LUIS GUSTAVO LIMA NASCIMENTO

CASEIN HYDROGELS: INTERACTION WITH BIOACTIVE COMPOUNDS AND VEGETABLE PROTEINS

Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Antônio Fernandes de Carvalho

Co-adviser: Federico Casanova

VIÇOSA - MINAS GERAIS
2022

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
N244c
2022

Nascimento, Luis Gustavo Lima, 1993-
Casein hydrogels: interaction with bioactive compounds and vegetable proteins / Luis Gustavo Lima Nascimento. – Viçosa, MG, 2022.
1 tese eletrônica (189 f.): il. (algumas color.).

Texto em inglês.
Orientador: Antônio Fernandes de Carvalho.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, 2022.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2022.604
Modo de acesso: World Wide Web.

1. Hidrogéis. 2. Caseína. 3. Transglutaminases. 4. Compostos bioativos. 5. Gelificação. 6. Ervilha. I. Carvalho, Antônio Fernandes de, 1964-. II. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. III. Título.

CDD 22. ed. 664.8

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

LUIS GUSTAVO LIMA NASCIMENTO

CASEIN HYDROGELS: INTERACTION WITH BIOACTIVE COMPOUNDS AND VEGETABLE PROTEINS

Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: August 29, 2022.

Assent:

Luis Gustavo Lima Nascimento
Author

Antônio Fernandes de Carvalho
Adviser

To my mother

ACKNOWLEDGMENTS

In the first place, I would like to thank my mother, Elzimar Soares, my inexhaustible source of inspiration, for her lifetime support and for always believing in me.

I also would like to thank:

my sister, Marília Lima, for the encouragement.

My advisor, Professor Antônio Fernandes de Carvalho for the opportunity to work in the INOVALEITE laboratory and for the shared knowledge, advices, and friendship.

Professor. Evandro Martins for the teachings, motivations, encouragements, and friendship.

All people I met in Inovaleite, especially Luana, Jean, Camila, Raiane, and Sidney, who have turned tiring days of work into many hours of joy and fun.

My girlfriend, Laurine Deldicque, for her full-time support and motivation during this journey.

My French housemates, Lauriane Bleuzé and Kevin Ory, for their friendship and for helping me in adapt to France.

All the people I met in the PIHM team, especially Luisa Scudeller, Sakhr Alhuthali, Angela Velasquez, and Manon Hiolle, for making the adaption into the new workgroup easier. And also, Federico Casanova, for giving me the opportunity to work at DTU. And to Lucas Queiroz, for easier my adaptation in Denmark.

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for granting the scholarship.

To the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), for granting the scholarship.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), to granting the scholarship.

ABSTRACT

NASCIMENTO, Luis Gustavo Lima, D.Sc., Universidade Federal de Viçosa, August, 2022. **Casein hydrogels: Interaction with bioactive compounds and vegetable proteins**. Adviser: Antônio Fernandes de Carvalho. Co-adviser: Federico Casanova.

Hydrogels are three-dimensional networks able to entrap a high amount of water. They can be formed by a wide range of polymers alone or in combination and have different applications depending on their composition and rheological features. In the food industry, hydrogels are mainly designed to work as a carrier system of bioactive compounds or to tailor the texture, mouthfeel, and water retention of foods. The facility to modulate Casein micelles (CMs) structure and interactions by application of physical, chemical, or enzymatic treatments, makes it an excellent protein matrix for the hydrogel's formulation. Moreover, the use of casein hydrogels can be also a way to incorporate more plant proteins into human food. The mixtures of plant proteins with caseins have been viewed as a more sustainable alternative to a diet based mainly on animal proteins. Since, in the mixture, the drawbacks of pure plant protein products, such as beany taste and low solubility, could be potentially diminished by the presence of caseins. Nevertheless, the CMs' interactions with micro molecules such as bioactive compounds or macromolecules such as proteins can alter the features of the gel. Thus, this study proposed the utilization of casein-based hydrogel in two distinct applications, i. in association with bioactive compounds extracted from Jabuticaba fruit with the use of transglutaminase for modulation of gels' microstructure and ii. in association with pea proteins (in different ratios) submitted to process conditions usually applied in the food industry such as thermal treatment and acidification, in addition, high-intensity ultrasounds also were applied. The addition of the bioactive extract in the gels decreased the gel elasticity and increase the pore sizes. However, these effects were contra-balanced by using transglutaminase as the crosslinking agent, which could modulate the release of the bioactive extracts from the gel. In the CMs: pea proteins systems, the heat treatment increased the elasticity of the systems with a higher impact in the systems with more pea protein. The network reinforcement is caused mainly by physical interactions between pea proteins, with disulfide bonds occurring only between proteins of the same source. During acidification, the replacement of 20 and

40% of CMs for pea protein disturbed the initial steps of CMs network formation, however, the final gel elasticity was higher than pure CMs gel due to the formation of the pea's network. In general, the proteins of different sources form independent protein networks even in high concentrations. Despite the reduced interaction between CMs and pea proteins, their distribution in the gel is responsible for modulating the final stiffness. In addition, the application of high-intensity ultrasound in the mixed suspensions increased the elasticity of the acid gels up to 10 times, depending on the protein ratio. This study shows that the association of CMs with bioactive molecules or pea proteins in gelled systems has the potential for the development of functional foods or foods with totally new rheological features.

Keywords: Hydrogels. Casein. Transglutaminases. Bioactive compounds. Gelification.

RESUMO

NASCIMENTO, Luis Gustavo Lima, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Hidrogéis de caseína: interação com compostos bioativos e proteínas vegetais.** Orientador: Antônio Fernandes de Carvalho Coorientador: Federico Casanova.

Hidrogéis são redes tridimensionais capazes de reter uma grande quantidade de água. Eles podem ser formados por uma ampla gama de polímeros, sozinhos ou em combinação, e têm diferentes aplicações dependendo de sua composição e características reológicas. Na indústria alimentícia, os hidrogéis são projetados principalmente para funcionar como um sistema transportador de compostos bioativos ou para ajustar a textura, mastigabilidade, e retenção de água dos alimentos. A facilidade de modular a estrutura e as interações das micelas de caseína (CMs) pela aplicação de tratamentos físicos, químicos ou enzimáticos, fazem delas uma excelente matriz proteica para a formulação de hidrogéis. Além disso, o uso de hidrogéis de caseína também pode ser uma forma de incorporar mais proteínas de origem vegetal na alimentação humana. As misturas de proteínas vegetais com caseínas têm sido vistas como uma alternativa mais sustentável à uma dieta baseada principalmente em proteínas animais. Uma vez que, com a mistura, os inconvenientes dos produtos de proteína vegetal pura, como gosto de feijão e baixa solubilidade, podem ser potencialmente diminuídos pela presença de caseínas. No entanto, as interações dos CMs com micromoléculas como compostos bioativos ou macromoléculas como proteínas, podem alterar as características do gel. Assim, este estudo propôs a utilização de hidrogel à base de caseína em duas aplicações distintas, i. em associação com compostos bioativos extraídos de Jabuticaba, usando transglutaminase para modulação da microestrutura dos géis e ii. em associação com proteínas de ervilha, em diferentes proporções, submetidas a condições de processo usualmente aplicadas na indústria alimentícia como tratamento térmico e acidificação. Além disso, ultrassom de alta intensidade também foi aplicado. A adição do extrato bioativo nos géis diminuiu sua elasticidade e aumentou o tamanho dos poros. No entanto, esses efeitos foram contrabalanceados pelo uso da transglutaminase como agente de reticulação, que modulou a liberação dos extratos bioativos do gel. Nos sistemas CMs: proteínas de ervilha, o tratamento térmico aumentou a elasticidade dos

sistemas com maior impacto nos sistemas com mais proteína de ervilha. O fortalecimento do gel é causado principalmente por interações físicas entre as proteínas da ervilha, com ligações dissulfeto ocorrendo apenas entre proteínas da mesma fonte. Durante a acidificação, a substituição de 20 e 40% de CMs por proteína de ervilha causou perturbação nas etapas iniciais de formação da rede tridimensional de CMs. Porém, as elasticidades finais dos géis foram maiores que no gel puro de CMs, devido à gelificação das proteínas de ervilha. Em geral, as proteínas de diferentes fontes formam redes de proteínas independentes mesmo em altas concentrações. Apesar da reduzida interação entre CMs e proteínas de ervilha, suas distribuições no gel é responsável por modular a rigidez final. Além disso, a aplicação de ultrassom de alta intensidade nas suspensões mistas aumentou a elasticidade dos géis ácidos em até 10 vezes, dependendo da proporção proteica. Este estudo mostra que a associação de CMs com moléculas bioativas ou proteínas de ervilha em sistemas gelificados tem potencial para o desenvolvimento de alimentos funcionais ou de alimentos com características reológicas totalmente novas.

Palavras-chave: Hidrogéis. Caseína. Transglutaminases. Compostos bioativos. Gelificação.

SUMMARY

GENERAL INTRODUCTION

1. Context

Hydrogels are three-dimensional networks able to entrap a high amount of water (Klement, Lord, & Parker, 1960). They can be formed by one or more polymers combined to produce rheological systems with modulated features (Nascimento et al., 2020). In the food industry, these hydrogels can be used for different purposes such as i. the entrapment of biomolecules for the creation of functional foods, ii. in tailoring the texture and mouthfeel properties of food systems, iii. improve the water retention by food matrix and iv. increase the food stability over time by preventing phase separation (Zhang, Zhang, & Yuan, 2020). Polysaccharides such as agar, gellan gum, alginate, pectin, and proteins such as whey and soy, are widely used in hydrogel formulations (Zhang, Zhang, & Yuan, 2020; Abaee, Mohammadian, & Jafari, 2017); however, casein micelles (CMs) stand out among them due to the facility to modulate their techno-functional properties by using physical, chemical and enzymatic treatments Broyard & Gaucheron, 2015).

Casein micelles (CMs) compose proximately 80% of cow's milk and they are supramolecular structures able to interact with water molecules, minerals, polyphenols, vitamins, carotenoids, lipids, and proteins (Casanova et al., 2021). In addition, under acid conditions or by the action of specific enzymes such as chymosin, the CMs can form a hydrogel; as can be observed during the production of fermented milk and cheeses (Li & Zhao, 2019). The formulation of CMs gels with macromolecules such as lipids, carbohydrates, and proteins is usually applied in the development of dairy products with specific fluidity and elastic features. Among the macromolecular interactions, the use of casein micelles in a combination of other proteins, mainly plant proteins is gaining increased attention in the last years (Hinderink et al., 2021; Alves & Tavares, 2019). According to the United Nations report (United Nations, 2015), the human population will reach around 10 billion people by 2050, increasing the demand for proteins. Thus, the use of alternative sources of proteins, such as soy, chickpea, lentils, rice, and pea proteins are seen as a potential solution to supply the new protein demand.

The production of plant proteins produces less environmental impact, with reduced use of water, energy, and land compared to animal-origin proteins (Fasolin et al., 2019). However, the main drawbacks are their reduced techno-functional properties and low sensory acceptance, which limits their larger application. As an

alternative to increasing the use of plant proteins, their utilization in mixed systems is promising (Guyomarc'h et al., 2021). The mix of plant and milk proteins presents several advantages. In one way, it can increase the applicability of plant proteins, diversifying the variety of products containing proteins from plant origin. On the other way, it increases the portfolio of dairy industries, creating products with totally new features.

The gelation of milk proteins has been studied for a long time, as well as several teams work on improving plant-proteins gelling properties. However, the mixture of proteins of different origins is complex and it is a lack of studies in mixed systems, mainly in high concentrated systems (> 10% (w/w) (Schmitt et al., 2019).

2. Objectives

This study aims to develop caseins- based hydrogels for two distinct applications: i. in association with bioactive molecules (anthocyanins) to study hydrogel ability to work as carrier systems for the delivery of bioactive compounds; and ii. in association with plant proteins (pea proteins) to understand the impact of the protein's interactions in the final properties of the gel.

3. Manuscript organization

Chapter I. Literature review

This chapter is divided into two parts, the first part presents overall information about the interactions between caseins and food bioactive compounds. Casein interactions with vitamins, polyphenols, lipids, and proteins were reviewed, as well as the type of delivery systems that can be formed using caseins. In the second part, the interactions between caseins and plant proteins were discussed to understand how these interactions can be useful in the development of new food products. The first part of this review is published in the *Food Chemistry* journal: *Interactions between caseins and food-derived bioactive molecules: A Review.* The second part of this review is prepared to be submitted to the *Future foods* Journal: *Combination of milk and plant proteins to develop novel food systems: A Review.*

Chapter II. Casein hydrogel to carry bioactive compounds from Jabuticaba.

Casein hydrogel was developed to entrap and control the release of anthocyanins-rich Jabuticaba extract under pH 2.0, 4.5, and 7.0. The effects of the

extract addition on the casein gel rheological properties were evaluated and compensated using transglutaminase as a crosslinking agent. The enzyme application also modulated the ratio of extract diffusion from the gel matrix. The contents of this chapter are published in the *Food Hydrocolloids* Journal: with the title "*Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (Myrciaria cauliflora) extract*"

Chapter III. Colloidal systems formed by CMs and pea proteins

Aqueous suspensions with high concentrations of CMs, and pea proteins were formulated and the effect of protein ratios and thermal treatment on their rheological properties were evaluated. Next, the suspensions were acidified with glucono-δ-lactone to gather information about how the different protein ratios interfere with the gel properties. This chapter will be valorized in the form of two articles, both to be submitted to the Food Hydrocolloids: "*Impact of protein ratio and thermal treatment on the rheological properties of high-concentrated casein micelles: pea protein suspensions*" and "*Acid gelation of high-concentrated casein micelles: pea protein mixed systems*".

Chapter IV. High-intensity ultrasound to improve gelling properties of CMs: pea mixed systems

Emergent green technology was applied to improve the gelling properties of the CMs: pea mixed systems. The ultrasounds treatment was applied in two different process routes and the effect in the suspensions, as well as in the formed gels were evaluated. The content of this chapter will be submitted to the Food Chemistry journal with the title: *High-intensity ultrasound treatment on casein: pea mixed systems: effect on gelling properties*.

General conclusions and perspectives

The main conclusions derived from the study are lined out with the future research needed to increment the knowledge about the mixed systems.

4. References

Abaee, A., Mohammadian, M., & Jafari, S. M. (2017). Whey and soy protein-based hydrogels and nano-hydrogels as bioactive delivery systems. Trends in Food Science & Technology, 70, 69-81.

Alves, A. C., & Tavares, G. M. (2019). Mixing animal and plant proteins: Is this a way to improve protein techno-functionalities?. Food Hydrocolloids, 97, 105171.

Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: A scientific and technological challenge. Dairy science & technology, 95(6), 831-862.

Casanova, F., Nascimento, L. G. L., Silva, N. F., de Carvalho, A. F., & Gaucheron, F. (2021). Interactions between caseins and food-derived bioactive molecules: A review. Food Chemistry, 359, 129820.

Fasolin, L. H., Pereira, R. N., Pinheiro, A. C., Martins, J. T., Andrade, C. C. P., Ramos, O. L., & Vicente, A. A. (2019). Emergent food proteins–Towards sustainability, health, and innovation. Food Research International, 125, 108586.

Guyomarc'h, F., Arvisenet, G., Bouhallab, S., Canon, F., Deutsch, S. M., Drigon, V., ... & Gagnaire, V. (2021). Mixing milk, egg, and plant resources to obtain safe and tasty foods with environmental and health benefits. Trends in Food Science & Technology, 108, 119-132.

Hinderink, E. B., Boire, A., Renard, D., Riaublanc, A., Sagis, L. M., Schroën, K., ... & Berton-Carabin, C. C. (2021). Combining plant and dairy proteins in food colloid design. Current Opinion in Colloid & Interface Science, 56, 101507.

Li, Q., & Zhao, Z. (2019). Acid and rennet-induced coagulation behavior of casein micelles with modified structure. Food chemistry, 291, 231-238.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., de Carvalho Teixeira, A. V. N., & de Carvalho, A. F. (2020). Casein-based hydrogels: A mini-review. Food chemistry, 314, 126063.

Schmitt, C., Silva, J. V., Amagliani, L., Chassenieux, C., & Nicolai, T. (2019). Heat-induced and acid-induced gelation of dairy/plant protein dispersions and emulsions. Current Opinion in Food Science, 27, 43-48.

Stojkov, G., Niyazov, Z., Picchioni, F., & Bose, R. K. (2021). Relationship between structure and rheology of hydrogels for various applications. Gels, 7(4), 255.

United Nations, D. o. E. a. S. A, Population Division (2015). Revision of world population prospects. United Nations

Wichterle, O., & Lim, D. (1960). Hydrophilic gels for biological use. Nature, 185(4706), 117-118.

Zhang, H., Zhang, F., & Yuan, R. (2020). Applications of natural polymer-based hydrogels in the food industry. In Hydrogels based on natural polymers (pp. 357-410). Elsevier.

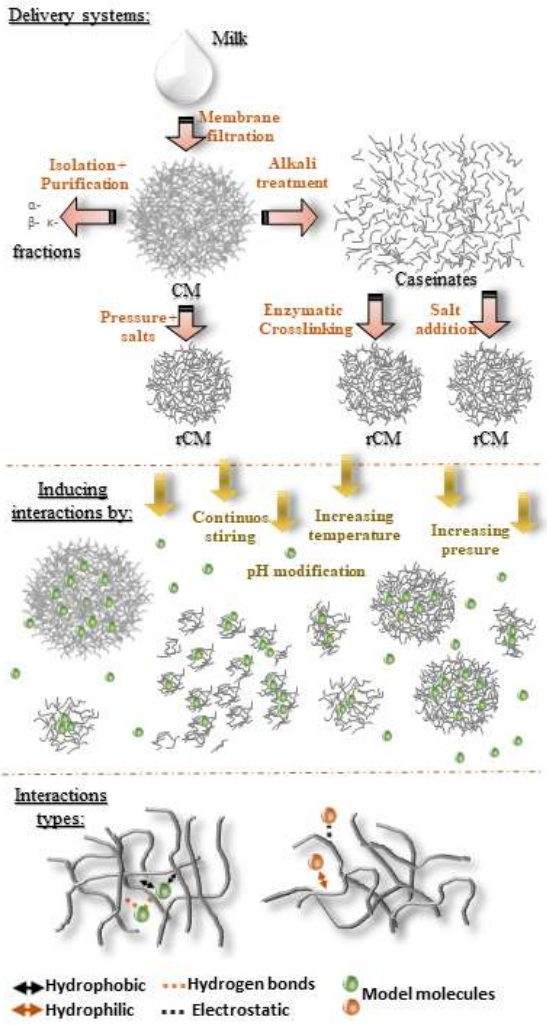
CHAPTER I: LITERATURE REVIEW

PART A: Interactions between caseins and food-derived bioactive molecules: A Review

The content of this part has been published in:

Food Chemistry Journal

Federico Casanova, Luis Gustavo Lima Nascimento, Naaman F. N. Silva, Antonio F. de Carvalho, Frédéric Gaucheron



1. Introduction

Bioactive molecules are defined as compounds that are able to interact with one or more components in live tissues and produce health benefits (Biesalski et al, 2009). These compounds are not essential to primary nutritional needs but they have been shown to provide beneficial effects when ingested in moderation (Guaadaoiu, Benaicha, Elmajdoub, Bellaoui, & Hamal, 2014). The health benefits associated with bioactive compounds include anti-cancer, anti-inflammatory and anti-obesity properties, a reduced risk of heart disease, and better eye health (Campos, 2018).

Although bioactive molecules offer health benefits, they are sensitive to environmental conditions including high temperatures, extreme pH values, exposure to light and/or oxygen, and enzyme degradation (Sinela et al., 2017; Mahmoodani, Perera, Abernethy, Fedrizzi, & Chen, 2018). Moreover, some bioactive molecules demonstrate low solubility in aqueous or lipid media (Rezaei, Fathi, & Jafari, 2019). The scientific challenge in food processing and formulation is therefore how to find ways to add, protect and deliver bioactive molecules using food products (Tripodi, Lazidis, Norton, & Spyropoulos, 2019). To succeed, the following requirements must be met: (i) A significant interaction must take place between the food component and the bioactive molecule; (ii) the food component must maintain and protect the biological activity of the bioactive molecule; (iii) the food component must deliver the bioactive molecule to the physiological target in the ingesting organism (Nowak, Livney, Niu, & Singh, 2019). Caseins show promise because they can bind hydrophobic, hydrophilic and charged molecules, they can interact with other biopolymers, and they can stabilize emulsions, form gels, and, to some extent, retard oxidation (Damodaran, Parkin, & Fennema, 2008; Horne, 2020). Moreover, caseins are recognized as safe for consumption, are easy to prepare on an industrial scale, and offer a high biological value for a relatively low production cost (Abd El-Salam & El-Shibiny, 2012; Sabliov, Chen, & Yada, 2015).

Caseins represent about 80 % of total cow milk proteins. They are rheomorphic proteins that exhibit an open and flexible conformation (Holt, Carver, Ecroyd, & Thorn, 2013; Lucey & Horne, 2018). There are four primary casein molecules: α-S1, α-S2, β and κ-caseins. The mass proportion of casein molecules in milk are 30, 10, 36 and 14 % for αS1, αS2, β and κ–caseins, respectively (Davies & Law, 1980). These four caseins have different amino acid sequences and exhibit additional heterogeneous

behaviors due to two post-translational modifications - phosphorylation, for all casein molecules, and glycosylation, for κ-casein (Holland, 2009). In milk, the casein molecules are naturally aggregated in the presence of calcium phosphate forming casein micelles (CMs). Caseins represent about 94 % of CMs dry matter. The remaining 6 % correspond to minerals, primarily colloidal calcium phosphate (CCP) and trace amounts of magnesium and citrate (Holt, Carver, Ecroyd, & Thorn, 2013). CMs have an average diameter of about 200 nm, precipitate at pH 4.6 and have a porous structure that contains about 3.3 g water / g protein (Huppertz et al., 2017; Dalgleish, 2011).

Due to their unique structural and physicochemical properties, caseins have been used as vehicles for bioactive molecules over the past decade (Ranadheera, Liyanaarachchi, Chandrapala, Dissanayake, & Vasiljevic, 2016). Various molecular arrangements of caseins have been studied, including: isolated and purified casein molecules, particularly β-casein; sodium caseinate (CasNa); re-assembled casein micelles (rCMs); native casein micelles (CMs); and casein nanoparticles. The structural aspects of these molecular arrangements have recently been reviewed by Nascimento et al. (Nascimento, Casanova, Silva, Teixeira, & Carvalho, 2020) and Rehan et al. (Rehan, Ahemad, & Gupta, 2019).

The objective of the present study is to review the interactions that occur between caseins and food-derived bioactive molecules such as vitamins, polyphenols, lipids and proteins. We have also covered the aggregation states of casein molecules and the techniques used to produce and study the particles thus formed. Table 1 summarizes bioactive molecule types, casein molecular arrangements, and methods used to study casein-bioactive interactions. This review aims to support the development of new and innovative functional foods in which caseins can be used as a designated delivery system.

2. Casein delivery systems

There are many ways to create casein aggregates that can work as a delivery system. This review will focus on the delivery systems formed by CMs, rCMs, CasNa and pure casein fractions. A summarized representation of casein delivery systems discussed in this paper is depicted in Figure 1. Although all these systems present casein molecules as the fundamental units, their preparation and the encapsulation

strategy of the bioactive compounds vary between them. Different types of casein products can be produced from milk by distinguishing ways, such as isoelectric precipitation, membrane filtration and rennet coagulation (Badem & Uçar). In this sense, the main forms of casein aggregates used to encapsulate bioactive molecules are described below, focusing on the characteristics that are important for the molecules' delivery.

The use of microfiltration techniques to perform the separation of CMs leads to structures very close to those that are naturally found in milk, which is used called native casein micelle (O'Mahony and Fox, 2013). In this case, raw milk is centrifuged to remove fat and dirty. After that, the skimmed milk is microfiltrated using a membrane of molecular cut off that allows the separation of the other milk constituents from CMs. The CMs-rich fraction is dialyzed to remove lactose and salts, is reconcentrated by microfiltration, and then can be spray dried (Schuck, 1994). In general, the strategy used to encapsulate bioactive compounds using native CMs is simple and consists of rehydrating the powder of CMs in a specific medium and environmental conditions, followed by the addition of the bioactive compound (Zhou, S., Seo, S., Alli, I., & Chang, Y. W. 2015; Haratifar, S., Meckling, K. A., & Corredig, M. 2014a), which can be further spray-drying, depending on the aim of the study (Nogueira et al., 2020; Khanji et al., 2018a). Since CMs present a porous supramolecular structure (O'Mahony and Fox, 2013), whose pores are several times larger than the sizes of bioactive molecules, there is always the possibility of the bioactive compounds interact internally in the CMs in a liquid media (Nascimento et al., 2020b). In general terms, the most the bioactive molecule is in the core of the casein aggregates higher is the protective effect (Jarunglumlerta, K. Nakagawab, S. Adachi, 2015). Naturally, the CMs works as a carrier system for delivery minerals for newborn, but CMs also can be modified to improve their physicochemical stability and their capacity of encapsulation (Nogueira et al., 2019; Nascimento, 2020b).

Caseinates also have been employed to develop delivery systems. Caseinates are produced by treating acid precipitated casein with alkali substances, such as NaOH, Ca(OH)2, KOH, which are subsequently spray-dried (Badem, & Uçar, 2017). Sodium and calcium caseinate are the main forms industrially produced. The former is more soluble than the latter (Thomar, P., Nicolai, T., Benyahia, L., & Durand, D. 2013), and the majority of studies uses sodium caseinate as delivery systems (Ghayour et al.,

2019; Penalva et al., 2015; Casanova et al., 2018). The caseinates are composed of all casein fractions, however, they do not form a supramolecular structure as in CMs. Thus, the approach to develop a delivery system changes in accordance with the casein fundamental units, i.e., the CMs is the supramolecular structure that can be modified, but it is already "constructed", while the CasNa is composed by the fractions that make CMs, but they can interact to form a different structure. When suspended, the organization of the casein molecules depends on environmental factors, such as pH, ionic strength, presence of divalent cations and temperature (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008). When the rearrangements of CasNa have a higher degree of organization that is close to the structure of native CMs, the systems are called reassemble casein micelle (rCMs) or reconstructed casein micelle (Knoop, Knoop, & Wiechen, 1979). Generally, the production of rCMs consists of the addition of tri-potassium citrate, K_2HPO_4 and $CaCl_2$ in CasNa suspensions (Semo, Kesselman, Danino, & Livney, 2007; Knoop, Knoop, & Wiechen, 1979). The type and concentration of the salts affect directly the characteristics of the rCMs, mainly their size and stability (Loewen, Chan, & Li-Chan, 2018). Besides the addition of specific salts, rCMs have been constructed by crosslinking of casein fractions with transglutaminase enzyme after CMs disruption by alkaline agent (Duerasch, Wissel, & Henle, 2018) and also by submitting CMs to high-pressure treatment followed by the addition of calcium and phosphate ions (Menéndez-Aguirre et al., 2011). Despite the use of casein aggregates composed of all casein fractions, delivery systems containing only α- and β-caseins also have been proposed (He, Xu, Zeng, Qin, & Chen, 2016; Zhang et al., 2014; Bourassa, Bariyanga, & Tajmir-Riahi, 2013). There are several methods to produce casein pure fractions, in general, the fractions have to be isolated from CMs and followed by purification processes, which were reviewed by Atamer et al. (2016). It is worth mentioning that β- casein is a protein that naturally presents the hydrophobic C-terminal well separated domain from the hydrophilic N- terminal domain, and this characteristic allows the association of β-casein with hydrophobic bioactive compounds and the formation of soap-like micelles (Swaisgood, 2003).

As discussed here, many casein delivery systems can be applied to encapsulate bioactive compounds. Thus, the best type of casein system used to encapsulate a specific bioactive compound cannot be simply predicted. The most suitable strategy

for encapsulating such compound with casein molecules or aggregates needs to be confirmed by experimental results. However, independently of the adopted strategy, it is important to consider the chemical interactions between caseins and bioactive molecules, which are reviewed in the next section.

3. General aspects of caseins and bioactive molecules interactions

Caseins can interact with a wide variety of bioactive molecules (Tavares, Croguennec, Carvalho, & Bouhallab, 2014) by different means such as hydrophobic, hydrophilic, and electrostatic interactions. Most of the studies that use casein systems to deliver bioactive compounds focus on the flowing topics in higher or low extensions: promotion of the encapsulation (Ghatak & Iyyaswami, 2019); measuring of the binding constants (Bourassa et al., 2013); observation of changes in the protein structure by the complex formation (He et al., 2016); studying the biomolecule stability under storage conditions (Yi, Fan, Yokoyama, Zhang, & Zhao, 2016); incorporating the vehicle in a food matrix (Loewen, Chan, & Li-Chan, 2018); investigating the changes that occur in the food properties (rheological, physicochemical and sensorial changes) (Moeller, Martin, Schrader, Hoffmann, & Lorenzen, 2018); evaluation of the bioaccessibility of the bioactive compound in vitro or in vivo (Cohen et al., 2017).

The strength of the interaction between caseins and the bioactive molecules is usually measured by fluorescence spectroscopy. In caseins, the change in the intrinsic fluorescence of tryptophan caused by the presence of additional molecules is used to determine the binding constants. The reduction in the intrinsic fluorescence can be a result of simple collisions between the caseins and the bioactive compound (dynamic quenching) or by complexation between these two (static quenching). Thermodynamical models are applied to determine which phenomenon occurs, and also to determine the binding's constants in the case of complexation (Lakowicz, 2013). Each molecule has different binding constants and sites and it varies when changes in pH, ionic strength and temperature occur (Casanova et al., 2018). Thus, the knowledge of these constants and how they change according to environmental parameters is a useful tool to develop a delivery system. The interaction of the bioactive molecule with the caseins does not guarantee a suitable delivery system. Consequently, investigations that aim to evaluate modifications in the protein structure or conformation caused by the complex formation are required, since these modifications

can directly impact the characteristics of the food systems (Guri, Haratifar, & Corredig, 2014). At a molecular level, spectroscopy analysis such as circular dichroism (CD), FTIR, and SAXS are valuable tools to follow the protein changes (Semenova et al., 2016; Antonov et al., 2017; Arroyo-Maya et al., 2016). However, changes in the protein structure after complexation with the bioactive molecule do not always occur (Gorji et al., 2015). Accordingly, the absence of a general rule makes necessary the study of those interactions and their micro and macro consequences in the carrier and food system.

The delivery systems can be applied in different fields as pharmaceutical, agricultural, and food. Generally, the encapsulated bioactive molecule is expected to increase its stability when applied in the food system in comparison with the free bioactive compound (Moeller et al. 2018; Kumar et al., 2016). However, good encapsulation efficiency and increased stability of the bioactive compound in a model system do not ensure the same results in a real food system. The complexity of a real food system can add new variables that can destabilize the carrier, invalidating its protective effect. Thus, It is possible that the enrichment of food with the encapsulated bioactive has the same results of stability as it was applied without the carrier material (Loewen, Chan, & Li-Chan, 2018). Another point to take into consideration concerning the application of a carrier system in food is its digestion. As a protein, caseins are degraded by pepsin and pancreatic enzymes (Cohen et al., 2017) which can cause the release of the encapsulated bioactive that can be metabolized. Generally, in vitro methods are applied to evaluate the capacity of caseins to protect and release bioactive compounds. Despite being a simplification of in vivo studies, there have been reported similarities in the casein degradation comparing in vivo and in vitro methods (Miralles et al., 2021). However, it lacks more in vivo studies concerning the bioactive molecules, and if encapsulation plays an important role in their activity.

Due to the difficulty in making comparisons among the published papers, once the parameters change according to casein aggregation, strategy of encapsulation, and type of bioactive compounds, a more detailed description of the recent papers published in the field is presented in the following sections.

4. Casein-vitamin interactions

Vitamins are a group of diverse, organic, nutritionally essential compounds that may induce health issues when deficiencies in the human body occur. Some vitamins are sensitive to light, oxygen, pH, and temperature (Gazzali, 2016), which is why different methods have been proposed to create protein-vitamin systems that protect the vitamins and improve their bioavailability (Katouzian & Jafari, 2016).

4.1. Vitamin A

Vitamin A molecules are liposoluble, unsaturated molecules found in different forms, including retinoic compounds and provitamin A carotenoids. Interactions between CasNa and lutein, an oxygenated carotenoid, have been studied by Yi et al. (Yi, Fan, Yokoyama, Zhang, & Zhao, 2016) using UV and fluorescence spectrometry (FS) and circular dichroism (CD). The authors observed that the lutein solution's turbidity decreased when CasNa was added, an effect attributed to lutein binding to casein molecules. However, the binding between lutein and CasNa had little impact on the caseins' secondary structures. According to the fluorescence results, caseins interacted with lutein by hydrophobic interactions with a constant association magnitude of 10^5 M^{-1}, and stoichiometry of about one bound lutein molecule per casein molecule. The interactions enabled the caseins to protect the lutein molecules against oxidation and decomposition during 16 days of storage at 25 °C. The chemical stability of β-carotene also increased after encapsulation with caseins, however, the protection degree varied depending on the casein aggregation (Jarunglumlerta, K. Nakagawab, S. Adachi, 2015). β-carotene is a carotenoid precursor of vitamin A which is used as a colorant in the food industry. Light, heat treatment and oxygen exposure can all cause degradations in β-carotene. As discussed previously, the structure of the casein aggregates impacts directly the vitamin-protein interactions. Jarunglumlert et al. (Jarunglumlerta, K. Nakagawab, S. Adachi, 2015) compared CasNa aggregates at pH 6.0 and rCMs on the encapsulation efficiency and chemical stability of β-carotene. The strategy consisted of stirring the protein suspensions added of β-carotene from 0 to 120 h. After complex formation was completed, the suspensions were spray-dried and the β-carotene's stability was evaluated over 21 days. The authors showed that the CasNa aggregates formed at pH 6.0 more efficiently encapsulated β-carotene molecules compared to rCMs. This result was attributed to a denser casein aggregate structure formed by CasNa compared to the rCMs structure. Also, it was found that

longer complex formation times improved encapsulation efficiency for CasNa aggregates, which was not the case for rCMs aggregates. The higher binding efficiency reflected in the stability of β-carotene during 21 days of storage at 60°C. The results of these studies demonstrate the caseins' potential as a carrier for liposoluble vitamins, which can be further improved by modulating the chemical environment in a simple way, i. e., by controlling pH, salt types and their concentrations and complex formation time.

Other strategies to increase encapsulation efficiency of casein systems were used by Blayo et al. (2014). Ultra-high-pressure homogenization at 14 °C or 24 °C, and isostatic high pressure at 14 °C or 34 °C for 15 min, both at 300 MPa were applied to encapsulate retinyl acetate in CMs. The authors evaluated the amounts of retinyl acetate in the CMs by their precipitation with ammonium sulfate. The authors showed that 2 – 5 nmol of retinyl acetate were carried per mg of precipitate casein. However, retinyl acetate concentrations in the control samples were similar to those found in the high-pressure treated samples, regardless of the type of high-pressure treatment. Therefore, the interactions between retinyl acetate and CMs caseins are not influenced by high-pressure technologies. In other words, solubilizing the retinyl acetate in native CMs suspension was enough to spontaneously encapsulate it.

In addition to systems composed solely of bioactive molecules and caseins, other biopolymers have been used to improve the protective role of caseins (Nascimento et al, 2020a). Jain et al. (Jain, Thakur, Ghoshal, Katare, & Shivhare, 2016) developed a carrier with gum tragacanth (a natural gum obtained from the dried sap of several species of Middle Eastern legumes in the Astragalus genus) to protect and allow for sustained release of β-carotene via a coacervation of casein. At a protein/gum ratio of 2/1, the optimal pH for complex coacervation was pH 4.3. At this pH, the intensity of electrostatic interaction was at its maximum. According to the authors, the particle size of the β-carotene-loaded coacervates ranged around 159.7 ± 2.2 nm. The coacervates presented low porous surfaces with no cracking, the coacervation yield was 82.5 ± 0.4 %, and the entrapment efficiency 79.4 ± 0.5 %.

4.2. Vitamin B

The interaction between isolated β-casein and folic acid, a hydro-soluble synthetic B vitamin, has been investigated by Zhang et al. (Zhang et al., 2014) using

FS, absorption spectroscopy and CD. The authors reported that folic acid binds to β-casein by hydrophobic interaction with a dissociation constant of ~105 M-1. They also showed that binding of folic acid to β-casein inhibited the vitamin's photodecomposition. Penalva et al. (Penalva et al., 2015) manufactured casein nanoparticles for oral delivery of folic acid. These nanoparticles were prepared using a coacervation process and stabilized with either lysine or arginine. Briefly, CasNa was suspended in pure water with lysine or arginine. Then, a solution of folic acid was added, followed by the addition of calcium chloride. The system was ultrafiltrated for purification, then spray-dried. The casein nanoparticles that formed presented a mean diameter close to 150 nm and folic acid content of 25 μg/mg of casein. In vitro and in vivo release studies showed that the oral bioavailability of the folic acid was around 52% when it was administered along with casein nanoparticles, i.e. 50% higher than in an aqueous solution. The results demonstrate the protective role caseins can play for water-soluble vitamin B.

These findings pointed to the potential use of caseins as a way to carry and protect folic acid. However, there is still little information available about the casein delivery of B vitamins.

4.3. Vitamin D

High-pressure treatment was applied to CMs to increase the loading capacity of Vitamin D2. The high-pressure treatments (0.1, 200, 400 and 600 MPa) combined with temperature variations (10 - 50 °C) were able to create rCMs with casein fractions that reassemble after been released from CMs (Menéndez-Aguirre et al., 2014). Contrary to the results found by Blayo et al. (2014) for retinyl acetate. The authors observed that the vitamin D2 load per casein increased from 2.2 ± 0.2 μg/mg (CMs) to 10.4 ± 0.2 μg/mg (rCMs) when 600 MPa at 50 °C was applied to a suspension of CMs. These findings highlight the suitability of high-pressure treatments for incorporating hydrophobic vitamin D molecules into rCMs. However, vitamin stability studies still need to be carried out before any recommendations can be made. In another study, a higher loading capacity for vitamin D in rCMs was achieved using response surface methodology to find the best salt concentrations (Loewen, Chan, & Li-Chan, 2018). Loewen et al. (Loewen, Chan, & Li-Chan, 2018) found the optimal vitamin D loading (13.8 – 14.6 mg/mg rCMs) using 4.9 mM phosphate, 4.0 mM citrate and 26.1 mM

calcium. The vitamin D stability also was evaluated. The powders from rCMs presented greater vitamin D preservation levels than the CasNa control powders during ambient (25 °C and 25 – 50 % humidity) and accelerated (37 °C and 75 % humidity) storage for 96 hours. However, when applied in fluid milk, it was observed that after 21 days of storage at 4° C under light exposure, vitamin D loss was not different for fluid milk with rCMs powder (loaded with vitamin D) and the control samples of fluid milk with direct addition of vitamin D (Loewen, Chan, & Li-Chan, 2018).

Usually, the delivery systems are used to fortified foods aiming the application in the food industry. The use of rCMs as a way to deliver vitamin D was also tested for fat-free yogurt production. Fat-free yogurt was enriched with vitamin D3 encapsulated in either rCMs or Polysorbate-80, a synthetic emulsifier (Levinson, Ish-Shalom, Segalb, & Livney, 2016). The yogurt samples were compared for in vivo bioavailability of vitamin D3. In vivo bioavailability of vitamin D3 was evaluated by clinical trial and no significant difference was observed between the two enrichment methods. In another study also with yogurt, an original approach was used by Moeller et al. (Moeller, Martin, Schrader, Hoffmann, & Lorenzen, 2018) to encapsulate and protect vitamin D2 in native CMs. The authors induced vitamin D2 encapsulation in an alcoholic solution by mixing the solution with an acidified suspension of native CMs at 2 °C and pH 5.5. Subsequently, the pH of the suspensions was adjusted to neutral. The suspensions were then spray-dried or freeze-dried. It was observed the maintenance of vitamin D2 content for 4 months of storage after the encapsulation. The authors also observed an increase in the in vitro bioavailability of vitamin D2 encapsulated in CMs, where 90% vitamin D2 (in CMs) remained active compared to only 67% in the free vitamin D2 yogurt samples. According to the authors, this result suggests that vitamin D2 can remain available in the lumen, but in vivo experiments must be done to confirm it.

Cohen et al. (Cohen, M. Levi, U. Lesmes, M. Margier, E. Reboul, Y. D. Livney, 2017) studied the use of rCMs as vehicles for vitamin D and evaluated the vitamin retention during simulated digestion and posterior in vitro bioavailability. The results showed that rCMs improved protection of Vitamin D3 during simulated digestion and demonstrated a significant increase in vitamin retention for 1 h under gastric conditions. Vitamin absorption by Caco-2 cells from digested rCMs was similar to free vitamin absorption. However, the bioavailability of the vitamin combined with rCMs was four times higher than that of the free vitamin.

The results above show that casein organization directly influences caseins' capacity to encapsulate and protect bioactive molecules. In addition to native CMs, developed rCMs represent another way to retain fat-soluble molecules, such as vitamin D. It is also important to note that both methods - homogenization and salt addition for rCMs production have shown positive results for protecting vitamin D. These principles offer potential as future research topics on enriching dairy products with other fat-soluble vitamins.

5. Casein-polyphenol interactions

Polyphenolic compounds are included in functional foods for their antioxidant, anti-inflammatory, antimicrobial, anti-amyloid, and anti-tumor properties (Martins, Barros, & Ferreira, 2016). Chemically speaking, polyphenolic molecules are characterized by the presence of one or several phenolic groups in their structure (Jia, Dumont, & Orsat, 2016). However, polyphenolic compounds are low soluble in aqueous solutions, which can lead to poor bioavailability and limit their clinical effectiveness. In addition, polyphenols present low stability when exposed to different pH values, light and high temperatures (Faridi Esfanjani & Jafari, 2016). One way to increase their solubility and maintain their stability until ingestion is to encapsulate them in caseins. In this sense, polyphenols' fluorescence, UV and visible spectra absorption are important elements for studying casein-polyphenol interaction.

5.1. Curcumin

Curcumin has low intrinsic toxicity and is credited with antioxidant, anti-inflammatory, antimicrobial, anti-amyloid, antitumor and anticancer pharmacological properties (Maheshwari, Singh, Gaddipati, & Srimal, 2006; Ono, Hasegawa, Naiki, & Yamada, 2004). Khanji et al. (Khanji et al., 2018a) used FS analysis to demonstrate that interactions in the solutions were primarily hydrophobic. From pH 7.4 to pH 5.0 during the gelation process, the binding site numbers varied from 1.25 to 1.49, and the binding constant varied from 3.9 to 7.5x104 M−1. In another study with the purified casein fractions, Bourassa et al. (Bourassa, Bariyanga, & Tajmir-Riahi, 2013) founded that curcumin and α- and β-caseins formed complexes through hydrophilic and hydrophobic interactions with binding constants comprised between 10^5 and 10^4 M^{-1}. Bound curcumin molecule counts were 1.43 per α-casein molecule and 1.27 per β-

casein molecule. These interactions were stabilized by a hydrogen bonding network with a free binding energy of −8.89 kcal per mol of α-casein and −10.70 kcal per mol of β-casein.

Besides the complexation of curcumin with CMs, in the study conducted by Khanji et al. (Khanji et al., 2018a), it was also observed that the ζ-potential value of CMs was not changed by curcumin addition. Acid gelation was examined using oscillation rheology and static multiple light scattering at 20 and 35 °C and led to similar behaviors for native and curcumin-doped CM suspensions. The authors demonstrated that the colloidal and functional properties of CMs remained unchanged when doped with curcumin during acidification. These results are interesting because they showed that the industrial process (at lab scale) to produce acid milk gels is not disturbed by the presence of curcumin molecules. Khanji et al. (Khanji et al., 2018a), studied CMs – curcumin interactions in powder systems. The sample was prepared by mixing 8 L of rehydrated CMs (15.5 % dry matter) with 290 mL of ethanolic curcumin solution (1 mg/mL) for 1 h. The sample was then centrifuged (5000 rpm, 10 min) and spray-dried. The mixture was atomized in the drying chamber at an inlet temperature of 180 °C. The powder particles were separated from the drying air at an outlet temperature of 90 °C. Powders were analyzed by small-angle x-ray scattering (SAXS) to determine possible CMs structure changes following interactions with curcumin. No differences in the internal CMs structure were observed after interaction with curcumin. In addition, the curcumin's antioxidant activity, monitored with ABTS and FRAP, was preserved for 60 days of storage at 40°C and remained at ~ 82 % and ~ 84 % levels, respectively. These findings open new possibilities for curcumin-doped CMs powders.

Ghayour et al. (Ghayour et al., 2019) investigated interactions between curcumin and quercetin with CasNa and studied their respective re-assembled micellar nanostructures or casein nanoparticle formations. During the study, CasNa was dispersed in buffer solutions at pH 7.4. Curcumin and quercetin were prepared in absolute ethanol at 200 and 600 μM, respectively. Spectrofluorometry was used to determine quercetin and curcumin binding, which were 0.96 and 0.78, respectively. The binding constants were 3.2×10^4 for quercetin and $0.92 \times 10^4\ M^{-1}$ for curcumin. The changes in the relative viscosity of the samples during the re-assembly process confirmed the formation of micellar nanostructures. In addition, the authors revealed the entrapment efficiency was greater than 90 % for both systems.

The interactions and gelation properties that occur between curcumin, CMs, Lactobacillus delbrueckii bulgaricus (Lb) and Streptococcus thermophilus (St) were evaluated by Khanji et al. (Khanji et al., 2018b). CMs were obtained by milk microfiltration then spray-dried. Suspensions were prepared at 50 g/L in a buffer at pH 7.4. Analysis by FS was carried out to investigate interactions between curcumin and St and Lb. A decrease in fluorescence intensity, from 1.7 A.U to 1.20 and 1.40 A.U confirmed the quenching process. The gelation process was studied using particle size analysis with multiple light scattering (Turbiscan) and rheometry. The results showed that curcumin adsorption did not affect Lb and St growth or milk acidification rate. For the first time, the authors demonstrated that curcumin interacted with lactic bacteria without modifying its growth or milk gelation properties.

Kumar et al. (Kumar et al., 2016) examined (i) curcumin preparations in oil-in-water nanoemulsions stabilized with CasNa and (ii) a subsequent addition of these to ice cream. The authors dissolved different concentrations of CasNa (1 – 7 %) in aqueous solutions with milk fat (5 – 9 %), curcumin (0.12 – 0.6 %) and medium-chain triglycerides (1 – 5%). The nanoemulsions were obtained by homogenization and their physicochemical properties were studied. The optimal ratio was observed when milk fat was at 8 %, medium chain triglycerides were at 2 %, curcumin was at 0.24 % and CasNa was at 6 %. In these conditions, the nanoemulsions presented a particle size of ~ 330 nm, a ζ-potential of ~ − 44 mV with an encapsulation efficiency of ~ 97 %. An in vitro release of curcumin under simulated digestion revealed that nanoemulsions remained stable against pepsin digestion (5.25 % release of curcumin) and pancreatic action (16.12 % release of curcumin). Following these experiments, ice cream was prepared by mixing the dry ingredients (skim milk powder, stabilizer, agar emulsifier and sugar) with milk and cream. The mixture was then homogenized and the O/W nanoemulsions were added. The ice cream base was homogenized, pasteurized and stored overnight at 5 °C. The next day, mango flavor was added to the ice cream mix and transferred to a batch freezer. The ice cream with added nanoemulsions was put to a taste test with a panel of volunteers. Results revealed no significant sensory attribute difference between the control ice cream sample and the ice cream sample prepared with a curcumin nanoemulsion. The authors suggest ice cream may be an ideal food system for delivering curcumin nanoemulsions.

Encapsulation technology which utilizes the pH-dependent solubility properties of curcumin and the self-assembly properties of CasNa was explored by Pan et al. (Pan, Luo, Gan, Baekcand, & Zhong, 2014). Curcumin was deprotonated by dissolution in a CasNa dispersion at pH 12 at 21 °C for 30 min. Capsule creation was achieved by re-associating CasNa after the neutralization of the dispersion (pH 7.0), as confirmed by dynamic light scattering (DLS) and analytical ultracentrifugation. The bioactivity capacity of curcumin to restrict the proliferation of human colorectal cancer cells (HCT-116) and human pancreatic cancer cells (BxPC3) was tested. Results showed that curcumin encapsulated in casein nanoparticles demonstrate greater anti-proliferation activity for both HCT-116 and BxPC3 cells compared to free curcumin pre-dissolved in a polar solvent.

