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NEW ADDED-VALUE DAIRY PRODUCTS INCORPORATIING CHEESE SURPLUSES: TECHNICAL FOUNDATIONS, FORMULATIONS AND PHYSICOCHEMICAL CHARACTERIZATION

Thesis submitted to Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology, with specialization in Food Technology

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Supervisor: Prof. João Paulo Ferreira
Co-supervisor: Prof. Ana Maria Gomes

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Dedicatória

Esta tese é dedicada a todas as pessoas que me conhecem verdadeiramente, que me apoiam diariamente, mesmo sem saber, e me fazem sentir leve, como se o peso da rotina que todos carregamos não passasse de uma pequena flor-de-leão.

Aos meus, à minha família de coração, vocês sabem perfeitamente quem são, e à de sangue que não deixa que o sangue se dilua à custa das vicissitudes e atitudes da nossa existência, aquela que não diz adeus...
A Deus e àqueles que já cá não estão...

Obrigada.

Resumo

O setor dos lacticínios tem uma grande importância na economia mundial, tendo sido extremamente competitivo em termos de portefólio de produtos. De modo a conseguir dar resposta às novas tendências do consumidor, tais como produtos com perfis nutricionais muito particulares e produtos com benefícios de saúde associados, esta indústria teve de adaptar esse portefólio em concordância com as mesmas. Mais ainda, os números relacionados com o desperdício alimentar são muito altos, sendo que, mundialmente, cerca de 1/3 dos alimentos destinados ao consumo humano são desperdiçados; em Portugal, é estimado que 17% da produção anual de alimentos se converte em desperdício alimentar. No setor da produção de queijos este desperdício pode ser encontrado em excedentes de produção ou em itens com defeito que não são colocados à venda. Neste projeto, os subprodutos do queijo foram valorizados, tendo servido de ingredientes base para o desenvolvimento de um grupo de produtos láteos inovadores, com novos perfis de sabor e características nutricionais melhoradas.

Isto foi alcançado formulando bases de queijo fundidas *(MCB),* pastas de queijo derretido dispersas em amido gelatinizado (ou noutros hidrocolóides incluindo goma xantana e goma guar), sendo o leite utilizado como fluido base. Estes MCBs foram o ingrediente característico para a incorporação posterior nos produtos finais, onde o queijo Emmental foi o tipo de queijo selecionado para grande parte da investigação, devido às suas propriedades. Além de queijo, os MCBs também incluíram na sua formulação agentes capazes de os estabilizar após o seu arrefecimento. De modo a evitar a inclusão de sais emulsificantes, sais esses utilizados na formulação de queijos processados, foram utilizados hidrocolóides como alternativa. Os principais agentes foram o amido de milho nativo (CS) e o amido de arroz ceroso (WRS), que também funcionaram como espessantes e estabilizadores dos MCBs. Foi provado que a suspensão de amido em leite deve ser aquecida para além da sua temperatura de gelatinização (85 ºC para o CS e 90 ºC para o WRS) antes da adição de queijo, para que a formulação final da MCB revele características texturais adequadas.

Foi realizado um estudo sobre as interações envolvidas na manutenção da estrutura proteica do queijo (Emmental), usando soluções de diferentes agentes dissociantes a diferentes concentrações e combinações. De modo a quantificar a proteína solubilizada, foi desenvolvido um método espetrofotométrico modificado, que pode ser aplicado na quantificação da proteína total em queijos, apresentando resultados estatisticamente semelhantes ($p>0.05$) aos resultados obtidos com método de Kjeldahl. Os nossos resultados revelaram que as ligações das caseínas no queijo Emmental são mantidas através de um conjunto de interações hidrofóbicas, pontes de hidrogénio e ainda interações electroestáticas, incluindo ligações iónicas, tendo as pontes de hidrogénio um papel mais importante, em comparação com as interações hidrofóbicas, uma conclusão que não é usualmente reportada em estruturas de queijo.

A parte final deste projeto consistiu no desenvolvimento de dois produtos láteos inovadores, nomeadamente, os *yogucheeses*, i.e. iogurtes fortificados com queijo, e queijos frescos tipo ricotta.

Para os *yogucheeses*, foi utilizado queijo (Emmental) e CS ou WRS na formulação das MCBs. Estas MCBs foram misturadas com leite em diferentes proporções, antes da adição da cultura bacteriana e da fermentação, de modo a criar amostras com diferentes proporções de MCB para análise posterior.

Os produtos obtidos foram caracterizados em termos de composição macronutricional e propriedades físicas e químicas.

No final, a incorporação de CS e WRS, combinada com a incorporação de queijo Emmental, criou amostras de iogurte com uma estrutura de gel mais firme, uma sinérese mais baixa e uma viscosidade mais alta. Todas as amostras de iogurte se mostraram estáveis durante um período de 14 dias, não mostrando alterações a nível do aspeto visual ou alteração visível na sinérese.

Para os queijos frescos tipo ricotta, as MCBs foram formuladas com recurso a dois tipos de queijo (Emmental e queijo de ovelha) e foram usados CS ou WRS. De seguida, a MCB foi diluída com leite e coagulada, para que o queijo fresco pudesse ser obtido.

Estas amostras foram caracterizadas em termos de composição macronutricional, propriedades físicas e químicas e propriedades sensoriais.

Os queijos frescos desenvolvidos apresentaram um conteúdo nutricional equilibrado, bem como uma textura similar à de muitos queijos frescos, ou queijos de barrar disponíveis no mercado. A formação da estrutura de gel da mistura inicial foi mais difícil de obter acima de um certo nível de incorporação de queijo maturado na MCB, no entanto, esta dificuldade conseguiu ser superada com a adição de leite em pó desnatado (SMP) à preparação inicial. Tanto os amidos como o SMP reduziram a sinérese do gel coagulado. Em relação aos atributos sensoriais, de acordo com um painel de consumidores, houve uma preferência maior para uma textura do produto mais sólida e com sabor a queijo tradicional de vaca.

Ambos os produtos foram produzidos utilizando a fórmula da MCB. Esta estratégia alternativa para a utilização de queijo pode contribuir para a minimização de subprodutos, ao mesmo tempo que pode trazer nutrientes adicionais e perfis de sabor específicos aos mesmos produtos. Já que foram utilizados neste projeto ingredientes compatíveis com designações de “rótulo limpo ou natural”, e não foram usados adoçantes de qualquer espécie, os produtos produzidos vão ao encontro das tendências atuais dos consumidores e estão alinhados com os objetivos de desenvolvimento sustentável.

Concluindo, este projeto de dissertação apresenta uma linha de investigação que conduziu diretamente à formulação de uma linha de produtos lácteos inovadores, económica e ambientalmente interessantes, estando ainda alinhado com as tendências e exigências recentes dos consumidores para alimentos mais sustentáveis e promotores da saúde e bem-estar. Tudo isto foi feito contribuindo para uma economia circular, visto que a formulação dos produtos utilizou subprodutos e excedentes de queijo, o que, em última instância, visa a redução do desperdício alimentar.

Palavras-chave: Valorização de excedentes de queijo; Interação das proteínas do queijo; Iogurte fortificado com queijo; Queijos frescos inovadores; Análise e caracterização físico-química

Abstract

The dairy sector is of great economic importance worldwide, and it has always been extremely competitive in terms of product portfolio innovation. In order to respond to new consumer trends, such as products with particular nutritional profiles and products with added health benefits, this industry has had to adapt its portfolio accordingly. Also, the numbers concerning food waste are very high, with about 1/3 of the food destined to human consumption being lost worldwide; in Portugal, 17% of the annual food production is estimated to become food waste. In the cheese production sector, generated waste includes cheese surpluses or their off-standards items. These cheese by-products were valorised in this dissertation project by serving as basic ingredient for the development of a group of novel dairy products, with innovative taste features and nutritionally enhanced characteristics.

That was achieved by the formulation of a melted cheese base (MCB) - a paste of melted cheese in gelatinized starch (or other hydrocolloids including xanthan gum and guar gum), having milk as the fluid vehicle. These MCBs were the distinctive ingredient for ulterior incorporation in the final products, where Emmental cheese was the cheese type selected for most of the research work, due to its properties. Besides cheese, the MCBs also included agents able to stabilize its dispersed state after cooling. In order to avoid the emulsifying salts common in processed cheeses, hydrocolloids were used instead. The main ones were native corn starch (CS) and waxy rice starch (WRS), that also worked as thickeners and stabilizers of the MCBs. It was shown that the starch suspension in milk should be heated past the gelatinization temperature (85 ºC for CS and 90 ºC for WRS) before cheese addition, so that the final MCB formulation could reveal ideal textural characteristics.

A study on the interactions involved in holding the protein structure in cheese (Emmental) was also made using solutions of different dissociating agents at different concentrations and combinations. To quantify the solubilized protein, a modification of a spectrophotometric-based method that can be employed to quantify total protein in cheese was developed, with statistically similar results ($p>0.05$) to those obtained by the Kjeldahl method. The results showed that caseins in the Emmental cheese are held together by a set of hydrophobic interactions, hydrogen bonds, and electrostatic bonds, including ionic bonds, with hydrogen bonds having an important role, comparable to hydrophobic interactions, a conclusion not commonly reported for cheese structures.

The last part of this project entailed the development of two novel dairy products, namely, yogucheeses corresponding to yogurts fortified with cheese, and novel ricotta-type fresh cheeses.

For the yogucheeses, cheese (Emmental) and CS or WRS were used in the MCB formulations. These MCBs were mixed with further milk in different proportions, before addition of starter culture and fermentation, in order to create samples with different proportions of MCB to further analyse.

The products were characterised in terms of macronutrient composition, physical and chemical properties.

In the end, the incorporation of CS and WRS, combined with the incorporation of ripened cheese, created yogurt samples with a firmer gel structure, lower syneresis, and higher viscosity. All the yogurts

were stable throughout a period of 14 days, with no alterations in their visual aspect and syneresis behaviour.

For the ricotta-type fresh cheeses, MCBs were made using two cheese types (Emmental and Ewe's cheeses) and using CS or WRS. Afterwards, the MCB wase diluted with milk and then renneted, so that the novel fresh cheeses could be obtained.

These samples were characterised in terms of macronutrient composition, physical and chemical properties, as well as sensory properties.

The fresh cheese developed had a balanced nutritional content, and a texture similar to many commercial fresh cheese types, or spreadable processed cheeses. Gel formation of the initial mixture was hindered above a certain incorporation of ripened cheese, but this was overcome by the addition of skim milk powder (SMP) to the preparation. Starch and SMP both reduced syneresis of the renneted gel. As for the sensory attributes of the products, according to a consumer panel, the preference was higher for a more solid texture and with the flavour of a traditional cow's cheese.

Both these products were manufactured using our MCB formulation. This alternative strategy for utilization of cheese can contribute to food surplus minimization, while bringing additional nutrients and specific flavours to these products. Since ingredients that are not compatible with clean label designation were not used, as well as sweeteners of any kind, these products are consistent with the consumer trends existing nowadays and are in alignment with the sustainable development goals.

In conclusion, this thesis project presents research that led to a novel line of dairy products, economically and environmentally interesting, as well as rewarding, being also in line with the recent consumers' trends and demands for more sustainable and health-promoting foods, all while contributing to the circular economy, since the product formulation uses cheese surpluses, which ultimately reduces food waste.

Keywords: Cheese surpluses valorisation; Cheese protein interactions; Cheese-fortified yoghurt; Novel fresh cheese; Physicochemical analysis

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Chapter 1. General Introduction and Project Framework

1.1. Cheese and Yogurt Market Trends

The dairy sector is of great economic importance worldwide, and it has always been extremely competitive in terms of product portfolio innovation. In order to respond to new consumer trends, such as better-for-you products and products with added health benefits, the dairy industry has to adapt its product portfolio accordingly. Added-value products, such as high-protein or full-fat formulations (Canning, 2020) were introduced, increasing its competitiveness in recent years. Therefore, dairy companies are pressed to maintain or increase their markets and revenues by improving quality, reducing costs, and permanently developing new products.

It is predicted that the cheese sector will increase in value 2% annually, until 2024, according to the International Dairy Federation (N.A., 2016); and that the global cheese consumption will increase continuously, projected to have an overall increase of 13.5% between 2016 and 2025 (Lamichhane, Kelly, & Sheehan, 2018).

According to data from Nielsen Consulting, the total cheese sales in Portugal increased 2% in volume and 1% in value in the period January to September 2015, in comparison with the homologous data from the previous year (Clara, 2017b). According to January 2016 data from the Portuguese National Institute of Statistics (INE), cheese imports during the 12-month previous period had increased 12% in comparison with analogous previous period, with the remarkable value of 4182 tonnes (Clara, 2017b). On the other hand, in 2016, cheese exportation increased 13% (in value and quantity) (N.A., 2017).

Regarding yogurt, the global yogurt market is projected to register a growth rate of 4.5% during the forecast period of 2020 to 2025 (Mordor Intelligence, 2020). Currently, set yogurt represents the most popular product type, holding the largest market share (IMARC, 2019). The approximate estimate of annual per capita yogurt consumption clearly indicates that European population has a higher consumption than other regions of the world, making this a target market for yogurt manufacture and distribution (Allied Market Research, 2017).

According to an analysis from (Statista, 2021) the yogurt market in Portugal is expected to grow annually by 0.90% and the revenue in the yogurt segment amounts to €369 millions in 2021. With consumer trends of valorising products that can improve health and that use natural ingredients, novel

yogurt products can constitute a good market opportunity. In Portugal, in 2019, Nielsen Consulting concluded that the yogurt segment with greater dynamism was the added value one, with emphasis on the biological, lactose free, probiotic, skyr and high-protein yogurts (Sousa, 2019). In fact, a 4.2% in growth of yogurt sales was registered in the high-protein products (Sousa, 2019).

According to INE, in 2015, yogurts and cheese each represented 33.3% of the total value of the national dairy products importations (Instituto Nacional de Estatística, 2016).

With the end of the European dairy quotas, Portuguese industry faces strong challenges in order to survive in an unbalanced market. Experts say that one of the possible measures to undertake is the exportation of products with higher added value, such as cheese and yogurts, taking advantage of the quality and differentiation of the national production (Clara, 2017a). Complementing this there is a high demand from consumers for enhanced cheese products in terms of their physico-chemical properties, sensory and nutritional quality. This is primarily driven by a growing consumer awareness of the role of diet in health and well-being, the potential to use structure to influence flavour release and sensory experience, and the extensive use of cheese as an ingredient in food retail applications (Lamichhane et al., 2018). These recent awareness of the consumers calls for differentiation and improvement of already existing products, or for novel innovative products.

1.2. Food Waste: Statistics and Solutions

The numbers concerning food waste have been increasing and, to counteract this trend, 2016 was considered the National Year of Fight Against Food Waste (Oliveira & Oliveira, 2016). According to the 2011 FAO report “Global Food Losses and Food Waste - Extent, Causes and Preventions”, 54% of food waste worldwide occurs in the primary production, manipulation after harvesting, and during storage of the products (Oliveira & Oliveira, 2016). Nowadays, about 1/3 of the food destined to human consumption is lost worldwide, which corresponds to about 1.3 billion tonnes/year (Pintado & Teixeira, 2015).

In Portugal, it is estimated that food waste represents 17% of the annual food production (about 1 million tonnes), divided along all different steps of the supply chain. As observed in Figure 1.1., the dairy sector is highly affected by food waste (Oliveira & Oliveira, 2016).

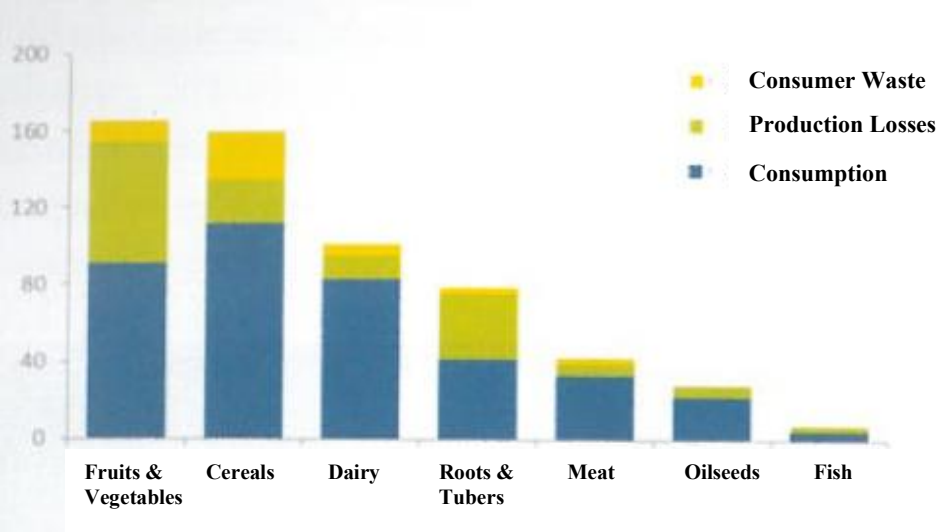

Figure 1.1. – Losses and waste of food in Portugal (Oliveira & Oliveira, 2016).

The food industry has been taking measures to reduce waste, including technical innovations, improvements in logistics, and avoiding waste by recycling and finding new uses for the potential waste (Oliveira & Oliveira, 2016).

In fact, the European Commission establishes that until 2030 all food waste must be managed as a resource, therefore it should be reused and recycled in a way also economically attractive for the industry (Pintado & Teixeira, 2015).

1.3. Project Framework

The project of this dissertation aims to translate into a market opportunity, with the development of a group of novel dairy products, which has as a basic ingredient the surpluses of cheese production, or their off-standards items, or yet the pieces remaining after slicing large items. The new products to develop should have innovative taste features and have nutritionally enhanced characteristics. The ready-to-eat food sector is an expanding market, consequence of the current lifestyle (Hernández-Carrión et al., 2011), and the products developed in this work fall in this category.

Recent studies approached the structure, stability, rheological behaviour (textural and viscoelastic properties), and sensorial behaviour of processed cheeses, where different dry matter contents were tested and different dairy ingredients were added to the cheese (Černíková et al., 2017; Kelimu et al., 2017).

Other research studies analysed the formulations, and the resultant rheological properties, microstructure and sensory aspects, as well as heat stability studies, of white sauces prepared from

starches, vegetable oil and powder milk (Arocas, Sanz & Fiszman, 2009a, 2009b; Arocas et al., 2010; Hernández-Carrión et al., 2015). Albeit not incorporating ripened cheese, these sauces have other resemblances to products here presented.

Other studies contemplated the production of several proteins and peptides, or lactose, among others, from milk or whey (Pintado & Teixeira, 2015). In 2012, researchers at Universidade do Minho developed a solution to add value to surpluses of the dairy industry, more specifically, cheese whey surpluses. They managed to transform it in to several products, such as protein concentrates, prebiotics, bioethanol, refined lactose, ultrapure water, and salts (Oliveira & Oliveira, 2016). This case demonstrates the need of cooperation between research and industry, sharing theoretical and technical knowledge, in order to answer industry needs and concerns (Oliveira & Oliveira, 2016).

In this project, the planned basic ingredient for ulterior incorporation in the final products is a melted cheese base (MCB), that is, a melted cheese paste or slurry. Currently, there is limited research on the viability of incorporating cheese surpluses in food product development (apart from processed cheeses).

The MCB as an ingredient is a differentiating factor of this project, being an innovation when compared to previous research. Details on MCB formulation process and microscopic analysis are described in Chapter 3.

MCBs are intended to be prepared with soft curd cheeses (e.g. Serra da Estrela), semi-hard cheeses (e.g. Emmental, Gouda), and hard curd ones (e.g. Cheddar, Parmigiano-Reggiano). Even though, in an exploratory phase of the project, MCBs were prepared with different types of cheese, the final choice of the principal cheese to use in this project was the Emmental, due to its properties, as described in Chapter 2, section 2.1.

Besides cheese, the MCBs included agents able to stabilize its dispersed state after cooling. In order to avoid the emulsifying salts common in processed cheeses, hydrocolloids were used. Hydrocolloids are colloidal substances with an affinity for water and that have a wide range of properties (Wüstenberg, 2015). For this project, native corn starch and waxy rice starch were used as thickeners and stabilizers of the MCBs. The properties of these starches are described in Chapter 2, section 2.3.

From the MCBs, a set of alternative dairy products can be produced, such as: cheese-fortified yogurts (*yogucheeses)*, ricotta-type cheese products, cheese sauces, cheese spreads, etc. In this work we developed cheese-fortified yogurts and ricotta type cheeses using MCBs with Emmental cheese and the two different types of starch referred above (native corn starch and waxy rice starch). These products' formulation and physico-chemical, rheological and textural characterization, as well as the sensory analysis process are described in Chapters 5 and 6.

1.4. Project Objectives

Based on the outline described in the present chapter, this doctoral project has the following general objectives, summarized in Figure 1.2.:

I. Contribution to the knowledge of the protein interaction properties in selected cheeses.

II. Development of research activities regarding the preparation of MCBs and their structural and chemical characterization.

III. Development and characterization of new dairy products derived from the MCBs, with added-value and that can be competitive in the market. These require positive consumer evaluation, and technical and economic feasibility.

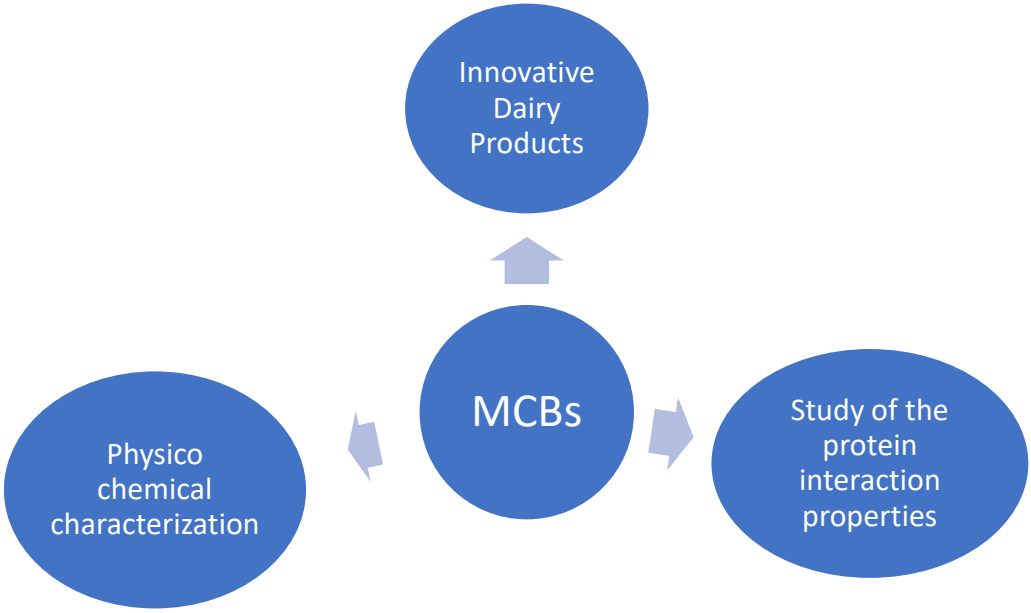

Figure 1.2. – Diagram of the thesis objectives.

