ATALITA DOS SANTOS DEVAUD

CASEIN MICELLES CROSS-LINKED WITH TRANSGLUTAMINASE: FOAMING PROPERTIES AS A FUNCTION OF pH

Dissertation presented to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa as part of the requirements to obtain the title of Magister Scientiae.

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Ao meu pai, Michel Devaud (in memoriam)

“No que diz respeito ao desempenho, ao compromisso, ao esforço, à dedicação, não existe meio termo. Ou você faz uma coisa bem-feita ou não faz. ”

Ayrton Senna

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TABLE OF CONTENTS

ABSTRACT viii

RESUMO x

1. GENERAL INTRODUTION 1

2. OBJECTIVE 2

CHARPTER 1: CASEINS AS FOAM STABILIZERS – KEY FACTORS AND TRANSGLUTAMINASE CROSSLINKING AS AN ALTERNATIVE FOR IMPROVING THEIR POTENTIAL 3

ABSTRACT 5

3. INTRODUCTION 6

4. FOAM STRUCTURE 7

5. CASEINS 11

5.1 **α_{s1}-casein** 11

5.2 **α_{s2}-casein** 12

5.3 **β-casein** 12

5.4 **κ-casein** 13

5.5 **Casein micelle** 14

5.6 **Caseins as foam stabilizers** 17

6. FACTORS AFFECTING THE FOAMING PROPERTIES OF CMS 19

6.1 **Temperature** 19

6.2 **Particle size** 21

6.3 **pH** 23

7. PERSPECTIVES 25

8. REFERENCES 29

CHARPTER 2: EFFECT OF PH ON THE STABILITY OF FOAMS BASED ON CASEIN MICELLES CROSS-LINKED WITH TRANSGLUTAMINASE 44

ABSTRACT 46

9. INTRODUCTION 47

10. MATERIALS AND METHODS 48

10.1 **Milk and ultrafiltrate** 48

10.2 **Casein suspensions preparation** 49

10.3 **Sample acidification** 50

10.4 **Zeta Potential** 50

10.5 **Z-average diameter** 51

10.6 **Spectrophotometry** 52

10.7 Surface tension 52
10.8 Foam stability 52
10.9 Statistical Analysis 53
11 RESULTS AND DISCUSSION 53
11.1 Zeta Potential 53
11.2 Z-average diameter 55
11.3 Spectrophotometry 59
11.4 Surface tension 61
11.5 Foam stability 62
12. CONCLUSION 66
13. REFERENCES 67
14. GENERAL CONCLUSIONS AND PERSPECTIVES 71

ABSTRACT

DEVAUD; Atalita dos Santos, M.Sc., Universidade Federal de Viçosa, February, 2019. **Caseins micelles crosslinked with transglutaminase: foaming properties as a function of pH.** Advisor: Antônio Fernandes de Carvalho. Co-advisors: Evandro Martins and Naaman Francisco Nogueira Silva.

Caseins represents 80% of the cow's milk proteins and are widely used in food industry due to their recognized foaming properties. The casein term refers to a mixture of four different casein fractions known as αs1, αs2, β, and κ-casein that are organized in structures called casein micelles (CMs). The foam properties of casein micelles are closely related to their structure, which is influenced by several factors, including pH variations. In this context, enzymatic crosslinking with transglutaminase (Tgase) can increase the stability of CMs against pH collapse. This work initially does a review on foam structure and the main factors influencing the CMs structure and its relation to foam stability. In addition, it approaches how Tgase enzymatic crosslinking increase CMs stability against pH variations. The hypothesis of expanding the use of CMs as foam stabilizers by Tgase crosslinking was raised. Thus, the objective of this study was to compare pH stability of CMs cross-linked with Tgase (CMs-Tgase) and native CMs, concerning foam stability. Different suspensions of native CMs (MCS-CMs) and CMs-Tgase (MCS-CMs-Tgase) were prepared at 27.5 g/L in deionized type 1 water with 2 mM $CaCl_2$. The ssamples were acidified at pH values ranging from pH 7.0 to pH 2.0, at intervals of 0.5. The samples obtained were analyzed in terms of particle size, charge, surface tension, absorbance and foam stability. Tgase did not induce to a significant difference between the diameter of native CMs and CMs-Tgase. However, it led to greater stability in acid destabilization: while native CMs precipitated below pH 5.5, CMs- Tgase precipitated only from pH 3.5 to 4.5. This occurred in part due to the change in the isoelectric point (pI) of the CMs-Tgase, pH 4.7 (found for native CMs) to pH 4.3. Foams with MCS-CMs-Tgase presented higher half-life time than those with MCS- CMs from pH 5.0 to 2.0, forming foams that last for 48 hours at pH 5.0. The MCS- CMs-Tgase foams had lower half-life time at pH 7.0 to 5.5, compared to the MCS- CMs foams. The higher half-life time at pH 5.0 to 2.0 can be attributed to a greater amount of dispersed CMs and small suspended aggregates, which, because of

particle size and protein concentration, were able to prolong the life of foams interfacial films. In relation to the lower stability found in the range of pH 7.0 to 5.5, it can be explained by the reduction of the amount of CMs able to unfold the air- water interfaces due to crosslinking, and to repulsive forces between particles at air/ water interface.

RESUMO

DEVAUD, Atalita dos Santos, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Micelas de caseínas reticuladas com transglutaminase: propriedades espumantes em função do pH.** Orientador: Antônio Fernandes de Carvalho. Coorientadores: Evandro Martins e Naaman Francisco Nogueira Silva.

As caseínas representam 80% das proteínas do leite de vaca e são amplamente utilizadas na indústria de alimentos devido às suas reconhecidas propriedades espumantes. O termo caseína refere-se a uma mistura de quatro frações diferentes de caseína conhecidas como αs1, αs2, β e κ-caseína que são organizadas em estruturas chamadas micelas de caseína (CMs). As propriedades da espuma das micelas de caseína estão intimamente relacionadas à sua estrutura, que é influenciada por vários fatores, incluindo variações de pH. Neste contexto, a reticulação enzimática com transglutaminase (Tgase) é capaz de aumentar a estabilidade de CMs contra o colapso por abaixamento do pH. Este trabalho inicialmente faz uma revisão sobre a estrutura da espuma e os principais fatores que influenciam a estrutura das CMs e sua relação com a estabilidade da espuma. Além disso, aborda como a reticulação enzimática Tgase aumenta a estabilidade dos CMs contra variações de pH. A hipótese de expandir o uso de CMs como estabilizadores de espuma pela reticulação de Tgase foi levantada. Assim, o objetivo deste estudo foi avaliar se a maior estabilidade ao pH de CMs reticulados com Tgase (CMs-Tgase) leva a maior estabilidade da espuma, comparativamente às CMs nativas. Diferentes suspensões de CMs nativas (MCS-CMs) e CMs-Tgase (MCS-CMs-Tgase) foram preparadas a 27.5 g / L em água deionizada tipo 1 com 2 mM de $CaCl_2$. As suspensões foram acidificadas a valores de pH variando de pH 7.0 a pH 2.0, em intervalos de 0.5. As amostras obtidas foram analisadas em termos de tamanho de partícula, carga, tensão superficial, absorbância e estabilidade da espuma. Tgase não induziu uma diferença significativa entre os valores de diâmetro de CMs nativas e CMs-Tgase. No entanto, a reticulação levou a uma maior estabilidade à desestabilização ácida: enquanto as CMs nativas precipitaram abaixo de pH 5.5, a CMs-Tgase precipitaram apenas de pH
3.5 a 4.5. Isso ocorreu em parte devido à mudança no ponto isoelétrico (pi) da CMs-Tgase, que passou de pH 4.7 (encontrado para CMs nativas) para pH 4.3. As espumas com MCS-CMs-Tgase apresentaram maior tempo de meia-vida que aquelas com MCS-CMs, em pH 5.0 a 2.0, formando espumas que duram 48 horas em pH 5.0.

As espumas formadas a partir de MCS-CMs-Tgase tiveram menor tempo de meia- vida em pH 7,0 a 5,5, em comparação com as espumas de MCS-CMs. A maior estabilidade em pH 5.0 a 2.0 pode ser atribuída a uma maior quantidade de CMs dispersas e pequenos agregados suspensos, que devido ao efeito do tamanho de partícula e concentração de proteína foram capazes de prolongar a vida útil dos filmes interfaciais da espuma. Em relação à menor estabilidade encontrada em pH
7.0 a 5.5, isso pode ser explicado pela redução da quantidade de CMs capazes de se desdobrar as interfaces ar-água, devido à reticulação e às forças repulsivas entre as partículas na interface ar / água.

1. GENERAL INTRODUTION

Milk foamed foods such as ice cream, frozen yogurts, chantilly, mousses and meringues are appealing for consumers due to their sensorial properties such as flavor, texture, appearance and mouthfeel. For dairy industry, they represent a very attractive sales market. However, the shelf-life of these products can be limited due to the foam instability phenomena. The food industry is always looking for new methods of stabilizing these products, increasing their shelf life without impairing the sensorial characteristics valued by consumers. Caseins, in the micellar form or as monomers, have been used for years in the food industry due to their known properties of foaming and foam stabilization. However, its use is limited by situations where the stability of these proteins is compromised, leading to precipitation. In this context, acid destabilization is highlighted, since many food products have a pH value below the natural pH of milk (pH 6.8). The use of methods to increase the stability of CMs represents an opportunity to increase their performance as foam stabilizers.

This dissertation is organized as follows: first, a chapter giving a literature review that presents the structure of foams, highlighting the role of proteins in their formation and stability. This chapter describes the casein micelles (CMs), their structure, and the main factors that alter its structure, addressing their role as foam stabilizers. Second, it presents transglutaminase (Tgase) crosslinking as a method for improving CMs against several destabilization factors, focusing in pH collapse, and points out to the perspective of using cross-linked casein micelles (CMs-Tgase) to improve the performance of CMs as foam stabilizers.

Then a second chapter is devoted to the experimental part of the work, which comprises a study of the use of CMs-Tgase as foam stabilizers, as a function of pH. In this chapter, the materials and methods, results and discussions thereof and the conclusions are brought in the form of an article manuscript. Finally, there is a general conclusion in which the main results of the work and new perspectives for the area are presented.

2. OBJECTIVE

The objective of this study was to evaluate if CMs cross-linked with Tgase presents better foam stability compared to native CMs at pH values ranging from pH 7.0 to pH 2.0.

CHARPTER 1: CASEINS AS FOAM STABILIZERS – KEY FACTORS AND TRANSGLUTAMINASE CROSSLINKING AS AN ALTERNATIVE FOR IMPROVING THEIR POTENTIAL

CASEINS AS FOAM STABILIZERS – KEY FACTORS AND TRANSGLUTAMINASE CROSSLINKING AS AN ALTERNATIVE FOR IMPROVING THEIR POTENTIAL

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ABSTRACT

Milk proteins are widely used to produce foamed products in food industry, playing a key role on their quality and stability of these products. Foam stability is related to interfacial properties and to the characteristics of liquid film between adjacent air bubbles. Caseins, that represent about 80% of the proteins from cow's milk, are widely used in food industry due to their recognized foaming properties. However, the supramolecular structure of the casein micelle (CM) is influenced by several factors such as temperature, pH, pressure and ionic environment, which can narrow CM application in aerated food matrices. The search for longer-lifetime foams boosts the investigation of new methods to improve the use of these proteins as foam stabilizers. Therefore, review focuses on the foam ability of CMs, addressing the key factors in their applicability in aerated food products. In particular, we report how the enzymatic crosslinking of CM, using transglutaminase (TGase), increase CMs stability against different destabilization factors which could indicate possibilities to expand the range of use of CMs as foam stabilizers.