5.2. Anthocyanins

Anthocyanins are plant pigments found throughout the natural world whose color depends on the environment's pH. The pigments can assume a range of colors from blue to red, passing through shades of purple and orange (He & Giusti, 2010). Aside from their use as colorants, anthocyanins have garnered attention for their effectiveness as bioactive compounds (Diaconeasa, Leopold, Rugină, Ayvaz, & Socaciu, 2015). Several works have attributed health characteristics to anthocyanins. These include reduced risk of heart disease, stroke, cancer, and obesity (Wrolstad, 2004). Anthocyanins' potential use in food formulations is limited by their high susceptibility to degradation (de Moura, Berling, Germer, Alvim, & Hubinger, 2018). The pigments are prone to degradation in the presence of light, oxygen, ascorbic acid and other factors. Therefore, it is essential to find technologies that can protect and maintain their benefits during destabilizing conditions (de Moura et al., 2018).

Malvidin-3-O-glucoside is the primary anthocyanin present in grape skin anthocyanin extract. The molecule contains several phenolic cycles that are sensitive to oxidation. He et al. (He, Xu, Zeng, Qin, & Chen, 2016) studied the interactions between malvidin-3-O-glucoside and α- and β-caseins. Using FS analysis, the authors showed that α- and β-caseins bind to malvidin-3-O-glucoside via both hydrophilic (van der Waals forces and hydrogen bonding) and hydrophobic interactions, with binding affinities ~ 10^3 M^{-1}. FTIR and CD indicated the caseins' secondary structures changed after binding. The authors also showed that a casein concentration of 0.1 mg/mL

promoted a decrease in anthocyanin degradation rates of 37.61 %, 18.40 %, and 29.37 % in solutions at pH 6.3 under thermal (80 °C / 2 h), oxidation (0.005 % H2O2 / 1 h) and photo illumination (5000 Lux / 5 d) treatments, respectively. It was concluded that complex malvidin-3-O-glucoside - caseins can be used as natural colorants in food systems that may be exposed to light or high temperatures with the preservation of the biological activity of anthocyanin molecules.

The interaction mechanisms that occur between cyanidin-3-O-glucoside (C3G) and CasNa nanoparticles at pH 7 and 2 were studied by Casanova et al. (Casanova et al., 2018) using FS and DLS. The authors found a complex formation between C3G – CasNa with static interaction. C3G interacted with two sets of binding sites with association constants of 10^6 and 10^5 M^{-1}. Electrostatic interactions were predominant at pH 7, while hydrophobic effects were the main force at pH 2. DLS analysis showed a slight modification in the CasNa without any alteration in its surface charge. The complexation of C3G molecules and CasNa occurred within the internal casein structure of the particles. The authors proposed CasNa as a putative nanocarrier for anthocyanins in soft drinks when an acidic pH is needed. Nascimento et al. (Nascimento et al., 2020b) investigated the effect of C3G-rich jaboticaba (Myrciaria cauliflora) extract on the rheological properties of CMs hydrogels. The addition of jaboticaba extract decreased the hydrogels' elasticity and produced hydrogels with larger pore sizes compared to control samples. The authors proposed the use of transglutaminase (an enzyme that promotes the formation of covalent bonds between glutamine and lysine residues) as a way to balance out the fruit extract's effect on the hydrogels' structure. The transglutaminase would also modulate the release rate of the encapsulated anthocyanins. The anthocyanin release was evaluated for three pH values, and the authors found a higher release rate at pH 7.0. These results highlight the potential use of CMs hydrogels for controlled anthocyanin release in the small intestine.

Interactions between pelargonidin, an anthocyanidin present in pomegranate fruit (Noda, Kaneyuki, Mori, & Packer, 2002) and β-lactoglobulin, WPI, and CasNa were investigated by Arroyo-Maya et al. (Arroyo-Maya, Campos-Terán, Hernández-Arana, & McClements, 2016). FS experiments demonstrated that pelargonidin quenched milk proteins. The authors showed that the secondary structure of all proteins evaluated was not significantly affected by pelargonidin. Analysis of fluorescence data indicated

that β-lactoglobulin and caseinate, but not WPI, bound the pelargonidin at both pH 7.0 and 3.0 with a binding constant of 1.0 x 10^5 M^{-1}.

5.3. Resveratrol

Resveratrol is a polyphenol commonly found in grapes and peanuts (Karthikeyan, Prasad, Ganamani, & Balamurugan, 2013). Studies attribute certain health benefits to resveratrol. These include anti-cancer, anti-inflammatory, neuroprotective and anti-diabetes activity (Bastianetto, Menard, & Quirion, 2015; Varoni, Faro, Sharifi-rad, & Iriti, 2016; Szkudelski & szkudelska, 2015; Bonnefont-Rousselot, 2016). Many authors have attempted to incorporate resveratrol into food systems (Pando, Beltrán, Gerone, Matos, & Pazos, 2015; Davidov-Pardo & Mcclements, 2014; Sessa et al., 2014). However, the polyphenol's poor water solubility presents a drawback and a carrier agent is necessary for its incorporation into a food product.

Insertion of resveratrol into an oil phase followed by casein stabilization is another way to encapsulate resveratrol. A CasNa / maltodextrin conjugate was developed by Consoli et al. (Consoli et al., 2018). A Maillard reaction using a wet-heating procedure was used to induce the conjugate formation. The conjugates that formed after different reaction times (3, 6, 9, 12 and 24 h) were used to stabilize resveratrol emulsions dispersed in palm oil. The authors showed that longer reaction times lead to the formation of higher molecular weight conjugates. These conjugates increased emulsion stability because they could cover a larger area than smaller conjugates. At the same time, higher reaction times also led to higher antioxidant activity. The authors concluded that CasNa / maltodextrin conjugates formed by the Maillard reaction can be used to stabilize emulsions containing resveratrol and retain the resveratrol's antioxidant properties.

Cheng et al. (Cheng, Fan, Liu, Wusigale, & Liang, 2020), dispersed resveratrol in sunflower oil and added CasNa with pectin or gum arabic to stabilize the O/W emulsion. The authors observed a decrease in wavelength intensity in the resveratrol emission spectra due to a change in the resveratrol environment which became more hydrophobic after the CasNa addition. The presence of gum Arabic did not influence the emission spectra, however, increasing the concentration of pectin gradually decreased the resveratrol's fluorescence intensity. In addition, the CasNa associated

with pectin increased the stability of resveratrol after 42 days of storage with stability levels at 84 % compared to 76 % of the CasNa sample without the carbohydrate. The association between CasNa and pectin improved protection against degradation for resveratrol.

5.4. Others polyphenols

Further investigations have been carried out on the relationships between caseins and other types of polyphenols compounds. Zhou et al. (Zhou, Seo, Alli, & Chang, 2015) studied interactions between caseins and two major phenolic acid compounds in cocoa: protocatechuic acid and coumaric acid. This study was performed in a model system, then compared to caseins extracted from milk and white chocolate. Electrophoresis analysis revealed that interactions between caseins and phenolic acids were induced by incubation at 55 °C, with a formation of hydrophobic interactions and hydrogen bonding. Moeiniafshari et al. (Moeiniafshari, Zarrabi, Bordbar, 2015) investigated the interactions between naringenin, a nutraceutical flavanone present in tomatoes and citrus fruits, and β-casein. Using fluorescence quenching methods, the authors found the constant of the complex at 10^5 M^{-1} whereas a thermodynamic analysis showed that the interaction was spontaneous with contributions of van der Waals forces, hydrogen bonds and hydrophobic interactions. The interactions between rosmarinic acid, a phenolic acid found in certain members of the Lamiaceae family, and α-s1, β and k-casein were investigated by Ferraro et al. (Ferraro et al., 2015). The analysis was performed in water at pH 3.0 and 4.5 and showed that hydrophobic, hydrogen bonding and dipole-dipole interactions occur, and the stabilization of these interactions is pH-dependent.

Several studies have been done in the interaction of tea catechin, especially epigallocatechin-gallate (EGCG), with CMs (Haratifar, Meckling, & Corredig, 2014a; Haratifar, Meckling, & Corredig, 2014b; Haratifar & Corredig, 2014) and Guri et al. (Guri, Haratifar, & Corredig, 2014). The authors showed that EGCG was able to bind to casein via both hydrophilic and hydrophobic interactions. A binding constant between CMs and EGCG was calculated to be between 10^{-4} and 10^{-3} M^{-1}. However, these interactions increased the milk gelation time after rennet addition probably because these interactions limited the access of the chymosin to the κ-casein fraction (Haratifar & Corredig, 2014). The bioavailability of this complex in fighting colon cancer

cell HT-29, was also investigated. The authors showed that CMs binding did not affect the bioavailability of EGCG, and the in vitro model showed a decrease in proliferation of cancer cells without any reduction in their bioavailability, thus confirming that CMs are an appropriate delivery system of phenolic acid.

Gorji et al. (Gorji et al., 2015) studied Interactions between resveratrol and β-casein. A static interaction with a binding constant of 7.33 x 10^5 M^{-1} was observed by FS analysis. In addition, the authors suggested the presence of hydrogen bond formations between the compound and - NH2, - OH and - SH groups that are located near the tryptophan residue (position 143). However, CD analysis revealed that resveratrol did not cause any significant change to the proteins' secondary structures.

6. Casein-lipid interactions

Lipids are energy sources and membrane constituents (Calder, 2015). The structural characterization of the interaction between caseins and lipids is important to understanding how lipids may be transported by caseins and few studies have covered this topic.

Cheema et al. (Cheema, Mohan, Campagna, Jurat-Fuentes, & Harte, 2015) clarified associations between hydrophobic molecules and native CMs to provide a better understanding of their biological distribution in raw milk. Hydrophobic and hydrophilic extractions followed by ultra-performance liquid chromatography analysis were performed on protein fractions obtained from size exclusion fractionation of raw skim milk. The authors showed that hydrophobic compounds, including phosphatidylcholine, lyso-phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, demonstrated strong associations exclusively with CMs.

The associations that occur between lipids, such as cholesterol (CHOL), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dioctadecyldimethyl - ammoniumbromide (DDAB) and dioleoylphosphatidylethanolamine (DOPE), and α- and β-caseins were studied by Bourassa et al. (2013) using FTIR, FS, CD and molecular modeling. Structural analysis showed that lipids were bound to casein primarily via hydrophobic interactions, with a constant association that ranged from 10^3 and 10^4 M^{-1} and binding sites that varied from 0.7 to 1.1 lipid per protein. Docking calculations showed different binding sites for α- and β-caseins with free binding energies varying from −10 to −13 kcal/mol. According to the authors, casein

conformation was altered by lipid interactions and yielded a reduction of α-helix and β-sheet and an increase in random coil and turn structures, suggesting a partial protein unfolding.

Panja et al. (Panja, Khatua, & Halder, 2018) explored the changes that occur in CMs microenvironments when fatty acids are present. Using fluorescence analysis, the authors observed that the unsaturation of fatty acids affects the CMs structure in contrast to hydrophobic interaction forces, which is followed by a decrease in electrostatic interactions of various amino acids. Alterations in these forces are responsible for an increase in aggregate size, modifications in secondary protein structures, and different CMs morphologies. Fluorescence lifetime imaging microscopy (FLIM) analysis indicated that the CMs microstructures become more compact when unsaturated fatty acids are present. According to the authors, the results provide useful information on the binding properties of fatty acids and may help evaluate other fatty acid behaviors.

The particles formed between covalent conjugates of CasNa and maltodextrins and either soy phosphatidylcholine liposomes or soy lysophosphatidylcholine micelles in an aqueous medium at pH 7.0 were investigated by Semenova et al. (Semenova et al., 2016) using DSC, ESR and SAXS. A high encapsulation value (> 95 %) was found for these soy phospholipids formed by the conjugates. More highly-soluble complex particles formed with higher densities and higher thermodynamic affinities for an aqueous medium compared to the control samples. The results have shown that CasNa can carry hydrophobic compounds.

7. Casein-protein interactions

Different studies have described interactions between caseins and various proteins. In general, the objectives of these studies have been to create new assemblies with various techno-functionalities such as texturing and emulsifying (Broyard & Gaucheron, 2015). In the present review, we discuss only research carried out on proteins with health benefits, i.e., lysozyme and lactoferrin.

Lysozyme is a small globular enzyme that contains 129 amino acids. It has bacteriostatic effects that inhibit gram-positive bacteria through the cleavage of the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer (Wu et al., 2019). Lysozyme's isoelectric pH is close to 11 and

consequently, at a neutral pH, the protein's charge is positive and interactions with negative parts of casein molecules remain possible. Antonov et al. (Antonov, Moldenaers, & Cardinaels, 2017) studied lysozyme's structural and morphological complexation using CasNa and native CMs. Their results showed that lysozyme forms complexes with caseins from pH 7.0 up to pH 11. Lysozyme binding with CasNa and CMs leads to a disruption of the lysozyme's secondary structure.

Lactoferrin is a globular glycoprotein that is widely present in secreted bodily fluids, such as milk, saliva, tears, and nasal secretions. Lactoferrin exhibits antibacterial, antiviral, antifungal, anti-inflammatory and anti-carcinogenic properties (Wang, Timilsena, Blanch, & Adhikari, 2019). Anema and de Kruif (Anema & (Kees) de Kruif, 2016) investigated the phase separation and composition of coacervates of lactoferrin and caseins. The authors showed that optimum complexation occurs at pH 6.55 and is characterized by maximum turbidity. Two different behaviors for coacervates were observed: the kinetics of complex formation between lactoferrin with κ or β - casein is rapid and appears to occur through a nucleation and coalescence process. However, the kinetics of complex formation between lactoferrin and αs–casein is much slower. The complex formations between caseins and proteins with health benefits offer potential as a way to protect the proteins' bioactive characteristics and also incorporate and deliver other molecules with nutritional and/or health benefits in the near future.

8. Conclusions and perspectives

Caseins constitute the major protein group present in milk. For the past several years, the formation of complexes between caseins and bioactive compounds has received attention in the academic sector because it represents an effective way to encapsulate and protect the biological activity of bioactive molecules. However, caseins' aggregation states can influence their encapsulation and protective properties. In general, different types of casein aggregates may be sensitive to physicochemical environmental conditions and consequently restrict their application in food development. Cross-linking agents offer one method for overcoming these limitations. Cross-linked caseins present higher stability than non-modified caseins when exposed to high temperatures, acid conditions and light (Casanova et al., 2017;

Nogueira et al., 2019). Future studies on the in vivo effects of bioactive molecules delivered by caseins are recommended.

9. References

Abd El-Salam, M. H., & El-Shibiny, S. (2012). Formation and potential uses of milk proteins as nano delivery vehicles for nutraceuticals: A review. *International Journal of Dairy Technology*, 65(1), 13–21.

Anema, S. G., & (Kees) de Kruif, C. G. (2016). Phase separation and composition of coacervates of lactoferrin and caseins. *Food Hydrocolloids*, *52*, 670–677.

Antonov, Y. A., Moldenaers, P., & Cardinaels, R. (2017). Complexation of lysozyme with sodium caseinate and micellar casein in aqueous buffered solutions. *Food Hydrocolloids*, 62, 102-118.

Arroyo-Maya, I. J., Campos-Terán, J., Hernández-Arana, A., & McClements, D. J. (2016). Characterization of flavonoid-protein interactions using fluorescence spectroscopy: Binding of pelargonidin to dairy proteins. *Food Chemistry*, *213*, 431–439.

Atamer, Z., Post, A. E., Schubert, T., Holder, A., Boom, R. M., & Hinrichs, J. (2017). Bovine β-casein: Isolation, properties and functionality. A review. International dairy journal, 66, 115-125.

Badem, A., & Uçar, G. (2017). Production of caseins and their usages. Int. J. Food Sci. Nutr, 2, 4-9.

Bahri, A., Henriquet, C., Pugnière, M., Marchesseau, S., & Chevalier-Lucia, D. (2019). Binding analysis between monomeric β-casein and hydrophobic bioactive compounds investigated by surface plasmon resonance and fluorescence spectroscopy. *Food chemistry*, 286, 289-296.

Bastianetto, S., Ménard, C., & Quirion, R. (2015). Neuroprotective action of resveratrol. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1852(6), 1195-1201.

Benzaria, A., Maresca, M., Taieb, N., & Dumay, E. (2013). Interaction of curcumin with phosphocasein micelles processed or not by dynamic high-pressure. *Food chemistry*, 138(4), 2327-2337.

Biesalski, H. K., Dragsted, L. O., Elmadfa, I., Grossklaus, R., Müller, M., Schrenk, D., ... & Weber, P. (2009). Bioactive compounds: Definition and assessment of activity. *Nutrition*, 25(11-12), 1202-1205.

Blayo, C., Puentes-Rivas, D., Picart-Palmade, L., Chevalier-Lucia, D., Lange, R., & Dumay, E. (2014). Binding of retinyl acetate to whey proteins or phosphocasein micelles: Impact of pressure-processing on protein structural changes and ligand embedding. *Food Research International*, *66*, 167–179.

Bohin, M. C., Vincken, J. P., Westphal, A. H., Tripp, A. M., Dekker, P., van der Hijden, H. T., & Gruppen, H. (2014). Interaction of flavan-3-ol derivatives and different caseins is determined by more than proline content and number of proline repeats.

Food chemistry, 158, 408-416.

Bonnefont-Rousselot, D. (2016). Resveratrol and cardiovascular diseases. *Nutrients*, 8(5), 250.

Bourassa, P., Bariyanga, J., & Tajmir-Riahi, H. A. (2013). Binding sites of resveratrol, genistein, and curcumin with milk α- and β-caseins. *Journal of Physical Chemistry B*, 117, 1287–1295.

Bourassa, P., Bekale, L., & Tajmir-Riahi, H. A. (2014). Association of lipids with milk α- and β-caseins. *International journal of biological macromolecules*, 70, 156-166.

Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: a scientific and technological challenge. *Dairy Science & Technology*, *95*, 831–862.

Calder, P. C. (2015). Functional Roles of Fatty Acids and Their Effects on Human Health. *Journal of Parenteral and Enteral Nutrition*, *39*, 18S-32S. https://doi.org/10.1177/0148607115595980

Campos, M. R. S. (Ed.). (2018). *Bioactive compounds: health benefits and potential applications*. Woodhead Publishing.

Casanova, F., Chapeau, A. L., Hamon, P., de Carvalho, A. F., Croguennec, T., & Bouhallab, S. (2018). pH-and ionic strength-dependent interaction between cyanidin-3-O-glucoside and sodium caseinate. *Food chemistry*, 267, 52-59.Casanova, F., Silva, N. F. N., Gaucheron, F., Nogueira, M. H., Teixeira, A. V., Perrone, I. T., ... & de Carvalho, A. F. (2017). Stability of casein micelles cross-linked with genipin: A physicochemical study as a function of pH. *International Dairy Journal*, 68, 70-74.

Cheema, M., Mohan, M. S., Campagna, S. R., Jurat-Fuentes, J. L., & Harte, F. M. (2015). The association of low-molecular-weight hydrophobic compounds with native casein micelles in bovine milk. *Journal of Dairy Science*, *8*, 5155–5163.

Cheng, H., Fan, Q., Liu, T., Wusigale, & Liang, L. (2020). Co-encapsulation of α-tocopherol and resveratrol in oil-in-water emulsion stabilized by sodium caseinate: Impact of polysaccharide on the stability and bioaccessibility. *Journal of Food Engineering*, *264*(July 2019), 109685. https://doi.org/10.1016/j.jfoodeng.2019.109685

Cohen, Y., Levi, M., Lesmes, U., Margier, M., Reboul, E., & Livney, Y. D. (2017). Re-assembled casein micelles improve in vitro bioavailability of vitamin D in a Caco-2 cell model. *Food & Function*, 8(6), 2133-2141.

Condict, L., Kaur, J., Hung, A., Ashton, J., & Kasapis, S. (2019). Combined spectroscopic, molecular docking and quantum mechanics study of β-casein and ferulic acid interactions following UHT-like treatment. *Food Hydrocolloids*, 89, 351-359.

Consoli, L., Dias, R. A. O., Rabelo, R. S., Furtado, G. F., Sussulini, A., Cunha, R. L., & Hubinger, M. D. (2018). Sodium caseinate-corn starch hydrolysates conjugates obtained through the Maillard reaction as stabilizing agents in resveratrol-loaded emulsions. *Food Hydrocolloids*, *84*, 458–472.

https://doi.org/10.1016/j.foodhyd.2018.06.017

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft Matter*, 7(6), 2265-2272.

Damodaran, S., Parkin, K. L., Fennema, O. R. (2008). *Fennema's Food Chemistry* (4th ed.), Taylor and Francis group, CRC press.

Davidov-Pardo, G., & McClements, D. J. (2014). Resveratrol encapsulation: Designing delivery systems to overcome solubility, stability and bioavailability issues. *Trends in Food Science & Technology*, 38(2), 88-103.

Davies, D. T., & Law, A. J. (1980). The content and composition of protein in creamery milks in south-west Scotland. *Journal of Dairy Research*, 47(1), 83-90.

de Moura, S. C. S. R., Berling, C. L., Germer, S. P. M., Alvim, I. D., & Hubinger, M. D. (2018). Encapsulating anthocyanins from Hibiscus sabdariffa L. calyces by ionic gelation: Pigment stability during storage of microparticles. *Food Chemistry*, *241*, 317–327. https://doi.org/10.1016/j.foodchem.2017.08.095

Diaconeasa, Z., Leopold, L., Rugină, D., Ayvaz, H., & Socaciu, C. (2015). Antiproliferative and antioxidant properties of anthocyanin rich extracts from blueberry and blackcurrant juice. *International Journal of Molecular Sciences*, *16*(2), 2352–2365. https://doi.org/10.3390/ijms16022352

Duerasch, A., Wissel, J., & Henle, T. (2018). Reassembling of alkali-treated casein micelles by microbial transglutaminase. Journal of agricultural and food chemistry, 66(44), 11748-11756.

Faridi Esfanjani, A., & Jafari, S. M. (2016). Biopolymer nano-particles and natural nano-carriers for nano-encapsulation of phenolic compounds. *Colloids and Surfaces B: Biointerfaces*, *146*, 532–543. https://doi.org/10.1016/j.colsurfb.2016.06.053

Ferraro, V., Madureira, A. R., Fonte, P., Sarmento, B., Gomes, A. M., & Pintado, M. E. (2015). Evaluation of the interactions between rosmarinic acid and bovine milk casein. *RSC Advances*, 5(107), 88529-88538.

Gazzali, A. M., Lobry, M., Colombeau, L., Acherar, S., Azaïs, H., Mordon, S., ... & Frochot, C. (2016). Stability of folic acid under several parameters. *European Journal of Pharmaceutical Sciences*, 93, 419-430.

Ghatak, D., & Iyyaswami, R. (2019). Selective encapsulation of quercetin from dry onion peel crude extract in reassembled casein particles. *Food and bioproducts processing*, 115, 100-109.

Ghayour, N., Hosseini, S. M. H., Eskandari, M. H., Esteghlal, S., Nekoei, A. R., Gahruie, H. H., ... & Naghibalhossaini, F. (2019). Nanoencapsulation of quercetin and curcumin in casein-based delivery systems. *Food Hydrocolloids*, 87, 394-403.

Gorji, E. G., Rocchi, E., Schleining, G., Bender-Bojalil, D., Furtmüller, P. G., Piazza, L., ... & Toca-Herrera, J. L. (2015). Characterization of resveratrol–milk protein interaction. *Journal of Food Engineering*, 167, 217-225.

Guaadaoui, A., Benaicha, S., Elmajdoub, N., Bellaoui, M., & Hamal, A. (2014). What

is a bioactive compound? A combined definition for a preliminary consensus. *International Journal of Nutrition and Food Sciences*, 3, 174-179.

Guri, A., Haratifar, S., & Corredig, M. (2014). Bioefficacy of tea catechins associated with milk caseins tested using different in vitro digestion models. *Food Digestion*, 5(1-3), 8-18.

HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids*, 22(8), 1460-1466.

Haratifar, S., Meckling, K. A., & Corredig, M. (2014a). Antiproliferative activity of tea catechins associated with casein micelles, using HT29 colon cancer cells. *Journal of Dairy Science*, 97(2), 672-678.

Haratifar, S., Meckling, K. A., & Corredig, M. (2014b). Bioefficacy of tea catechins encapsulated in casein micelles tested on a normal mouse cell line (4D/WT) and its cancerous counterpart (D/v-src) before and after in vitro digestion. *Food & Function*, 5(6), 1160-1166.

Haratifar, S., & Corredig, M. (2014). Interactions between tea catechins and casein micelles and their impact on renneting functionality. *Food Chemistry*, *143*, 27–32.

He, J., & Giusti, M. M. (2010). Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annual Review of Food Science and Technology*, *1*(1), 163–187. https://doi.org/10.1146/annurev.food.080708.100754

He, Z., Xu, M., Zeng, M., Qin, F., & Chen, J. (2016). Interactions of milk α- and β-casein with malvidin-3-O-glucoside and their effects on the stability of grape skin anthocyanin extracts. *Food Chemistry*, *199*, 314–322.

Holland, J. W. (2009). Post-translational modifications of caseins. In H. Thompson, A., Boland, M., Singh (Ed.), *Milk Proteins: From Expression to Food* (pp. 107–132). San Diego.

Horne, D. S. (2020). Casein micelle structure and stability. In H. Thompson, A., Boland, M., Singh (Ed.), *Milk Proteins: From Expression to Food* (pp. 213-250). Academic Press.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *Journal of Dairy Science*, 96(10), 6127-6146.

Jain, A., Thakur, D., Ghoshal, G., Katare, O. P., & Shivhare, U. S. (2016). Characterization of microcapsulated β-carotene formed by complex coacervation using casein and gum tragacanth. *International Journal of Biological Macromolecules*, 87, 101-113.

Jarunglumlert, T., Nakagawa, K., & Adachi, S. (2015). Influence of aggregate structure of casein on the encapsulation efficiency of β-carotene entrapped via hydrophobic interaction. *Food Structure*, 5, 42-50.

Jia, Z., Dumont, M. J., & Orsat, V. (2016). Encapsulation of phenolic compounds present in plants using protein matrices. *Food Bioscience*, *15*, 87–104.

https://doi.org/10.1016/j.fbio.2016.05.007

Jin, T. R. (2018). Curcumin and dietary polyphenol research: Beyond drug discovery. *Acta Pharmacologica Sinica*, *39*(5), 779–786. https://doi.org/10.1038/aps.2017.179

Karthikeyan, S., Prasad, R. R., Ganamani, A., & Balamurugan, E. (2013). Anticancer activity of resveratrol-loaded gelatin nanoparticles on NCI-H460 non-small cell lung cancer cells. *Biomedicine and Preventive Nutrition*, *3*(1), 64–73. https://doi.org/10.1016/j.bionut.2012.10.009

Katouzian, I., & Jafari, S. M. (2016). Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins. *Trends in Food Science and Technology*, *53*, 34–48.

Kaur, J., Katopo, L., Hung, A., Ashton, J., & Kasapis, S. (2018). Combined spectroscopic, molecular docking and quantum mechanics study of β-casein and p-coumaric acid interactions following thermal treatment. *Food chemistry*, 252, 163-170.

Khanji, A. N., Michaux, F., Jasniewski, J., Petit, J., Lahimer, E., Cherif, M., ... & Banon, S. (2015). Structure and gelation properties of casein micelles doped with curcumin under acidic conditions. *Food & Function*, 6(12), 3624-3633.

Khanji, A. N., Michaux, F., Petit, J., Salameh, D., Rizk, T., Jasniewski, J., & Banon, S. (2018a). Structure, gelation, and antioxidant properties of curcumin-doped casein micelle powder produced by spray-drying. *Food & Function*. https://doi.org/10.1039/C7FO01923H

Khanji, A. N., Michaux, F., Salameh, D., Rizk, T., Banon, S., & Jasniewski, J. (2018b). The study of curcumin interaction with micellar casein and lactic acid bacteria cell envelope. *LWT - Food Science and Technology*, *91*(September 2017), 293–302. https://doi.org/10.1016/j.lwt.2018.01.067

Knoop, A. M., Knoop, E., & Wiechen, A. (1979). Sub-structure of synthetic casein micelles. Journal of Dairy Research, 46(2), 347-350.

Kumar, D. D., Mann, B., Pothuraju, R., Sharma, R., Bajaj, R., & Minaxi. (2016). Formulation and characterization of nanoencapsulated curcumin using sodium caseinate and its incorporation in ice cream. *Food & Function*, *7*(1), 417–424. https://doi.org/10.1039/c5fo00924c

Lakowicz, J. R. (Ed.). (2013). Principles of fluorescence spectroscopy. Springer science & business media.

Levinson, Y., Ish-Shalom, S., Segal, E., & Livney, Y. D. (2016). Bioavailability, rheology and sensory evaluation of fat-free yogurt enriched with VD 3 encapsulated in re-assembled casein micelles. *Food & function*, 7(3), 1477-1482.

Loewen, A., Chan, B., & Li-Chan, E. C. Y. (2018). Optimization of vitamins A and D3 loading in re-assembled casein micelles and effect of loading on stability of vitamin D3 during storage. *Food Chemistry*, *240*, 472–481.

Lucey, J. A., & Horne, D. S. (2018). Perspectives on casein interactions. *International Dairy Journal*, 85, 56–65. https://doi.org/10.1016/j.idairyj.2018.04.010

Maheshwari, R. K., Singh, A. K., Gaddipati, J., & Srimal, R. C. (2006). Multiple biological activities of curcumin: A short review. *Life Sciences*, *78*(18), 2081–2087.

Mahmoodani, F., Perera, C. O., Abernethy, G., Fedrizzi, B., & Chen, H. (2018). Lipid oxidation and vitamin D3 degradation in simulated whole milk powder as influenced by processing and storage. *Food Chemistry*, 261, 149-156.

Mantovani, R. A., Hamon, P., Rousseau, F., Tavares, G. M., Mercadante, A. Z., Croguennec, T., & Bouhallab, S. (2020). Unraveling the molecular mechanisms underlying interactions between caseins and lutein. *Food Research International*, 138, 109781.

Martins, N., Barros, L., & Ferreira, I. C. F. R. (2016). In vivo antioxidant activity of phenolic compounds: Facts and gaps. *Trends in Food Science and Technology*, *48*, 1–12. https://doi.org/10.1016/j.tifs.2015.11.008

Menéndez-Aguirre, O., Kessler, A., Stuetz, W., Grune, T., Weiss, J., & Hinrichs, J. (2014). Increased loading of vitamin D2 in reassembled casein micelles with temperature-modulated high pressure treatment. *Food research international*, 64, 74-80.

Miralles, B., Sanchón, J., Sánchez-Rivera, L., Martínez-Maqueda, D., Le Gouar, Y., Dupont, D., ... & Recio, I. (2021). Digestion of micellar casein in duodenum cannulated pigs. Correlation between in vitro simulated gastric digestion and in vivo data. *Food Chemistry*, 343, 128424.

Moeiniafshari, A. A., Zarrabi, A., & Bordbar, A. K. (2015). Exploring the interaction of naringenin with bovine beta-casein nanoparticles using spectroscopy. *Food Hydrocolloids*, 51, 1-6.

Moeller, H., Martin, D., Schrader, K., Hoffmann, W., & Lorenzen, P. C. (2018). Spray- or freeze-drying of casein micelles loaded with Vitamin D2: Studies on storage stability and in vitro digestibility. *Lwt*, *97*, 87–93.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., Teixeira, A. V. N.C., & Carvalho, A. F. (2020a). Casein-based hydrogels: A mini-review. *Food Chemistry*, 314, 126063.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., de Carvalho Teixeira, Á. V. N., Júnior, P. P. D. S. P., Vidigal, M. C. T. R., ... & de Carvalho, A. F. (2020b). Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (*Myrciaria cauliflora*) extract. *Food Hydrocolloids*, 105872.

Noda, Y., Kaneyuki, T., Mori, A., & Packer, L. (2002). Antioxidant activities of pomegranate fruit extract and its anthocyanidins: Delphinidin, cyanidin, and pelargonidin. *Journal of Agricultural and Food Chemistry*, *50*(1), 166–171. https://doi.org/10.1021/jf0108765

Nogueira, M. H., Tavares, G. M., Casanova, F., Silva, C. R., Rocha, J. C., Stringheta, P. C., ... & de Carvalho, A. F. (2020). Cross-linked casein micelle used as encapsulating agent for jaboticaba (Plinia jaboticaba) phenolic compounds by spray drying. International Journal of Dairy Technology.

Nogueira, M. H., Tavares, G. M., Silva, N. F. N., Casanova, F., Stringheta, P. C.,

Gaucheron, F., ... & Carvalho, A. F. (2019). Physico-chemical stability of casein micelles cross-linked by transglutaminase as a function of acidic pH. *Food structure*, 19, 100103.

Nowak, E., Livney, Y. D., Niu, Z., & Singh, H. (2019). Delivery of bioactives in food for optimal efficacy: What inspirations and insights can be gained from pharmaceutics?. *Trends in Food Science and Technology*, 91, 557–573. https://doi.org/10.1016/j.tifs.2019.07.029

O'mahony, J. A., & Fox, P. F. (2013). Milk proteins: Introduction and historical aspects. In Advanced dairy chemistry (pp. 43-85). Springer, Boston, MA.

Ono, K., Hasegawa, K., Naiki, H., & Yamada, M. (2004). Curcumin has potent anti-amyloidogenic effects for Alzheimer's β-amyloid fibrils in vitro. *Journal of neuroscience research*, 75(6), 742-750.

Pando, D., Beltrán, M., Gerone, I., Matos, M., & Pazos, C. (2015). Resveratrol entrapped niosomes as yoghurt additive. *Food chemistry*, 170, 281-287.

Panja, S., Khatua, D. K., & Halder, M. (2018). Investigations on the Effect of Fatty Acid Additives on Casein Micelles: Role of Ethylenic Unsaturation on the Interaction and Structural Diversity. *ACS Omega*, *3*(1), 821–830. https://doi.org/10.1021/acsomega.7b01741

Pan, K., Chen, H., Davidson, P. M., & Zhong, Q. (2014). Thymol nanoencapsulated by sodium caseinate: physical and antilisterial properties. *Journal of Agricultural and Food Chemistry*, *62*, 1649–1657.

Pan, K., Luo, Y., Gan, Y., Baek, S. J., & Zhong, Q. (2014). pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter*, 10(35), 6820-6830.

Penalva, R., Esparza, I., Agüeros, M., Gonzalez-Navarro, C. J., Gonzalez-Ferrero, C., & Irache, J. M. (2015). Casein nanoparticles as carriers for the oral delivery of folic acid. *Food Hydrocolloids*, *44*, 399–406.

Ranadheera, C. S., Liyanaarachchi, W. S., Chandrapala, J., Dissanayake, M., & Vasiljevic, T. (2016). Utilizing unique properties of caseins and the casein micelle for delivery of sensitive food ingredients and bioactives. *Trends in Food Science & Technology*, 57, 178-187.

Rehan, F., Ahemad, N., & Gupta, M. (2019). Casein nanomicelle as an emerging biomaterial—A comprehensive review. *Colloids and Surfaces B: Biointerfaces*, 179, 280–292. https://doi.org/10.1016/j.colsurfb.2019.03.051

Rezaei, A., Fathi, M., & Jafari, S. M. (2019). Nanoencapsulation of hydrophobic and low-soluble food bioactive compounds within different nanocarriers. *Food Hydrocolloids*, 88, 146–162. https://doi.org/10.1016/j.foodhyd.2018.10.003

Sabliov, C., Chen, H., & Yada, R. (Eds.). (2015). *Nanotechnology and functional foods: effective delivery of bioactive ingredients*. John Wiley & Sons.

Sahu, A., Kasoju, N., & Bora, U. (2008). Fluorescence study of the curcumin− casein micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules*, 9(10), 2905-2912.

Semenova, M. G., Zelikina, D. V., Antipova, A. S., Martirosova, E. I., Grigorovich, N. V., Obushaeva, R. A., ... & Kasparov, V. V. (2016). Impact of the structure of polyunsaturated soy phospholipids on the structural parameters and functionality of their complexes with covalent conjugates combining sodium caseinate with maltodextrins. *Food Hydrocolloids*, 52, 144-160

Semo, E., Kesselman, E., Danino, D., & Livney, Y. D. (2007). Casein micelle as a natural nano-capsular vehicle for nutraceuticals. Food hydrocolloids, 21(5-6), 936-942.

Sessa, M., Balestrieri, M. L., Ferrari, G., Servillo, L., Castaldo, D., D'Onofrio, N., ... & Tsao, R. (2014). Bioavailability of encapsulated resveratrol into nanoemulsion-based delivery systems. *Food Chemistry*, 147, 42-50.

Szkudelski, T., & Szkudelska, K. (2015). Resveratrol and diabetes: from animal to human studies. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1852(6), 1145-1154.

Sinela, A., Rawat, N., Mertz, C., Achir, N., Fulcrand, H., & Dornier, M. (2017). Anthocyanins degradation during storage of Hibiscus sabdariffa extract and evolution of its degradation products. *Food Chemistry*, 214, 234-241.

Swaisgood, H. E. 2003. Chemistry of the caseins. In: P. F. Fox & P. L. H. McSweeney (Eds.), Advanced Dairy Chemistry (Vol. 1, pp. 139–202). New York

Tavares, G. M., Croguennec, T., Carvalho, A. F., & Bouhallab, S. (2014). Milk proteins as encapsulation devices and delivery vehicles: Applications and trends. Trends in Food Science and Technology, 37,5–20.

Tripodi, E., Lazidis, A., Norton, I. T., & Spyropoulos, F. (2019). Food Structure Development in Emulsion Systems. In *Handbook of Food Structure Development* (pp. 59-92).

Thomar, P., Nicolai, T., Benyahia, L., & Durand, D. (2013). Comparative study of the rheology and the structure of sodium and calcium caseinate solutions. International Dairy Journal, 31(2), 100-106.

Varoni, E. M., Lo Faro, A. F., Sharifi-Rad, J., & Iriti, M. (2016). Anticancer molecular mechanisms of resveratrol. *Frontiers in Nutrition*, 3, 8.

Wang, B., Timilsena, Y. P., Blanch, E., & Adhikari, B. (2019). Lactoferrin: Structure, function, denaturation and digestion. *Critical Reviews in Food Science and Nutrition*, 59(4), 580-596.

Wrolstad, R. E. (2004). Anthocyanin pigments—Bioactivity and coloring properties. *Journal of Food Science*, 69(5), C419-C425.

Wu, T., Jiang, Q., Wu, D., Hu, Y., Chen, S., Ding, T., ... & Chen, J. (2019). What is new in lysozyme research and its application in food industry? A review. *Food Chemistry*, 274, 698-709.

Yi, J., Fan, Y., Yokoyama, W., Zhang, Y., & Zhao, L. (2016). Characterization of milk proteins-lutein complexes and the impact on lutein chemical stability. *Food Chemistry*, *200*, 91–97.

Zhang, J., Liu, Y., Liu, X., Li, Y., Yin, X., Subirade, M., ... & Liang, L. (2014). The folic acid/β-casein complex: Characteristics and physicochemical implications. *Food Research International*, 57, 162-167.

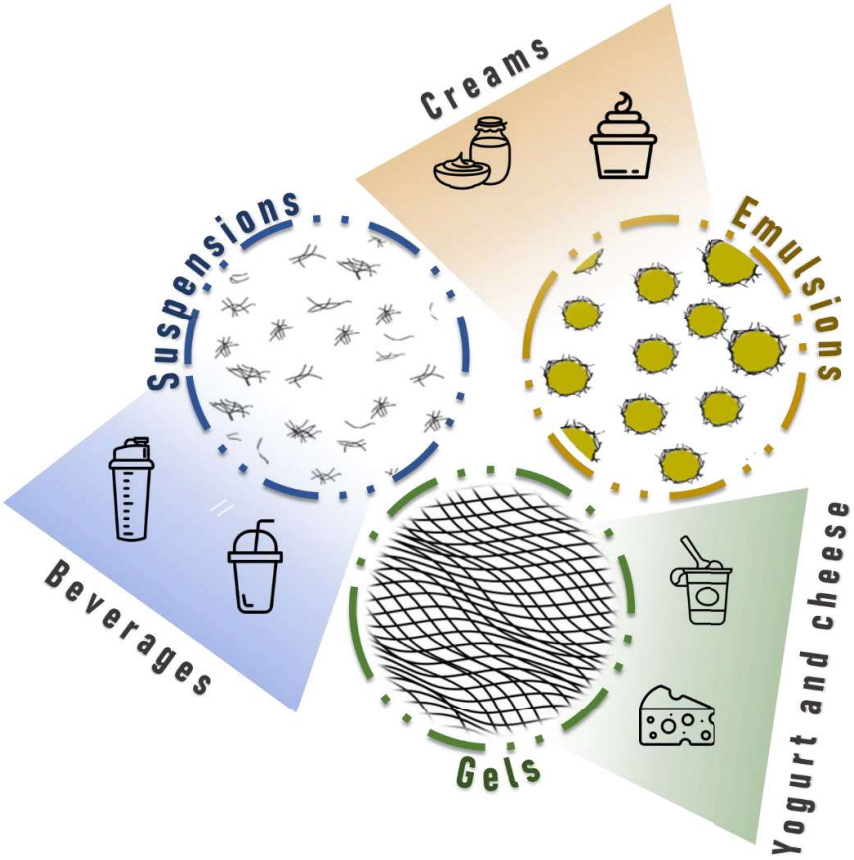
Zhou, S., Seo, S., Alli, I., & Chang, Y. W. (2015). Interactions of caseins with phenolic acids found in chocolate. *Food Research International*, *74*, 177–184.

PART B: Combination of milk and plant proteins to develop novel food systems: A Review.

The content of this part is being prepared for submission in:

Future Foods Journal

Luis Gustavo Lima Nascimento, Davide Odelli, Evandro Martins,Federico Casanova, Naaman Francisco Nogueira Silva, Paulo Peres de sá Peixoto Júnior, Guillaume Delaplace, Antônio Fernandes de Carvalho



1. Introduction

The human population is continuously growing, and it is estimated to reach 9.7 billion people in 2050, which will naturally increase the demand for animal protein for human nourishment (United Nations, 2015). A report conducted by Poore and Nemecek (2018) considered the environmental footprint of the production of 90% of global proteins based on land use, freshwater usage, GHG emissions, and chemical emissions in soil and water. The authors showed that proteins from animal sources (meat, dairy, eggs, and aquaculture) use ~ 83% of the world's available farmland and are responsible for 56-58% of general emissions, providing in the end only 37% of food protein supply (Poore & Nemecek, 2018). Thus, considering the crescent demand for proteins and the deployment of the Glasgow Climate Pact (UNFCCC, 2021), the development of sustainable production systems for the production of alternative protein sources is required.

Inside this scenario, plant proteins are good candidates to partially substitute the animal proteins in food, once it has a lower production cost and a low greenhouse effect (Clune, Crossin, & Verghese, 2017). Beyond this, plant proteins are less allergenic (Matsumiya & Murray, 2016). The consumer's increasing awareness of healthy and sustainable food products has recently enhanced the demand for plant-based proteins as food ingredients worldwide, and, only in the United States 83% of North American consumers are adding plant-based foods to their diets to improve health (NDC, 2019). Proteins from various vegetables have been already studied and employed as animal protein replacers in meat and dairy analog products (Bergsma, 2017; Lipan et al., 2020; Schreuders et al., 2019).

Many researchers have highlighted the positive nutritional aspects of this kind of protein which, among the others, include a reduced glycemic index, reduced the incidence/probability of developing cardiovascular diseases, obesity, and metabolic syndromes; conditions that reduce the overall all-cause mortality (Budhathoki et al., 2019; Lonnie & Johnstone, 2020; Qian et al., 2019). Therefore, the incorporation of plant proteins is not only a necessity, but also a consumer tendency to keep the well-being and healthy (Sá, Moreno, & Carciofi, 2020).

Despite the advantages in the use of plant proteins for human nutrition, their pronounced taste and poor solubility limit their applicability in the food industry (Nasrabadi, Doost, & Mezzenga, 2021). To overcome this techno-functional drawback,

association with animal proteins, such as milk proteins, can be an interesting strategy to increase the use of plant proteins with low compromising of food sensorial aspects. Among the potential animal proteins that can be combined with the plant ones, milk proteins stand out due to high productions, easy isolation and purification by membranes filtration systems, stability in the dry form, techno-functionality in dairy and no dairy products and good acceptability for consumers (Goulding, Fox, & O'Mahony, 2020; Uluko et al., 2016; Pouliot, 2008). The milk and plant proteins association can improve the sensorial and nutritional aspects of foods, increase the intake of plant proteins in processed foods, reduce costs in ingredients, decrease phase separation or syneresis in dairy gels (Oliveira et al., 2022; Guyomarc'h et al., 2021). It is desired that plant proteins addition in milk-based foods can improve some properties of the product, however, this addition can alter significatively the characteristics of product resulting in rejection by consumers.

Thus, the impact of this associations as well as the optimization of protein interactions must be better understood for the development of innovative products with sensory characteristics suited to the needs of consumers. In the recent years, consistent research has been delivery to study these associations in different colloidal states such as dispersions, foams, gels and emulsions (Hinderink et al., 2019). Indeed, these interactions depend of several aspects such as type of proteins, protein ratio, pH, ionic strength, presence of salts, besides industrial process that can cause protein modification such as temperature, acids and enzymes (Ben-Harb et al., 2018)

In this context, this review aims to describe the scientific advances regarding how the mixing of milk and plant protein change the features of protein systems and how these new characteristics can be useful in the formulation of foods with new textural and sensorial aspects.

2. Milk Proteins

In general, the main milk constituents are water (85 – 87%), lipids (3.8 – 5.5), lactose (4.8 – 5.0), and proteins (2.9 – 3.5 %) (Foroutan et al., 2019). The raw fluid milk can be transformed into a variety of food products such as ice cream, concentrated milk, milk powders, yogurt, cheese, etc. These transformations come mainly by manipulating the structure and organization of milk proteins, which influence taste, appearance, texture, color, and stability of these products (Walstra, 2006). The milk

protein fraction can be grouped into two main classes: the caseins which are thermal resistant and have isoelectric point in pH around 4.6 and the whey proteins, which are soluble at their isoelectric point (~pH 4.8 - 5.0) but are precipitated by increasing the ionic strength temperature (Horne, 2020; Edwards et al., 2020).

The structures and functional properties of the two main groups of milk protein will be better discussed in the following topics.

2.1. Caseins

Caseins compose about 80% of total milk proteins and they are represented by four main fractions: αs1-, αs2-, β-, and k- caseins in a molar ratio of 11:3:10:4, respectively (Walstra, 2005). In natural milk conditions, these fractions interact with each other by hydrophobic and electrostatic interactions, and calcium phosphate nanoclusters forming supramolecular structures named casein micelles (CMs). κ-casein fraction contributes to the electrostatic and steric repulsion among CMs, being the main responsible for stabilizing and maintaining CMs in suspensions (Holt et al., 2013).

Commercially, the separation of caseins from other constituents of milk, occurs by isoelectric precipitation, ultra and microfiltration, and rennet coagulation (Carter at al., 2021; Carr & Golding, 2016). Caseins can be precipitated by adjusting milk pH to 4.6, after that, a centrifugation step can separate the fractions. As a food ingredient, casein is generally applied in the form of sodium or calcium caseinate. They are produced from CMs by the addition of NaOH or CaOH to skimmed milk. The resulting product is more soluble and has better water holding capacity (WHC) compared to native CMs. Casein in different configurations, *i.e*. CMs, caseinates, acid caseins and rennet casein (caseins enzymatically precipitated) can be incorporated as food ingredients in a variety of food products such as waffles, cake mixtures, bread, cream liqueurs, coffee whiteners, processed meat and fish products and also dairy products such as cheese analogs, ice cream yogurt, among others (Carr & Golding, 2016). Despite their nutritional features, the main reasons for casein applications are their suitable functional properties such as an emulsifier agent, fat replacer, texture and water retention improver (Hammam, Martínez-Monteagudo, & Metzger, 2021). These properties are due to the casein possibility of modification and formation of different colloidal systems such as dispersions, emulsions, and gels (Nascimento et al., 2020).

2.2. Whey proteins

In the past, whey was considered a waste created by the cheese and caseins production but the panorama has changed since that, mainly due to the discovery of its nutritional and techno-functional properties, which boost whey applications in the food industry (Smithers, 2018). Whey proteins account for approximately 20% of milk proteins and are composed mainly of β-lactoglobulin (60% w/w) and α-lactalbumin (20% w/w) with lower contents of immunoglobulins (10% w/w), bovine serum albumin (3% w/w) and lactoferrin (< 0.1% w/w) (Farrell al., 2004). Contrarily to the caseins, whey proteins are globular proteins with well-defined secondary, tertiary, and quaternary structures that can be modified depending on medium conditions such as pH and ionic strength, temperature, pressure, ultrasounds treatments, pulsed electric field and enzymes (Edwards et al., 2020). When the whey proteins are heated above their denaturation temperature, the molecular structure is unfolded and the formation of new hydrophobic interactions, hydrogen bonds and disulfide bounds is favored (Guyomarc'h et al., 2015)

Ultra and microfiltration are applied largely in the manufacture of whey protein. The advantage of this technology is the use of low temperatures and the absence of chemicals or enzymes added to the milk, which results in whey proteins close their natural conformation. In the food industry, the main products obtained from whey processing are whey protein concentrates (WPCs) and whey protein isolates (WPIs). Those products can be used as ingredients in food formulations due to the ability to strengthened food gels, stabilize emulsions and foams. Also, WPCs and WPIs can be directly consumed by the final consumer after powder resuspension; thus, protein solubility plays an important role (Smithers, 2008).

