fig. 6). Afterward, the antioxidant capacity of the infusions was evaluated.

Figure 6 Green tea infusions. From up to down: "Gorreana", "Happy Flora", "Chá Camélia" (Leaves), "Chá Camélia" (Flowers). From right to left: first, second, third and fourth infusions.

4.3 Preparation of green tea extracts

4.3.1 Solid-Liquid Extraction

Initially, the *C. sinensis* samples were ground, homogenized, and sieved. 5g of each sample (AS 220.R1 PLUS scale) were subjected to solid-liquid extraction with 50mL of absolute ethanol (99.8%) and were placed in a horizontal shaker, Edmund Bühler KL2, for 30 min at 400 rpm, followed by centrifugation (Sigma 3-16K centrifuge) at 5000g for 15 min at 15°C. Afterward, the supernatant was removed into a pyriform flask and, using a rotary evaporator, the ethanol was evaporated entirely at 40°C. Finally, the extract was removed from the flask, using a spatula, and stored in a closed container at 5°C. Afterward, a solution was prepared using 10mg of the extract and 10ml of ethanol. Posteriorly the antioxidant capacity was evaluated.

4.3.2 Soxhlet Extraction

A Soxhlet device can also be used to do a solid-liquid extraction. Soxhlet has several advantages, including the ease of introducing fresh solvent in contact with the solid matrix and that no filtration is necessary at the end of the process. It is important to remark that from the perspective of the food sector, ethanol and water are the most suitable solvents, as they have GRAS (Generally Recognized as Safe) classification (Lourenço *et al*., 2019).

For this extraction, the *C. sinensis* samples were ground, homogenized, and sieved. Afterward, 5g of each sample was weighed into a Soxhlet cartridge, and this was placed in a Soxhlet extractor for 6h, using 150ml of absolute ethanol (99.8%) as solvent. Afterward, using a rotary evaporator, the solvent was evaporated entirely at 40°C. Finally, the extract was removed from the distillation flask, using a spatula, and stored in a closed container at 5°C (Fig. 7).

A) B) C)

Figure 7 Schematic representation of the solid-liquid extraction using a soxhlet apparatus. A) soxhlet extraction. B) evaporation of the solvent using a rotary evaporator. C) distilation flask with the extract.

Afterward, a solution was prepared using 10mg of the extract and 10ml of ethanol. Posteriorly the antioxidant capacity was evaluated.

4.4 Preparation of whey-based protein films

The method of preparing whey protein films was adapted from Ribeiro-Santos *et al.* (Ribeiro-Santos *et al.*, 2018). Initially, ultrapure water was added to the whey protein concentrate (Myprotein-"impact whey protein"). Then, homogenization was carried out using a magnetic stirrer, obtaining a solution (8%, w/w protein). The pH was, when necessary, adjusted to 7.0 using 1M NaOH. Subsequently, the protein was denatured by heating the solution in a thermostatic bath for 30 minutes at 80°C. The solution was rapidly cooled in an ice bath to room temperature (+/-23°C). Glycerol 1:1 (protein: glycerol) was added and mixed into the solution. Furthermore, 2.5% (w/w) of the extract was incorporated into the formulation, followed by its homogenization using an Ultra-Turrax, at 14,000 rpm for 2 minutes. The solution was distributed in Petri dishes (13.5 mL/100cm^2). Finally, the Petri dishes were incubated in an oven (Memmert 854 Schwabach) at 40°C for 24h (Fig. 8). A film without extract incorporation in the film-forming solution was used as a blank (control).

In order to enable the antioxidant evaluation of the films, a migration assay was performed. 6cm^2 of each film (blank and active) were immersed into 10ml of ethanol (95%) and incubated at 40°C for 10 days. After the incubation, the antioxidant capacity was immediately evaluated.

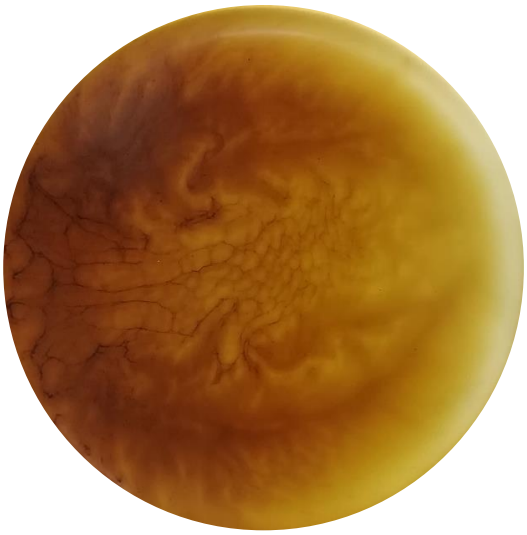

Figure 8 Whey protein-based active film incorporated with Green tea extract.

4.5 Characterization of the antioxidant capacity

4.5.1 β-carotene bleaching assay

The procedure of this assay followed the method described by Miller and adapted by Andrade *et al.* (Andrade *et al.*, 2018). Initially, a solution was prepared by dissolving β-carotene in chloroform (0.2mg/ml). Next, in a round-bottomed flask, an emulsion of β-carotene and linoleic acid was prepared using 20mg of linoleic acid, 200mg of Tween® 40, and 1ml of the previously prepared solution. Chloroform was evaporated at 40°C on a rotary evaporator. After that, 50ml of oxygenated ultrapure water was added, and the solution was vigorously stirred (Fig. 9). After preparation of the emulsion, 5ml was added to 200µL of the sample. The samples were kept in a Gerhardt SV24 thermostatic bath (1500w) at 50°C for 120 minutes. Using a Hitachi U-3900 spectrophotometer, the absorbances of the samples were measured at 470nm, against water. The Antioxidant Activity Coefficient (AAC) was calculated using equation 1.

$$AAC = \frac{AS - AC2}{AC0 - AC2} \times 1000 \qquad (1)$$

Where "AS" represents the absorbance of the samples, "AC0" represents the absorbance of the control before heating, and "AC2" represents the absorbance of the control after heating.

Figure 9 Emulsion of β-carotene and linoleic acid

4.5.2 DPPH radical scavenging method

The DPPH radical (2,2-diphenyl-1-picryl-hydrazyl) assay is an easy and quick method to assess a given sample's *in vitro* antioxidant activity. Briefly, 2mL of a methanolic solution of DPPH (14.6µg/mL) was added to 50µL of the sample. After homogenization, the solutions

were protected from light for 30 min. Absorbance was measured at 515nm using a Hitachi U-3900 spectrophotometer. Inhibition percentage was measured by using equation 2.

$$IP\ (\%) = \frac{AC-AS}{AC} \times 100 \qquad (2)$$

Where "AC" represents the absorbance of the control and "AS" represents the absorbance of the sample. The method applied follows the method described by Blois (1958) with some adaptations. A trolox calibration curve was drawn up. Results were expressed as μg trolox equivalents / g or ml of sample.

4.5.3 Determination of total flavonoids content (TFC)

In order to determine the TFC, the method described by Yoo *et al.* (Yoo *et al.*, 2008) was applied. Briefly, 4ml of ultrapure water was added to 1ml of sample. Then, 300µL of aqueous sodium nitrite (5% w/v) solution was added, and the mixture was homogenized. After 5 min, 600µL of an aqueous aluminum chloride solution (10%, w/v) was added, and the solution was homogenized again. After 6 min, 2ml of an aqueous sodium hydroxide solution (1M, w/v) and 2.1ml of ultrapure water were added. Finally, the samples were homogenized, and the absorbance was measured at 510nm using a Hitachi U-3900 Spectrophotometer. An epicatechin calibration curve was drawn up. Results were expressed in mg epicatechin equivalents / g or ml of sample.

4.5.4 Determination of total phenolic compounds (TPC)

The content of TPC was determined by the method described by Singleton & Rossi (1965). Briefly, 1ml of each sample was mixed with 7.5ml of Folin-Ciocalteu Reagent (10%, v/v). After 5 minutes, 7.5ml of a 60mg/ml (w/v) aqueous sodium carbonate solution was added. After homogenization, the solutions were kept in the dark for 120 minutes. Finally, absorbance was measured using a Hitachi U-3900 spectrophotometer at 725nm. A gallic acid calibration curve was drawn up. Results were expressed in mg gallic acid equivalents / g or ml of sample.

4.6 Microbiological analysis

The antimicrobial capacity of active packages, when in contact with two types of fresh cheese (prepared with goat milk and with goat/sheep milk mixture), was analyzed by counting the microorganisms present in the cheese.

In order to observe the antimicrobial capacity of active packages various parameters were determined: total microorganisms at 30°C according to ISO 4833-1:2013, total of psychrophiles according to ISO 17410:2019, count of E. coli according to ISO 16649-2:2001 and count of coagulase-positive staphylococci according to ISO 6888-1.