Keywords: casein, casein micelles, crosslinking, transglutaminase, foaming capacity, foam stability, foam stabilizers.

3. INTRODUCTION

Food proteins are addressed in manystudies due to their functionalities widely used by food industry such as ability to develop stable networks as gels and films, hold water, absorb fat, and stabilize foams and emulsions (Aryee, Agyei & Udenigwe, 2017; Patel, 2018). Proteins are molecules with well recognized interfacial activity (McClements, 2016; Sarkar & Singh, 2016) and this particular characteristic is mainly attributed to the existence of both hydrophilic and hydrophobic patches distributed along their structures (Dickinson, 2003). It justifies why proteins are widely used in food industry to stabilize emulsions (dispersion of two immiscible liquids) (McClements, 2016) and foams (dispersions of gas in liquids) (Weaire & Hutzler, 1999; Cantat, Cohen-Addad, Elias, Graner, Hohler, Pitois, Rouyer, Saint-Jalmes, 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018).

Foams in a high variety of food products is stabilized by proteins, such as in beer, breads, cakes, extruded and expanded cereal-based products, whipped cream, ice cream, aerated chocolate bars, meringue, pavlova, soufflé, marshmallow, meat foams or vegetable paste foams, fruit fools, sorbets among many others (Campbell & Mougeot, 1999). Food foams are important in the food industry because they confer a texture characteristic valued by consumers and they represent an economically interesting alternative, since they use air as an ingredient at low cost (Narchi, Vial & Djelveh, 2009). In addition, foams are a technological approach in the search for reducing fat content on formulated products, providing a creaminess sensation, in agreement with the market trend of healthy lifestyle (Santos-Murphy, Green & Cox, 2016; Patel, 2018).

Given the importance of setting the texture of foamed foods on long time periods, which is directly related to the stability of its structure, the foaming formulation is of great importance. In this regard, proteins are usually applied in food industry in the search of long - term stable foams due to their ability to slow or even prevent destabilizing phenomena (Narsimhan & Xiang, 2018).

In this context, milk proteins are extensively applied as functional ingredients for formation and stabilization of foamed systems (Rouimi, Schorsch, Valentini, Vaslin, 2005). Milk is composed of casein micelles (CMs), whey proteins, lipids,

lactose, and salts (White & Davies, 1958). During foaming of whole milk, caseins and whey proteins adsorb at interfaces, forming a layer around the air bubbles (Dickinson & Patino, 1999). Due to its large use, the adsorption mechanism of milk proteins at interfaces has been comprehensively studied and it is well known that isolated caseins, CM, and whey proteins present different behaviors during the formation of interfacial layers (Rouimi et al., 2005). Consequently, the use of each type of protein will present different challenges and advantages, and produce foams with varied properties characteristics (Marinova, Basheva, Nenova, Temelska, Mirarefi, & Campbell, 2009; Amine, Dreher, Helgason, Trados, 2014; Narsimhan & Xiang, 2017).

In this review, we will briefly present the structure of foams, the casein relative fractions and their assembly in casein micelles. Further, we discuss various relevant works regarding the employment of CMs and its characteristics concerning foaming capacity and foam stability. Then we present the factors setting the stability of casein stabilized foams; finally, a possibility for extending and improving the use of CMs as foam stabilizers will be pointed.

4. FOAM STRUCTURE

A foam is a dispersion of a large amount of gas into a much smaller volume of a liquid (Weaire & Hutzler, 1999; Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018). As the gas bubbles represent the major part of the volume, foams have a large interfacial area per volume unit. The amount of liquid in a foam is defined as the liquid volume fraction, expressed by the ratio of the liquid volume to the total volume of a foam (Exerowa & Kruglyakov, 1998).

As showed in Figure 1, three different types of foam structure are identified depending on liquid fraction: a bubbly liquid, a wet foam or a dry foam (Weaire & Hutzler, 1999; Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018).

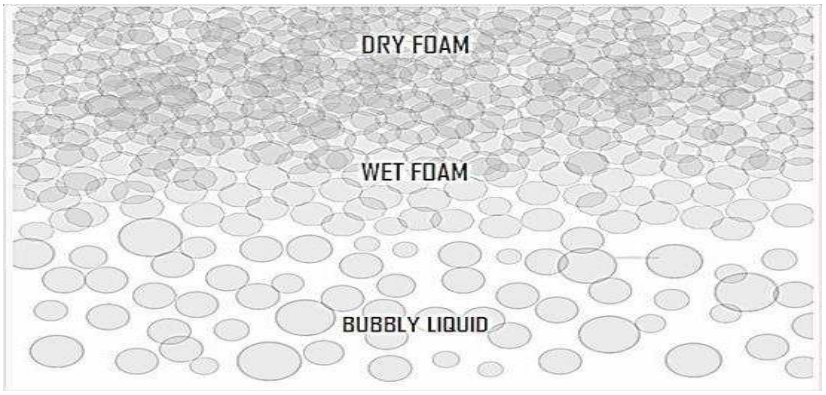

Figure 1. Schematic representation of foam structure depending on liquid fraction, based on Cantat et al., 2013.

A bubbly liquid corresponds to a dispersion of well-separated spherical bubbles. In a wet foam, bubbles are all in contact but only softly packed, so that their shape remains close to a sphere, only with flat parts where bubbles are in contact; A dry foam is found at low liquid fractions, where bubbles get polyhedral shapes due to their compaction. There is no definitive criterion to define the transition between wet and dry foams, but it can be considered that a dry foam has a liquid fraction of less than 10% (Patel, 2018). In that respect, concerning aerated food products, most of them could be classified as wet foams (Patel, 2018).

The thin continuous liquid layer between two bubbles in contact is called a liquid lamella. This liquid lamella is limited by the two interfacial layers, coming from each bubble and made of adsorbed molecules. The adsorption of active molecules at the gas-liquid interface is essential for producing and stabilizing any type of foam (Walstra, 1989; Weaire & Hutzler, 1999; Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018). In food industry, the use of proteins together with low molecular weight surfactants, is very common for this purpose (Patel, 2018). The figure 2 is a schematic representation of a foam structure at different length scales. At the smallest scale, one finds the surface active (amphiphilic) molecules: they are soluble in bulk, and can adsorb at interfaces with their polar head, in contact with water, and their hydrophobic part in contact with air, then, the liquid lamellae separates two bubbles, with typical thickness ranging from 10 to hundreds of nm (Cantat et al., 2013). The menisci formed by the encounter of three lamellae are called Plateau borders (Pb) in homage to the Belgian physicist Joseph Plateau (1801–1883), who demonstrated that lamellae always meet by three at Pbs, with constant angle of 120 °. The nodes are encounters of four plateau

borders (Weaire & Hutzler, 1999; Cantat et al., 2013). Pbs and nodes are typically in the range of ten to one hundred of microns. Altogether, Pbs and nodes make the liquid skeleton of a foam.

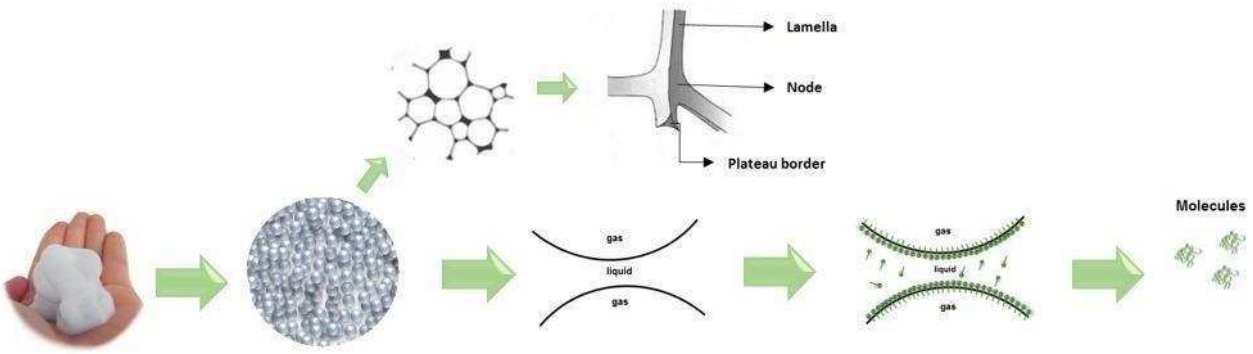

Figure 2. Foam structure from different perspectives. Based on the information contained in the works of Cantat et al., (2013), Fameau & Salonen (2014), and Rio, Drenckhan, Salonen, & Langevin (2014).

The study of foams stabilized by proteins can be done at all the different length scales previously discussed. The main features evaluated in each level, are listed below.

- Level 1 – molecular scale: protein molecular size, chemical nature of amino acids, secondary, tertiary, and quaternary structures, and the hydrophobic-hydrophilic balance.
- Level 2 – interfacial scale: dynamical and equilibrium conditions of protein absorption, interfacial tension reduction, rheological properties.
- Level 3 – lamella scale: thickness, uniformity, permeability, stability.
- Level 4 – bubble scale: mean bubble size and distribution of bubbles sizes, dynamics of bubble rearrangements.
- Level 5 – foam scale: liquid fraction, stability, rheology: viscoelastic behavior, yielding behavior, steady-flow behavior.

According to Zayas (1997), proteins are ideal for making foams when acting (i) effectively in low concentrations, (ii) in a pH range found in various types of foods, and (iii) even in the presence of foaming inhibitors such as fat and alcohol.

Physicochemical factors related with good foaming behavior for a protein are adsorption rate, ability to efficiently decrease the surface tension and its ability to increase the lifetime of a lamella (Marinova, Basheva, Nenova, Temelska, Mirarefi, & Campbell, 2009).

The foaming capacity can be defined as the foam volume produced under fixed conditions or by the time necessary to produce a definite volume of foam (Sarkar & Sight, 2016) while foam stability can be understood as the variation of foam volume with time (Wilde & Clark, 1996). While foaming capacity depends on the rate at which the surface-active molecules can adsorb to the air-liquid interface (Damodaran, 1997), foaming stability depends on the viscoelasticity of the interfacial film, and the existence of other stabilizing factors able to prevent or slow down the usual destabilizing phenomena in foams (McSweeney & O'Mahony, 2016). For comparison, low molecular weight surfactants can easily diffuse from the bulk to the interface due to the low adsorption/desorption energy, facilitating the rapid production of foams. However, the surfactant monolayer has no viscoelastic properties, and cannot reduce coarsening or bubble coalescence (Patel, 2008). On the other hand, proteins have much higher adsorption energies which slows down the molecular exchange but the interfaces are relatively more viscoelastic than those made up of surfactants, leading to higher foam stability (Patel, 2008).