3. Plant proteins

3.1. Sources

Plant proteins are characterized by a different structure and morphology than animal proteins, which highly influence their functionality (Loveday, 2020). During their evolution history, the plants have developed the ability to biosynthesize a large number of proteins for different purposes and can be generally classified into two different groups: "metabolic" and "storage" proteins. The first one represents the crucial proteins for the development of the plant, while the second one consists of the reservoir of vital

amino acids to sustain plant life (Tan, Nawaz, & Buckow, 2021). These groups represent an important nutritional source for both humans and livestock or animal feed thanks to the presence of essential amino acids which can satisfy their nutritional requirements (Bessada, Barreira, & Oliveira, 2019; Hara-Nishimura, Shimada, Hatano, Takeuchi, & Nishimura, 1998). Plant proteins are generally obtained by dry or wet extraction methods as co- or by-products from various starting materials of the oil and starch extraction industries. More than 30 plant protein sources are currently used in food formulation and overall they can be organized into 3 general groups: legumes, cereals, and oilseeds (Loveday, 2020) (Figure 1). Among the legumes, soybean and green peas are the most employed nowadays, but also proteins from other beans such as fava beans, chickpeas, and lentils are commonly requested by the food industry (Boye, Zare, & Pletch, 2010; Gumus, Decker, & McClements, 2017). Regarding the cereals group, the main sources of proteins are provided by wheat gluten, corn zein, and rice, while proteins from oilseeds are separated from the oil, starch, and fibers of products such as canola, sunflower, peanut, rapeseed, and flaxseed (Aachary et al., 2014; Loveday, 2019; Mohammed et al., 2018; Pereira et al., 2020).

Figure 1. Main plant-based sources and some of their applications

Protein ingredient	Application examples
LEGUMES  soybean, peas, fava beans, chickpeas, lentils	**MEAT PRODUCTS** Sausages, cakes bologna, meat patties, meatballs, hamburgers, nuggets. **BAKERY PRODUCTS** Pancakes, bread, doughnuts and cookies. **VEGAN PRODUCTS AND OTHERS** Gravies and soups, meat analogs, pizza toppings, bean curd, vegan cheese, and vegan ice cream.
CEREALS  wheat, corn, rice	Bakery products, zein coating, hams and turkey rolls, pizza toppings sausages, whipping agents, gluten films, adhesives, plasticizer, protein-based nanocarriers, meat replacement, and fish feeds .
OILSEEDS  canola, sunflower, peanut, rapeseed, flaxseed	Meat emulsion, sausages, tofu, ice cream, bakery products, gravy .

Adapted from (Akharume et al., 2021).

3.2. Structure and Functionality

Proteins from plants display a specific morphology when they are biosynthesized, which allows them to express their biological functions. The natural 3D structure is obtained through the folding and interaction of the protein-peptide chains, driven by several natural forces such as van der Waals and hydrophobic attraction (Tan, Nawaz, & Buckow, 2021). Hydrogen bonds, disulfide bonds, electrostatic and steric attraction/repulsion, torsional angles, and solvent interactions also participate in the morphology of the amino acid chains within a protein, but the same interaction can occur also within protein molecules. For this reason, it is reasonable to believe that proteins physiologically exist in different states which can range from monomers to oligomers and at a certain concentration, to assemblies and aggregates, all characterized by this kind of natural forces (Roberts, 2014; Schmitt et al., 2021).

The extraction method employed highly influences the colloidal state of the proteins and their functionality. It has been proven that proteins extracted from the same source with different methods may present a greater functionality variation than proteins extracted from different sources with the same method (Nicolai & Chassenieux, 2019). For instance, with a dry extraction, proteins tend to maintain their native organization, while with a wet extraction, different solvents are adopted such as water or an alkali, acid, or a salt solution which interact with the native proteins causing a potential disruption and rearrangement of their structures (Schmitt et al., 2021). Therefore, it is fundamental to adapt this process to obtain the desired characteristics of the proteins.

When added as functional ingredients, plant proteins exhibit many roles in food matrices influencing for example their texture and structure but also their organoleptic properties such as flavor, color, odor, and appearance. Indeed, thanks to their amphipathic nature they can interact with the other macronutrients like carbohydrates and fats but also with water and air, working as gelling and thickening agents, stabilizers of foams and emulsions, film-forming polymers, and binding agents for fat and water (Akharume, Aluko, & Adedeji, 2021). Moreover, they could also have biological properties exhibiting antimicrobial and antioxidant effects (Nasrabadi, Doost, & Mezzenga, 2021). Knowing therefore how each protein works in specific conditions is fundamental to designing new food products with the desired characteristics.

4. Protein-protein interactions to modify food techno-functional properties

Protein techno-functionality can be described as the protein behavior during the food processing and in a food system, the latter being based only on its physicochemical properties, without necessarily including its biological and nutritional activities (Foegeding, 2015). For example, the interactions between caseins and whey proteins play an important role in yogurt manufacturing. The protein colloidal state can be manipulated during food processing, in which other ingredients are added into the formulation and physical, chemical, or enzymatic treatments are employed. As complex systems, foods are usually composed of more than one colloidal state. Therefore, the knowledge of proteins behavior in each state is precious to food formulation, once it is a tool to predict and tailor the final product features.

4.1. Milk:plant proteins suspensions

A dispersion is a colloidal system where a solid material is dispersed into a liquid, being the solid the dispersed phase, and the liquid the continuous phase (Milani & Golkar, 2019). Thus, the formulation of beverages arises as to the direct application of the knowledge gained in these studies. Also, dispersions must be made before the other systems, *i.e.*, gels, emulsions, and foams, and the type of interactions, as well as the dispersion properties as viscosity, particle sizes, and solubility, will affect the final product (Alrosan et al., 2021). By the dispersion's definition, solubility is the most important factor and can be understood as the resultant of the protein-protein and protein-solvent interactions (Nick Pace et al., 2004). The challenge increases when it is required a high percentage of protein dispersed, as observed in high-protein beverages, mainly designed for the market of sports drinks (Shire, Shahrokh, & Liu, 2010). The solubility of plant proteins is lower than milk proteins and can even be worse when high temperatures are used for the protein extraction. In mixed systems, the presence of a different protein can impact the overall system solubility. Ben-Harb et al. (2017) observed an antagonistic effect in the solubility of mixed pea: milk proteins, where the mixture of pea and milk proteins was less soluble compared to each protein individually. However, other treatments can improve the solubility of mixed systems as demonstrated by Wang, Xu, Chen, Zhou and Wang (2019) by the application of a pH-cycle technique. By variation of dispersion pH from 12.0 to 7.0, the authors observed an increase of proximately 30 times in rice protein solubility when it was associated with WPI (1:1) compared with the pure rice suspension. The main reason for the observed phenomenon was attributed to the protein's complexation drive mainly by the formation of hydrogen bonds. Using the same method, Wang, Yue, Xu, Wang and Chen (2018) observed an increase proximately 52 times in the solubility of rice protein when combined with sodium caseinate in the ratio 1:0.01. In addition, the increase in sodium caseinate content until 1:1 ratio did not change significantly the solubility of the systems, however, the reduction of the milk protein caused a reduction in the rice protein solubility.

The viscosity and the particle size of the proteins in dispersion also change regarding the protein combination ratios, which directly impacts the process parameters. It was observed by Singh, Prakash, Bhandari and Bansal (2019) that the mixture of milk protein concentrate (MPC) with soy protein hydrolyzed (SPH) resulted

in dispersions with higher viscosities when compared with the systems formed only by one type of protein at equal protein concentrations. The coagulation time of the systems also was impacted, SPH doesn't coagulate when exposed to 145 ℃ for 15 min, and the MPC took 14 min to show the first sign of coagulation. After the mix, depending on the ratio, the coagulation time decreases for less than 2 min. It shows that the general processing make in the food industry for systems with only a protein source cannot be directly applied in mixed systems.

4.2. Milk:plant proteins gels

A gel can be defined as a colloidal system where there is a solid material in the continuous phase and liquid material in the dispersed phase (Shaw, 1992). In rheological studies, the gel characterization originates from the material response to an applied force. Thus, a system where its elastic modulus (G') is higher than its viscous modulus (G") is defined as a gel (Gunasekaran & Ak, 2000). The gelation properties of protein gels depend on intrinsic and extrinsic factors, such as amino acid composition, presence of disulfide bonds, hydrophobic and electrostatic interactions, protein concentration, ionic strength, temperature, pressure, and pH (Phillips, 2013). Particularly in a mixed system, the type of proteins, their concentration, and ratios affect the final gel properties. For example, the minimal protein concentration to achieve thermal and acid gelation was determined for mixed pea and β-lactoglobulin systems (Chihi, Sok, & Saurel, 2018). In the pure systems, the minimal concentration required for thermal gelation was 7 and 5 % for pea proteins and β-lactoglobulin, respectively. The mixed systems minimal thermal gelation varied according to the protein ratio, being 5% the least concentration between the mixed samples for 1:4 pea: β-lactoglobulin protein ratio. Smaller values were found by Wong, Vasanthan and Ozimek (2013) where the least gelation concentrations diminished when different protein rates were mixed. It was required 3% of total protein concentration to form whey and pea gels separately. However, because of the synergistic enhancement of 1:4 pea: whey, the gelation occurred at 2% of total protein at this specific protein ratio.

4.2.1. Heat-induced milk: plant proteins gels

Protein gelation can occur when a sufficient amount of energy in form of heat is applied to a system. Generally, at high temperatures, globular proteins unfold,

exposing their hydrophobic residues that were hidden in the natural conformation. Once exposed, the amino acids can associate by hydrophobic interaction, Van-der-walls forces, and hydrogen bonds or can associate more strongly with disulfide bonds (Nicolai & Chassenieux, 2019). These new interactions between the protein chains lead to aggregation and a three-dimensional structure starts to form. In milk processing, heat treatment is used to promote aggregation between whey proteins and CMs, which in turn leads to stiffer gels after acidification. Thus, when plant proteins are added to milk, the first question that appears is if plant proteins can aggregate with CMs as whey proteins?

Some authors have investigated the interactions between CMs and pea and soy proteins after heat treatment (Silva et al., 2019; Silva, Cochereau, Schmitt, Chassenieux, & Nicolai, 2019; Silva, Cochereau, Schmitt, Chassenieux, & Nicolai, 2018, Mession, Roustel, & Saurel, 2017). The common approach to access this information is using small-amplitude rheology technique to follow G' during heat application. Silva et al. (2018) studied the gelation profile of suspensions composed by CMs alone or in the presence of whey, pea, or soy protein at pH 5.8. As expected, a reinforcement of CMs gels was observed in the presence of whey protein, which was attributed to their co-aggregation. However, no reinforcement of the gel was observed even at high temperatures for both pea and soy protein, which suggests the absence of co-aggregation. Also, the protein ratios, *i.e*, the proportion between CMs and plant proteins, or protein concentrations, did not lead to their co-aggregation. In mixed systems where both CMs and plant proteins can form gels, the gel features are driven mainly by the protein that is in high concentration and it can be noted that the presence of two independent three-dimensional structures leads to less stiff gels (Silva, Cochereau, Schmitt, Chassenieux, & Nicolai, 2019). Indeed, Mession, Roustel, and Saurel (2017) studied the aggregation patterns of CMs and two fractions of pea protein, i.e., legumin, and vicilin, at pH 7.2 using reducing and non-reducing electrophoresis, DSC, and liquid chromatography. They concluded that during heat treatment, denaturation of both pea protein fractions took place, followed by the formation of protein aggregates. This aggregation occurs differently in each protein fraction, with the formation of disulfide bonds for legumin and non-covalent interactions for vicilin. However, the CMs did not participate in aggregation.

Despite the absence of co-aggregation between CMs and pea and soy proteins, the presence of the plant proteins impacts the availability of free calcium in the mixed systems, which seems to increase the CMs gelation temperature (Tgel) (Silva et al., 2018). Tgel is defined as the temperature where occurs the sol-gel transition, and in the case of CMs suspensions, it is affected by free calcium concentration in the medium (Nicolai & Chassenieux, 2021). As the temperature increases the calcium solubility decreases, which leads to calcium precipitation on the CMs surface. As consequence, CMs destabilization occurs and ultimately leads to aggregation (Huppertz & Nieuwenhuijse, 2022). Thus, the less calcium available to precipitate harder will be to aggregation occurs. As observed by Silva et al. (2018), pea and soy protein can bind calcium from the medium, where soy proteins bind more calcium than pea proteins, which resulted in higher gelation temperature of CMs in the systems where soy proteins were present. Thus, the authors argued that these plant proteins work as a chelating agent in mixed system increasing the heat stability of mixed systems in comparison to the suspensions of pure/isolated/native CMs.

The studies of how the plant protein specifically interacts with CMs are important to understand the potential application of mixed systems in the food industry. Ben-Harb et al. (2018) studied heat-induced gelation in mixed milk/pea suspensions at pH 6.33. They found that 14.8% (w/w) mixed systems gel in protein ratios of 1:1 showed G' as high as pea protein alone, while the sample containing solely milk did not gel at 7.4% (w/w) concentration and formed a weak gel at 14.8% (w/w). The data indicates that pea proteins were responsible for gel structuration, since CMs do not form gels when heated at pH as high as 6.33 (Nicolai & Chassenieux, 2021). Nevertheless, pea proteins could not be the unique responsible for gel structure, since the mixed systems only with 7.4% (w/w) of pea protein showed gel stiffness as high as the 14.8% (w/w) pea gels. Thus, interactions between whey and pea proteins may take place. Indeed, Wong, Vasanthan and Ozimek (2013) studied the gel formation achieved by heating pea and whey protein in different rations, concentrations, and pH values. The best synergistic enhancement in G' was achieved by 16% (w/w) total protein concentration, 2:8 pea: whey ratio at pH 6.0. In general, small amounts of pea protein increased the gel stiffness, but it varies upon pH and protein concentration. Each protein has its isoelectric point and solubility, thus a pH value that promotes a similar aggregation profile of both proteins leads to the formation of a more homogeneous network. Also,

the decrease in the electrostatic repulsion caused to pHs close to the protein isoelectric point leads to an increase in protein-protein interaction (Wong, Vasanthan, & Ozimek, 2013).

The mechanism of the interaction between β- lactoglobulin and pea after heat treatment at pH 7.2 was hypothesized by Chihi, Mession, Sok, and Rémi Saurel (2016). The authors suggested the unfolding of both protein types after heating, which exposed thiol groups and previously buried hydrophobic groups. Then, the proteins start to self-aggregate, and potentially aggregation between β- lactoglobulin and legumin occur by disulfide bonds. Then, those small protein aggregates interact mainly by hydrophobic and/or electrostatic interactions, which increases their sizes. Despite the differences between soy and pea proteins, it is reasonable to think that the interactions with whey proteins for both plant proteins are similar. Indeed, the formation of disulfide bonds after heat treatment for 6% (w/w) soy-whey protein mixed systems have been proposed (Roesch & Corredig, 2005). The authors showed that when high amounts of whey are present, the incorporation of soy proteins occurs, and the formed aggregate is composed of both proteins. However, the presence of low amounts of whey proteins also led to the formation of aggregates formed solely by whey proteins. These different profiles led to differences in the gel network, having a more homogeneous network, as well as presetting higher G', the gels formed when a higher amount of whey protein was present. The same feature of mixed soy-whey protein gels was observed, even at 12 and 16% (w/w) total protein concentration. Thus, in mixed systems, the whey protein is responsible for gel formation, while soy proteins appear as filler material within the gel structure (McCann, Guyon, Fischer, & Day, 2018). In resume, the incorporation of soy protein in whey gels decreases the G' and changes the network structure. It allows the creation of 16% (w/w) protein gels with the same strength of 6% (w/w), only modeling the soy-whey protein ratio (McCann, Guyon, Fischer, & Day, 2018).

4.2.2. Acid induced milk: plant proteins gels

The acid gelation is induced by pH modification toward the isoelectric point of the proteins. During the pH decreasing of protein suspension, the electrostatic and steric repulsion between the proteins is reduced, which causes approximation between them, formation of new interactions, aggregation, and ultimately the formation of a continuous three-dimensional network (Totosaus et al., 2002). In milk, the

solubilization of calcium phosphate cannot be neglected, once it causes protein rearrangement of the gel matrix. Acid gelation is widely applied in the dairy industry, mainly in the production of fermented milks and cheeses to develop desirable textural properties (Lucey, 2020).

In mixed protein systems, the difference in protein origins and properties interfere in gel formation during acidification. For example, the pH where the gelation starts for each protein impacts directly the composition of the gel network (Grygorczyk, Alexander, & Corredig, 2013). In the acid gelation of pea and milk proteins, Ben-Harb et al. (2017) observed that pea protein plays a major role in the first stages of gel formation because it goes forward the isoelectric point at a higher rate, due to its lower buffer capacity. Chihi, Sok, & Saurel (2018) showed that the rates of acidification were equal for single and mixed systems composed by β-lactoglobulin and pea protein at 4% protein concentration. According the authors, pea protein gelation occurred after 24 min of acidification in pH 6.6, while β-lactoglobulin gelation occurred 58 min after of acidification at pH 5.7 in single systems. Thus, the increase of β-lactoglobulin in the mixed systems resulted in a decrease in gelation pH. The same was observed for an acid gel formed by mixing soy and cow's milk at 4.5% total protein (Grygorczyk, Alexander, & Corredig, 2013). Soy gelation pH is around 6.0, while milk did not form gels by acidification in pH higher than 5.6. Thus, a gel network formed by the mixture of soy and milk in pH above 5.6 will be composed only of soy protein. In addition, the presence of milk proteins interfered in the soy network formation. If a rennet treatment is applied, the milk gelation pH rises to around 6.1; in this way, the formed gel network counts either with soy protein or milk protein contributions. The gelation of both proteins occurring at the same time increases G' and forms a more homogeneous network compared to cow's milk not treated with chymosin. However, the gels formed with only cow's milk or soy milk presented higher G' compared to the mixed systems. It indicates that there is no co-aggregation of the proteins and a network formation interferes in the other (Grygorczyk, Alexander, & Corredig, 2013).

The presence of plant proteins in a dairy product requires evaluation of the changes during the production process and the interferences caused by the presence of lactic acid bacteria (LABs). Yousseef, Lafarge, Valentin, Lubbers & Husson (2016) developed a pea: milk yogurts with several LABs. In those systems, altering the pea: milk protein ratio from 0:100 to 40:60 at 4.5% total protein led to faster gel formation

and increased the product acidity. The same occurred with the addition of lentil flour (Zare, Boye, Orsat, Champagne & Simpson, 2011). This phenomenon was explained by the lower buffer capacity in the systems with less casein content. Another effect after increasing pea protein amount was the increase in gels' syneresis, which was related to the differences in gel network formation. The presence of pea protein decreased the firmness of the mixed gels compared to milk gels. It was suggested that the pea proteins prevent the formation of the most homogeneous casein network, thus weakening the resulted gel. This behavior highlights the possibility to develop gelled products of similar firmness with higher protein content using vegetable proteins.

It is usual in the dairy industry the supplementation of milk with milk protein powder to increase the solid content aiming the development of a more elastic gel. The substitution of milk protein powder for lentil flour as a source of solids was evaluated by Zare, Boye, Orsat, Champagne & Simpson (2011). The syneresis of yogurts supplemented with 3% lentil flower was similar to the samples with 3% of milk powder. However, the syneresis increased when less quantity of lentil flour was added 1 and 2%. The increase in protein content in the samples with the addition of more solids, as a consequence more protein, lead to more water retention in the gel matrix compared to control samples. After 28 days of storage, the samples containing lentil flour presented G' comparable to the samples supplemented with milk proteins, showing the potential of replacement of milk proteins for plant proteins.

The formulation of an acid-induced gel system does not exclude the application of a pre-treatment before gelling. Indeed, thermal treatment of milk is generally applied before fermentation in yogurt production, which increases the gel stiffness of the final product. The pre-treatments such as heat are useful for modifying the proteins and the types of interactions between them, changing the building blocks of the acid gel. These building blocks are the foundation of the gel and their size and organization can be modulated by modifying the processing parameters such as pH, protein ratio, and the order of heat treatment, *i.e*., heating proteins separated with posterior mixing or mix the proteins with posterior heating (Mession, Roustel, & Saurel, 2017). The effect of the pre-heat treatment in the gel composed of sodium caseinate (CasNa), an important milk ingredient used in the dairy industry in several applications, and soy proteins were studied by Martin, Marta & Pouvreau (2016). The pH of the suspension during heat impacted the acid gel structure. In general, the heat treatment in lower pHs leaded

values to more fragile and coarse gels. Also, the addition of soy protein without any heat treatment resulted in a gel with a coarser microstructure. Concerning to the processing order, the heat treatment of only the soy protein with posterior mixing with CasNa lowed the mechanical properties of the gel in comparison with CasNa alone. However, mixing the proteins before heat treatment increased the gel's mechanical properties to a value close to the CasNa alone. Similar results were observed by Chihi, Sok, & Saurel (2018) studying mixed β-lactoglobulin: pea protein gels. The authors showed that when the proteins were heated separated and after mixed, the gels show a more open and disordered structure with lower WHC compared when the proteins that were heated together before acidification. However, Mession, Roustel, and Saurel (2017) studying casein: pea gels observed that heat treat the proteins separated followed by their mixture produced more elastic gels, being the type of pea protein fraction utilized a critical factor to the final gel stiffer. Thus, a general rule for all mixed milk: plant protein systems cannot be established, once the nature of the proteins changes completely the characteristics of the gel.

4.2.3. Gelation induced by other methods

Another physical method to modify proteins conformation is the use of ultrasounds. The utilization of ultrasound treatment is increasing in the food industry as a way to develop products with new features. Opposing the results reported by Mccan et al. 2018 who used heat treatment, Cui et al. 2020 developed a whey- soy-based gel with higher hardness compared to the gels produced from the isolated proteins sources. However, the authors used a combination of ultrasound treatment with transglutaminase (Tgase). While ultrasounds treatment promotes the exposure of hidden amino acid residues, Tgase can promote a cross-link reaction between them. The higher hardness was recorded when the system was sonicated for 45 min. The ultrasound treatment also influenced the water holding capacity (WHC) of all systems being mixed or not, the maximum WHC was recorded 30 min of ultrasound treatment and the mixed and separated systems did not differ. In the enzymatic gelation, the caseins are the main responsible for gel formation, once they are more susceptible to chymosin and Tgase action. The mixed gels have a lower store modulus compared to pure milk gels because the presence of other proteins can impair the action of the enzymes (Cui et al. 2020).

4.3. Mixed milk: plant proteins emulsions

Emulsions are colloidal systems formed by two immiscible liquids, where one liquid is scattered in small droplets, the dispersed phase, in the other liquid, the continuous phase. Naturally, these systems are unstable, being necessary molecules able to adsorb in the interphases to decrease the interfacial tension and increase their stability (Derkach, 2009). In foods, emulsion systems are currently found, both water dispersed in oil (w/o emulsion) or oil dispersed in water (o/w emulsion). Margarine and butters are practical examples of the former, while mayonnaise and creams of the latter (Milani & Golkar, 2019). Milk by itself is an emulsion, where the lipids are finely dispersed in the continuous water phase, and stabilized by phospholipids, CMs and whey proteins (Singh & Gallier, 2017).

The combination of sodium caseinate and soy protein with 5% oil fraction at 2% protein concentration in a 1:1 ratio was performed Ji et al. (2015). The emulsions showed an average droplet size of 250 nm and a zeta potential of -45 mV at pH of 6.8. This high zeta potential value associated with the small droplet's sizes conferred remarkable stability to the emulsion. The long-term stability of the emulsions stabilized by mixed proteins was higher than that of single proteins. After two weeks at room temperature, the droplet's sizes grow from 250 nm to more than 1100 nm for a single protein emulsion, while it did not change for mixed systems. Similar results were found by by Hinderink, Munch, Sagis, Schroen and Berton-Carabin (2019), where emulsions stabilized by combination of pea: WPI and pea: CasNa presented better stability after 14 days storage compared to emulsions where only one kind of protein was present. Showing the synergic effect of the protein blends in the emulsion stability. The mixed emulsions layer was denser than the single proteins, and it may be a reason for better emulsion stabilization, where the systems were mainly stabilized by steric repulsion (Ji et al., 2015). In the mixed systems, both proteins are absorbed in the interfacial layer with a low amount of proteins in the aqueous phase. However, during the storage time, a displacement of interfacial proteins can occur, as observed by Hinderink et al. (2019), where whey proteins could substitute pea proteins in the interface, as well as pea protein displaced CasNa, but without stability loss.

Liang, Wong, Pham & Tan (2016) studied emulsions formed by mixing CMs, pea, soy, and whey protein with a protein total concentration of 10% w/w, which is high

if compared to the concentration of emulsifiers generally used. CMs mixed with plant proteins showed lower droplet size compared to a combination of CM-whey. Higher the amount of whey, the higher the droplet size. Concerning heat stability, the systems containing soy protein presented better results in comparison to the systems formed by pea and whey.

The emulsions can also work as delivery system for sensible hydrophobic bioactive molecules, which can be applied in the fortification of foods. The mixed system CasNa: Soy proteins showed better protection properties compared to single protein systems, showing retention of vitamin A around 93% after three months of storage (Ji et al., 2015). This protection over Vitamin A is due mainly 2 factors: i. proteins' light deviation which diminishes Vitamin A light exposure and ii. ability of protein of binding metal in the aqueous phase. Milk: plant protein blends were also used as emulsifiers in lycopene emulsions (Ho, Schroën, San Martín-González, & Berton-Carabin, 2018).

. The blends containing whey-soy and whey-pea presented better emulsion stability than the proteins alone. However, an antagonistic effect was observed in the blends of CasNa and the plants, which cause emulsion destabilization after 7 days of production, probability caused by competitive absorption in the oil-drop surface between CasNa and pea (Ho, Schroën, San Martín-González, & Berton-Carabin, 2018).

The process that milk undergoes to develop the milk products changes the protein structure and interaction. The understanding of the different usual processes in the dairy industry in mixed systems is relevant to give a more concrete idea of the potential uses. Besides temperature, homogenization plays an important role during milk processing. The impact of the homogenization order, *i.e.* homogenize cow's milk with cream followed by soy milk addition, or homogenize soy milk with cream followed by cow's milk addition or homogenize both kinds of milk together was studied by (Grygorczyk, Duizer, Lesschaeve, & Corredig, 2014). The homogenization order modulate which protein will be predominant in the fat globule interfaces. When soy milk is homogenized with milk cream in absence of cows' milk, soy proteins are the major constituents in the fat globule interface. The same occurs when milk is homogenized in absence of soy milk. However, when both milks and cream are homogenized

together the fat globule interface is composed mainly of milk proteins. The homogenization process did not have an impact on the fat droplet's sizes.

5. Sensory attributes of mixed systems

An important feature of any food is its sensorial attributes. It is required consumer intent, desirable texture, taste, flavor, among others. The studies regarding the sensory evaluation of mixed proteins systems are still scarce. Zare et al. (2011) compared smoothness, graininess, flavor, color, and overall acceptance of two supplemented yogurts. The replacement of skim milk powder with lentil flour was evaluated sensorially. The yogurt supplemented with 1,2 and 3% of lentil flour showed no significant difference for smoothness, graininess, flavor, and overall acceptance when compared to yogurts supplemented with 1,2 and 3% of skim milk powder. However, in the color parameter, yogurts added with 2 and 3% of lentil flour were different from 2 and 3% skim milk yogurt. Thus, the impact of the addition of vegetable protein was not perceived by the consumers in the concentration studied, indicating that lentil flour can be used to fortify yogurts without sensorial loss. The concentration of plant protein added in dairy products must be high enough to cause desirable changes in the functional properties and at the same time cause minimum interference in the sensorial attributes. Thus, the sensorial impact caused by increasing concentration of pea protein in yogurts produced using several starter cultures was evaluated by (Yousseef et al., 2016). As pea concentration increases the intensity of the terms: pea, earth, smoked and vinegar increased, which are considered negative sensory characteristics, while the positive terms dairy and creamy decreased in intensity. Among the pea concentrations studied, yogurts containing from 20 to 40 % of pea protein were characterized as products with undesirable features. Being the 10% pea protein the closest to the control yogurt sample. Thus, the sensory changes promoted by the addition of plant proteins cannot be underestimated and studies regarding the maximum quantity of protein addition should be performed. The fermentation process shows the potentiality of decreasing the undesirable beany flavor of mixed milk-pea gels (Pua et al., 2022). However, the type of metabolites, as well as the microorganism growth depends on the composition of the gel matrix (Harper et al., 2022). Thus, it is essential the optimization the process by choosing a suitable microorganism.

Grygorczyk et al. (2013) using napping methodology investigated the effect of the order of homogenization in the texture of systems formed by soy milk and cow's milk. The homogenization of milk with cream in the presence or absence of soymilk leads to yogurts with high thickness, roughness, and mouthcoating. When the cream was homogenized in soymilk with posterior addition of skim milk, the formed gel exhibits thinner and watery features. The perception of fatty also was influenced by the homogenization order, once the fat content of all samples was the same. The samples where the aggregation of milk proteins started first had more fatty-related attributes, the opposite happened when the aggregation of the protein occurred at the same time. Showing that how the fat globules are disposed of in the matrix can influence the perception of fatty/oil in the product.

6. Conclusion and perspectives

The studies address evidence of concept indicate that combination of plant and milk proteins can be used to module colloidal systems with direct application in the food industry. The mixed systems can be applied in the formulations of several products such as protein beverages, yogurt, and cheeses. However, it is necessary the improvements of the functional properties of these systems focusing in the plant proteins. Thus, emergent approaches to modify the colloidal properties of dairy-plant proteins such as ultrasound, pulsed electric filed, ohmic heating and high hydrostatic pressure that still have reduced application, maybe the key to improve the binary systems. In addition, there is a lack of robust studies on an industrial scale and the sensory features of these products is still an important challenge.

7. References

Akharume, F. U., Aluko, R. E., & Adedeji, A. A. (2021). Modification of plant proteins for improved functionality: A review. Comprehensive Reviews in Food Science and Food Safety, 20(1), 198-224.

Alrosan, M., Tan, T. C., Easa, A. M., Gammoh, S., & Alu'datt, M. H. (2021). Molecular forces governing protein-protein interaction: Structure-function relationship of complexes protein in the food industry. Critical reviews in food science and nutrition, 1-17.

Ben-Harb, S., Panouille, M., Huc-Mathis, D., Moulin, G., Saint-Eve, A., Irlinger, F., ... & Souchon, I. (2018). The rheological and microstructural properties of pea, milk,

mixed pea/milk gels and gelled emulsions designed by thermal, acid, and enzyme treatments. Food Hydrocolloids, 77, 75-84.

Bessada, S. M., Barreira, J. C., & Oliveira, M. B. P. (2019). Pulses and food security: Dietary protein, digestibility, bioactive and functional properties. Trends in Food Science & Technology, 93, 53-68.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. Food research international, 43(2), 414-431.

Carr, A., & Golding, M. (2016). Functional milk proteins production and utilization: Casein-based ingredients. Advanced dairy chemistry, 35-66.

Carter, B. G., Cheng, N., Kapoor, R., Meletharayil, G. H., & Drake, M. A. (2021). Invited review: Microfiltration-derived casein and whey proteins from milk. Journal of Dairy Science, 104(3), 2465-2479.

Chihi, M. L., Mession, J. L., Sok, N., & Saurel, R. (2016). Heat-induced soluble protein aggregates from mixed pea globulins and β-lactoglobulin. Journal of Agricultural and Food Chemistry, 64(13), 2780-2791.

Chihi, M. L., Sok, N., & Saurel, R. (2018). Acid gelation of mixed thermal aggregates of pea globulins and β-lactoglobulin. Food Hydrocolloids, 85, 120-128.

Clune, S., Crossin, E., & Verghese, K. (2017). Systematic review of greenhouse gas emissions for different fresh food categories. Journal of Cleaner Production, 140, 766-783.

Corredig, M. (2006). Protein-protein interactions in food. FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-, 154, 283.

Cui, Q., Wang, G., Gao, D., Wang, L., Zhang, A., Wang, X., ... & Jiang, L. (2020). Improving the gel properties of transgenic microbial transglutaminase cross-linked soybean-whey mixed protein by ultrasonic pretreatment. Process Biochemistry, 91, 104-112.

Derkach, S. R. (2009). Rheology of emulsions. *Advances in colloid and interface science*, *151*(1-2), 1-23.

Edwards, P.B., Creamer, L.K., Jameson, G. B. (2020). Structure and stability of whey proteins. In Milk proteins (pp. 213-250). Academic Press.

Farrell Jr, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., ... & Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk—Sixth revision. Journal of dairy science, 87(6), 1641-1674.

Foegeding, E. A. (2015). Food protein functionality—a new model. Journal of food science, 80(12), C2670-C2677.

Foroutan, A., Guo, A. C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., ... & Wishart, D. S. (2019). Chemical composition of commercial cow's milk. Journal of agricultural and food chemistry, 67(17), 4897-4914.

Goulding, D. A., Fox, P. F., & O'Mahony, J. A. (2020). Milk proteins: An overview. Milk proteins, 21-98.

Grygorczyk, A., Alexander, M., & Corredig, M. (2013). Combined acid-and rennet-induced gelation of a mixed soya milk–cow's milk system. International journal of food science & technology, 48(11), 2306-2314.

Grygorczyk, A., Duizer, L., Lesschaeve, I., & Corredig, M. (2014). Gelation of recombined soymilk and cow's milk gels: Effect of homogenization order and mode of gelation on microstructure and texture of the final matrix. Food Hydrocolloids, 35, 69-77.

Gumus, C. E., Decker, E. A., & McClements, D. J. (2017). Impact of legume protein type and location on lipid oxidation in fish oil-in-water emulsions: Lentil, pea, and faba bean proteins. Food Research International, 100, 175-185.

Gunasekaran, S., & Ak, M. M. (2000). Dynamic oscillatory shear testing of foods—selected applications. Trends in Food Science & Technology, 11(3), 115-127.

Guyomarc'h, F., Arvisenet, G., Bouhallab, S., Canon, F., Deutsch, S. M., Drigon, V., ... & Gagnaire, V. (2021). Mixing milk, egg and plant resources to obtain safe and tasty foods with environmental and health benefits. Trends in Food Science & Technology, 108, 119-132.

Guyomarc'h, F., Famelart, M. H., Henry, G., Gulzar, M., Leonil, J., Hamon, P., ... & Croguennec, T. (2015). Current ways to modify the structure of whey proteins for specific functionalities—a review. Dairy Science & Technology, 95(6), 795-814.

Hammam, A. R., Martínez-Monteagudo, S. I., & Metzger, L. E. (2021). Progress in micellar casein concentrate: Production and applications. Comprehensive Reviews in Food Science and Food Safety, 20(5), 4426-4449.

Hara-Nishimura, I., Shimada, T., Hatano, K., Takeuchi, Y., & Nishimura, M. (1998). Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. The Plant Cell, 10(5), 825-836.

Harper, A. R., Dobson, R. C., Morris, V. K., & Moggré, G. J. (2022). Fermentation of plant-based dairy alternatives by lactic acid bacteria. Microbial Biotechnology, 15(5), 1404-1421.

Hinderink, E. B., Münch, K., Sagis, L., Schroën, K., & Berton-Carabin, C. C. (2019). Synergistic stabilisation of emulsions by blends of dairy and soluble pea proteins: Contribution of the interfacial composition. Food Hydrocolloids, 97, 105206.

Ho, K. K., Schroën, K., San Martín-González, M. F., & Berton-Carabin, C. C. (2018). Synergistic and antagonistic effects of plant and dairy protein blends on the

physicochemical stability of lycopene-loaded emulsions. Food Hydrocolloids, 81, 180-190.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. Journal of dairy science, 96(10), 6127-6146.

Horne, D. S. (2020). Casein micelle structure and stability. In Milk proteins (pp. 213-250). Academic Press.

Huppertz, T., & Nieuwenhuijse, H. (2022). Constituent fouling during heat treatment of milk: A review. International Dairy Journal, 126, 105236.

Jarpa-Parra, M., Tian, Z., Temelli, F., Zeng, H., & Chen, L. (2016). Understanding the stability mechanisms of lentil legumin-like protein and polysaccharide foams. Food Hydrocolloids, 61, 903-913.

Ji, J., Zhang, J., Chen, J., Wang, Y., Dong, N., Hu, C., ... & Wu, C. (2015). Preparation and stabilization of emulsions stabilized by mixed sodium caseinate and soy protein isolate. Food Hydrocolloids, 51, 156-165.

Liang, Y., Wong, S. S., Pham, S. Q., & Tan, J. J. (2016). Effects of globular protein type and concentration on the physical properties and flow behaviors of oil-in-water emulsions stabilized by micellar casein–globular protein mixtures. Food Hydrocolloids, 54, 89-98.

Loveday, S. M. (2020). Plant protein ingredients with food functionality potential. Nutrition Bulletin, 45(3), 321-327.

Loveday, S. M. (2019). Food proteins: technological, nutritional, and sustainability attributes of traditional and emerging proteins. Annual review of food science and technology, 10, 311-339.

Lucey, J. A. (2020). Milk protein gels. In Milk proteins (pp. 599-632). Academic Press.

Martin, A. H., Marta, L., & Pouvreau, L. (2016). Modulating the aggregation behaviour to restore the mechanical response of acid induced mixed gels of sodium caseinate and soy proteins. Food Hydrocolloids, 58, 215-223.

McCann, T. H., Guyon, L., Fischer, P., & Day, L. (2018). Rheological properties and microstructure of soy-whey protein. Food Hydrocolloids, 82, 434-441.

Mession, J. L., Roustel, S., & Saurel, R. (2017). Interactions in casein micelle–Pea protein system (part I): Heat-induced denaturation and aggregation. Food Hydrocolloids, 67, 229-242.

Milani, J. M., & Golkar, A. (2019). Introductory chapter: some new aspects of colloidal systems in foods. In Some New Aspects of Colloidal Systems in Foods. IntechOpen.

Nasrabadi, M. N., Doost, A. S., & Mezzenga, R. (2021). Modification approaches of plant-based proteins to improve their techno-functionality and use in food products. Food Hydrocolloids, 118, 106789.

Nick Pace, C., Trevino, S., Prabhakaran, E., & Martin Scholtz, J. (2004). Protein structure, stability and solubility in water and other solvents. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 359(1448), 1225-1235.

Nicolai, T., & Chassenieux, C. (2021). Heat-induced gelation of casein micelles. Food Hydrocolloids, 118, 106755.

Nicolai, T., & Chassenieux, C. (2019). Heat-induced gelation of plant globulins. Current Opinion in Food Science, 27, 18-22.

Oliveira, I. C., de Paula Ferreira, I. E., Casanova, F., Cavallieri, A. L. F., Lima Nascimento, L. G., de Carvalho, A. F., & Nogueira Silva, N. F. (2022). Colloidal and Acid Gelling Properties of Mixed Milk and Pea Protein Suspensions. Foods, 11(10), 1383.

Phillips, L. G. (2013). Structure-function properties of food proteins. Academic Press.

Pouliot, Y. (2008). Membrane processes in dairy technology—From a simple idea to worldwide panacea. International Dairy Journal, 18(7), 735-740.

Pua, A., Tang, V. C. Y., Goh, R. M. V., Sun, J., Lassabliere, B., & Liu, S. Q. (2022). Ingredients, Processing, and Fermentation: Addressing the Organoleptic Boundaries of Plant-Based Dairy Analogues. Foods, 11(6), 875.

Roesch, R. R., & Corredig, M. (2005). Heat-induced soy− whey proteins interactions: Formation of soluble and insoluble protein complexes. Journal of agricultural and food chemistry, 53(9), 3476-3482.

Sá, A. G. A., Moreno, Y. M. F., & Carciofi, B. A. M. (2020). Plant proteins as high-quality nutritional source for human diet. Trends in Food Science & Technology, 97, 170-184.

Shaw, J. D. (1992). Introduction to colloid and surface chemistry. Oxford Butterworth-Heinemann. ISBN: 0-7506-1182-0.

Schmitt, C., Bovetto, L., Buczkowski, J., Reis, G. D. O., Pibarot, P., Amagliani, L., & Dombrowski, J. (2021). Plant proteins and their colloidal state. Current Opinion in Colloid & Interface Science, 56, 101510.

Shire, S. J., Shahrokh, Z., & Liu, J. U. N. (2010). Challenges in the development of high protein concentration formulations. Current trends in monoclonal antibody development and manufacturing, 131-147.

Sethi, S., Tyagi, S. K., & Anurag, R. K. (2016). Plant-based milk alternatives an emerging segment of functional beverages: a review. Journal of food science and technology, 53(9), 3408-3423.

Silva, J. V., Balakrishnan, G., Schmitt, C., Chassenieux, C., & Nicolai, T. (2018). Heat-induced gelation of aqueous micellar casein suspensions as affected by globular protein addition. Food Hydrocolloids, 82, 258-267.

Silva, J. V., Cochereau, R., Schmitt, C., Chassenieux, C., & Nicolai, T. (2019). Heat-induced gelation of mixtures of micellar caseins and plant proteins in aqueous solution. Food Research International, 116, 1135-1143.

Silva, J. V., Balakrishnan, G., Schmitt, C., Chassenieux, C., & Nicolai, T. (2018). Heat-induced gelation of aqueous micellar casein suspensions as affected by globular protein addition. Food Hydrocolloids, 82, 258-267.

Silva, J. V., Jacquette, B., Amagliani, L., Schmitt, C., Nicolai, T., & Chassenieux, C. (2019). Heat-induced gelation of micellar casein/plant protein oil-in-water emulsions. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 569, 85-92.

Singh, H., & Gallier, S. (2017). Nature's complex emulsion: The fat globules of milk. *Food Hydrocolloids*, *68*, 81-89.

Singh, J., Prakash, S., Bhandari, B., & Bansal, N. (2019). Ultra high temperature (UHT) stability of casein-whey protein mixtures at high protein content: Heat induced protein interactions. Food Research International, 116, 103-113.

Smithers, G. W. (2008). Whey and whey proteins—from 'gutter-to-gold'. International dairy journal, 18(7), 695-704.

Tan, M., Nawaz, M. A., & Buckow, R. (2021). Functional and food application of plant proteins–a review. Food Reviews International, 1-29.

Totosaus, A., Montejano, J. G., Salazar, J. A., & Guerrero, I. (2002). A review of physical and chemical protein-gel induction. International journal of food science & technology, 37(6), 589-601.

Uluko, H., Liu, L., Lv, J. P., & Zhang, S. W. (2016). Functional characteristics of milk protein concentrates and their modification. Critical Reviews in Food Science and Nutrition, 56(7), 1193-1208.

UNFCCC (2021). Glasgow Climate Pact. United Nations climate changes. Acessed on 15 June 2022, https://unfccc.int/documents/310475.

Vall-Llosera, M., Jessen, F., Henriet, P., Marie, R., Jahromi, M., Sloth, J. J., ... & Casanova, F. (2021). Physical Stability and Interfacial Properties of Oil in Water Emulsion Stabilized with Pea Protein and Fish Skin Gelatin. Food Biophysics, 16(1), 139-151.

Villarino, C. B. J., Jayasena, V., Coorey, R., Chakrabarti-Bell, S., & Johnson, S. K. (2016). Nutritional, health, and technological functionality of lupin flour addition to bread and other baked products: Benefits and challenges. Critical reviews in food science and nutrition, 56(5), 835-857.

Walstra, P., Wouters, J. and Wageningen, T. G. (2005). Chp 2 Milk Components. In: Book Dairy Science and Technology. CRC Press, The Netherlands.

Wang, R., Xu, P., Chen, Z., Zhou, X., & Wang, T. (2019). Complexation of rice proteins and whey protein isolates by structural interactions to prepare soluble protein composites. LWT, 101, 207-213.

Wang, T., Yue, M., Xu, P., Wang, R., & Chen, Z. (2018). Toward water-solvation of rice proteins via backbone hybridization by casein. Food chemistry, 258, 278-283.

Wong, D., Vasanthan, T., & Ozimek, L. (2013). Synergistic enhancement in the co-gelation of salt-soluble pea proteins and whey proteins. Food chemistry, 141(4), 3913-3919.

Yousseef, M., Lafarge, C., Valentin, D., Lubbers, S., & Husson, F. (2016). Fermentation of cow milk and/or pea milk mixtures by different starter cultures: Physico-chemical and sensorial properties. LWT-Food Science and Technology, 69, 430-437.

Zare, F., Boye, J. I., Orsat, V., Champagne, C., & Simpson, B. K. (2011). Microbial, physical and sensory properties of yogurt supplemented with lentil flour. Food Research International, 44(8), 2482-2488.

CHAPTER II: CASEIN HYDROGEL TO CARRY BIOACTIVE COMPOUNDS FROM JABUTICABA

Preamble

Jabuticaba is a fruit cultivated in the tropical regions of the planet, which presents a high amount of bioactive compounds, mainly anthocyanins. Despite the health benefits of anthocyanins ingestion, these compounds are very sensitive to light, pH variation, oxygen, among others. Thus, their entrapment is an alternative to the delivery of these compounds with the minimal loss of their functions. The use of hydrogels to target the delivery molecules of interest is gained attention and the open structure of casein micelles allows the interaction of several molecules. Thus, the casein micelle gels associated with anthocyanin-rich extract can have direct application in the development of functional dairy products.

Questions:

- Can CMs hydrogel entrap anthocyanin-rich extract from Jabuticaba?
- What are the consequences of this association for the hydrogel rheological features?
- Enzymatic crosslinking can improve gels elasticity and modulate extract release?

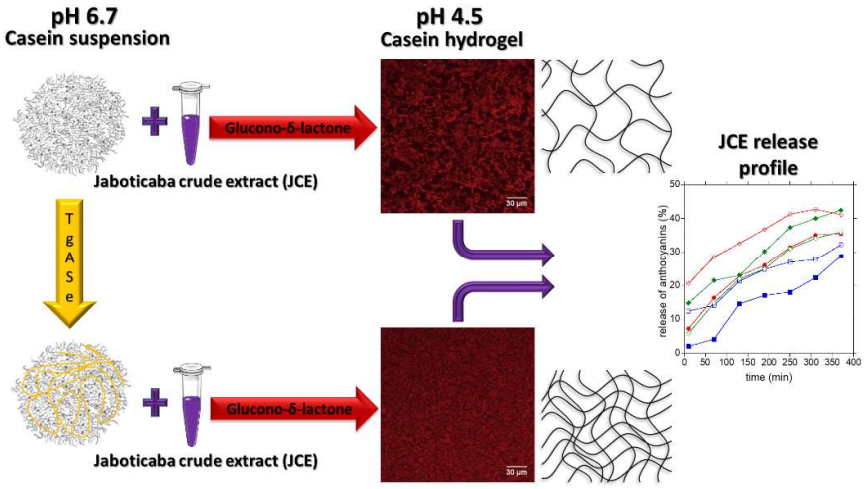
The experimental part of this chapter was conducted at INOVALEITE, in the Department of Food Science and Technology of the Universidade Federal de Viçosa (Brazil)

Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (Myrciaria cauliflora) extract

The content of this chapter has been published in:

Food Hydrocolloids Journal

Luis Gustavo Lima Nascimento, Federico Casanova, Naaman Francisco Nogueira Silva, Álvaro Viana Novaes de Carvalho Teixeira, Paulo Peres de Sá Peixoto Júnior, Márcia Cristina Teixeira Ribeiro Vidigal, Paulo Cesar Stringheta, Antônio Fernandes de Carvalho



Abstract

Casein micelle hydrogels were developed using transglutaminase (Tgase) as a crosslinking agent in order to encapsulate anthocyanins from jaboticaba fruit (*Myrciaria cauliflora*). Spray dried casein micelles (CMs) powder was rehydrated in ultrapure water at a concentration of 4.5% w/w, and Tgase was added at 3 units/g of casein. The suspensions were incubated at 45 °C for 1 h, followed by enzyme deactivation at 85 °C for 5 min. Jaboticaba extract (JE), obtained from jaboticaba peel, was added to the suspensions at a concentration of 2 % (w/w) at 25 °C. In the suspensions, Tgase promoted a reduction in CMs size and an increase in the degree of casein polymerization. The presence of JE did not affect CMs size or charge. The hydrogel samples were produced by acidification of the suspensions using 2% w/w of glucono – δ – lactone until pH 4.5 at 30 °C. The hydrogels were analyzed using small deformation rheology and confocal laser scanning microscopy. Tgase treatment promoted the formation of a more compact protein matrix compared to the samples without the enzyme. The presence of JE decreased hydrogel elasticity and increased hydrogel pore size. The JE release profile was evaluated by immersing the hydrogels in three buffer solutions at pH 2.0, 4.5 and 7.0. The release rates for the hydrogels with Tgase were lower for all pH values. Solutions with higher pH values induced faster release rates. These findings can be applied to specific delivery systems, such as the transport of JE in an intestinal environment.