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Chapter 2. State of the Art

2.1. Cheese Overview

Cheese is the fresh or ripened solid or semisolid product obtained after coagulation of milk, cream, skimmed or partly skimmed milk, buttermilk, whey cream, or mixture of these products, through the action of rennet, specific bacteria, organic acids, or other suitable coagulating agents, and by the partial drainage of the whey resulting from such coagulation (César & Paula, 2009; Walstra, Wouters, & Geurts, 2006).

Cheeses can be made exclusively with cow, sheep or goat milk (among less common others), or from a mixture of two or more of those three origins (Vieira de Sá & Barbosa, 1990). The essential ingredients of common cheese are milk, coagulating enzyme, bacterial cultures, and salt (Goff, 2016). The composition of final cheese is comprised of casein as dominant protein, water, fat and salt. Lactose is only present at the beginning of ripening, being almost completely removed and metabolized during cheese manufacturing (Vieira de Sá & Barbosa, 1990).

The general cheese manufacturing steps are milk standardization and heat treatment or pasteurization, milk cooling, starter and non-starter bacteria inoculation, ripening, rennet addition and curd formation, curd cutting and heating, whey drainage, curd texturizing, salt or brine addition, cheese molding, storing, aging, and final packaging (Fox et al., 2004; Goff, 2016; Milk Facts, 2016).

Being a nutrient-dense dairy product, rich in proteins, vitamins and minerals, particularly calcium and phosphorous, cheese can be consumed as the main component of a meal, as a dessert, a snack, a condiment, or as a food ingredient (El-Bakry & Sheehan, 2014). This makes cheese a highly versatile product, which can also be used in multiple ways in food research. Cheeses can be unripened (*fresh cheeses*) or ripened. Within the ripened ones, three families can be identified: soft-ripened, semi-hard, and hard curd cheeses.

In soft-ripened cheeses, coagulation is achieved primarily by rennet, the amount of lactic acid bacteria (LAB) in the inoculum is large, and the fermentation period before rennet addition is rather extended. Furthermore, the step of curd cutting is delayed to further encourage acidification and demineralization. That procedure is carried out with very large knives or with paddles, in order to minimize moisture and minimize solid losses (Goff, 2016).

The cut curd is then placed in molds and let stand in a warm room for several hours. This enables acidification to continue until the accumulation of lactic acid inhibits significant culture

growth. This growth is also influenced by the timing and level of salting. The final pH is about 4.4-4.6, and moisture level is typically between 45 and 60% (Goff, 2016).

In semi-hard cheeses, the cut curd is typically washed, which leaches lactose from it. The goal is to attain a pH of 5.0-5.2, at least, and a moisture content between 40 and 50% (Goff, 2016).

Being part of this category, Emmental cheese was chosen as a model in this work.

Hard cheeses are characterized by lower moisture contents (35-39%) than the above ones, which allows for the removal of sufficient lactose by syneresis, avoiding the need for curd washing. The acid development is controlled mainly by the extent of syneresis, but keeping the objective of attaining a minimum pH of 5.0-5.2 within 1-3 days after manufacturing (Goff, 2016).

All cheeses have a few common points: the casein and the milk fat are concentrated in the cheese; their shelf-lives are much longer than that of raw, pasteurized, or even fermented milks; and cheese has a distinct and characteristic flavour due to a great number of flavour compounds formed during ripening (Walstra et al., 2006). These flavour compounds contribute to the sensory characteristics of the products made with cheese.

All types of cheese, including the processed ones, are highly nutritious, as they contain proteins, vitamins and minerals (Tunick, 2014). Recent studies have shown that, in humans, spermidine ingestion, a substance present in ripened cheese, is inversely associated with the risk of both fatal heart failure and clinically overt heart failure (Eisenberg et al., 2016). These are important factors when considering the manufacture of a new nutritious, added-value products.

Cheese can be described as a bi-continuous gel structure consisting of a porous protein matrix (casein) interspaced with localized domains of fat (Vogt et al., 2015). The end-product characteristics, such as flavour, functional properties (texture and melting), and overall quality of cheese are significantly affected by its microstructure (El-Bakry & Sheehan, 2014).

On heating, several structural changes occur within cheese, such as: (a) the electrostatic and hydrophobic interactions between proteins increase in strength; (b) other bonds and interactions decrease in number and strength; and (c) there is a transition of the fat to a liquid state (Vogt et al., 2015).

Processed (or process) cheese started as a subproduct of the production of cured cheeses, however it rapidly became a product of great interest. The huge acceptance by consumers worldwide of this type of cheese has led to particular attention from manufacturers, not only to improve its intrinsic quality, but also to use safer and more economical packages, and conceiving industrial equipment to assure a more rigorous and controlled process (Vieira de Sá & Barbosa, 1990).

Traditional processed cheese is made from a mixture of cheeses, fat, water, and emulsifying salts. After stirring and heating of the ingredients until melting temperature (90-100°C), the hot mixture is poured into cups and left cool down to below 8 °C (Hladká et al., 2014).

Emulsifying salts are added due to their ability of sequestering calcium ions from the cheese protein matrix, which contributes to its disintegration. They are also responsible for improving the texture of the final product. However, traditional emulsifying salts contain a high concentration of sodium and phosphorous and a high amount of these substances in human nutrition is considered a risk factor for many diseases, whilst also affecting the flavour of some types of cheeses (Černíková et al., 2010). Therefore, the successful substitution of traditional emulsifying salts by hydrocolloids allows for: (i) the reduction of the sodium and phosphorous concentration; (ii) the utilization of biodegradable additives from renewable sources instead of phosphorus, an excess of which is generally considered a risk factor for the environment; (iii) establishment of new products with health benefits (Černíková et al., 2010).

Hence, in order to improve the sensorial, nutritional and economical value of cheese, emulsifying salts have been tentatively replaced by hydrocolloids with emulsifying properties (Hladká et al., 2014). This work followed that line and used starches instead of emulsifying salts.

2.2. Yogurt Overview

Two major types of yogurts are available on the market. One has a firm gel-like structure (set type) and the other is a liquid with thick consistency (stirred type). In set type yogurt, milk is inoculated with the yogurt starter, transferred to the final package and fermentation takes place; in stirred type, fermentation takes place in large vessels, after which the yogurt gels are disrupted by stirring (Oraç & Akın, 2019), and only then the final packaging takes place.

Manufacture of yogurt is a multi-step process and includes milk treatment, standardization, homogenization, and heat treatment, in order to prepare the milk and make it safe and ideal for yogurt production; fermentation, where the starter culture acts through biochemical reactions and inductively causes the formation of the yogurt curd and the development of its characteristic flavour components; and cooling to around 5 °C, in order to inhibit the growth and metabolic activity of the starter culture, which would lead to a rise in acidity (Sfakianakis & Tzia, 2014).

Yogurt is commonly made by fermenting cow's milk using a 1:1 ratio of the lactic acid bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* under controlled temperature and environmental conditions. The starter culture for the production of yogurt has a huge influence

on the flavour, texture, appearance and the overall attributes of the finished product, as referred in the previous paragraph (Das, Choudhary, & Thompson-Witrick, 2019).

During fermentation, the concentration of lactic acid in milk increases and the pH decreases, leading to a dissociation of the carboxyl groups, and an ionization of serine phosphate, culminating in the increase of the negative charge between casein micelles. However, the presence of calcium phosphate neutralizes this negative charge, keeping electrostatic repulsion down to a level where attractive forces between the protein molecules are dominant. Due to these attractive forces, the casein micelles aggregate and eventually coagulate into a network of small chains, being ultimately responsible for the increase of viscosity and formation of the yogurt coagulum (Sfakianakis & Tzia, 2014).

Yogurt is an excellent source of protein, calcium, phosphorous, riboflavin, thiamine, vitamin B12, folate, niacin, magnesium, and zinc (Das et al., 2019), it is associated with a high nutritional image, therapeutic and health benefits, such as digestion enhancement, immune system boosting, anticarcinogenic activity, and reduction in serum cholesterol (Nastaj et al., 2019).

Instrumental methods have been applied to the analysis of yogurt, and they can be categorized into two groups, namely chemical analysis methods (including flavor and milk constituents analysis), and structural analysis methods (including textural and rheological analysis, as well as microstructural analysis) (Mortazavian, Rezaei, & Sohrabvandi, 2009).

The variation in protein and fat content among commercial yogurts and concentrated fermented milks leads to a great variation in physical and sensory properties among yogurts on the market (Jørgensen et al., 2018), since the sensory properties of dairy products are largely dependent upon the balance of flavours derived from the proteins, carbohydrates, and fats found in the milk (Das et al., 2019).

Being yogurt one of the most consumed dairy products in the world (Fang & Guo, 2019), it is highly important to differentiate any novel yogurt-based product from the ones already existing in the market. For instance, defining the textural properties and their relative magnitude, and defining the final protein and fat contents, with respect to other similar products, will increasingly become a critical criterion for food manufacturers seeking to design new products, maintain the quality of current ones, or understand their strengths and weaknesses relative to their competitors (Kealy, 2006).

2.3. Hydrocolloid Overview

Hydrocolloids are a diverse group of long chain polysaccharide-based or protein-based biopolymers, distinguished by their ability to form viscous dispersions and/or gels when placed in water, via the presence of hydroxyl groups in their structure, which makes them hydrophilic compounds (Figure 2.1. A and B) (Milani & Maleki, 2012; Rubel et al., 2019).

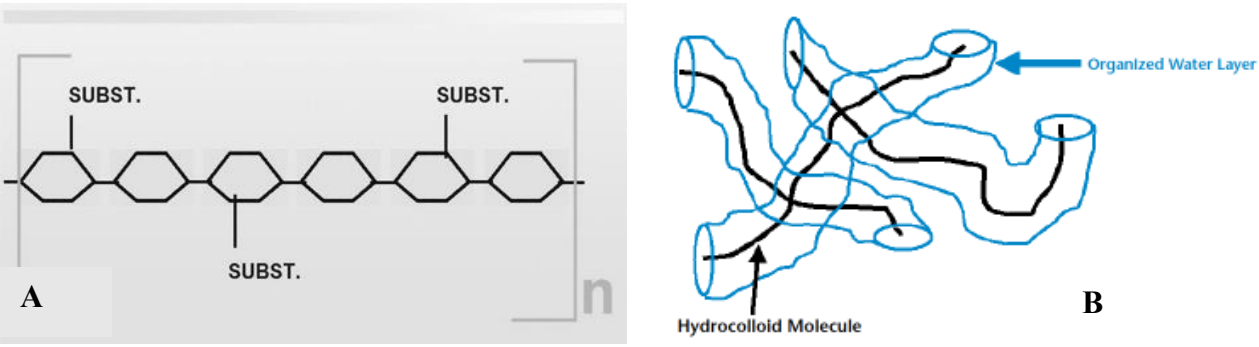

Figure 2.1. Schematic representation of a typical food hydrocolloid (A); and hydrocolloid molecules surrounded by "organized" water (from Hoefler, 2004).

Hydrocolloids can have linear or branched structures, and the molecules can have side units, such as sugar units, carboxyl, sulphate, or methyl ether groups, among others, and these have in general a great influence in the properties of the hydrocolloid (Hoefler, 2004).

They are used in technical applications to thicken and stabilize formulations in different products, such as food, pharmaceuticals, cosmetics, or textiles (Wüstenberg, 2015). In the food industry, hydrocolloids have a wide range of functions: they are added for controlling stability, texture, colour and appearance (Laaman, 2011). Due to the legislation applied in each region/country, some hydrocolloids may not be locally allowed in food (and/or in other products).

Among food polysaccharides, native and modified starches are the main ones, and the remaining are collectively known as non-starch polysaccharides (NSP). The term *hydrocolloid* often includes both categories (Stephen, Philips, & Williams, 2006).

Hydrocolloids can be classified into four different groups: (i) hydrocolloids isolated from plants; (ii) those obtained from fermentation; (iii) plant-derived but chemically modified; and (iv) hydrocolloids from animal origin (Milani & Maleki, 2012).

In Table 2.1., the main functions and applications of some hydrocolloids used in the food sector are described.

Table 2.1. – Functions and applications of hydrocolloids used in the food industry (Stephen et al., 2006).

Hydrocolloid	*Function*	*Application*
Guar and locust bean gums	Stabilizer, water retention, thickener	Dairy products, non-dairy ice cream, desserts, bakery, seasonings, beverages, cured meats
Carrageenans	Stabilizer, emulsifier, thickener, gelation agent	Ice cream, flans, meat products, dressings, instant puddings, cheeses
Agars	Gelation, stabilizer	Dairy, confectionery, meat products, beverages
Pectins	Gelation, thickener, stabilizer	Jams, preserves, beverages, confectionery, dairy
Xanthan gum	Stabilizer, thickener	Dressings, beverages, dairy, bakery, sauces
Carboxylmethyl cellulose	Stabilizer, thickener, water retention	Ice cream, batters, syrups, cake mixes, meats
Modified starches (Processes used)		
Oxidation	Stabilization; adhesion gelling, clarification	Formulated foods, batters, gum, confectionery
Dextrinization	Binding, coating, encapsulation; high solubility	Confectionery, baking (gloss), flavourings, spices, oils, fish pastes
Cross-linking	Thickening, stabilization, suspension, texturizing	Pie fillings, breads, frozen bakery products, puddings, infant foods, soups, gravies, salad dressings
Esterification	Stabilization, thickening, clarification; combined with cross-linking; alkali sensitive	Candies, emulsions, products gelatinized at lower temperatures
Etherification	Stabilization; low-temperature storage	Soups, puddings, frozen foods
Dual modification	Combinations of properties	Bakery, soups and sauces, salad dressings, frozen foods

In this work native corn starch and a modified starch, waxy rice starch, were the main hydrocolloids employed.

Native starches belong to the group of hydrocolloids isolated from plants and is one of the most widely used ingredients in the food industry (He et al., 2019). Native or modified starches can be used in the dairy industry as texture and structure providers, helping the emulsion stabilization, and the thickening of the aqueous phase (He et al., 2019; Polowsky, 2016). In particular, in fermented dairy, they are often used to prevent whey syneresis and to increase viscosity.

Chemically, starch is composed of two types of glucose macromolecules, namely amylose and amylopectin. Amylose is mostly a linear polysaccharide where glucose is linked by α-1,4 bonds, and amylopectin a much larger and branched structure, linked by both α-1,4 and α-1,6 bonds (Yang et al., 2020).

Native untreated starches are insoluble in cold water and form opaque suspensions with a strong tendency to sediment, due to the higher density of the granules (Obadi & Xu, 2021; Wüstenberg, 2015). However, when heated starch forms colloidal solutions, which form a gel upon cooling, called starch paste, in a process called gelatinization (Obadi & Xu, 2021; Wüstenberg, 2015). After cooling, intermolecular interactions involving amylose and amylopectin molecules occur, increasing the medium viscosity and forming a gel. Differences in behaviour are originated by the compositions of the different starches (Diamantino et al., 2019).

The tendency of a gelatinized starch to retrograde can be studied from its pasting behaviour, usually by observing changes in pasting viscosities during programmed heating and cooling of a starch suspension, using a viscograph or a Rapid Visco Analyzer (RVA) (Wang, Li, Copeland, Niu, & Wang, 2015). From these experiments, five characteristic parameters can be obtained: peak viscosity, trough viscosity, breakdown viscosity, final viscosity, and setback viscosity. This last one is defined as the reflection of the retrogradation tendency of amylose in a starch paste (Wang et al., 2015). A typical RVA pasting curve is presented in Figure 2.2.

The viscosity parameters during pasting are cooperatively controlled by the properties of the swollen granules and the soluble materials leached out from the granules (Sandhu & Singh, 2007).

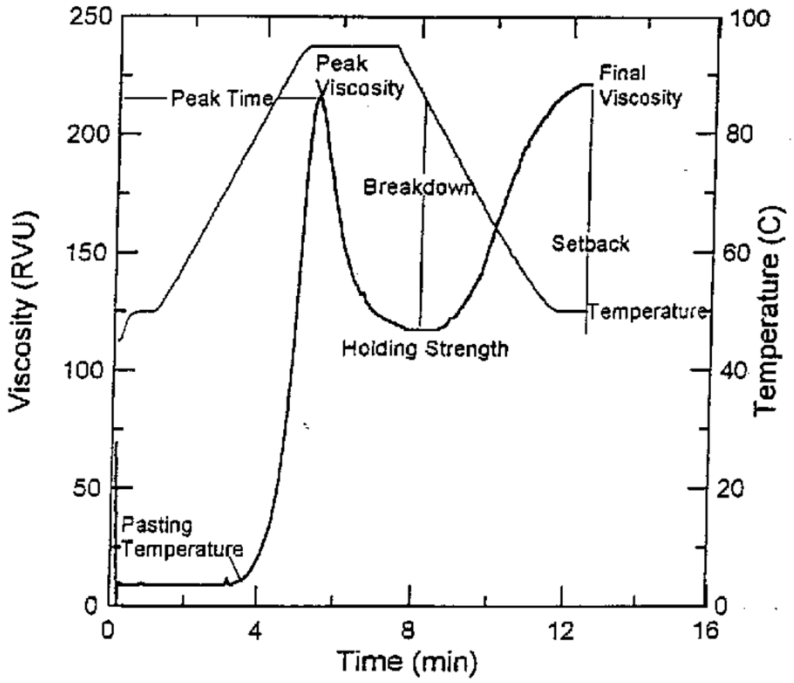

Figure 2.2. – Typical RVA pasting curve identifying characteristic parameters (from Zhou, Robards, Glennie-Holmes, & Helliwell, 1998).

Amylose content can vary within the same plant source, affecting the size and uniformity of the granules, and pasting characteristics (Obadi & Xu, 2021). Regular maize starches normally contain between 25% and 28% amylose and produce consistent gels after cooking. Waxy starches generally contain little or no amylose (less than 1%), and produce weak, clear, and sticky gels with a high peak viscosity and low retrogradation tendency. High-amylose starches normally contain more than 50% amylose (reaching up to 70%), present high gelatinisation temperatures, and capacity of forming films (Diamantino et al., 2019). The amylose content of the starches used in this work, waxy rice starch and corn starch, are less than 2% and up to 50%, respectively (Obadi & Xu, 2021).

Starch has been added to an array of dairy products such as cheese sauces, fresh cheeses, and yogurts, in order to stabilize and thicken the gel structure, while avoiding excessive syneresis and promoting particular textural and rheological features (Arocas, Sanz, & Fiszman, 2009; Lobato-Calleros et al., 2014; Palyvou-Gianna, Paula Vilela, Gomes, & Ferreira, 2021; Paula Vilela, Gomes, & Ferreira, 2021).

As said above, starches will be the main hydrocolloids used in this work. CS and WRS were the selected ones since they were easily available and showed the ability to form viscous gels without phase separation whilst being soluble in milk.

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Chapter 3. MCB Formulation and Microscopy

3.1. Chapter Introduction

As briefly referred to in chapter 1, in this work, the central ingredient for ulterior incorporation in the final products is a melted cheese base (MCB), that is, a paste or slurry of melted cheese in gelatinized starch (or other hydrocolloid), having milk as the fluid vehicle.

The basic observation is that ripened cheese can be dispersed into sub-millimetre particles by mixing it in a hot paste of gelatinized starch. These MCBs have been under study in our laboratory, aiming the application in different dairy products. The ripened cheese will provide added nutritional value and flavour to the final products. Simultaneously, this utilization of cheese can contribute to preventing potential food waste and boosting circular economy in the food sector.

In this chapter, the optimization of the formulation and the preparation process of the MCB at a laboratorial scale are presented. Furthermore, the endeavours of optical microscopy of the MCB will also be analysed.

MCBs formulas were optimized in terms of ingredients considering the following parameters: nature of hydrocolloid, its concentration, type of cheese, and its amount in the MCB.

The cheese selected for the majority of the tests was Emmental (EM), a semi-hard, Swiss type cheese. In this kind of cheese, the matrix acidification occurs after pressing of the curd, i.e., when most of the whey has already been expelled. Hence, the concentration of colloidal calcium increases proportionally with the concentration of casein at drainage, leading to the formation of a highly cohesive and mineralised matrix (Gagnaire, Trotel, Graët, & Léonil, 2002). Two other cheeses were also tested: a soft-curd type, Queijo da Bica (QB); and a hard-curd type, Parmigiano Reggiano (PR). All of the cheese types were successfully incorporated onto the MCB, if grated or cut into small enough pieces.

In the initial stages of the project, five hydrocolloids were tested and optimized in terms of final concentration in the MCB: xanthan gum (XG), guar gum (GG), carboxymethyl cellulose (CMC), corn starch (CS) and waxy rice starch (WRS).

XG and GG are fast-hydrating, water-soluble hydrocolloids that can be dissolved at room temperature or at higher temperatures, and they have the ability to develop extremely high viscosities even at low concentrations (Wüstenberg, 2015). As for their milk solubility, some studies have used XG dispersed in cold and heated milk with concentrations up to 0.5% (w/w) (Hematyar et al., 2012; Nguyen et al., 2017; Pang, Deeth, & Bansal, 2015), while other have used GG dispersed in heated milk with concentrations up to 0.5% (w/w) (Everett & McLeod, 2005; Heyman et al., 2010).

CMC is a derivative of cellulose and is an anionic hydrocolloid, Therefore, it can interact with the positive charges on the surface of casein micelles to strengthen the casein network and reduce syneresis in dairy products (Hematyar et al., 2012). (Leite et al., 2012) found also that sodium-CMC at concentrations up to 0.45% (w/w), used in conjunction with cassava starch, were soluble in water.

Native and modified starches have been added to an array of dairy products, such as cheese sauces, fresh cheeses, and yogurts, in order to stabilize and thicken the gel structure, while avoiding excessive syneresis and promoting innovative textural and rheological features (Arocas, Sanz, & Fiszman, 2009; Lobato-Calleros et al., 2014; Palyvou-Gianna, Paula Vilela, Gomes, & Ferreira, 2021; Paula Vilela, Gomes, & Ferreira, 2021).