Figure 10 Latin-style fresh cheese wrapped with the whey protein-based edible film (Blank).

Figure 11 Latin-style fresh cheese wrapped with the whey protein-based edible film incorporated with the green tea extract.

The counts of the different microorganisms were carried out on fresh cheese goat cheese and fresh mixture (Sheep and goat) cheese produced on the day. Furthermore, the two kinds of cheese were wrapped with the edible active films (Fig. 10) and with the control films (without green tea extract - Fig. 11) and stored at 5°C, in order to simulate the standard storage conditions. After one week of storage at refrigerated temperatures, the counts of the microorganisms were carried out.

4.7 Statistical analysis

For the statistical analysis, the software "Prism – GraphPad" (Version 9.1.2) was used. One-way ANOVAs were performed using a significance level of 0.05. Additionally, Tukey's multiple comparison test was performed.

V. Results & Discussion

5.1 *In vitro* evaluation of the antioxidant capacity

In this research study, as previously described, the antioxidant capacity was evaluated and TPC and TFC of tea infusions from different green tea varieties were determined. Subsequently, the same evaluation was performed on different green tea extracts. Finally, we evaluated the antioxidant capacity and determined the TPC and TFC of the active edible film.

To assess the antioxidant capacity of the active edible film and green teas infusions and extracts, DPPH free radical scavenging and β-carotene bleaching methods were selected. For the DPPH assay, a calibration curve ($y = 0.6051x + 7.0233$, $r^2 = 0.9979$) was built by plotting different concentrations of Trolox (5 - 150µg/ml) in order to express the results in Trolox equivalents (TE).

For the evaluation of TPC the Folin–Ciocalteu's reagent assay was used. In order to evaluate the TFC, a spectrophotometric assay based on the production of aluminum complexes and their spectrophotometric determination was used. For the TPC method a gallic acid calibration curve ($y = 0.0064x + 0.0463$, $r^2 = 0.9929$) was built by plotting different concentrations of gallic acid (5 - 150µg/ml) in order to express the results in Gallic Acid equivalents (GAE). A calibration curve ($y = 0.0027x – 0.0017$, $r^2 = 0.9972$) was also built for the TFC assay by plotting different concentrations of epicatechin (5 - 200µg/ml) in order to express the results in Epicatechin equivalents (ET).

5.1.1 Evaluation of the Antioxidant Capacity Among Different Green Tea Infusions

This study evaluated the antioxidant capacity of green tea infusions from two Portuguese brands: "Gorreana" and "Chá Camélia". From the "Gorreana" tea brand, the "Hysson" variety was chosen, and from the "Chá Camélia" brand, one infusion was prepared from the leaves of *Camellia sinensis* L., and another from the flowers of *C. sinensis*. In addition, the antioxidant capacity of an Asian green tea marketed by "Happy Flora" was also assessed. Furthermore, the potential of retention of the antioxidant capacity was also evaluated through the antioxidant analysis of infusions made by reusing the same leaves/flowers.

Figures 12-15 show that, in general, the analyzed infusions of green tea from Portuguese plantations (G and CA) have superior antioxidant capacity than the analyzed infusion of Asian green tea (H). Furthermore, tea from the "Chá Camélia" plantation, obtained from the leaves of *C. sinensis* (CA), has shown to have the highest potential to retain the antioxidant capacity.

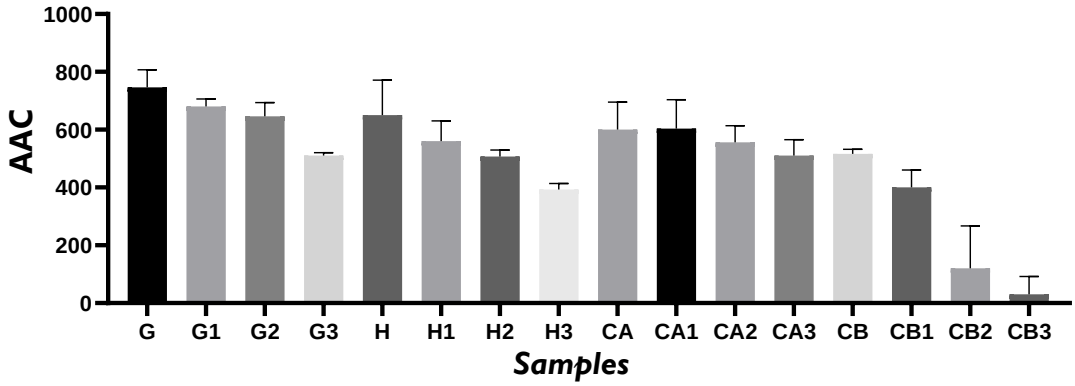

Figure 12 Comparison of the antioxidant activity of different green teas and C. sinensis flowers' infusion. Results of β-carotene bleaching inhibition test expressed as antioxidant activity coefficient (AAC). G=Gorreana; H=Happy Flora; CA=Chá Camélia (Leaves); CB=Chá Camélia (Flowers). 1,2 and 3 stands for the 2nd, 3rd and 4th infusion obtained by reusing the green tea leaves.

Upon analyzing in more detail, it is concluded that Figures 13, 14, 15 corroborate the previously established assumptions. By observing figure 12, it is possible to verify that the "Gorreana" tea has a greater capacity to inhibit lipid peroxidation (A=746.7). However, there are no significant differences (P>0.05) among the teas "Gorreana" (G), "Chá Camélia" (AAC_{CA}=600), and "Happy flora" (AAC_{H}=650).

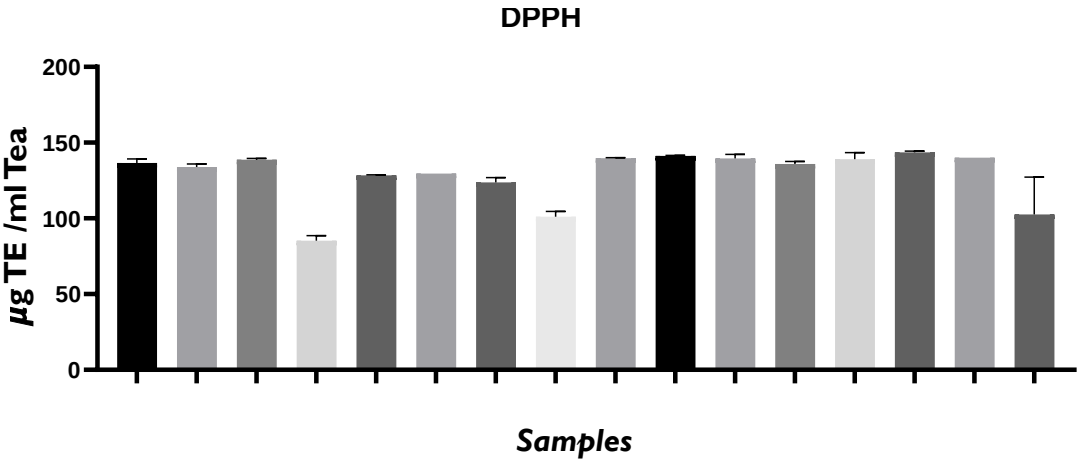

Figure 13 Comparison of the antioxidant activity of different green teas and C. sinensis flowers' infusion. Results of DPPH radical inhibition test with results expressed in mg Trolox equivalents per g of tea (*µ*g TE /ml Tea). G=Gorreana; H=Happy Flora; CA=Chá Camélia (Leaves); CB=Chá Camélia (Flowers). 1, 2 and 3 stands for the 2nd, 3rd and 4th infusion obtained by reusing the green tea leaves.

Additionally, considering the AAC of teas produced from fresh leaves and *C. sinensis* flowers' infusion (G, H, CB, CA) and teas/ herbal infusions obtained from their reuse (*1*, 2*,3), it appears that the green tea obtained from the leaves of the "Chá Camélia" tea plantation shows the highest capacity to retain the antioxidant activity (85%), compared to the other samples ("Gorreana" ≈68.3% / "Chá Camélia" (Flowers) ≈5.8% / "Happy flora" ≈60.5%). As far as we know, the β-carotene bleaching assay was not used to evaluate the capacity of green tea infusions to inhibit lipid peroxidation, and therefore, it is not possible to compare our results to results from other researches.

The analysis of Figure 13 shows that the green tea obtained from the leaves of the "Chá Camélia" tea plantation has the highest capacity to capture DPPH● free radicals (≈139.8 μg T.E./ml). However, excluding tea/herbal infusions produced through the third reuse of leaves/flowers, there were no significant differences ($P<0.05$) between tea varieties regarding antioxidant capacity. Regarding all tea infusions, it is possible to observe that the infusion of "Chá Camélia" green tea obtained from leaves has a higher retention potential of the antioxidant capacity (≈97.3%) when compared to the infusions obtained from flowers (≈73%) or from the green teas "Gorreana" (≈62.4%) and "Happy Flora" (≈78.8%). Several studies used DPPH radical to evaluate the antioxidant capacity of green tea infusions. However, those publications reported the results of the DPPH assay in different units, which we were unable to compare to the current study.