1. Introduction

Jaboticaba is a fruit that is high in polyphenols with elevated levels of anthocyanins, especially in the peel (De Castro, Da Silva, De Oliveira, Desobry, & Humeau, 2014). Several studies reviewed by Smeriglio, Barreca, Bellocco & Trobetta (2016) attribute health characteristics to anthocyanins which include reduced risk of heart disease, stroke, cancer, and obesity. There are several methods for extracting anthocyanins from jaboticaba peel these include use of acidified ethanol, acidified methanol, ultrasounds, supercritical fluids, pressurized liquid and microwave exposure (Silva, Costa, Calhau, Morais, & Pintado, 2017). The potential applications for anthocyanins in enriched food systems and biomedical applications is limited by the anthocyanins' susceptibility to degradation. Anthocyanins are sensitive to light, oxygen, temperature and pH changes (Moura, Cunha, Alezandro, & Genovese, 2018). Therefore, techniques that can protect and maintain the anthocyanins' ability to resist destabilizing conditions are essential for future applications.

Hydrogels are defined as three-dimensional networks that can entrap high volumes of water in their structures (Klement, Lord, & Parker, 1960). Hydrogels can be classified into many categories according to source, polymeric composition, configuration, crosslink type, network electrical charge and physical appearance (Ahmed, 2015). Use of hydrogel systems in biomedical applications such as tissue engineering, delivery systems and absorption materials has been studied over the years (Ahmed, 2015; Caló & Khutoryanskiy, 2015). Hydrogels can be made from a wide range of polymers, such as proteins (Khodaverdi, Maftouhian, Aliabadi, & Hassanzadeh, 2018; Li, Fu, & Zhang, 2014; Loewen, Chan, & Li-chan, 2018), carbohydrates (Bera & Dutta, 2017) and polymer combinations (Ozel, Cikrikci, Aydin, & Oztop, 2017; Zhang, Decker, & McClements, 2014; Ma et al., 2016; Xu, Fan, Duan, & Gao, 2018). Polymer toxicity levels are determining factors when it comes to using hydrogels in live organisms. In this context, caseins have been considered as suitable polymer source because they are biodegradable, biocompatible and non-toxic (Tavares, Croguennec, Carvalho, & Bouhallab, 2014).

Caseins are phosphorylated proteins, accounting for 80 % (m/m) of total milk proteins. In milk, caseins are naturally present as casein micelles (CMs). CMs are supramolecular structures formed through the interaction of four main caseins

fractions, α- s1, α- s2, β, κ – casein (40, 10, 35, 15% w.w-1). They are joined through hydrophobic interactions, hydrogen bonds, electrostatic forces and calcium phosphate bonds to form spherical like structures (Broyard & Gaucheron, 2015). The average CMs diameter ranges from 50 to 500 nm (Holt and Kruif, 2003) and presents higher polydispersity levels compared to other protein assemblies. CMs are highly hydrated and hold approximately 3.3 g water per g of protein (Huppertz et al., 2017).

Although CMs remain relatively stable in mild physicochemical conditions, the change in the pH toward casein's isoelectric point (~pH 4.6) promotes the destabilization of the CMs and the formation of a casein hydrogel (Holt & Kruif, 2003). The rheological properties of these hydrogels, such as the firmness, water holding capacity and porosity are dependent on the physicochemical state of the CMs (Silva et al., 2018). One possibility to improve the rheological properties of casein hydrogels is the crosslinking of CMs.

CMs crosslinking consists in the formation of covalent bonds within or between proteins (Ercili-Cura et al., 2012). Several crosslinking agents such as glutaraldehyde (Migneault et al., 2004; Xu, Teng, & Wang, 2016), genipin (Casanova et al., 2017; Elzoghby, Helmy, Samy, & Elgindy, 2013), and transglutaminase (Tgase) (Schorsch et al., 2000; Salcedo-Sandoval et al., 2015) have been studied previously. Tgase is a transferase produced by Streptoverticilium which acts by catalyzing the formation of covalent bonds between lysine and glutamine residues (Huppertz & Kruif, 2008). The advantage it has over other crosslink agents is that it is a GRAS ingredient which increases its potential uses in food systems (Raak, Rohm, & Jaros, 2017).

Recently, Casanova et al. (2017) showed that cyanidin-3-O-glucoside, an anthocyanin from Jaboticaba peel, binds spontaneously to sodium caseinate with a pH-dependent mechanism. The authors demonstrated that hydrophobic-driven interactions were predominantly at pH 2, whereas ionic strength-sensitive electrostatic interactions were the main binding forces dominant binding forces at pH 7. Given the above, the purpose of this study was to develop a hydrogel using acid gelation from cross-linked CMs that would be capable of storing and producing a controlled release of Jabuticaba extract (JE). The effects of both JE and Tgase in the CMs characteristics and in the hydrogel properties were studied. The JE release profiles in response to pH 2.0, 4.5 and 7.0 were also examined.

2. Materials and Methods

2.1. Materials

A CMs powder containing 85.70% total protein, 84.78% casein, 8.25% ash, 3.80% moisture and 0.83 % lactose was obtained according to the methods described by Schuck et al. (1994). The milk was centrifuged in an industrial centrifuge to remove fat and dirt sediment. The skimmed milk was double tangentially microfiltrated. The first tangential filtration was performed with a 1.4 μm membrane pore size to remove microorganisms and any remaining fat. The second microfiltration was performed with a membrane pore size 0.1 μm to promote caseins and whey protein separation. After protein separation, the casein-rich retentate was diafiltrated (membrane pore size 0.1 μm) five times using deionized water to remove lactose. The resulting dispersion was spray dried in a lab-scale single state spray dryer (Niro atomizer, GEA, Germany) with 180 °C inlet air, 85 °C outlet air temperatures and 18.3 Kg/h feed flow rate. The CMs powder samples were vacuum-sealed in aluminum foil-wrapped bags and stored at 4 °C until ready to use.

Jaboticaba extract (JE) was obtained according to the methods described by Rocha et al. (2017) with some minor modifications. Briefly, jaboticaba peel was manually removed from fresh fruits, washed with clean water and crushed using a food processor. It was placed in an extracting solution of 70% (v/v) acidified ethanol (pH 2.0). The proportions for jaboticaba peel to ethanol was 1:4 w/w. The mixture was treated by ultrasounds for 10 min and kept at 4 °C overnight to allow polyphenol extraction. The resulting suspension was vacuum filtrated using a 0.45 μm pore size filter. After filtration, the suspension was concentrated to 10 % of its initial volume at 40 °C in a rotative evaporator (MA 120/2057, Marconi). The JE presented a pH level of 2.0, a total polyphenol content of 11.64 g/L as determined by the Folin-Ciocalteau method (Larrauri, Rupérez, & Saura-Calixto, 1997), and a monomeric anthocyanin content of 8.39 g/L as determined by the pH differential method (Lee, Durst, & Wrolstad, 2005). The JE sample was stored at -18 °C until further use.

Microbial transglutaminase (Tgase) Activa® YG (Tgase, EC. 2.3.2.13) was provided by Ajinomoto Foods SAS (Mesnil- Saint-Nicaise, France). According to Ajinomoto, the product presents activity levels of 100 units/g of powder. Glucono-δ-lactone (GDL) was purchased from Sigma-Aldrich (São Paulo, Brazil). All other chemical reagents used were analytical grade.

2.2. Sample preparation

The CMs powder was rehydrated in ultrapure water with 2 mM of sodium chloride to reach a casein concentration of 4.5% (w/w). The CMs suspension was kept under magnetic stirring at 900 rpm at room temperature until the CMs were completely rehydrated. Hydration time took approximately 72 hours. Rehydration was confirmed by verifying particle size with a Zetasizer Nano-S (Malvern Instrument, Worcestershire, UK). The suspension was considered to be completely rehydrated when only one population of particles smaller than 400 nm was detected (data not shown). Sodium azide in a concentration of 0.03% (w/w) was added to prevent microbial growth.

The CMs suspension pH was adjusted to 6.7 using 1 M HCl or 1 M NaOH. It was then divided in four samples, as can be seen in Table 1. Tgase was rehydrated in ultrapure water at a concentration of 10% (w/w). It was then added to the CMs samples to a concentration of 3 U per g of CMs. Crosslink reactions occurred at 45 °C after 1 hour and were halted by incubating the samples at 85 °C for 5 min. The samples were then put in a 0 °C ice bath to lower their temperatures to 4 °C. The samples were allowed to return to room temperature, i.e. 25 °C. The CMs samples were stirred continuously in a magnetic stirrer while JE was added with a dropper to a concentration of 2% (w/w). The samples were stirred at 1000 rpm in a magnetic stirrer for 10 min at 25 °C to allow the JE compounds and CMs to interact.

Acid hydrogels were produced by chemical acidification of the samples. GDL at concentration of 2% (w/w) was added to all suspension samples and stirred until complete GDL solubilization was reached. The gelation process occurred at 30 °C when the samples' pH reached 4.5. The pH drop was monitored as a function of time using a pH-meter Hanna Instrument HI2223 (São Paulo, Brazil). Data were recorded using Real-Time Logging HI 9200e5.0.26 Hanna Instrument (São Paulo, Brazil). Acidification monitoring began right after complete GDL dissolution. The treatment designation is presented in Table 1.

Table 1. Treatment designation.

	A	B	C	D
Transglutaminase (u/g)	-	3	-	3
Jaboticaba extract (%)	-	-	2	2
Glucono – δ – lactone (%)	2	2	2	2

(A) Casein micelles without transglutaminase or jaboticaba extract, (B) Casein micelles with transglutaminase but without jaboticaba extract, (C) Casein micelles without transglutaminase but with jaboticaba extract, (D) Casein micelles with transglutaminase and jaboticaba extract.

2.3. Suspension characterization

The suspensions were evaluated for their polymerization degrees prior to gelation using SDS-PAGE electrophoresis, hydrodynamic diameter (Dh) measurement, and zeta potential determination of the particles (ζ).

2.3.1. SDS-PAGE Electrophoresis

SDS-PAGE electrophoresis in the polyacrylamide gel was determined according to the method described by Judd (1994) in 3 phase gel. The electrophoresis tests were run in an electrophoresis vertical cube (Mini-Protean Tetra System, Bio-Rad California, U.S.A) using a Tris-glycerin buffer solution (pH 8.6). The CMs samples were prepared in a concentration of 4.5% w/w. An aliquot of each suspension was allowed to react with a 2-mercaptoetanol buffer solution (1:4). The molecular weight marker (Precision Plus Protein™, BIO-RAD, São Paulo, Brazil), casein standard and 8 µL of each suspension were placed on top of the polyacrylamide gel. It was applied at 100 V for 10 minutes, 80 V for 15 minutes and 60 V for 300 minutes using a power source (PowerPacTM Basic, Bio-Rad California, U.S.A). The gels were dyed using Coomassie® Brilliant Blue R-250 dissolved at 0.3% in a methanol: acetic acid: water solution (4.5:1:4.5 v/v) at 25 °C for 4 hours. After the dyeing phase, the gels were immersed in methanol to obtain a clear contrast between the bands and the background.

2.3.2. Particle size by dynamic light scattering (DLS)

The particles' hydrodynamic diameters were determined with a Zetasizer Nano-S (Malvern Instrument, Worcestershire, UK). The analysis was carried out at 173° scattering using a 632.8 nm wavelength excitation angle. Samples were diluted to 1:20 in the same solution used for CMs rehydration and allowed to rest at 25 °C for 5 min. The pH level of all the samples was adjusted to 6.7. Suspension viscosity (η) was 1.033 mPa.s-1; each cell reading was performed in triplicate.

Particle Dh was determined using the Stokes-Einstein equation with a diffusion coefficient (Dt) extracted from the correlation curve using the cumulant method, as follows:

$$Dh = \frac{KbT}{3\pi\eta Dt} \qquad (1)$$

Where Kb is Boltzmann's constant and T is temperature and η the solvent viscosity (Pa s^{-1})

2.3.3. ζ-potential measurements

The zeta potential (ζ) of particles was determined using a Zetasizer Nano-S (Malvern Instrument, Worcestershire, UK) under the same conditions described for the DLS measurements above. Capillary cells were used to perform the analysis. The measurements were carried out using 50 volts, and the calculations were made according to the Henry equation, Equation 2.

$$\zeta = \frac{3\eta\mu}{2\varepsilon f(kRh)} \qquad (2)$$

where η is the solvent viscosity (Pa s^{-1}) (1.033 mPa s^{-1}), μ is the electrophoretic mobility (V Pa^{-1} s^{-1}), ε is the medium dielectric constant (dimensionless) and $f(kRh)$ is Henry's function. Because the particle analysis was conducted in aqueous media, the Smoluchowski approximation value of 1.5 was adopted for $f(kRh)$.

2.4. Hydrogel characterization

The hydrogel samples were evaluated for oscillatory dynamic rheology and water holding capacity (WHC). Their microstructures were determined using confocal laser scanner microscopy and the pore sizes were determined by 2D- correlation analysis of the CLSM images. The JE sample release profiles were measured under different pH conditions.

2.4.1. Dynamic rheological measurements

Shear stress and frequency sweep tests were performed in the casein hydrogel samples. CMs samples were prepared at concentration of 4.5% w/w using ultrapure water with 2 mM of calcium chloride. The GDL was incorporated into the CMs samples at a 2% w/w concentration to promote samples acidification. After the GDL was added,

the CMs samples were stirred for 30 seconds. The hydrogel samples were allowed to gel at 30 °C in a 50 mL cylindrical glass. Once their pH reached 4.5, the hydrogel samples were carefully placed in a modular advanced rheometer system (Haake Mars, Thermo Electron Corp., Karlsruhe, Germany), equipped with a thermostatic bath (Phoenix 2C30P, Thermo Electron Corp., Karlsruhe, Germany). First, the hydrogels samples' linear viscoelastic regions were evaluated through shear stress sweep test. The shear stress had increased from 0.01 to 10000 Pa at a constant frequency (1 Hz). The hydrogel samples linear viscoelastic regions were determined when their loss tangent assumed constant values. Second, a frequency sweep test was carried out applying a variable frequency from 0.1 to 10.0 Hz in the hydrogel samples. Both experiments were carried out at 37 °C. This temperature was chosen because it is close to the human body temperature and thus represents a potential environmental application for the hydrogels studied. In both assays, a stainless-steel cone-and-plate geometry (Cone C35/2° Ti) with a gap of 1 mm was used.

2.4.2. Water holding capacity (WHC)

The water holding capacity (WHC) was determined according to the method described by Silva et al. (2018). Each hydrogel sample was allowed to form in 50 mL centrifuge tubes at 30 °C until their pH reached 4.5. The hydrogels were centrifuged at 3000 g (Heraus megafuge 8R centrifuge, Thermo scientific) at 37 °C for 15 min. The supernatant was carefully separated and weighed. The WHC of the hydrogel samples was calculated using equation 3.

$$WCH\% = \frac{(mi - ms)}{mi} X\ 100 \qquad (3)$$

Where mi is the initial mass and ms is the supernatant mass.

2.4.3. Confocal scanning laser microscopy (CLSM)

CLSM was used to study the hydrogel sample microstructures according to the method described by Andoyo, Guyomarc'h, Cauty, & Famelart (2014). Suspension samples were labeled using 0.2 g/kg of rhodamine B isothiocyanate (RITC) (Sigma Sigma-Aldrich, Sao Paulo, Brazil). The suspensions were stirred for 5 min at room temperature to ensure RITC solubilize)ion. Hydrogel formations occurred in petri dishes (MatTek Corporation, Ashland, USA) at 30 °C when 2% (w/w) GDL was added.

After the hydrogels formed, the samples were studied using a Helion-Neon laser (excitation at 543 nm and emission at 625 nm) in a Zeiss LSM 510 Meta Confocal microscope.

2.4.4. Pore size determination

Pore size was determined by 2-D correlation analysis as described by Silva et al. (2018). Briefly, CLSM images (CLSM) were digitalized on a grey scale as 512 x 512 pixel matrix. Each pixel was given a greyscale range from 0 (black) to 216 (white). Proteins appear in the images as clear spots while pores appear as the darker regions. When a physical value depends on an extension, the value can be analyzed using a correlation function. The spatial coordinate function used to analyses the images is written as equation 4.

$$C\left(\vec{\delta}\right) \equiv \frac{I\left(\vec{r}\right) I\left(\vec{r}+\vec{\delta}\right)}{I^2} - 1 \qquad (4)$$

2.5. Controlled release

Ten grams of the hydrogel samples containing JE were allowed to form in closed shot flasks under the same conditions described in section 2.2. The hydrogel samples' release of anthocyanins was determined by immersing the hydrogels in three buffer solutions at different pH levels. To mimic a human stomach pH, a buffer solution of 0.1 M KCl:HCl was adjusted to pH 2. To mimic a pH value close to the small intestine, a buffer solution of 0.1 M HEPES at pH 7.0 was used. The release experiments were also performed at pH 4.5, which is the hydrogels pH, using a buffer solution of 0.1 M acetic acid: sodium acetate. The hydrogel samples were placed in closed shot flasks and covered with 20 mL of each buffer solution (Figure 7.1). The systems were incubated in a water bath at 37 °C without light. An aliquot of 60 µL of the supernatants was withdrawn from each sample to determine total monomeric anthocyanin release.

2.6. Monomeric anthocyanin measurements

Monomeric anthocyanin measurements were carried out according to Lee, Durst, & Wrolstad (2005) with adaptations made for a microplate reader as proposed by Lee, Rennaker, & Wrolstad (2008). The total monomeric anthocyanin levels were calculated using Equation 5. The 100% level was determined by placing the same

quantity of fruit extract used in the hydrogel sample formulation into 20 mL of each buffer solution. All experiments were performed in triplicate. The results are expressed in percentages of the released anthocyanins.

$$Ant = \frac{A\ MW\ DF\ 1000}{\varepsilon\ 1} \qquad (5)$$

Where Ant is the anthocyanin content in terms of cyanidin-3-glucoside equivalent expressed in mg/L, A is (Absorbance at 520 nm - Absorbance at 700 nm) at pH 1 subtracting the (Absorbance at 520 nm - Absorbance at 700 nm) at pH 7, MW is the molecular weight of cyanidin-3-glicoside (449.2 g/mol), DF is the dilution factor used (5), ε is the molar extinction coefficient for cyanidin-3-glucoside (26,900 L mol^{-1} cm^{-1}), 1000 is the conversion factor from g to mg, and 1 is the path length in cm.

2.7. Statistical analysis

The data were processed using variance analysis (ANOVA) with SAS university edition software to access the influence of Tgase and JCE in the hydrogel's features. The Tukey HSD test was applied to compare the mean values. $p < 0.05$ was used to determine significance. All experiments were performed in triplicate.

3. Results and discussion

3.1. Suspension characterization

3.1.1. Electrophoresis

Figure 1 shows SDS-PAGE electrophoresis analysis results. The CC sample represents the control CMs sample without the reticulation process, which had also been submitted to the heat treatments for Tgase crosslinking and inactivation as well the subsequent ice bath cooling phase. Even though the CMs powder treatment process was designed to eliminate all serum proteins, it is possible to observe low molecular weight molecules in the samples, as can be seen in the band formed between 10 and 15 KDa. When sample CC is compared to sample A, no polymerization degree modification caused by heating can be observed. Samples A and C presented similar bands, as well as samples B and D. The presence of Tgase induced certain differences between bands. The samples treated with Tgase showed polypeptide presence and had higher molecular weights than the non-treated samples. All the casein monomer bands in samples B and D were less dark than samples A and C. At the same time, a darker path was observed between 75 KDa and 37 KDa in samples B and D due to the formation of casein oligomers. Similar results were found by Chen, Li, Han, Yuan, & Zhang (2018) when they studied the crosslink effects when

Tgase was added to a milk protein concentrate (MPC). They observed a gradual decrease in the protein bands when a longer reaction time was allowed. It is worth noting that the presence of JE did not change the caseins' polymerization degrees, as observed in previous studies (He, Xu, Zeng, Qin, & Chen, 2015), interactions between anthocyanins and caseins did not change the proteins' primary structure. Thus, the same band patterns were observed in the both the samples with and without JE.

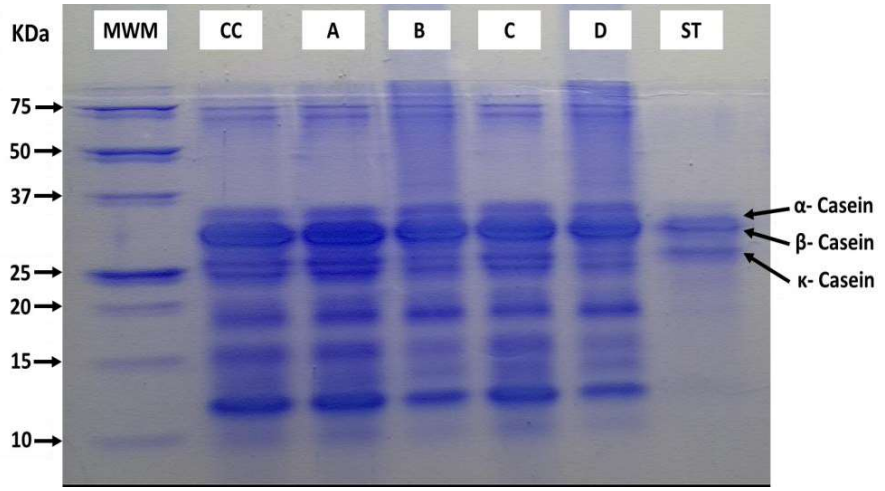


Figure 1. SDS-PAGE electrophoresis images of four samples showing molecular weight markers and casein fraction standards. MWM: Molecular weight marker; CC: Casein dispersion before any treatment; (A) CMs without Tgase or JE; (B) CMs with Tgase; (C) CMs without Tgase but with JE; (D) CMs with both Tgase and JE; ST: Casein fraction standards.

3.1.2. Dynamic light scaterring

CMs size distribution in the sample suspensions are shown in Figure 2 and Table 2. The main differences were observed between native (samples A and C) and crosslinked CMs (samples B and D). Crosslinked CMs (samples B and D) had hydrodynamic diameter of around 160 nm, whereas samples without Tgase (A and C) had diameters of approximately 175 nm. Similar results were founded by Nogueira et al. (2018) when studying CMs suspensions at pH levels close to milk pH. Tgase promotes a covalent bond formation between glutamine and lysine residues (Huppertz & Kruif 2008). Tgase favors crosslinks between κ and β- casein fractions in CMs over crosslinks with α- caseins (Smiddy, Martin, Kelly, Kruif, & Huppertz, 2006). This difference in crosslink levels is due to casein fraction position in the micelle structure. Because α-s caseins are located within the CMs, lower Tgase activity was observed compared to Tgase activity for β and κ- casein fractions (Smiddy et al., 2006).

Alternately, the κ- casein's location on the CMs surface makes it more accessible to crosslinked reactions. Therefore, an increase in the polymerization degree of the k-casein reduced the CMs size (Duerasch, Wissel, & Henle, 2018).

Table 2. Hydrodynamic diameter and ζ-potential of casein micelles.

	A	B	C	D
Hydrodynamic diameter (nm)	174.3 ± 5.5^{a}	161.4 ± 1.6^{b}	177.2 ± 5.2^{a}	160.2 ± 1.5^{b}
ζ-potential (mV)	-12.8 ± 0.1^{a}	-12.7 ± 0.2^{a}	-12.8 ± 0.1^{a}	-12.5 ± 0.1^{a}

Means followed by the same letter in the lines do not differ significantly ($p > 0.05$) according to Tukey's HSD test. (A) Casein micelles without transglutaminase or jaboticaba extract, (B) Casein micelles with transglutaminase but without jaboticaba extract, (C) Casein micelles without transglutaminase but with jaboticaba extract, (D) Casein micelles with transglutaminase and jaboticaba extract.

JE presence did not interfere with CMs size distributions. In previous studies, favorable interactions between casein and anthocyanins, i.e. Gibbs energy assuming negative values, have been reported. He et al. (2015) used β and α-s caseins to encapsulate anthocyanins from grape skins, and it has been suggested that the hydrophobic interactions between malvidin-3-O-glucoside with β- casein and the van-der-walls and hydrogen bonds formed between α- casein and the anthocyanin are usually predominant. Casanova et al. (2017) showed that cyanidin-3-O-glucoside interacted internally with sodium caseinate through electrostatic forces at pH 7. Therefore, it is likely the interaction between the JE anthocyanins occurred internally without causing any modification in the CMs surface charge.

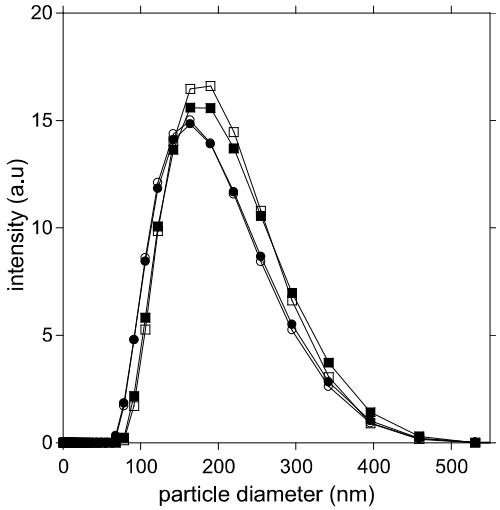


Figure 2. Intensity (a.u.) as a function of particle diameter (nm) obtained by DLS measurement of each suspension. A (□), B (○), C (■), D (●).

The ζ- potential did not change in any of the samples (Table 2). The unaltered Tgase CMs crosslinks are in accordance with Silva et al. (2018) and Kruif & Hold (2003). Tgase was able to catalyze a covalent bond formation between lysine and glutamine resides by acyl transition reactions (Jaros, Partschefeld, & Henle, 2006). This bond formation occurred in the CMs themselves. Thus, it did not cause any polymerization between adjacent CMs. The majority of JE anthocyanins were cyanidin-3-O-glucoside and delphinidin-3-glucoside (De Castro et al., 2014) and their charges varied according to the pH of the medium used. In acidic pH mediums, the anthocyanins were positively charged (Dangles & Fenger, 2018). The presence of positively charged anthocyanins in JE did not influence the CMs charges. Casanova et al. (2017), He et al. (2015) and Wei et al. (2018) found favorable interactions between anthocyanins and caseins, i.e. Gibbs free energy lower than zero. The ζ-potential and size results found in this study in combination with the results detailed in previous literature suggest an internal complexation of JE within the CMs.

3.2. Hydrogel characterization

3.2.1. Acidification monitoring

The CMs pH variations observed after the GDL addition are shown in Figure 3. All curves represent behavior characterized by a rapid decrease in pH after the first 20 min of the acidification process, followed by a slower pH decrease rate in the following minutes. The presence of Tgase did not affect the acidification profiles of the samples,

as has been shown by Raak, Rohm, & Jaros (2017). Indeed, the curves for samples C and D are located below the curves for samples A and B, but their tendencies were similar. JE has a low pH (pH 2), therefore incorporating JE in the CMs samples caused a pH drop at the beginning of the acidification process. The hydrogel samples with JE thus reached a pH 4.5 forty minutes before the hydrogels without JE. The initial pH levels recorded for samples without JE were 6.49 (without Tgase) and 6.43 (with Tgase), while the first recorded pH levels in samples with JE were 5.99 (without Tgase) and 5.89 (with Tgase). For this reason, a shift in the acidification curves in the dispersions containing JE can be observed, as can be seen in Figure 3.

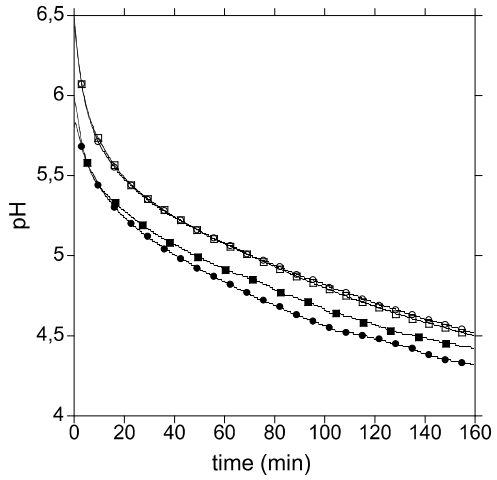


Figure 3. Acidification profile curves. A (□), B (○), C (■), D (●).

3.2.2. Dynamic oscillatory rheological measurements

The rheological behavior of acid milk gels, which are similar to the casein hydrogels proposed in this study, depends on the quantity and strength of the interactions that occur between caseins (Lucey, 2017). Shear stress and frequency sweep results are shown in Figures 4.1 and 4.2; both results are expressed as loss tangents. Loss tangent is defined as the ratio between viscous and elastic moduli. Lower loss tangent values indicate hydrogels with a prominently elastic modulus compared to those with a viscous modulus. A shear stress sweep experiment (Figure 4.1) was conducted to determine the linear viscoelastic region of the hydrogel samples and the critical shear stress values the hydrogels can withstand without permanent structure loss. The end of the linear viscoelastic region occurred when applied stress was important enough to break the bonds within the protein matrix (Everett & Olson,

2000). The hydrogel samples all behaved similarly where the linear viscosity region ends. This behavior was characterized by an abrupt change in loss tangent curves. It can be explained by types of interactions found on hydrogel structures. Caseins associate mainly by hydrophobic interaction, and there is no mechanism to relieve the stress that occurs. For this reason, a hydrogel can withstand certain stress levels, but when the interactions among caseins start to break and crack the surface, propagation occurs rapidly (Ma et al., 2016). Hydrogel samples treated with Tgase which contained JE showed shorter linear viscoelastic regions compared to the other hydrogel samples. This indicates lower structure stability (Tunick, 2010). This phenomenon occurred only in the hydrogel sample with Tgase. Anthocyanin interactions with caseins promote changes in the caseins' secondary structure (He et al., 2015), which may impact the protein-protein interactions. However, the structure of the hydrogel samples without Tgase were more flexible which facilitated casein rearrangements and allow the samples to withstand greater stress levels until the interactions break. The opposite was the case for hydrogels with Tgase. Tgase promoted the formation of covalent bonds within the hydrogel matrix. The greater number interactions in the protein matrix made the matrix more inflexible (Rohm, Ullrich, Schmidt, Löbner, & Jaros, 2014). Therefore, the modified protein-protein interactions resulted in fewer protein rearrangements and brought about a shorter linear viscoelastic region.

In general, all samples showed a correlation between loss tangent and frequency, Figure 4.2. The hydrogel sample with Tgase but no JE showed lower loss tangent values compared to other samples, while the hydrogel without Tgase or JE showed the highest loss tangent values in the frequency sweep, Figure 4.2. The samples can be classified by two factors: enzymatic crosslinking and JE loading. The Tgase treatment diminished loss tangent values of the samples as compared to the untreated hydrogel samples, as can be observed by comparing samples A - B, and C - D. The hydrogel samples with JE presented higher loss tangent values, as can be observed by comparing samples A - C and B - D. Tgase increased the hydrogel samples' elasticity, while the presence of JE decreased their elasticity. There are two explanations for this behavior. First, variations in acidification rates caused by the addition of JE also influenced the frequency sweep of the hydrogel samples with JE. Jacob, Nobel, Jaros, & Rohm (2011) studied the effects of acidification rates in acid milk gels using a rheological approach and used a system similar to the one in this

study. They found a significant decrease in gel stiffness due to higher rates of acidification for both crosslinked and non-crosslinked milk. As shown in Figure 3, samples containing JE began acidification at lower pH levels than the other samples because of the pH decrease caused by the addition of JE. The JE extract had to be held at lower pH values (pH 2.0) in order to keep the anthocyanins stable. Therefore, samples with JE (C and D) reached a pH 4.5 level forty minutes before the samples without JE (A and B).

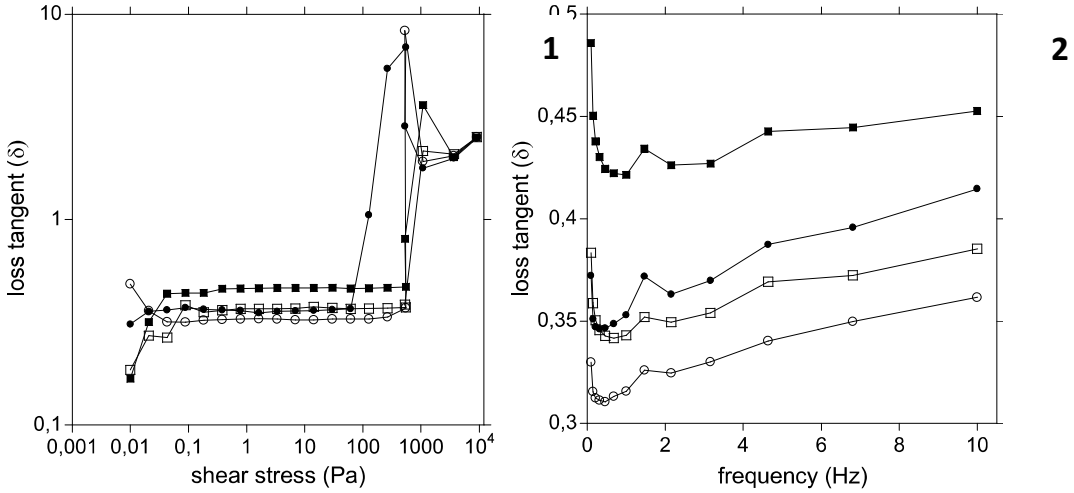


Figure 4. (1) Shear stress sweep of the four hydrogel samples. (2) Frequency sweep of the four hydrogel samples. Samples designation: Casein micelles without transglutaminase or jaboticaba extract (□), Casein micelles with transglutaminase but without jaboticaba extract (○), Casein micelles without transglutaminase but with jaboticaba extract (■), Casein micelles with transglutaminase and jaboticaba extract (●).

Nevertheless, the effects of the polyphenols present in the JE should not be overlooked. In this study, the interactions between the JE and the caseins did not cause significant changes in the ζ-potential or the CMs hydrodynamic diameter. CMs were stabilized internally by hydrophobic interactions and colloidal calcium phosphate (Lucey & Horne, 2018). Thus, these two forces counterbalanced the JE's tendency to alter casein-casein interactions. However, the colloidal system state change from suspension to gel was followed by CCP solubilization (Lee & Lucey, 2004). Thus, the destabilized forces caused when the JE was added were no longer balanced out by the CCP, which ultimately produced hydrogels with higher loss tangent values. Silva et al., 2017 have studied the impact on the rheological properties of yogurt when grape extract was added. The authors showed that an addition of 3% of grape extract

containing 962 mg GAE/g of phenolic compounds increased acidification time, but significantly decreased gel strength. Similar results have been found by Pereira et al. (2016). The authors found that adding a JE with a concentration of 0.5% to Petit Suisse cheese decreased its G' and increased its loss tangent. This suggests an interference in protein network formation when JE was present. Recent studies have demonstrated that the changes in the secondary structure conformation of α- and β- caseins are caused by an interaction with anthocyanins (Lang et al., 2019; Wei et al., 2018; He et al., 2016). Lang et al. (2019) observed 2.86 times more α- helix and 1.17 times more β-sheet in β- casein suspensions when malvidin-3-O-glucoside was present. These changes in secondary structure may cause a decrease in milk gel strength when polyphenol extracts are present.

3.2.3. Water Holding Capacity (WHC)

In these experiments, the hydrogel samples ability to hold water was measured under centrifugal force. Higher values were observed for samples treated with Tgase. The crosslinked hydrogels showed 21.34 and 22.33% WHC when JE was absent or present, respectively. However, without the enzymatic treatment, the WCH dropped to 12.36 and 12.33% when JE was absent or present, respectively. Sun et al. (2018) found an 82.78% WHC using a CMs powder that had a similar protein profile. The differences can be attributed to the methodology used as they applied a centrifugal force of 1200 g for 15 min, which is 60% less intense then the centrifugal force applied in the current study. The WHC ratio of non-crosslinked to crosslinked casein hydrogels are in accordance with the results published by Silva et al. (2018). Tgase treatment promotes a denser and more homogeneous network formation in the casein hydrogel samples (Figure 5) because it catalyzes the formation of covalent bonds within the protein matrix. The Tgase action resulted in greater interaction between the proteins. The higher crosslink point extensions conferred a protein network with a smaller pore size for the hydrogel samples, as shown in Figure 6.2. The water diffusion was made more difficult when the hydrogel matrix was more compact (Ercili-Cura, et al., 2012). The presence of JE did not affect the WHC of the hydrogel samples.

3.2.4. Hydrogel microstructure

The images taken of hydrogel sample microstructures both in the presence of JE and Tgase were obtained by CLSM and are presented in Figure 5. It can be observed that the network density of samples B and D is more homogeneous and compact than that of samples A and C. The CLSM images were submitted to 2D-correlation analysis in order to numerically estimate the pore size of each sample (Figure 6.1 and 6.2). The hydrogel samples without an enzymatic treatment showed a mean pore size of 1.40 ± 0.14 μm for sample A, and 1.72 ± 0.12 μm for sample C. In the samples treated with Tgase, pore size ranged from 0.63 ± 0.02 for sample B, and 0.68 ± 0.01 for sample D. According to previous literature (Silva et al., 2018; Ercila-Cura et al., 2012), Tgase causes a porosity reduction in acid milk gels. Thus, similar behavior in the CMs hydrogel samples was to be expected. Jacob, Jaros, & Rohm (2011) have argued that the presence of Tgase promotes the formation of casein oligomers. These act as a skeleton for gel development as acidification occurs. Hence, if the acidification rates occur over a sufficiently long time period to allow the caseins to regroup into a 3D structure, the formed gel will be denser and more homogeneous.

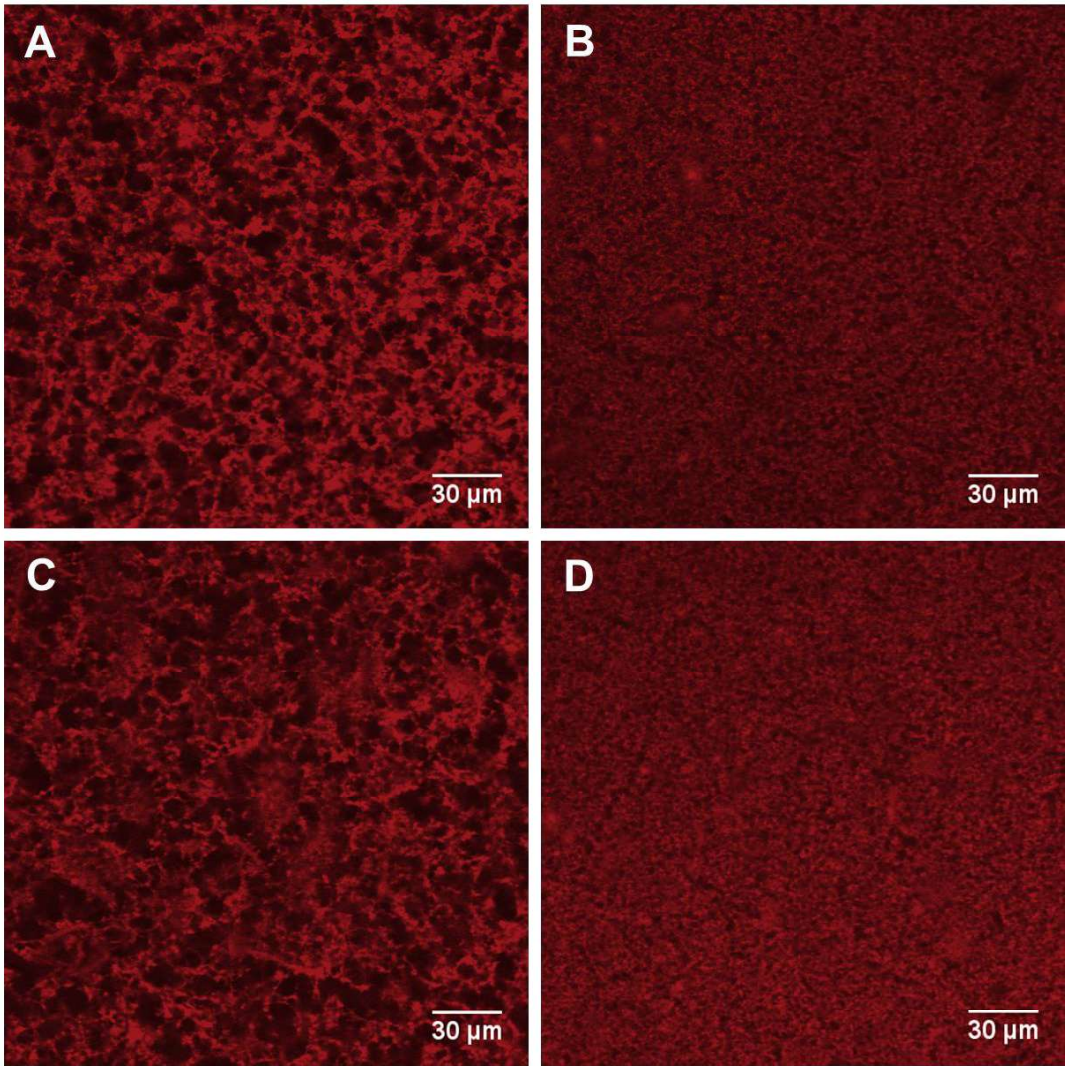


Figure 5. Confocal scanning laser images of casein hydrogel samples. (A) Casein micelles without transglutaminase or jaboticaba extract, (B) Casein micelles with transglutaminase but without jaboticaba extract, (C) Casein micelles without transglutaminase but with jaboticaba extract, (D) Casein micelles with transglutaminase and jaboticaba extract.

The addition of JE significantly increased ($p < 0.05$) the pore size of the casein hydrogel samples without Tgase (Figure 6.2). The fast acidification rate promoted a coarser protein matrix structure. However, no significant difference ($p > 0.05$) in pore size was observed in the hydrogel samples treated with Tgase. Therefore, Tgase plays an essential role in maintaining hydrogel structure when JE is entrapped.

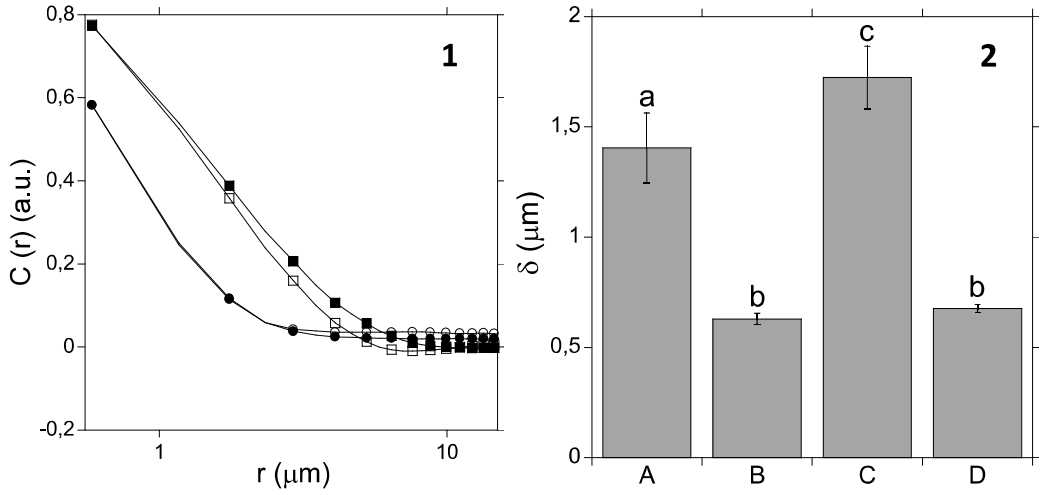


Figure 6. (1) Plot correlation as a function of pore diameter for A (□), B (○), C (■), D (●). The lines are determined by simple exponential decay. (2) Hydrogel sample mean pore size (μm). Different lower-case letters at the top of the columns indicate 5 % difference in the significance level as determined by Tukey HSD test. Samples designation: (A) Casein micelles without transglutaminase or jaboticaba extract, (B) Casein micelles with transglutaminase but without jaboticaba extract, (C) Casein micelles without transglutaminase but with jaboticaba extract, (D) Casein micelles with transglutaminase and jaboticaba extract.

3.3. Controlled release

The release profiles of the hydrogel samples both with or without Tgase at three pH values (2.0, 4.5 and 7.0) is presented in Figure 7.2. The Tgase-treated hydrogel samples presented slower release rates than the non-treated hydrogel samples at all pH levels evaluated. Song, Zhang, Shi, & Li (2010) found similar results. In their study, Tgase was applied to hydrogel samples in two different concentrations and the hydrogel sample with the greater amount of Tgase showed a slower delivery rate of Vitamin B12 at a pH level of 7.4. Using GP as crosslink agent, Song, Zhang, Yang, & Yan (2009) also found a slower release rate when the amount of GP in the system was increased. As can be observed in the CLSM images, the hydrogel samples treated with Tgase presented a compact structure, with smaller pore size. This ultimately decreased anthocyanin diffusion and lowered their release rate.

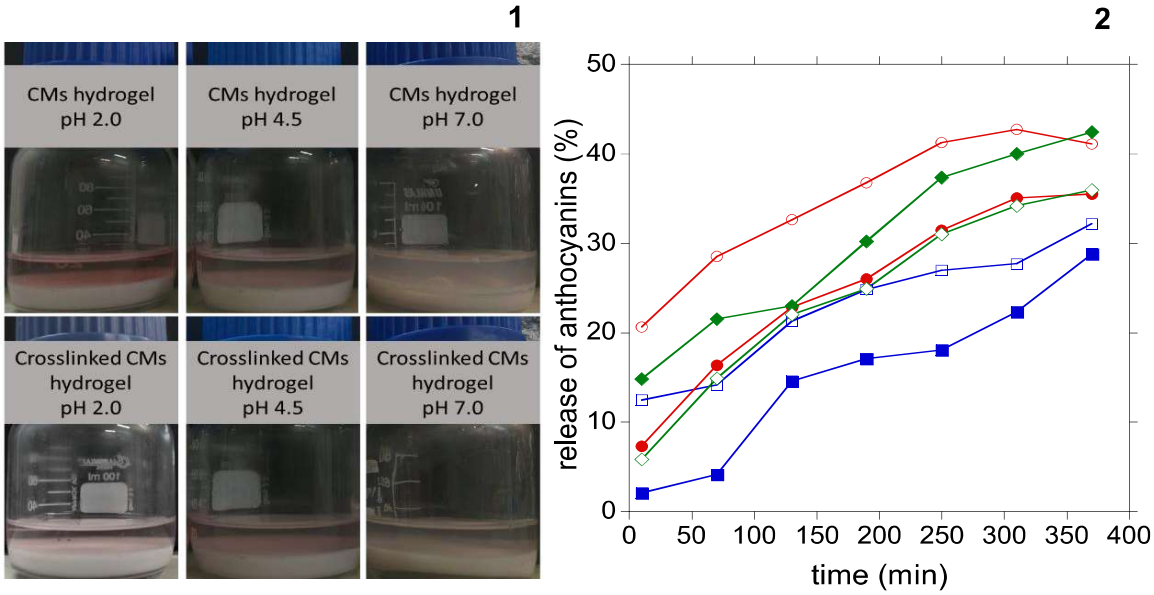


Figure 7. (1) On the left: shot flasks containing casein hydrogels in different pH conditions. (2) anthocyanin release profile of crosslinked, sample D (full symbols) and non-crosslinked, sample C (empty symbols) hydrogels at pH 2.0 (■□), 4.5 (●○) and 7.0 (♦◊).