The amylose content of native CS and of WRS are up to 50% and less than 2%, respectively (Obadi & Xu, 2021). After the selection of ingredients, the physical parameters in the preparation process of the MCBs were also optimized, with the use of those two types of starch (CS and WRS). Controlled temperature-time heating tests were carried out, with different agitation paddles - a helix paddle and a U-shaped paddle. In these tests, the agitation profile was varied and optimized, as well as the time allowed for starch gelatinization.

For the visualization of the MCB microstructure, both light and fluorescence microscopy techniques were used, which ultimately allowed us to distinguish between all the macrocomponents present, i.e., proteins, starch, and lipids.

3.2. Materials and Methods

3.2.1. Materials

The materials used were semi-skimmed (1.5 % fat) HTST milk (Vigor, Portugal), commercial grated Emmental cheese (Milbona, Germany), Queijo da Bica (Lactimercados, Portugal), Parmigiano Reggiano (Lovilio, Germany), corn starch (Maizena, Unilever, Portugal), waxy rice starch (Remyline XS, BENEO GmbH Germany, kindly provided by Nutripar, Portugal), guar gum, sodium carboxymethyl cellulose, xanthan gum (all three from Sigma-Aldrich, USA), Rhodamine B (Sigma-Aldrich, USA), Sudan III (Sigma-Aldrich, USA).

3.2.2. MCB Formulation

3.2.2.1. Ingredients and Preparation Procedure

All hydrocolloids, except guar gum, were dispersed in cold or room temperature milk at the ratios described in Table 3.1. Guar gum was dispersed in milk heated to 50 °C, with constant agitation, following the protocols of other studies that also dispersed this gum in milk (Brennan & Tudorica, 2008; Everett & McLeod, 2005). At this stage, only Emmental cheese was used. The mixtures were prepared in a milk base, instead of a water base, since preliminary studies showed that MCBs prepared with water only dispersed cheese partially, leading to phase separation and visible, macroscopic pieces of cheese in the MCB samples.

Table 3.1. – Formulation of MCBs samples using different hydrocolloids, as well as the final heating temperature of the mixture.

	Hydrocolloid type	Hydrocolloid (g)	Milk (g)	Emmental Cheese (g)	Final Heating Temperature (°C)
XG	Xanthan gum	0.05	100	5	85
GG	Guar gum	1.20	100	5	92
CMC	Carboxymethyl cellulose	5	100	5	80
CS	Corn starch	5	100	5	85
WRS	Waxy rice starch	5	100	5	92

Afterwards, the mixtures of milk and hydrocolloid were heated for 5 minutes, using a hotplate, with continuous stirring, until the final temperature targeted was reached (Table 3.1.), after which point the grated cheese was added, at a ratio of 5 g cheese per 100 g milk. The mixtures were immediately removed from the hotplate and manually stirred until the cheese was fully dispersed, with no visible, macroscopic pieces. The MCB samples were left to cool down to room temperature (ca. 21 °C).

Judging by the ease of preparation, viscosity of the MCBs, cheese dispersibility, consumer and industry acceptance, from these early trials it was concluded that the best hydrocolloids to use in further work were the native starches, CS and WRS (more on section 3.3), at a ratio of 5 g cheese per 100 g milk, since this was the ratio that provided MCB with ideal consistency, cheese dispersibility in the matrix, and no phase separation after stabilization. MCB samples prepared with water instead of milk provided a low capability to disperse cheese, that is, they only dispersed cheese partially. That could be explained by the fact that water lacks components for a full interaction with the cheese protein matrix. In that medium, the addition of dissociating agents is needed, in order for the cheese to fully disperse (Paula Vilela, Gomes, & Ferreira, 2020).

The next step taken was to vary the cheese type, using either CS or WRS for the MCB preparation, according to Table 3.2. Cheese types used were Emmental cheese (EM), a semi-hard cheese; Queijo da Bica cheese (QB), a soft-curd cheese; and Parmigiano Reggiano cheese (PR), a hard-curd cheese.

Table 3.2. – Formulations for the MCB samples with different cheese types and two different starches.

	Milk (g)	Hydrocolloid (g)	Hydrocolloid type	Cheese (g)	Cheese type
EM_CS	100	5	Corn starch	5	Semi-hard
EM_WRS	100	5	Waxy rice starch	5	Semi-hard
QB_CS	100	5	Corn starch	5	Soft-curd
QB_WRS	100	5	Waxy rice starch	5	Soft-curd
PR_CS	100	5	Corn starch	5	Hard-curd
PR_WRS	100	5	Waxy rice starch	5	Hard-curd

3.2.2.2. Physical Parameters Adjustment: Time-temperature Curves

After the analysis of the MCB formulations in 3.2.2.1., the physical parameters of the process were also optimized, as described in this section. For this stage, the most convenient MCB formula was the one with 100 g milk, 5 g CS or WRS, and 5 g Emmental cheese (EM_CS and EM_WRS).

For the preparation, starch was dispersed in cold or room temperature fluid milk. Then, under constant agitation, the temperature was gradually increased during the heating stage (approximately 7 minutes) until 85 °C (for CS), or 90 °C (for WRS) (Tester & Morrison, 1990). With WRS, a holding period at 90 °C of approximately 2 minutes with stirring was provided, in order to guarantee a full gelatinization of the starch (Grazyna Bortnowska et al., 2016). Then the cheese was added and, after about 1.5 minutes of stirring, the cheese was completely dispersed (as judged visually) in the slurry, after which the MCBs were left to cool down to room temperature (ca. 21 °C). At this stage, the stirring was provided using an overhead stirrer, with a rotor (Eurostar 60 digital, IKA, Germany), and one of two types of paddles tested: a helix-shaped (R 1342 Propeller stirrer, 4-bladed, IKA, Germany) paddle and a U-shaped paddle (R 1375 Paddler stirrer, IKA, Germany).

Two factors were varied: the agitation speed vs time of the rotor, and the type of paddle used while mixing. The agitation speed-time profile was optimized to the one presented in Table 3.3., which enabled to obtain a stable MCB with no phase separation, no macroscopic pieces of

cheese, and with adequate consistency. In the table below, the parameters evaluated are shown. All test runs were performed in duplicate.

Table 3.3. – Speed-time profiles and paddle types used for the physical parameters optimization on the MCB formulation.

Sample	Rotor speed vs. time Speed (rpm)			Paddle type	Starch type
	First 10 s	During starch gelatinization (about 5 min)	After cheese addition (about 1.5 min)		
CS_H	550	160	300	Helix	Corn starch
CS_U	550	160	300	U-shaped	
WRS_H	550	160	300	Helix	Waxy rice starch
WRS_U	550	160	300	U-shaped	

3.2.3. Light and Fluorescence Microscopy

The microstructure of MCBs prepared with 5 g CS or WRS and 5 g grated Emmental cheese per 100 g of milk was analysed using an Olympus BX51 fluorescence optical microscope (Olympus, Japan), with a 10 x objective lens, and the images were captured using an Olympus EP50 camera (Olympus, Japan).

Samples were stained with Rhodamine B (1 g/L), in order to visualize starch and protein (fluorescence microscopy); and with Sudan III (1 g/L), for visualizing fat globules (light microscopy). Afterwards, samples were transferred to concave slides and covered with a cover slip.

All samples were equilibrated at room temperature for 15 minutes prior to microscopic analysis and all observations were performed in duplicate, with at least four pictures taken per sample. In order to obtain the sizes of protein agglomerates, the software ImageJ (version 1.51 m9, Wayne Rasband, National Institute of Health, USA) was used.

3.3. Results and Discussion

3.3.1. MCB Formulation

3.3.1.1. Ingredients Testing

Regarding the analysis of MCB formulation, the preparations made with XG did not disperse the cheese completely. The dissolution of XG in milk was also a time-demanding process. (Benichou, Aserin, & Garti, 2002).

The formulations made with GG and CMC, on the other hand, had a good capacity for dispersing the cheese. Lower concentrations of CMC than the ones referred above were tested (data not shown), nevertheless the cheese only dispersed at a minimum of 5% (w/w). However, with this concentration of CMC resulted an extremely viscous MCB, that presented phase separation after 24 hours, i.e., a whey phase at the top and a sedimented slurry at the bottom. GG produced an MCB with adequate viscosity, albeit the process of solubilization in milk being somewhat elaborate, i.e., milk had to be warmed to about 40 ºC, then GG added slowly with constant stirring and left with mixing overnight; finally, in order to achieve full solubilisation, milk had to be heated slowly until about 70 ºC.

MCBs made with CS and WRS, at a concentration of 5 g per 100 g of milk produced a formulation with adequate viscosity and consistency, no visible phase separation after 48 hours, and very good cheese dispersibility.

Regarding the cheese type, in Figure 3.1. A-C and Figure 3.2. A-C, we can observe samples of the MCBs made with the three different cheeses tested and using CS or WRS.

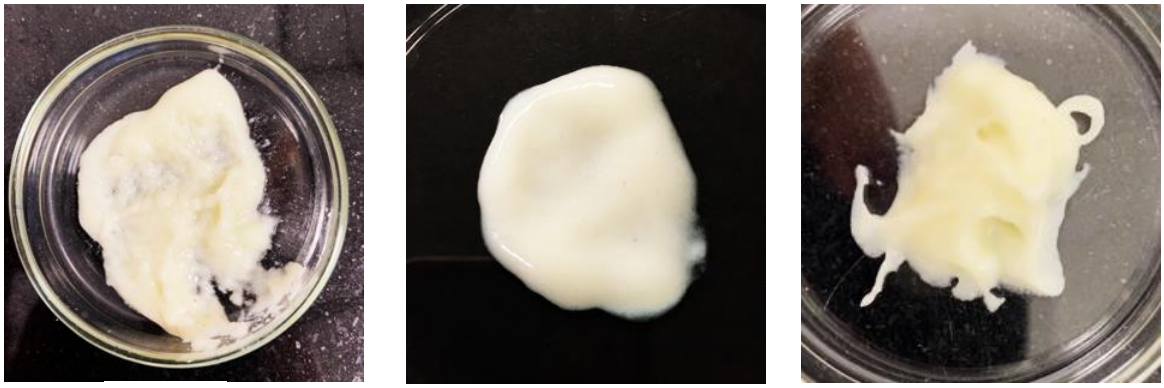

Figure 3.1. – Images of the samples EM_CS (A), QB_CS (B), and PR_CS (C).

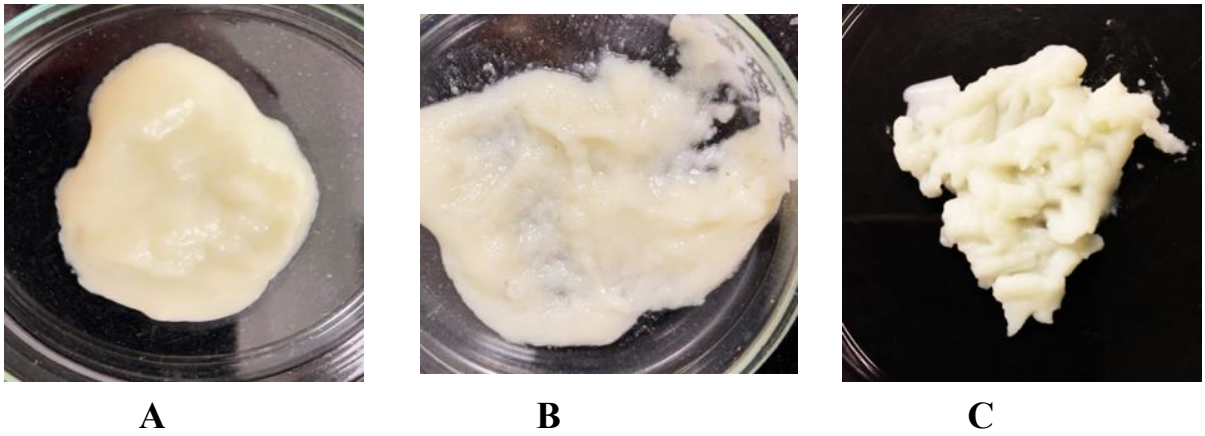

Figure 3.2. – Images of the samples EM_WRS (A), QB_WRS (B), and PR_WRS (C).

Samples with WRS were less fluid than the CS counterparts. This was expected since WRS, being a waxy starch, allows for increased swelling and improved water-holding capacity, leading to more viscous gels than the ones obtained with non-waxy starches (Hsieh et al., 2019).

The samples with Emmental cheese and Queijo da Bica cheese produced MCBs with adequate consistency (as judged by hand handling and mixing) and were deemed able to be incorporated in future products. Emmental cheese was the one that showed an easier dispersion and was also easier to handle. Queijo da Bica, a soft cheese, on the other hand, had to be manually cut into small pieces before addition to the milk-starch paste and the final MCB had to be put through a mesh, in order to separate reminiscent macroscopic pieces of cheese. For this reason, Emmental cheese was the chosen one for further experiments.

3.3.1.2. Time-temperature Curves

Time-temperature curves are presented in Figure 3.3. A and B for CS samples and WRS samples respectively. CS_U and WRS_U profiles were obtained using a U-shaped paddle in the rotor, while CS_H and WRS_H profiles were obtained using a helix-shaped paddle. The heating temperature and the time allowed for starch gelatinization were the ones reported in Table 3.3., in the previous section.

The results presented are average points between duplicate experiments done.

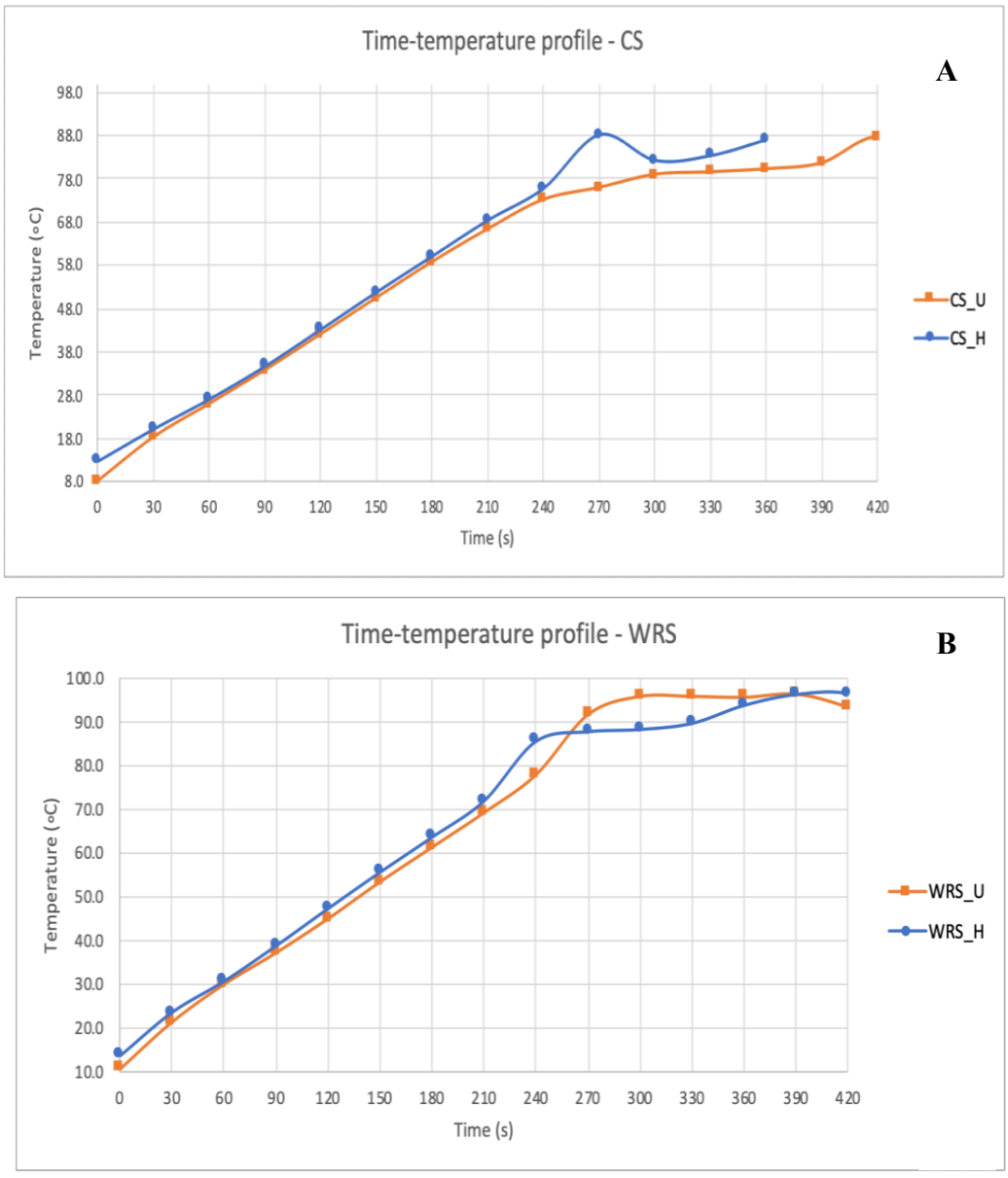

Figure 3.3. – Time-temperature curves for CS samples (A) and WRS samples (B), using either U-shaped (CS_U and WRS_U) or helix-shaped paddle (CS_H and WRS_H).

Analysing Figure 3.3. A, it can be seen that using the U-shaped paddle (CS_U) takes longer to achieve the gelatinization temperature of CS than with the helix paddle (CS_H).

Analysing Figure 3.3. B, both paddles gave similar heating curves till close to the gelatinization temperature for WRS samples; in the final stages, however, the helix paddle (WRS_H) provided smoother temperature profiles, as depicted between 240 s and 270 s.

In fact, it was observed that the helix paddle provided a more homogeneous mixing and lead to MCBs with better texture and consistency, both for CS and WRS samples. The optimum final temperature found for procedures with corn starch was 85 ºC and with waxy rice starch was 90 ºC. These values are as expected, since they are in the range of gelatinization temperatures found in literature for these types of starch (Tester & Morrison, 1990). As a curious note, the onset gelation temperature of native rice starch is higher than 90 ºC, however WRS presents a lower gelatinization temperature than the native rice counterpart (Park et al., 2007). This can be explained by the fact that WRS, having more amylopectin, has a larger crystalline region, requiring less energy to begin melting than the native rice starch. The polymeric mixture of amylose and amylopectin of this last starch, with a semi-crystalline structure, requires more energy to begin melting because the crystalline regions restricts hydration of the non-crystalline, amorphous regions, and delays initiation of swelling and gelatinization (Park et al., 2007).

As referred in section 3.2.2.2, in order to allow for WRS to complete its gelatinization, the temperature was held for 2 minutes above 90 ºC (Bortnowska et al., 2016), and only afterwards was the cheese added. This allowed for further thickening of the mixture before cheese addition and made for a better cheese dispersion and incorporation, with no macroscopic pieces detectable.

3.3.2. Light and Fluorescence Microscopy

Figure 3.4. A–C are microscopic images of MCB samples, prepared with 5 g CS or WRS and 5 g Emmental cheese per 100 g milk.

The dye Rhodamine B (Figure 3.4. A and Figure 3.4. C) stains proteins red and starch yellow, and the dye Sudan III (Figure 3.4. B and Figure 3.4. D) stains starch brown. In both MCBs, one can observe an intertwined protein-starch matrix, with sporadic starch granules remnants. The cheese protein aggregates have a size range from 5 to 200 μm in diameter. In some of these

aggregates, starch layers can be seen covering their surfaces. This surrounding starch points to a strong interaction with the cheese caseins.

The dye Sudan III (Figure 3.4. B and Figure 3.4. D) stains fat globules in red and the starch is stained brown. The protein rich areas correspond to the white areas. The fat globules were entrapped in the MCB matrix, with sizes ranging from < 1 μm up to 100 μm and are spherical in shape, found in aggregate form. Some smeared red staining was observed in some areas of the matrix, corresponding to fat dispersed by the high temperature and shearing used in the preparation of the MCBs (Macdougal et al., 2019).

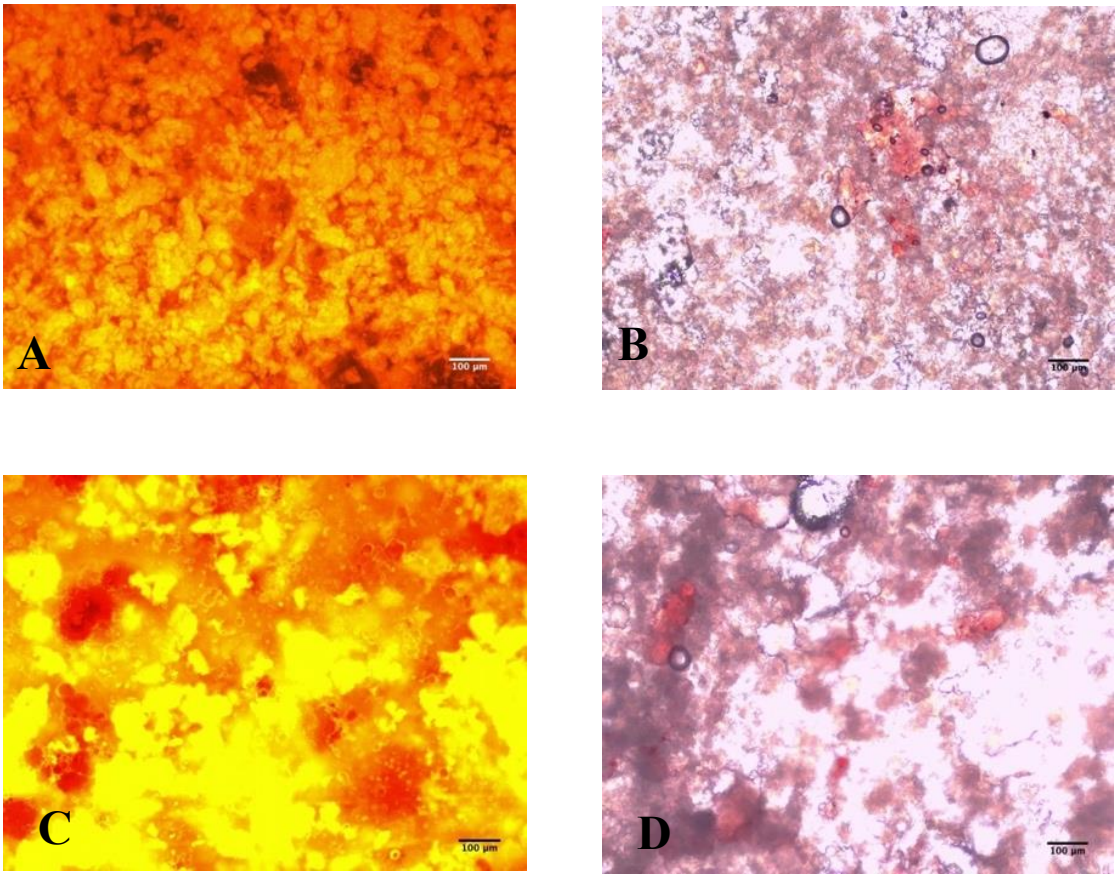

Figure 3.4. – Optical microscopy images of MCB samples prepared with CS (A and B) or WRS (C and D) and Emmental cheese, stained with Rhodamine B (A and C) and Sudan III (B and D).