Despite that the interfacial properties have been extensively related to foam stability, it is also dominated by the stability of thin liquid lamella between bubbles (Langevin, 1999; Wierenga, Norél & Basheva, 2009; Chen et al., 2018). With time, coarsening, drainage and coalescence are the main mechanisms leading to the destruction of a foam instability (Weaire & Hutzler, 1999; Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018). Coarsening involves the transport of gas between the bubbles of different sizes leading to a reduction in the number of bubbles and to an increase in the average bubbles radius (Weaire & Hutzler, 1999; Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018)). The gas diffuses from bubbles to bubbles due to differences in Laplace pressure (Weaire & Hutzler, 1999; Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018). Drainage is characterized by the flow of the foaming liquid through the foam, due to gravity, causing a decrease of the foam mean liquid fraction (Saint-Jalmes, 2006). Coalescence occurs by ruptureof the film (lamella) between

two neighboring bubbles, eventually resulting in a total foam destruction, by a reduction of the total number of bubbles (Weaire & Hutzler, 1999 Cantat et al., 2013; Exerowa, Gochev, Platikanov, Liggieri, Miller, 2018). Consequently, the production and the long-term stability of aqueous foams are mainly dependent on the ability of the formulation to delay or to arrest these destabilizing phenomena.

5. CASEINS

Caseins are a group of milk-specific proteins and represent 80% of bovine milk protein (Hamhrreus & LOnnerdal, 2003). The term refers to a mixture of four different casein fractions known as α_{s1}, α_{s2}, β, and κ-casein (Martin, Ferranti, Leroux, Addeo, 2003; Qi, 2007). There are also other minor casein fractions resultant of hydrolytic activity of a milk endogenous enzyme, called plasmin (Swaisgood, 2003). Caseins are not present as monomers in milk, but are associated in so called micelles (Walstra, Wouters, & Geurts, 2006). The four main casein fractions lie in the following proportions within the CM: α_{s1}-casein 40%, α_{s2}-casein 10%, β–casein 35% and κ-casein 15% (Dalgleish, 2011). Although sharing many physicochemical similarities, the casein fractions present distinctions that will be discussed below.

5.1 α_{s1}-casein

There are currently nine variants of α_{s1}-casein. Two of them are predominant and have the same amino acid sequence, differing only in the number of phosphorylation sites (Farrel et al., 2004). These two most common states in bovine milk have 8 (α_{S1}-CN-8P) and 9 (α_{S1}-CN-9P) phosphorylated serine residues (Bijl, Vries, van Valenberg, & Hooijdonk 2014), the α_{S1}-CN-8P in triple concentration relative to the α_{S1}-CN-9P fraction (Heck, Faccio, Richter, Thony-Meyer, 2013). Thus, the reference protein for this family is α_{S1}-CN B-8P fraction (Farrel et al., 2004) and it contains 199 amino acids, and a molecular mass of ~23.6 kDa after the phosphorylation of 8 serine residues (Huppertz, 2018). This fraction is moderately hydrophobic and negatively charged at milk natural pH (Singh & Singh, 2016), with 25 amino acids residues capable of carrying a positive charge (Arg/His/Lys) and 40

capable of carrying a negative charge (Asp/Glu/SerP) (Huppertz, 2018). The major casein fraction, it presents a high percentage of proline residues (17) and the complete absence of cysteine residues (Huppertz, 2018) which breaks α-helix and β- sheet secondary structure (Dickinson, 1989). Additionally, the α_{s1}-casein fraction hydrophilic and hydrophobic amino acids residues are found in segregated agglomerates, leading to expressive peaks in the hydrophobicity profiles of this protein, conferring it a distinct amphipathic nature (Horne, 2014).

5.2 α_{s2}-casein

There are four variants of α_{s2}-casein in bovine milk. The differences between them concern to their degree of phosphorylation (Farrell et al., 2004). The reference protein has 11 phosphorylated serine residues and an internal disulfide bond (α_{S2}- CN-11P) (Singh & Singh, 2016). This is the most phosphorylated and, consequently, the most hydrophilic of all casein fractions (Singh & Singh, 2016). The α_{S2}-casein structure has three anionic phosphoserine clusters that result in a higher sensibility to ionic strength and Ca^{2+} activity (Swaisgood, 2003), what influence in the calcium- binding capacity of α_{S2}-casein (Singh & Singh, 2016). This casein fraction has only 10 proline residues, not as much as the other casein fractions, and has 2 cysteine residues (Huppertz, 2018), that participate on the intramolecular disulfide bond (Farrell et al., 2004). The primary structure of α_{S2}-casein contains 207 amino acids and a molecular weight of ~ 25.2 kDa for the 11P variant (Huppertz, 2018). According to Farrell, Malin, Brown, & Mora-Gutierrez (2009), an examination of α_{s2}-casein linear amino acid sequence shows that the molecule has 3 segments highly negatively charged, mainly due to phosphoseryl and glutamyl residues, and a C- terminal part positively charged.

5.3 β-casein

This casein fraction has 12 variants in bovine milk, but β-CN A2-5P is reference protein for this family (Farrell et al., 2004). The β-casein has 209 amino acids and a molecular mass of 24.0 kDa considering the reference protein primary structure with phosphorylation of 5 serine residues (Huppertz, 2018). This casein

fraction has a strongly amphipathic nature (Huppertz, 2018) due to the high contrast amino acid sequence: while the N-terminal part is highly charged, working as a polar domain, the C-terminal has zero net charge and high hydrophobicity (Swaisgood, 2003). As this protein also has the hydrophilic and hydrophobic residues in isolated regions, it also presents peaks in the hydrophobicity profiles (Horne, 2014). β-casein has 35 proline residues, the higher amount of all fractions, and a lack of cysteine residues (Huppertz, 2018). Just like the α_{s1}-casein fraction, the amino acid profile works breaking α-helix and β-sheet secondary structure (Dickinson, 1989). Because of its amino acids sequence, the structure of β-casein is significantly open and flexible, and it is the most hydrophobic of all caseins (Singh & Singh, 2016), with its hydrophobic regions particularly rich in proline residues (Horne, 2017).

5.4 κ-casein

The κ-casein is known for its quite heterogeneous composition with respect to both phosphorylation and glycosylation (Jensen et al., 2015). The κ-casein fraction can be found in 11 variants (Farrell et al., 2004), but there are 2 most common ones, called A and B, with A variant being predominant in most dairy breeds (Ng-Kwai- Hang & Grosclaude, 2003). The reference protein for *κ*-casein family, called κ-CN- A-1P, holds only one phosphoseryl residue (Singh & Singh, 2016). Due to its low degree of phosphorylation, this protein is not very sensible to Ca^{2+} presence (Leman, Kinsella & Kilara, 1989; Swaisgood, 2003), what plays an important role on integrity of CMs structure and colloidal stability (Waugh & von Hippel, 1956; Walstra, Geurts, Noomen, Jellema, & van Boekel 1999). Besides, k-casein is located on CMs surface, and, consequently, it controls the total micellar surface area and the CMs size (Horne, 2014). Considering its location, this casein fraction works as an interfacial agent between the hydrophobic CMs core and the hydrophilic aqueous environment (Creamer, Plowman, Liddell, Smith & Hill, 1998). The *κ*-casein is the smallest of the caseins, containing 169 amino acids residues, and a molecular mass of ~19.1 kDa for the reference protein after the phosphorylation of 1 serine residue (Huppertz, 2018). This fraction has 20 proline residues, and 2 cysteine residues, what may create a complex disulphide bonding design between κ-casein molecules (Huppertz, 2018). This casein fraction has an important peculiarity that differentiates

it from other caseins: it is glycosylated, with sialic acid residues in the 106-169 polypeptide portion, conferring a hydrophilic character (Singh & Singh, 2016). The 106 to 169 sequence, called the glycomacro-peptide due to glycosylation, presents polar amino acids that confer even higher hydrophilic properties to the molecule and a strong negative charge (Bylund, 1995). The N-terminal part containing the residues 1–105, called para-κ-casein, has a hydrophobic characteristic, is insoluble, and has a positive net charge at natural milk pH (Singh & Singh, 2016; Huppertz, 2018). In this way, the hydrophobic part of the κ -casein works as an anchor block, while the hydrophilic part provides steric stabilization to the CMs (Schorsch Carrie & Norton, 2000).

5.5 Casein micelle

CMs, despite the name, cannot be considered as true micelles taking the traditional micelle definition found in literature, that describes micelles as dynamical multimolecular assemblies of amphiphilic molecules (Fox & Brodkorb, 2008; Dalgleish, 2011). According to Dickinson (1992), this definition could not be applied since CMs are irreversibly formed over normal time-scales. Instead, CMs are complex spherical colloidal associations formed by the interaction of caseins fractions, aggregated with calcium phosphate, and surrounded by a hairy layer formed by *κ*-casein (Dalgleish, 2011; Horne, 2014). Although natural systems are generally size homodisperse, the CMs of bovine milk are polydisperse when milk is obtained from different cows (de Kruif, 1998). Authors present different ranges for micelle diameter, most of them close to 80 nm for the lower limit and to 500 nm for the upper limit, with an average size of 150 to 200 nm (de Kruif, 1998; Fox & Brodkorb, 2008; Balde & Aider, 2016; Chen et al., 2016).

Caseins fractions tend to associate to form the micelle structure through different molecular mechanisms including hydrophobic interaction, hydrogen bonding, electrostatic interaction, and calcium bridging (Fox & Brodkorb, 2008). Each fraction contributes to the formation and maintenance of the micellar structure. As it was showed in the former section, all caseins are phosphorylated at some degree. Casein phosphorylation is a post-translational modification catalyzed by kinase enzymes that attach phosphate groups to specific amino acids in the protein

sequence (Bijl et a., 2014). This modification is one of the key factors responsible for the stabilization of calcium phosphate in CMs and for the internal structure of the CMs (Bijl et a., 2014).

Phosphoseryl groups have high affinity for divalent ions, such as Ca^{2+}. A calcium ion can bind simultaneously to two phosphoseryl groups, leading to the formation of inter and intra protein bonds responsible for the aggregation of the casein molecules. Higher the degree of molecule phosphorylation (α_{s2}> α_{s1}> β> κ) (Huppertz, 2018), higher the association with calcium and, consequently, greater the stability of the aggregate formed. The regions in which an intense association between the phosphoseryl residues and precipitated calcium phosphate occur in the CM are called nanoclusters of calcium phosphate or colloidal calcium phosphate (Walstra et al., 2006).

Due to the high amount of phosphoseryl groups, the α_s-casein fractions associate strongly between each other. Since β and κ-caseins have a lower degree of phosphorylation (Fox, 2003; Huppertz, 2018) and a high proportion of proline, their secondary structures are limited. The β-caseins can bind to only one calcium phosphate nanocluster at a time, thus, the hydrophobic interactions between α_s and β- caseins are the most relevant (Croguennec, Jeantet & Brulé, 2008). Since κ-caseins have low phosphorylation but high amphiphilic character, they associate with α_s- casein aggregates essentially through hydrophobic interactions. In this configuration, the hydrophobic portion of k-casein is oriented towards the interior of the micelle while its hydrophilic tail is directed towards the surface, forming a kind of hairy cover with negative surface charge.