Release rate increases were observed at higher pH levels in the mediums used. Two factors should be taken into consideration: (i) protein matrix behavior in the given pH conditions, and (ii) the strength of the interactions between the anthocyanins and the protein matrix at pH 2.0, 4.5 and 7.0. The buffer solution was placed over the casein hydrogel samples. For this reason, the only part of the hydrogel samples that came into contact with the buffers was their surface. This means that the contact area for anthocyanin diffusion was the transversal area of the flask containing the hydrogel sample. The first layer of the hydrogel samples come into contact with the specific buffer pH value which was different from the hydrogel samples' pH. Thus, changes in the protein charges were expected. JE release at pH 7.0 and 4.5 levels were similar. At pH 7.0, a lower concentration of hydrogen ions in the buffer solution was observed. Thus, the protein chains acquired a negative charge. This negative charge induced an increase in repulsive forces among the protein chains, which weakened the hydrogel matrix (Lucey, 2009) and ultimately freed the JE from the hydrogel samples. At pH 4.5, the caseins were close to their isoelectric point, therefore the overall charges were close to zero. The possibility for similar release rates came not from protein matrix relaxation, but from a decrease in the strength of interactions between the JE and casein at this particular pH level. However, more studies are required to confirm this

hypothesis. At pH 2.0, a charge modification in the surface of the hydrogel samples was also to be expected, but contrary to the conditions at pH 7.0, the protein chains were protonated and assumed positive charges because the pH was below their isoelectric point of 4.6 pH (Horne, 2009). However, at pH 2.0 the anthocyanin release rate was minimal. This behavior can be explained by the interactions that can occur between the proteins and anthocyanins. Casanova et al. (2017) found that binding forces between cyanindin-3-O-glucoside and sodium caseinate were higher at pH 2.0 than at pH 7.0 at 36 °C. The forces changed in degree and nature when the pH of the medium was modified. At an acid pH, the complexes formed between sodium caseinate and cyanindin-3-O-glucoside were dominated by hydrophobic forces, whereas van der Waals bonds and electrostatic interactions determined the nature of the complexation between anthocyanins and proteins at pH 7.0. Cyanindin-3-O-glucoside was the primary anthocyanin present in the JE and it interacted more strongly with proteins at acid pH levels. Thus, this strong interaction contributed to a slower release rate at pH 2.0. Due the complexity of the systems in this study, the thermodynamic parameters were not determined, however our knowledge of them from previous studies has been essential to understanding the release properties demonstrated by the JE.

4. Conclusion

This study has shown that crosslinked casein hydrogels can be used to carry and provide controlled delivery of JE. The rate of release can be modulated by the presence of Tgase. JE did not alter the CMs structure in suspension. In general, the crosslinked systems showed slower JE release rates at all pH levels evaluated, with a maximum release at pH 7.0 and a minimum at pH 2.0. This behavior gave the hydrogel samples the ability to retain or release JE, depending upon the pH of the medium studied. Additional studies under simulated and/or in vivo digestion conditions must be carried out to determine CMs hydrogels' ability to protect JE and potentially increases its bioavailability.

5. References

Ahmed, E. M. (2015). Hydrogel: Preparation, characterization, and applications: A review. *Journal of Advanced Research*, *6*(2), 105–121. https://doi.org/10.1016/j.jare.2013.07.006

Andoyo, R., Guyomarc'h, F., Cauty, C., & Famelart, M. H. (2014). Model mixtures evidence the respective roles of whey protein particles and casein micelles during acid gelation. *Food Hydrocolloids*, *37*, 203–212.

Bera, S., & Dutta, D. (2017). Encapsulation and release of a bacterial carotenoid from hydrogel matrix: Characterization, kinetics and antioxidant study. *Engineering in Life Sciences*, *17*(7), 739–748. https://doi.org/10.1002/elsc.201600238

Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: a scientific and technological challenge. *Dairy science & technology*, 95(6), 831-862.

Caló, E., & Khutoryanskiy, V. V. (2015). Biomedical applications of hydrogels: A review of patents and commercial products. *European Polymer Journal*, 65, 252-267.

Casanova, F., Chapeau, A. L., Hamon, P., de Carvalho, A. F., Croguennec, T., & Bouhallab, S. (2017). PH- and ionic strength-dependent interaction between cyanidin-3-O-glucoside and sodium caseinate. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2017.06.081

Casanova, F., Nogueira Silva, N. F., Gaucheron, F., Nogueira, M. H., Teixeira, A. V. N. C., Perrone, I. T., Alves, M. P., Fidelis, P.C., & Carvalho, A. F. (2017). Stability of casein micelles cross-linked with genipin: A physicochemical study as a function of pH. *International Dairy Journal*, 68, 70–74. https://doi.org/10.1016/j.idairyj.2016.12.006

Chen, L., Li, Y., Han, J., Yuan, D., Lu, Z., & Zhang, L. (2018). Influence of transglutaminase-induced modification of milk protein concentrate (MPC) on yoghurt texture. *International Dairy Journal*, *78*, 65–72. https://doi.org/10.1016/j.idairyj.2017.10.001

Dangles, O., & Fenger, J. A. (2018). The chemical reactivity of anthocyanins and its consequences in food science and nutrition. *Molecules*, 23(8), 1970.

De Castro, V. C., Da Silva, P. H. A., De Oliveira, E. B., Desobry, S., & Humeau, C. (2014). Extraction, identification and enzymatic synthesis of acylated derivatives of anthocyanins from jaboticaba (Myrciaria cauliflora) fruits. *International Journal of Food Science and Technology*, *49*(1), 196–204. https://doi.org/10.1111/ijfs.12298

Duerasch, A., Wissel, J., & Henle, T. (2018). Reassembling of Alkali-Treated Casein Micelles by Microbial Transglutaminase. *Journal of agricultural and food chemistry*, 66(44), 11748-11756.

Elzoghby, A. O., Helmy, M. W., Samy, W. M., & Elgindy, N. A. (2013). Spray-dried casein-based micelles as a vehicle for solubilization and controlled delivery of

flutamide: Formulation, characterization, and in vivo pharmacokinetics. *European Journal of Pharmaceutics and Biopharmaceutics*, 84(3), 487–496. https://doi.org/10.1016/j.ejpb.2013.01.005

Ercili-Cura, D., Lille, M., Legland, D., Gaucel, S., Poutanen, K., Partanen, R., & Lantto, R. (2012). Structural mechanisms leading to improved water retention in acid milk gels by use of transglutaminase. *Food Hydrocolloids*, *30*(1), 419–427. https://doi.org/10.1016/j.foodhyd.2012.07.008

Everett, D. W., & Olson, N. F. (2000). Dynamic Rheology of Renneted Milk Gels Containing Fat Globules Stabilized with Different Surfactants. *Journal of Dairy Science*, *83*(6), 1203–1209. https://doi.org/10.3168/jds.S0022-0302(00)74985-X

He, Z., Xu, M., Zeng, M., Qin, F., & Chen, J. (2015). Interactions of milk a - and b - casein with malvidin-3- O -glucoside and their effects on the stability of grape skin anthocyanin extracts. *Food Chemistry*, *199*, 314–322. https://doi.org/10.1016/j.foodchem.2015.12.035

Holt, C., & Kruif, C. G. (Kees) De. (2003). Casein micelle structure, functions and interactions. In P. F. FOX & P. L. H. Mcsweeney (Eds.), *Advanced Dairy Chemistry* (3rd ed.). https://doi.org/10.1007/978-1-4419-8602-3

Horne, D. S. (2009). Casein micelle structure and stability. In: Thompson, A., Boland, M., & Singh, H (eds). *Milk Proteins: From Expression to Food* (pp. 133 – 162). San Diego: Elsevier.

Huppertz, T., & de Kruif, C. G. (2008). Structure and stability of nanogel particles prepared by internal cross-linking of casein micelles. *International Dairy Journal*, 18(5), 556–565. https://doi.org/10.1016/j.idairyj.2007.10.009

Huppertz, T., Gazi, I., Luyten, H., Nieuwenhuijse, H., Alting, A., & Schokker, E. (2017). Hydration of casein micelles and caseinates: Implications for casein micelle structure. *International dairy journal*, 74, 1-11.

Jacob, M., Nöbel, S., Jaros, D., & Rohm, H. (2011). Food Hydrocolloids Physical properties of acid milk gels: Acidification rate significantly interacts with cross-linking and heat treatment of milk. *Food Hydrocolloids*, *25*(5), 928–934. https://doi.org/10.1016/j.foodhyd.2010.09.003

Jaros, D., Partschefeld, C., Henle, T., & Rohm, H. (2006). Transglutaminase in dairy products: chemistry, physics, applications. Journal of texture studies, 37(2), 113-155.

Judd, R. C. Electrophoresis of peptides (1994). In: Walker JM (ed.), *Methods in molecular Biology*, vol. 32, Basic Protein and Peptide Protocols (pp. 49-57). Totowa: Humana Press Inc.

Khodaverdi, E., Maftouhian, S., Aliabadi, A., Hassanzadeh-Khayyat, M., Mohammadpour, F., Khameneh, B., & Hadizadeh, F. (2018). Casein-based

hydrogel carrying insulin: preparation, in vitro evaluation and in vivo assessment. *Journal of Pharmaceutical Investigation*, 1-7.

Klement, W., Lord, H., & Parker, R. (1960). Hydrophilic Gels for Biological Use. *Nature*, *188*, 929–930.

Lang, Y., Gao, H., Tian, J., Shu, C., Sun, R., Li, B., & Meng, X. (2019). Protective effects of α-casein or β-casein on the stability and antioxidant capacity of blueberry anthocyanins and their interaction mechanism. *LWT*, 115, 108434.

Larrauri, J. A., Rupérez, P., & Saura-Calixto, F. (1997). Effect of Drying Temperature on the Stability of Polyphenols and Antioxidant Activity of Red Grape Pomace Peels. *Journal of Agricultural and Food Chemistry*, 45(4), 1390–1393. https://doi.org/10.1021/jf960282f

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC international*, 88(5), 1269-1278.

Lee, J., Rennaker, C., & Wrolstad, R. E. (2008). Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry*, 110(3), 782–786. https://doi.org/10.1016/j.foodchem.2008.03.010

Lee, W. J., & Lucey, J. A. (2004). Structure and physical properties of yogurt gels: effect of inoculation rate and incubation temperature. *Journal of Dairy Science*, 87, 3153–3164.

Li, N. N., Fu, C. P., & Zhang, L. M. (2014). Using casein and oxidized hyaluronic acid to form biocompatible composite hydrogels for controlled drug release. *Materials Science and Engineering C*, *36*(1), 287–293.

Loewen, A., Chan, B., & Li-Chan, E. C. (2018). Optimization of vitamins A and D3 loading in re-assembled casein micelles and effect of loading on stability of vitamin D3 during storage. *Food chemistry*, 240, 472-481.

Lucey, J. A (2009). Milk protein gels. In: Thompson, A., Boland, M., & Singh, H (eds). *Milk Proteins: From Expression to Food* (pp. 458 – 464). San Diego: Elsevier.

Lucey, J. A. (2017). Formation, structural properties, and rheology of acid-coagulated milk gels. In: McSweeney, P. L. H., Fox, P. F., Cotter, P. D., Everett, D. W. (eds). *Cheese: Chemistry, physics & microbiology* (pp. 179 – 197). London: Elsevier Academic Press.

Lucey, J. A., & Horne, D. S. (2018). Perspectives on casein interactions. *International Dairy Journal*, 85, 56-65.

Ma, J., Lee, J., Han, S. S., Oh, K. H., Nam, K. T., & Sun, J.-Y. (2016). Highly Stretchable and Notch-Insensitive Hydrogel Based on Polyacrylamide and Milk

Protein. *ACS Applied Materials & Interfaces*, *8*(43), 29220–29226. https://doi.org/10.1021/acsami.6b10912

Migneault, I., Dartiguenave, C., Bertrand, M. J., & Waldron, K. C. (2004). Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques*, 37(5), 790–802.

Moura, M. H. C., Cunha, M. G., Alezandro, M. R., & Genovese, M. I. (2018). Phenolic-rich jaboticaba (Plinia jaboticaba (Vell.) Berg) extracts prevent high-fat-sucrose diet-induced obesity in C57BL/6 mice. *Food Research International*, 107, 48–60. https://doi.org/10.1016/j.foodres.2018.01.071

Nayak, S., & Andrew Lyon, L. (2005). Soft nanotechnology with soft nanoparticles. *Angewandte Chemie - International Edition*, *44*(47), 7686–7708. https://doi.org/10.1002/anie.200501321

Nogueira, M. H., Tavares, G. M., Silva, N. F. N., Casanova, F., Stringheta, P. C., Gaucheron, F., ... & Carvalho, A. F. (2019). Physico-chemical stability of casein micelles cross-linked by transglutaminase as a function of acidic pH. *Food Structure*, 19, 100103.

Ozel, B., Cikrikci, S., Aydin, O., & Oztop, M. H. (2017). Polysaccharide blended whey protein isolate-(WPI) hydrogels: A physicochemical and controlled release study. *Food Hydrocolloids*, *71*, 35–46. https://doi.org/10.1016/j.foodhyd.2017.04.031

Pereira, E. P. R., Cavalcanti, R. N., Esmerino, E. A., Silva, R., Guerreiro, L. R. M., Cunha, R. L., ... & Cruz, A. G. (2016). Effect of incorporation of antioxidants on the chemical, rheological, and sensory properties of probiotic petit suisse cheese. *Journal of dairy science*, 99(3), 1762-1772.

Raak, N., Rohm, H., & Jaros, D. (2017). Cross-linking with microbial transglutaminase: Isopeptide bonds and polymer size as drivers for acid casein gel stiffness. *International Dairy Journal*, *66*, 49–55. https://doi.org/10.1016/j.idairyj.2016.10.015

Rocha, J. de C. G., Procópio, F. R., Mendonça, A. C., Vieira, L. M., Perrone, Í. T., Barros, F. A. R. de, & Stringheta, P. C. (2017). Optimization of ultrasound-assisted extraction of phenolic compounds from jussara (Euterpe edulis M.) and blueberry (Vaccinium myrtillus) fruits. *Food Science and Technology*, *38*(1), 45–53. https://doi.org/10.1590/1678-457x.36316

Rohm, H., Ullrich, F., Schmidt, C., Löbner, J., & Jaros, D. (2014). Gelation of Cross-Linked Casein under Small and Large Shear Strain. *Journal of texture studies*, 45(2), 130-137.

Salcedo-Sandoval, L., Cofrades, S., Ruiz-Capillas, C., Matalanis, A., McClements, D. J., Decker, E. A., & Jiménez-Colmenero, F. (2015). Oxidative stability of n-3 fatty acids encapsulated in filled hydrogel particles and of pork meat systems containing them. *Food Chemistry*, 184, 207–213. https://doi.org/10.1016/j.foodchem.2015.03.093

Schorsch, C., Carrie, H., Clark, A., & Norton, I. (2000). Cross-linking casein micelles by a microbial transglutaminase conditions for formation of transglutaminase-induced gels. *International Dairy Journal*, 10(8), 519–528.

Schuck, P., Piot, M., Méjean, S., Le Graet, Y., Fauquant, J., Brulé, G., & Maubois, J. L. (1994). Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane. *Le lait*, 74(5), 375-388.

Silva, N. F. N., Casanova, F., Gaucheron, F., Teixeira, A. V. N. de C., da Silva, G. M., Minim, L. A., & Carvalho, A. F. de. (2018). Combined effect of transglutaminase and sodium citrate on the microstructure and rheological properties of acid milk gel. *Food Hydrocolloids*, *82*, 304–311. https://doi.org/10.1016/j.foodhyd.2018.03.038

Silva, S., Costa, E. M., Calhau, C., Morais, R. M., & Pintado, M. E. (2017). Anthocyanin extraction from plant tissues: A review. *Critical Reviews in Food Science and Nutrition*, *57*(14), 3072–3083. https://doi.org/10.1080/10408398.2015.1087963

Smeriglio, A., Barreca, D., Bellocco, E., & Trombetta, D. (2016). Chemistry , Pharmacology and Health Benefits of Anthocyanins. *Phytotherapy Research*, 30, 1265–1286.

Smiddy, M. A., Martin, J. G. H., Kelly, A. L., Kruif, C. G. De, & Huppertz, T. (2006). Stability of Casein Micelles Cross-Linked by Transglutaminase. *Journal of Dairy Science*, *89*(6), 1906–1914. https://doi.org/10.3168/jds.S0022-0302(06)72258-5

Song, F., Zhang, L. M., Shi, J. F., & Li, N. N. (2010). Novel casein hydrogels: formation, structure and controlled drug release. *Colloids and Surfaces B: Biointerfaces*, 79(1), 142-148.

Song, F., Zhang, L. M., Yang, C., & Yan, L. (2009). Genipin-crosslinked casein hydrogels for controlled drug delivery. *International journal of pharmaceutics*, 373(1-2), 41-47.

Sun, J., Ren, F., Chang, Y., Wang, P., Li, Y., Zhang, H., & Luo, J. (2018). Formation and structural properties of acid-induced casein – agar double networks : Role of gelation sequence. *Food Hydrocolloids*, *85*, 291–298. https://doi.org/10.1016/j.foodhyd.2018.07.030

Sung, H.-W., Huang, R.-N., Huang, L. L. H., & Tsai, C.-C. (1999). In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. *Journal of Biomaterials Science, Polymer Edition,* 10(1), 63–78.

Tavares, G. M., Croguennec, T., Carvalho, A. F., & Bouhallab, S. (2014). Milk proteins as encapsulation devices and delivery vehicles: Applications and trends. *Trends in Food Science and Technology*, 37(1), 5–20. https://doi.org/10.1016/j.tifs.2014.02.008

Tunick, M. H. (2010). Small-strain dynamic rheology of food protein networks. *Journal of Agricultural and Food Chemistry*, 59(5), 1481-1486.

Wrolstad, R. E. (2004). Anthocyanin pigments - Bioactivity and coloring properties. *Journal of Food Science*, *69*(5), C419–C421. https://doi.org/10.1111/j.1365-2621.2004.tb10710.x

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). Dairy Science and Technology (Second Edi., Vol. 4). Taylor & Francis.

Wei, J., Xu, D., Yang, J., Zhang, X., Mu, T., & Wang, Q. (2018). Analysis of the interaction mechanism of Anthocyanins (Aronia melanocarpa Elliot) with β-casein. *Food hydrocolloids*, 84, 276-281.

Xu, J., Fan, Z., Duan, L., & Gao, G. (2018). A tough, stretchable, and extensively sticky hydrogel driven by milk protein. *Polymer Chemistry*, *9*(19), 2617–2624. https://doi.org/10.1039/c8py00319j

Xu, R., Teng, Z., & Wang, Q. (2016). Development of tyrosinase-aided crosslinking procedure for stabilizing protein nanoparticles. *Food Hydrocolloids*, 60, 324–334. https://doi.org/10.1016/j.foodhyd.2016.04.009

Zhang, Z., Decker, E. A., & McClements, D. J. (2014). Encapsulation, protection, and release of polyunsaturated lipids using biopolymer-based hydrogel particles. *Food Research International*, 64, 520–526. https://doi.org/10.1016/j.foodres.2014.07.020

CHAPTER III: COLLOIDAL SYSTEMS FORMED BY CMS AND PEA PROTEINS

Preamble

In the last chapter, it was demonstrated the potentiality of using CMs hydrogels to carry and control the release of bioactive compounds. For this, it was used only CMs to formulate the hydrogel, however, the association with other polymers can create gels with totally new rheological features. Currently, products based on plant proteins are highly demanded due to sustainability and health reasons. Among the plant alternatives, pea stands out since it is widely cultivated, presents balanced amino acid content and it is non-allergenic. However, pea proteins have lower functional properties and sensory acceptance, which limit their widespread use in the food industry. Thus, the association with CMs is a strategy to overcome their drawbacks, and at the same time, create new foods. The formulation of protein food products typically uses thermal and acidification treatments and their impact on systems formed by CMs and pea proteins is not well understood, with scarce information about how the proteins interact and how it interferes with the final gel characteristics.

Questions:

- Is there a synergistic effect caused by CMs:pea interactions in the properties of the systems?
- Is the heat treatment able to improve the interactions between proteins from different sources?

The experimental part of this chapter was conducted at PIHM- UMET- INRAE (France)

PART A: Impact of protein ratio and thermal treatment on the rheological properties of high-concentrated casein micelles: pea protein suspensions

The content of this part is being prepared for submission in:

Food Hydrocolloids Journal

Luis Gustavo Lima Nascimento, Amandine Descamps, Xavier Trivelli, Federico Casanova, Rodolphe Marie, Evandro Martins, Antônio Fernandes de Carvalho, Guillaume Delaplace, Paulo Peres de Sá Peixoto Junior

Abstract

Currently, there is an increase in the consumption of plant-based products for healthy and sustainable reasons, which increases the demand for plant proteins, such as pea. However, the low techno-functionality and undesirable flavor make their incorporation into food systems challenging. Thus, the association of these proteins with a more consumer-accepted product, such as milk, is an alternative. However, it is necessary to understand the properties of such mixed systems. This study aimed to investigate the physicochemical properties of mixed casein micelles (CMs): pea protein systems at different protein ratios (80:20, 60:40, 40:60, 20:80 w/w) in high-concentrated protein systems, before and after thermal treatment. Thus, rheological assays, dynamic light scattering, electrophoresis, and nuclear magnetic resonance were applied as analytical tools. A synergistic effect in the elasticity and viscosity results at the 20:80 ratio was observed. It was probably caused by stronger interactions among the pea proteins at this specific ratio. Since the same phenomenon was observed only after thermal treatment in less concentrated suspensions. Thus, it is hypothesized that the small amount of CMs present within the pea network influences the organization of pea aggregates, inducing more pea-pea non-covalent interactions. These results bring new insight into the formulation of mixed milk: plant protein systems.

1. Introduction

The population is expected to reach 9.7 billion people by 2050 (United Nations, 2015) and, to support the upcoming demand, the industry needs to increase food production. However, the food industry should propose solutions that englobe sustainability, economic and environmental questions, as well as to attend the consumer desire (Liu et al., 2022). Among the food constituents, the proteins are essential macromolecules that present several physiological roles such as in the construction of tissues, muscle contraction, catalyzing reaction, immune system, and nitrogen storing, among others (Damodaran, 2008). For these reasons, this nutrient needs to be daily consumed through the intake of meats, eggs, milk, and vegetables. Thus, in the near future, it is expected to be a higher demand for protein sources and, consequently, an intensification of animal handling. However, the expansion of animal handling is accomplished by increasing deforested areas, greenhouse gas emissions, water consummation, and energy costs (Ismail et al., 2020). One possible way to get around these environmental problems consists in incorporating more plant proteins into human food. The demand for pea proteins in the world market tends to grow by about 12% per year in the coming years (Grandviewresearch, 2019). It is partly explained by its low allergenicity and high nutritional value with a good balance of amino acids. In contrast, pea proteins have a beany taste and low solubility, factors that make their application in the food industry challenging (Boukid, Rosell, & Castellari, 2021). Therefore, for pea proteins to be effectively incorporated into food formulations, some technological strategies must be applied to mitigate these inconveniences. A strategy to increase the consumption of pea is mixing it with other protein sources that possess more consumer acceptability.

It is clear the change in the consumption habits to a more sustainable and plant-based. However, this transition may take time due to lower the acceptability of pea. Currently, milk is one of the most important sources of animal protein and can be used as a raw material in the production of various processed foods such as UHT milk, cheese, yogurt, powdered milk, cream, butter, and ice cream (Walstra et al., 2005). The wide variety of dairy products is possible due to the functionalities of milk proteins. Therefore, the creation of mixed systems using the combination of pea proteins and milk proteins could be an alternative to increase the consumption of vegetable protein, as well as to develop dairy products with totally new sensorial and techno-functional

characteristics (Chihi et al., 2018). However, the incorporation of another protein source significantly changes the final result of the product and how the two types of proteins interact in mixed systems is not well elucidated, and studies in the area are still scarce (Alves & Tavares, 2019). Thus, the impact of this association as well as the optimization of protein interactions must be better understood for the development of innovative products.

Studies involving the formation of casein micelles (CMs) -pea protein thermal and acid gels, in general, demonstrate an absence of synergism in the rheological properties of these protein sources. It is probably due to the existence of a thermodynamic impartibility between these proteins causing the formation of independent systems, microphase separation, and, gels with less elastic structure and greater syneresis (Ben-Harb et al., 2018); Mession, Roustel, & Saurel, 2017). However, there is an absence of studies of mixed systems in higher protein concentrations, which can possibly favor the interactions between the protein sources.

This study aims to create high concentrated mixed casein micelle: pea suspensions and understand the effect ratio and concentration of proteins on the rheological properties of the systems. Also, a thermal treatment was applied to follow the changes caused in the systems after the application of a common unit operation in the food industry.

2. Materials and methods

2.1. Materials

The micellar casein (CMs) powder (Promilk 85B) was kindly provided by Ingredia SA (Arras, France) and pea protein powder (F85S) was provided by Roquette SA (Lestrem, France). All others reagents were of analytical grade.

2.2. Sample preparation

Both protein powders were separately rehydrated in deionized water at 12, 14, and 16% $w.v^{-1}$ concentrations. These concentrations were defined in the pre-test and chosen based on the highest concentration that the pea protein could be solubilized. The hydration process occurred overnight at 25 ℃ using a magnetic stirring plate at 600 rpm. After complete rehydration, the proteins were mixed in four ratios of CMs:

Pea, being 80:20, 60:40, 40:60, and 20:80, and systems formed only with CMs or Pea proteins were also analyzed. After the mixture, the mixed protein systems were stirred at 30 ℃ for 2 hours at 600 rpm.

2.3. Effect of thermal treatment in the mixed systems

Each protein mixture produced as described in section 2.2 was placed in centrifuge tubes and put in a water bath at 85 ℃ for 1 h. After that, the samples were immersed in an ice bath to decrease rapidly the temperature to 30 ℃. The long thermal treatment time was chosen to produce denaturation in the pea proteins aiming to potentialize possible protein interactions.

2.4. Dynamic light scattering (DLS)

The particle size distribution analysis for each sample was performed according to Silva et al. (2018) with some modifications. A DLS apparatus (DynaPro Nanostar, Wyatt, CA, USA) at a 90° scattering angle and 658 nm wavelength excitation was used. Briefly, before and after the thermal treatment, the protein suspensions were diluted 100 times, in the same solvent used to rehydrate the protein powders. After dilution, the samples were put in a polystyrene cuvette. The analysis was performed at 30℃.

2.5. Electrophoresis

The electrophoresis was carried out to gather information about the formation of disulfide bonds in the samples. The suspensions were analyzed in polyacrylamide gels in reduced and non-reduced conditions, as described by Veloso, Teixeira, and Ferreira (2020), with slight modifications. The samples were diluted with deionized water at 8 mg/mL. For the reducing condition, 2-mercaptoethanol buffer solution (1:4) was added. Then, 10 µm of samples were put on the top of the polyacrylamide gel composed of a stacking gel of 4% and a separation gel of 12% acrylamide. The protein migration was performed in a SE 600 Series Vertical Slab Gel unit (Hoefer Scientific instrument, San Francisco, US), using a Tris-glycerin buffer solution at pH 8.8. A voltage of 30 V was applied in the first 30 min of analysis, followed by the application of 90 V until the last band reach approximately 4/5 of the gel. After the protein migration, the gels were dyed by immersion in a solution of 0.3% of Coomassie® Brilliant Blue R-250 dissolved in acetic acid: methanol: water (1: 4.5: 4.5) for 4 h at 25

℃. After, the gel was immersed in methanol: acetic acid solution to decolorate and promote a contrast between the protein bands and the background. The gels were digitalized and the images were analyzed using ImageJ software (Hermanto, Sholaikah, & Mulyani, 2016).

2.6. Apparent viscosity and flow curve

Before the thermal treatment, the rheological behavior of the samples and apparent viscosity were evaluated regarding their flow behavior in a strain-controlled rheometer (ARES, TA Instruments, USA) using a cone-plate geometry with an angle of 0.04 rad and 0.0457 mm gap. The analysis was performed at 30 ℃ by varying the shear stress from 0.1 until 300 s^{-1} in three cycles (to detect possible thixotropic behavior), being the first and third upward and the second downward. The third curve was used to determine the flow behavior and consistency index using the power-law model (equation 1).

$$\sigma = k.y^n \quad (1)$$

Where $\sigma\ (Pa)$ is the shear stress, k ($Pa.s^n$) is the consistency index, $y\ (s^{-1})$ is the shear rate, and n (dimensionless) is the behavior index.

2.7. Small amplitude oscillatory shear (SAOS) test

SAOS test was carried out to get elastic and viscous properties of the pure and mixed protein systems. The effect in the sample structure during and after the thermal was analyzed according to Costa et al. (2021) with slight modifications. The suspensions were carefully placed in the rheometer (ARES, TA Instruments, USA) equipped with cone-plate geometry (60 mm, 2°) using a gap of 0.54 mm. The sample excess was removed with a spatula and paraffin oil was put on the edges of the geometry to avoid water evaporation during the experiments. The time and temperature sweeps were performed within the linear viscoelastic region, at 1 Hz frequency and 0.1 % amplitude. The sample was allowed to equilibrate for 5 min. Then, a temperature sweep test was performed from 30 ℃ until 85 ℃ with a heating rate of 8℃/ min (the same rate achieved in the water bath). As soon as the temperature reached 85℃, a time sweep test was performed for 1h. After, a second temperature sweep is applied from 85 ℃ until 30 ℃ at a rate of 15 ℃/min. After, a time sweep for 5

min at 30 ℃ was applied to verify complete gel formation. In the end, a frequency sweep test (0.1 to 50 Hz, at 0.1 % amplitude) was applied to the samples.

2.8. Nuclear magnetic resonance (NMR)

NMR was applied to gather information about the water dynamics in the samples. The NMR experiments were recorded on a Bruker AVANCE NEO 900 spectrometer equipped with a 5 mm TCI cryo-probe at 85 °C. Two types of experiments were run: standard 1D-^{1}H, and 2D-^{1}H-diffusion (*ledbpgp2s* pulse program, recycling delay=10 s, d20=120 ms, p30=1,5 ms, 8 scans, 16 gradients values from 5 to 95 % with a linear variation, duration 24 minutes). The samples were prepared according to section 2.2 with the replacement of water for deuterium oxide and transferred into standard 5 mm NMR tubes. 70 µL of mineral oil used in PCR application were then added to avoid solvent evaporation (Wieruszeski et al., 2006).

2.9. Confocal scanning laser microscopy (CSLM)

CSLM was applied to access the samples microstructure and it was performed according to Andoyo, Guyomarc'h, Cauty, & Famelart (2014) with slight modifications. 0.2 g/kg of rhodamine B isothiocyanate (RITC) (Sigma Sigma-Aldrich) was used to label the proteins The suspensions were stirred for 5 min at 25 ℃ to ensure RITC solubilization. The samples were poured into an 8- well chamber slide (Ibidi GmbH, Germany) that was thermally treated in a water bath in the same conditions described in section 2.3. After thermal treatment, the samples were visualized using an inverted microscope (Nikon Ti2) equipped with a sCMOS camera (Photometrics Prime95b).

2.10. Statistical analysis

The samples were compared regarding ratio, concentration, and thermal treatment by variance analysis (ANOVA). When a significant difference ($p < 0.05$) was found, the Tukey HSD test with 5% significance was applied to differentiate means. All the experiments were performed at least in duplicate and the data was evaluated utilizing SAS software student edition.

3. Results and discussion

3.1. Apparent viscosity and flow behavior

The apparent viscosity for all the studied suspensions decreased when the shear rate applied increased (Figure 1A, 1B, 1C). The shear-thinning behavior was more evidenced in systems with a higher amount of pea proteins and in higher protein concentrations. The shear-thinning is usually explained by the increase in the molecular orientation when the shear rate increases, which decreases the degree of molecular entanglement (Varesano, Aluigi, Vineis, & Tonin, 2008). The apparent viscosities at 200 s^{-1} were chosen as the parameter for a sample comparison, once this is a common shear rate applied in the food industry and encountered in mouth (Hubbe et al., 2017). Viscosity is understood as the friction between the fluid layers, or the resistance of a fluid to flow (Bourne, 2002). Comparing the protein ratios at the same protein concentration, the 100:0 suspension presented the lowest apparent viscosity for all the concentrations studied. In the mixed samples, it was noticed the rise of synergism in the protein ratio of 20:80 in higher protein concentration suspensions. At 12%, the apparent viscosity increased in a continuous way from pure CMs to pure pea suspensions, thus the 0:100 presented the highest apparent viscosity (Figure 1A). At 14%, the increase in apparent viscosity became less continuous, with the viscosity at 0:100 and 20:80 being equal (Figure 1B). Finally, at 16%, the viscosity at 20:80 was higher than in pure pea suspensions (0:100) (Figure 1C).

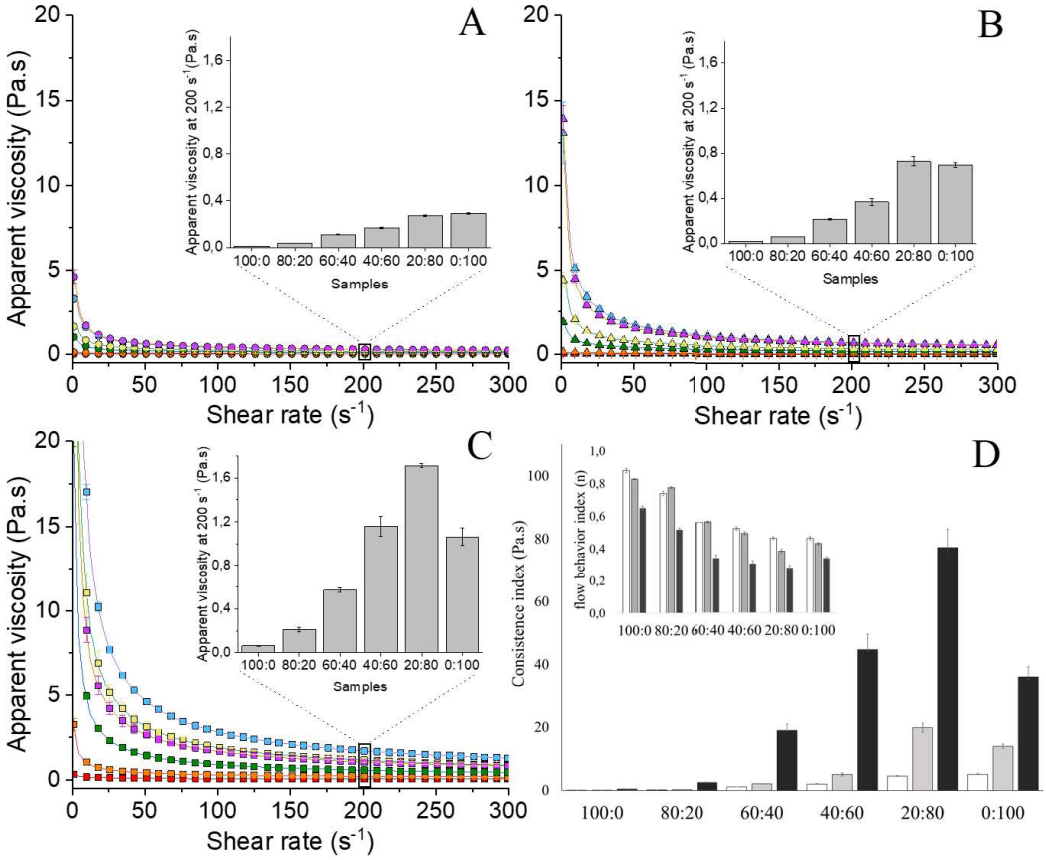


Figure 1. Apparent viscosity as a function of shear rate and apparent viscosity at 200 s^{-1} shear rate (inserted graphs) for protein suspensions at 12 (○) (A), 14 (△) (B), and 16% (□) (C) total protein concentration. CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100. (D) consistency index and flow index (inserted graph) for 12 (white), 14 (light gray) and 16% (dark gray) protein suspensions

This synergism in the apparent viscosity in the 20:80 protein ratio may be related to the specific organization of the proteins in the systems at higher protein concentrations. It has been already reported a synergism effect in protein blends at a ratio where one of the proteins is much more abundant. Wong, Vasanthan, & Ozimek (2013) observed a synergistic effect between pea protein and whey protein at 20:80 blend ratio in the elastic modulus (G'), hardness, and viscosity parameters, which varies depending on protein concentration and pH. Tomé, Pires, Batista, Sousa, and Raymundo (2014) observed synergism in the G' of a protein mixed emulsion formed by the mixture of pea protein with hake protein at 20:80 ratio at pH 7.0. The authors argue that it may be caused by the optimum balance between repulsive and attractive forces in the system and at this specific ratio the protein-protein interactions are optimized. Comfort and Howell (2002) found synergism in G' for thermal gels formed

by the combination of whey and soy proteins at 17:1 and 16:2 ratios, the further addition of soy caused a decrease in G'. However, the authors argue that the synergism is most likely caused by gelled inclusions of the plant protein present in lower concentration into the continuous phase of the protein present in higher concentration. Thus, the synergism is due to the preference of a protein source to interact with itself than with the other protein source. In the present study, the mechanism of interaction seems to be closer to the one reported by Comfort and Howell (2002) once the presence of a small amount of casein micelles may force the pea protein to be closer, reinforcing pea-pea interactions, which could increase the viscosity of the system.

The flow behavior of the suspensions is depicted in Figure 1D, where the power law was used to fit the shear strain/shear rate curves. All the samples were classified as pseudoplastic fluids, for the range 0 – 300 s^{-1} with r^2 higher than 0.999, as the flow index (*n*) was found to be lower than 1 (inserted graphics in Figure 1D). The consistent index of the suspensions followed the same behavior found for the apparent viscosity. In the same way, the *n* decreased following the increase of pea protein until the minimum value in the 20:80 ratio at 16% protein concentration. Showing the reinforcement of pseudoplastic nature at this specific ratio and concentration.

The heat treatment is commonly applied to modify proteins in the food industry seeking the increase proteins' techno-functional properties. Thus, how the protein systems pure or mixed behave after heat treatment was studied as the next step.

3.2. Small amplitude oscillatory shear test

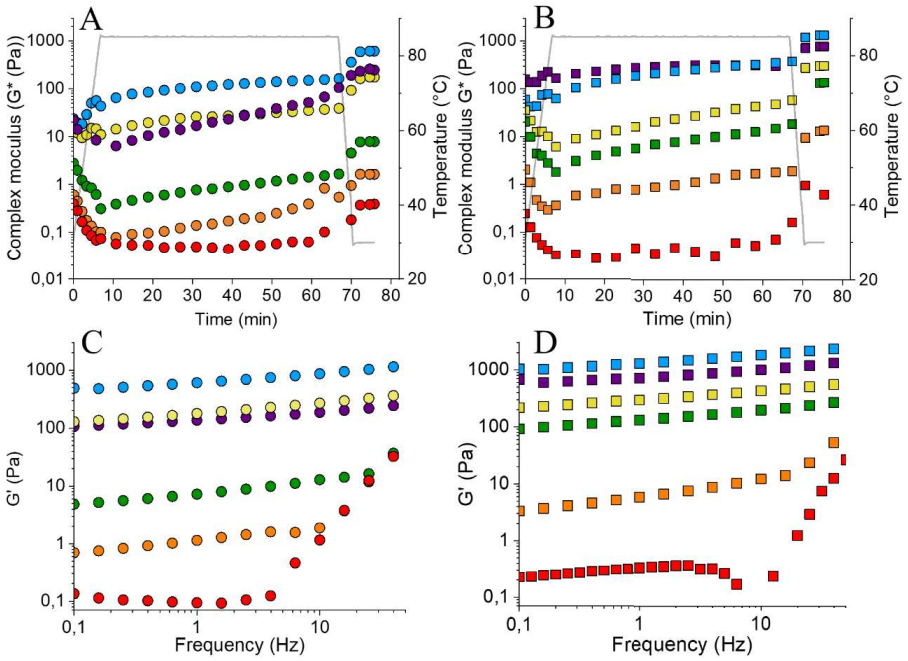


Figure 2. G* as a function of thermal treatment for casein micelle: pea protein suspensions at 12 (○A) and 16% (□B). Frequency dependence of mixed casein micelle: pea protein suspensions at 12 (○C) and 16% (□D). CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100.

SAOS rheology was performed to follow the thermal treatment employed in the suspensions of lower and higher concentrations, 12 and 16% total protein (Figure 2). The analysis can be divided into 4 regions, marked by the changes in temperature (gray line). The first is the increase in temperature from 30 ℃ to 85 ℃, which happened in the first 8 minutes. The second region is characterized by keeping the temperature constant at 85 ℃ for one hour. After, the temperature is decreased until the initial temperature (region iii) followed by maintaining the temperature at 30 ℃ (region iv) for 5 minutes. The results are presented in terms of complex modulus (G*). It was chosen as a parameter to compare the samples because it takes into consideration both elastic (G') and viscous (G'') modulus and makes easier the analysis.

The casein micelle suspension (0:100) presented the lowest G* among all samples. During the heat ramp, it was observed a decrease in G* in both 12 and 16% suspensions. It is explained by the expansion of the samples in the rheometer during the fast increase in temperature caused by the increase in the molecular motion, which disrupt hydrogen bonds (Tomé, Pires, Batista, Sousa, and Raymundo, 2014). After reaching the treatment temperature, the 100:0 G* did not change during the treatment

time. It was expected since the absence of tertiary structure in the casein micelles makes it stable for heat treatment (Schäfer et al. 2017). Schäfer et al (2017) were able to detect a sol-gel transition in casein suspensions only at pH lower than 5.4. Thus, in the studied conditions of pH and calcium content, it is unlike to observe significant changes detectable by SAOS in casein micelles upon heat. In the cooling step, it was observed an increase in G* probably due to the shrink in the system caused by the reduction of molecular movements, which increases the proximity of proteins. In addition, the formation of hydrogen bonds can take place as well (Yi et al., 2013). Thus, after the heat treatment, the difference in G* for the sample composed only of casein micelle is negligible.

The opposite behavior was observed for suspensions formed only by pea protein (0:100). The G* varies during the heat ramp in a higher degree for 12% suspensions and a lower degree in 16%. The increase in molecular movements also was present in 0:100, but the beginning of pea protein unfold creates new interactions between the protein in the system, which balance the changes caused by swelling. During the heat treatment, the G* increased gradually. Pea proteins are globular proteins that are heat sensitive and, after achieving a certain temperature, some amino acids buried in the protein's natural conformation are exposed (Clark, Kavanagh, & Ross-Murphy, 2001). The exposure of hydrophobic amino acids leads to the formation of new interactions, mainly hydrophobic, but also hydrogen and disulfate bonds occur, depending on the pea protein fraction involved (Sun & Arntfield, 2012). If the newly formed interactions are sufficient to form a network and a continuous phase, a gel is established (Shanda, Yaa, Pietrasika, & Wanasundaraab, 2017).

Comparing the studied concentration at the same protein ratio, it was observed an increase in the G* when the concentration increased from 12% to 16% (Figure 2C, 2D). It shows that the higher concentration of proteins makes easier their interactions, which increases the system G*. However, the changes compared before and after heat treatment were more pronounced in 12%, probably because in less concentrated suspension there is more space for protein unfolding before interactions, thus more new interactions between the proteins counterparts are formed.

In the protein blends, the G* increased following the addition of pea protein and the thermal treatment applied. The difference increased when the amount of pea

protein increased, showing that is the pea the main protein source involved in the modifications caused by heat. However, at the end of the thermal treatment, the 20:80 protein ratio presented the highest G* compared to the other samples independently of protein concentration in the system (Figure 2A, 2B). These results increment the finds in the apparent viscosity analysis since the synergism effect in the 20:80 ratio was observed only at 16% (Figure 1C). Thus, considering that pea protein was responsible for the system structuration and the new interactions formed after heat are probably formed among pea proteins, the results indicate that the synergism at 20:80 is derived from the pea-pea interaction with the CMs in the between pea aggregates.

After samples rest time, a frequency sweep test was performed to gather more information about gel networks (Figure 2C, 2D). The frequency sweep test can be applied to gather information about the type of interactions in a food gel, *i.e* if the polymers are simply entangled, or there is the participation of covalent bonds or only physical interactions (Tunick, 2011). A strong gel is characterized by low-frequency dependence, while a weak gel presents high-frequency dependence (Tunick, 2011). The 100:0 suspension presented highly frequency dependence, where the curves change drastically inclination before 4 Hz at 12% and 16%. The frequency results are aligned with the previous data and explained by the inability of casein micelles to form a gel in the applied conditions. Regarding the concentration, in 12% protein concentration, a more stable curve in the frequency sweep test only appears when pea protein was in majority, *i.e*., 40:60 sample (Figure 2C). The protein system 60:40 showed stability during the entire test at 16%, indicating that the higher the protein concentration, the higher the number of protein interactions. The increase in pea protein proportion decreases the sample's frequency dependence at 12 or 16%, which indicates the formation of stronger interaction in the protein matrixes with more pea protein. It was observed that the 20:80 sample presented higher G*, but the 0:100 presented lower frequency dependence (Figure 2D). This suggests that both protein ratios present bounds of the same nature, evidencing that the synergism effect comes from a specific network organization and not from newly formed pea-casein interactions.

3.3. Particles size distributions

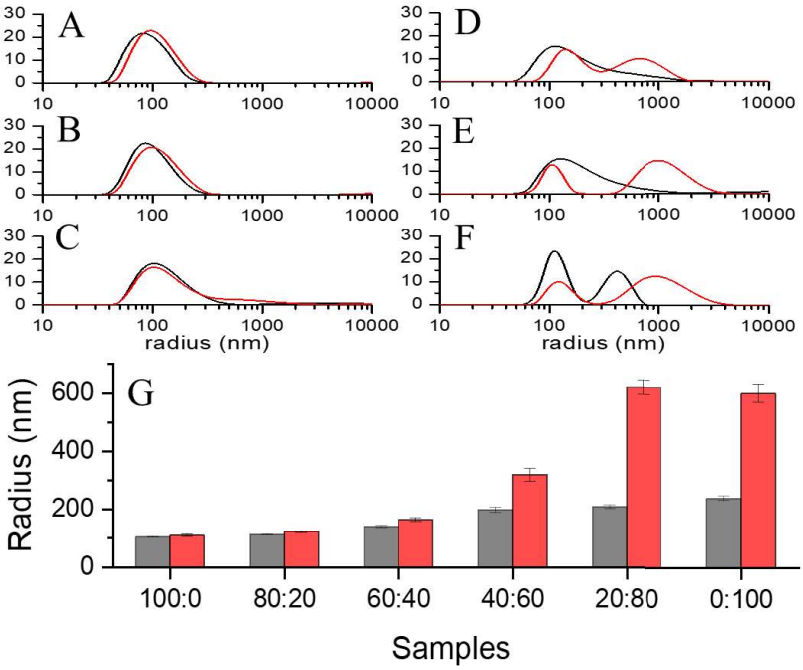


Figure 3. Particle size distribution of mixed protein suspensions before (black line) and after (red line) heat treatment at 12%, (A) 100:0, (B) 80:20, (C) 60:40, (D) 40:60, (E)20:80, and (F) 0:100. (G) Average particle size before (dark gray) and after (red) heat treatment at 12%.

The particle size of the suspensions at 12% was analyzed by DLS. It was observed one particle population in the casein suspensions with a hydrodynamic diameter of 213.95 ± 9.5 (Figure 3A). This diameter is in accordance with the results generally found for casein micelles in the pH condition studied (Nascimento et al., 2020; Silva et al., 2018). The thermal treatment applied did not cause any change in the casein micelle size, which explains the absence of change observed in the SAOS analysis. Contrarily, the pea proteins presented two particle sizes population, the first around 180 nm and the second around 690 nm (Figure 3F). The pea protein monomers have a size of around 15 nm (Wu, Wang, Ma, Cai, Wang, 2020), thus, the particle sizes found in this study are much bigger than the expected values for the proteins present in pea proteins. The absence of monomers or oligomers in the evaluated pea protein suspension suggests that the majority of proteins are aggregated. It was probably caused by the harsh production process applied in the extraction of the pea protein (Adebiyi & Aluko, 2011), which impacts their functional properties. Kornet et al. (2021) also found large protein aggregates in the commercial pea protein, compatible with the

aggregates profile found in this study. The authors also observed the absence of a denaturation peak in the commercial pea protein, showing that all the proteins were already denatured.

The thermal treatment led to an additional increase in the population sizes, mainly in the largest particle population. The pea proteins are globular proteins that unfold and aggregate by different interactions such as hydrophobic, electrostatic, and disulfide bonds (Sun, & Arntfield, 2012). Thus, the increase in the G* observed in the gels where pea protein was present is linked to the increase in the protein particle sizes. The gradual replacement of casein micelles for pea protein increased the particle sizes in the samples, which was more evidenced after heat treatment. The differences caused by the thermal treatment started to be significant ($p < 0.05$) at 40:60 protein ratio, being the pea protein the main responsible for the formed aggregates. However, the increase in average size is not linear, and the 20:80 protein ratio presented an average size higher than 0:100, mainly due to the second peak of particle population, which was slightly higher in the 20:80 ratio.

To understand the nature of the aggregates in the system, a electrophorese analysis in reduced and non-reduced conditions was performed.