We have demonstrated that the matrix of this same Emmental cheese is primarily held by a combination of hydrophobic and electrostatic interactions, including hydrogen bonds (elaborated in Chapter 4). Starch presents a high density of hydrogen bonds, but it also has a hydrophobic character, provided by the segments of double helices formed by glucose chains (Bortnowska & Goluch, 2018; Considine et al., 2010; Noisuwan, 2009; Stephen, Philips, & Williams, 2006). The fact that the starch must be well gelatinized in order to have cheese dispersing capacity

corroborates this hypothesis: during pasting, starch fragments composed primarily of amylose, although some amylopectin can also be present, leach out from the granules providing double helices segments that can interact with the cheese proteins via the above two types of bonds (Considine et al., 2010; Noisuwan, 2009; Sandhu & Singh, 2007). Therefore, we can speculate that the interactions between starch and the cheese protein aggregates can be dominated by electrostatic and hydrophobic bonds (Considine et al., 2010; Sun et al., 2016).

Heat is necessary in order to achieve cheese dispersibility and cheese melting. The increase in temperature can have a two-fold effect: it enables cheese melting; and can also enhance the formation and stability of protein-polysaccharide complexes, since an increase in temperature enhances hydrophobic interactions, favouring the interactions between biopolymers (Ye, 2008). In our experiments, we found that a previously gelatinized mixture of starch in milk at room temperature does not disperse cheese, moreover indicating that heat is, in fact, needed for a MCB formation.

Future studies are deserved to study specifically this feature and a more detailed section on interaction between proteins and hydrocolloids can be found in Chapter 4.

3.4. Conclusions

We conclude that an easy to prepare and to handle MCB formula can be obtained using milk as a base fluid, CS or WRS as a hydrocolloid, and grated or finely cut curd cheese. The MCBs have good texture and consistency, with no visible, macroscopic, pieces of cheese.

The starch suspension in milk should be heated past the gelatinization temperature (85 ºC for CS and 90 ºC for WRS) before cheese addition. Furthermore, for WRS, the high temperature must be held for 2 minutes, in order for the mixture to thicken at its maximum.

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Chapter 4. The Effect of Dissociating Agents on the Dispersion of a Grated Cheese

4.1. Chapter Introduction

4.1.1. Probes for studying interactions in gel systems

Urea, sodium dodecyl sulphate (SDS), and ethylenediaminetetraacetic acid (EDTA) solutions are among the most commonly used protein denaturants. Urea, which is usually used at concentrations up to 9 M, disrupts both intra- and intermolecular hydrogen bonds and weakens hydrophobic interactions, leading to protein denaturation and solubilization (von Hagen, 2008). A few studies (Medronho & Lindman, 2014a, 2014b; Schmid et al., 2015) have also used substituted ureas, such as thiourea, concluding that some of them could be more efficient in breaking hydrophobic interactions. However, in the same studies, urea was shown to be more efficient than the substituted compounds in breaking hydrogen bonds, demonstrating that its overall solubilization power was greater (Rabilloud, 2002).

Several studies conducted in different protein gel matrices, such as surimi gels (Shiku et al., 2004; Zhang et al., 2018), lamb myofibrillar protein gels (Ni et al., 2014), sardine with added proteins (egg white, soy, casein and gluten) gels (Gómez-Guillén, Borderías, & Montero, 1997), alkali-induced ovalbumin gels (Zhao et al., 2016), and egg yolk gels (Yang et al., 2019), stated that urea, at a concentration of 1.5 M, disrupts hydrogen bonds; at a concentration of 8 M, urea also disrupts hydrophobic interactions. For example, the study conducted with a sardine gel with added sodium caseinate, concluded that, for those gels prepared at 50 – 60 °C, the solubilities in 1.5 M urea were low, suggesting a small participation of hydrogen bonds in gel formation; but high values of solubility were obtained in 8 M urea, taken as a measure of hydrophobic interactions (Gómez-Guillén et al., 1997).

It is worth mentioning that, in spite of the vast research on the subject, there are still different views about how urea acts as a protein unfolding agent. Some authors propose that it interacts directly with protein side chains to start the unfolding process via hydrophobic interactions (direct interactions between urea and the protein) (Steinke et al., 2017). Others

propose that urea acts indirectly, by changing the bulk water-water interactions, leading to a decrease in protein hydration, which destabilizes the protein, causing it to unfold and expose the hydrophobic parts (Steinke et al., 2017). A combined mechanism between these two theories is also proposed by some authors (Steinke et al., 2017; Stumpe & Grubmu, 2007).

SDS is a surfactant detergent that gives a negative charge to all proteins (Hamada, Arakawa, & Shiraki, 2009), and acts as a denaturant by preventing interactions among hydrophobic domains, within and among proteins (von Hagen, 2008). This surfactant molecule is supposed to interact with both charged and nonpolar groups of the side chains of proteins (Lefebvre-Cases et al., 1998).

Ionic bonds are traditionally disrupted by increasing the ionic strength of the media, e.g., with addition of NaCl (Gómez-Guillén et al., 1997; Ni et al., 2014; Strange, Van Hekken, & Holsinger, 1994; Zhang et al., 2018). EDTA is a chelation agent with high affinity for calcium ions. Hence, the addition of this substance to e.g. casein containing samples, such as cheese, is expected to contribute for the destruction of the micelles by the disruption of micellar calcium phosphate nanoclusters (Gaucheron, 2005).

The above agents are used individually or combined, in order to evaluate their cumulative effect in, presumably, different types of interactions.

4.1.2. Chemical Interactions in Milk Gels

In cheese making, milk fat and caseins are concentrated, leading to a complex and heterogeneous system (Gagnaire et al., 2002). Cheese can be described as a bi-continuous gel structure consisting of a porous protein matrix (casein) interspaced with localized domains of fat (Vogt et al., 2015). The way this matrix is formed is a key variable, since it plays a role in the final microstructure of cheese and, consequently, its texture, flavour, and overall quality (Gagnaire et al., 2002; El-Bakry & Sheehan, 2014).

Emmental cheese, the one used in this work, is a Swiss-type, semi-hard cheese. In these cheeses, the matrix acidification occurs after pressing of the curd, i.e., when most of the whey has already been expelled. Hence, the concentration of colloidal calcium increases proportionally with the concentration of casein at drainage, leading to the formation of a highly mineralized, cohesive para-κ-casein matrix, with a calcium-casein ratio close to that of the casein micelles in milk (Gagnaire et al., 2002). Previous studies suggest that, within the casein micelles, several interactions take place among the individual casein chains, such as: (1) weak hydrophobic interactions; (2) salt bridges; and (3) calcium binding to caseins that result in the formation of colloidal calcium phosphate (CCP) (Hinrichs & Keim, 2007; Lucey & Horne, 2018; Stankey, Johnson, & Lucey, 2011). In gel-like systems based on casein, such as cheese, literature

proposes that self-association of casein micelles is also driven by different interactions, such as hydrophobic interactions and electrostatic ones (Horne, 1998). However, as far as Emmental cheese is concerned, and to the best of our knowledge, there are no studies on the relationship between such possible interactions and the cheese structure.

A study that looked at different types of fermented milk structures, determined that, for fresh rennet casein gels, specifically Gouda curd grains and Mozzarella cheese, calcium bonds were the dominant interaction in stabilization of the protein structure; for ripened cheeses, such as Camembert or Gouda, hydrogen and other electrostatic bonds were the main interactions (Hinrichs & Keim, 2007). Another study on rennet-induced gels concluded that hydrophobic interactions and calcium bonds were the main forces acting, whereas, for acid-induced gels, hydrophobic, hydrogen bonds, and electrostatic interactions were homogeneously distributed (Liu et al., 2014).

The studies conducted by Lefebvre-Cases et al. (1998) identified interactions among casein gels using urea, SDS, and EDTA. It was determined that hydrophobic interactions and calcium bonds were the main ones in rennet milk gels; while hydrophobic, electrostatic, and hydrogen bonds were the major ones in acid milk gels. The roles of these interactions were reiterated in the study by Zamora, Trujillo, Armaforte, Waldron, & Kelly (2012), where calcium and hydrogen bonds, as well as hydrophobic interactions, were found to be involved in the protein matrix of drained rennet curds.

In a study by Gagnaire et al. (2002), 2 M urea and 100 mM EDTA were used to disrupt the molecular interactions in fresh Emmental pressed curd. It was determined that the disruption of hydrogen bonds, and possibly some hydrophobic interactions, did not completely disintegrate the paracasein matrix, being the calcium-crosslinking interactions of utmost importance. Based on their findings, the authors hypothesized a joint interaction of those different forces in the maintenance of the curd matrix.

Regarding processed cheese, some authors suggest that hydrophobic interactions play a crucial role in the protein structure (Lucey & Horne, 2018), with some studies indicating that they are the main interaction in this type of cheese (Fu & Nakamura, 2017). However, other studies suggest that different types of interactions must act in cooperation with each other. Namely, hydrophobic, hydrogen bonding and other electrostatic interactions, and disulphide bonds, are all appointed as responsible for stabilizing the protein network in processed cheese (Lucey & Horne, 2018; Marchesseau, Gastaldi, & Lagaude, 1997; Schmid et al., 2015).

Studies on whey protein gel matrices also concluded that different chemical interactions acted together in order to provide structure. For instance, in heat-induced gels of whey protein isolate, the hydrophobic interactions and the intermolecular disulphide bonds were found both responsible for the firmer gel structure with increasing protein concentration (Shimada & Cheftel, 1988). Disulphide bonds were also stated to be important in the early stages of aggregation of

whey protein mixtures that formed hydrogels; yet, these hydrogels were found to be mostly stabilized by noncovalent interactions (Mercadé-Prieto et al., 2018).

From the above overview, we conclude that different works point to different interactions as the main ones in maintenance of cheese structure (at the curds stage, cured, or processed type). Our experimental work aimed to clarify the contribution that each type of interaction has in ripened Emmental cheese. For that purpose, we used the dissociating agents referred above, evaluating their ability to solubilize protein (Shukla & Trout, 2010), under a careful experimental protocol.

4.1.3. Protein Quantification Methods

Although several other methods can be used to quantify the protein content in milk and other dairy products, the Kjeldahl method is still the standard one. However, it is very time-consuming, expensive to run, and has some safety issues (Liu & Pan, 2017). Furthermore, this method cannot be used on samples containing urea (since it measures the nitrogen content of the sample). Colorimetric methods commonly used for protein quantification are not as accurate, due to the various interferences associated therewith, including SDS or high concentrations of urea (Redmile-Gordon, Armenise, White, Hirsch, & Goulding, 2013). Another possible option is through UV absorbance, which is a direct method using a simple calibration curve (Olson & Markwell, 2007; Zheng et al., 2017). However, it can be applied only in quite pure protein solutions.

The alkalinisation of milk systems, e.g. by the addition of NaOH, results in changes in the physical properties of the casein micelles, namely destabilization and ultimately their disruption (Lam et al., 2018). Studies show that NaOH can disrupt various chemical bonds in milk systems, including disulphide bonds (Florence, 1980; Reichardt & Eckert, 1991). In this work, in order to quantify the protein in cheese, a modification of the method reported by Reichardt & Eckert (1991) was used. Samples were dissolved in 0.1 M NaOH and, afterwards, the soluble protein was evaluated by UV absorbance. The protein present in the different dissociating media was quantified also by the same method.

4.2. Materials and Methods

4.2.1. Materials

The dissociating agents used were urea (molecular biology grade, Sigma-Aldrich, USA), SDS (ultrapure, ITW, USA), EDTA (Fluka, USA), NaOH (Eka, Sweden) and NaCl (Fluka, USA). The cheese was commercial grated Emmental (Milbona, Germany). Casein was from bovine milk (Sigma-Aldrich).

4.2.2. Experimental Methods

4.2.2.1. Determination of Total Protein in Cheese

The total protein in the Emmental cheese was determined by the Kjeldahl method (AOAC, 1990) and by a modification of the method by Reichardt & Eckert (1991). In this, approximately 1.3 g of cheese was dispersed in 30 mL 0.1 M NaOH, and the mixture was warmed up to 70 °C. After cooling down, the samples were centrifuged at 5000 rpm (4410 x *g*), 4 °C, for 30 minutes. After centrifugation, the lipid layer was removed, and the middle clear solution was separated from the pellet, which was discarded. Then, the absorbance of the solution at 280 nm was determined, as detailed below. The absorbance values were converted to protein concentration using a calibration curve of casein in 0.1 M NaOH. Three replicates were made.

4.2.2.2. Determination of Solubilized Protein by Different Dispersing Media

Aliquots of 35 mL of 0.17 M NaCl solution were drawn into 100 mL glass beakers and then different dissociating agents were dissolved in the saline. Additionally, solutions of NaCl at 50 mM and 0.6 M were also prepared. The dissociating agents used and respective concentrations are listed in Table 4.1

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Table 4.1. – Dissociating agents and their concentrations used in the study.

Dissociating Agent	Concentrations
NaCl	0.05 M and 0.6 M
Urea	1.5 M and 6 M
SDS	2.5 %
EDTA	4 mM and 50 mM
Urea + SDS	6 M + 2.5 %
Urea + EDTA	6 M + 4 mM
Urea + SDS + EDTA	6 M + 2.5 % + 4 mM

1.33 ± 0.5 g of cheese was added to each beaker, and each solution was slowly heated to 70 °C (in about 10 minutes), with constant magnetic agitation. This step of heating the cheese suspension was included in order to mimetize the conditions found in the preparation of several cheese-containing foods, such as sauces or processed cheeses, among others. However, in order to study the contribution of heating on solubilization of the cheese proteins, the test with 6 M urea was also carried out at room temperature.

The cheese suspensions were allowed to cool down and the dispersions were centrifuged in 50 mL tubes for 30 minutes, at 5000 rpm (4410 x *g*), and at 4 °C. That supernatant was left at room temperature overnight, in order to account for any possible alterations that might still occur.

The protein concentration of this solution was then evaluated by UV absorbance at 280 nm. For this measurement, 920 μL of 0.1 M NaOH was mixed with 80 μL of the sample solution in a 1 mL quartz cuvette. The corresponding blank was made with the same dissociating solution, and with the same dilution in 0.1 M NaOH.

Preliminary tests of UV spectra of the blanks were carried out, in order to check if the dissociating agents would interfere with the method. There were no significant interferences for any of the agents, at the concentrations employed, and at the wavelength of 280 nm.

The amount of solubilized protein was evaluated based on the calibration curve of casein, and the result compared with the total protein in cheese.

Each experiment was repeated three times.

4.2.2.3. Statistical Analysis

A t-student test was applied to the results of total protein in cheese evaluated by Kjeldahl method and by the dissolution in 0.1 M NaOH, as reported here. An ANOVA one-way was used for the statistical analysis of the effect of the different dissociating agents.

4.3. Results and Discussion

4.3.1. Determination of Total Protein Concentration in Emmental Cheese

Total protein in the Emmental cheese was determined by two methods: the reference Kjeldahl and by dissolution in 0.1 M NaOH followed by UV measurement. The calibration curve for the latter method can be found in Figure 4.1.

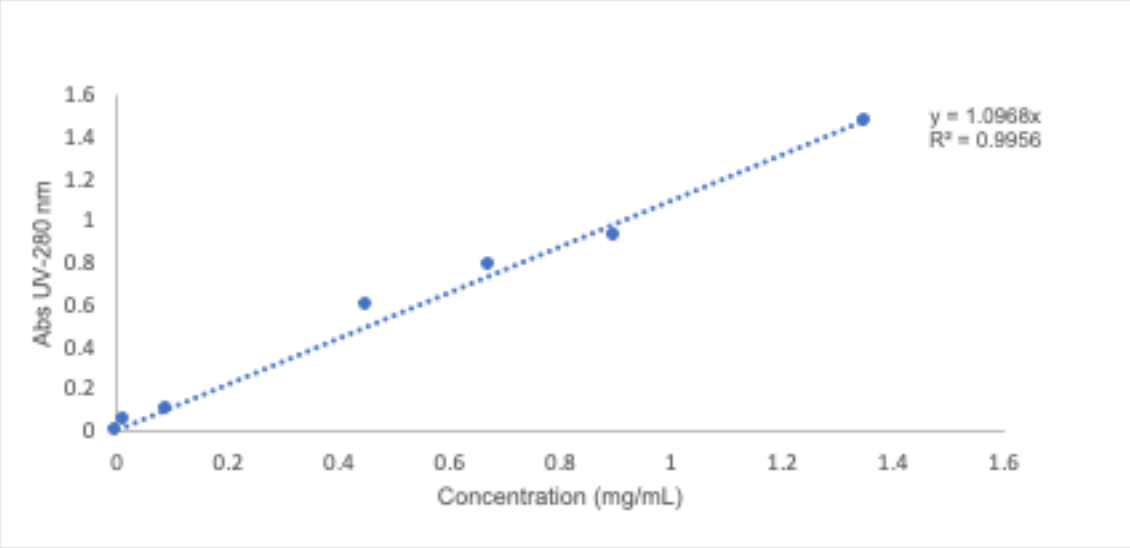

Figure 4.1. - Calibration Curve of Casein Dissolved in 0.1 M NaOH

The calibration curve of casein in 0.1 M NaOH has shown to be linear up to 1.4 mg/mL concentration, with a value of R^2 of 0.9903, as shown in the figure above.

The mean value of protein content of the cheese by these two methods was 0.297 ± 0.030 and 0.315 ± 0.010 g protein/g of cheese, respectively, indicating no statistically significant difference ($p > 0.05$). This shows that the NaOH/UV method can be conveniently applied, enabling a quite precise determination of protein in cheese. Based on these observations, we believe that this method is likely to be applicable to several other dairy matrices, which is a subject of ongoing work.

4.3.2. Determination of Solubilized Protein by Dissociating Agents

The solubilized protein by each dissociating medium was evaluated by the NaOH/UV method, and the results compared to the total protein in cheese. The values are reported in Table 4.2.

Table 4.2. – Dissociating media and values of respective solubilized cheese protein.

Dispersing medium	Soluble Protein (g protein/g cheese)	Solubilized Protein (%)
Deionized Water	0.0319 ± 0.006^{a}	9.97 ± 1.90^{a}
NaCl 50 mM	0.0439 ± 0.003^{a}	13.70 ± 0.95^{a}
NaCl 0.6 M	0.0746 ± 0.011^{a}	23.33 ± 3.52^{a}
Urea 1.5 M	0.167 ± 0.018^{b}	52.10 ± 5.47^{b}
Urea 6 M	0.269 ± 0.025^{c}	85.30 ± 6.13^{c}
Urea 6 M (no heating)	0.261 ± 0.016^{c}	82.57 ± 2.66^{c}
EDTA 4 mM	0.0905 ± 0.0079^{a}	28.67 ± 2.02^{a}
EDTA 50 mM	0.0730 ± 0.0063^{a}	22.83 ± 1.96^{a}
SDS 2.5 %	0.213 ± 0.007^{b}	67.37 ± 1.40^{b}
Urea 1.5 M + SDS 2.5%	0.282 ± 0.012^{c}	88.20 ± 3.60^{c}
Urea 6 M + SDS 2.5 %	0.270 ± 0.016^{c}	85.40 ± 2.40^{c}
Urea 6 M + EDTA 4 mM	0.275 ± 0.008^{c}	87.27 ± 3.56^{c}
Urea 6 M + SDS 2.5 % + EDTA 4 mM	0.279 ± 0.022^{c}	92.13 ± 10.61^{c}

Samples with the same superscript letter do not present statistical differences among them, according to the Tuckey test.

From the results, we can conclude that ionic interactions might play some role in this cheese matrix, since 0.6 M NaCl can dissolve up to 23 % of cheese proteins. In contrast, deionized water and 50 mM NaCl, which have low ionic strength, disperse up to 13 % protein, which can represent “free” protein in cheese, particularly non-drained whey proteins. Despite these slight variations, the results obtained with deionized water and with NaCl (50 mM and 0.6 M) were not statistically different ($p > 0.05$).

Urea at 6 M, with or without heating to 70 °C, solubilized a great part (up to 85 %) of the cheese proteins. This result is in line with a previous study, that reports that the proteins and large polypeptides in cheese are completely soluble in 4 to 6 M urea (McSweeney & Fox, 1997). However, 1.5 M urea dissolves considerably less protein. As stated previously, some published analyses on protein gels and films (Gómez-Guillén et al., 1997; Zhang et al., 2018) used that concentration as a means to probe hydrogen bond interactions, and urea 8 M to measure, additionally, hydrophobic interactions. Our results can be interpreted under the same principles, albeit a clear dependence of urea concentration on each type of bond can still be questioned.

Hydrophobic interactions are known to strengthen with temperature (Alessi et al., 2007). However, some authors (Gómez-Guillén et al., 1997) point out that temperatures beyond 58 °C can, instead, weaken hydrophobic interactions, by destabilizing the hydrogen bonds among water molecules, which would facilitate hydrophobic hydration and make it easier for protein denaturation. Furthermore, temperature increases molecular motions, facilitating separation of aggregated proteins, such as in cheese. Notably, in our experiments with Emmental cheese, there was no significant difference ($p > 0.05$) between the amount of solubilized protein by 6 M urea heated to 70 °C (85%) and at room temperature (82%) (Table 4.2.).

EDTA, as a chelating agent, at both 4 mM and 50 mM, only dispersed up to 28 % of the cheese proteins, a value similar to the one obtained with 0.6 M NaCl solution. This suggests that, in this matrix, the calcium bonds do not require a specific chelating agent for their disruption (Lefebvre-Cases et al., 1998). Furthermore, there was no significant difference between EDTA 4 mM and 50 mM ($p > 0.05$).

SDS, as opposed to EDTA, dispersed up to 67% of the cheese proteins, which shows that hydrophobic interactions must play an important role in this matrix (Lefebvre-Cases et al., 1998). This is not unexpected given the significant number of nonpolar regions found along the casein polypeptide chain. Solutions with combinations of urea (6 M or 1.5 M) with SDS, or with EDTA, dissolve practically the same amount of protein as 6 M urea alone (up to 88%). In fact, there were no significant differences observed between the results of any pair of the solutions 6 M urea, 6 M urea + SDS, 1.5 M urea + SDS, 6 M urea + EDTA, and 6 M urea + SDS + EDTA ($p > 0.05$). The fact that 6 M urea was similar to 1.5 M urea with SDS, supports the view that, at high concentrations, urea disrupts hydrophobic interactions.