Over the years, several models have been proposed in an attempt to explain CM structure. Despite the huge number of models and reviews of models proposedso far (Waugh, 1958; Rose, 1969; Waugh, Creamer, Slattery & Dresdner, 1970; Farrell, 1973; Slattery & Evard, 1973; Schmidt, 1982; Walstra & Jenness, 1984;
Holt, 1992; Visser, 1992; Horne, 1998; Dalgleish, 1998; Walstra, 1999, Horne, 2003; de Kruif & Holt, 2003; Tuinier & de Kruif, 2002; McMahon & Oomen, 2008; Fox & Brodkorb, 2008; Dalgleish, 2011) there is still debate about the actual structure of CMs. Formerly, one of the most accepted model was the submicelle model, proposed by Walstra in 1984 and reviewed in 1999. The submicelle model attempts to explain

the structure of the CMs based on the interactions that occur between the casein molecules. In this model, the CMs are approximately spherical, made by two types of submicelles, one composed by α_{S1}, α_{S2}, and β-caseins, and other composed by α_{S1}, α_{S2} and κ-caseins. The hydrophobic portions are accommodated within the sphere while the hydrophilic (c-terminal part of the k-caseins) portions are oriented outwardly. The submicelles are bound together by calcium phosphate nanoclusters and hydrophobic interactions between the proteins. In this structure, submicelles have different positions, the first located in the center and the former, located in the outside of the micelle, forming a "hairy layer" closely related to the steric stabilization of the micelle. The figure 3 brings a schema of the submicelle model of Walstra.

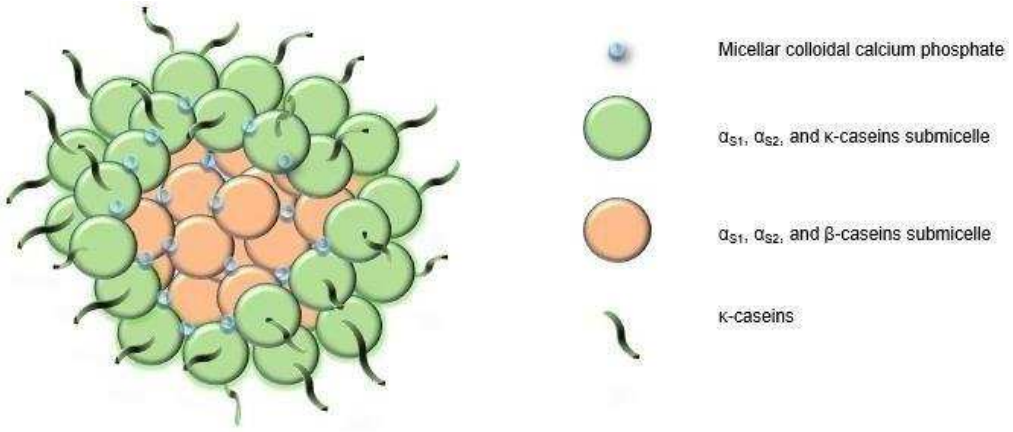

Figure 3. Schematic overview of the submicelle model of Walstra, Geurts, Noomen, Jellema, & van Boekel (1999).

Although this model had been the subject of numerous studies (Slattery & Evard, 1973; Schmidt, 1982; Walstra & Jenness 1984) the electron microscopy techniques used to find this structure creates artifacts that lead to inaccurate interpretation of the existence of submicelles (McMahon & McManaus, 1998; Holt, de Kruif, Tuinier, Timmins, 2003; Dalgleish, Spagnulo & Goff, 2004).

The open-structure model, which is currently the most widely recognized, states that CMs are formed by an entangled protein network between α and β-caseins stabilized by colloidal calcium phosphate nanoclusters and hydrophobic interactions between molecules. The surface of this protein network is covered by κ-casein molecules whose hydrophilic portions are oriented towards the aqueous environment.

The result of this model is also a spherical and open structure covered by a hairy layer formed by the k-caseins (Holt et al., 2003; de Kruif, Huppertz, Urban Petukhov, 2012). Despite the differences, both models agree on this aspect: the high occurrence of k-casein on the surface of the micelle, and its absence in the interior.

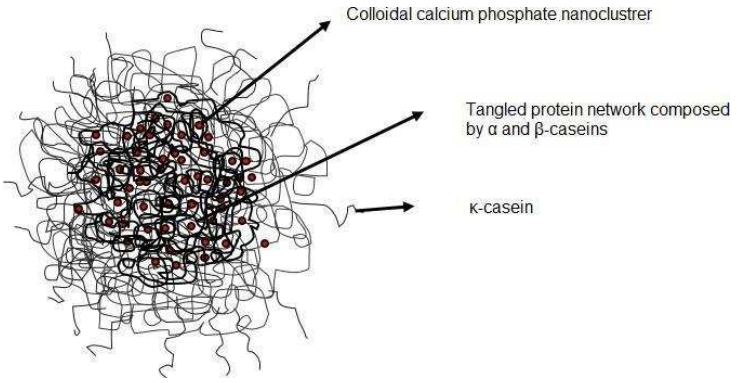

Figure 4. Schematic representation of the CM open structure model proposed by Holt et al., 2003.

5.6 Caseins as foam stabilizers

Milk proteins are one of the most common stabilizers in food systems, including whole milk, whey proteins, and caseins (McClements, 2016). Caseins are known since the beginning of colloidal science as molecules with remarkable surface activity and good stabilization properties due to their unique physicochemical characteristics (Dickinson, 2006) such as their low secondary structure (Walstra et al., 2006; Dalgleish, 2011; McClements, 2016). It means that caseins are highly flexible molecules capable of undergoing large conformational changes, what is relevant concerning interface adsorption capacity (Damodaran, 2008). This characteristic is mainly driven by the high amount of proline residues present in these proteins, an amino acid with rigid cyclic structure, that turns difficult molecule's twisting, playing an important role in determining protein conformation (Biedermannova, Riley, Berka, Hobza, & Vondrasek, 2008).

Despite of purified fractions being expensive and not representative of the reality of food systems they are often used in research studies because they simplify the comprehension of protein functional behavior (McClements, 2016). In this way, it is important to acknowledge that the different fractions of caseins present different

surface activities. The two fractions with the highest percentage representation, α_{s1} and β-casein, have a recognized high surface activity. The amino acid profile and the distribution of the hydrophilic and hydrophobic groups of α_{s1} and β-caseins, described in the previous section, justifies its high capacity to stabilize interfaces (Horne, 2014). To the date of this review, no studies about isolated α_{s2}-casein surface properties were found. Concerning κ-casein, due to the disulphide bonds that confer a relatively ordered structure and a higher resistance to complete unfolding at the interface (Graham & Phillips, 1976; Wong, Camirand, Pavlath, Parris & Friedman, 1996), this fraction has limited surface activity when compared with other caseins (Dickinson, 1989; Murphy and Fox, 1991).

Compared to whey proteins, caseins present similar viscoelastic behavior at interfaces when it concerns to stabilizing aerated systems (Williams & Prins, 1996; Silva, Saint-Jalmes, de Carvalho, Gaucheron, 2014). However, surface tension decreases and adsorption kinetics are better for caseins than whey proteins (Rouimi et al., 2005). A classic example of use of caseins stabilization ability is milk homogenization, in which the caseins, together with whey proteins, adsorb at lipid-water interface forming a protein film substituting the former lipoprotein fat globule membrane, making it more stable than previously due to the better stability of this new film (Walstra et al., 2006; Damodaran, 2008). In this case, caseins prevail at the interfaces as a result of their higher number of hydrophobic residues and their more flexible structure (Dalgleish, Goff, Brun, & Luan, 2002). Evidence suggests that the adsorption at interfaces is directly proportional to their concentrations in the aqueous phase and caseins represent most milk proteins (Hunt & Dalgleish, 1994). Caseins adsorption dynamics, as all proteins, is dependent of many factors such as accessible surface area, the time scale, and the protein concentration, among others. Depending on the conditions, nearly the complete unfolding of the peptide chain may occur, or the protein may preserve its native structure (Walstra & de Roos, 1993).

There is still no consensus among researchers regarding the interfacial properties of CMs. The early works on the surface aspects of milk bubbles using electron microscopy suggested that CMs adsorb at air–serum interfaces (Brooker, Anderson, & Andrews, 1986; Goff, Verespej, & Smith, 1999) forming a two-dimensional network (Mulder & Walstra, 1974). Some works, however, do not agree with this concept: Borcherding, Lorenzen, Hoffmann, & Schrader (2008) suggest that

CMs are not part of the immediate monolayer at the air-serum interface, or the primary air-serum interface, on foamed skimmed milk. Chen Sala, Meinders, van Valenberg, van der Linden, & Sagis, (2017) corroborates these results concluding that on foams formed by CMs dispersions, CMs may be randomly incorporated into the interface, or bonded in a primary layer formed by the peptides, caseins and smaller micelles. Another interpretation for the role of CMs on foams stabilization is given by Case et al. (2005) and Silva, Saint-Jalmes, de Carvalho, & Gaucheron (2014). These authors describe the adsorption of CMs at air-water interface as being composed of two stages: a fast initial decrease of surface tension caused by the arrival of CMs at the interface; a progressive and slower reduction of surface tension due to gradual dissociation of CMs which is followed by the adsorption of casein molecules at the interface.

6. FACTORS AFFECTING THE FOAMING PROPERTIES OF CMS

Environmental changes can modify CM structure and consequently change their performance as foams stabilizers (Zhang, Dalgleish & Goff, 2004; Borcherding, Lorenzen, Hoffmann, & Schrader, 2008; Chen et al., 2016;2017;2018). This review focus on temperature, pH, and particle size as factors influencing the properties of CMs as foams stabilizers.

6.1 Temperature

CMs are not fixed but a dynamic structure (Horne, 2014) with casein molecules being able to diffuse in and out of each micelle, depending on environmental conditions (Walstra et al., 2006). The amounts of non-micellar and micellar caseins are influenced by temperature, what has been seen a great influencer on the stability of the liquid films between the bubbles. Thus, as consequence of protein mobility, alterations on temperature impact CMs composition (Zhang et al., 2018) and consequently CMs foaming properties (Kamath, Huppertz, Houlihan & Deeth, 2008 a; Huppertz, 2010).

The modifications caused by an increase in temperature of CMs environment are the strength of hydrophobic interactions, the decrease in the solubility of colloidal calcium phosphate and higher calcium binding, which decrease protein net charge hence the electrostatic repulsion (Fox, 2003).

Heating milk until 100 °C do not lead to a significant change on micellar size, unless in the presence of whey proteins (Anema & Li, 2003). High heat treatments do not make casein molecules to show denaturation, as expected for most proteins, because they have little secondary structure, but at temperatures above 120 °C, caseins can gradually become insoluble (Walstra et al., 2006), mainly by the thermal dissociation of κ-casein, what can lead to CMs aggregation (O'Connell & Fox, 2003). Still, considering its natural pH, fresh milk can be kept at a temperature of 140 °C for over 10 minutes before coagulation happens (Huppertz, 2016). Heating can lead to caseins dissociation from CMs, the extent of dissociation increasing with temperature, although at 45 °C, 95% of caseins are in the form of micelles (Singh, 1995).