3.4. Electrophoresis

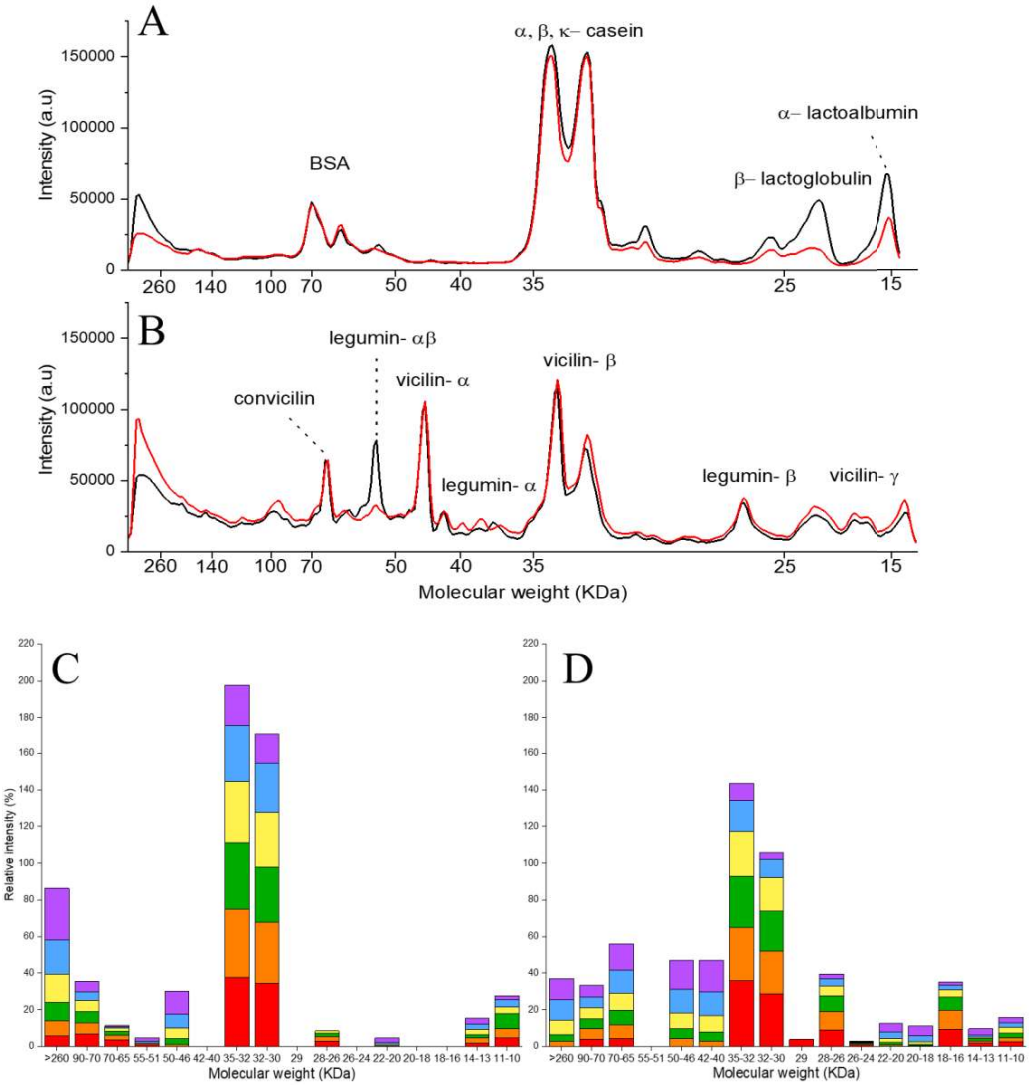


Figure 4. (A) 100:0 and (B) 0:100 protein band intensities before (black line) and after (red line) heat treatment at 12% protein concentration. Relative protein band intensities in non-reduced (C) and reduced (D) conditions. CMs: pea protein ratios: (red) 100:0; (orange) 80:20; (green) 60:40; (yellow) 40:60; (blue) 20:80; (purple) 0:100.

The electrophorese peak intensities profile for the suspension at 12% protein concentration is shown in Figure 4. The protein band profile in non-reducing conditions was analyzed for pure casein and pea system before and after thermal treatment as can be observed in Figures 4A and 4B. The casein powder used in the experiment presented a small amount of whey proteins, as can be seen in the peaks between 18 and 10 KDa, which correspond to β-lactoglobulin and α-lactalbumin (Tarhan, & Kaya, 2021). In the sample, the casein band appeared at 35 – 32 KDa, also it was visualized as a small amount of BSA at 50 – 72 KDa molecular weight (Figure 4A). It was opted

to use a commercial casein powder in the experiment to facilitate the transference of the results found here to applications in the food industry. Thus, it was admitted a small amount of serum proteins in the sample, which did not interfere with the overall sample physicochemical properties as can be seen by the results reported previously in section 3.2. The thermal treatment did not cause changes detectable by electrophoresis in the casein fractions; however, the serum proteins peak decreased after heating, probably due to the formation of new interactions with k-casein by thiol-disulfide interchange reactions (Anema, 2021).

The pea protein powder presented several protein fractions, which include convicilin, legumin- αβ, vicilin-α, legumin-α, vicilin-β, legumin-β, and vicilin-γ, which can be visualized in Figure 4B. It was observed a decrease in the intensity of legumin- αβ after heat treatment. It is explained by the unfolding, followed by aggregation of the legumin fraction, which also resulted in the increase of protein aggregates with molecular weight higher than 260 KDa, probably linked by disulfide bonds (Mession, Roustel, Saurel, 2017). It was observed the absence of differences in the other protein fractions. However, the increase in G* after thermal treatment cannot be completely explained by the s-s linkages, once the amount of legumin- αβ corresponded only to 16% of pea protein bands. Thus, part of the gel structuration also was led by non-covalent interactions.

In the mixed suspensions, the electrophoresis was made only after heat treatment, once it is unlike the formation of any covalent interaction before the heat treatment. The samples were analyzed in non-reduced and in reduce conditions to have a better idea of the interactions in the gels. The proteins share similar protein molecular weights, and the relative band intensities were calculated and plotted in Figures 4C and 4D.

The comparison between the protein bands in the two different reducing conditions brings light to the type of interactions formed in the samples. Most of the high molecular weight aggregates disappeared in the reducing conditions, which confirms the formation of aggregates by disulfide bonds after heat treatment. The remaining aggregates are probably insoluble, and enable to go through the gel. The increase in the intensity of band 70-65 KDa is probably due to the convicilin fraction that can also interact via disulfide bond (Lam, Karaca, Tyler, & Nickerson, 2016) and

was probably in the high-molecular-weight aggregates. The increase and arise in the protein bands with molecular weight around 50-40 KDa is due to the legumin- α, which comes from the breakdown of the disulfide bond that kept the legumin fractions α and β together. Thus, the legumin-β can be visualized by the increase in the band 22-18 KDa. For casein, the most remarkable difference is the increase in the peak bands 28-26 and 18-15 KDa, which is related to κ- casein, β-lactoglobulin, and α-lactalbumin, confirming the formation of S-S linkages in these proteins after heat treatment. Despite the clear interaction between the proteins with their counterparts, the bands in the mixture systems seem to be resulted only from the simple addition of the protein, since they share protein bands with the same molecular weight. Thus, it is reasonable to attest that there is no covalent interaction between pea proteins and casein micelles, as proposed by other authors in less concentrated protein suspensions (Silva et al., 2019; Mession, Roustel, & Saurel, 2017).

3.5. Nuclear Magnetic Resonance (NMR)

The water dynamics in the samples at 12% before and after heat treatment is shown in Figures 5A and 5B, this set of samples was chosen because it presents more water compared with 14 and 16%, and the synergism effect was observed after heat treatment. The dynamics decreased when more pea protein was added to the suspensions whatever before or after heat treatment. It indicates that the pea network presents lower pore sizes, which limits the water molecules' freedom as confirmed in the microstructure analysis after heat treatment (Figure 6F). In addition, pea proteins integrate water molecules within the protein network, with almost no water in the interstitial region of the protein matrix (Peters, Vergeldt, Boom, Goot, 2017). It is notorious a difference in the water peaks after the replacement of 20% casein micelle for pea proteins (80:20) before and after heat treatment. However, it did not happen in the opposite way, *i.e*., when 20% of the pea protein dispersion was replaced by casein micelles (20:80). It was observed a gradual decrease in the water dynamics before the heat treatment when pea protein proportion increase, however, the water peak for 20:80 and 0:100 protein ratios after the thermal treatment are equal (Figure 5B). It indicates that the casein micelles present in 20:80 dispersions are finely distributed within the pea protein network.

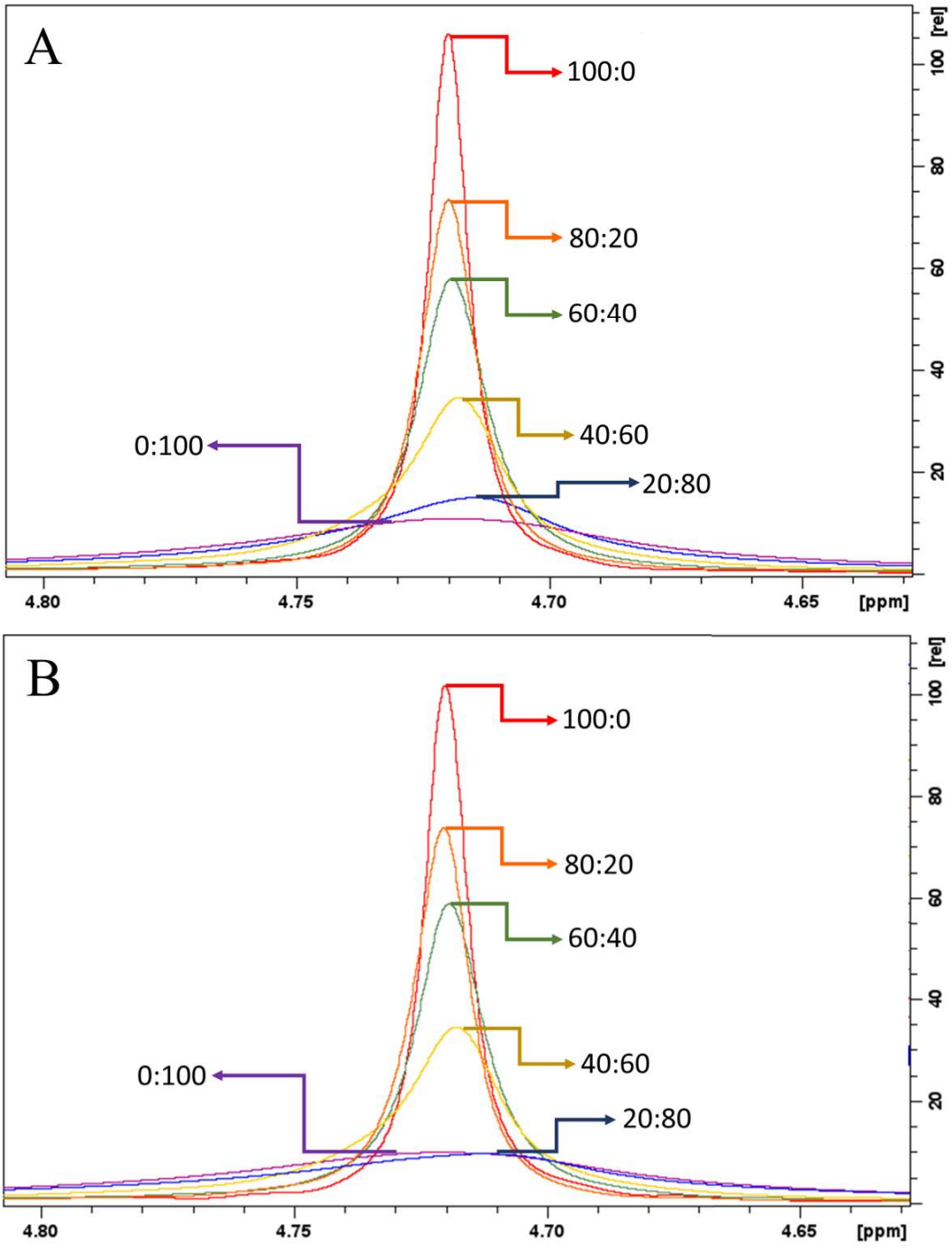


Figure 5. Water peak measured by liquid NMR analysis at 12% protein concentration before (A) and after (B) thermal treatment. CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100.

3.6. Confocal scanner laser microscopy (CLMS)

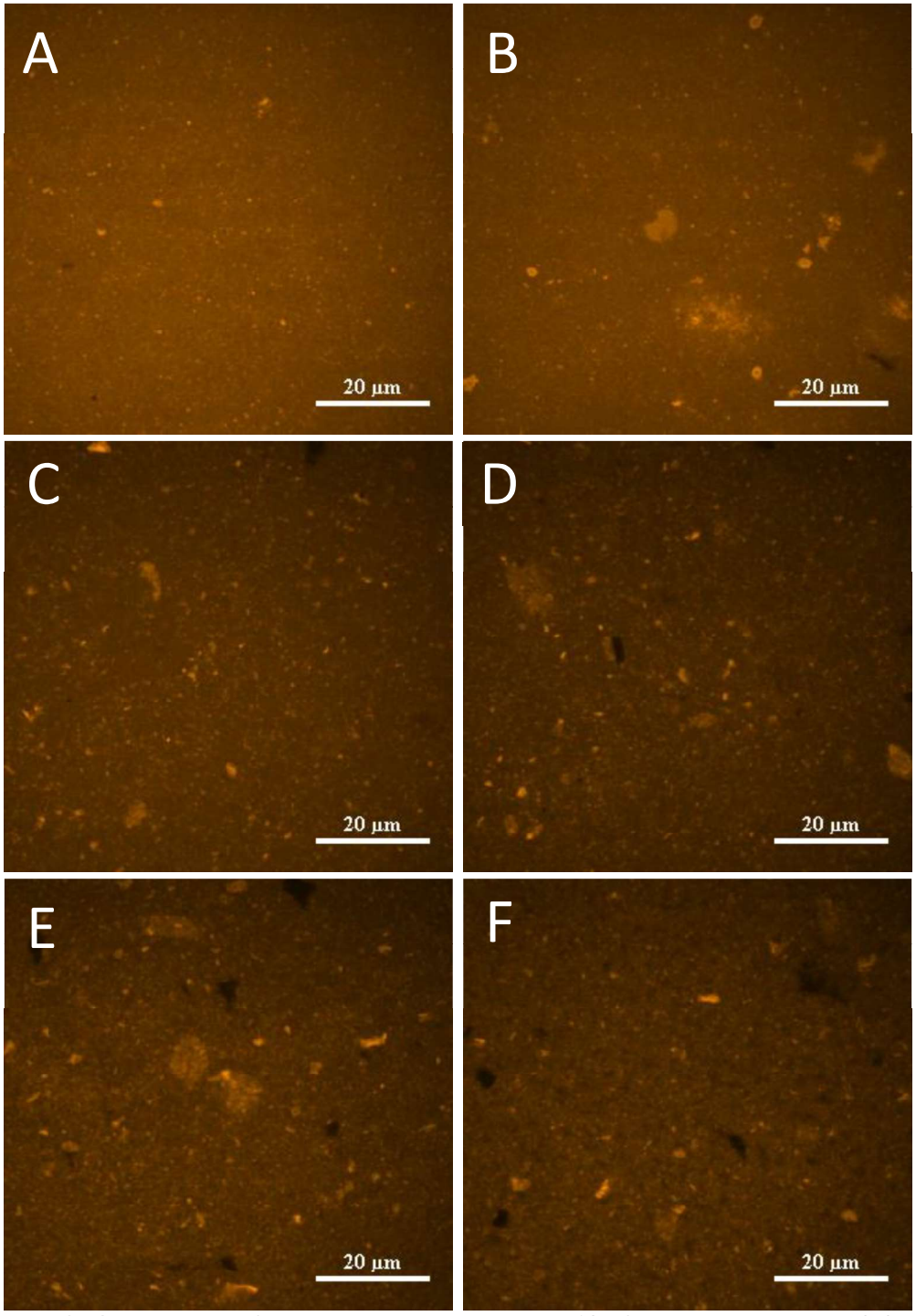


Figure 6. CLSM images of protein suspensions at 12% after heat treatment. (A) 100:0, (B) 80:20, (C) 60:40, (D) 40:60, (E)20:80, and (F) 0:100.

The images show the microstructure of the samples after heat treatment at 12% concentration. The samples were labeled with rhodamine B that attaches to both

proteins, which makes impossible a color differentiation. However, it is notorious the difference in the sample microstructure when the protein ratios vary. It is known that the solubility of commercial casein protein is an issue in the food industry, and even applying a high rehydration time, some aggregates still remained. The thermal treatment did not affect significantly the sample containing only casein micelles (100:0). Thus, in Figure 6A, it was observed a typical liquid system, which is characterized by the absence of a porous structure or strands. In opposition, the formation of a structure with small pore sizes is visualized in the sample containing solely pea protein (Figure 6F), indicating the formation of a thermal gel.

In the mixed systems, the microstructure depended on the protein ratio as can be seen in Figure 6B, 6C, 6D, and 6E. larger protein aggregates highly spaced were formed when 20% of the casein micelle was replaced for pea protein. These aggregates are probably formed by pea protein and they were not able to form a continuous network, which agreed with the rheology results for the 80:20 protein blend. The increase in the pea protein proportion in the mixed systems promoted a more homogeneous distribution of the protein aggregates. The samples 20:80 and 0:100 seem to have more protein aggregates better distributed in the gel structure

Therefore, the 20:80 ratio has the ideal combination of repulsive and attractive forces. Independently of the protein ratio, the nature of the interactions did not change, however, the spatial distribution of the different proteins in the system can modulate its rheological properties.

4. Conclusion

In this study, rheological features of mixed casein micelles: pea proteins in high concentration suspensions were evaluated. Casein micelles and pea proteins seem to prefer interacting with themselves even in high protein suspensions. The thermal treatment increased the interaction between the pea proteins, which increased the elasticity of the systems. The formation of disulfide bonds occurs, but only between milk-milk and pea-pea proteins. Despite the absence of disulfide bonds between pea and casein micelles, a synergism in the complex modulus and in the apparent viscosity of protein mixtures starting at 16% before thermal treatment and at 12% after thermal treatment was observed for 20:80 protein ratio. Thus, this study showed that the presence of a small amount of CMs interferes in the organization of the proteins and

can induce more interactions in the overall system if a specific balance of forces is achieved. The next step of the investigation is the acid gelation of the mixed systems, once this process is widely applied in the fabrication of milk products such as yogurt.

5. References

Adebiyi, A. P., & Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (Pisum sativum L.) seed protein isolate. Food Chemistry, 128(4), 902-908.

Alves, A. C., & Tavares, G. M. (2019). Mixing animal and plant proteins: Is this a way to improve protein techno-functionalities?. Food Hydrocolloids, 97, 105171.

Anema, S. G. (2021). Heat-induced changes in caseins and casein micelles, including interactions with denatured whey proteins. International Dairy Journal, 122, 105136.

Bourne, M. (2002). Food texture and viscosity: concept and measurement. Elsevier.

Boukid, F., Rosell, C. M., & Castellari, M. (2021). Pea protein ingredients: A mainstream ingredient to (re) formulate innovative foods and beverages. Trends in Food Science & Technology, 110, 729-742.

Chihi, M. L., Sok, N., & Saurel, R. (2018). Acid gelation of mixed thermal aggregates of pea globulins and β-lactoglobulin. Food Hydrocolloids, 85, 120-128.

Clark, A. H., Kavanagh, G. M., & Ross-Murphy, S. B. (2001). Globular protein gelation—theory and experiment. Food Hydrocolloids, 15(4-6), 383-400.

Comfort, S., & Howell, N. K. (2002). Gelation properties of soya and whey protein isolate mixtures. Food Hydrocolloids, 16(6), 661-672.

Costa, J. N., Nascimento, L. G. L., Leal, A. R., Danalache, F., Leite, B. S. M., Figueiredo, R. W., ... & de Sousa, P. H. M. (2021). Effect of agar and gellan gum on structured guava (Psidium guajava L.): Rheological behavior and gastrointestinal digestion in vitro. Food Bioscience, 42, 101165.

Damodaran, S. (2008). Amino acids, peptides and proteins. Fennema's food chemistry, 4, 217-329.

Grandviewresearch. Pea protein market size & share | industry growth report, 2019-2025. Retrieved June 10, 2022, from https://www.grandviewresearch.com/industry-analysis/pea-protein-market

Hermanto, S., Sholaikah, M. I., & Mulyani, S. S. (2016). Identification of Differentially Expressed Protein from Electrical Stunning of Broiler Chickens Meat Protein. Molekul, 11(1), 71-79.

Hubbe, M. A., Tayeb, P., Joyce, M., Tyagi, P., Kehoe, M., Dimic-Misic, K., & Pal, L. (2017). Rheology of nanocellulose-rich aqueous suspensions: a review. BioResources, 12(4), 9556-9661.

Ismail, B. P., Senaratne-Lenagala, L., Stube, A., & Brackenridge, A. (2020). Protein demand: Review of plant and animal proteins used in alternative protein product development and production. Animal Frontiers, 10(4), 53-63.

Kornet, R., Shek, C., Venema, P., van der Goot, A. J., Meinders, M., & van der Linden, E. (2021). Substitution of whey protein by pea protein is facilitated by specific fractionation routes. Food Hydrocolloids, 117, 106691.

Lagrange, V., Whitsett, D., & Burris, C. (2015). Global market for dairy proteins. Journal of food science, 80(S1), A16-A22.

Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2018). Pea protein isolates: Structure, extraction, and functionality. Food reviews international, 34(2), 126-147.

Liu, F., Li, M., Wang, Q., Yan, J., Han, S., Ma, C., ... & McClements, D. J. (2022). Future foods: Alternative proteins, food architecture, sustainable packaging, and precision nutrition. Critical Reviews in Food Science and Nutrition, 1-22.

Mession, J. L., Roustel, S., & Saurel, R. (2017). Interactions in casein micelle–Pea protein system (part I): Heat-induced denaturation and aggregation. Food Hydrocolloids, 67, 229-242.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., de Carvalho Teixeira, Á. V. N., Júnior, P. P. D. S. P., Vidigal, M. C. T. R., ... & de Carvalho, A. F. (2020). Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (Myrciaria cauliflora) extract. Food Hydrocolloids, 106, 105872.

Peters, J. P., Vergeldt, F. J., Boom, R. M., & van der Goot, A. J. (2017). Water-binding capacity of protein-rich particles and their pellets. Food Hydrocolloids, 65, 144-156.

Schäfer, J., Läufle, I., Schmidt, C., Atamer, Z., Nöbel, S., Sonne, A., ... & Hinrichs, J. (2018). The sol–gel transition temperature of skim milk concentrated by microfiltration as affected by pH and protein content. International journal of dairy technology, 71(3), 585-592.

Silva, J. V., Cochereau, R., Schmitt, C., Chassenieux, C., & Nicolai, T. (2019). Heat-induced gelation of mixtures of micellar caseins and plant proteins in aqueous solution. Food research international, 116, 1135-1143.

Silva, N. F. N., Casanova, F., Gaucheron, F., de Carvalho Teixeira, A. V. N., da Silva, G. M., Minim, L. A., & De Carvalho, A. F. (2018). Combined effect of transglutaminase and sodium citrate on the microstructure and rheological properties of acid milk gel. Food Hydrocolloids, 82, 304-311.

Sun, X. D., & Arntfield, S. D. (2012). Molecular forces involved in heat-induced pea protein gelation: Effects of various reagents on the rheological properties of salt-extracted pea protein gels. Food Hydrocolloids, 28(2), 325-332.

Tarhan, Ö., & Kaya, A. (2021). Investigation of the compositional and structural changes in the proteins of cow milk when processed to cheese. LWT, 151, 112102.

Tomé, A. S., Pires, C., Batista, I., Sousa, I., & Raymundo, A. (2015). Protein gels and emulsions from mixtures of Cape hake and pea proteins. Journal of the Science of Food and Agriculture, 95(2), 289-298.

Tunick, M. H. (2011). Small-strain dynamic rheology of food protein networks. Journal of Agricultural and Food Chemistry, 59(5), 1481-1486.

Varesano, A., Aluigi, A., Vineis, C., & Tonin, C. (2008). Study on the shear viscosity behavior of keratin/PEO blends for nanofibre electrospinning. Journal of Polymer Science Part B: Polymer Physics, 46(12), 1193-1201.

Walstra, P., Wouters, J. and Wageningen, T. G. (2005). Dairy Science and Technology. CRC Press, The Netherlands.

Wieruszeski, J.-M., Landrieu, I., Hanoulle, X., Lippens, G. (2006) ELISE NMR: Experimental liquid sealing of NMR samples. J. Magn. Reson. 181, 199-202.DOI: 10.1016/j.jmr.2006.04.007

Wong, D., Vasanthan, T., & Ozimek, L. (2013). Synergistic enhancement in the co-gelation of salt-soluble pea proteins and whey proteins. Food chemistry, 141(4), 3913-3919.

Wu, C., Wang, J., Ma, W., Cai, Y., & Wang, T. (2020). Preheat-stabilized pea proteins with anti-aggregation properties. International journal of biological macromolecules, 155, 1288-1295.

Preamble

In Part A of this chapter, the effect of thermal treatment on the interactions between CMs and pea proteins was evaluated. In Part B, the same mixture systems will be submitted to acidification, since acidification of proteins is present in the formulation of several food products. Thus, the impact of the mixtures on the gels network during and after acidification was evaluated.

Questions:

- Does the previous heat treatment improve the gelling properties of the mixed systems?
- Is there synergism in the complex modulus for the mixed systems?
- Does stronger interactions between caseins and pea proteins occurs?

The experimental part of this chapter was conducted at PIHM- UMET- INRAE (France)

PART B: Acid gelation of high-concentrated casein micelles: pea protein mixed systems

The content of this part is being prepared for submission in:

Food Hydrocolloids Journal

Luis Gustavo Lima Nascimento, Bertrand Doumert, Evandro Martins, Federico Casanova, Rodolphe Marie, Antônio Fernandes de Carvalho, Guillaume Delaplace, Paulo Peres de Sá Peixoto Junior.

Abstract

The increased demand for plant-based products brings a new challenge to the food industry. Proteins from soy, chickpea, and pea are highly demanded. However, they still present some drawbacks such as poor techno-functional properties and remarkable beany flavor that hamper their wider application. Contrarily, milk products such as yogurt and cheeses are highly consumed and accepted worldwide. Therefore, the association of plant proteins, such as pea with milk protein is an interesting strategy to incorporate more plant-based proteins into people's diets. However, the addition of another protein within the gel changes the gel formation and final structure. Thus, the aim of this study was to create mixed CMs: pea protein gel at high concentrations in four protein ratios, 80:20, 60:40, 40:60, 20:80 (CMs: pea) by acidification. The effect of a thermal treatment prior to gelation was also evaluated. The replacement of CMs for pea proteins disturbed the gel formation at the beginning of acidification, with an increased effect when the amount of pea protein increased. Despite this, the final gel elasticity was higher in the presence of pea proteins for the ratios 80:20 and 60:40, probably due to the formation of the pea network. Interestingly, the pea pure gel showed the highest G*, but the ratio of 20:80, which was the most elastic before acidification, presented the lowest complex modulus at the end of acidification. It is hypothesized that pea proteins can form a network when surrounded by CMs, however, CMs restrict the pea proteins aggregation. Therefore, the final characteristics of mixed gels can be tailored by changing the protein ratios and applying thermal treatment prior to acidification, which opens the possibility for the development of new food products.

1. Introduction

Currently, it is a tendency toward consuming more proteins of vegetable origin. This tendency is due to higher consumer awareness about the health aspects of food and the increase in environmental concerns (Sá, Moreno, & Carciofi, 2020). Indeed, the production of animal proteins demands more land, and water and contributes more to greenhouse effects than the production of plant proteins (Thavamani, Sferra, & Sankararaman, 2020). Also, with the increased population growth, alternative sources of proteins need to be explored to achieve the demanded protein production with the minimum environmental impact. Thus, the exploration of plant protein from soy, lentils, chickpea, and pea is an alternative (Aschemann-Witzel et al., 2021)

Pea stands out among the possible legume to be explored for protein production. Pea production increased in the last 30 years, being produced in several countries. Pea presents between 20 to 24% of proteins, with starch and fiber composing the rest of the seed composition. (Ge et al., 2020; Lu et al., 2020; Burger & Zhang, 2019). In absolute value, pea presents less protein than soybean, however, the pea proteins are non-allergenic and lysine-rich (Ge et al., 2020; Senthilkumaran et al., 2022). Its digestion generates peptides bioactive, which present antioxidant properties (Duffuler et al., 2022). The main drawbacks of pea proteins concern their techno-functional properties and sensory acceptance, which are lower in comparison to milk proteins.

Milk is worldwide consumed due to its proteins' techno-functional properties and pleasant taste, with a prediction of an increase of 58% in the demand in the next 30 years (Fasolin et al., 2019). Over the course of the years, the food industry has developed technology and process to transform milk into several food products and have used milk proteins as ingredients in several other food applications (Walstra, 2005). The proteins from milk can be divided into two groups, the serum proteins, and the caseins. The caseins are the most abundant, comprising around 80% of the total proteins. There are four fractions of caseins, α-s1, α-s2, β, and κ, that self-assemble mainly by hydrophobic and calcium phosphate bonds, forming structures called casein micelles (CMs) (De Kruif, 2014). In the food industry, the gels formed by CMs destabilization are the base for the production of yogurt and cheeses.

Thus, the creation of products combining milk and plant proteins is an interesting strategy (Guyomarc'h et al., 2021). Since it uses the established dairy market to increase the consumption and application of pea protein, mitigating their drawbacks, and at the same time, increasing the dairy product diversity. However, the replacement of a protein changes completely the rheological properties of the gels and the studies regarding the acidification properties of these mixed systems are scarce (Alves & Tavares, 2019). The majority of the studies focus on the impacts of the combination of CMs with pea proteins during the acidification were performed in low protein concentrations, without exploring the different protein ratio combinations (Ben-Harb et a., 2018; Message, Roustel, & Saurel, 2017[b]; Roesch et al., 2004).

Therefore, the aim of this study was to investigate the gelling properties of high concentrated mixed systems formed by CMs and pea protein. It was studied different protein ratios of CMs: pea protein (80:20, 60:40, 40:60, 20:80). The effect of thermal treatment prior to acidification on the gelling properties of the systems also was evaluated.

2. Material and Methods

2.1. Materials

The Casein micelle (CMs) powder Promilk 85B was kindly provided by Ingredia SA (Arras, France). The pea protein powder (F85S) was provided by Roquette SA (Lestrem, France). All the other used reagents were analytical grade.

2.2. Sample preparation

The CMs and pea protein powders were resuspended in deionized water at 12 and 16% total concentrations. The suspensions were stirred using a magnetic stirring at 25 ℃ overnight to allow complete protein rehydration. To prevent microbial growth, sodium azide at 0.003 % (w.w-1) was added to the suspensions. After complete rehydration, the suspensions were mixed in different CMs: pea protein ratios, which were 80:20 (80% CMs and 20% pea), 60:40 (60% CMs and 40% pea), 40:60 (40% CMs and 60% pea) and 20:80 (20% CMs and 80% pea). The pure systems of CMs (100:0) and pea protein (0:100) were also analyzed. After mixing, the samples were stirred for additional 3 hours at 25 ℃. Then, the mixed and pure systems were submitted to thermal treatment consisting of heating the protein suspensions at 85℃

for 1 hour using a water bath, followed by a fast temperature decrease until 30 ℃ using an ice bath.

Then, the samples were acidified with glucono-δ-lactone (GDL). Due to the differences in the buffer capacity of the proteins, GDL was added to the samples in different amounts aiming to standardize the time when all the systems reach pH 4.6 (supplemented material). The acidification occurred at 30 ℃ and the samples reached pH 4.6 after 4.5 hours.

2.3. Small-amplitude oscillatory shear (SAOS) test

The formation of the acid gels was followed using the small-amplitude oscillatory shear (SAOS) test. After the addition of GDL, the samples were placed in a controlled stress rheometer (ARES, TA Instruments, USA) equipped with a 40 mm steel cone-plate geometry with an angle of 2° in a gap of 0.57 mm. The edges of the geometry were covered with silicon oil to prevent water evaporation during the test hours. After setting the gap, the suspensions were allowed to equilibrate for 2 min, and a time sweep test at 0.1 % amplitude, 1 Hz, and at 30℃ was performed. The chosen parameters were within the linear viscoelastic region (LVR), previously determined by an amplitude sweep from 0.01 to 100% at 1Hz and 30℃ (data not shown). After the time sweep, a frequency sweep test was performed without disturbing the formed gels. The frequency varied from 0.1 to 50 Hz at 0.1% amplitude at the same test temperature.

2.4. Water holding capacity

The gels' water holding capacity (WHC) was performed as described by Nascimento et al. (2020). The gels were allowed to form in 15 mL centrifuge tubes at 30 ℃. After 4.5 hours, the gels were centrifugated using a g force of 4.000 *g* for 15 min at 30℃. After that, the released water was carefully removed and the protein pellet was weight. The WHC was calculated according to equation 1.

$$WHC(\%) = \frac{mi - ms}{mi}\ x\ 100 \quad (1)$$

Where mi is the suspension mass before centrifugation and ms is the mass of removed water.

2.5. Water dynamics and free phosphate measurements by NMR

Solid-state nuclear magnetic resonance spectroscopy (NMR) was applied in the course of the acidification to access the evolution in the water dynamics and free phosphate content. ^{1}H NMR and ^{31}P NMR spectra were performed on a 9.4T AVIII Bruker spectrometer using a 4 mm probe operating at a spinning frequency of 700 Hz and temperature of 30°C. The spectra were recorded at the Larmor frequency of 400.13 MHz. Adamantane was used as an external referenced compound (δ_{iso} = 1.76ppm). The recycle time was 2 s and the 90° pulse was 2.7 µs. The spin-lattice relaxation times T_1 were calculated by saturation-recovery pulse sequence (90°-τ-90°) using a delay time in the range of 0.1 to 20 s.

2.6. Confocal laser scanner microscopy (CLSM)

The protein suspensions were prepared as described in section 2.2. Then, rhodamine B at 85g.L^{-1} was added to each sample, which was mixed for 30 s to complete the rhodamine solubilization. Then, the required amount of GDL was added to the samples to decrease the samples' pH. The samples were poured into an 8- well chamber slide (Ibidi GmbH, Germany) and put carefully in a water bath at 30 ℃. After complete gel formation, the samples were visualized using an inverted microscope (Nikon Ti2) equipped with an sCMOS camera (Photometrics Prime95b).

2.7. Statistical analysis

The samples were compared by Analysis of variance (ANOVA). The effect of protein ratio was verified before and after heat treatment. Then, the effect of the heat treatment on each protein ratio was verified. When a significant difference ($p < 0.05$) was found, the Tukey HSD test with 5% significance was applied to differentiate means. All the experiments were performed, at least, three independent times, and the data was evaluated utilizing *SAS* software student edition.

3. Results and discussion

3.1. Time sweep analysis

The effect of acidification in the complex modulus (G*) of the samples is depicted in Figure 1. For pure systems, it can be observed a step increase in the G* modulus in the first minutes of acidification in both studied concentrations (Figure 1 A,

a and F, f). It is explained by the faster acidification rates after the addition of GDL. GDL is a weak acid that dissociates slowly until reaches a plateau. However, the pH drops faster at the beginning of acidification (Zouari et al., 2018). After the first 1.5 hours of acidification, a larger difference between the pure systems was observed. While pure pea protein gels kept a slower but crescent increase in G*, the pure CMs gel presented a decrease in G* around pH 5.2, followed by a posterior increase in G* starting at pH 4.9. The differences in the curve are linked to the differences in the protein structures and mechanisms of stabilization. In suspensions, the CMs are stabilized mainly by their k-casein fraction, which is present on the micelle surface, conferring steric and electrostatic stabilization to CMs (Dalgleish, 2011). When the pH goes down, the overall charges approach zero, and the k-casein shirk. Hence, the CMs approach each other, and aggregation takes place, the process goes on until the formation of a three-dimensional protein network. This phenomenon is perceived as the increase in the G*. Ouanezar, Guyomarc'h, and Bouchoux (2020) observed by AFM analysis the CMs morphology during acidification, where it presented a rough surface at pH 6.8, and a sphere-like form after the loose of the k-casein layer at pH 5.0. The posterior decrease is explained by internal rearrangements in the CMs trigged by solubilization of calcium phosphate at lower pHs (Andoyo et al., 2014). When all the colloidal calcium phosphate is completely solubilized, the G* starts to increase due to the reinforcement of the three-dimensional network. This phenomenon is better visualized by plotting the loss tangent values (inserted graph), where it is observed a peak at pH 4.9 for CMs gels. On the other hand, the loss tangent keeps slowly decreasing for 0:100 samples, without any peak, explained by the absence of calcium phosphate bonds in the pea proteins. In addition, the formation of pea acid gels is due to the decrease in the surface charge of the proteins, leading to aggregation and gel formation (Klost & Drusch, 2019). The final G* is another remarkable difference between the pure systems. The pea gel possessed a G* of 19,884 Pa at the end of acidification (pH 4.6), while pure CMs gel reached 1,425 Pa, more than 10 times lower at 12% total protein concentration. Ben-Harb et al. (2018) studying mixed milk: pea acid gels at 14.8 % of protein, found that pea gels presented elastic modulus, at least, eight times higher compared to milk gels.

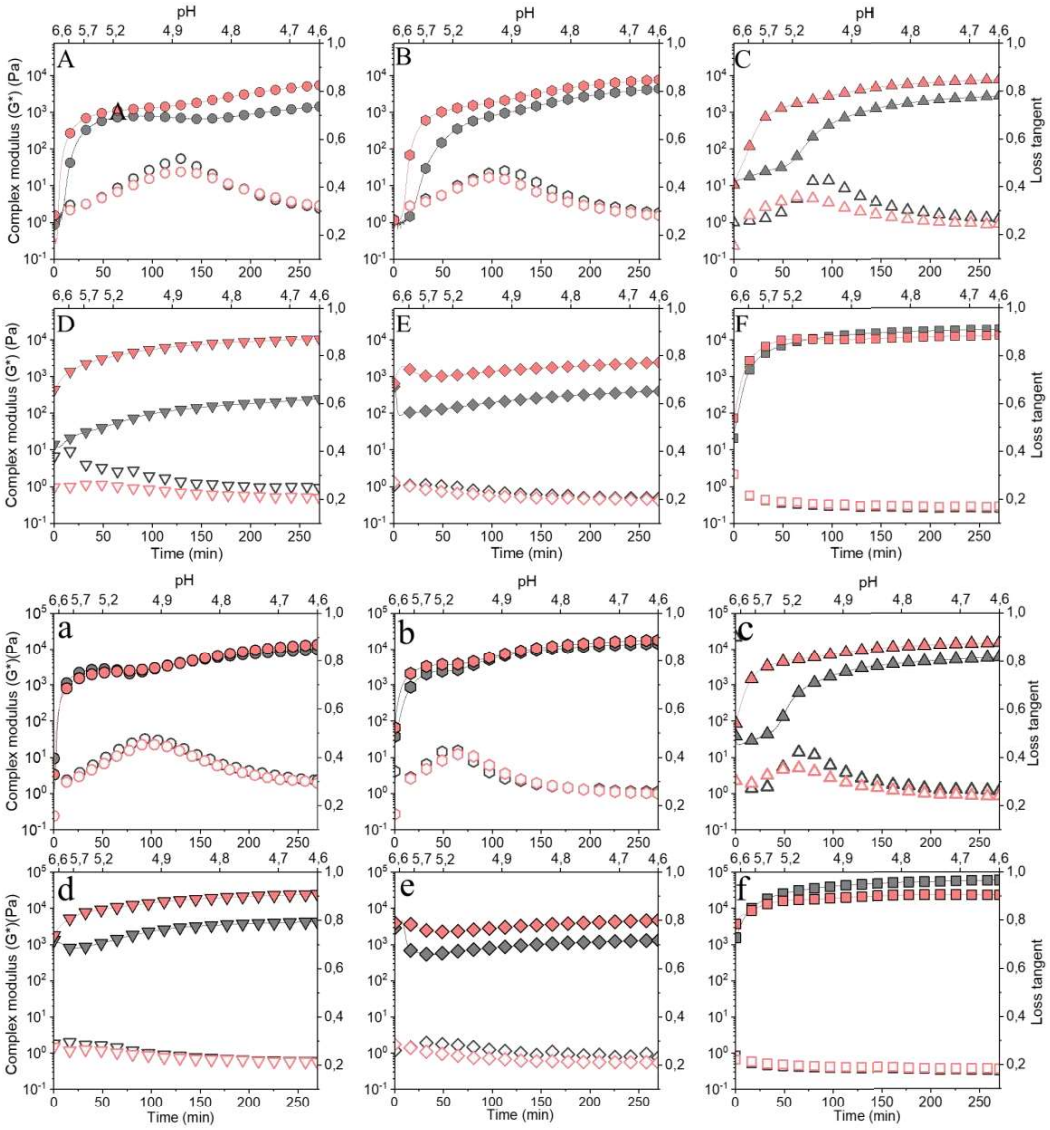


Figure 1. Complex modulus (full symbols) and loss tangent (empty symbols) as a function of acidification time for the samples without thermal treatment (gray symbols) and with thermal treatment (red symbols) prior to acidification. CMs:pea protein ratios (Aa) 100:0, (Bb) 80:20, (Cc) 60:40, (Dd) 40:60, (Ee) 20:80, (Ff) 0:100. The high-case letters stand for 12% and lower-case letters for 16% total protein concentration.

In the mixed systems, the gel formation depends on the protein ratio. Taking the 100:0 sample as a reference, when 20% of CMs are replaced by pea protein (80:20) retardation of G* increases occur (Figure 1B). After the first 30 min of acidification, 100:0 showed G* close to 295 Pa, while 80:20 showed G* around 19 Pa. However, at the end of the acidification process, the 80:20 gel possesses G* three times higher than 100:0. In resume, the replacement of CMs by a small amount of pea protein disturbs the gel formation at the beginning of acidification, but increased gel elasticity

after complete gel formation. Thus, interactions formed in the middle of acidification is responsible for the final gel elasticity. The inserted graphs show that in the samples at 80:20 ratio, the peak in loss tangent occurs 16 min before and in lower intensity compared to the 100:0 sample. The further increase in the pea protein content in relation to CMs promotes an even shorter loss tangent peak time and intensity (Figure 1C - E). Thus, the pea presence seems to alter the calcium phosphate balance, which can be due to a simple dilution effect caused by the gradual replacement of CMs by pea protein, or the pea protein interferes directly in the loose of calcium phosphate by the CMs. In the further replacement of 40% CMs for pea protein (60:40 sample) (Figure 1C), the same curve behavior was observed, however with more intensity, as can be shown by the G* after 60 min of acidification. At this time, 100:0, 80:20 and 60:40 samples presented 700, 300, and 48 Pa, respectively, showing an even higher disturbance effect in the gel formation caused by the presence of pea protein. However, the final G* for 60:40 was still higher compared to 100:0, 2440, and 1440 Pa, respectively. Thus, despite the impact of pea in the CMs gel, the formation of the pea network by itself is able to promote an increase in the final gel elasticity.

The dependence of the protein ratio in the systems is clearer when the pea protein becomes the major protein component. Until 60:40, despite the final G* being lower than G* for purely pea protein gel, it was higher compared to purely CMs protein gel. However, in 40:60 and 20:80 protein ratios, the final G* is lower compared to any pure system (Figure 1D and 1E). In the 40:60 ratio, the first G* point plotted in the graph is as high as in the 60:40 sample, however, when the pH keeps approaching the isoelectric point, the G* increases in much lower intensity compared to 60:40 or 80:20, and finished at 270 Pa. In the 20:80 sample, the first G* was higher than any other protein ratio or pure system. However, when the pH decreased, its G* also decreased, reaching a final G* of 250 Pa. Apparently, there is the formation of two independent networks as observed by other authors (Ben-Harb et al., 2018, Roesch, Juneja, Monagle, & Corredig, 2004), where a protein disturbs another's protein network formation. When CMs are the majority, the CMs gel disturbance is balanced by the formation of the pea network, resulting in an increase in G*. However, when the pea protein is present in a higher amount, it is hypothesized that CMs are homogeneously distributed within the pea protein network. Due to their thermodynamic incompatibility, there is a tendency for more pea-pea interactions at this ratio. As a consequence, the

formation of larger pea aggregates occurs, strengthening the system elasticity before acidification. However, during acidification, the CMs formed network restricts the interactions between the pea protein aggregates, which decreases the final gel elasticity.

The increase in the total concentration in the systems promoted a general increase in the G* for all studied ratios (Figure 1a – 1f). In the pure systems, the final G* was increased proximately 6.5 and 3 times for CMs and pea protein, respectively. The lower increase in pure pea gel is probably due to system saturation. The proteins are already very close and the network is highly connected, thus the addition of more pea protein caused less effect comparing 12 to 16% total concentration. Regarding the G* curves, the profile observed for samples containing 16% protein is very similar to 12% protein. The replacement of CMs for pea protein showed the same tendency compared to 12% total concentration, *i.e.*, a disturbance of the initial aggregation stages in the CMs gelation. However, the 80:20 ratio is the only mixed gel that showed the final G* higher than the G* of pure CMs. Thus, even at 60:40 where the CMs were the majority protein present, the final G* was lower. It shows that the gel behavior depends not only on the ratio of the proteins but also on their concentrations.

The temperature is a useful tool to promote modification in the proteins and sometimes promotes interactions between two different proteins, as in the case of β-lactoglobulin and κ- casein (Andoyo, 2014). The formation of disulfide bond previous to acidification between β- lactoglobulin and κ- casein promoted by thermal treatment is applied in the yogurt production technology to increase final gel elasticity and decrease syneresis (Walstra, 2006). Despite having the required amino acids, such interactions do not take place between CMs and pea proteins (Mession, Roustel, Saurel, 2017). However, the thermal treatment still impacts the pea protein structures and pea-pea interactions. Thus, the effect of heat treatment in the suspensions prior to acidification was evaluated (Figure 1). The effect of thermal treatment was similar in both protein concentrations studied. For CMs pure gel, it was observed a slight increase in G* in the sample treated thermally, probably delivered by the denaturation of the small amount of serum protein present in the powder (Alting, Hamer, de Kruif, & Visschers, 2000). Interestingly, the pea protein gel with previous thermal treatment showed a final G* slight lower compared to pea gels without thermal treatment (Figures 1F and 1f). As pointed out previously, the pea proteins in both 12 and 16%

concentrations are very close to each other. The heat treatment caused protein denaturation and more interactions were formed, as can be evidenced by the higher G* at time zero. These new interactions may reduce the protein's freedom to rearrange, thus, during the acidification, fewer connections were formed, resulting in a less rigid gel.

In a mixed system, besides the increase in the final G*, the thermal treatment reduced the gel formation time mainly in the 60:40 protein ratio. Before thermal treatment, after 30 min of acidification, the G* was 40 Pa for the 60:40 gel. When the suspension was heated prior to acidification, the G* increased to 3 KPa at the same acidification time. Thus, thermal treatment is a useful tool to increase gelation properties of mixed systems, even if no strong interaction between the proteins of different sources takes place. This phenomenon is explained by the partial pea protein aggregation occurring during heat treatment, which gives additional protein connections to the formed network (Shand, Ya, Pietrasik, & Wanasundara, 2007).

3.2. Frequency sweep

The gels frequency dependence varies according to the nature of the network interactions. Gels with simply entangled polymer networks present higher frequency dependence when compared to gels with covalent bonds within the network (Tunick, 2011). A frequency sweep test was performed to determine the sample's frequency dependence and construct a better understanding of the protein interactions. The slope of the G' curves in the double logarithmic plot was calculated and is depicted in Figure 2. As the samples have a predominant gel nature, *i.e.* a higher G' compared to G'', only the G' was used to characterize the samples. During the entire frequency sweep no crossover of G' and G'' was observed (data not shown), showing the solid-like nature of the sample even in small observation times (50Hz) (Stojkov, Niyazov, Picchioni, & Bose, 2021). CMs and pea protein pure gels presented the highest and lowest frequency dependence, respectively. The mixed systems decreased the frequency dependence as the amount of pea protein increased in both studied protein concentrations. The decrease in the frequency dependence seems to be caused only by a simple additive effect, which evidences the absence of newly formed strong interactions between the CMs and pea proteins and the formation of separated protein networks. The application of thermal treatment did not cause a significant difference in

the frequency dependence in the samples, probably due to the absence of new stronger interaction within the protein matrix.