The effect of temperature of the dispersion media was also carried out with 1% and 2.5% SDS, and with 4 mM EDTA solutions. Unlike urea, EDTA solutions only dispersed a significant amount of the cheese proteins when heated to 70 °C (up to 28%); at room temperature, no significant amounts (ca. 6%) were solubilized. With 2.5% SDS solution at room temperature some cheese was dispersed, however, after the centrifugation, a phase separation occurred, and the turbidity of the solution turned quantification inviable. At 1% SDS concentration, no phase separation was noticeable, but the solution remained quite opaque for UV measurement. These problems were not observed when heating to 70 °C was included. Therefore, apart from the case of urea, heating was needed in order to attain significant levels of protein solubilization. An

interpretation of such observation is that the thermal agitation of the molecular chains in the protein aggregates in cheese complements the action of the dispersing agents.

We also monitored the pH of the separated supernatants of the dispersion media. All values ranged between 5.5 for 0.6 M NaCl and 6.64 for 6 M urea + 2.5% SDS solution; when dispersing cheese in 0.1 M NaOH, the corresponding pH was 8.6. Except in this last case, the pH should not have a significant impact on the extent of protein solubilization, as backed by previous works (Lam et al., 2018; Marchesseau et al., 1997).

We note that the literature uses the mentioned dissociating solutions as “sensors” of specific interactions, but clearly several of these solutions might disrupt more than one type of interaction, with no absolute specificity. Therefore, the amounts of solubilized protein should be interpreted as indicators of the influence of the different types of interactions in a structure.

4.4. Conclusions

Even though a consensus has not yet been reached regarding the main molecular interactions acting in the casein matrix of cheese, this work was able to provide some insights, for the case of ripened Emmental cheese.

We proved that the caseins in this matrix are maintained by a set of hydrophobic interactions, hydrogen bonds, and other electrostatic interactions, including ionic bonds. The results obtained with urea suggest that hydrogen bonds have an important role, comparable to hydrophobic interactions, a conclusion not commonly reported for cheese structures. Rather surprisingly, and different from what is often stated, calcium bonding seems not to have a relevant role in this case.

Solutions without urea required heating for solubilization of proteins to some extent, a fact interpreted as thermal agitation helping separation of the protein chains in aggregates.

Finally, in terms of protein quantification, we point out that 0.1 M NaOH dissolves the totality of cheese proteins and this fact can be explored for their quantification, as previously reported (Reichardt & Eckert, 1991). However, we introduced analytical improvements in the method that enabled results quite close to the standard Kjeldahl one.

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Chapter 5. Yogucheeses – Yogurts Fortified with Dispersed Cheese

5.1. Chapter Introduction

Yogurt is one of the most common dairy products and its consumption has been increasing in the last years. It is prepared by fermenting cows', goats' or other ruminants' milk using lactic acid-producing bacteria, under a controlled temperature and environmental conditions (Das, Choudhary, & Thompson-Witrick, 2019).

Two types of yogurts are available on the market: with a firm gel-like structure (set type), or with a thick liquid consistency (stirred type). In set type yogurt, milk is inoculated with the starter, placed in the final package and subsequently fermented whereas in stirred type yogurt, the fermentation takes place in larger vessels, and then the gels are disrupted by vigorous stirring, before being transferred to the final package (Oraç & Akın, 2019).

Yogurt holds a high nutritional and health benefits connotation, such as digestion enhancement, immune system boosting, anticarcinogenic activity, and reduction in serum cholesterol (Nastaj et al., 2019). Besides its nutritional value, the viscosity and rate of syneresis are important indexes of sensory qualities and stability of yogurt products (Fang & Guo, 2019). In recent times, the market has valorised products with increased protein content, with a thicker consistency, and that do not whey-off during storage. Furthermore, another recent trend is the avoidance of chemical additives, and the use of clean label ingredients.

In order to attend to the above features and trends, the formulation of yogurts frequently includes supplementary dairy protein powders and stabilizing hydrocolloids. Among these are native and modified starches, gelatine, or xanthan gum, among others. They can provide structural stabilization, reduce syneresis, and can also have emulsification properties (He et al., 2019).

In order to increase the protein content, whey and whey concentrates, caseinates, and skim milk powders are also commonly included (Damin et al., 2009). For example, (Bong & Moraru, 2014) evaluated the addition of micellar casein concentrate to fortify Greek-style yogurts. All samples showed a shear-thinning behaviour associated with a weak-gel structure, with the fortified yogurt samples presenting lower water-holding capacity than the controls.

A review study also states that the addition of skimmed milk powder (SMP) produces good quality yogurt, with increased viscosity and gel strength; furthermore, the addition of whey protein

favours the final product viscosity, firmness, gel strength (G′), and syneresis (Karam et al., 2013).

Lobato-Calleros et al., 2014 showed that the addition of native or chemically modified starches, from different origins, to reduced-fat yogurts contributed to the formation of more stable milk gels. Najgebauer-Lejko et al., 2007 studied the effect of the addition of different starches in set-style yogurts and reported products with lower acidity and higher resistance to whey separation. Goncalvez et al., 2005 added gelatin and starch, separately, to stirred yogurt and observed that the addition of these thickeners resulted in a significant increase in viscosity, ropiness, mouthfeel, and creaminess, while also reducing syneresis. Pang, Deeth, Prakash, & Bansal, 2016 concluded that the combination of whey protein isolate and gelling polysaccharides (starch, carrageenan, and xanthan/locust bean gum) as gelatin replacers in yogurts induced stronger gels with higher water-holding capacity.

In this work, we aimed to produce protein-fortified yogurts using cheese as ingredient. This cheese can possibly come from surpluses from the industry and retail sector, which would contribute to a sustainable added-value strategy for reducing food waste and encourage a circular economy system. A significant part of surplus cheese is used for the manufacture of processed cheese; however, besides natural cheese, these composites also contain additional sodium-containing chemicals, colours, and flavours for aroma, taste and texture. Targeting a more natural approach and benefiting from the additional health benefits of yoghurt as a fermented product, in comparison with processed cheese, our study offers the opportunity to enhance the nutritional profile of yoghurt using natural cheese surplus. In addition to the sustainable character of such solution, it must be highlighted that besides protein, the cheese also provides several other nutrients, such as minerals and fat, as well as flavour compounds.

Cheese can be dispersed into sub-millimeter particles by mixing it in a hot paste of gelatinized starch in milk. This *melted cheese base* (MCB) can be an ingredient for the development of novel dairy products incorporating ripened cheese, which is the subject of ongoing work in our group.

We selected grated Emmental cheese and corn starch (CS) or waxy rice starch (WRS) for the preparation of the MCBs (more on this selection can be found in Chapter 3). This was mixed with further milk in different proportions, before addition of starter culture and fermentation. The resultant cheese-fortified yogurts (*yogucheeses*) were then characterized in terms of macronutrient composition, pH and titratable acidity, syneresis, textural and rheological properties. To the best of our knowledge this is the first study using cheese surpluses to enrich protein content of yogurt matrices, creating added nutritional value in a sustainable manner.

5.2. Materials and Methods

5.2.1. Materials

The materials used were commercial grated Emmental cheese (Milbona, Germany), commercial native corn starch (Maizena, Unilever, Portugal), waxy rice starch (Remyline XS, BENEO GmbH Germany, kindly provided by Nutripar, Portugal), semi-skimmed (1.5 % fat) HTST milk (Vigor, Portugal), and commercial standard yogurt cultures containing *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* (Condi, Malveira, Portugal), which is a lyophilised, direct vat set culture.

5.2.2. Experimental Methods

5.2.2.1. MCB Preparation

CS or WRS was dispersed in cold milk, at a ratio of 5 g starch per 100 g milk, and the mixture was heated for 5 minutes, with continuous stirring, until 85 °C (for CS), or 90 °C (for WRS). At this point, the gelatinization of the starch was noticeable, and the grated cheese was added, at a ratio of 34 g cheese per 100 g milk. The mixture was then removed from the hotplate and stirred until the cheese was fully dispersed, with no visible, macroscopic pieces. The MCB was left to cool down to room temperature (ca. 21 °C).

5.2.2.2. Preparation of Yogurt Samples

The milk used to prepare all the following samples was previously heat treated at 90 °C for 10 min, and then cooled to 43 °C, in order to promote denaturation of whey proteins (Torres et al., 2018). In order to enable the preparation of samples with different incorporations of ripened cheese, while using the same MCB and keeping constant the starch concentration, we mixed in different proportions of heat-treated milk, milk with 3.6% gelatinised starch, and MCB prepared as above. The amounts are reported in Table 5.1. The mixtures (100 g) were prepared in small glass flasks (60 mm diameter, 65 mm height). Three different control samples (without MCB addition) were prepared: one containing plain milk (CL) and two containing milk with 2.0% (w/w) final concentration of either CS (CL_CS) or WRS (CL_WRS).

For the cheese-fortified yoghurt samples, three different incorporations were tested, corresponding to adding MCB at 20%, 40% and 60% (w/w) of overall mixture (A20, A40 and A60).

Except for the milk control (CL), all other samples had CS or WRS at a final concentration of 2% (w/w). Commercial starter was used at the recommended level of 7 g per L of preparation.

Incubation of the yoghurt samples was carried out at 40 °C for about 4.5 h, the time required for the pH to reach 4.6. The samples were then stored at 4 °C.

Table 5.1. – Preparation of control and yogucheese samples.

	Milk (g)	Milk + 3.6% starch (g)	MCB (g)
CL	100	0	0
CL_CS	40	60	0
CL_WRS			
A20_CS	40	40	20
A20_WRS			
A40_CS	40	20	40
A40_WRS			
A60_CS	40	0	60
A60_WRS			

5.2.2.3. Physicochemical Analysis

5.2.2.3.1. Cheese Protein Content Determination

Total protein of Emmental cheese was determined using a novel method based on the one proposed by (Reichardt & Eckert, 1991), but with modifications (Paula Vilela, Gomes, & Ferreira, 2020). About 1.33 g of cheese cut in small pieces was placed in 30 mL of 0.1 M NaOH and left overnight. The following day, the mixture was placed in a waterbath (TW20, JULABO GmbH, Seelbach, Germany) at 40 °C, for 10 min and mixed well. After cooling down, it was centrifuged (Universal 320R, Andreas Hettich GmbH, Germany, centrifuge) at 4000 x g, 4 °C, for 10 min. The top layer of fat was removed, the underlying supernatant was collected, and its volume evaluated. The absorbance at 280 nm was measured by diluting 80 µL of the supernatant with 920 µL of 0.1 M NaOH. The concentration was calculated from a casein calibration curve, and the protein content in cheese was then calculated. All measurements were done in duplicate.

We have shown that this method gives results that are not statistically different from those obtained with the standard Kjeldahl method (Paula Vilela et al., 2020).

5.2.2.3.2. Cheese Fat Content Determination

Fat content of Emmental cheese was evaluated using the Van Gulik method (ISO 3433 2008). All measurements were done in duplicate.

5.2.2.3.3. Time – pH Curves and Determination of Titratable Acidity

The pH of selected samples was recorded throughout the fermentation process, using a pH meter (SensION^{+} PH31, Hach, USA) equipped with a probe SensION+ pH gel combination electrode, with automatic temperature compensation. The same set was used for all other pH measurements. All evaluations were carried out in duplicate.

Titratable acidity (TA) was determined 24 hours after fermentation, following the method from (Bong & Moraru, 2014). 9 ± 0.5 g of yogurt sample was diluted in 18 g of deionized water. Then, a titration was carried out using a standard 0.1 M NaOH solution and 0.5 ml phenolphthalein as an indicator. TA, expressed as % of Lactic Acid, was calculated using the equation (Nielsen, 2017):

$$TA(\%\ Lactic\ Acid) = \frac{M\ NaOH \times V\ NaOH \times 90.08}{w_{sample}} \times 100\% \qquad \text{(Eq. 5.1)}$$

Where *M* is the molarity of NaOH solution; *V* NaOH is the total volume (L) of NaOH used in the titration; 90.08 g.mol^{-1} is the molecular weight of lactate; and w_{sample} (g) is the weight of the sample. All measurements were made in duplicate.

5.2.2.3.4. Evaluation of Syneresis

20 ± 0.5 g of yogurt sample (at 4 ± 1 °C) was placed in 50 mL falcon tubes and centrifuged (Universal 320R, Andreas Hettich GmbH, Germany, centrifuge) at 220 x *g* for 15 min, at 4 °C. The clear supernatant was poured off, weighed, and syneresis was expressed as the ratio between the weights of supernatant and original yogurt sample (Lobato-Calleros et al., 2014). These measurements were made in duplicate.

5.2.2.4. Textural Properties

Texture of the samples was analysed using a TA.TX.plus texture analyser (Stable Micro Systems, Godalming, UK), calibrated with a 30 kg loading cell. The texture profile analysis (TPA) was performed with the samples pre-equilibrated at 22 ± 0.5 °C for 15 min. The tests were carried out keeping the samples in the vials in which they were originally prepared, thus avoiding gel disturbance with transfers. The equipment had a cylinder probe (36 mm diameter and 34 mm height), and the test was carried out with a trigger force of 5 g, speed of 1 mm/s, and a penetration distance of 12 mm, which corresponds to a maximum deformation of about 40% of the sample height. Hardness, adhesiveness, cohesiveness and springiness of the samples were determined using the Exponent PC software (version 5) (Texture Technologies Corp., Hamilton, MA, USA). All measurements were carried out in duplicate samples.

5.2.2.5. Rheological Properties

The rheological properties of the yogurt samples were determined by oscillatory amplitude and frequency sweep tests, using a Bholin Gemini rheometer (Malvern Panalytical, Malvern, UK), with a cone and plate geometry probe. The samples, prepared 24 h before and kept at 4 °C, were slightly homogenized by hand mixing with a spoon before each test, then 1 mL aliquots were transferred to the probe. Samples were equilibrated at 7.5 °C before measurements. The tests were performed in duplicate.

An amplitude sweep with a strain range from 0.0001 to 0.1, at the frequency of 1 Hz, was performed, in order to find the linear viscoelastic region (LVR).

Afterwards, a frequency sweep from 0.01 to 10 Hz was applied with a strain of 0.001, shown to be in the LVR, in order to determine several viscoelastic properties, including the storage modulus (G'), the viscous or loss modulus (G''), and the dynamic viscosity curve (η).

5.2.2.6. Product Physicochemical Stability Tests

A representative group of samples was selected for a stability study: a control sample, a control sample with starch, and samples with 40% (w/w) MCB incorporation, prepared with either CS or WRS. The samples were maintained at 4 °C and their stability was evaluated over the following two weeks, for visual aspect of the gel and whey separation (syneresis).

5.2.2.7. Statistical Analysis

One-way ANOVA tests were used for the statistical analysis of the physicochemical, textural and rheological data, with the application of the Tuckey test for pairwise comparisons between particular samples. The normality of the data, as well as the homogeneity of variances was verified, and the SPSS software (version 26) was used for the statistical analysis of the results.

5.3. Results and Discussion

5.3.1. Macronutrient Composition of the Yogurt Samples

Based on the corresponding compositions of the raw materials, the macronutrient compositions of the yogucheese samples are presented in Table 5.2. The ingredient with the highest influence on the protein and fat levels is the cheese added, so these were subjected to analyses. For the milk, we considered the supplier's nutritional values. We found the analyses of ingredients, in particular those of cheese, to be more reliable than the analyses of the yogucheeses, as these are more prone to interferences by the starch, particularly in the case of the fat determination.

The protein and fat contents of the Emmental cheese were determined as 31.5 ± 0.01 % (Paula Vilela et al., 2020) and 29 ± 0.07 % (w/w), respectively (Palyvou-Gianna et al., 2021).

Table 5.2. – Macronutrient composition of yogucheese samples. All values are given as %(w/w).

	Proteins	**Lipids**	**Total carbohydrates**
CL	3.40	1.60	4.90
CL_CS **CL_WRS**	3.28	1.54	4.72
A20_CS **A20_WRS**	4.61	2.91	4.77
A40_CS **A40_WRS**	5.81	4.22	4.64
A60_CS **A60_WRS**	7.02	5.53	4.51

In a typical commercial yogurt, protein values most often range from 2.5 to 4 % (w/w), and lipids range from 1.5 to 4% (w/w) (Bullard, St-Gelais, & Turgeon, 2018; Faihst et al., 2017; Lobato-Calleros et al., 2014; Moore, Horti, & Fielding, 2018; Torres et al., 2018). In Greek yogurts, these values increase up to 11 % (w/w) for proteins and up to 10% (w/w) for fat (Bong & Moraru, 2014; Moore et al., 2018). Some research studies produced yogurts with values up to 5.6 % in

proteins by fortifying them with whey proteins microparticulated powder or skim milk powder (Faihst et al., 2017; Lobato-Calleros et al., 2014; Pang et al., 2016; Tamime, Barrantes, & Sword, 1996; Torres et al., 2018). Hence, the range of protein and lipid values obtained for our cheese-fortified yogurts are at, and above, the ones of full-fat commercial yogurts, even matching the ones of some Greek yogurts in the samples A60. Moreover, current consumer trends in terms of snacking products are also changing, with cheese-containing snacks being one of the biggest opportunities for creative new product developments, business growth, and improved profitability (NewNutrition Business, 2020). Cheese offers consumers a high-protein, low-sugar alternative to sweet or carbohydrate-heavy snacks. As seen from Table 5.2., our product can be considerably high in protein and the cheese incorporation can be a key factor in the final consumer acceptance.

Regarding total carbohydrates, the starch incorporation was fixed as 2%, and the difference between these two values is the lactose content, provided mainly by the milk. The absence of sugar or other sweeteners, or any *artificial* ingredient, should also be highlighted.

An issue can be raised regarding the salt level found in the yogucheeses, as ripened cheeses can have a considerable concentration. In this case, Emmental has a particularly low salt content of only 0.6% (w/w). Considering the yogucheese samples with the highest cheese incorporation (A60), the cheese would contribute to just 0.1% salt, which is a fairly low level. If cheeses with higher salt concentrations were used, for example a rather high value of 1.9%, then those samples would carry 0.28% salt, corresponding to 0.11 g sodium /100 g, a value that still does not raise health concerns. Recall that the World Health Organisation recommends that adults consume less than 2 g of sodium per day as part of a healthy eating pattern.

5.3.2. Time-pH Curves

Figures 5.1. and 5.2. show the time-pH profiles during the fermentation period. The two control samples with starch (CL_CS and CL_WRS) start at slightly higher pH values than the plain milk control (CL), but the difference shortens considerably at under 1-hour incubation time. Samples with a low amount of cheese (A20_CS and A20_WRS) start with pH close to the CL control and reach pH 4.6 at about the same time (240 minutes). The observed fermentation times are within the usual range for yogurts, and similar time-pH profiles can be found in previous works (Bong & Moraru, 2014; Singh & Byars, 2009; Singh & Kim, 2009).

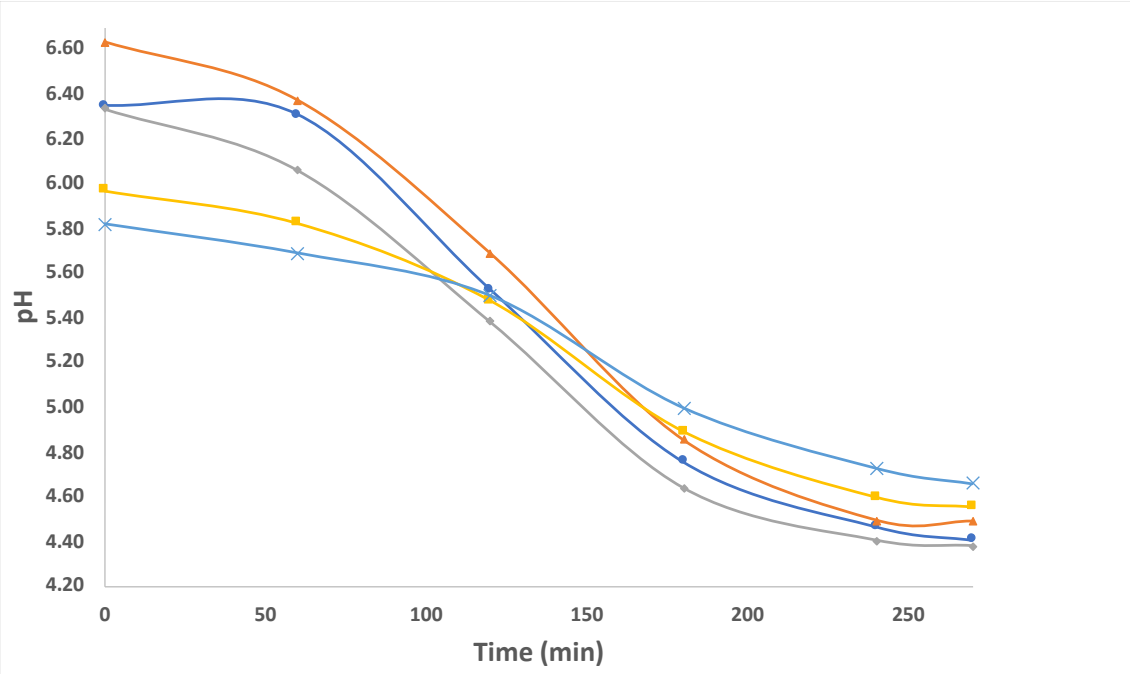

Figure 5.1. – Time-pH curve for yoghurt samples made with corn starch (CS).

CL, CL_CS, A20_CS, A40_CS and A60_CS.