In this way, at higher temperatures, around 40 °C, a longer foam lifetime is found (Walstra et al., 2006). According to Kamath et al. (2008a), the foaming capacity of skim milk increases gradually with increasing foaming temperatures because skim milk surface tension and viscosity are inversely proportional to the temperature rise, what can greatly contribute to faster adsorption of milk proteins, including caseins, at air–serum surface. The authors found better foam stability found at 45 °C and suggest that it is due to the lower concentration of non-micellar caseins is at this temperature, what combined with the lack of denatured whey protein, could lead to the formation of a strong interfacial layer consisting predominantly of CMs. Borcherding, Lorenzen, Hoffmann, & Schrader (2008) studied the effect of foaming temperatures of skimmed milk ranging from 4 to 60 °C and found most stable foams at 50–60 °C. In this case, the authors correlated the increased foam stability to a decrease in surface tension and viscosity and higher adsorption velocity at higher temperatures.

Conversely, according to Walstra & Jenness (1984), a decrease in temperature promotes modifications such as the weakening of hydrophobic interactions and increase in the solubility of calcium phosphate promoting a weaker

binding of individual casein molecules in the micelles. As hydrophobic bonds become much weaker, there is an increase in the volume of CMs. The amount of free casein molecules, mainly β-casein, increases considerably, and below 5 °C no association of β-casein at CMs is found, the molecule remains unfolded, in solution, increasing milk viscosity and contributing to a higher CMs steric repulsion (Walstra et al., 2006). In this way, at low temperatures around 4 °C, skim milk foams readily, but the foam has a short life (Borcherding, Lorenzen, Hoffmann, & Schrader, 2008). The easy foamability and worse foam stability found at low temperatures could be explained by the fact that although being able to quickly adsorb on the interfaces, free proteins with random structure, such as α and β-casein, show no detectable surface viscosity (Boyd, Mitchell, Irons, Musselwhite & Sherman, 1973). It means that the foams formed by caseins molecules can be relatively unstable, since the adsorbed film with the low surface viscosity has little mechanical strength (Dickinson, 1989).

6.2 Particle size

In recent years, studies have shown that milk proteins foaming properties, including caseins, are dependent on the size and on the ratio between aggregates and non-aggregated proteins (Davis & Foegeding 2004; Rullier, Axelos, Langevin, & Novales, 2009, 2010; Fameau & Salonen, 2014; Chen, 2017; Schmitt, Gunes, Gehin-Delval & Leser, 2018). In this new perspective, during milk proteins adsorption, the primary interface, is composed by low molecular weight products, such as caseins and whey protein molecules, monomers and oligomers (Borcherding, Lorenzen, Hoffmann, & Schrader, 2008), casein peptides, and small micelles (Chen et al., 2017). Non-aggregated caseins, whey proteins and peptides can adsorb at the film surfaces and act as anchors for the aggregates (Borcherding, Lorenzen, Hoffmann, & Schrader, 2008; Rullier et al., 2009, Chen et al., 2017), that crosslink the two thin interfacial films of adjacent bubbles, leading to more stable films (Rullier et al., 2009). In the case of whole milk proteins used as stabilizers, CMs and whey protein aggregates are attached to this primary layer, using the no-aggregated proteins as anchors (Borcherding, Lorenzen, Hoffmann, & Schrader, 2008; Rullier et al., 2009). When using only one type of protein (caseins or whey proteins) the same behavior is observed (Chen et al, 2017; Rullier et al., 2010). The figure 5 brings a schematic

representation of CMs using low molecular weight products as anchors to form adsorbed layers.

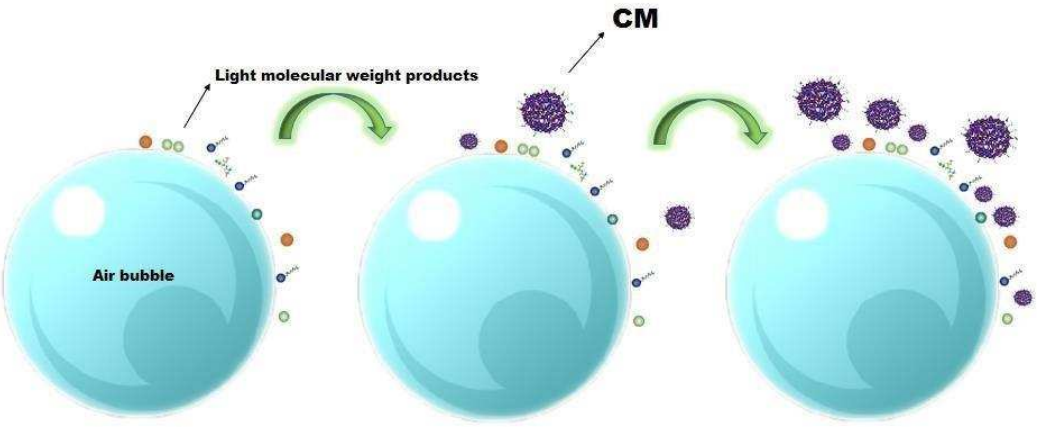

Figure 5. Schematic representation of CMs adsorption at a primary interface formed by low molecular weight products.

In this way, while small molecules main contribution for foam stabilization is related with its interfacial properties by causing interfacial tension decrease (Bos & Vliet, 2001), CMs stay trapped in the lamellar phase and plateau borders, due to their bigger size and the low surface activity, forming a gel network (Chen et al, 2017). This was found to be strong correlated with increasing foam stability, by increasing film life time, since the gel network formed is able to slow down drainage (Chen et al, 2017; Chen et al., 2018) and decrease coarsening and coalescence (Fameau & Salonen, 2014). The figure 6 brings a comparison of the aspect of interfaces between simple monolayers, formed by low molecular weight products, and the gel network, formed by particles and aggregates.

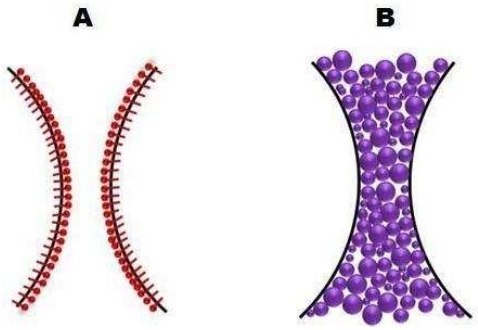

Figure 6. Scheme of interfaces stabilized by different molecules adapted from the information of Fameau & Salonen, 2014. A: Light molecular weight products forming simple monolayers. B: Big particles and aggregates forming a gel network.

Chen et al. (2016,2017,2018) compared the interfacial properties and size of CMs aggregates on foam stability of CMs dispersions. The results of the series of studies showed that the average size of colloidal particles in a dispersion have a significant role in foam stability, larger particles leading to more stable foams. Furthermore, foam stability as well as thin film stability increased with increasing bulk concentration of CMs aggregates. However, no correlation between surface rheological properties and better foam stability was found. Better foam stability was attributed to delayed drainage and less film ruptured due to the presence of casein aggregates, acting as hydrophilic particles, entrapped within the lamella. This does not mean that the interfacial properties are irrelevant for foam stability, but indicates that the thin film properties are probably prevailing.

6.3 pH

Casein molecules can only associate in the form of micelles in pH values ranging from 2.0 to 3.0 and 5.5 to 12.0 (Liu & Guo, 2008).

At pH values lower than 3.0, all the calcium phosphate nanoclusters are solubilized, and the casein molecules assembly in micellar structure happens through hydrophobic interactions, and also through hydrogen bonding (Liu & Guo, 2008). The CMs structure at low pH is more compact by the decrease in the electrostatic charge repulsion between casein molecules, what enhances the hydrophobic interaction between them. This suggests that the calcium phosphate nanocluster is not an essential factor in the formation of CMs (Zhong, Daubert, & Velev, 2007; Liu & Guo, 2008). Despite such evidence, some authors still consider the solubilization of calcium phosphate critical concerning integrity of CMs (Schorsch et al., 2000; Silva, Piot, de Carvalho, Violleau, Fameau, & Gaucheron, 2013; Gonzalez-Jordan, Thomar, Nicolai & Dittmer, 2015). Although related to temperature, the lowering of pH always leads, in greater or lesser extent, to the release of casein molecules from the micellar structure with the decrease of calcium binding (Dalgleish & Law, 1989; Singh Roberts,Munro, & Teo,1996). Zhang et al. (2004) studied the relationship between whippability and pH of skim milk powder (SMP), with pH varying from 3.0 to 8.0. The authors found best foamability of SMP at pH 3.0 and attributed this to the high amount of dissociated casein molecules at this pH value, which contributes to

the quick formation of adsorbed layers at the interfaces. To the date, no study on the stability of casein foams at low pH was found.

Approximating the pH to the isoelectric point of caseins (pI = 4.6) affects surface charges of κ-caseins and interferes in its role on steric stabilization of the CMs. The C-terminal part of κ -casein has high amount of Glutamate and aspartame negative residues, and a low amount of positively charged residues, resulting on a high negative net charge (Hupperzt, 2018). The pH lowering makes the negatively charged residues to become gradually protonated, occasioning collapse of κ-casein brush (de Kruif, 1999; Dalgleish, 2011). In this way, the repulsion becomes overwhelmed by the van der Waals attraction, which leads to a strong net attraction resulting in CM aggregation (Tuinier & de Kruif, 2002).

Caseins foams are unstable near to isoelectric point of caseins since coagulation of CMs leads to decrease of adsorption rate, delay of dynamic surface tension drop is decline of film lifetime (Marinova et al., 2009). Studying the effect of pH on SMP foaming capacity, Zhang et al. (2004) observed that it was very low in pH values between 4.0 and 5.0. The same was observed by Marinova et al. (2009) using sodium caseinate solutions. Both studies attribute the poor foamability to the precipitation of caseins and consequent lack of caseins able to adsorb at the surfaces and, thus, stabilize the foams (Marinova, 2009).

At high pH values, up and around 5.5, more carboxylic groups are ionized and the consequent electrostatic repulsion results in relaxing of the hydrophobic domains (Liu & Guo, 2008). Therefore, CMs become voluminous due to weaker hydrophobic interactions and stronger repulsive electrostatic interactions (Liu & Guo, 2008) and part of casein molecules dissociate from CMs (Dombrowski, Mattejat, & Kulozik, 2016). Dombrowski et al. (2016) found that foamability increase constantly from pH 6.0 to pH 11.0, with a maximum at pH 9.0 which the higher amount of free casein molecules leads to a quick and efficient covering of the air/water interface. However, the results indicate that although foam stability increase progressively from pH 6.0 to pH 9.0, it decreases from pH 10.0 to pH 11.0. The authors suggest that at pH values higher than 9.0 there are important structural changes, inclundind the loss of the micellar structure, meaning that most of casein molecules are present in the serum phase. The better foam stability found in pH 6.0

to 9.0 can be credited by (i) increased CMs diameter that reduced the reducing drainage and increases the interface viscoelasticity, (ii) increased negative net charge, that contribute by rising the distance between adjacent bubbles by electrostatic repulsion, avoiding coalescence and disproportionation rate.