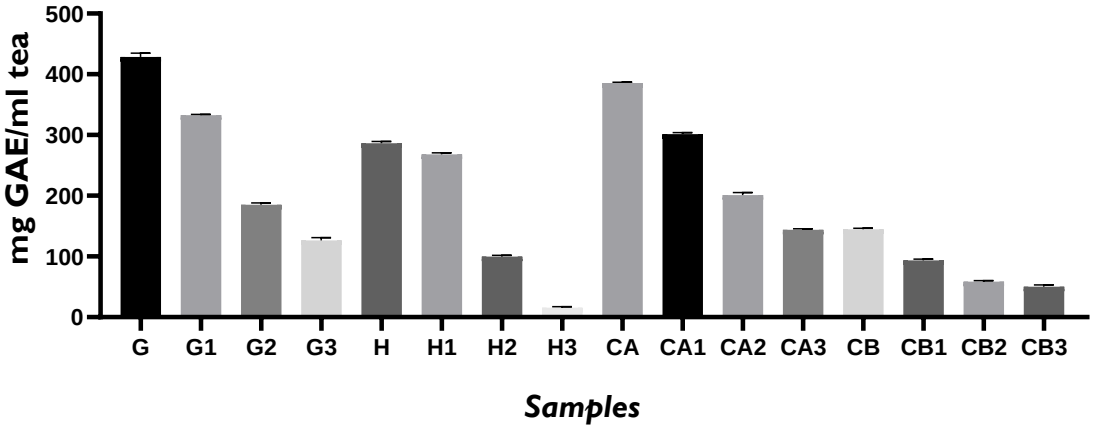

Figure 14 Comparison of the total phenolic compounds of different green teas and C. sinensis flowers' infusion. Results of TPC are expressed in mg of gallic acid equivalents per g of tea (mg GAE/g tea). G=Gorreana; H=Happy Flora; CA=Chá Camélia (Leaves); CB=Chá Camélia (Flowers). 1,2 and 3 stands for the 2nd, 3rd and 4th infusion obtained by reusing the green tea leaves.

After the analysis of Figure 14, it can be observed a significant superiority ($p<0.05$) of the Portuguese tea samples, obtained from leaves, in terms of total phenolic compounds content, with the "Gorreana" being considerably ($p< 0.05$) richer in phenolic compounds

(≈428.4 mg GAE/ml tea), compared to "Chá Camélia" (≈385.5 mg GAE/ml tea). Expectedly, the "Chá Camélia" tea has shown the highest potential to retain phenolic compounds (≈37.4%). However, the difference is not as discrepant as the "Gorreana" tea (≈30%). It should be noted that, despite presenting the second-highest potential for retention of phenolic compounds (≈34.5%), the infusion, obtained from flowers of *C. sinensis* ("Chá Camélia"), presented much lower values (≈144.9 mg GAE/ml tea) compared to the two aforementioned green teas.

A revision of the literature allowed to verify that, even though the TPC is a widely used parameter to evaluate the antioxidant activity of tea infusions, it is challenging to compare TPC values with other studies. Even though the Folin-Ciocalteu reagent method is one of the main techniques used to determine TPC, many procedures are applied. For instance, Kodama *et al*. (Kodama *et al*., 2010) and Almeida *et al*. (Almeida *et al*., 2019) determined the TPC of tea infusions using the Folin-Ciocalteu reagent but according to Singleton, Orthofer and Lamuela-Raventos (1998). Komes *et al*. (2010) used the same reagent but determined the TPC according to the modified method of Lachman, Hosnedl, Pivec, and Orsák (1998). In addition, the units in which the results are displayed also vary among studies. For instance, Kodama *et al*., 2010 expressed the results as mg of catechin equivalents.200 mL^{-1}.

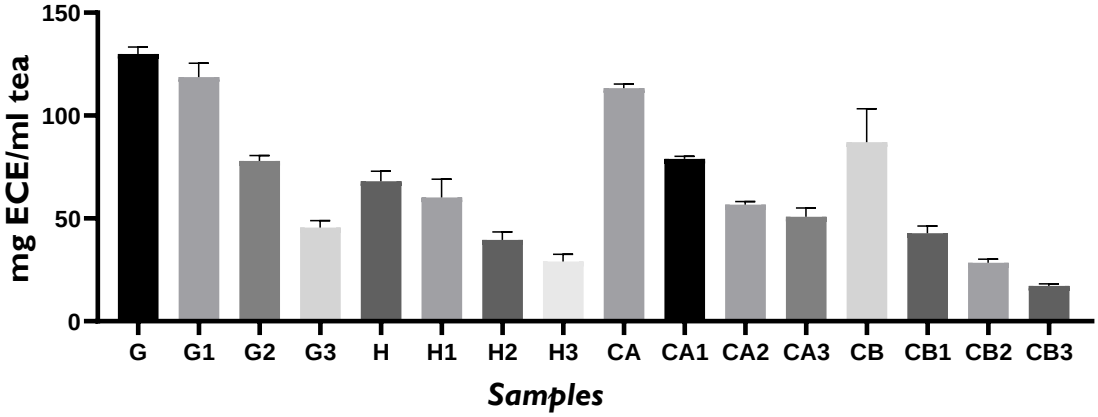

Figure 15 Comparison of the total flavonoids compounds (TFC) of different green teas and C. sinensis flowers' infusion. Results of TFC are expressed as mg of epicatechin equivalent per g of tea (mg ECE/g Tea). G=Gorreana; H=Happy Flora; CA= Chá Camélia (Leaves); CB= Chá Camélia (Flowers). 1,2 and 3 stands for the 2nd, 3rd and 4th infusion obtained by reusing the green tea leaves.

Nevertheless, some authors express their results in gallic acid equivalents, allowing comparisons. Our tea infusions have a higher concentration of phenolic compounds than all teas evaluated by Atoui *et al*. (Atoui *et al*., 2005), including Chinese green tea (1216 ± 32.0 mg GAE/200ml). Furthermore, our infusions also demonstrate higher TPC values than the green and black tea infusions of Almeida *et al*. (Almeida *et al*., 2019), even when compared to their highest TPC value (0.52±0.02 GAE mg/ml). Moreover, when compared to Matcha green teas, our results presented higher concentrations of phenolic compounds(Jakubczyk *et al*., 2020).

However, it is important to remark that tea preparation procedures and the type of water used to brew tea vary, which may influence the final phenolic content of the infusions (Almeida *et al*., 2019).

Finally, observing the results obtained in Figure15, it is possible to see values similar to those in Figure 3, which was expected. Thus, Portuguese teas obtained from the leaves have higher total flavonoids content. However, although "Gorreana" tea is richer in flavonoids (≈130 ECE/ml tea), there are no significant differences (P>0.05) compared to "Chá Camélia" (≈113.3 mg ECE/ml tea). Once again, "Chá Camélia" is superior in retention potential (44.9%) compared to other samples. It should be noted that although "Happy flora" has a higher retention potential than "Gorreana" (approximately 42.8% and 35%, respectively), the total flavonoids content in "Happy flora" tea (68 mg ECE/ml tea) are much lower than "Gorreana." Our infusions presented higher total flavonoids content compared to the Matcha green teas, evaluated by Jakubczyk *et al*.. In addition, the TPC and TFC values of our infusions were higher than the infusions evaluated by Komes *et al*. (2010) in which the green tea blend Rose of the Orient had the highest TPC and TFC (2560 and 1920 mg/L GAE, respectively), whereas the green tea bagged Twinings of London (2560 and 1920 mg/L GAE, respectively) and powdered green tea Matcha (2230 and 1630 mg/L, respectively) had the lowest TPC and TFC (Komes et al., 2010).

5.1.2 Evaluation of the Antioxidant Capacity of Green Tea Extracts

In the present study, solid-liquid extractions and Soxhlet extractions were performed simultaneously, using the teas previously evaluated, in order to compare the antioxidant capacity of green tea extracts as well as their yield. "Chá Camélia" tea varieties were excluded from this trial due to the limited number of samples available.

In a brief analysis of Figures 16, 17, 18, and 19, it is possible to conclude that the extracts obtained through the method described by Andrade *et al.* demonstrate superior antioxidant capacity than the extracts obtained through solid-liquid extraction with the Soxhlet apparatus. In a more detailed analysis, by analyzing Figure 16, it is possible to strengthen the previous statement. By observing Figure 16 it is confirmed that the extracts obtained by the conventional method inhibit lipid peroxidation more efficiently, presenting

AAC values around 1500. However, there are no substantial differences among the analysed green tea samples when the same extraction method was applied (P>0.05).