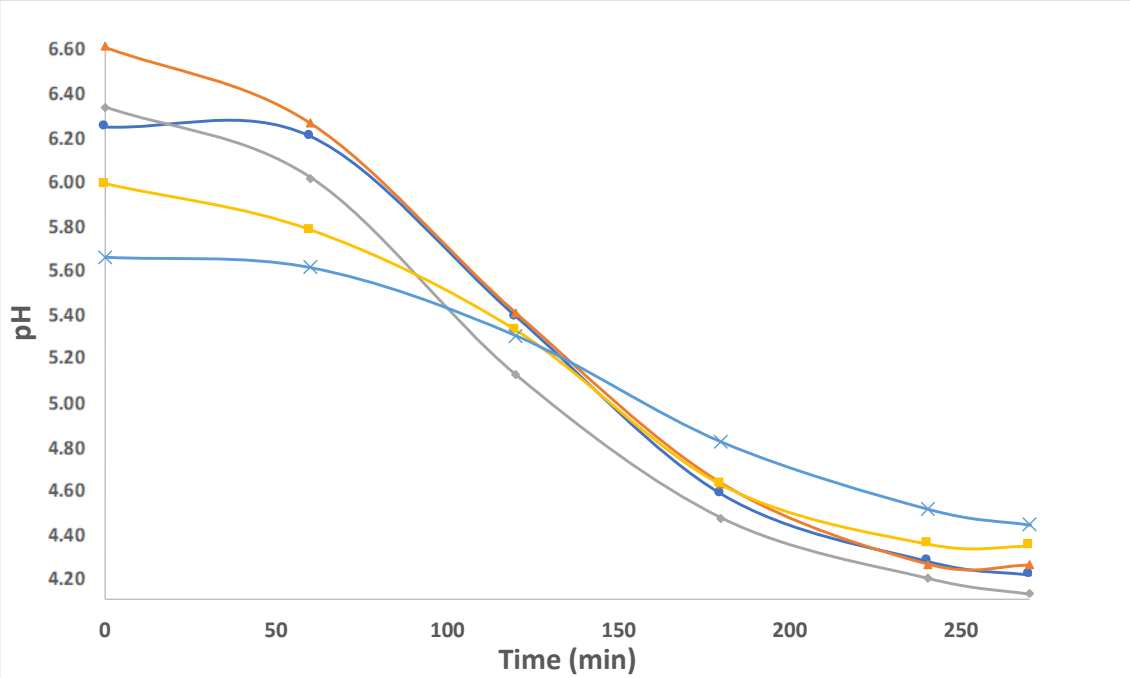

Figure 5.2. – Time-pH curve for yoghurt samples made with waxy rice starch (WRS).

CL, CL_WRS, A20_WRS, A40_WRS and A60_WRS.

Interestingly, the curves for the samples with higher amount of cheese (A40 and A60) start at considerably lower pH values but show higher resistance to decrease the pH at late fermentation stages. At about 2 hours, these curves cross the control curves, showing higher pH values thereafter. CL and A20 samples reach pH 4.6 at about 4 hours, while A40 and A60 take 30 minutes longer. This behaviour can be explained by the buffering capacity provided by the cheese proteins. If fermentation is stopped at 4 hours, for instance, the samples with more cheese will have a higher pH, a feature that can eventually be valorised sensorially by consumers. These samples also represent an example of discrepancy in the results of pH and titratable acidity (next section).

5.3.3. Syneresis and TA

Table 5.3. presents the values of syneresis and titratable acidity (TA) of the various yogucheese samples.

Table 5.3. – Syneresis and titratable acidity (TA) values of the yogurt samples (n = 4).

	Syneresis (%)	**Titratable Acidity (% Lactic Acid)**
CL	22.9 ± 2.4 ^{a}	0.82 ± 0.03 ^{a,b}
CL_CS	37.8 ± 4.8 ^{b}	0.77 ± 0.01 ^{a}
CL_WRS	34.8 ± 4.4 ^{b}	0.75 ± 0.01 ^{a}
A20_CS	31.6 ± 3.4 ^{b}	0.87 ± 0.05 ^{b}
A20_WRS	24.5 ± 3.5 ^{a}	0.96 ± 0.02 ^{d}
A40_CS	24.6 ± 2.0 ^{a}	1.02 ± 0.01 ^{c,d}
A40_WRS	11.8 ± 1.1 ^{c}	1.14 ± 0.05 ^{e}
A60_CS	13.4 ± 0.4 ^{c}	1.05 ± 0.05 ^{c}
A60_WRS	1.9 ± 0.8 ^{d}	1.25 ± 0.06 ^{f}

Samples with the same superscript letter, within the same column, do not present statistical differences among them, according to the Tuckey test.

Regarding syneresis, a comparison among the yogurt samples of plain milk (CL) and milk plus starch (CL_CS and CL_WRS), suggests that the incorporation of starch gives gels with lower whey retention. This might be due to a less regular protein gel structure in the presence of starch. We note that these values of syneresis are consistent with others reported for yogurts prepared with starch as a thickener (Ares et al., 2007). However, the addition of dispersed cheese leads to a significant reduction in syneresis. This effect was particularly strong in the samples with WRS. It is possible that the cheese proteins, and complexes of cheese proteins with starch, lead to free water binding; or that some of the dispersed cheese proteins take part in the continuous acid gel structure. The differences in syneresis between samples with CS and with WRS might originate from differences in the structure of the starch – cheese protein complexes and how they interact, or take part, in the continuous acid gel network. It has been reported that products that contain starch release water mainly due to retrogradation of amylose (Guardeño et al., 2012); as WRS has a very low percentage of amylose, this can then be a preponderant factor for the lower syneresis observed in the samples with this starch. Future studies, with a detailed characterization of these yogurt structures are justified.

Regarding TA, it is observed that, the more cheese is incorporated, the higher the TA values. This is an expected result since cheese itself provides acidity. This can also be seen in Figures 5.1. and 5.2., with lower initial pH values for samples with more cheese. Overall, the TA values obtained are consistent with the ones in literature for other yogurt samples (Bong & Moraru, 2014).

5.3.4. Textural Properties

The textural parameters observed for the yogucheese samples are reported in Table 5.4. The original time – force curves (not shown) indicated that all samples, except CL, resisted fracture under the test conditions.

Table 5.4. – Textural parameters of the yogurt samples (n = 3).

Sample	Hardness (g)	Adhesiveness (g.s)	Springiness	Cohesiveness
CL	218.44 ± 19.31 [a,c]	-150.06 ± 11.93 [a]	0.973 ± 0.002 [a]	0.410 ± 0.002 [a]
CL_CS	93.93 ± 29.27 [b]	-54.19 ± 26.34 [a]	0.986 ± 0.012 [a]	0.520 ± 0.023 [a,c]
CL_WRS	80.84 ± 10.75 [b]	-63.59 ± 11.35 [a]	0.976 ± 0.026 [a]	0.547 ± 0.015 [a,c]
A20_CS	90.60 ± 28.87 [b]	-50.68 ± 15.46 [a]	0.958 ± 0.018 [a]	0.521 ± 0.032 [a,c]
A20_WRS	90.56 ± 16.30 [b]	-70.69 ± 20.70 [a]	0.959 ± 0.002 [a]	0.544 ± 0.020 [a,c]
A40_CS	91.57 ± 16.80 [b]	-63.80 ± 28.46 [a]	0.957 ± 0.012 [a]	0.605 ± 0.010 [b,c]
A40_WRS	118.28 ± 7.86 [a,b]	-99.18 ± 6.18 [a]	0.933 ± 0.004 [a]	0.618 ± 0.021 [b,c]
A60_CS	123.26 ± 44.96 [a,b]	-64.14 ± 36.11 [a]	0.760 ± 0.138 [a]	0.605 ± 0.122 [b,c]
A60_WRS	224.39 ± 3.97 [c]	-171.54 ± 48.86 [a]	0.936 ± 0.011 [a]	0.602 ± 0.018 [b,c]

Samples with the same superscript letter, within the same column, do not present statistical differences among them, according to the Tuckey test.

Coincidently, that control sample of plain milk (CL) has significantly higher hardness ($p < 0.05$) than all the others, except for sample A60_WRS. Addition of starch might interfere with continuity and/or regularity of the acid gel, leading to lower hardness. Mounsey & O'Riordan, 2008, for instance, showed that the addition of starch to imitation cheeses decreased hardness values. Similarly, Mounsey & O'Riordan, 2001; Ye, Hewitt, & Taylor, 2009 concluded that the addition of waxy rice starch to a model processed cheese and an imitation cheese also decreased hardness. Although revealing a similar trend, one must be aware of the differences between the products being compared. Other studies (He et al., 2019; Tavakolipour, Vahid-Moghadam, & Jamdar, 2014) have reported that the addition of modified and resistant starches to yogurt samples increased hardness when comparing with control samples. It is important to refer, however, that there are differences in starch type, methodology of yogurt preparation, and sample handling between these studies and the one presented herein.

In our samples, the addition of ripened cheese led to an increase in hardness, more pronounced in samples with WRS. In fact, sample A60_WRS showed a hardness value similar to that of the milk-control. Although it could be speculated that the cheese protein – starch complexes could hinder regular gel formation, it is also possible that those complexes interact with the acid gel, functioning as active fillers, as stated above.

Adhesiveness varies among samples with a trend similar to hardness, albeit being unrelated properties. The springiness of all samples was high and comparable; the lower value of sample A60_CS is not statistically different from the others. The addition of starch increased cohesiveness relative to the milk control, and further addition of cheese did not alter the effect.

This is another sign of the active interaction between the acid gel formed by the milk protein and the starch-cheese protein complexes from the MCB.

5.3.5. Rheological Properties

The frequency dependence of the rheological parameters elastic modulus (*G'*), viscous modulus (*G''*) and dynamic viscosity for the yogurt samples made with CS and WRS are presented in Figures 5.3. to 5.5. (A and B).

Analysing Figures 5.3. and 5.4 reveals that both *G'* and *G''* have weak frequency dependences, with slight increases, for all samples. This and the fact that the elastic modulus is higher than the viscous modulus (*G'* > *G''*), are characteristics of systems that have a weak gel network (Bong & Moraru, 2014; Lobato-Calleros et al., 2014; Singh & Byars, 2009).

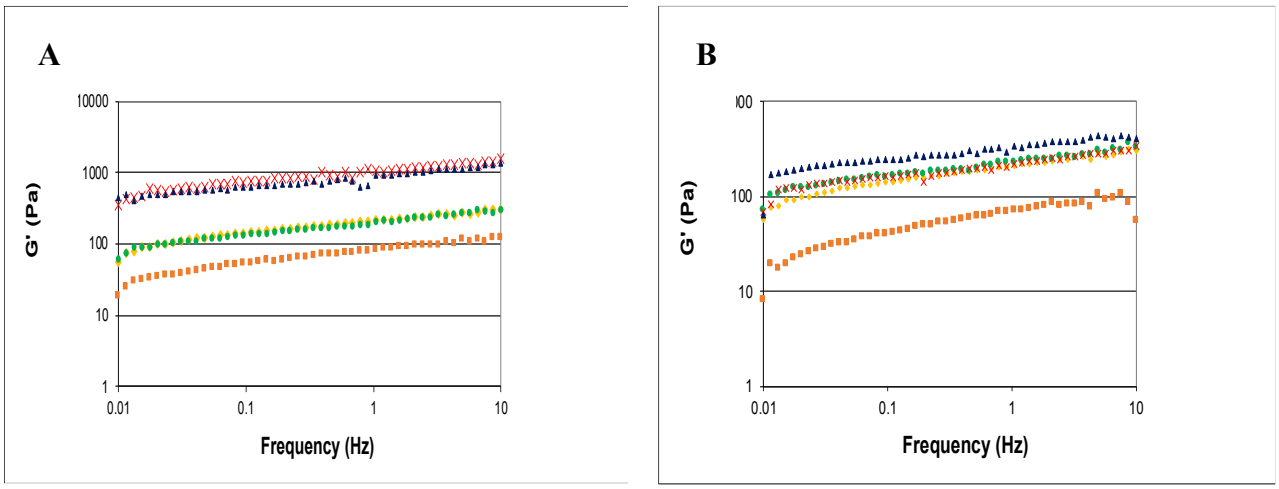

Figure 5.3. – Elastic modulus (*G'*) for yogurt samples containing corn starch (CS) (5.3. A) or waxy rice starch (WRS) (5.3. B) (*n* = 4).

♦ CL, ■ CL_CS, ● A20_CS, × A40_CS and ▲ A60_CS.

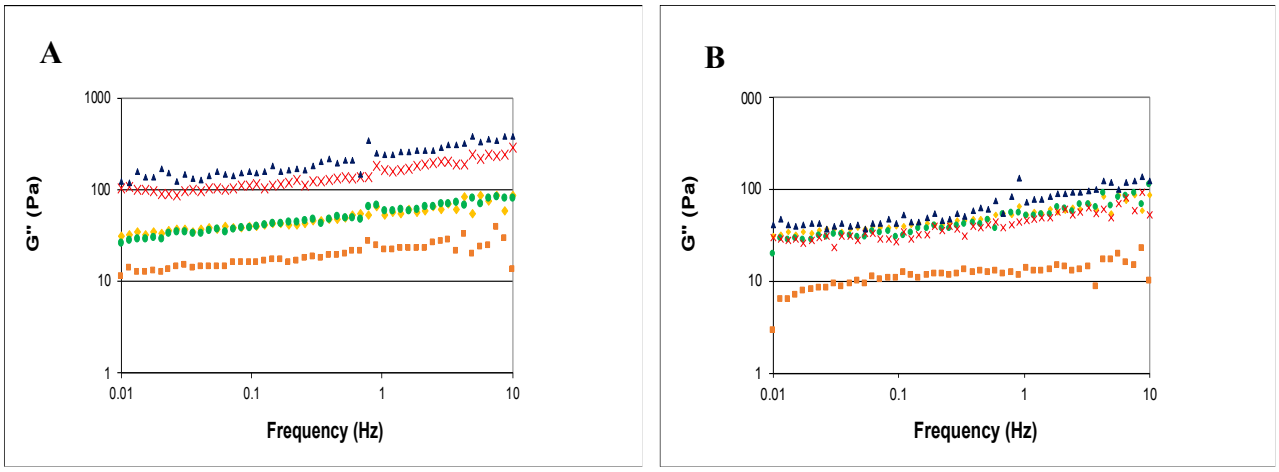

Figure 5.4. – Viscous modulus (*G''*) for yogurt samples containing corn starch (CS) (5.4. A) or waxy rice starch (WRS) (5.4. B) (*n* = 4).

♦ CL, ■ CL_CS, ● A20_CS, × A40_CS and ▲ A60_CS.

The addition of starch, either CS or WRS, leads to lower *G'* values than that of the milk-control sample (CL). The incorporation of cheese increases *G'*, with samples A40 and A60 surpassing CL, particularly when combined with WRS. The differences between CL_CS and A60_CS, and between CL_WRS and A60_WRS, are statistically significant ($p < 0.05$). These last two observations are in line with those regarding hardness by textural analysis discussed above.

The reason for the rheological parameters *G'* and *G''* being slightly higher for the samples with CS, can be due to the higher amylose content of this starch, compared to WRS. Mounsey & O'Riordan, 2008 found that gels from amylose-containing starch had higher elasticity than from amylose-free starch, attributed to the increased rigidity and decreased swelling power of the amylose-containing starch granules.

As seen in Figure 5.5., for all samples, the viscosity decreases with frequency, a characteristic of shear thinning behaviour (Bong & Moraru, 2014). Yogurts have been characterized as a pseudoplastic material with this behaviour being credited to weak electrostatic and hydrophobic interactions within the yogurt matrix, which are easily disrupted by shear (Bong & Moraru, 2014; Lobato-Calleros et al., 2014). CL_CS and CL_WRS have lower viscosity than CL. Regarding the controls, significant differences were found between CL and CL_CS and between CL and CL_WRS, with the difference being higher in this last case. Incorporation of cheese increased viscosity; sample A60_CS showed statistically higher dynamic viscosity ($p < 0.05$) than the control samples CL and CL_CS.

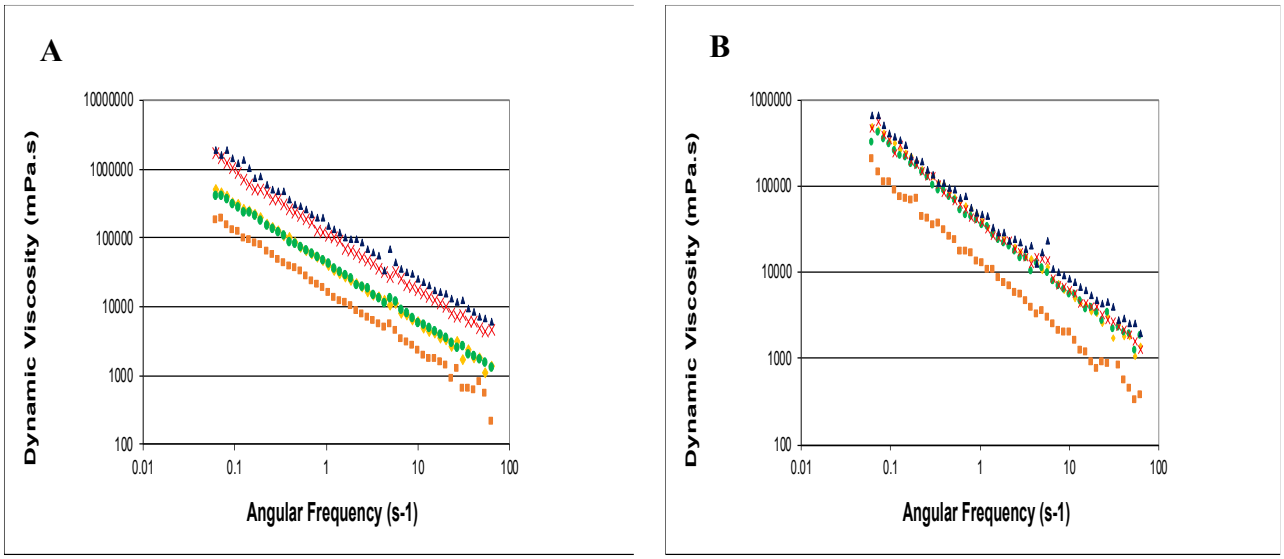

Figure 5.5. – Dynamic viscosity for yogurt samples containing corn starch (CS) (A) or waxy rice starch (WRS) (B) (*n* = 4).

♦ CL, ■ CL_CS, ● A20_CS, × A40_CS and ▲ A60_CS (A)

♦ CL, ■ CL_WRS, ● A20_WRS, × A40_WRS and ▲ A60_WRS (B)

5.3.6. Physicochemical Stability Tests

In order to determine the stability of the samples during storage, syneresis was measured after 14 days of refrigerated storage for a group of representative yogucheese samples.

The results in Table 5.5 show that, after 14 days, the syneresis of the samples remained unaffected ($p > 0.05$). As for the visual aspect of the yogucheeses, also no alterations were noticeable, showing a good stability in all cases.

Table 5.5. – Syneresis values (weight %) for a group of selected yogurt samples, 24 hours and 14 days after fermentation (*n* = 4).

Sample	24 hours	14 days
CL	22.89 ± 2.36 [a]	18.11 ± 3.16[a]
CL_CS	37.78 ± 4.82 [b]	34.77 ± 2.88[b]
CL_WRS	34.81 ± 4.39 [b]	35.19 ± 4.82[b]
A40_CS	24.61 ± 2.03 [a]	25.32 ± 1.19[a]
A40_WRS	11.79 ± 1.13 [c]	13.77 ± 1.67[c]

Samples with the same superscript letter, within the same column and line, do not present statistical differences among them, according to the Tuckey test.

5.4. Conclusions

A successful novel product, a cheese-fortified yogurt, was developed. This was possible by melting the cheese in a hot paste of gelatinized starch. This alternative strategy for utilization of cheese can contribute to food surplus minimization, while bringing additional nutrients and specific flavors to these yogucheeses. Ingredients that are not compatible with clean label designation are not used, as well as sweeteners of any kind. The preparation process requires minimum ingredients, and it is quite straightforward. The adaptation of manufacturers to this novel variant of yogurt would also be relatively simple. Therefore, yogucheeses can potentially represent a novel line of dairy products, economically and environmentally interesting and rewarding, and in line with the recent consumers' trends and demands for more sustainable and health-promoting foods.

The incorporation of CS and WRS, combined with the incorporation of ripened cheese, created yogurt samples with a firmer gel structure, lower syneresis, and higher viscosity. All the yogurts were stable throughout a period of 14 days, with no alterations in their visual aspect and syneresis behavior.

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Chapter 6. A Starch-Milk Paste Enables the Incorporation of Ripened Cheese in Novel Fresh Cheese

Important note: My contributions to the work in this chapter were the following: establishment of parameters of MCBs preparations; support in the realization of certain analytical techniques, such as cheese protein determination and texture analysis; statistical treatment of data; support for the writing of the chapter, in particular data handling.

6.1. Chapter Introduction

Cheese consumption in the European Union (and other parts of the World) has been increasing, with a yearly consumption per capita in 2019 close to 15 kg (CLAL, 2020), as referred in chapter 1. Of this, a significant share comes from different varieties of fresh cheese (e.g. fromage blanc, crème fraîche, cottage cheese, quark, queso fresco, etc.), and from spreadable processed cheese. These products are appreciated for a variety of factors, such as freshness, lightness, versatility to be mixed with a variety of other foods or even beverages, etc. As they do not undertake a ripening stage, their flavour is generally bland.

The production process of fresh cheese is relatively simple and fast: after pasteurization and cooling, the milk, or milk-cream mixture, is standardized, and the coagulation takes place. The coagulation can be acid- and/or rennet-induced. Along with the coagulating factors, other ingredients, such as preservatives and emulsifiers, can be introduced (Litopoulou-Tzanetaki, 2007). In order to remove the whey from the curd, draining or centrifugation of the coagulum can be carried out. In order to avoid the removal of whey at this stage, a more recent technology can be applied, namely ultrafiltration of the starting milk, which separates the smaller lactose, water, minerals, and vitamins molecules, from the larger proteins and fat globules. This results in a milk product concentrated in protein and lower in lactose content (FDA, 2017; Pombo, 2020) but this process also generates a stream of nutritionally valuable liquid (Gaber et al., 2021).

The current research study aimed at the development of a novel renneted fresh cheese product incorporating (ripened) cheese. The latter may come from the surpluses of the dairy industry and/or the retail sector, thus representing a needed mitigation of food waste, since food

waste in the dairy sector for Europe can be over 10%, and for North America and Oceania over 20% (Gustavsson, Cederberg, & Sonesson, 2011). The ripened cheese can provide additional nutritional value to the fresh cheese, particularly added protein, lipids and minerals. It will also bring a multitude of flavour and aroma compounds, as well as varying textures.

Ripened cheese can be dispersed into submillimetre particles when mixed in a hot paste of gelatinized starch, MCBs, as referred in chapter 3. Such dispersions are used, with variants, in the preparation of some simple cheese sauces (Barham, 2012; A. Brown, 2011; Stiavetti & McCord, 2013). This technique for dispersing matured cheese is an interesting alternative to the use of emulsifying salts, namely polyphosphates, generally employed in processed cheese (A. Brown, 2011; Stiavetti & McCord, 2013). Polyphosphates can be health deleterious, at least for certain groups, such as bone disease patients (Černíková et al., 2010), besides imparting a metallic tone to the processed cheese (Stieger & Velde, 2013).