Thus, given the impact of pH on the micelles structure, is possible to suggest that foam stability is increased when the difference between pH of foaming and the isoelectric point of the caseins is amplified.

7. PERSPECTIVES

Milk aerated foods are attractive for consumers because of their sensorial properties such as structure, texture and appearance. However, the shelf-life of these products can be limited due to the foam instability phenomena. Many studies have been done on the production and stability of foams using caseins, approaching variables such as casein fraction, effect of protein concentration, micelles size and presence of micelles aggregates, stress factors such as pH, temperature, Ca^{2+} chelating agents, and ethanol, among others (Ahmed & Dickinson, 1990; Murphy & Fox, 1991; Zhang et al, 2004; Sanchez & Patino, 2005; Borcherding, Lorenzen, Hoffmann, & Schrader, 2008; Marinova et al., 2009; Silva et al., 2013; Amine, Dreher, Helgason, & Tadros, 2014; Fameau & Salonen, 2014; Martínez-Padilla, García-Mena, Casas- Alencáster, & Sosa-Herrera 2014; Chen et al., 2016;2017;2018; Dombrowski, Dechau, & Kulozik, 2016). Still, some limitations of these proteins and their organization in the micellar structure impose barriers that narrow their use as foam stabilizers in the food industry.

Therefore, the improvement of CMs functionality is not only a challenge but also a necessity and an opportunity for food industry. In this regard, the increase in CMs stability against different stress situations was found to be achieved by crosslinking (Mounsey, O'Kennedy & Kelly, 2005; Heck et al., 2013; Silva, Saint-Jalmes, de Carvalho, & Gaucheron, 2014; Casanova, Silva, Gaucheron, Nogueira, Teixeira, Perrone, 2016). Protein crosslinking by either chemical, enzymatic, or chemoenzymatic ways comprehends the establishment of covalent bonds between polypeptides (Heck et al., 2013). In food industry, the controlled enzymatic

crosslinking reaction has proven to be especially interesting since enzymes are suggested as protein crosslinking agents in consequence of their high specificity, mild reaction conditions and low risk of toxic products formation (Stojadinovic, Pieters, Smit, Velickovic, 2014).

In the early 1980's, Ikura, Kometani, Yoshikawa, Sasaki & Chiba first studied the crosslinking of caseins by transglutaminase (TGase). Today, it is the most common enzyme used for protein crosslinking in food industry. The fact that caseins are good substrates for TGase has been credited to their flexible structure and the absence of disulphide bonds in the α_{s1}- and β-caseins, exposing reactive groups to TGase (O'Connell & de Kruif, 2003; Bonisch, Tolkach, & Kulozik, 2006). TGase catalyzes acyl transfer reactions between the γ-carboxamide group of glutamine and primary amines such as ε-amino groups of lysine, and intermolecular reactions between protein-bound glutamine and lysine residues (Raak, Abbate, Lederer, Rohm, Jaros, 2018). Simplifying, the major mechanism of TGase action involves polymerizations, which modifies proteins hydrophobicity (Camolezi Gaspar & Pedroso de Góes-Favoni, 2015). The rate order of crosslinking in a raw milk system was found to be κ-casein > β-casein > αs-casein (Smiddy, Martin, Kelly, de Kruif & Huppertz, 2006) and is seems to be related with accessibility of caseins in CMs (Hinz, Huppertz, & Kelly, 2012). TGase can polymerize casein components, by formatting intermolecular crosslinks, without impairing functional properties displayed in both presence and the absence of calcium ions. This indicates that TGase can hold the casein molecules inside the CM structure and change caseins rheological properties improving their functional properties (Ikura et al., 1980).

Commercially, transglutaminase (TGase) is an enzyme produced by the microorganism Streptoverticillium moboarense (Yokoyama, Nio, & Kikuchi, 2004). Since 1998, TGase has been recognized as a Generally Recognized as Safe (GRAS) substance by the Food and Drugs Administration (FDA), what makes it very interesting for food industry (Aalami & Leelavathi, 2008). Besides, different levels of protein modification can be achieved by altering conditions such as temperature, pH, enzyme concentration, substrate accessibility and specificity (Romeih & Walker, 2017)

Many works have applied TGase in the search for better understanding of casein crosslinking prospects and limitations and how it could be applied in favor of food products. Smiddy et al. (2006) found that treatment of milk with TGase increased the stability of casein micelles against disruption of hydrophobic interactions, solubilization of micellar calcium phosphate and high-pressure treatment. Mounsey, O'Kennedy & Kelly (2005) found not only that TGase treated CMs had increased stability against dissociation by heat in alkali pH, and higher ethanol stability, but also that TGase increased stability of CMs during the acidification. Besides, milk proteins crosslinking using TGase seems to be an efficient alternative to advance sensorial and economic characteristics of dairy products such as cheeses (Cozzolino, Pierro, Mariniello, Sorrentino, Masi, Porta, 2003), ice cream (Kuraishi, Yamazaki & Susa, 2001; Rossa, de Sá, Burin, & Bordignon-Luiz, 2011) and yogurt (Lorenzen, Neve, Mautner, & Schlimme, 2002).

Considering the great impact of protein functionality in the development of organoleptic and nutritional characteristics of dairy products, TGase represents a promising tool to expand the functional properties such as gelation, solubility, water-holding capacity, emulsifying capacity, foaming, viscosity, elasticity and protein content (Romeih & Walker, 2017).

Since the crosslinking of the CMs makes it more stable at high temperatures, low pH values, and do not reduce the particle size of CMs, comparatively to the native CMs (Mounsey, O'Kennedy & Kelly, 2005), the stabilization of foams using cross-linked CMs can open new possibilities for food industry. Some studies have been done on interfacial and foaming properties of sodium caseinate and individual casein fractions treated with TGase (Han & Damodaran, 1996; Faergemand & Murray, 1998; Partanen et al., 2009; Zeeb, Beicht, Eisele, Gibis, Fischer & Weiss, 2013). However, studies about the effect of TGase on interfacial and foaming properties of casein micelles are still lacking.

It is essential to emphasize the fact that TGase treatment do not reduce the particle size of CMs, indicating very little inter-micellar crosslinking (Mounsey, O'Kennedy & Kelly, 2005). This is particularly important since the size of CMs has been reported to be closely related with foam stability. Besides, since TGase can hold the casein molecules inside the CM structure it is reasonable to think that the enzyme

may alter the balance between micellar and non-micellar casein, which also has a huge impact on foaming properties of CMs. Unique characteristics and benefits like the possibility produce “clean-label” dairy products, without the requirement of synthetic stabilizers, and the favorable influence in the texture of low-fat products (Nielsen, 1995) can be obtained by enzymatic crosslinking using TGase, hence, it has a great potential for the food industry (Gauche, Tomazi & Bordignon-Luiz, 2008).

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CHARPTER 2: EFFECT OF PH ON THE STABILITY OF FOAMS BASED ON CASEIN MICELLES CROSS-LINKED WITH TRANSGLUTAMINASE

EFFECT OF PH ON THE STABILITY OF FOAMS BASED ON CASEIN MICELLES CROSS-LINKED WITH TRANSGLUTAMINASE

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ABSTRACT

Enzymatic crosslinking with transglutaminase (Tgase) is a method capable of increase the stability of casein micelles (CMs) against pH collapse. The objective of this study was to evaluate if the higher pH stability of CMs cross-linked with Tgase (CMs-Tgase) influence foam stability, comparatively to native CMs. The micellar suspensions of native CMs (MCS-CMs) and CMs-Tgase (MCS-CMs-Tgase) were prepared at 27.5 g/L in deionized type 1 water with 2 mM $CaCl_2$, and acidified at pH values ranging from pH 7.0 to pH 2.0. The samples obtained were analyzed in terms of particle size, charge, surface tension, absorbance and foam stability. The size and the charge (ζ-potential) were determined by dynamic light scattering. There was found no significant difference between the diameter values of native CMs and CMs- Tgase. Dispersed CMs-Tgase are stable in pH 2.0–3.0 while native CMs precipitates at this pH range. Native CMs precipitated below pH 5.5; CMs-Tgase only precipitated from pH 3.5 to 4.5. The isoelectric point (pI) of native CMs and CMs- Tgase was found to be pH 4.7 and 4.3, respectively. The highest foam stability was observed at pH 3.5 for control samples and at pH 5.0 for CMs-Tgase, where the foam kept stable for over 48 hours. The foams with CMs-Tgase were more stable than the ones with native CMs from pH 5.0 to 2.0 and less stable between pH 7.0 to 5.5. These results shows that cross-linking by Tgase increases the foam stability of CMs at acid pH, what may be of interest for the manufacturing of products such as fruit ice cream, frozen yogurt, and other acid desserts.

Key words: Casein micelles, transglutaminase, crosslinking, foam stability.

9. INTRODUCTION

Proteins have been classically studied from the nutritional perspective and capacity to form colloidal structures. However, in food industry context, proteins have more than nutritional purposes and the colloidal structure formed has a great impact in texture and shelf life of food products (Foegeding & Davis, 2011). Foaming properties of proteins received attention due to their molecular characteristics related to adsorption kinetics and interfacial properties, and are the usual molecules used to form and stabilize food foams (Wierenga & Gruppen, 2010).

Caseins and whey proteins present similar behavior at interfaces (Williams & Prins, 1996; Nogueira Silva, et al., 2014), but the decrease in surface tension and the adsorption kinetics are better for caseins than for whey proteins (Rouimi et al., 2005). Caseins are a group of milk-specific proteins in a mixture of four different casein fractions associated in so called casein micelles. Caseins are highly flexible molecules, capable of undergoing large conformational changes (Damodaran, 2008) but casein micelles (CMs) structure can be modified by changes in their physicochemical environment (Nogueira et al., 2019). Through controlled physical, chemical or enzymatic methods, the structure and the assembly of casein molecules in CMs can be adjusted, altering their interfacial characteristics, what may lead to positive or negative changes in their foaming properties (Nogueira Silva et al., 2013).

Near to pI of caseins (pH 4.6), the micellar structure collapses and consequent coagulation occurs (Huppertz, Fox & Kelly, 2018). Thus, the casein foams near to the pI become unstable because coagulation of CMs leads to decrease of adsorption rate, delay in drop of dynamic surface tension and shortening of film lifetime (Marinova et al., 2009).

In previous studies conducted by our laboratory, it was demonstrated that CMs cross-linked with transglutaminase (CMs-Tgase) presented higher stability against acid pH, comparated to native CMs (Nogueira et al., 2019). Tgase is an enzyme found in various animal tissues and plants, commercially produced by the microorganism Streptoverticillium moboarense (Yokoyama, Nio, & Kikuchi, 2004) and Generally Recognized as Safe (GRAS) by the Food and Drugs Administration (FDA) (Aalami & Leelavathi, 2008). This enzyme has been widely used in food industry due to its ability to form crosslinks between proteins, to improve firmness, elasticity, viscosity, heat stability, and water-holding capacity of food such as seafood, meat products, noodles, baked goods, and dairy products, among others (Romeih & Walker, 2017).