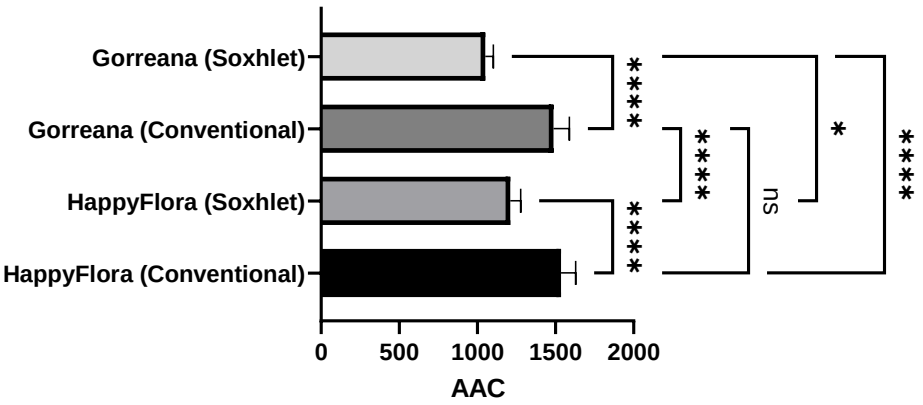

Figure 16 Comparison of green tea extracts prepared by two different extraction methods. β-carotene bleaching inhibition test with results expressed as AAC). ****=P≤0.0001,*=P≤0.05, Ns= P>0.05.

Furthermore, Figure 17 demonstrates that the extract of "Gorreana" obtained by conventional solid-liquid extraction contains a higher content on total flavonoids (701.5 mg ECE/ g extract). However, Figure 18 results do not support the previously established premise. By analyzing Figure 18, it can be inferred that the extracts obtained through Soxhlet extraction have a greater capacity to capture free radicals DPPH● ("Gorreana" ≈ 138.2 μg E.T./g / "Happy Flora" ≈ 131.2 μg E.T./g) than extracts obtained by the conventional solid-liquid extraction method ("Gorreana" ≈ 118 μg E.T./g / "Happy Flora" ≈ 114 μg E.T./g), with no differences among green tea extracts when applied the same extraction method (P>0.05).

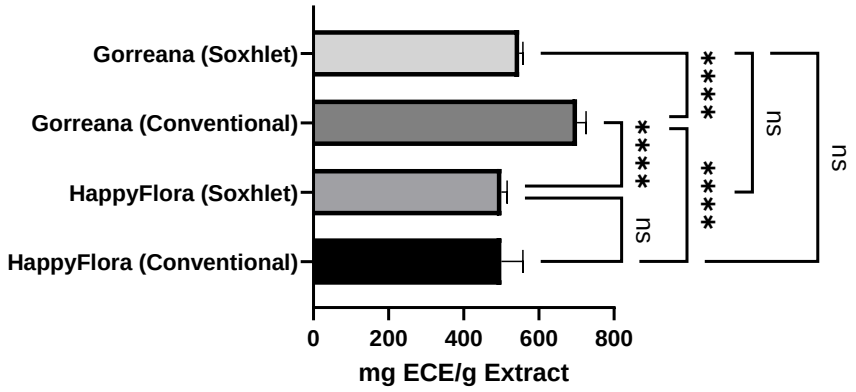

Figure 17 Comparison of green tea extracts prepared by two different extraction methods. Total Flavonoids content expressed in mg of epicatechin equivalent per g of extract (mg ECE/g extract). ****=P≤0.0001, Ns= P>0.05.

Furthermore, regarding total phenolic compounds content, there were no significant differences (P>0.05) between "Gorreana" extracts, even when different extraction methods were applied. However, through Figure 19, significant differences (P<0.05) among the "Happy

Flora" extracts can be observed. Furthermore, there are considerable differences between the "Happy flora" extract obtained by the conventional solid-liquid extraction (≈758.3 mg GAE/g extract) and the "Gorreana" extract obtained through the same method (≈863.1 mg GAE/ g extract). In addition, there are considerable differences between the previously mentioned "Happy Flora" extract and the "Gorreana" extract obtained by Soxhlet extraction (≈833.7 mg GAE/g extract).

In the literature, many publications have evaluated green tea extracts' antioxidant capacity. However, the evaluations of Martins *et al*. (2018) and Castro *et al*. (2019) are the most adequate to perform more accurate comparisons, as they chose similar methods to assess the green tea extracts antioxidant capacity and expressed the results in units that allow comparisons.

Martins *et al*. (2018) did an *in vitro* determination of the antioxidant capacity of green tea extracts obtained through solid-liquid-extraction. In their research, they evaluated the antioxidant capacity of extracts from four green tea varieties: (1) commercial green tea; (2) green tea from capsules; (3) Hysson green teas; and (4) "Encosta da Bruma" green tea.

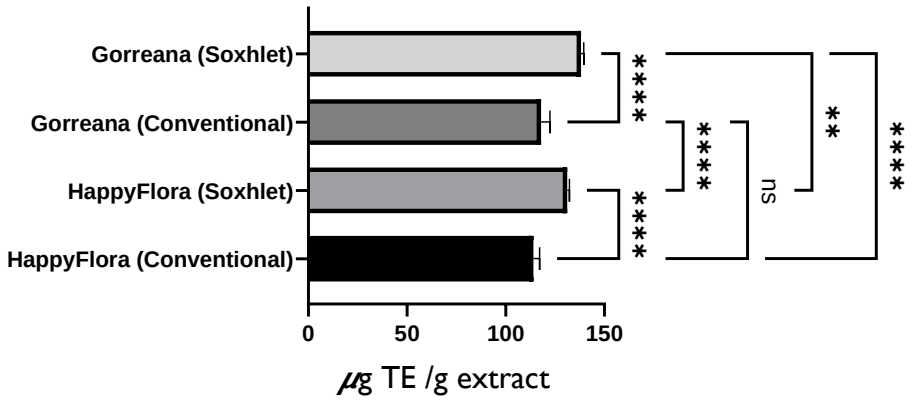

Figure 18 Comparison of green tea extracts prepared by two different extraction methods. Results of DPPH radical inhibition test expressed as mg Trolox equivalents per g of extract (*μ*g TE /g extract). ****=P≤0.0001,**=P≤0.01, Ns= P>0.05.

Overall, the antioxidant capacity of our extracts was higher than the antioxidant capacity of the extracts from the Martins *et al*. (2018) study. All of our extracts presented higher AAC, except Gorreana extracts obtained through Soxhlet extraction. According to our results, Happy flora extract (conventional solid-liquid extraction) exhibited an AAC of 1536; the Gorreana extract exhibited an AAC of 1488, and the Happy flora extract (Soxhlet) exhibited an AAC of 1210, while according to the results of Martins *et al*., extracts from

commercial green tea, green tea from capsules, Hysson green, and "Encosta da Bruma" green tea exhibited an AAC of 918, 1079, 1144 and 1178 respectively. Only the Gorreana extracts obtained through Soxhlet extraction exhibited a lower, but still high, AAC (1051) than the

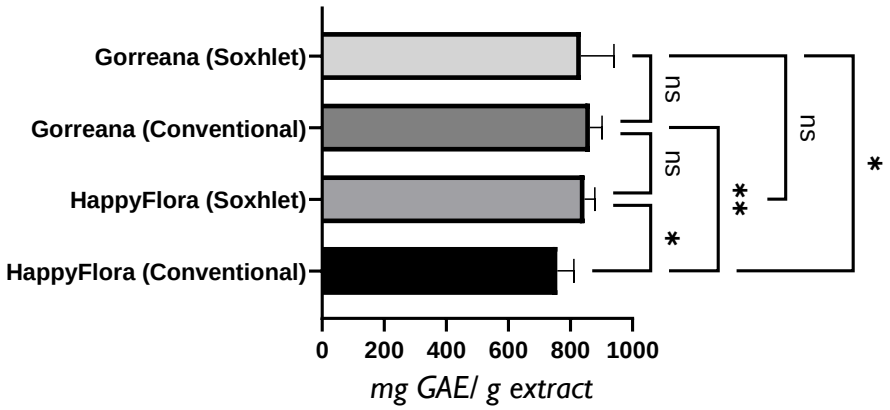

Figure 19 Comparison of green tea extracts prepared by two different extraction methods. Content of total phenolic compounds with results expressed as mg of gallic acid equivalents per g of extract (mg GAE/g extract). **=P≤0.01, *=P≤0.05, Ns= P>0.05.

green tea extracts from the previously mentioned study. In addition, our DPPH results were also very high. Extracts from Gorreana (conventional), Gorreana (Soxhlet), Happy flora (conventional), and Happy Flora (Soxhlet) exhibited a TEAC (***μ***g TE /g extract) of 117.9; 138.2; 114 and 131.2 respectively. According to Martins *et al*., extracts from commercial green tea, green tea from capsules, Hysson green, and "Encosta da Bruma" green tea exhibited a TEAC (mg TE/g extract) 0.917 ± 0.09; 0.560 ± 0.02; 0.602 ± 0.12 and 0.693 ± 0.07 respectively.