Furthermore, the process here proposed dispenses the step of cutting the coagulum and removal of whey, which can be time consuming and costly, and that produces abundant whey wastage. In this process, the milk whey is incorporated in the final product, contributing with minerals and proteins of high biological value.

Therefore, the aim of this study was the development and testing of a new fresh cheese incorporating ripened cheese, dispersed in a paste of gelatinized starch. The nature of the ripened cheese and the type of starch (corn or waxy rice starch) were studied.

6.2. Materials and Methods

6.2.1. Materials

For the sample preparation, the milk used was semi-skimmed (1.5 % (m/m) fat) HTST milk (Vigor, Portugal). The starches employed were regular corn (Maizena, Unilever) and waxy rice (Remyline XS, BENEO GmbH, Mannheim, Germany, kindly provided by Nutripar, Porto, Portugal). The types of cheese were Emmental (Milbona, Bissingen, Germany), goats' (Queso de Cabra, Lácteas García Baquero S.A., Alcázar de San Juan (Ciudad Real), Spain), ewes' (Queijo Serra Valmadeiros, Indulac - Industrias Lácteas S.A., Oliveira de Azeméis, Portugal), and mature Cheddar (Valley Spire, Dale Farm Ltd., Belfast, UK). Skim milk powder was from Regilait (Nestlé S.A, Vevey, Switzerland). The rennet was a liquid preparation of *Rhizomucor miehei* enzyme (Britex, Lusocoalho, Montes da Senhora, Portugal).

6.2.2. Experimental Methods

6.2.2.1. MCB Preparation

For a typical preparation, corn starch was dispersed in cold milk, at a ratio of 5 g per 100 g milk, in a small non-adherent pan, and the mixture was heated on a hotplate for approximately 5 minutes, with continuous hand stirring, until reaching 85 ^{o}C. Temperature was controlled with a kitchen electronic thermometer (LACOR, Bergara, Spain). At this point, the gelatinization of the starch was noticeable and 20 g of grated or finely cut cheese was added to the milk-starch slurry. The mixture was removed from the hotplate and hand stirred for approximately 30 seconds, until the cheese was fully dispersed, with no visible macroscopic pieces. This *melted cheese base* was left to cool down to room temperature (approx. 21 ^{o}C).

When using waxy rice starch, the procedure was similar, but the milk-starch mixture was heated to 90 ^{o}C and this temperature maintained for 2 minutes, in order to achieve complete starch gelatinization, before cheese addition.

6.2.2.2. Preparation of Fresh Cheeses

Four different control mixtures (without cheese) using milk and the two types of starch were prepared, namely, just HTST milk (sample CT), milk with 2 % (m/m) CS (CT.CS.1) or 2 % (m/m) waxy rice starch (CT.WRS.1), milk with 4 % (m/m) starch (CT.CS.2 and CT.WRS.2), and milk with 2 % (m/m) starch and 2.8 % (m/m) skim milk powder (CT.CS.3 and CT.WRS.3). In order to prepare these samples, milk, milk with 4 % (m/m) gelatinized starch, and milk with 5.7 % (m/m) skim milk powder, were used and mixed in different proportions, according to Table 6.1. The latter was prepared by adding 6 g skim milk powder to 100 g fluid milk, and the solution left 24 hours at 4 ºC, for the powder to fully rehydrate.

For samples containing ripened cheese, we further used a melted cheese base incorporating 4% (m/m) corn starch and 16 % (m/m) of Emmental cheese. Then, samples with an amount of cheese varying between 3.2 and 8.0 % (m/m) (samples EM.CS.1 to 5) were prepared (Table 6.1.). One of the samples (EM.CS.5) included also skim milk powder at 2.8 % (m/m). Similar samples were prepared with waxy rice starch (EM.WRS.1 to 5) (Table 6.1.).

Two other sets of samples were prepared with ewes' cheese, using either corn starch (EW.CS.1 to 5), or waxy rice starch (samples EW.WRS.1 to 5) (Table 6.1.).

Table 6.1. – Mixing of different preparations in the formulation of control and fresh cheese samples. Percentages are (w/w).

Sample	Milk (g)	Milk + 4 % Starch (g)	Milk + 5.7 % SMP (g)	MCB 16 % Cheese (g)
CT	100	0	0	0
CT.CS.1 CT.WRS.1	50	50	0	0
CT.CS.2 CT.WRS.2	0	100	0	0
CT.CS.3 CT.WRS.3	0	50	50	0
EM.CS.1 EM.WRS.1	50	30	0	20
EM.CS.2 EM.WRS.2	50	20	0	30
EM.CS.3 EM.WRS.3	50	10	0	40
EM.CS.4 EM.WRS.4	50	0	0	50
EM.CS.5 EM.WRS.5	0	0	50	50
EW.CS.1 EW.WRS.1	50	30	0	20
EW.CS.2 EW.WRS.2	50	20	0	30
EW.CS.3 EW.WRS.3	50	10	0	40

EW.CS.4	50	0	0	50
EW.WRS.4				
EW.CS.5	0	0	50	50
EW.WRS.5				

CT: milk control; CT.CS and CT.WRS: milk control with corn starch (CS) or waxy rice starch (WRS); EM.CS.1 to 5 and EM.WRS.1 to 5: fresh cheese samples with varying amounts of Emmental cheese (EM), with CS or WRS; EW.CS.1 to 5 and EW.WRS.1 to 5: fresh cheese samples with varying amounts of ewes' cheese (EW); some samples also have skimmed milk powder (SMP).

The renneting of all samples was done by adding 240 µL liquid rennet per 100 g of mixture, pre-equilibrated in a thermostable waterbath (TW20, JULABO GmbH, Seelbach, Germany). The temperature was maintained at 35 ºC for 15 minutes (until coagulation was completed). The flasks with the samples were then transferred to 4 ºC.

Several batches of all samples were prepared, in order to prepare the following tests.

6.2.2.3. Physicochemical Analysis

6.2.2.3.1. Determination of Total Solids

The determination of the total solids was carried out for a representative group of samples, namely for the ewes' and Emmental ripened cheese, and for fresh cheese samples EW.CS.4 and EM.CS.4. Aluminium dishes were pre-dried (105 ºC, 1 h) and weighed. 3-5 g of sample were placed in the dishes and the weight recorded. The samples were dried in an oven to constant weight (Drying Oven d-6450, Heraeus Thermo Fisher Scientific, Waltham, MA, USA), for 24 h, at 105 ºC. The samples were cooled in the desiccator and the total solids was determined following the AOAC Official Method 925.23 (AOAC, 1990), using Eq. 6.1:

$$\%\, Total\ Solids = \frac{dry\ weight}{wet\ weight} \times 100 \qquad \text{(Eq. 6.1)}$$

Two batches of each sample type were subjected to analysis and, in each case, duplicate measurements were taken. Results are averages ± SD.

6.2.2.3.2. Protein Content Determination

The total protein was determined for the Emmental and ewes' cheese in duplicate samples, using a non-conventional method. It is based on the one proposed by Reichardt & Eckert (Reichardt & Eckert, 1991), but with modifications (Paula Vilela, Gomes, & Ferreira, 2020). A standard casein solution was prepared by adding 0.405 g of bovine casein, (Sigma-Aldrich, St. Louis, MO, USA) in 50 mL of deionized water, followed by heating to 40 ^{o}C and gently stirring. The absorbance at 280 nm of this solution and several dilutions were determined (Spectronic Helios Gamma UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) and a calibration curve of protein concentration was thus obtained.

For the determination of the protein content of cheese, about 1.33 g of cheese cut in small pieces was placed in 30 mL of 0.1 M NaOH and left overnight. The following day, the mixture was placed in a waterbath (TW20, JULABO GmbH, Seelbach, Germany) at 40 ^{o}C, for 10 min and mixed well. After cooling down, it was centrifuged (Universal 320R, Andreas Hettich GmbH, Germany, centrifuge) at 4000 x *g*, 4 ^{o}C, for 10 min. The top layer of fat was removed, the underlying supernatant was collected, and its volume evaluated. The absorbance at 280 nm was measured by diluting 80 µL of the supernatant with 920 µL of 0.1 M NaOH. The concentration was calculated from the casein curve, and the protein content in cheese then calculated. The determinations were done in duplicate samples of cheese and the averages ± SD were calculated.

We have shown that this method gives results that are not statistically different from those obtained with the standard Kjeldahl method (Paula Vilela et al., 2020).

6.2.2.3.3. Fat Content of Ripened Cheese Determination

The fat content of the different types of ripened cheese was measured by the Van Gulik method (ISO 3433, 2008). The cheese was grated or cut in small pieces, and precisely 3 g were placed in the lower part of the Van Gulik butyrometer (cheese butyrometer 0 – 40%, Gerber Instruments, Effretikon, Switzerland). 15 ml of sulfuric acid 62 %, d =1,522 ± 0.005, according to Van Gulik, were added, the butyrometer was tapped and put in a waterbath at 65 ^{o}C until the cheese was fully digested. Then, 1 ml of isoamyl alcohol was added and the contents were mixed thoroughly. Additional sulfuric acid was added, up to the point that the liquid reached the 35 % mark of the butyrometer scale. The butyrometer was tapped and the contents were mixed thoroughly once again. The samples were centrifuged in a Gerber centrifuge (NormMilk centrifuge, International PVI, Milan, Italy) for 6 min, at 1200 rpm and 65 ^{o}C. After the centrifugation, the reading of the fat content was done directly on the butyrometer. The determinations were done in duplicate and the average ± SD were calculated.

6.2.2.3.4. Determination of Syneresis

Control samples and fresh cheese samples EM.CS.2, EM.CS.4, EW.WRS.2 and EW.WRS.4 were centrifuged at 1,500 x *g* for 15 min, at 20 ºC (Zamora et al., 2012), and the mass of the pellet and supernatant (separated whey) were measured. The syneresis was calculated as seen in Eq. 6.2 (Wolfschoon-Pombo, Dang, & Chiriboga, 2018):

$$\%\ Syneresis = \frac{Amount\ of\ whey\ separated}{Amount\ of\ sample\ added} \times 100 \qquad \text{(Eq. 6.2)}$$

Two batches of each sample type were subjected to analysis and, in each case, duplicate measurements were taken. Results are averages ± SD.

6.2.2.3.5. Textural Profile Analysis

For the texture profile analysis, the renneted samples rested at 4 ºC for 24 hours, and then they were equilibrated at 15 ºC. A Stable Micro Systems TA.XT Plus Texture Analyzer (Surrey, UK) with a cylindrical probe of 36 mm diameter was used. The fresh cheese samples were tested in the glass flasks of approximately 6 cm-diameter in which they were renneted, without any transfer to avoid gel breakage. A typical texture profile analysis cycle was used, with a trigger force of 5 g. The probe test speed was 1 mm/s and the maximum deformation was set at 12 mm, which corresponded to 33-40 % of sample height. The waiting time between the two compressions was 5 seconds (Khanal et al., 2019; Pons & Fiszman, 1996). The measurements were done in samples of three distinct batches, as this is a destructive test. The average ± SD of each sample type was calculated.

6.2.2.3.6. Sensory Analysis

The sensory analysis was carried out by a consumer panel of 40 people from the University community, with ages between 15 and 45. Fresh cheese (600 mL each, prepared in 1 L glass flasks) incorporating 6.4 % of four different (ripened) types of cheese were prepared: goats' cheese (sensorial sample S.GT), ewes' (S.EW), and two with Cheddar cheese, without and with skim milk powder at 2.8 % (sensorial samples S.CH and S.CH.SMP). The starch selected for this purpose was the waxy rice, as preliminary trials showed that it led to fresh cheese with a smoother texture and no perceptible starch taste. Also, albeit the physicochemical studies here reported used Emmental cheese, the sensorial tests employed the stronger flavoured Cheddar, and included a goats' cheese. After resting overnight at 4 ºC, the fresh cheese samples were equilibrated in a waterbath (TW20, JULABO GmbH, Seelbach, Germany) at 8 ºC, and

maintained at this temperature throughout the session. The panellists were served with a spoonful of each sample in a small plastic cup, upon arrival. The samples were identified by randomized codes. The panellists were asked to give ratings on a 9-point hedonic scale for each product, regarding the following attributes: appearance, odour, texture, flavour, and overall evaluation of the product. In addition, they were asked to evaluate the intensity of the flavour of each sample, as too weak, ideal or too strong.

6.2.2.3.7. Statistical Analysis

The statistical analysis of these results was made using an ANOVA one-way test, with the application of the Tuckey test for pairwise comparisons between particular samples. The normality of the data, as well as the homogeneity of variances was verified, and the SPSS software (SPSS® Statistics version 27.0.1 for Windows®) was used for the statistical analysis of the results.

6.3. Results and Discussion

6.3.1. Key Variables in Fresh Cheese Preparation

In this research, a novel renneted fresh cheese was developed based on melted cheese bases. Figure 6.1. shows an overall view of a sample.

Figure 6.1. – Image of a fresh cheese sample, incorporating Emmental cheese and skimmed milk powder (sample EM.CS.5).

Fresh cheese is generally produced by renneting milk, or a mixture of milk and cream, and then draining the curd, in order to obtain higher concentrations of protein and fat. Alternatively, a few dairy industries start by concentrating the milk by ultrafiltration before renneting. In this latter case, the coagulation can be carried out already in the final package, and, in order to achieve a product safety and a longer shelf life, the product can be further subjected to heat treatment. In either process, though the product has an increased protein (and fat) content, the flavour is bland compared to ripened cheese. Our technique enables the inclusion of ripened cheese - which could eventually become food waste - into the matrix of the novel fresh cheese, providing additional high value protein, fat, salts, texture and flavour to the product. The production and consumption of fresh cheese varieties have been facing a steady and continuous increase over the past years and constitute a major proportion of the cheese consumed in many countries, including Portugal, where the traditional form of fresh cheese is designated "*queijo fresco*". Fresh cheese varieties may vary between a softer creamy texture to a more continuous

gel suitable for cutting. Extreme softness, brittleness or graininess, and lower yields are some of the drawbacks pointed out in conventional fresh cheese manufacture; our technique enables the achievement of a more consistent and continuous curd at higher yields. It should also be noted that the quality and nutritional balance of fresh dairy products, including fresh cheese varieties, the versatility of their consumption (home cooking and processing use) and longer shelf-life strategies have been pointed out as important features to contribute to increasing EU net exports of fresh dairy products by 2030 (European Comission, 2020). The novel fresh cheese developed herein contributes to this product competitiveness where an improved nutritional profile (corresponding to the evolving consumers demands), a higher and much appreciated sensorial quality, an improved versatility of consumption and a cost-effective production adaptability with reduction of waste leading to higher yields, are all contributing features, and in the medium-long term they lead to higher *per capita* consumption.

From a technological point of view, differences were observed in the production of conventional and melted cheese base - related renneted fresh cheese; the addition of fungal rennet to plain semi-skimmed milk resulted, after 20 minutes, in a soft gel that, when cut and slightly squeezed, surfaced whey, whereas the renneting of preparations with MCBs was completed in approximately 15 minutes, a shorter time relative to the above control. No surfacing of whey was observed when these gels were cut.

Preliminary trials were carried out varying the amount of cheese and that of starch in the final mixture. Incorporation of cheese above a final mass concentration of approximately 8 % (m/m) resulted in a too soft, almost fluid, texture of the fresh cheese. The conclusion being that the incorporation of dispersed ripened cheese inhibited gel formation. In fact, in the melted cheese base, the sub-millimetre cheese particles are coated with starch, as revealed by microscopy. It is thus understandable that these particles act as inert fillers, hindering gel formation by the renneted milk caseins. Therefore, the microstructure of the fresh cheese is envisaged as a typical fresh rennet gel (Arocas et al., 2010; Brown, McManus, & McMahon, 2012) that incorporates dissolved starch fragments and dispersed starch-coated ripened cheese particles.

Having an amount of starch above 2 % in the final mixture, although leading to stronger gels, can also lead to a perceptible starch taste in the product, particularly when corn starch is used. Therefore, based on these observations, standard fresh cheese samples had a fixed final starch concentration of 2 % and an 8 % limit for ripened cheese incorporation.

6.3.2. Total Solids in Ripened Cheese and in Fresh Cheese Samples

The determination of dry matter content (total solids) was carried out for two types of ripened cheese and two representative fresh cheese samples incorporating 6.4 % (m/m) of those same cheese varieties. These samples were selected for having identical content of ripened cheese as the ones in sensorial analysis. Duplicate measurements were carried out and the average ± SD are presented in Table 6.2.

Table 6.2. – Dry matter content (w/w) of two ripened cheeses and two fresh cheese samples (n = 4).

Samples	Total solids ± SD (%)
Emmental cheese	61.6 ± 0.64[a]
Ewes' cheese	57.8 ± 0.21[a]
EM.CS.4	15.1 ± 0.02[b]
EW.CS.4	15.1 ± 0.17[b]

Samples with the same superscript letter, within the same column, do not present statistical differences among them, according to the Tuckey test. EM.CS.4 and EW.CS.4: fresh cheese samples containing either Emmental cheese (EM) or ewes' cheese (EW), and corn starch (CS).

As the total solids of the Emmental and ewes' cheese were similar, the two fresh renneted types of cheese also had identical results, as expected. ANOVA-one way test was applied between the pairs of samples Emmental / Ewes' cheese and EM.CS.4 / EW.CS.4, showing that the samples of each pair were not significantly different ($p < 0.05$) in this regard. Regarding the fresh cheese samples, we note that the level of total solids is within typical values of several varieties of conventional fresh cheese varieties, such as cottage cheese, ymer, or *fromage blanc* (Cesbron-Lavau et al., 2017; Walstra, Wouters, & Geurts, 2006).

6.3.3. Total Protein in Ripened Cheese

Using our modified method of Reichardt & Eckert, 1991, with cheese dispersed in sodium hydroxide solution, followed by evaluation of the protein in the fat-free solution by UV absorbance, the protein content of Emmental cheese was 29.8 ± 0.25 % (w/w) and that of ewes' cheese was 21.3 ± 0.42 % (w/w). These results are very close to the values indicated in the product labels, namely 28 % and 23 %, respectively. This shows that the method employed is quite precise, and it can be used in alternative to the lengthy Kjeldahl one. In fact, previous work in our laboratory has shown a good agreement between the two methods (Paula Vilela et al., 2020).

6.3.4. Total Fat in Ripened Cheese

The Van Gulik method applied to Emmental, ewes', and goats' ripened cheeses indicated fat contents (± SD) of 29 ± 0.07 % (m/m), 27 ± 0.71 %, and 35 ± 0.00 %, respectively. These values are also close to the ones indicated on the products labels (28 %, 30 %, and 35 %, respectively).

The Van Gulik method was also carried out for samples of the fresh renneted cheeses. However, probably due to interference of the starch, the method was not successful.

6.3.5. Macronutrient Composition of Fresh Cheese Samples

Table 6.3. presents the macronutrient composition of fresh cheese samples incorporating EM and EW cheese. The results were determined based on the corresponding compositions of the raw materials - milk, cheese, and starches. We considered that the validation of the macronutrient composition of the starting materials – particularly the ripened cheese-, as described above, is sufficient for a reliable estimate of the corresponding composition of the fresh renneted cheese. These have a more complex matrix, with some analytical methods, in particular fat determination, being more prone to interferences.

Table 6.3. – Macronutrient composition of the fresh cheese samples.

Sample	Proteins (g/ 100 g)	Lipids (g/ 100 g)	Carbohydrates (g/ 100 g)
EM.CS.1	4.2	2.4	6.9
EM.CS.2	4.6	2.9	6.9
EM.CS.3	5.0	3.3	6.9
EM.CS.4	5.4	3.8	6.6
EW.CS.1	3.9	2.4	6.9
EW.CS.2	4.2	2.8	6.9
EW.CS.3	4.5	3.2	6.9
EW.CS.4	4.8	3.6	6.8
EW.CS.5	5.4	3.6	7.8
EM.WRS.1	4.2	2.4	6.8
EM.WRS.2	4.6	2.9	6.8
EM.WRS.3	5.0	3.3	6.7
EM.WRS.4	5.4	3.8	6.7
EM.WRS.5	6.1	3.8	7.6
EW.WRS.1	3.9	2.4	6.8
EW.WRS.2	4.2	2.4	6.8
EW.WRS.3	4.5	2.8	6.7
EW.WRS.4	4.8	3.2	6.7
EW.WRS.5	5.4	3.6	7.6

EM.CS1 to 5 and EM.WRS1 to 5: fresh cheese samples with varying amounts of Emmental cheese (EM), with corn starch (CS) or waxy rice starch (WRS); EW.CS1 to 5 and EW.WRS1 to 5: fresh cheese samples with varying amounts of ewes´ cheese (EW); samples with number 5 also have skimmed milk powder (SMP).

The above-mentioned experimental results for total solids of samples EM.CS.4 and EW.CS.4 (15.1 % for both) can be compared with the sum of macronutrients in these tables, 15.8 % and 15.2 %, respectively. Albeit these last numbers do not incorporate the (minor) ash contents, the proximity of results supports our methodology.

The composition of this fresh renneted cheese can be compared to those of commercial products. A well-known commercial spreadable processed cheese (Philadelphia, Kraft Foods) has a protein content around 5.4 % for the original version, and 7.4 % for the light version (Mendelez International, 2021). A review of different brands of *fromage blanc* in the French market points to an average protein content of 5.7 % (Cesbron-Lavau et al., 2017). In terms of fat content, the Original Philadelphia has 21 %, and the light version 11 % (Mendelez International, 2021), while the average of fromage blanc is 7.8 % (Cesbron-Lavau et al., 2017). Our samples have similar protein content, but the formulation can be easily adapted for higher protein, by simply increasing the amount of ripened cheese and/or skim milk powder (see below). In terms of fat, our fresh cheese has significantly lower content than the Philadelphia (or other brands) cream cheese, and also lower than the standard *fromage blanc*, which can be envisaged as a healthful feature. These observations are also in agreement with a recent study of six commercial cream cheeses (Macdougall et al., 2019).

An issue can be raised regarding the salt level on the fresh cheese, as ripened cheeses generally have a considerable concentration. As the only ingredient contributing significantly to salt level is the ripened cheese, attending that all varieties used have salt levels below 1.9 g/100 g, then it imparts in the fresh cheese, at the incorporations used, a salt level below 0.15 g/100 g, which is fairly low. We point out that there is no salting step in the preparation procedure.

The control samples of plain milk had a pH of 6.4, and those of milk plus starch of 6.6. The samples with ripened cheese had, as expected, slightly lower pH, with values reaching 5.8 for those with the highest incorporation. These values are higher than those of common fresh cheese varieties, which, in most cases, are within 4.5 – 5.0 (Walstra et al., 2006); as our samples did not undergo an acidification step, either by fermentation or acid addition.