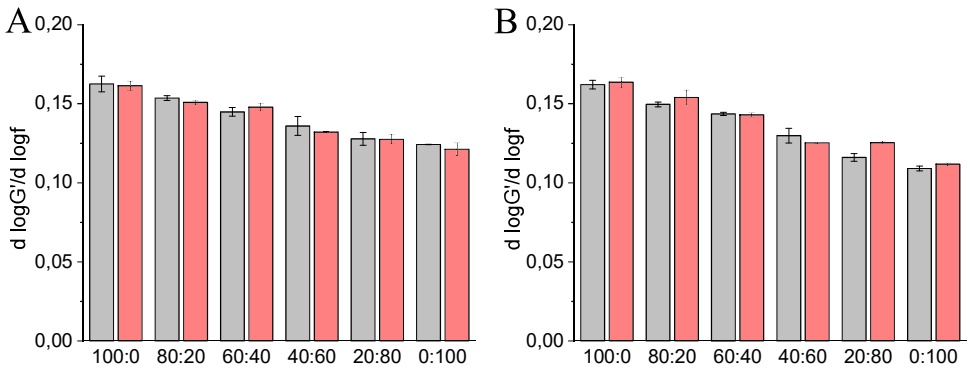


Figure 2. The slope of the double logarithmic curve in the frequency sweep test at 12% total protein concentration (A) and 16% total protein concentration (B), untreated (gray) or thermally treated (red) prior to acidification.

3.3. Water holding capacity (WHC)

The WHC of a protein gel is usually linked to the gel's microstructure. Stronger and more compact gels present high WHC, once the water diffusion becomes harder in this kind of structure (Cortez-Trejo, Gaytán-Martínez, Reyes-Vega, & Mendoza, 2021). In the pure systems, the CMs and pea gels presented 82.6 ± 1.9 % and 98.3 ± 0.8 %, respectively at 12% total protein concentration (Figure 4A). These results corroborate with the frequency sweep test, which indicates that pea protein gels possess a stronger interconnected network compared to CMs gels. As observed in the frequency dependence test, the progressive increase in the pea protein content increased the WHC of the systems, showing the role of the pea proteins in the gel network. In the 16% gels (Figure 4B), the WHC increased, however, little differences can be noted comparing the protein ratios. It is explained by the saturation of the gels caused by the high protein concentrations.

The application of heat treatment prior to acidification affected differently the WHC properties of the systems. While a WHC increase was observed for the systems where pea proteins were present, the opposite behavior was observed for CMs gels. It was probably caused by the faster protein rearrangement at the beginning of acidification in the 100:0 sample heat treated. The same phenomenon was observed by Lucey, Munro, & Singh (1998), studying acid milk formed by means of GDL

acidification. Despite being a practical method to infer the gel's microstructure. The WHC test presents low sensibility and evaluates the water-structure relation only after the complete gel formation. Thus, solid-state NMR was performed during the course of acidification to follow the changes in the water dynamics during the entire gelation process.

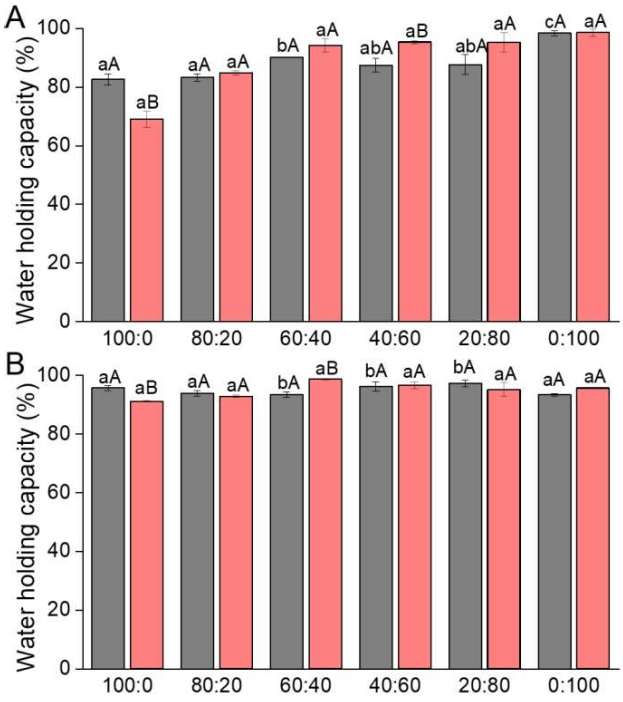


Figure 4. Water holding capacity at 12% total protein concentration (A) and 16% total protein concentration (B), untreated (gray) or thermally treated (red) prior to acidification.

3.4. Water dynamics measurement

The water dynamics for 16% of total protein gels during the acidification were determined by solid-state ^{1}H NMR (Figure 5A and 5B). The pure systems presented opposite behavior, being the final spin-lattice relaxation time T_1 for pure pea gels 73 % lower when compared to CMs gels. These observations are in accordance with WHC measurements. The higher water relaxation times imply in high water dynamics within the gel structure, which is related to a coarser microstructure with larger pore sizes for the CMs gels. On the other hand, the low water mobility in the pea gels indicates a more homogeneous microstructure with small pore sizes. Besides the final T_1, the acidification curve shape depends on the major protein fraction present. In the CMs gels, at the beginning of acidification, was observed an increase in T_1, *i.e.* an increase

in the water mobility until a peak at approximately pH 4.8. After that, it was observed a decrease in T_1 values. For the pure pea gels, the T_1 kept decreasing during the entire course of acidification. The initial increase in T_1 in 100:0 samples can be explained by the CMs' behavior in the course of acidification. As observed in the time sweep analysis, the solubilization of calcium phosphate occurs during acidification. It has been observed a positive correlation between calcium phosphate solubilization and the increase in water mobility, measured in terms of T_2 (Mariette & Marchal, 1996). After that, the CMs aggregate and form a tridimensional network, entrapping water, hence decreasing T_1. The pea proteins do not present the same salt composition and behavior, for this reason, the initial increase in T_1 was not observed. The decrease in pH decreased the overall charge of the proteins, which approach their isoelectric point causing aggregation with posterior network formation and decreasing the water dynamics.

In the mixed system, the T_1 at the beginning of acidification followed the behavior of the prominent protein fraction. Thus, in 80:20 and 60:40 gels it was visualized a slight decrease of T_1 in the first minutes of acidification, followed by its decrease when the acidification proceeds. In 40:60 and 20:80 samples, the curve profile is closer to pea protein gels, and the T_1 decrease during the entire acidification process. These results were also in agreement with the WHC test. Interestingly, the replacement of CMs for pea decreases the final T_1 in a no-linear way. For example, when 20% of CMs were replaced by pea protein a high drop in T_1 was observed, and the further increment of pea protein kept decreasing T_1, but to a lower degree.

The application of thermal treatment before suspension acidification also was evaluated by means of T_1 (Figure 5B). In general, the thermal treatment promoted a decrease in the T_1 for most studied samples, with more influence in the systems containing more pea protein. However, the opposite behavior was observed for CMs gels, which presented an increase in the final T_1 of CMs gels, indicating higher water mobility. These results are in agreement with the WHC, with heated casein micelles presenting decreased WHC. For the other gels, besides the lower final T_1, the heat treatment increased the rate of T_1 drop, which indicates a faster network formation, aggreing with the G* results. For 20:80 and 0:100 samples, the T_1 values kept stable after the first 2 hours of acidification, showing that the additional interactions caused by thermal treatment in the pea protein led to less protein rearranging during

acidification. These results correlate with the founds in WHC and G*, where only slight differences were observed before and after heat treatment for the samples containing a high amount of pea.

As observed in G* curves, the CMs' loss of calcium phosphate has an important role during CMs gels and the presence of pea proteins seems to anticipate this process. To better understand the relation between calcium phosphate distribution within the mixed systems, the free phosphate was evaluated at the beginning and at the end of acidification by NMR and it is discussed in the next section.

3.5. Free phosphate measurements

The ^{31}P NMR peak corresponding to free phosphate found in the samples water phase at the beginning and the end of acidification is shown in Figures 5C and 5D. The pure systems (only casein or only pea) present different free phosphate content at the beginning of acidification, being the amount of free phosphate in the pea protein pure gels is 33 times lower compared to CMs pure gel. Also, the pure systems present remarkable differences comparing the beginning with the end of the acidification process. In CMs pure gels, an increase of 57% in the amount of free phosphate was observed, corresponding mainly to the liberation of CM colloidal phosphate, while free phosphate content did not change for pure pea gels. The results agreed with the observations in G* curves, where calcium solubilization occurs during sample acidification for the CMs system. Concerning the mixed systems, the replacement of 20% of CMs for pea protein (80:20 sample) led to a decrease of 71% in the free phosphate content when compared to CMs pure gels, while the further increase of pea protein content, sample 60:40, only decrease 27% in free-phosphate content comparing to 80:20 sample. This non-linear effect of pea protein ratio increase is the same observed in section 3.5. It demonstrates the chelating effect caused by pea proteins, being a small quantity of pea inside a gel enough to bind a high quantity of phosphate.

The amount of free phosphate decreased for all samples, pure or in combination, after heat treatment, with a more intense effect in the CMs pure system (Figure 5D). However, the ranking between the beginning and end of the acidification kept unchangeable whatever before or after heat treatment, *i.e.*, it was observed an increase in free phosphate content for CMs gels, and no effect in pea systems was

reported observed. The decrease is explained by the reduction in solubility of phosphate with the application of heat treatment. The prolonged heat treatment leads to irreversible precipitation of the salts, which interacts with the CMs surface (Broyard & Gaucheron, 2015).

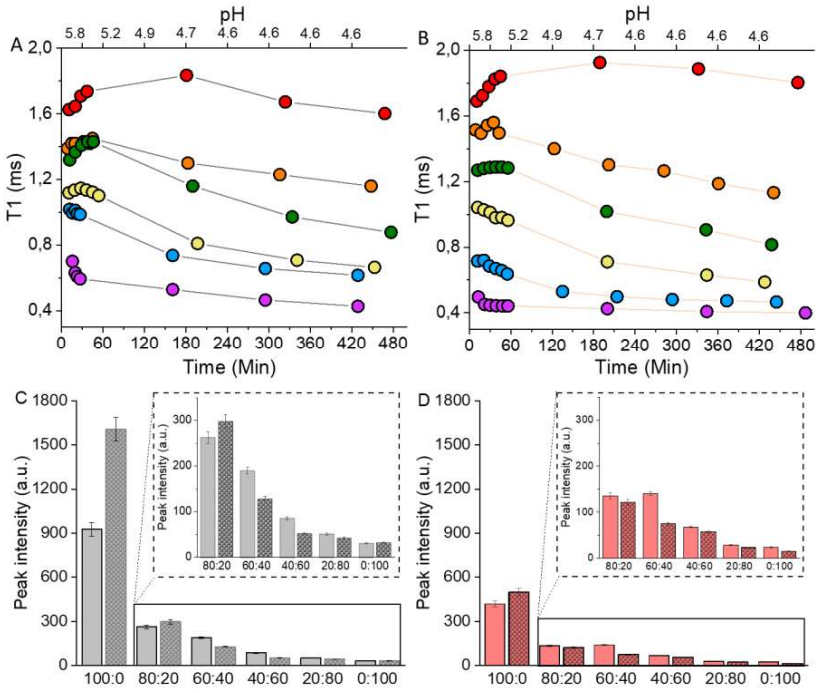


Figure 5. Spin-lattice relaxation times T_1 of water as function of acidification time for CMs: pea ratios at 100:0 (red), 80:20 (orange), 60:40 (green), 40:60 (yellow), 20:80 (blue), 0:100 (purple) samples at 16% protein content. (A) without heat treatment and (B) with heat treatment. Free phosphate at the beginning, (smooth bars), and at the end, (rough bars), of acidification for 16 % protein content without (C) and with heat treatment (D).

3.6. Confocal laser scanner microscopy (CLSM)

The gels' microstructure is shown in Figure 6. As the fluorophore attaches equally to both proteins it is impossible to differentiate them in a mixed system. However, the image of the mixed systems summed with the previous results is a valuable tool to understand the protein's interactions and arrangements. It is clear the differences between the pure gels. The CMs gels presented a microstructure similar to that found in the literature (Nascimento et al., 2020) with pores on the micro-scale. The CMs presented a coarser microstructure with larger pore sizes compared to pea gels. These results corroborate with the observations made in the WHC, final G*, and NMR. The bigger pore sizes facilitate the water dynamics, which is perceived physically as

reduced WHC. At the same time, the water molecules have more space to freely move in the CMs gels, which explains the higher water dynamics found in the NMR results. And the lower connection degree within the protein network is responsible for the reduced final G* compared to the pure pea gels.

Compering 80:20 and 100:0 gels, it is clear the inhomogeneity created in the gel microstructure promoted by pea protein presence, increasing the pore sizes in relation to the pure CMs gels. Nevertheless, the same CMs gel organization can still be visualized, however, with the presence of denser aggregates probably composed of pea protein, which were the responsible for the higher final G* compared to pure CMs system. Comparing the 20:80 ratio with the systems composed only of pea protein, it was observed a remarkable difference in the microstructure. The 20:80 sample microstructure is inhomogeneous with larger empty spaces. At the same time, the proteins are very closely packed and with the same nano-scale pore presented in the 0:100 sample. In the intermediated systems, 60:40 and 40:60, the microstructure seems to be a combination of the observed in pure CMs and pea gels. They present small protein aggregates uniformly distributed, more connected than CMs gels, and less connected than pure pea gels. Thus, the formation of two distinct networks is hypothesized. The application of thermal treatment increased the size of the protein aggregates as can be seen in Figure 5 A' – E', with no remarkable difference in pure pea proteins. The CMs gel microstructure also was affected by heat treatment, probably due to the small amount of whey proteins present in the casein powder used. The results of microstructure agree with the found in the rheological analysis.

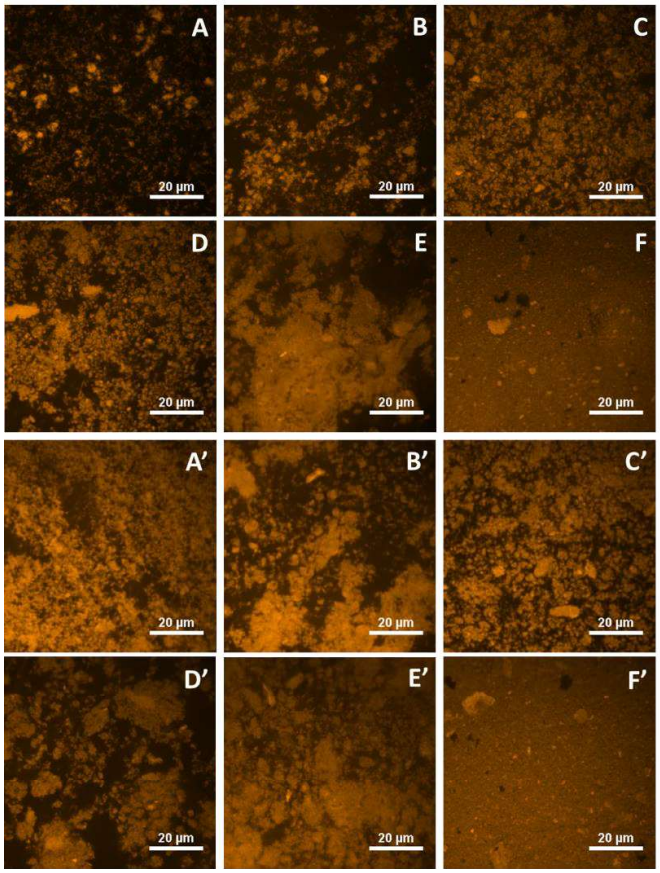


Figure 6. Microstructure of protein gels untreated (lower-case letters) and treated thermally (high-case letter) at 16% protein content. 100:0 (A, A'), 80:20 (B, B'), 60:40 (C, C'), 40:60 (D, D'), 20:80 (E, E'), 0:100 (F, F').

4. Conclusion

The applied techniques allowed a robust view of systems acidification, from a small scale, in the evaluation of water dynamics and free phosphate contents, to a macro scale in rheological experiments. In the gels with a higher amount of casein (80:20 and 60:40), the gradual replacement of CMs for pea proteins retarded the strengthening of the network. However, in the course of acidification, the gelation of pea proteins increased the gels' elasticity, generating more elastic systems compared with pure CMs gels. On the other hand, in the gels where pea proteins were the major component (40:60 and 20:80), the final gels presented lower elasticity compared to any pure protein gel. The thermal treatment prior to acidification increased the gels' elasticity, with stronger impacts in gels with a higher amount of pea proteins. Showing that the reinforcement of the network is caused by the pea-pea protein interactions.

Therefore, the major responsible for modulating the gel stiffness is the distribution of the proteins within the gel matrix and pea-pea interactions.

5. **Reference**

Alting, A. C., Hamer, R. J., de Kruif, C. G., & Visschers, R. W. (2000). Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. Journal of Agricultural and Food Chemistry, 48(10), 5001-5007.

Alves, A. C., & Tavares, G. M. (2019). Mixing animal and plant proteins: Is this a way to improve protein techno-functionalities?. Food Hydrocolloids, 97, 105171.

Andoyo, R., Guyomarc'h, F., Cauty, C., & Famelart, M. H. (2014). Model mixtures evidence the respective roles of whey protein particles and casein micelles during acid gelation. Food Hydrocolloids, 37, 203-212.

Aschemann-Witzel, J., Gantriis, R. F., Fraga, P., & Perez-Cueto, F. J. (2021). Plant-based food and protein trend from a business perspective: markets, consumers, and the challenges and opportunities in the future. Critical Reviews in Food Science and Nutrition, 61(18), 3119-3128.

Ben-Harb, S., Panouille, M., Huc-Mathis, D., Moulin, G., Saint-Eve, A., Irlinger, F., ... & Souchon, I. (2018). The rheological and microstructural properties of pea, milk, mixed pea/milk gels and gelled emulsions designed by thermal, acid, and enzyme treatments. Food Hydrocolloids, 77, 75-84.

Burger, T. G., & Zhang, Y. (2019). Recent progress in the utilization of pea protein as an emulsifier for food applications. Trends in Food Science & Technology, 86, 25-33.

Cortez-Trejo, M. C., Gaytán-Martínez, M., Reyes-Vega, M. L., & Mendoza, S. (2021). Protein-gum-based gels: Effect of gum addition on microstructure, rheological properties, and water retention capacity. Trends in Food Science & Technology, 116, 303-317.

Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: A scientific and technological challenge. Dairy science & technology, 95(6), 831-862.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. Soft matter, 7(6), 2265-2272.

Duffuler, P., Bhullar, K. S., de Campos Zani, S. C., & Wu, J. (2022). Bioactive Peptides: From Basic Research to Clinical Trials and Commercialization. Journal of Agricultural and Food Chemistry, 70(12), 3585-3595.

De Kruif, C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V. (2012). Casein micelles and their internal structure. Advances in colloid and interface science, 171, 36-52.

De Kruif, C. G. (2014). The structure of casein micelles: A review of small-angle scattering data. Journal of Applied Crystallography, 47(5), 1479-1489.

Fasolin, L. H., Pereira, R. N., Pinheiro, A. C., Martins, J. T., Andrade, C. C. P., Ramos, O. L., & Vicente, A. A. (2019). Emergent food proteins–Towards sustainability, health and innovation. Food Research International, 125, 108586.

Ge, J., Sun, C. X., Corke, H., Gul, K., Gan, R. Y., & Fang, Y. (2020). The health benefits, functional properties, modifications, and applications of pea (Pisum sativum L.) protein: Current status, challenges, and perspectives. Comprehensive Reviews in Food Science and Food Safety, 19(4), 1835-1876.

Guyomarc'h, F., Arvisenet, G., Bouhallab, S., Canon, F., Deutsch, S. M., Drigon, V., ... & Gagnaire, V. (2021). Mixing milk, egg and plant resources to obtain safe and tasty foods with environmental and health benefits. Trends in Food Science & Technology, 108, 119-132.

Klost, M., & Drusch, S. (2019). Structure formation and rheological properties of pea protein-based gels. Food Hydrocolloids, 94, 622-630.

Liu, Q., Li, Z., Pan, X., Dai, Y., Hou, H., Wang, W., ... & Dong, H. (2021). Effect of grinding on the structure of pea protein isolate and the rheological properties of its acid-induced gels. International Journal of Food Science & Technology, 56(7), 3455-3462.

Lu, Z. X., He, J. F., Zhang, Y. C., & Bing, D. J. (2020). Composition, physicochemical properties of pea protein and its application in functional foods. Critical reviews in food science and nutrition, 60(15), 2593-2605.

Lucey, J. A., Munro, P. A., & Singh, H. (1998). Whey separation in acid skim milk gels made with glucono-δ-lactone: Effects of heat treatment and gelation temperature. Journal of Texture Studies, 29(4), 413-426.

Mariette, F., & Marchal, P. (1996). NMR relaxation studies of dairy processes.

Mession, J. L., Roustel, S., & Saurel, R. (2017). Interactions in casein micelle–Pea protein system (part I): Heat-induced denaturation and aggregation. Food Hydrocolloids, 67, 229-242.

Mession, J. L., Roustel, S., & Saurel, R. (2017)[b]. Interactions in casein micelle-Pea protein system (Part II): Mixture acid gelation with glucono-δ-lactone. Food Hydrocolloids, 73, 344-357.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., de Carvalho Teixeira, Á. V. N., Júnior, P. P. D. S. P., Vidigal, M. C. T. R., ... & de Carvalho, A. F. (2020). Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (Myrciaria cauliflora) extract. Food Hydrocolloids, 106, 105872.

Ouanezar, M., Guyomarc'h, F., & Bouchoux, A. (2012). AFM imaging of milk casein micelles: Evidence for structural rearrangement upon acidification. Langmuir, 28(11), 4915-4919.

Peleg, M. (2017). The basics of solid foods rheology. Food texture, 3-33.

Roesch, R., Juneja, M., Monagle, C., & Corredig, M. (2004). Aggregation of soy/milk mixes during acidification. Food Research International, 37(3), 209-215.

Sá, A. G. A., Moreno, Y. M. F., & Carciofi, B. A. M. (2020). Plant proteins as high-quality nutritional source for human diet. Trends in Food Science & Technology, 97, 170-184.

Senthilkumaran, A., Babaei-Ghazvini, A., Nickerson, M. T., & Acharya, B. (2022). Comparison of protein content, availability, and different properties of plant protein sources with their application in packaging. Polymers, 14(5), 1065.

Shand, P. J., Ya, H., Pietrasik, Z., & Wanasundara, P. K. J. P. D. (2007). Physicochemical and textural properties of heat-induced pea protein isolate gels. Food Chemistry, 102(4), 1119-1130.

Stojkov, G., Niyazov, Z., Picchioni, F., & Bose, R. K. (2021). Relationship between structure and rheology of hydrogels for various applications. Gels, 7(4), 255.

Thavamani, A., Sferra, T. J., & Sankararaman, S. (2020). Meet the meat alternatives: The value of alternative protein sources. Current nutrition reports, 9(4), 346-355.

Walstra, P., Wouters, J. and Wageningen, T. G. (2005). Dairy Science and Technology. CRC Press, The Netherlands.

Zouari, A., Marchesseau, S., Chevalier-Lucia, D., Raffard, G., Ayadi, M. A., & Picart-Palmade, L. (2018). Acid gelation of raw and reconstituted spray-dried dromedary milk: A dynamic approach of gel structuring. International dairy journal, 81, 95-103.

CHAPTER IV: HIGH-INTENSITY ULTRASOUNDS TO IMPROVE GELLING PROPERTIES OF CMS: PEA MIXED SYSTEMS

Preamble

In the last chapter, the mixture systems were submitted to gelation, using heat treatment to improve the gelling properties. In this chapter, the use of emergent processing technology, high-intensity ultrasounds (HIUS), was applied to improve the final gels' elasticity. This technique has shown promising results in protein pure protein systems; however, it has never been applied in mixed casein micelles: pea proteins.

Questions:

- Is the HIUS able to improve CMs: pea interactions?
- Does the gelation properties of the systems are improved after HIUS treatment?
- Does the order of the HIUS treatment interfere in the system gelation?

The experimental part of this chapter was conducted at the Food Processing group, at the Technical University of Denmark (Denmark)

High-intensity ultrasound treatment on casein: pea mixed systems: effect on gelling properties

The content of this chapter is being prepared for submission in:

Food Chemistry Journal

Luis Gustavo Lima Nascimento, Heidi Olander Petersen, Rodolphe Marie, Mohammed Amin Mohammadifar, Paulo Peres de Sá Peixoto Júnior, Guillaume Delaplace, Antônio Fernandes de Carvalho, Federico Casanova

Abstract

The mix of pea with milk proteins is a strategy to increase the consumption of plant proteins. Since these proteins still possess low sensory acceptance and reduced techno-functional properties when compared to milk proteins. However, in general, these mixtures have lower gelling properties than pure systems. Therefore, this study aimed to investigate the suitability of the application of high-intensity ultrasounds to improve the gelling properties of mixed protein systems formed by pea and casein micelles (CMs). In the suspensions, the ultrasound treatment produced an increase in solubility, surface hydrophobicity, and a decrease in the samples' viscosity, with more remarkable differences in protein blends where pea protein was the major component. However, the replacement of 20% of CMs for pea proteins highly affected the gel elasticity. Hence, the creation of smaller and more hydrophobic building blocks before acidification due to the HIUS treatment increased the elasticity of the gels up to 10 times. Therefore, high-intensity ultrasounds are a suitable green technique to increase the gelling properties of CMs: pea systems.

1. Introduction

The expected population growth of 2 billion people in the next 30 years will increase worldwide the demand for proteins (United nations, 2015). Only for the proteins from animal sources such as milk and meat, the demand is expected to increase by 58 and 73%, respectively (Fasolin et al., 2019). However, the planet's limited resources combined with climate changes require more sustainable protein production. Thus, the wider utilization of proteins from vegetable origins suck as soybean, lentils, chickpea, and pea can diversify the protein production and make it more sustainable (Aiking & de Boer, 2020).

Pea is one of the largest legumes produced worldwide, being produced in over 84 countries. It is the most produced pulse with a production estimated at 35 million meters tons per year, comprising 36% of total pulse production (Lu et al., 2020; Burger & Zhang, 2019). Pea presents similar protein content compared to soy however, pea proteins stand out due to their non-allergenic status and a good balance of essential amino acids, being rich in lysine. The main drawback of pea proteins is their reduced techno-functional properties and their beany flavor when compared to milk proteins (Ge et al., 2020).

Milk is worldwide produced and consumed. Besides the fluid milk, the formulation of different dairy products is possible due to milk proteins' techno-functional properties (Walstra, 2006). Milk presents an average of 3.2% of proteins, being the 80% caseins, and 20% serum proteins. There are four fractions of caseins, α-s1, α-s2, β, and κ that self-assemble in supramolecular structures called casein micelles (CMs) (Goulding, Fox, & O'Mahony, 2020). The gelation of milk is usually achieved by the destabilization of the κ-casein, the fraction that confers electrostatic and steric repulsion for the CMs. Thus, the fabrication of milk products such as yogurt and cheese depends on the gelling properties of CMs (Li & Zhao, 2019).

In this way, the creation of mixed systems, which can incorporate two complementary protein sources is gained attention. The combination of milk proteins with pea proteins can minimize the undesirable effects of pea proteins, and at the same time increase the versatility of the dairy products (Guyomarc'h, 2021). However, a better comprehension of the protein behavior in these systems must be gained. The few studies focusing on the acidification of mixed CMs: pea protein resulted in gels

with reduced stiffness when compared to pure systems (Ben-Harb et al., 2018). The reduction in the gel elasticity is caused by the competitive behavior of the proteins during the gelation, and frequently two distinct protein networks are formed (Roesch et al., 2004). Thus, the application of pre-treatments such as sonication, prior to gelation may be an alternative to improve the gelling properties of mixed systems.

The high-intensity ultrasound (HIUS) treatment consists of the application of acoustic waves in series of compression and rarefaction cycles with frequencies higher than 20 kHz. At sufficient energy input, the formation of small gas bubbles that eventually violently implode occurs, generating a punctual increase in temperature, pressure, and shear forces (Chemat & Khan, 2011). In protein suspensions, HIUS usually impacts their tertiary and secondary structures, generating a decrease in the size of the aggregate, and an increase in the hydrophobicity and solubility, which impacts directly the techno-functional properties such as gelling, foaming, and emulsion stability (Gallo, Ferrara, & Naviglio, 2018). The use of HIUS shows promising results in increasing the gelling properties of a diversity of proteins such as sunflower, soy, chickpea, lentils, and pea (Bernardi et al., 2021).

Despite the promising results in pure suspensions, the application of HIUS has not been studied in mixed suspensions of milk and plant proteins. Thus, the aim of this study was to apply HIUS in mixed suspensions composed of CMs and pea proteins and evaluate the effect of the modifications in the improvement of the gelling properties.

2. Materials and Methods

2.1. Materials

Casein micelle powder (Promilk 85B) was provided by Ingredia S.A (Arras, France). The pea protein powder (F85F) was provided by Roquette (Lestrem, France). No additional purification step was applied to the protein powders. All the other reagents used in this study were of analytical grade.

2.2. Sample preparation

The protein powders were separated rehydrated in deionized water at 8% (w/w) and stirred overnight at 25 ℃. To prevent microbial growth, sodium azide at 0.003% (w/w) was added. Then two routes of ultrasound processing were applied. In route 1,

the pure protein dispersions were mixed in three ratios of casein micelles (CMs): pea protein (80:20, 50:50, 20:80). The blends were mixed for additional 2 hours under the same stirring conditions used to hydrate the powders. After that, the samples were submitted to high-intensity ultrasound treatment (HIUS) described in detail in the next section. In route 2, the CMs and pea protein suspensions were ultrasonicated individually, in the same conditions used in route 1, and then mixed in the three protein ratios 80:20, 50:50, and 20:80. The pure systems before and after ultrasound were also analyzed.

2.3. High-intensity ultrasound treatment (HIUS)

The samples (60 mL) were put in a beaker which was inserted in an ice bath to keep the sample's temperature always below 35℃, therefore, avoiding any temperature effects. HIUS treatment was performed using a sonifier apparatus operating at a constant frequency of 20 kHz (Emerson, St. Louis, MO, USA), using the same parameters as Kumar et al. (2022). Briefly, the ultrasound probe was inserted in the center of the beaker and at 2 cm distance from its bottom. Then, 495 W of power was applied for 15 min in pulsed mode with 5 s ON and 5 s OFF. The real energy input was calculated based on the calorimetry method described by Arzeni et al. (2012). The samples' temperature in the first 30 seconds of ultrasound treatment was recorded using a thermocouple (Pico Technology, St Neots, UK). Then, equation 1 was used to determine the acoustic power (P) in Watts (W) applied in the sample, and equation 2 was applied to calculate the acoustic intensity (I) in W/cm^2. The power and intensities for all protein suspensions are shown in the supplemented material.

$$P = mCp\frac{dT}{dt} \quad (1)$$

$$I = \frac{P}{Sa} \quad (2)$$

Where m (g) is the mass of the treated suspension, Cp (J/g °C) is the specific heat of the suspension, dT/dt is the change in temperature as a function of time, and Sa (cm^2) is the area of the emitting ultrasound surface.

The applied acoustic power and intensities are shown in Table 1 (supplemented material).

2.4. Suspension analysis

2.4.1. Viscosity measurement

The samples were put in a stress-controlled rheometer (Discovery HR-2, TA Instruments, USA) equipped with a concentric cylinder geometry. Then, the shear rate was varied in three cycles from 10 to 320 s^{-1} to check for thixotropy. Being, the first and last circles were ascendant and the second descendent. The analysis was carried out at 30℃. The apparent viscosity at 200 s^{-1} of the third curve was chosen to compare between samples.

2.4.2. Solubility

The solubility assay was performed according to Silventoinen and Sozer (2020), with slight modifications. The samples at the work concentration were centrifugated at 10,000 g for 15 min at 4℃. Then, the supernatant was withdrawn. The protein dispersion before centrifugation and the supernatant were analyzed for the protein content using the Dumas method using 6.25 as the nitrogen conversion factor. The equipment was previously calibrated using aspartic acid and wheat flour. The solubility was calculated according to equation 3.

$$Solubility\ (\%) = \frac{Ps}{Pt}\ x\ 100 \qquad (3)$$

Where Ps is the protein found in the supernatant and Pt is the total protein before centrifugation.

2.4.3. Particle size and ζ- potential

Dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) was used to determine particle size and ζ-potential of particles according to Nascimento et al. (2020). Briefly, the samples were diluted 100 times in deionized water and put in capillary cells (Malvern Instruments, Worcestershire, UK). The samples were allowed to equilibrate for 5 min and the analysis was recorded at 30 ℃.

2.4.4. Intrinsic fluoresce

The intrinsic fluorescence of tryptophan (Trp) in the samples was accessed using a SPECTRAmax GEMINI spectrophotometer (Molecular Devices, CA, USA) according to Yerramilli, Longmore, and Ghosh (2017). The samples were diluted in deionized water at 0.1 $mg.mL^{-1}$ and placed in a 96-well plate. The samples were excited at 280 nm wavelength and the emission was recorded between 340 and 400 nm wavelength.

2.4.5. Surface hydrophobicity (H_0)

The surface hydrophobicity (H_0) was determined using 1-anilino-8-naphthalenesulfonate (ANS) at 8 mM as described by Kumar et al. (2022) with slight modifications. In test tubes, the samples were diluted in four distinct concentrations (1, 0.75, 0.5, 0.25 $mg.ml^{-1}$) using deionized water as solvent. Then, 20 µL of ANS was added to 4 mL of each protein dilution. Then, the test tubes were vortex and kept in the dark for 15 min to allow reaction. After the reaction time, 200 µL were placed in a 96-well plate and put in a SPECTRAmax GEMINI spectrophotometer (Molecular Devices, CA, USA). The blanks were composed of deionized water with ANS and protein dilutions without ANS. The excitation wavelength was set to 390 nm, and the emission intensity at 468 nm was recorded. The fluorescence intensity results were plotted against the protein concentrations and a linear regression curve was calculated. The slope of the curve can be understood as the sample's surface hydrophobicity (H_0).

2.4.6. SDS-Page electrophoresis

SDS- Page electrophoresis in polyacrylamide gels was used to determine if the HIUS application would impact the primary structures of the proteins. A 12% polyacrylamide gel was formulated according to Queiroz et al. (2021). The gels were loaded with 10 µL of each sample, previously diluted in a buffer solution containing 125 mM Tris HCl (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM EDTA, and bromophenol blue. The gels were placed in a Mighty Small (Hoefer) and 100 V was applied for the first 15 min of running, after, the voltage was increased to 150 V and kept until the lower band achieve the last 1:4 of the gel height. Then, the gels were dyed by immersion in a solution containing Coomassie brilliant blue for 4 hours,

followed by discoloration in ethanol: water solution. The gels were scanned and the images were analyzed using ImageJ software to calculate the protein band intensities.

2.5. Gelling properties

2.5.1. Small amplitude oscillatory shear (SAOS) test

The gel formation was followed by SAOS test with the test parameters within the linear viscoelastic region (LVR). Glucono-δ-lactone (GDL) was added to the samples, followed by 1 min stirring to allow complete GDL solubilization. Then, the samples were placed in a stress-controlled rheometer (Discovery HR-2, TA Instruments, USA) equipped with a cone-plate geometry. Then, a time sweep test was performed for 5 hours at 1 Hz frequency and 1 % of amplitude at 30℃. The edges of the geometry were covered with silicon oil to avoid water loss during the experiment. After the 5 hours, without disturbing the formed gel, a frequency sweep test was performed from 0.1 to 50 Hz to determine the samples' frequency dependence. After that, a strain sweep was performed by varying the applied oscillation strain from 0.1 to 500 %.

In the conditions used in this experiment, the GDL was added in enough quantity to allow the samples to reach pH 4.6 at the same time (in 5 hours). The first point in the rheograms occurred 5 min after GDL addition for all samples. The delay is due to the equilibration step performed before the begging of the measurements. The gelation time (Tgel) was defined as the time where G* reached 1 Pa.

2.5.2. Water holding capacity (WHC)

The WHC was determined according to Nascimento et al. (2020). The gels were allowed to form in 50 mL centrifuge tubes. The centrifugation was performed at 4000 g at 4℃ for 10 min. The supernatant was carefully removed from the tube and weight. The percentage of the water entrapped in the gel was calculated according to equation 4.

$$WHC\ (\%) = \frac{mi - ms}{mi}\ x\ 100 \quad (4)$$

Where mi is the initial mass and ms is the supernatant mass.

2.5.3. Confocal laser scanner microscopy (CLSM)

CSLM was performed according to Andoyo, Guyomarc'h, Cauty, and Famelart (2014) with slight modifications. 0.2 $g.kg^{-1}$ of rhodamine B isothiocyanate (RITC) was added to the samples to label the proteins (Sigma Sigma-Aldrich). The suspensions were stirred for 5 min at room temperature to ensure RITC solubilization. Then, the required amount of GDL was added to the samples and stirred for 1 min to allow GDL solubilization. Then, the samples were carefully placed into an 8- well chamber slide (Ibidi GmbH, Germany), which was placed in a water bath at 30℃ to acidify. After acidification, the samples were visualized using an inverted microscope (Nikon Ti2) equipped with an sCMOS camera (Photometrics Prime95b).

2.6. Statistical analysis

The samples were compared by Analysis of variance (ANOVA). The effect of protein ratio was verified before and after HIUS treatment. Then, the effect of the HIUS application in each protein ratio was verified. When a significant difference ($p < 0.05$) was found, the Tukey HSD test with 5% significance was applied to differentiate means. All the experiments were performed, at least, two independent times, and the data was evaluated utilizing *SAS* software student edition.

3. Results and Discussion

3.1. Viscosity and Solubility

The viscosity at 200 s^{-1} was chosen based on the common shear rate found in the food industry processing (Hubbe et al., 2017). Observing the effect of the protein ratio before the HIUS treatment, the viscosity values increased with the increase of pea protein, being the dispersion of 0:100 presenting the highest value (Figure 1A). The same tendency was observed by Oliveira et al. (2022) in mixed milk: pea systems at concentrations higher than 7% (v/v). The authors argued that the presence of insoluble protein aggregates may be responsible for the increased viscosity. Despite the replacement of more CMs for pea proteins, it is interesting to note that viscosity values did not change significantly ($p > 0.05$), comparing 80:20 and 50:50 samples. Comparing the effect of HIUS for each protein ratio, it was observed a significant decrease in the viscosity ($p < 0.05$), for all samples. However, the effect of different processing routes was observed only for 50:50 and 20:80 ratios, where route 1 decreased more the samples' viscosity than route 2. The same reduction in viscosity after HUIS treatment was observed by O'Sullivan et al. (2016) (O'Sullivan, Murray,

Flynn, & Norton, 2016) evaluating four different animal and plant protein suspensions. In protein suspension, the size and surface properties of the particle play an important role in the final suspension viscosity (Kornet et al., 2020). The larger differences in the viscosity values among the protein ratios before HIUS application may be due to the differences in the size of the protein particles in the systems. The application of HIUS probably decreased the protein sizes making the variation among the protein ratios smaller, which also explains the absence of significant differences among the ratios after HIUS.

The viscosity of a suspension is understood as the friction between the suspension layers (Bourne, 2002). Thus, the presence of insoluble protein aggregates can increase the viscosity of the samples by increasing the protein volume fraction (McPhie, Daivis, & Snook, 2006). Thus, the changes in the solubility of the samples were verified (Figure 1B). Initially, the CMs presented solubility around 64.0 ± 0.9 %, which was more soluble than pea protein (42.8 ± 0.6 %). The low solubility of pea proteins is an industrial challenge that compromises their application in more food products (Alves & Tavares, 2019). Compering the protein ratios before ultrasound treatment, it is interesting to note that the solubility of the systems did not change with the addition of pea protein until it became the major protein present. These observations are similar to the trends observed in the viscosity results. After the ultrasound treatment, the solubility increased until 85.6 ± 0.2 % for pure CMs dispersions and until 97.5 ± 0.4 % for systems formed solely by pea proteins (Figure 1B). An increase in solubility of rapeseed proteins was also reported by Li et al. (2020), where sonication increased almost 6 times their solubility. The HIUS was also efficient in increasing protein solubility in mixed systems, the 50:50, and 20:80 samples presented comparable solubility to 0:100 after HIUS application ($p < 0.05$). Thus, the modifications caused by HIUS treatment are a tool to increase protein solubility in mixed systems, which may impact directly their techno-functionality such as gelling, emulsion, and foaming properties (Kumar et al., 2022; Ma et al., 2019; Higuera-Barraza, Del Toro-Sanchez, Ruiz-Cruz, & Márquez-Ríos, 2016). However, no differences were detected concerning the processing routes.

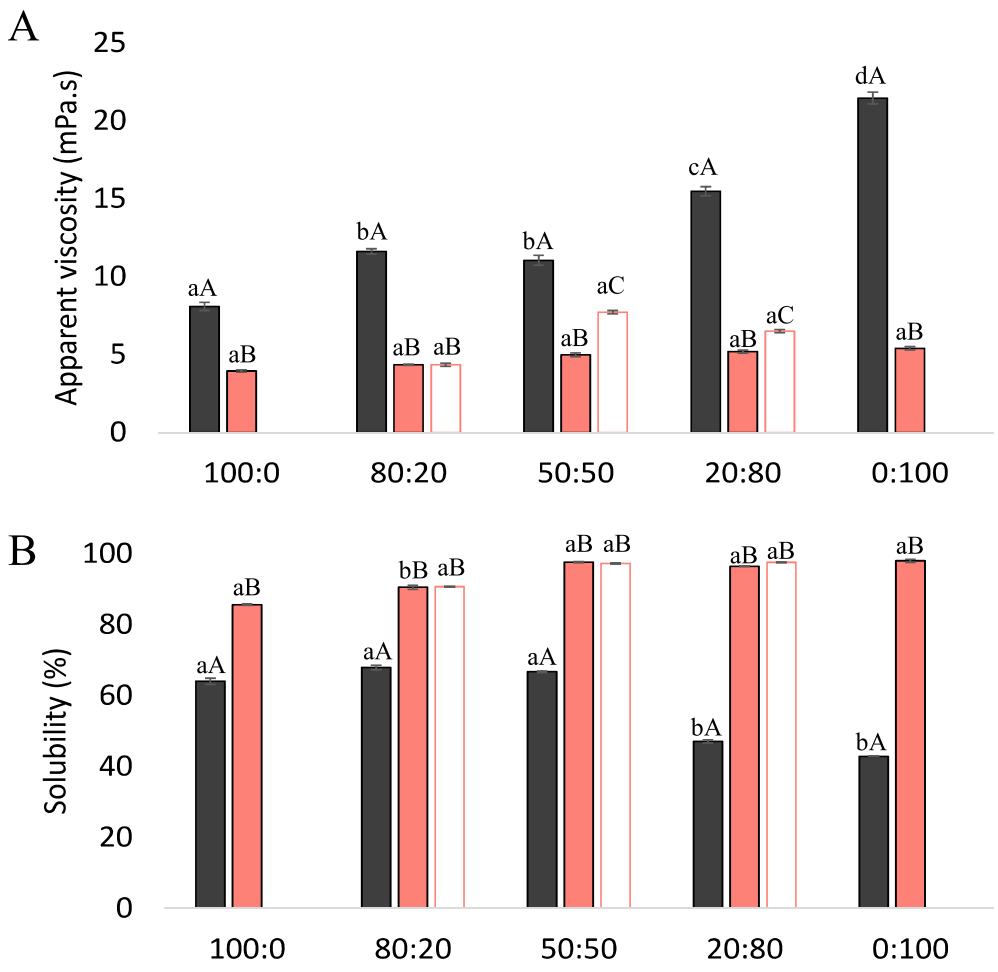


Figure 1. (A) Apparent viscosity at 200 s^{-1} shear rate. (B) solubility of protein suspensions. (■) Without HIUS application, HIUS application in route 1 (■) and route 2 (□). Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%.

3.2. Particle size

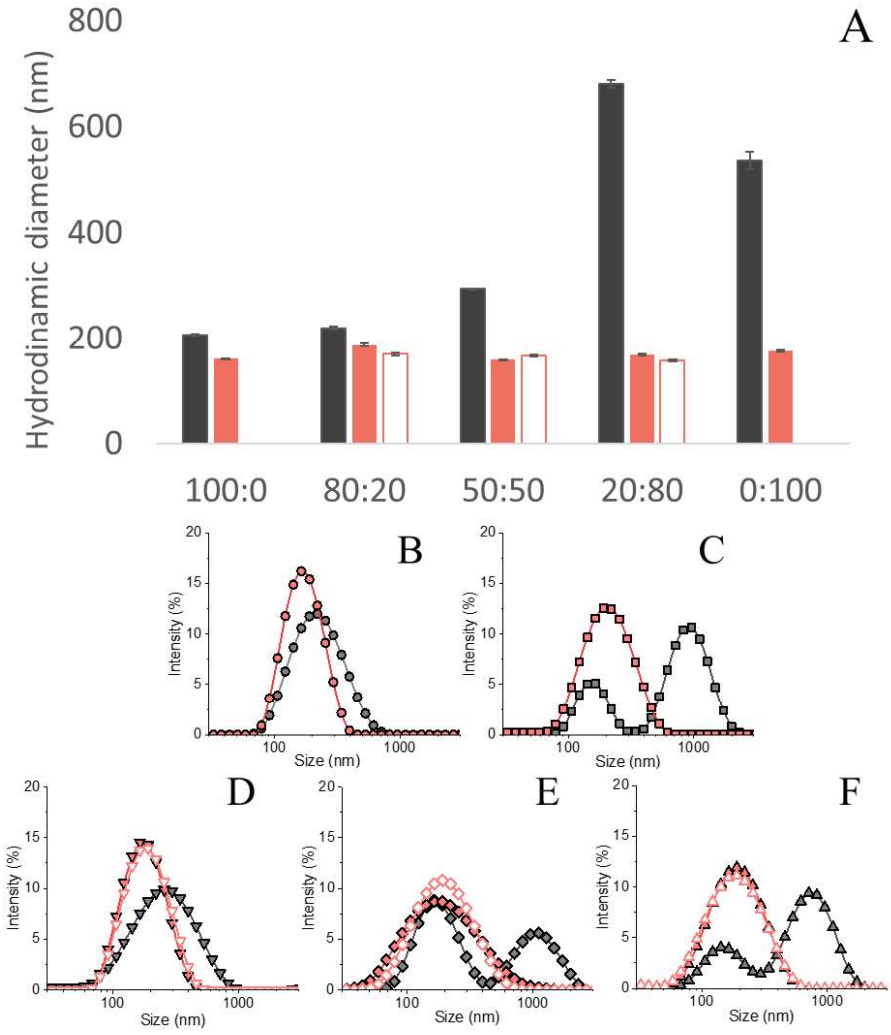


Figure 2. (A) mean particle size of protein suspensions without (■) and with HIUS application. for route 1 (■) and route 2 (□). Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%. (B) Protein particle size distributions B- 100:0, C- 0:100, D- 80:20, E- 50:50, F- 20:80.

Before HIUS treatment, it was observed a distinct particle distribution comparing CMs dispersion and pea protein dispersion. The CMs dispersion was characterized by a unique population with the main hydrodynamic diameter of 206 ± 1.2 nm (Figures 2B1 and 2A). While, the pea protein dispersion presented two populations, the first peak at 157.0 ± 2 nm and the second at 973.5 ± 7.7 nm (Figure 2B2). The observed particle distributions are due to pea protein aggregates, probably formed due to the harsh processing condition applied to extract the pea proteins from the bean (Tanger,

Engel, & Kulozik, 2020). The pea protein aggregates sizes can vary depending on the extraction method, and the values found here aggress with the results found by Oliveira et al. (2020) who found aggregates as bigger as 2 μm.

For the protein blends, it was observed a gradual increase in the particle sizes of the dispersions when more CMs were substituted for pea protein (Figure 2A), which corroborates with the viscosity results. However, at the ratio of 20:80 the particle size population mean was 680.4 ± 4.6 nm, which was higher than the mean for the pea protein alone (535.6 ± 12 nm). This increase in the mean is due to the increase of the second peak population, indicating that the presence of CMs in this particular ratio increases the size of the larger pea aggregates. After the HIUS treatment, the particle size decreased for all the studied suspensions, being the more remarkable difference in the ratios where the pea was the major protein component, which indicates that the observations in viscosity results are linked to protein size in the dispersions. The CMs dispersion particle size decreased by around 21% compared to CMs before ultrasounds application, at the time that the 0:100 sample decreased to 68.9 %, and the 20:80 ratio to 76.61%. As can be seen in Figure 2B1 - 5, the ultrasound treatment broke the larger aggregates in pea protein dispersion, making disappear the second particle peak. Also, it was observed that the 0:100 system presented a higher particle size mean than the 20:80 ratio, 157.1 ± 0.2 nm, and 175.1 ± 1.47 nm respectively, confirming that the previous aggregates were formed by interactions among the pea proteins. No differences were observed regarding the HIUS process routes.

The energy input was able to reduce the size of the aggregates, however, it is important to verify if the HIUS interfered in the primary structure of the proteins.