Nevertheless, our extracts were richer in total polyphenols and total flavonoids than those evaluated by Martins *et al*. (2018). As previously mentioned, extracts from Gorreana (conventional), Gorreana (Soxhlet), Happy flora (conventional), and Happy Flora (Soxhlet) exhibited a TPC of 863.1; 833.7; 758.3; 845.3 mg GAE/ g extract respectively. While extracts from commercial green tea, green tea from capsules, Hysson green, and "Encosta da Bruma" green tea exhibited a TPC of 416 ± 9.95; 272 ± 2.34; 330 ± 4.68 and 361 ± 3.22 mg GAE/ g extract, respectively. Regarding the flavonoids content, extracts from Gorreana (conventional), Gorreana (Soxhlet), Happy flora (conventional), and Happy Flora (Soxhlet) exhibited a TFC of 701.5; 547.6; 500.2; 500.0 mg ECE/ g extract. Extracts from commercial green tea, green tea from capsules, Hysson green, and "Encosta da Bruma" green tea exhibited a TFC of 148 ± 0.2; 139 ± 9.59; 184 ± 0.64 and 165 ± 7.03 mg ECE/ g extract.

Furthermore, compared to the extract from Castro *et al*. (2019), our extracts were much higher, as their extract presented an AAC of 379.24 ± 10.43, a TPC of 443.55 ± 10.00 mg GAE/g of extract, and a TFC of 119.81 ± 11.70 mg ECE/g of extract.

5.2 Antioxidant and Antimicrobial Properties of the Active Film

After a global analysis of the previously mentioned tests, "Gorreana" green tea extract was chosen to be incorporated in the whey protein film. Theoretically, the Chá Camelia extract (Leaves) would also be an excellent option due to the tea's high retention potential and sensory characteristics. Additionally, solid-liquid extraction with Soxhlet apparatus was selected as the extraction method since it presented a higher yield (43%) than conventional solid-liquid extraction (5.6%).

The films obtained showed good mechanical properties. The active films were characterized by an olive-green hue and a light green tea aroma. Additionally, there was no solubilization between the films and the fresh cheeses.

5.2.1 Evaluation of the Antioxidant Capacity of the Whey-Protein Based film

After producing the active films, migration tests were carried out, and their antioxidant capacity was evaluated.

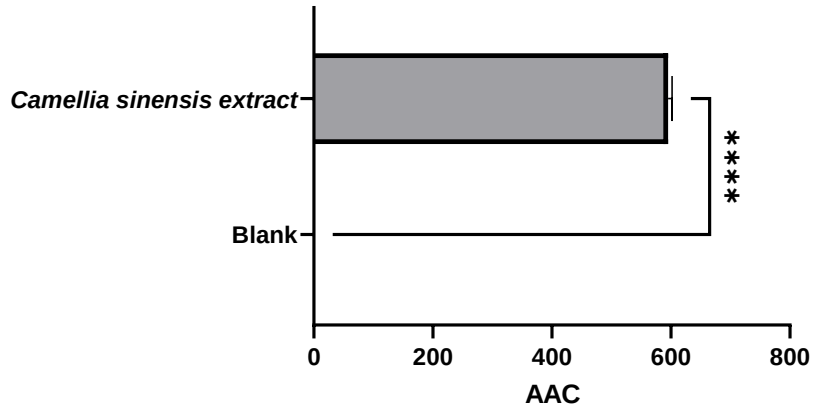

Figure 20 Results of the antioxidant capacity regarding β-carotene bleaching inhibition test of the active films after a migration test at 40°C for 10 days (Active Film - C. sinensis extract vs Control - Blank film). β-carotene bleaching inhibition test with results expressed as AAC. ****=P≤0.0001.

As expected, the active film incorporated with 2.5% green tea extract from the "Gorreana" plantation exhibited a higher antioxidant capacity relative to the blank film (no added antioxidant extract). By observing Figures 20 and 21, it can be seen that it has not only a high capacity to inhibit lipid peroxidation (≈595.4 AAC) but also a greater capacity to capture DPPH● free radicals (≈121 μg ET.g). However, compared to the results obtained in the

evaluation of the extract of "Gorreana" obtained by Soxhlet extraction, there is a reduction of 43% and 11%, in relation to the β-carotene bleaching and DPPH radical tests, respectively.

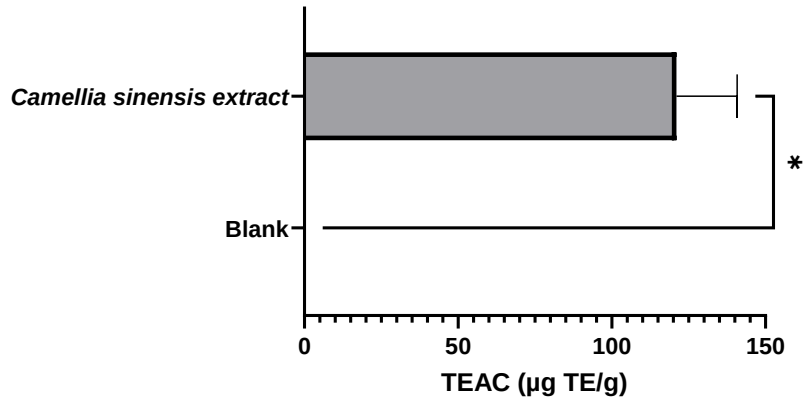

Figure 21 Results of the antioxidant capacity of the active films after a migration test at 40°C for 10 days (Active Film - C. sinensis extract vs Control - Blank film). DPPH radical inhibition test with results expressed in mg Trolox equivalents per g of film (μg T.E./g film). *=P≤0.05.

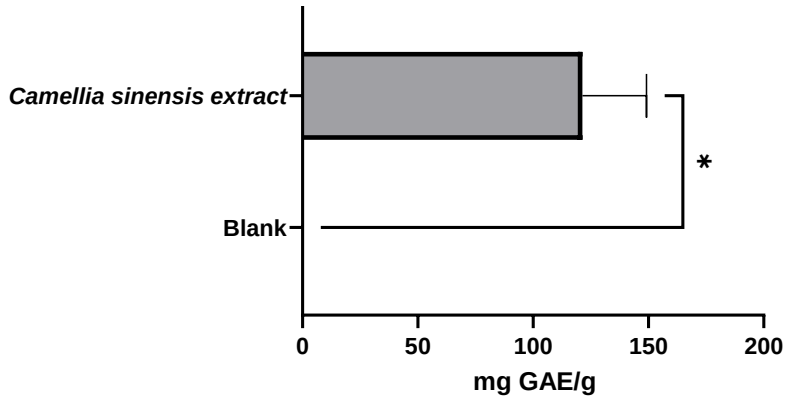

Figure 22 Results of the total phenolics content of the active films after a migration test at 40°C for 10 days (Active Film - C. sinensis extract vs Control - Blank film). Total phenolic count with results expressed in mg Gallic Acid equivalents per g of film (mg GAE/g film). *=P≤0.05.

After a migration test carried out using ethanol 95% (v/v) as food simulant (simulant of fatty foods), it can be concluded that the new active film, presented a TPC of 121.3mg GAE/g and a TFC of 55.4mg ECE/g (Figures 22 and 23). However, comparing with the extract of "Gorreana" obtained by Soxhlet extraction, there is a decrease of 93.3% and 76% regarding TPC and TFC, respectively.

Besides films prepared with green tea extract, there were also films prepared with green tea. Regarding these films, the values obtained in the β-carotene bleaching and DPPH radical tests are very similar to those obtained during the evaluation of the antioxidant capacity of "Gorreana" green tea. However, there is a reduction of 71.7% and 57.4% regarding the content of phenolic compounds and flavonoids, respectively. However, it should be noted that the active films developed from the incorporation of green tea did not demonstrate any antioxidant capacity.