6.3.6. Level of Syneresis

The determination was done for a meaningful set of samples, in duplicate, and the average ± SD of the measurements is presented in Table 6.4. The control sample CT, containing only renneted milk, shows the highest syneresis values. Incorporation of corn starch or waxy rice starch (CT.CS.1-3 and CT.WRS.1-3) lead to a decrease in syneresis, with the difference to the control CT being statistically significant ($p < 0.05$) at the 4 % (w/w) concentration. Corn starch and waxy rice starch, at equal concentrations, showed no significant differences among them ($p > 0.05$), therefore, we can conclude that starch addition contributes to water retention in the gel and lower syneresis.

Table 6.4. – Average values for syneresis of fresh cheese samples. All percentages are (w/w) (n=4).

Samples	Starch (%)	Ripened Cheese (%)	SMP (%)	Syneresis ± SD (%)
CT	-	-	-	(48 ± 2.8)[a]
CT.CS.1	2	-	-	(23 ± 0.1)[a,c]
CT.CS.2	4	-	-	(11 ± 2.1)[b,c]
CT.CS.3	2	-	2.8	(21 ± 3.6)[a,c]
CT.WRS.1	2	-	-	(36 ± 0.1)[a,c]
CT.WRS.2	4	-	-	(10 ± 0.3)[b,c]
CT.WRS.3	2	-	2.8	(22 ± 1.8)[a,c]
EW.CS.2	2	4.8	-	(37 ± 0.1)[a,c]
EW.CS.4	2	8	-	(39 ± 1.3)[a,c]
EW.WRS.2	2	4.8	-	(42 ± 0.0)[a]
EW.WRS.4	2	8	-	(40 ± 0.2)[a,c]

Samples with the same superscript letter, within the same column, do not present statistical differences among them, according to the Tuckey test. CT: milk control; CT.CS.1 and 2 and CT.WRS.1 and 2: milk control with corn starch (CS) or waxy rice starch (WRS); CT.CS.3 and CT.WRS.3 also have skimmed milk powder (SMP); EW.CS.2 and 4 and EW.WRS.2 and 4: fresh cheese samples with ewes´ (EW) cheese.

Addition of 2.8 % (m/m) skim milk powder also seemed to contribute to an additional reduction in syneresis, when samples with the same amount of either corn starch or waxy rice starch (2 %) are compared, although with no statistical significance ($p > 0.05$). This observation deserves further studies using a higher concentration of skim milk powder.

The addition of ripened cheese to fresh cheese samples prepared with corn starch lead to higher syneresis values (though not statistically significant), and in samples with waxy rice starch the addition of ripened cheese affected syneresis minimally, comparing with corresponding controls. In both cases, increasing from 4.8 % to 8 % cheese incorporation did not change syneresis significantly ($p > 0.05$). Therefore, one can conclude that the presence of starch is the factor with the higher impact on syneresis, as seen also in previous studies (Azim et al., 2010; Rodríguez-Hernández et al., 2006).

6.3.7. Textural Analysis

TPA measurements were done in triplicate samples and the average ± SD of the three measurements is presented in Table 6.5. The parameters selected were hardness, adhesiveness, springiness, and cohesiveness.

Table 6.5. – Results of textural analysis for fresh cheese samples (n=3).

Sample	Hardness (g)	Adhesiveness (g.s)	Springiness	Cohesiveness
CT	(139.71 ± 11.18)^{a,c,d,f}	(-31.13 ± 7.43)^{a}	(0.97 ± 0.01)^{a}	(0.47 ± 0.03)^{a}
CT.CS.1	(98.08 ± 3.30)^{a,c,d,e}	(-63.63 ± 3.17)^{a}	(095 ± 0.01)^{a}	(0.51 ± 0.00)^{a,b}
CT.CS.2	(166.51 ± 12.34)^{a,b,f}	(-190.52 ± 16.56)^{b,c}	(0.93 ± 0.01)^{b}	(0.50 ± 0.01)^{a,b}
CT.CS.3	(146.78 ± 16.9)^{a,c,f}	(-110.59 ± 21.30)^{a}	(0.95 ± 0.00)^{a}	(0.48 ± 0.02)^{a,b}
CT.WRS.1	(104.92 ± 15.81)^{a,c,d,e}	(-65.21 ± 12.05)^{a}	(0.95 ± 0.02)^{a}	(0.62 ± 0.0)^{c}
CT.WRS.2	(147.66 ± 76.8)^{a,c,f}	(-258.99 ± 151.91)^{b}	(0.91 ± 0.03)^{b}	(0.5 ± 0.00)^{b}
CT.WRS.3	(227.30 ± 27.58)^{b,f}	(-209.75 ± 1.75)^{b,d}	(0.94 ± 0.00)^{a,b}	(0.52 ± 0.00)^{a}
EM.CS.1	(94.05 ± 7.02)^{a,c,d,e}	(-63.1 ± 8.70)^{a,c,e}	(0.96 ± 0.00)^{a,b}	(0.48 ± 0.00)^{a,b}
EM.CS.2	(77.38 ± 6.14)^{c,d,e}	(-50.9 ± 11.8)^{a,c,e}	(0.96 ± 0.00)^{a,b}	(0.51 ± 0.00)^{a,b}
EM.CS.3	(67.61 ± 0.39)^{d,e}	(-43.24 ± 3.6)^{a,c}	(0.96 ± 0.00)^{a,b}	(0.53 ± 0.00)^{a,b}
EM.CS.4	(54.74 ± 1.45)^{e}	(-26.69 ± 1.5)^{a,c}	(0.97 ± 0.00) ^{a,b}	(0.61 ± 0.00)^{a,b}
EM.CS.5	(99.11 ± 4.76)^{a,c,d,e}	(-69.84 ± 12)^{a,c,d}	(0.95 ± 0.00)^{a,b}	(0.52 ± 0.00)^{a,b}
EW.CS.1	(84.43 ± 2.97)^{c,d,e}	(-49.82 ± 7.75)^{a,c,e}	(0.96 ± 0.01)^{a,b}	(0.52 ± 0.04)^{a,b}
EW.CS.2	(79.53 ± 4.79)^{c,d,e}	(-54.41 ± 2.98)^{a,c,e}	(0.96 ± 0.00)^{a,b}	(0.51 ± 0.03)^{a,b}
EW.CS.3	(63.2 ± 8.84)^{d,e}	(-31.54 ± 11.24)^{a,c}	(0.97 ± 0.00)^{a,b}	(0.56 ± 0.01)^{a,b}
EW.CS.4	(57.33 ± 3.27)^{e}	(-24.55 ± 9.29)^{a,c}	(0.97 ± 0.01)^{a,b}	(0.58 ± 0.03)^{a,b}
EW.CS.5	(118.52 ± 2.03)^{a,c,d,e,f}	(-84.59 ± 12.28)^{a,c,d}	(0.97 ± 0.02)^{a,b}	(0.46 ± 0.01)^{a,b}
EW.WRS.1	(107.61± 10.03)^{a,c,d,e,f}	(-92.5 ± 134.58)^{a,c,d}	(0.94 ± 0.00)^{a,b}	(0.53 ± 0.02)^{a,b}

EW.WRS.2	(97.63 ± 6.52)^{a,c,d,e}	(-77.25 ± 115.76)^{a,c,d}	(0.94 ± 0.01)^{a,b}	(0.53 ± 0.01)^{a,b}
EW.WRS.3	(85.66 ± 4.53)^{c,d,e}	(-63.89 ± 99.92)^{a,c,e}	(0.95 ± 0.00)^{a,b}	(0.54 ± 0.02)^{a,b}
EW.WRS.4	(72.81 ± 3.57)^{c,d,e}	(-47.09 ± 79.53)^{a,c,e}	(0.95 ± 0.00)^{a,b}	(0.57 ± 0.00)^{a,b,c}
EW.WRS.5	(183.04 ± 2.8)^{f}	(-184.89 ± 31.33)^{b,d,e}	(0.94 ± 0.00)^{a,b}	(0.47 ± 0.00)^{a,b}

Samples with the same superscript letter, within the same column, have no statistical differences among them, according to the Tuckey test. CT: milk control; CT.CS and CT.WRS: milk control with 2% corn starch (CS) or waxy rice starch (WRS); EM.CS.1 to 5 and EM.WRS.1 to 5: fresh cheese samples with varying amounts of Emmental cheese (EM), with CS or WRS; EW.CS.1 to 5 and EW.WRS.1 to 5: fresh cheese samples with varying amounts of ewes´ cheese (EW); some samples also have skimmed milk powder (SMP).

The force – deformation curves (not shown) revealed that all samples fractured near or at the hardness level (where the maximum force was applied), except sample CT.WRS.3, which did not fracture. The majority of the cheese samples fractured between 55 and 100 g applied force. The fact that CT.WRS.3 did not fracture resides in the particular resistance of this gel, provided by both the gelatinized waxy starch and the additional rennetable casein from skim milk powder.

Regarding hardness, for control samples, addition of 2% starch (CT.CS.1 or CT.WRS.1) lead to slightly lower values than those of the milk control (CT), but 4 % starch (CT.CS.2 or CT.WRS.2) reverted the trend; however, the differences are not statistically significant ($p > 0.05$).

In control samples added with skim milk powder (CT.CS.3 and CT.WRS.3), the one prepared with waxy rice starch showed a significant difference ($p < 0.05$) with the other control samples. As said above, skim milk powder increases the protein content, which creates a higher concentration of rennetable, gel-forming casein molecules, resulting in a more rigid structure.

Among the samples with ripened cheese, a common trend is observed, namely, increasing the amount of cheese results in lower values for hardness. The dispersed ripened cheese acts as an inert filler, therefore hindering the formation of a more continuous and homogeneous gel network, thus explaining the impact on hardness. But, as with the control samples without cheese, addition of skim milk powder increased considerably the hardness of the gel, particularly in the presence of waxy rice starch.

The initial slope of the force – deformation curve gives an apparent modulus of elasticity of the gels (Shirvani, Ghanbarian, & Ghasemi-Varnamkhasti, 2014). We have evaluated such slopes for a representative number of samples (data not shown). While the control CT sample had a slope of 20 N/cm, in the controls with starch (CT.CS.1 and CT.WRS.1) the number decreased to 10-12 N/cm, that is, resulted in a softer gel. However, inclusion of skim milk powder

(CT.CS.3 and CT.WRS.3) results in an almost 3-fold higher value (36-39 N/cm). Therefore, the inclusion of skim milk powder makes the gel significantly stiffer. Samples incorporating ripened cheese have lower elasticity than CT, indifferently of having corn starch or waxy rice starch. These results are in alignment with those previously reported for hardness.

Regarding adhesiveness, control samples with starch, either corn starch or waxy rice starch, lead to increased (negative) values. On the other hand, addition of SMP decreased adhesiveness of control samples. Samples incorporating ripened cheese showed lower adhesiveness than corresponding controls, and without a significant variation with the level of cheese incorporation. We note that the relatively high values of standard deviation reported for adhesiveness in same samples is a common feature of this analysis, particularly with heterogeneous matrixes, as it is the case (Kealy, 2006; Sandoval-Castilla et al., 2004). But one can conclude that starch is the ingredient with the main impact on this property.

As for the cohesiveness values, controls and samples with ripened cheese showed similar values among them, albeit the statistical analysis showed differences in a few cases. For instance, CT and CT.WRS.2, and CT and CT.WRS.1, have statistically significant differences ($p < 0.05$), indicating that samples with 2 % and 4 % waxy rice starch are more cohesive than the basic milk control.

All samples showed springiness values close to 1, which indicates that they sprung back almost entirely after the first compression, albeit the above-mentioned gel breakage.

6.3.8. Sensory Analysis

A total number of 40 participants were included in the sensory analysis of the fresh cheese samples, predominantly young people, reflecting a university campus: 40 % were below 24 years-old, 37 % between 25 and 34, 10 % between 35 and 45, and 13 % were above 45.

The panel of participants reported a frequent consumption of dairy products, including cheese (83 % of the participants), milk (75 %), and yoghurt (65 %), with a slightly higher preference for cheese, which makes them a good consumer panel for this product.

Regarding the different modes of consumption of fresh cheese, 63 % of the consumers of the panel slice it over bread or toast, 55 % consume it in salads, and 43 % might also eat it by itself. The attributes most enjoyed in fresh cheese were reported to be freshness (75 %), followed by texture (55 %), and flavour (48 %). 30 % of the participants are also interested in its low caloric content.

For the purpose of these sensorial tests, we selected the same ripened ewe´s cheese used in the above studies but substituted the (cows') Emmental for Cheddar cheese (CH), for its stronger flavour, and also included a goat´s cheese (GT). All three were used at 6.4 % (m/m) in

the fresh cheese samples. A fourth sample, made with Cheddars', included also skim milk powder for a firmer texture.

The fresh cheese tested were rated for their appearance, odour, texture, flavour, and overall evaluation in a 9-point hedonic scale (in which 1 - dislike extremely, 2 - dislike very much, 3 - dislike moderately, 4 - dislike slightly, 5 - neither like or dislike, 6 - like slightly, 7 - like moderately, 8 - like very much, 9 - like extremely). The participants showed preference for the sample with Cheddar cheese and added with skim milk powder (S.CH.SMP), in all the attributes (Figure 6.2. A). This fresh cheese also had the highest overall evaluation, with an average of 6.3 points, followed by sample S.GT, with a rating of 5.5.

Regarding the intensity of flavour (Figure 6.2. B), 50 % of the participants replied that sample S.CH.SMP was just right, and 45 % had the same opinion for sample S.CH. 50 % found sample S.EWE having a too strong flavour, and 42.5 % found sample S.GT having a weak flavour. In fact, ewe's cheese frequently has an intense flavour, to which many consumers are not familiarized, and/or not expect to find in a fresh cheese. The goats' cheese used had a milder flavour intensity than either the Cheddar or the ewes' cheese.

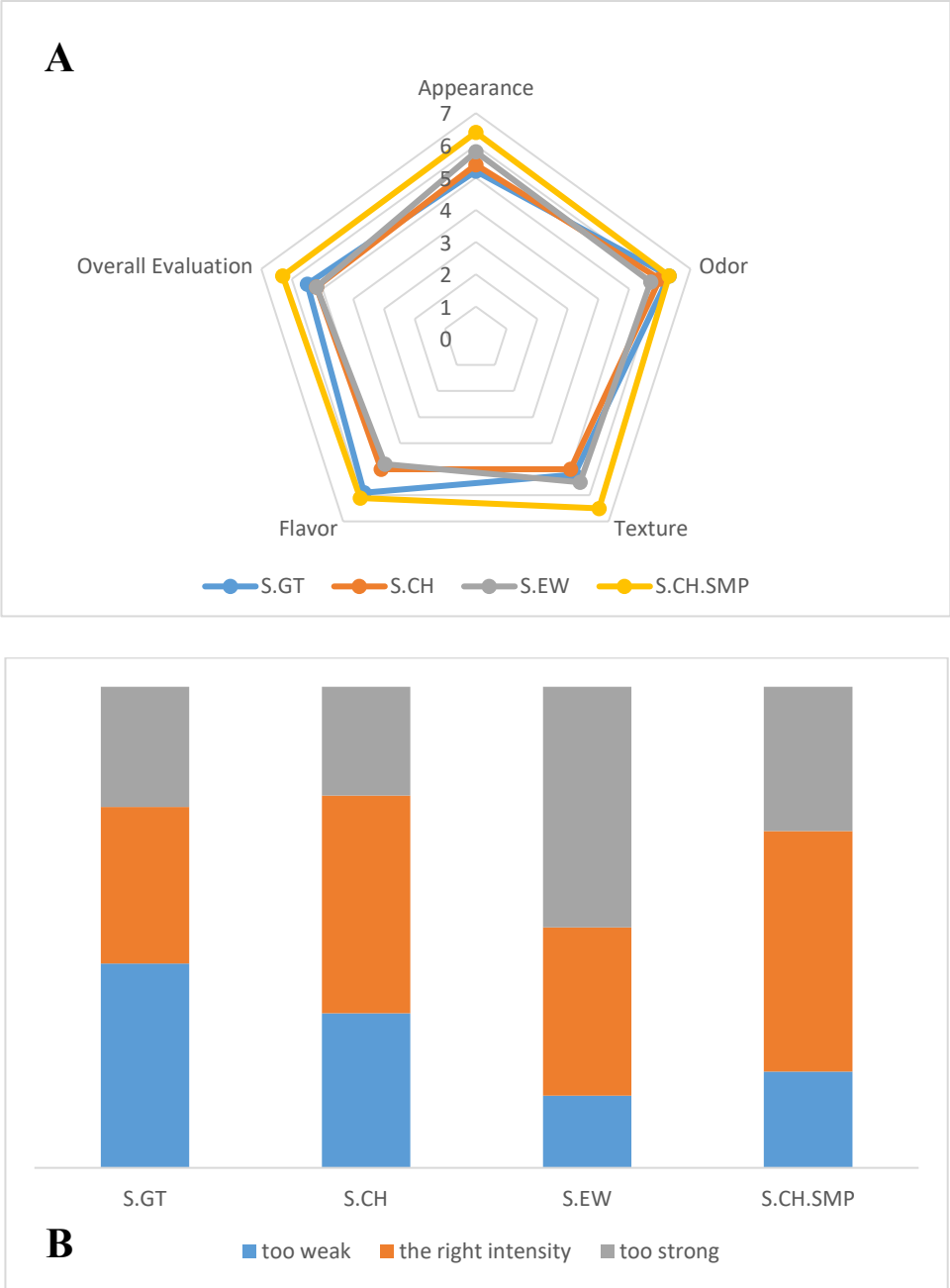

Figure 6.2. – Panel evaluation of: (A) different attributes and overall acceptability, and (B) flavour intensity of the different fresh cheese samples. S.GT, S.EW, S.CH are fresh cheese samples incorporating goats', ewes', or Cheddar cheese; S.CH.SMP also incorporates skimmed milk powder (SMP).

An additional comment made by 10 participants was that, regarding texture, the samples were more similar to yoghurt or quark cheese, rather than to a characteristic Portuguese fresh cheese, which has a denser texture. The relevance given to this property justifies that the sample with highest rating was the one with skim milk powder (S.CH.SMP), which leads to a product with a considerably more consistent and cohesive texture. A recent work points as well to a correlation between results of textural parameters and sensorial evaluation for acid coagulated fresh cheeses (Skarlatos et al., 2021).

6.4. Conclusions

Ripened cheese can be dispersed in hot gelatinized starch in milk and the resulting slurry (melted cheese base) can be an ingredient for a variety of food products. This represents a valorisation strategy for ripened cheese that could eventually become food waste. In this work, the melted cheese base was diluted with further fluid milk and then renneted, in order to obtain novel fresh cheese. That ingredient not only adds protein and fat to the fresh cheese, but it also adds minerals and flavour. A step of whey drainage is not included and the overall process is extremely simple. The fresh cheese developed have a valuable and balanced nutritional content, and a texture similar to many commercial fresh cheese types, or spreadable processed cheese. Furthermore, no emulsifying salts or any non-natural ingredient are used, enabling the classification of “clean label” and alignment with the sustainable development goals.

Gel formation of the initial mixture is hindered above a certain incorporation of ripened cheese, but this can be overcome by the addition of skim milk powder (or rennetable casein) to the preparation. Starch and skim milk powder both reduce syneresis of the renneted gel. Starch seems to decrease gel hardness, but addition of skim milk powder has a strong opposite effect. The sensory attributes of the product, such as texture and flavour, can be modulated by varying the amount and type of ripened cheese, and of extra casein (from skim milk powder or other). According to a consumer panel, the preference was higher for a more solid texture and with the flavour of a traditional cows’ cheese.

Therefore, the viability and versatility of this novel fresh cheese is here demonstrated. Further work can focus on minor adjustments of composition and in adjusting production to the pilot scale level.

6.5. References

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Chapter 7. Future Work and Final Considerations

7.1. Future Work

Regarding MCB formulations, it would be interesting to achieve stable formulations by: (1) improving the preliminary formulations with xanthan gum and with guar gum; (2) use other types of hydrocolloids, such as carboxymethylcellulose, or other types of native and modified starches; and (3) use other types of cheese for the creation of a set of alternative flavors.

As for the products developed in this thesis with the use of MCBs, yogucheeses and fresh cheeses, a deeper knowledge would be advantageous, in order to unlock and understand various features of these novel products. Some testing could intel: (1) more detailed analyses of the microstructures of these products, as well as of the MCB structures, using confocal and/or scanning electron microscopy techniques; (2) microbiological studies of bacterial growth during fermentation and their evolution during the product storage period, as well as total counts at several critical time points during preparation; evaluation of consumers' acceptance through carefully designed sensorial tests, although preliminary ones were already carried out, that would include samples with different cheeses, at varying levels of incorporation, with the ultimate goal of getting the product adjusted to the consumers' market.

Besides the two major products involved in this thesis, one can envision other ones. Cheese snacks are a growing market trend, so novel cheese snacks incorporating MCBs can be a promising route to explore. A different one can be the drinks sector: ready-to-drink dairy products with (real) cheese flavor; or mixed drinks, in particular coffee drinks, incorporating melted/dispersed cheese.

7.2. Final Considerations

At the end of this project, with all of its ups and downs, amazing learnings and self-improvements, and its cross through a Global Pandemic, the main conclusion can be summarized as in the following paragraphs.

The development of a group of novel dairy products, yogucheeses and fresh cheeses, with nutritionally enhanced characteristics and innovative taste features, obtained from a cheese paste, the MCB, was achieved. Since the MCBs can be formulated using cheese surpluses, or off-standard items, the manufacture of this product would contribute to the reduction of food waste, and for innovation of the dairy industry portfolio. This novel line of products is economically and environmentally interesting, being in line with the recent consumers' trends and demands for more sustainable and health-promoting foods, since all the ingredients used were clean label ones, in particular without the use of emulsifying salts.

Regarding the MCBs formulations, it was concluded that the ingredients should include milk as a base fluid, a hydrocolloid, and cheese that should previously be grated or finely cut, in order to produce an MCB with good textural characteristics. Moreover, when using a starch suspension in milk, it should be heated past the gelatinization temperature before cheese addition, in order to enable its full dispersion.

Regarding the interactions holding protein structure in Emmental cheese, by using solutions of different dissociating agents, at different concentrations and combinations, we were able to quantify the solubilized cheese protein in these solutions, using a novel method for total protein quantification. Our results pointed out that caseins in the Emmental cheese are held by a set of hydrophobic interactions, hydrogen bonds, and other electrostatic ones, including ionic bonds, with hydrogen bonds apparently having an important role, comparable to hydrophobic interactions, a conclusion not commonly reported for cheese structures.