The formation of food foams may occur at different pH ranges and in certain cases the casein foams cannot be formed or are unstable due to the micelle structure (Rouimi et al., 2005). Although not yet explored, the treatment of CMs with transglutaminase may be an alternative to overcome this inconvenience by increasing the stability of casein foams in acidic food systems.

The objective of this study was to compare CMs cross-linked with Tgase to native CMs concerning foam stability, at pH values ranging from pH 7.0 to pH 2.0, at intervals of 0.5. MCS-CMs and MCS-CMs-Tgase were analyzed in terms of particle size, charge, surface tension, absorbance and foam stability.

10. MATERIALS AND METHODS

10.1 Milk and ultrafiltrate

Fresh raw milk was skimmed by a cream separator and micro filtrated twice. The first micro filtration used a 1.4 µm pore sized membrane and was applied to remove microorganisms. The second one used a 0.1 µm pore sized membrane and was used to separate caseins from whey proteins. After protein separation, lactose was removed from casein rich retentate using diafiltration with deionized water. The retentate was diafiltrated 5 times in a 0.1 µm pore sized membrane. The resulting dispersion was spray dried using lab-scale single state spray dryer (Minor production, Niro atomizer, GEA, Germany) with 180 °C inlet air and 85 °C outlet air temperature. The micelar casein powder (MCP) obtained (85.7 % total protein, 8.25 % ash, 3.8 % moisture) was sealed in aluminum vacuum covered bags and stored at 4°C until its use.

10.2 Casein suspensions preparation

Micelar casein suspensions (MCS) at 2.75 % (w/w) were obtained by rehydrating MCP in deionized type 1 water with 2 mM of calcium chloride at pH 6.7. Sodium azide 0.3 g/kg (Synth, Diadema, SP) was added to prevent microbial growth. MCS was kept under stirring at 700 rpm at room temperature (25 ± 2°C) for 72 hours or until completely rehydration of casein micelles, checked by Dynamic Light Scattering (DLS) determined on a Zetasizer Nano-S (Malvern Instrument, Worcestershire, UK). Since CM diameter ranges from close to 80 nm to 500 nm, with an average size of 150 to 200 nm (de Kruif, 1998; Fox & Brodkorb, 2008; Balde & Aider, 2016; Chen et al., 2016), it was considered a complete rehydration when 90 % of powder particles presented equal or lower size to 0.2 µm (Fialho et al., 2017).

Microbial transglutaminase (Tgase) (Activa ®) with declared 100 U/g, purchased from Ajinomoto Food ingredients, was rehydrated in type 1 water at concentration of 10% (w/w), and added in a concentration of 3 U per gram of casein in half of every volume of MCS prepared. The control sample consisted of MCS without Tgase. The Tgase treated samples and the control samples were incubated in a water bath at 45 ºC for 1 hour, followed by inactivation of the enzyme by heat treatment at 85 ºC for 5 minutes. Then, samples were cooled down rapidly to 4 ºC and stored at this temperature prior to acidification.

10.3 Sample acidification

MCS-CMs and MCS-CMs-Tgase were aliquoted and acidified with HCl (1 M, 0.05 M or 0.025 M) at pH values ranging from 7.0 to 2.0, at intervals of 0.5, at 4°C to prevent precipitation. Then, temperature was raised to 25 °C before analysis.

10.4 Zeta Potential

Zeta potential measurements were determined with particle electrophoresis instrument (Zetasizer Nano-ZS, Malvern Instruments, UK) using capillary cells equipped with gold electrodes. Dispersion was previously diluted (1/10) in type 1 water on same pH of the samples at room temperature (25 ± 2 °C). The measurements were performed with an applied voltage of 50 V. Zeta potential (ζ) was calculated from the electrophoretic mobility (μ) using Henry equation, as follows:

$$\zeta = \frac{3\square\square}{2\square\square(\square\square_{\square})}$$

Where:

η represents the solvent viscosity (Pa/s),

μ is the electrophoretic mobility (V/Pa s),

ε is the medium dielectric constant (dimensionless),

κ^{-1} is the Debye length (measured thickness of the double electric layer around the molecule) (nm),

R_H is the particle radius (nm)

f $f(\kappa R_H)$ is the Henry function (dimensionless)

The electrophoretic mobility conversion in zeta potential is made automatically by the Malvern software data analysis. Each individual result was calculated as two measurements average, each with three sub-measures.

10.5 Z-average diameter

Hydrodynamic diameter (D_h) was determined by dynamic light scattering (DLS) (Zetasizer Nano-S, Malvern Instrument, UK). Samples were appropriately diluted (1/10) in type 1 water, on same pH of the MCS, at room temperature (25 ± 2 °C). The viscosity of the MCS was considered the same of pure water (1.033 mPa/s). Each individual result was calculated from two measurements average, each with three sub-measures.

10.6 Spectrophotometry

The amount of dispersed CMs in MCS was assessed by spectrophotometry using a 96 Well Microplate reader (Multiskan 60, Thermo Scientific) for all treatments. The samples were acidified and kept still for 2 hours at room temperature (25° C ± 2 °C) prior to analyses, to allow all the proteins not in suspension to precipitate. After, 200 µL of each sample's supernatant was placed in a micro-well and read at 400 nm wavelength. Measurements were performed in duplicate.

10.7 Surface tension

The surface tension (ST) of the casein suspensions were evaluated in a tensiometer (Dataphysics, model DCAT 21), at room temperature (25 ± 2 °C), by applying the Wilhelmy plate method, with the value of 0.03 as the limit of standard deviation. The samples were acidified and kept still for 2 hours at room temperature (25° C ± 2 °C) prior to analyses, to allow all the proteins not in suspension to precipitate. Data were obtained using SCAT software v. 3.2.0.84. Measurements were carried out in duplicate.

10.8 Foam stability

Foam stability was measured following the protocol described by Nogueira Silva et al. (2013). Two syringes containing 4 mL of samples and 4 mL of air, respectively, connected through a plastic connector, were used as a foaming device, by pushing alternatively the plungers of both syringes during 30 seconds. A metronome playing 130 beats per minute was used to standardize the speed of

pushing. The time of half-life of the foam as a function of pH was used to evaluate the foam stability. Measurements were performed in duplicate.

10.9 Statistical Analysis

All experiments were performed in duplicate and results expressed as mean and standard deviation. An analysis of variance (ANOVA) was used to compare the treatments. It was established at 0.05 the level of rejection of the null hypothesis. Data analyzes were performed using SAS software (version 9.0, SAS Institute Inc., Cary, NC, USA).

11 RESULTS AND DISCUSSION

11.1 Zeta Potential

Tgase has been recognized to improve the stability of CMs to pH variations (Nogueira et al., 2019). Such resistance can be related with the ability of Tgase to crosslink CMs, altering their pI, which can be measured by their charge (Tang et al., 2005). To observe if the CMs-Tgase in this study would differ from the native CMs in relation to the pI, ensuring greater pH stability, the ζ-potentials of these particles were determined in the pH range of 7.0 to 5.0 and 3.0 to 2.0 (Figure 7). From pH 4.5 to 3.5, measurements were not possible for both native CM and CM-Tgase due to the presence of unreliable intensity peaks in particle electrophoresis.

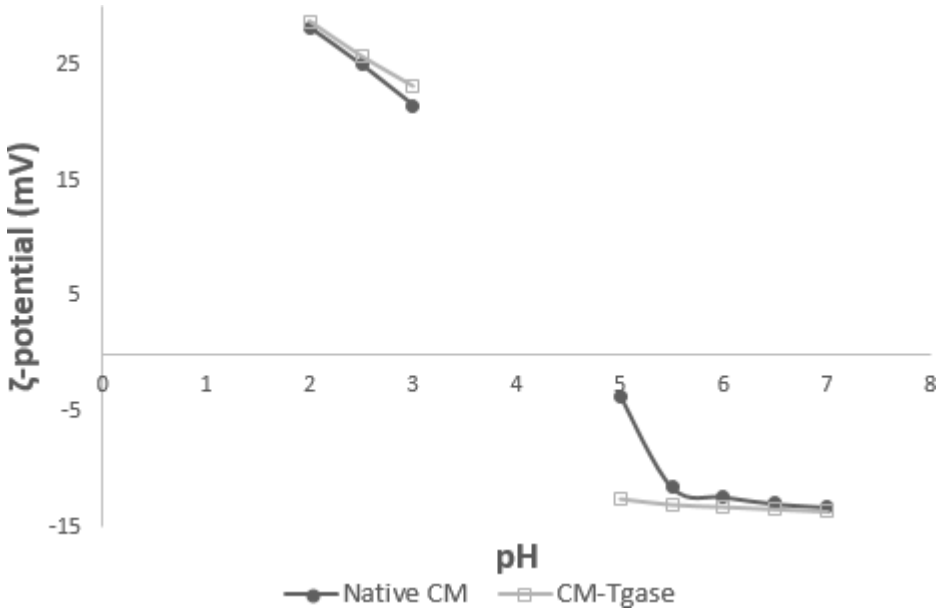

Figure 7. ζ-potential as a function of pH *of native CMs (●) and CMs-Tgase (○).*

The measurements ranged from – 13,2 mV to -3.2 mV for native CMs and -13.7 mV to -12.0 mV for CMs-Tgase in this pH range. These values are about 40% higher than the usual ζ-potential for CMs reported in the literature (Nogueira et al., 2019; de Kruif & Holt, 2003). Nevertheless, different diluents and measurement techniques have influence in ζ-potential values and should be considered when comparing reported results (Glantz et al., 2010). During acidification, the ζ-potential of native CMs and CMs-Tgase increase due to the neutralization of the negative charges of external κ-casein layer (Tuinier & de Kruif, 2002; Walstra et al., 2010). The pI of CMs and CMs-Tgase, seen as the intersection of x-axis curve on the z-axis at 0 mV, was calculated as 4.7 and 4.3, respectively. These results are in accordance with reported by Nogueira et al. (2019), working with Tgase, and Casanova et al. (2017), who found similar results for CMs cross-linked with genipin. A significant difference between ζ-potential for native and CMs-Tgase was found around the pI, in the pH 5.5 and 5.0. This can be understood because of Tgase crosslinking activity

that mainly happens in the κ-caseins (Tang et al., 2005; Huppertz & de Kruif, 2007a, 2007b), which are directly related to the CMs net charge and steric stabilization (Huppertz, Fox & Kelly, 2018). This effect is predominantly noticed around the pI, when Tgase impacts the neutralization of negative charges, which confers higher stability to CMs (Nogueira et al., 2019).

11.2 Z-average diameter

Studies have shown that casein foaming properties are dependent on the size of CMs and their aggregates (Chen et al., 2016; Chen et al., 2017). Larger particles can stay trapped in foam liquid channels, forming a gel network that increases foam stability (Chen et al, 2017; Fameau & Salonen, 2014). Thus, it is interesting to note if crosslinking will cause significant effects on the size of CMs, which may lead to changes, either positive or negative, in their foam stability.