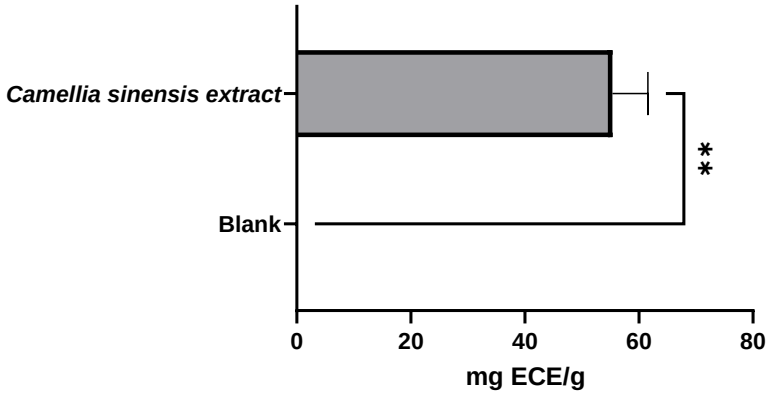

Figure 23 Results of the antioxidant capacity of the active films after a migration test at 40°C for 10 days (Active Film - C. sinensis extract vs Control - Blank film. Total Flavonoid count with results expressed in mg Epicatechin equivalents per g of film (mg ECE/g). **=P≤0.0.

According to the literature, very few studies evaluated the antioxidant capacity of whey protein-based films incorporated with green tea extracts. Pluta-Kubica *et al.* (2021) and Chalob & Abdul-Rahman (2018) developed edible films using whey-protein isolates as biopolymeric matrix and green tea extracts as antioxidant agents. However, the first study used both Furcellaran and whey protein isolate as a biopolymeric matrix and therefore is not possible to compare accurately the antioxidant capacity since we used only whey protein concentrate as a biopolymeric matrix. The second study developed a whey protein isolate based film and evaluated its antioxidant capacity using a procedure in which the film was cut into aliquots and dissolved in deionized water followed by centrifugation. A portion of the solution was mixed with a methanolic solution of DPPH, vortexed and then incubated. Finally, the solution was centrifuged, and the absorbance was measured. Unfortunately, this procedure is different of the one used in this assay to evaluate the antioxidant capacity and therefore does not allow comparison.

In addition, it is important to remark that both studies have used whey protein isolate in the edible films formulation and in this study we used whey protein concentrate which may influence the film antioxidant capacity. As far was we know, according to the available

bibliography, there are not any studies that fully compare the antioxidant activity of WPI and WPC films.

Moreover, these researches only assessed the antioxidant capacity through DPPH scavenging assay, and this parameter does not provide meaningful information about the film's actual antioxidant capacity when considered alone (Amorati & Valgimigli, 2015).

5.2.2 Antimicrobial Properties of Films

In this study, the total count of microorganisms at 30°C, *E. coli* and staphylococci coagulase-positive was carried out, since these are indicators not only of food quality but also of compliance, within the scope of good hygiene practices, during cheese production and

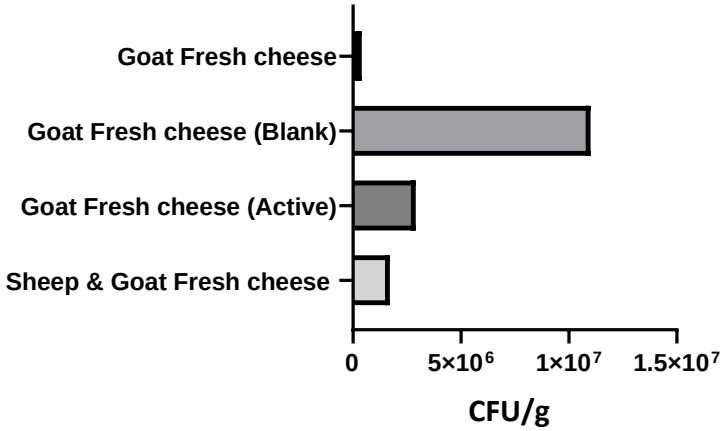

Figure 24 Results of total microorganisms count at 30°C. Results are expressed as colony-forming units per g of fresh cheese (CFU/g fresh cheese). Total microorganisms count was carried out on goat fresh cheese (Goat Fresh cheese) and mixture fresh cheese (Sheep & goat fresh cheese) produced on the day. Furthermore, both cheeses were wrapped with the edible active films (Goat Fresh cheese - Active | Sheep & goat fresh cheese - Active) and with the control films (Goat Fresh cheese - Blank | Sheep & goat fresh cheese - Blank) and stored at 5°C, in order to simulate the standard storage conditions. After one week of storage at refrigerated temperatures the Escherichia coli colony-count was carried out. The number of colonies of goat fresh cheeses active and blank were uncountable and therefore are not shown.

distribution. Additionally, since fresh cheeses are usually stored at refrigerated temperature (5°C), the psychrophile count was also carried out. The counts of the different microorganisms were carried out on goat fresh cheese and on fresh mixture cheese (Sheep and goat fresh cheese) produced on the day. Furthermore, the microorganisms count of the two kinds of fresh cheeses wrapped with active films (Active) and control films (without green tea extract - Blank), was also carried out after one week o storage at 5°C.

In terms of food quality, it can be concluded from Figure 24 that the active films effectively inhibited the growth of mesophilic microorganisms, and the goat cheese coated with the active film presented a significant lower count of CFU/g (2.9 x 106 CFU/g) than the blank coated cheese (1.1 x 107 CFU/g). In mixed cheese (goat and sheep), the colonies were countless; however, to the naked eye, the active film-coated sample appeared to have fewer colonies compared to the blank coated sample (fresh cheese coated with control film).

In the context of food safety, considering that despite working as a hygiene indicator, some strains of E. coli are pathogenic, when looking at Figures 25 and 26, it can be observed inhibition of the growth of microorganisms in the samples coated by the active film. In fact, in goat cheese (Fig. 25), there is even a significant reduction in the number of colonies (1.5 x 10 CFU/g) when compared to fresh cheese produced on the day (1.8 x 10^2 CFU/g) and, of course, to the blank coated cheese (2.2 x 10^2 CFU/g).

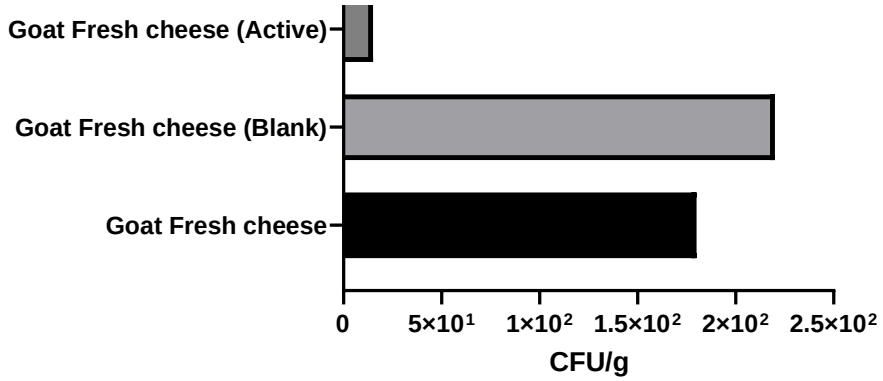

Figure 25 Results of the Escherichia coli colony-count in fresh goat cheese. Results expressed CFU per g of fresh cheese (CFU/g fresh cheese). Escherichia coli colony-count was carried out on goat fresh cheese produced on the day (Goat Fresh cheese). Furthermore the cheeese was wrapped with the edible active films (Goat Fresh cheese - Active) and with the control films (Goat Fresh cheese - Blank) and stored at 5 °C, in order to simulate the standard storage conditions. After one week of storage at refrigerated temperatures the Escherichia coli colony-count was carried out.

In the mixed cheese (Fig. 26), there is no decrease in the number of colonies when compared to fresh cheese produced on the day (1.6×10^4 CFU/g); however, the cheese coated by the active film had a lower number of CFU/g (3.2×10^5 CFU/g) compared to blank coated one (4.4×10^5 CFU/g). Note that coagulase + staphylococci or psychrophiles have not grown in any of the samples.

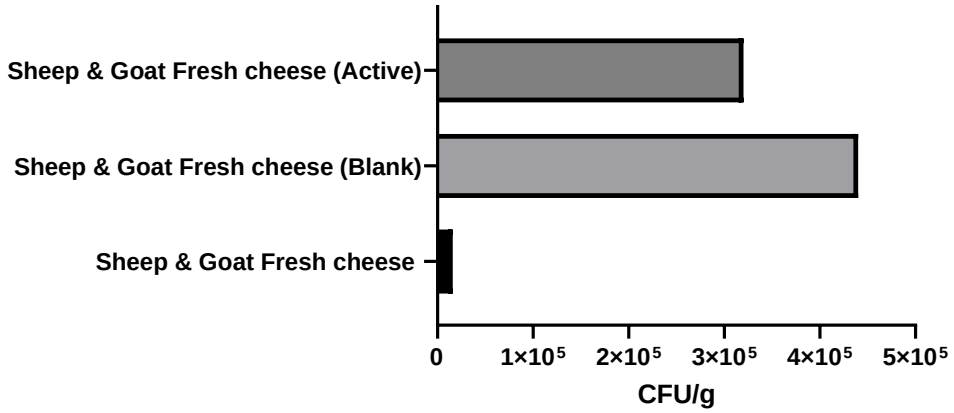

Figure 26 Fresh cheese prepared with mixed milk from sheep and goat. Escherichia coli colony-count. Results expressed as Colony-Forming Units (CFU) per g of fresh cheese (CFU/g fresh cheese. Escherichia coli colony-count was carried out on goat fresh cheese produced on the day (Sheep & Goat Fresh cheese). Furthermore the cheese was wrapped with the edible active films (Sheep & Goat Fresh cheese - Active) and with the control films (Sheep & Goat Fresh cheese - Blank) and stored at 5°C, in order to simulate the standard storage conditions. After one week of storage at refrigerated temperatures the Escherichia coli colony-count was carried out.