3.3. Electrophoresis

The electrophorese results were plotted in terms of band intensity (supplemented material). The pea proteins present several protein fractions including α- and β- legumin, α-, β- and γ – vicilin, and convicilin. The legumin fraction associates by S-S bonds and appears in ~ 65 KDa in electrophorese analysis (Jiang et al., 2017), however, the use of 2-mercaptoethanol cleavages the disulfide bonds, for this reason, legumin- αβ cannot be observed in our results. In the pure CMs systems, the four casein fractions present in the CMs appear in the electrophorese results. It was observed the presence of a small amount of whey proteins in the studied gels, which

remain in the powder after the purification process, but such small amount is not enough to interfere in the experimental results. Concerning the HIUS treatment, it was not observed the presence of new bands, nor the disappearance of previous bands. Similar results were found by Xiong et al. (2018) where even in higher HIUS intensities, no disruption in the amino acids backbone was observed. Despite the disappearance or formation of bands, it was noted an increase in the band's intensity after the HIUS application. The increased intensity is probably due to the disruption of protein aggregates and the increase in their solubility, which facilitated the entering of the protein in the polyacrylamide gel. Therefore, the HIUS applied did not cause modification in the primary structures of proteins, only disruption of the aggregates.

The HIUS application broke down the protein aggregates, which promoted the formation of new smaller protein particles impacting the solubility and viscosity of the systems. Hence, the surface of these protein aggregates may also be affected, thus the following analysis aimed to understand the modification in the protein particle surfaces caused by the different protein combinations and HIUS treatment.

3.4. Intrinsic fluorescence

The intrinsic fluorescence intensity of all the samples increased with the ultrasound application. The highest intensity was at 340 nm wavelength for all samples, without red or blue shifts. The applied wave-length excites mainly the Trp residue. Thus, the increase in fluorescence intensity can be explained by the modification of the Trp position in relation to the neighbor environment. It is known that the exposition of Trp to the solvent causes fluorescence quenching, which reduces the fluoresce intensity (Cheng & Cui, 2021). However, it was observed the opposite result. Thus, it is hypothesized that before ultrasound treatment, the Trp residue was buried inside the protein aggregate. After HIUS treatment, the aggregates were broken and the proteins suffer structural modifications which exposed more Trp to the solvent, but at the same time increase the distance from other quenching species, increasing the fluoresce intensity. Similar results were reported by Wang, Zhang, Xu, and Ma (2020), the authors observed an increase in fluorescence intensity in pea proteins extracted using HUIS, where the intensity increased with the prolonged sonication time until a maximum after 15 min of treatment. It is remarkable the difference in the fluorescence intensity for the systems composed mainly of CMs and pea proteins (supplementary

material), which is explained by the quantity of Trp, while CMs present ≅ 1.4 %, pea proteins have ≅ 0.9 %. Thus, the differences in the intrinsic fluorescence among the protein ratios may come from a simple additive effect caused by unbalance of Trp content. Therefore, the surface hydrophobicity was evaluated to have more complete insight into the difference in the systems before and after HIUS application.

3.5. Surface hydrophobicity (H_0)

In the surface hydrophobicity analysis, a fluorophore (ANS) is added to the protein suspensions and it interacts with the hydrophobic regions of the molecules (Kato & Nakai, 1980). The HIUS treatment promoted an increase in the H_0 for all the studied ratios. The turbulence applied in the systems due to the cavitation disturbed the protein aggregates, breaking them and promoting the exposure of hydrophobic regions that was before buried (Xiong et al., 2018). Similar results were found by Wang et al. (2020), where the HIUS increased the H_0 of chickpea protein suspensions, with better results after 20 min HIUS processing.

Concerning the processing routes, the observed result depends on the studied protein ratio. In unbalanced dispersions, *i.e*., when the amount of protein source was much higher than the other (80:20 or 20:80) route 1 presented higher H_0 than route 2. The inverse was observed in protein ratios of equal amounts of both proteins (50:50), being the route 2 producing slightly higher H_0. It hypothesized that when a small amount of one protein is dispersed in a higher amount of another, better homogeneity is achieved, and some degree of synergism can arise (Krentz, García-Cano, Ortega-Anaya, & Jiménez-Flores, 2022). In addition, the turbulence caused by cavitation may turn easier the CMs- pea interactions, and some of the pea protein, mainly the vicilin fraction, may be entrapped in the CMs structure (Krentz, García-Cano, Ortega-Anaya, & Jiménez-Flores, 2022). In the 50:50 ratio, the thermodynamic incompatibility of the proteins generates a stronger protein separation, and the HIUS imputed energy increased it.

3.6. ζ- potential

The ζ -potential measures the resultant surface charge of the particles, which is an indication of suspension electrostatic stability (Larsson, Hill, & Duffy, 2012). All samples presented negative ζ -potential which means that the surface of the particle

contained a higher amount of negative amino acids than positive ones. The results found for the pure CMs and pea are in agreement with other authors (Nascimento et al, 2020; Shevkani, Singh, Kaur, & Rana, 2015; Dalgleish, 2011). Comparing the effect of the ultrasound treatment in each ratio, it was observed that the blends 80:20 and 20:80 showed a significant difference ($p < 0.05$), which is an indication of specific protein interaction in these particular ratios, which aggresses with the results discussed in section 3.5. Nevertheless, ζ -potential slightly increased for the 80:20 sample and slightly decrease for the 20:80 sample, indicating differences in the nature of protein interactions. Cheng and Cui (2021) also observed a decrease in the ζ -potential of pea proteins after sonication, with a higher decrease when more intense treatments were applied. The authors explained their observation based on the increase in the protein interactions, which would decrease the ζ -potential. Thus, slight changes caused by HIUS in the ζ -potential are not sufficient to explain the improvements in viscosity and solubility, indicating that they were not strongly related to the surface charge but to the balance between the hydrophobic and hydrophilic amino acids.

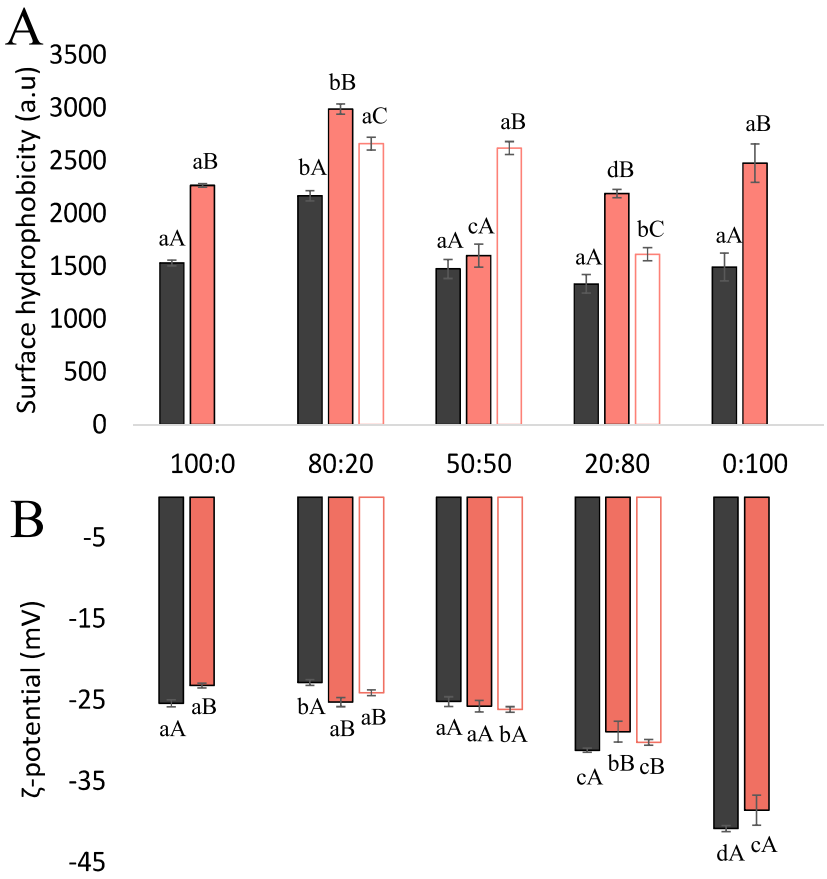


Figure 3. (A) surface hydrophobicity and (B) ζ -potential of protein particles before (■) and after HIUS application. for route 1 (■) and route 2 (□). Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%.

The modification in the proteins also can impact direct the gelling, emulsion, and foam properties of these proteins. Thus, the gelling properties were studied to understand the effect of the HIUS application.

3.7. Gelling properties

3.7.1. Gel formation

The gel formation after GDL addition of the pure systems before and after ultrasound treatment is shown in Figure 4A. The first minutes of acidification were marked by a fast increase in G* for both pure systems. At the end of minute 55, the pure CMs reached a maximum G*, however, in the next minutes of acidification, the G* decreased and only started to increase again in minute 155. This phenomenon can be also observed in terms of loss tangent (inserted graph), in which the peak happened in minute 155 after acidification. The observed decrease in G*, as well as the increase in loss tangent, can be explained by the solubilization of calcium phosphate (Andoyo et al., 2014). The CMs are supramolecular structures formed by four protein fractions. These fractions interact mainly by hydrophobic interaction and nanoclusters of calcium phosphate (Walstra, 2006). When the pH decreases, the calcium phosphate increases its solubility, causing the weakening of casein fractions interactions, which is noted by the decrease in G*. After, the pH kept decreasing, and the degree of protein interactions increased, which caused the re-increasing in G*. In the sonicated suspensions, it was observed a decrease in the G* at the very beginning of acidification, indicating that the application of HIUS increased the fluidity of the system, which aggress with the viscosity results shown in section 3.1. For pure systems, a decrease in gelation time (Tgel) occurred from 21.67 to 18.56 min for the 100:0 sample and from 16.53 min to 13.88 min for the 0:100 sample. Arzeni et al. (2012) also observed a decrease in the Tgel of whey protein systems treated with HIUS. The faster tridimensional network formation may be related to the higher surface hydrophobicity of the proteins, which increased the formation of hydrophobic interaction among them. The HIUS treatment increased the final G* for the CMs gel by more than 10 times and reduced the decrease in the G* during the protein rearrangement period. Chandrapala,

Zisu, Kentish, and Ashokkumar (2013) demonstrated the HIUS also increased the strength of CMs gels. However, Chandrapala, Martin, Zisu, Kentish, and Ashokkumar (2012) showed that in natural pH conditions the HIUS treatment only disrupts the protein aggregates and does not interfere with the integrity of the CMs. The pure pea system (0:100) also presented a step increase in the G* in the first minutes of acidification, however, the G* reaches a stable value around 105 minutes without any strong changes, only with protein network reinforcement, as can be also visualized in the constant decrease in loss tangent values (inserted graph). The HIUS application also increased the final G* for pure pea systems probably due to the decrease in the protein particle sizes and the increase in their surface hydrophobicity, which created a more homogeneous and interconnected network.

It was observed an effect caused by the protein ratios before the HIUS application (Figure 4B, C, D). The sample 80:20 (Figure 4D) presented the highest Tgel (32.74 min), an increase in 11 min compared with the 100:0 sample. Thus, the replacement of 20% of CMs for pea proteins impacted the initial aggregation steps of the CMs. In another hand, Tgel decreased in 9.5 min, comparing pure pea protein gels (0:100) with 20:80. This behavior supports the observation made in section 3.2, where the presence of small amounts of CMs intensifies the interactions among the pea proteins which increased the number of bigger aggregates at the begging of acidification, hence decreasing the Tgel in the 20:80 system. Thus, the pea protein seems to retard aggregation of CMs, while CMs seem to tune aggregation in pea systems. In the 50:50 ratio, where the protein of different sources is balanced, the G* was higher than 1 Pa before 5 min, but it was observed a slower G* development compared to the 100:0 sample. Thus, even if the presence of pea proteins disturbs the CMs network formation, the concomitant formation of the pea protein network contra-balances this effect, resulting in a gel stronger than 100:0 gel, but weaker than 0:100 gel. Grygorczyk, Alexander & Corredig (2013) studied the formation of an acid gel composed of a combination of cow and soy milk, the authors found that the differences between aggregation times led to the formation of independent protein networks. The formation of independent networks also was observed by Silva et al. (2019) in thermal gels formed between CMs and different plant proteins.

During acidification, the gel formation curve of the 20:80 sample was similar to 0:100, with a slightly lower G* caused probably by the presence of CMs. However, in

the 80:20 ratio, it was observed an antagonist effect in the final G*, which means that G* for 80:20 is lower than the lowest pure gel G* (100:0). As observed in Tgel, the presence of a small amount of pea seems to interfere strongly with the CMs gel formation. The CMs gel has probably a lower amount of junction zones compared to the 0:100 gel, for this reason, the presence of a different protein source caused a higher impact in Tgel and in the final G*.

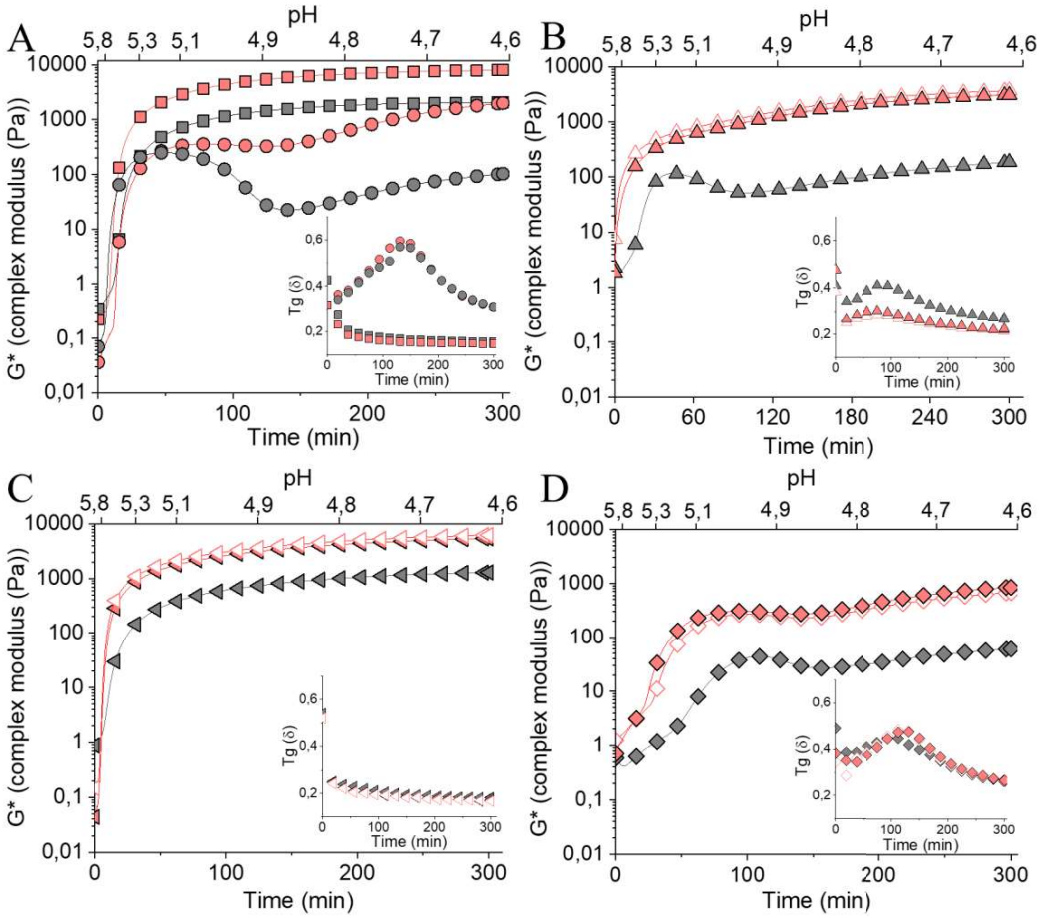


Figure 4. Complex modulus G* in the function of time after addition of GDL. (A) pure CMs (●. ●) and pure pea protein (■. ■). (B) 50:50, (C) 20:80, and (D) 80:20. The inserted graphs are the loss tangent plots in the function of time. Black symbols stand for suspensions before HIUS treatment. Colored full symbols stand for samples treated with HIUS with route 1 whereas colored empty symbols stand for samples treated with HIUS with route 2.

The application of HUIS in the protein suspensions before the acidification increased the final G* for all studied protein blends, with the more remarkable change observed in the 50:50 ratio. It was observed an increase proximately 4, 13, and 20 times in the final G* for the samples 20:80, 80:20, and 50:50 respectively, showing the efficiency of the sonication in improving the gelling properties of mixed protein systems.

Before HUIS treatment, the 50:50 sample acidification curve was similar to the pure CMs system, showing a pronounced decrease in G* around pH 5.0. However, after sonication, the curves are closer to those observed in pea pure systems with a lower reduction in G* during the calcium solubilization. This difference can be confirmed by observing the loss tangent plot (inserted graph), where was observed a smaller peak in the loss tangent values for the samples treated with HIUS. It can be explained by the reduction of the protein aggregates and the increase in their surface hydrophobicity before gel formation, which could increase the interaction between the pea proteins during acidification. Thus, the modifications in CMs internal structure were less remarked.

3.7.2. Frequency dependence and amplitude sweep

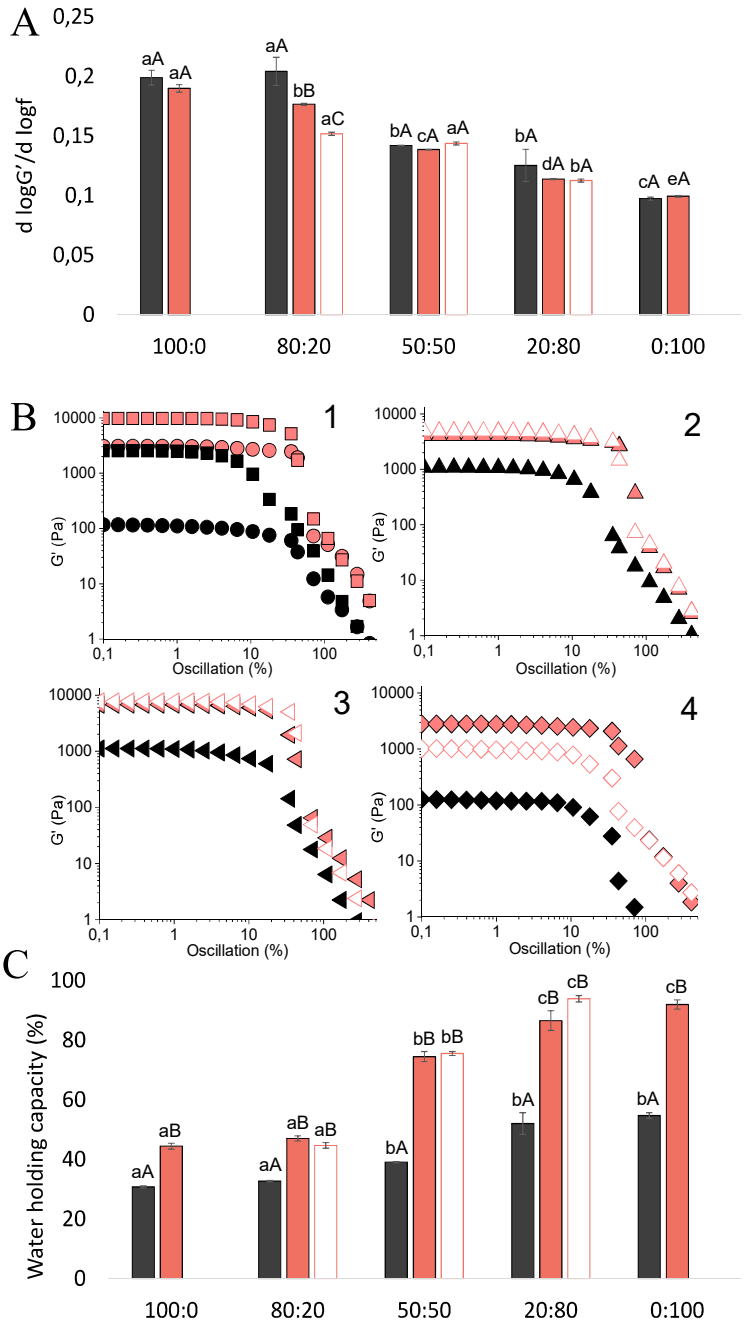


Figure 5. (A) Frequency dependence of the gels and (C) water holding capacity of the gels before (■) and after HIUS application, for route 1 (■) and route 2 (□). Different lower-case letters mark a significant difference between the protein ratios in the same sonication stage. Different upper-case letters mark a significant difference between the HUIS treatments at the same protein ratio. The significance of the Tukey test was 5%. (B) Strain sweep test of protein gels, B1- pure CMs (●, ●) and pure pea protein (■, ■), B2- 50:50, B3- 20:80, B4- 80:20. Black symbols stand for gels before HIUS treatment, colored full symbols stand for samples treated with HIUS with route 1, and colored empty symbols stand for samples treated with HIUS with route 2.

The pure pea gel (0:100) presented the lowest frequency dependence and the pure CMs gel (100:0) the highest (Figure 5A). These results agree with those discussed in section 3.7.1, where it was concluded that the interactions formed in CMs gels were more sensible to disturbing compared to the interactions in 0:100. In general, it was observed a decrease in frequency dependence when more pea protein was added to the samples, except for the 80:20 blend. These results corroborate with gel formation data, discussed in section 3.7.1. The presence of a different protein source disturbs the network formation of CMs and 20% of pea protein is not enough to establish a strong network by itself. It was also noticed that the presence of a small amount of CMs in the pea protein gel also impacted its frequency dependence, but it did not decrease severally the gel elasticity once a higher degree of disturbance is necessary to change the pea gel rheological properties, probably due to its higher connected network.

Amplitude sweep tests were performed to characterize the gels after their complete formation. The region where the G' of the samples shows non-significant deviation from a constant value is denoted as the linear viscoelasticity region (LVR) (Tunick, 2011). In LVR, the applied strain is not strong enough to break irreversibly the bonds within the gel structure (Tunick, 2011). Thus, the end of LVR can be used as a structural parameter, where a stronger gel presents a larger LVR (Bong and Moraru, 2014). The application of HIUS increased the size of the LVR region for all the studied samples (Figure 5B1 – B4). Despite the pea gels being less frequency-dependent and possessing a more interconnected network, the pure CMs gel presented a higher LVR (end in 35% deformation) than pure pea gels (end in 10% deformation). Thus, despite the more sensible aggregation, the CMs gels are more resistant to breaking when a stress is applied than pea gels, probably because the lower junction zones in CMs are stronger. Thus, the increase in pea protein content in the samples narrowed the LVR compared with the 100:0 sample. However, the decrease in LVR was not linear, once it was at 24, 33, and 13% deformation for samples 80:20, 50:50, and 20:80 respectively. As observed during the gel formation, the small amount of pea protein disturbs the initial aggregation of the CMs, which interferes in the Tgel and final G*, thus, the size of the LVR was also affected. In the 20:80 ratio, the amount of CMs is very small and gel behavior is close to the 0:100 sample. However, at the 50:50 sample the decrease in LVR was small compared to the 100:0 gel, showing that this specific

ratio allows the formation of a stable pea and CMs network. The HIUS treatment did not have a substantial effect on the sample frequency dependence, however, it increased the LVR for all samples, which indicates a reinforcement of the protein network, but with the same kind of intermolecular interactions.

3.7.3. Water holding capacity (WHC)

The WHC before the HIUS application increased when more pea protein was present in the samples, once the pea proteins presented a higher WHC as can be verified by comparing the WHC of both pure systems (Figure 5C). The WHC of all samples increased significantly after the application of HIUS, in a higher degree in the samples containing more pea proteins. The results found in the WHC of the gels are directly linked to the increase in protein solubility before gel formation. It was noticed that despite the higher WHC of pea proteins, the 80:20 mixed gel did not show a significant increase in the WHC compared to 100:0, reinforcing the finds that the small amount of pea is not able to contra-balance the disturbing effect caused in CMs network. It shows the high potential of HUIS treatment in conferring desirable features to mixed systems, once the ability to retain water inside the gel network, avoiding syneresis, is one of the stability problems found in yogurt.

3.7.4. Confocal laser scanner microscopy (CLSM)

The gel images obtained by CLSM are shown in Figure 6. Before the HIUS treatment, it is noted the presence of big protein aggregates and open and low connected structures in both pure CMs and pea gels. The same gel aspect was observed for the samples 80:20 and 20:80. Interestingly, the 50:50 gel presented a more homogeneous structure with smaller protein aggregates compared to all other samples before HIUS, showing that the presence of both proteins in the same amount led to better protein distribution by breaking down the previous big aggregates.

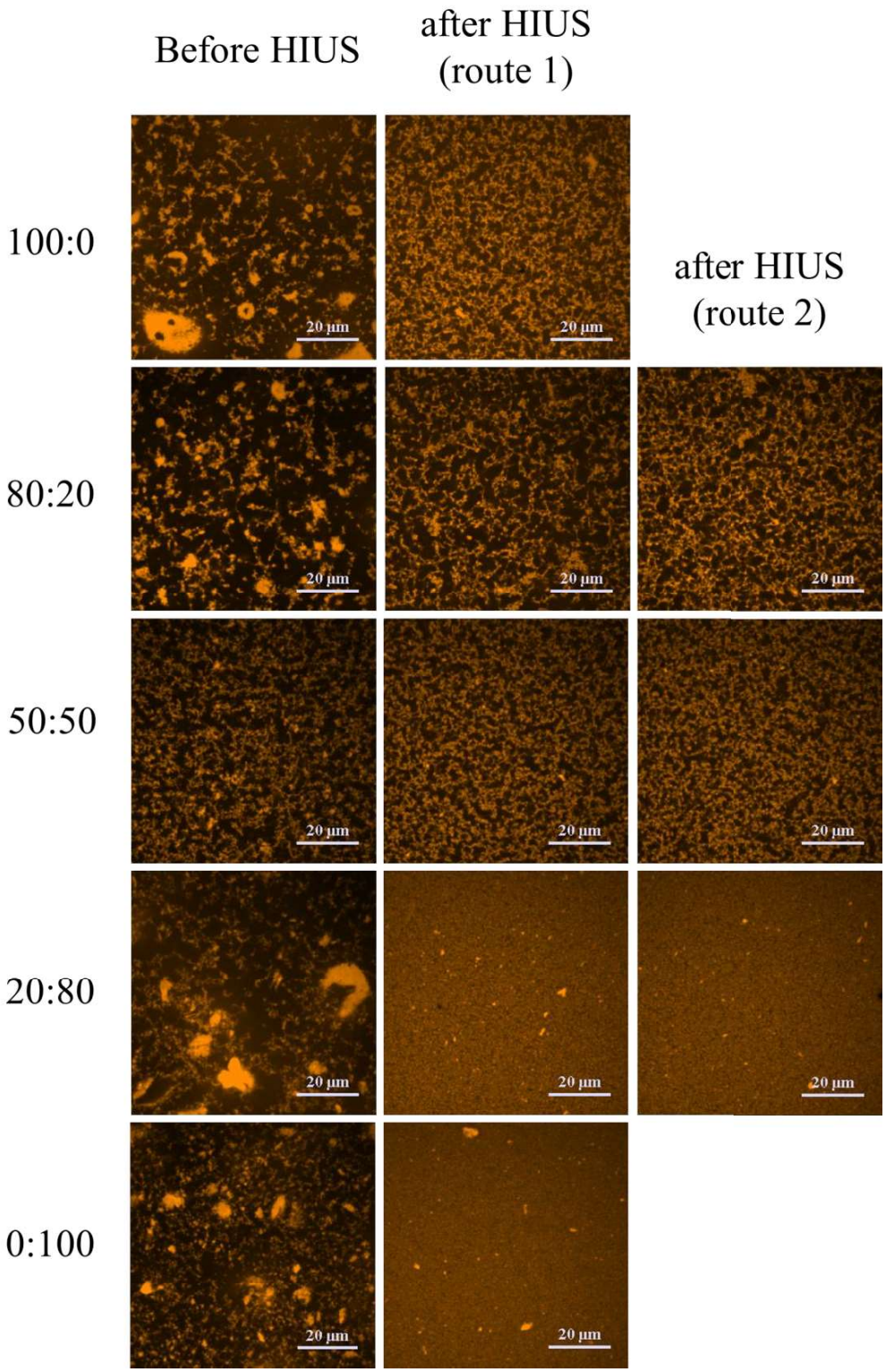


Figure 6. CLSM images of protein gels.

It was observed a remarkable change in the gel microstructure after applying sonication before suspension gelation. The CMs gels after HUIS were more

homogeneous and without the presence of big protein aggregates. The same was observed for pure pea protein gels, where it is visualized as a highly connected network. The results of CLSM aggress with the rheological results, where the pea gels showed higher final G* and lower frequency dependence. It can be noted that the 80:20 gel presents a coarser structure compared to 100:0, which also aggress with the rheological and WHC results. The 20:80 gel microstructure is similar to 0:100, but presents a slightly bigger pore size, showing that the CMs also disturb the pea protein aggregation, but the results are less pronounced in the rheological analysis since the network still rests highly connected. The 50:50 gel presents a structure similar to pure CMs, which aggress with the strain sweep results. It shows that in this specific ratio, the formation of an independent network occurs, but both proteins are in sufficient amounts to form stable networks. No visual differences were observed in the gel's microstructure concerning the process routes.

4. Conclusion

The HUIS breaks down the protein aggregates and increases their solubility and surface hydrophobicity without changing particle charges, which decreases the suspension's viscosities. This knowledge can be easily transferred to application in the production of protein beverages that can possess higher protein content with lower viscosity. At the same time, the protein modifications caused by HIUS decrease the suspension's viscosities, while increased the elasticity of all studied gels. Showing that the previously weak gels formed by the mixed systems can be more elastic after HIUS application. As a practical application, higher elastic acid gels can be produced using the same amount of protein. The process routes showed slight differences in the suspension analysis. However, it did not interfere with the gel rheological properties, showing that any of the routes are suitable for the application.

5. Reference

Aiking, H., & de Boer, J. (2020). The next protein transition. Trends in Food Science & Technology, 105, 515-522.

Alves, A. C., & Tavares, G. M. (2019). Mixing animal and plant proteins: Is this a way to improve protein techno-functionalities?. Food Hydrocolloids, 97, 105171.

Andoyo, R., Guyomarc'h, F., Cauty, C., & Famelart, M. H. (2014). Model mixtures evidence the respective roles of whey protein particles and casein micelles during acid gelation. Food Hydrocolloids, 37, 203-212.

Arzeni, C., Martínez, K., Zema, P., Arias, A., Pérez, O. E., & Pilosof, A. M. R. (2012). Comparative study of high intensity ultrasound effects on food proteins functionality. Journal of Food Engineering, 108(3), 463-472.

Ben-Harb, S., Panouille, M., Huc-Mathis, D., Moulin, G., Saint-Eve, A., Irlinger, F., ... & Souchon, I. (2018). The rheological and microstructural properties of pea, milk, mixed pea/milk gels and gelled emulsions designed by thermal, acid, and enzyme treatments. Food Hydrocolloids, 77, 75-84.

Bernardi, S., Lupatini-Menegotto, A. L., Kalschne, D. L., Moraes Flores, É. L., Bittencourt, P. R. S., Colla, E., & Canan, C. (2021). Ultrasound: A suitable technology to improve the extraction and techno-functional properties of vegetable food proteins. Plant Foods for Human Nutrition, 76(1), 1-11.

Bong, D. D., & Moraru, C. I. (2014). Use of micellar casein concentrate for Greek-style yogurt manufacturing: Effects on processing and product properties. Journal of Dairy Science, 97(3), 1259-1269.

Bourne, M. (2002). Food texture and viscosity: concept and measurement. Elsevier.

Burger, T. G., & Zhang, Y. (2019). Recent progress in the utilization of pea protein as an emulsifier for food applications. Trends in Food Science & Technology, 86, 25-33.

Chandrapala, J., Zisu, B., Kentish, S., & Ashokkumar, M. (2013). Influence of ultrasound on chemically induced gelation of micellar casein systems. Journal of dairy research, 80(2), 138-143.

Chandrapala, J., Zisu, B., Palmer, M., Kentish, S. E., & Ashokkumar, M. (2014). Sonication of milk protein solutions prior to spray drying and the subsequent effects on powders during storage. Journal of Food Engineering, 141, 122-127.

Chemat, F., & Khan, M. K. (2011). Applications of ultrasound in food technology: processing, preservation and extraction. Ultrasonics sonochemistry, 18(4), 813-835.

Cheng, J., & Cui, L. (2021). Effects of high-intensity ultrasound on the structural, optical, mechanical and physicochemical properties of pea protein isolate-based edible film. Ultrasonics Sonochemistry, 80, 105809.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. Soft matter, 7(6), 2265-2272.

Fasolin, L. H., Pereira, R. N., Pinheiro, A. C., Martins, J. T., Andrade, C. C. P., Ramos, O. L., & Vicente, A. A. (2019). Emergent food proteins–Towards sustainability, health and innovation. Food Research International, 125, 108586.

Gallo, M., Ferrara, L., & Naviglio, D. (2018). Application of ultrasound in food science and technology: A perspective. Foods, 7(10), 164.

Ge, J., Sun, C. X., Corke, H., Gul, K., Gan, R. Y., & Fang, Y. (2020). The health benefits, functional properties, modifications, and applications of pea (Pisum sativum L.) protein: Current status, challenges, and perspectives. Comprehensive Reviews in Food Science and Food Safety, 19(4), 1835-1876.

Goulding, D. A., Fox, P. F., & O'Mahony, J. A. (2020). Milk proteins: An overview. Milk proteins, 21-98.

Grygorczyk, A., Alexander, M., & Corredig, M. (2013). Combined acid-and rennet-induced gelation of a mixed soya milk–cow's milk system. International journal of food science & technology, 48(11), 2306-2314.

Guyomarc'h, F., Arvisenet, G., Bouhallab, S., Canon, F., Deutsch, S. M., Drigon, V., ... & Gagnaire, V. (2021). Mixing milk, egg and plant resources to obtain safe and tasty foods with environmental and health benefits. Trends in Food Science & Technology, 108, 119-132.

Higuera-Barraza, O. A., Del Toro-Sanchez, C. L., Ruiz-Cruz, S., & Márquez-Ríos, E. (2016). Effects of high-energy ultrasound on the functional properties of proteins. Ultrasonics Sonochemistry, 31, 558-562.

Hubbe, M. A., Tayeb, P., Joyce, M., Tyagi, P., Kehoe, M., Dimic-Misic, K., & Pal, L. (2017). Rheology of nanocellulose-rich aqueous suspensions: a review. BioResources, 12(4), 9556-9661.

Jiang, S., Ding, J., Andrade, J., Rababah, T. M., Almajwal, A., Abulmeaty, M. M., & Feng, H. (2017). Modifying the physicochemical properties of pea protein by pH-shifting and ultrasound combined treatments. Ultrasonics sonochemistry, 38, 835-842.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochimica et biophysica acta (BBA)-Protein structure, 624(1), 13-20.

Kornet, C., Venema, P., Nijsse, J., van der Linden, E., van der Goot, A. J., & Meinders, M. (2020). Yellow pea aqueous fractionation increases the specific volume fraction and viscosity of its dispersions. Food Hydrocolloids, 99, 105332.

Krentz, A., García-Cano, I., Ortega-Anaya, J., & Jiménez-Flores, R. (2022). Use of casein micelles to improve the solubility of hydrophobic pea proteins in aqueous solutions via low-temperature homogenization. Journal of Dairy Science, 105(1), 22-31.

Kumar, S., Queiroz, L. S., Marie, R., Nascimento, L. G. L., Mohammadifar, M. A., de Carvalho, A. F., ... & Casanova, F. (2022). Gelling properties of black soldier fly (Hermetia illucens) larvae protein after ultrasound treatment. Food Chemistry, 386, 132826.

Larsson, M., Hill, A., & Duffy, J. (2012). Suspension stability; why particle size, zeta potential and rheology are important. Annual transactions of the Nordic rheology society, 20(2012), 6.

Li, Q., & Zhao, Z. (2019). Acid and rennet-induced coagulation behavior of casein micelles with modified structure. Food chemistry, 291, 231-238.

Liu, J., Klebach, M., Visser, M., & Hofman, Z. (2019). Amino acid availability of a dairy and vegetable protein blend compared to single casein, whey, soy, and pea proteins: A double-blind, cross-over trial. Nutrients, 11(11), 2613.

Li, Y., Cheng, Y., Zhang, Z., Wang, Y., Mintah, B. K., Dabbour, M., ... & Ma, H. (2020). Modification of rapeseed protein by ultrasound-assisted pH shift treatment: Ultrasonic mode and frequency screening, changes in protein solubility and structural characteristics. Ultrasonics Sonochemistry, 69, 105240.

Lu, Z. X., He, J. F., Zhang, Y. C., & Bing, D. J. (2020). Composition, physicochemical properties of pea protein and its application in functional foods. Critical reviews in food science and nutrition, 60(15), 2593-2605.

Ma, W., Wang, J., Xu, X., Qin, L., Wu, C., & Du, M. (2019). Ultrasound treatment improved the physicochemical characteristics of cod protein and enhanced the stability of oil-in-water emulsion. Food Research International, 121, 247-256.

McPhie, M. G., Daivis, P. J., & Snook, I. K. (2006). Viscosity of a binary mixture: Approach to the hydrodynamic limit. Physical Review E, 74(3), 031201.

Nascimento, L. G. L., Casanova, F., Silva, N. F. N., de Carvalho Teixeira, Á. V. N., Júnior, P. P. D. S. P., Vidigal, M. C. T. R., ... & de Carvalho, A. F. (2020). Use of a crosslinked casein micelle hydrogel as a carrier for jaboticaba (Myrciaria cauliflora) extract. Food Hydrocolloids, 106, 105872.

Oliveira, I. C., de Paula Ferreira, I. E., Casanova, F., Cavallieri, A. L. F., Lima Nascimento, L. G., de Carvalho, A. F., & Nogueira Silva, N. F. (2022). Colloidal and Acid Gelling Properties of Mixed Milk and Pea Protein Suspensions. Foods, 11(10), 1383.

O'sullivan, J., Murray, B., Flynn, C., & Norton, I. (2016). The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. Food hydrocolloids, 53, 141-154.

Queiroz, L. S., Regnard, M., Jessen, F., Mohammadifar, M. A., Sloth, J. J., Petersen, H. O., ... & Casanova, F. (2021). Physico-chemical and colloidal properties of protein extracted from black soldier fly (Hermetia illucens) larvae. *International Journal of Biological Macromolecules*, *186*, 714-723.

Roesch, R., Juneja, M., Monagle, C., & Corredig, M. (2004). Aggregation of soy/milk mixes during acidification. Food Research International, 37(3), 209-215.

Shevkani, K., Singh, N., Kaur, A., & Rana, J. C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study. Food Hydrocolloids, 43, 679-689.

Silva, J. V., Cochereau, R., Schmitt, C., Chassenieux, C., & Nicolai, T. (2019). Heat-induced gelation of mixtures of micellar caseins and plant proteins in aqueous solution. Food research international, 116, 1135-1143.

Silventoinen, P., & Sozer, N. (2020). Impact of ultrasound treatment and pH-shifting on physicochemical properties of protein-enriched barley fraction and barley protein isolate. Foods, 9(8), 1055.

Soria, A. C., & Villamiel, M. (2010). Effect of ultrasound on the technological properties and bioactivity of food: a review. Trends in food science & technology, 21(7), 323-331

Tanger, C., Engel, J., & Kulozik, U. (2020). Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. Food Hydrocolloids, 107, 105949.

Tunick, M. H. (2011). Small-strain dynamic rheology of food protein networks. Journal of Agricultural and Food Chemistry, 59(5), 1481-1486.

Yerramilli, M., Longmore, N., & Ghosh, S. (2017). Improved stabilization of nanoemulsions by partial replacement of sodium caseinate with pea protein isolate. *Food Hydrocolloids*, *64*, 99-111.

Wang, Y., Wang, Y., Li, K., Bai, Y., Li, B., & Xu, W. (2020). Effect of high intensity ultrasound on physicochemical, interfacial and gel properties of chickpea protein isolate. Lwt, 129, 109563.

Wang, F., Zhang, Y., Xu, L., & Ma, H. (2020). An efficient ultrasound-assisted extraction method of pea protein and its effect on protein functional properties and biological activities. LWT, 127, 109348.

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). Dairy Science and Technology (Second Edi., Vol. 4). Taylor & Francis. doi:9780824727635

Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B., & Chen, Y. (2018). Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. Food Research International, 109, 260-267.

Supplementary material:

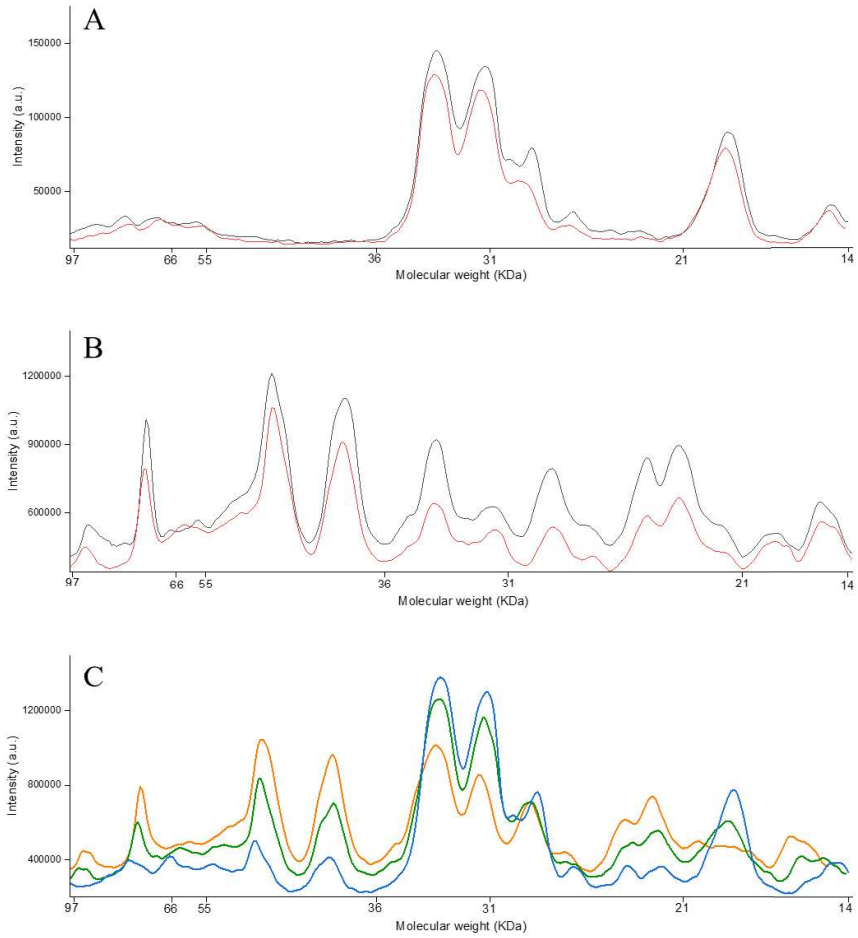


Figure S1. (A) CMs suspensions without (black) and with (red) HIUS application. (B) Pea protein suspensions without (black) and with (red) HIUS application. (C) CMs:pea suspensions HIUS treated at 80:20 (blue), 50:50 (gree) and 20:80 (yellow).

Table 1. HIUS treatment power and intensity.

	100:0	80:20	50:50	20:80	0:100
P (W)	26.56 ± 1.53	27.63 ± 0.20	32.53 ± 3.69	31.94 ± 2.61	30.82 ± 1.41
I (W/cm^2)	38.49 ± 2.21	40.04 ± 0.19	47.14 ± 5.34	46.29 ± 3.78	44.67 ± 2.05

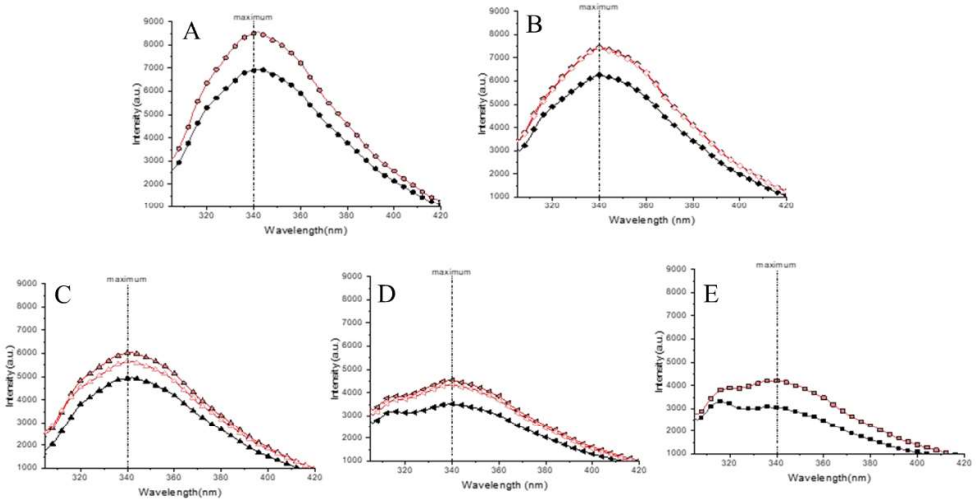


Figure S2. Intrinsic fluorescence. (A) pure CMs, (E) pure pea protein, (C) 80:20, (D) 50:50, (D) 80:20. Black symbols stand for gels before HIUS treatment, colored full symbols stand for samples treated with HIUS with route 1, and colored empty symbols stand for samples treated with HIUS with route 2.

GENERAL CONCLUSION E PERSPECTIVES

1. Conclusions

This study shows the versatility of the use of casein micelles (CMs) based hydrogels, which presented promising results for both designed applications.

The hydrogel was able to entrap the anthocyanin-rich extract from jaboticaba fruit and promote a controlled release of the compounds. The maximum rate of release was at pH 7.0, the same pH found in the place where anthocyanins are mostly absorbed (the small intestine). The bioactive compounds present in the Jabuticaba extract disturbed the hydrogel formation by weakening the protein-protein interactions. However, the use of transglutaminase promoted additional covalent bonds into the systems, which contra-balanced the disturbing effect caused by the bioactive compounds. The entrapment and modulated release of anthocyanin-rich Jabuticaba extract can be applied in the development of functional foods with the specific target release of the bioactive compounds, modulated by the environment pH.

The hydrogels created with the association of CMs and pea proteins showed a complex behavior, which depends mainly on the proportion between the proteins. The thermal treatment improved the elasticity of the protein systems mainly by the denaturation of pea proteins. The main forces driving the formation of the hydrogel are of physical nature, with limited disulfide bonds created only between proteins of the same source. Despite the absence of strong interactions between CMs and pea proteins, a synergistic effect in the elastic properties was observed in 20:80 (CMs: pea) protein ratio. Concerning the hydrogel formation by acidification, the replacement of CMs for pea proteins (80:20 and 60:40 ratios) retarded the development of the protein network at the begging of acidification. However, the probable formation of pea-pea interactions resulted in final elasticities higher than in pure CMs gels. The opposite effect was observed when pea proteins were the major protein component (40:60 and 20:80 ratios), where gels with lower elasticity than both CMs or pea protein pure gels were produced. It shows that in absence of strong interactions between proteins of different sources, the protein distributions within the gel matrix, which is related to the protein ratios, is the responsible for modulating the final gel properties.

In addition, the use of high-intensity ultrasounds showed promising effects in the improvement of the gelation properties of the mixed systems. The physical

treatment decreased aggregates sizes and increased solubility and surface hydrophobicity, which increased up to ten times the elasticity of the mixed gels.

The increased elasticity in the CMs gels derived for the partial replacement by pea proteins is useful in the formulations of acidified milk products such as yogurt, to produce a more consistent texture with the same protein content. Therefore, the results showed the potentiality of CMs hydrogel for the development of new products.

2. Perspectives

2.1. Strategies to increase CMs: pea proteins interactions

The use of transglutaminase emerges as a potential alternative since it catalyzes the formation of covalent bonds between the amino acids glutamine and lysine. However, may be necessary for a higher exposure of active sites for transglutaminase action, justifying the use of strategies for exposing these key amino acids. The employment of methods with low environmental impact is preferable, with emerging technologies such as ultrasound and high hydrostatic pressure and pH shift being promising.

2.2. Selection of specific pea protein fraction

The pea protein extract is formed by different types of proteins with particular functional properties. For a more assertive development of gels with desired features, a previous separation and selection of the pea proteins fraction is an alternative.

2.3. Use of lactic acid bacteria fermentation

The studied acid hydrogels were formulated using a weak acid to drive the pH drop. However, the industrial application of these systems will be in fermented products. The ratio of pH drops and the additional compounds produced during fermentation can address significant changes in the hydrogel features. Also, the changes in the conventional fermentation parameters caused by the association of these molecules must be studied.