At least six edible films have been developed, to date, for unripened cheeses, and their antimicrobial activity was assessed. Pluta-Kubica *et al*. (2020) developed active edible furcellaran/whey protein films with yerba mate and white tea extracts and applied them to fresh soft rennet-curd cheese. The microbiological quality of the cheese was determined through total counts of bacteria (TBC), yeast, molds, and coliforms. In 2021 Pluta-Kubica *et al*. published another article in which they applied furcellaran-whey protein Isolate films containing extracts (pu-erh or green tea) to an acid-curd cheese. This study determined Lactococcus, total counts of bacteria, yeast, molds, and coliforms. Furthermore, Santacruz & Castro (2018) developed a cassava starch edible coating incorporated with *Lactobacillus acidophilus* and applied it to Manaba fresh white cheese. The antibacterial activity of L. acidophilus was assessed against a *Salmonella* strain previously isolated from Manaba cheese. The mesophilic aerobic bacteria on cheese was also determined. Morevover, Di Pierro *et al*. (2011) developed a chitosan/whey protein coating to extend Ricotta cheese shelf-life and determined its antimicrobial activity against aerobic, mesophilic, and psychotropic

microorganisms. In addition, Mileriene *et al*. (2021) evaluated the effect of liquid whey protein concentrate–based edible coating enriched with cinnamon carbon dioxide extract on eastern European curd cheese's quality and shelf life. To assess the microbiological quality, they enumerated Total bacteria count (TBC), Lactic acid bacteria, *Staphylococcus spp*. Enterobacteria, Coliforms and yeast, and molds. Finally, Jutinico-Shubach *et al*. evaluated the antilisterial activity of chitosan-based edible coating incorporating cell-free supernatant from *Pediococcus pentosaceus* 147 on the preservation of Colombian fresh cheese and performed the microbiological analysis by determination of *L. monocytogenes*, mesophilic bacteria, psychrophilic bacteria, and total molds and yeasts count

Most of the previously mentioned research developed attractive edible films/coatings to apply to unripened cheese surfaces. However, the microbiological quality of the various unripened cheeses is evaluated through a wide variety of parameters, not allowing adequate comparisons. In addition, it is essential to remark that the microbiological quality varies between unripened cheeses, as some require starter cultures (preparations of living microorganisms, which are deliberately added to foods in order to take advantage of the compounds or products derived from their metabolism or enzymatic activity to be produced (García-Díez & Saraiva, 2021). Nevertheless, some authors evaluated the microbiological quality of cheeses through enumeration of mesophilic bacteria, allowing comparisons with the present study results.

In the study of Pluta-Kubica *et al*. (2020), within one week, the TBC of uncoated fresh soft rennet-curd cheese TBC diminished from 8.8 log CFU/g to 8.6 log CFU/g. In addition, after one week, the TBC of cheese coated with furcellaran-whey protein isolate exhibited 8.8 log CFU/g, cheese coated with furcellaran-whey protein isolate incorporated with yerba mate extract exhibited 8.6 log CFU/g, and cheese coated with furcellaran-whey protein isolate incorporated with white tea extracts exhibited 8.5 log CFU/g. Even though differences between the active films and the control are minimal or non-existent, they were able to get good results in the scope of their research because TBC, in fresh soft rennet-curd cheese, includes primarily lactic acid bacteria, which are not spoilage microorganisms. In 2021 Pluta-Kubica et demonstrated that within one week, the TBC of uncoated fresh Acid-curd cheese TBC diminished from 7.5 log CFU/g to 6.5 log CFU/g. In addition, after one week, the TBC of cheese coated with furcellaran-whey protein isolate exhibited 5.9 log CFU/g, cheese coated with furcellaran-whey protein isolate incorporated with green tea extract exhibited 5.9 log CFU/g, and cheese coated with furcellaran-whey protein isolate incorporated with pur-erh

extracts exhibited 6.8 log CFU/g. In this study, however, none of the edible films used improved the microbiological quality of an acid-curd cheese.

Furthermore, Santacruz & Castro (2018) demonstrated that Within ten days, mesophilic bacteria on Uncoated Manaba cheese raised from 8.60 log CFU/g to 9.20 log CFU/g. After ten days, Manaba cheese coated with cassava starch film containing free *L. acidophilus* presented 4.10 log CFU/g while Cassava starch film containing encapsulated *L. acidophilus* presented 5.90 log CFU/g. Moreover, According to Di Pierro *et al.* (2011), the viable numbers of mesophilic bacteria on the uncoated ricotta cheese after one week raised from ≈6.5 log CFU/g to ≈7.0 log CFU/g, while on the coated ricotta cheese, the viable numbers of mesophilic bacteria maintained (≈6.5 log CFU/g). Finally, in Mileriene *et al.* 2021 study, the TBC diminished from 7 to 4 log CFU/g in all samples during storage (31 days). However, after ten days of storage, the curd cheese TBC raised from 6.91 log CFU/g to 7.15 log CFU/g, and the coated curd cheese TBC raised from 6.92 log CFU/g to 7.26 log CFU/g. On the other hand, vacuum-packed curd cheese and coated and vacuum-packed curd cheese TBC diminished from 6.91 log CFU/g to 6.39 log CFU/g and from 6.90 log CFU/g to 6.47 log CFU/g, respectively.

Regarding the viable numbers of mesophilic bacteria, by converting CFU/g to log CFU/g, we can verify that after one week, our uncoated goat cheese presented ≈7.0 log CFU/g and coated goat cheese presented ≈6.5 log CFU/g. In addition, it is important to recall that Latin-style fresh cheese manufacture does not require starter cultures. This actively demonstrates that our whey protein-based edible film incorporated with green tea extract had a good performance compared to the formerly developed ones. However, this parameter alone does not allow any conclusions.

5.3 Textural and organoleptic changes of Fresh cheeses

This study also showed that whey protein-based film incorporated with Green tea extracts altered Latin-style fresh cheese's texture and organoleptic properties after one week. The significant evaluated effects were color changes and an increase in hardness. As shown in Figure 27, the active film altered the color of the goat cheese while both control and uncoated cheese maintained the color. In addition, all of the films presented an increase in hardness, with the goat cheese wrapped with an active film being the one with the highest hardness, followed by the fresh cheese wrapped with the control and uncoated fresh cheese.

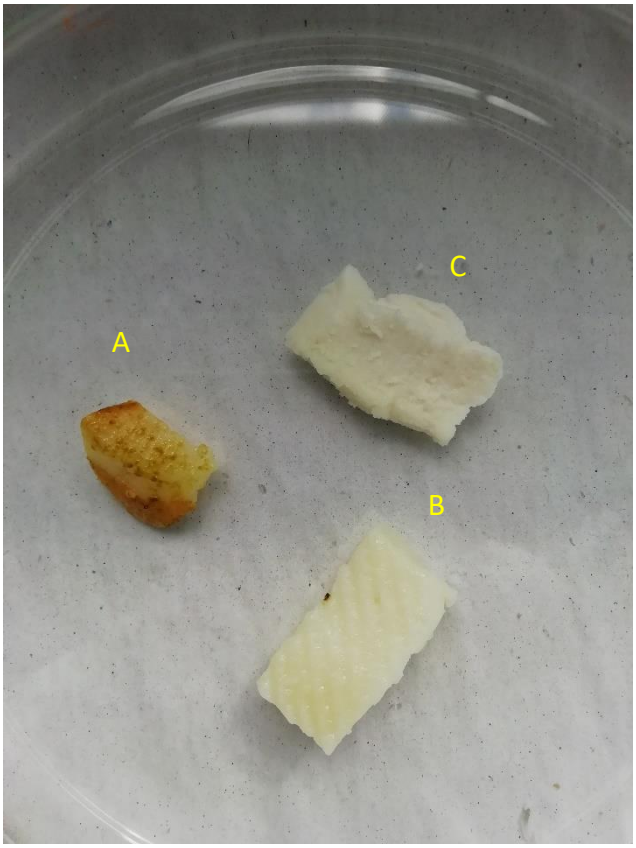

Figure 27 Colour changes of wrapped and unwrapped goat cheeses. A - Goat cheese wrapped wih active film (Whey protein-based film with green tea extract), B - Goat cheese wrapped with control film (Whey protein-based film), C - Unwrapped goat cheese.

VI. Conclusion & Future perspectives

In the present study, a screening of the antioxidant capacity of Portuguese and Asian green tea infusions was successfully performed. The green tea provided by "Chá Camélia" were characterized for the first time as well as the infusion prepared with flowers of *C. sinensis* and revealed a great antioxidant capacity and outstanding potential to retain the antioxidant capacity. Moreover, extracts from two brands (a Portuguese and an Asian brand) of green tea were prepared and their yield and antioxidant capacity were evaluated demonstrating their high antioxidant potential. Due to limitations in samples' availability, "Chá Camélia" samples were not used to prepare extracts. However, in the future these samples should be considered. In addition, a comparison between conventional solid-liquid extraction and Soxhlet extraction suggested that the former may be the most adequate due to its yield. However, the use of Soxhlet apparatus does not allow stirring, and may lead to the thermal degradation of compounds since this process exposes the samples to high temperatures for a long time (Lourenço *et al*., 2019).

Additionally, active whey protein-based films incorporated with green tea extracts (2.5%) were successfully developed, and their application as a packaging material for Latin-style fresh cheeses prolonged their shelf life. Tested over a week, the cheese samples wrapped with active edible films were characterized by lower counts of mesophilic bacteria and *E coli.* However, the active edible films altered the color and hardness of fresh cheeses. Further studies are required to fully evaluate the impact of active films developed on the organoleptic quality of Latin-style fresh cheese samples. Additional studies should be performed to fully evaluate the antimicrobial capacity concerning food-borne bacteria such as *Listeria monocytogenes.*

The evaluation of the antioxidant capacity of the developed films was also carried out and indicated that whey protein-based films incorporated with green tea extracts might successfully prevent or inhibit lipid oxidation of fresh cheeses. However, further studies are required to evaluate lipid oxidation during storage (e.g., TBARS, peroxide value) and, therefore, validate the antioxidant capacity in the cheese samples. Furthermore, the phenolics present in the Latin-style fresh cheese should be quantified in order to avoid biases.

The developed whey protein-based films incorporated with green tea extracts are disruptive packaging materials that have been successfully used to extend Latin-style fresh

cheese shelf life. Furthermore, this packaging is environmentally friendly as it is biodegradable and renewable and therefore generates zero waste. Moreover, their manufacture adds value to whey making it more profitable to companies. However, a greater understanding of the subject is required to better understand the magnitude of the benefits/cost ratio. Nevertheless, currently, edible coatings and films are already being explored by companies such as Improveat (Braga, Portugal), BecorBarbanxa (A Coruña, Spain), and Vink chemicals GmbH & Co (Kakenstorf, Germany). This actively demonstrates that these types of packaging are promising and should be continuously exploited to allow the translation from the laboratories to industry.

Future research could involve the discoloration of green tea extract (without loss of antioxidant and antimicrobial properties) in order to develop more transparent edible packages. Furthermore whey-protein coatings incorporated with green tea extracts to fruits as whey-protein films can constrain enzymatic browning of fresh-cut products (Ramos *et al*., 2012). Furthermore, nanotechnology could be an exciting area to focus on as nanostructures display a high surface-to-volume ratio and unique surface properties (Costa *et al*., 2018).

The manufacturing of edible packaging materials should be simple, fast, and easy to reproduce at an industrial scale at a low cost. In my opinion, in future research, instead of films, formulations involving whey protein concentrates and green tea extracts should be used to produce edible coatings, applied by dipping (in small industries, the brushing method could be used in order to avoid the use of large tanks required for dipping), to Latin-style fresh cheese, as this coatings are simple to manufacture, low cost and allow to cover all the surface by immersion (Costa *et al*., 2018). This way, instead of creating an active material, we would create a novel food. Therefore, this novel food would have an extended shelf-life and could be interesting to associate probiotics, as Latin-style fresh cheeses are attractive probiotic carriers. Furthermore, we could appeal to all tea consumers and the sports community, as the novel food would be produced with green tea extracts and whey protein concentrates.

Moreover, their marketing could include all the outstanding nutraceutical and nutritional properties provided by the fresh cheese with probiotics and whey-protein edible coating. It is important to remark that the purpose is not to replace traditional cheese but to develop a novel probiotic enriched food that does not require packaging as it is the packaging itself. This way, we could create a third more economical and possibly more profitable option for cheese industries to add value to whey.

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Annex I – Oral Communication

XV ENCONTRO DE QUÍMICA DOS ALIMENTOS

OC02: Development of an active whey protein film using Portuguese green tea (*Camellia sinensis* L.) extract to enhance fresh cheese shelf life

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Fresh cheese is a slightly acid and mildly salted non-ripened cheese, made from raw or pasteurized milk, widely consumed in Europe, in particular in Portugal. However, it's high pH (>5), water activity and lipid content favours not only the growth of microorganisms but also lipid oxidation, leading to a short shelf-life. [1–4]

Cheese making results in the production of whey, the main by-product of cheese industry. Unfortunately, if not managed correctly, cheese whey can become a major pollution factor, due to its high chemical and biochemical oxygen demand. Therefore, cheesemakers all around the world face both the perishability of fresh cheese and the management of cheese whey. [5]

Even though, formerly, cheese whey was considered waste, and treated as such, in recent years it has been acknowledged as a valuable by-product since it retains 55% of milk nutrients. Additionally, cheese whey protein comprises several bioactive compounds, that can be used by nutraceuticals' industry. [6–8]

Nowadays, there is a growing interest in "green packaging" due to the environment negative impact of conventional packaging materials. The advances in technology, made possible the development of membrane separation technologies, allowing whey to be processed into whey protein concentrates that can be used to produce biodegradable active films, in which antioxidant/antimicrobial agents can be incorporated in order to delay lipid oxidation and/or avoid post-process contamination. [9-12]

Green tea (*Camellia sinensis* L.), the second most consumed beverage in the world, is rich in bioactive compounds such as polyphenols, namely flavonoids and phenolic acids, therefore, it is well known for its antioxidant and antimicrobial properties. [10,13]

Portugal is one of the few producers of tea (*C. sinensis L.*) in Europe. "Gorreana" is one of the most well-known brands of Portuguese tea, and it is produced in São Miguel Island, in Azores. Lately a company, "Chá Camélia", started to produce green tea in a farm near by Vila do Conde. However, this tea has never been characterized before.

Thus, the main objective of this study was to compare different green teas ("Gorreana", "Chá Camélia" and "Happy flora") regarding their antioxidant capacity including Chá Camelia green tea from 2020 production. Moreover, this study developed a biodegradable and edible active film using whey protein and green tea or green tea extracts, in order to extend fresh cheese shelf-life.

In the frame of this study, solid-liquid and Soxhlet extractions were compared in order to obtain *C. sinensis* L. concentrated extracts. The antioxidant capacity of green teas and green teas extracts was assessed, through DPPH free radical scavenging activity, β-carotene bleaching assay. Moreover, total phenolics and flavonoids content were also determined. Furthermore, whey films incorporated with green tea and green tea extracts were produced. Additionally, through migration assays, the antioxidant capacity of the films was evaluated, using the previously mentioned methods.

Our results demonstrated that Soxhlet extraction had presented higher yield (43%) than solid-liquid extract (5.6%). Additionally, "Gorreana" green tea, produced in Azores, presented the highest antioxidant capacity, both as tea and as extract, when compared to "Chá Camélia" and "Happy flora". Extracts obtained from "Gorreana" green tea by Soxhlet method exhibited 137.4 µg ET/mL while those

obtained by "Happy Flora" green tea exhibited 130.8 µg ET/mL. Gorreana tea exhibited a higher Antioxidant Activity Coefficient (AAC=1038) when compared with the other tea samples. However, the new "Chá Camélia" tea, which was studied for the first time, also presented considerable antioxidant capacity (AAC=600).

The effectiveness of the new active edible film to enhance fresh cheese shelf life was also evaluated testing antimicrobial activity of the active films in contact with different types of fresh cheese. In addition, TBARS assay was performed in fresh cheese packaged with the new active film in order to evaluate the capacity of the film to inhibit lipid oxidation of fresh cheese.

In sum, this study suggests that the new whey protein film incorporated with Portuguese green tea extract has potential to be used to extend fresh-cheese shelf life. This will allow switching from an economical to a profitable whey management, while avoiding fresh cheese deterioration and, simultaneously, implementing a circular economy approach.

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