The D_h, of native and cross-linked CMs evaluated as a function of pH was assessed by dynamic light scatering. In the pH range from 7.0 to 5.5 the native CMs presented a D_h (161.1 ± 3 nm) while the cross-linked CMs presented a D_h (160.2 ± 2 nm). The values found for native CMs are in accordance with the literature (de Kruif, 1998; Fox & Brodkorb, 2008; Dalgleish & Corredig, 2012). Concerning the values found for CMs-Tgase, no significant difference between them and the ones found for control samples was identified. This indicates no substantial intermicellar crosslinking (Mounsey, O'Kennedy, & Kelly, 2005; Huppertz & de Kruif, 2007b). Some works showed that crosslinking lead to a decrease in the size of CMs (Schorsch, Carrie & Norton 2000; Vasbinder, Rollema, Bot, & de Kruif, 2003), but also studies in which crosslinking led to an increase (Mounsey, O'Kennedy, & Kelly,

2005) and still others in which it had no significant influence in the size of micelles (Smiddy et al., 2006; Bönisch, Lauber & Kulozik, 2007; Huppertz & de Kruif, 2007b; Nogueira et al., 2019), as happened in the present study. In cases where the variation was found, in increasing or decreasing the CMs size, this was in the order of 10%. In this way, the more important is that the results here found indicate that benefits or harms to the foam stability, achieved through CMs crosslinking will not be related with changes in CMs size at this pH range, since such changes were noticed when much larger size variations were studied (Chen et al, 2017; Fameau & Salonen, 2014).

At pH 5.0 only the CM-Tgase suspensions provided reliable data. At this pH value, it is not expected to find non-aggregated CMs, however, in this case pH 5.0 was far enough from pI (4.3) making possible to find dispersed CMs with D_h (156.2 ± 2.1 nm). In both suspensions, a small decrease in CMs size was observed with the pH lowered from 7.0 to 5.0. This behavior was also observed by Nogueira Silva et al., (2013) and can be understood because of solubilization of colloidal calcium phosphate combined with favoring of hydrophobic bonds resulting from the decrease in pH (Liu & Guo, 2008).

In the pH range from 4.5 to 3.5, measurements were not possible for any of the samples. Caseins carry a net negative charge at the pH of milk (Lucey & Horne, 2018). Near to the pI of caseins occurs a partial neutralization of negative charges in the κ-caseins hairy layer of CMs, which leads to a decrease in electrostatic repulsions between CMs (Tuinier & de Kruif, 2002; de Kruif & Holt, 2003). Besides, the progressive solubilisation of colloidal calcium phosphate with reduction of pH (Dalgleish & Law 1989; Mekmene et al. 2010) interferes in internal CMs stability by reducing the degree of interactions between the α_s-caseins (Lucey & Horne, 2018).

These two phenomena happening together results in inter-micelle interactions, with subsequent coagulation of caseins. The presence of visible precipitates caused unreliable intensity peaks in dynamic light scatering measurements (Figure 8).

From pH 3.0 to 2.0 measurements were not possible for control samples. Although many particles within the range of values for CMs were found in this pH range, measurements were not reliable since more than one particle size population was observed. These populations were both larger and smaller in size than the predominant population (Figure 8). The Figure 9 exemplifies an appropriate size distribution curve.

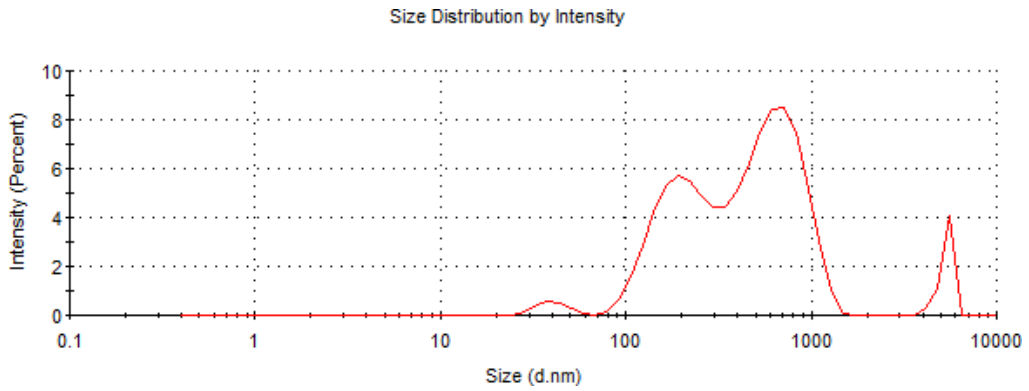

Figure 8. Unreliable size distribution curve for native CMs found in the pH range between 3.0 and 2.0

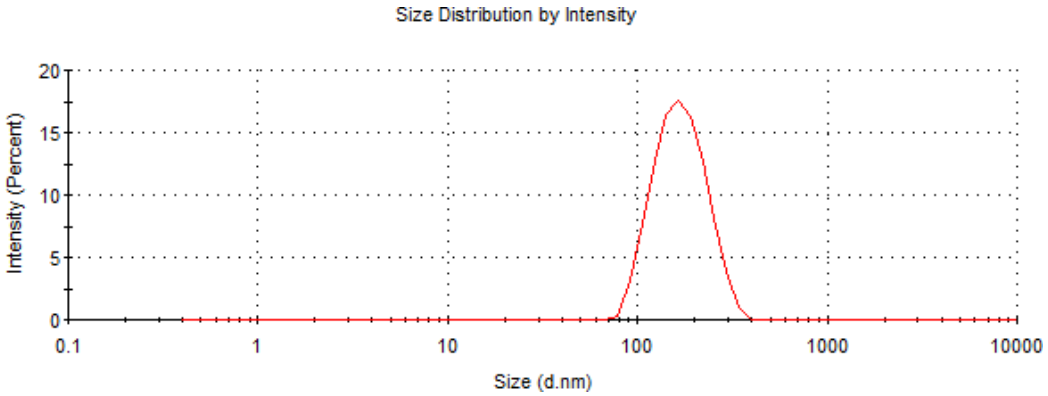

Figure 9. Desirable size distribution curve for native CMs.

Larger particle populations can be understood as aggregates of CMs, normal at low pH values (Sinaga, Bansal & Bhandari, 2016). As for the particles of less diameter, they exist thanks to the phenomenon of formation of small micelles at low pH, resulting from the solubilization of colloidal calcium phosphate and the strengthening of hydrophobic interactions (Nogueira Silva et al., 2013). CMs-Tgase kept stable even when all their colloidal calcium phosphate was solubilized and presented a D_h between 211±4nm and 205 ±2nm in this pH range (Figure 10). Similar results were found by Nogueira et al., (2019) and for Nogueira Silva et al. (2013) and Casanova et al. (2017), studying cross-linked CMs with genipin. However, this result goes against Liu and Guo findings (2008), who found more compact CMs structures at pH values below the pI. In any case, the presence of CMs

-Tgase at these pH values may be attributed to the formation of irreversible intra-casein linkages, that prevents the release of casein fractions into the serum phase at low pH values (Vasbinder, Rollema, Bot, & de Kruif, 2003).

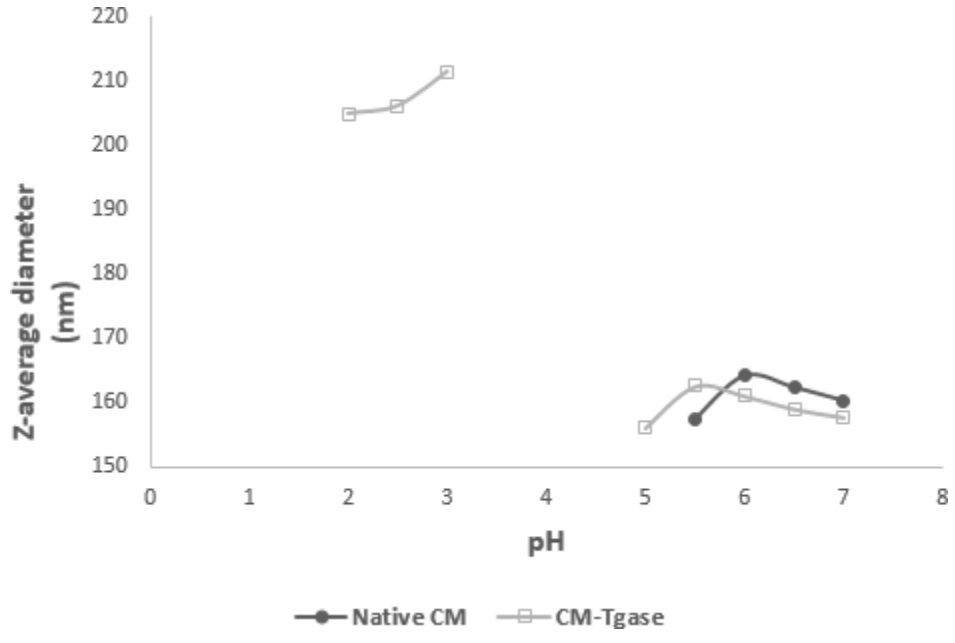

Figure 10 Z-*average diameter as a function of pH of native CMs (●) and CMs-Tgase (○).*

11.3 Spectrophotometry

This analysis is based on the quantitative measure of light absorption by solutions. In this study, due to the applied methodology, it was used as an indication of the amount of suspended CMs. The dispersed CMs are of importance for the stabilization of foams, since these dispersed proteins will be the ones available to adsorb in the interfacial film (Cantat et al., 2013).

The absorbance of dispersed CMs as a function of pH was assessed by spectrophotometry (Figure 11).

Figure 11. Absorbance as a *function of pH of native CMs (●) and CMs-Tgase (○).*

In the pH range from 7.0 to 5.5 there was no significant difference between MCS-CMs and MCS-CM-Tgase, that showed absorbance of 2.427 ±0.014 and 2.427 ± 0.038, respectively. These results confirm that native CMs are stable at this pH

values, with no precipitation, and that Tgase has no positive or negative effect on it at this pH range (Nogueira et al., 2019).

At pH 5.0, a significant difference of absorbance was detected betweentreated and no treated MCS (Figure 11). At this pH value the native CMs precipitate almost completely (0.215 ± 0.063), while the CM-Tgase remain suspended (2.244 ± 0.004). It was aligned with no visual precipitation. In pH values of 4.5 and 4.0, differences between MCS-CMs and MCS-CMs-Tgase are also identified. While the absorbance for MCS-CMs in this range is 0.227 ± 0.064, it is of 0.346 ± 0.200 for MCS-CM-Tgase (almost 50% higher). These data are in accordance with the fact that the dispersion was composed mostly by proteins which precipitate around the pI. The higher values for MCS-CM-Tgase agree with the fact that Tgase confers better pH stability to CMs, and decrease the pI, allowing a greater amount of casein micelles to remain in the supernatant at low pH values.

From pH 3.5 to 2.0, the significant difference between MCS-CMs and MCS-CMs-Tgase becomes more evident, being notice even visually. An interesting phenomenon can be observed at pH 3.5 in figure 11. At this pH value, both MCS- CMs an MCS-CMs-Tgase present a peak of absorbance (1.504 ± 0.029 and 1.912 ± 0.011, respectively). As dispersed CMs were not detected by DLS at this pH value neither for native CMs or CMs-Tgase suspensions, this result indicates that although in an aggregated state, the CMs were able to remain in suspension. Besides, the favoring of the hydrophobic bonds could lead to the formation of small CMs, which would maintain the micellar structure, despite the solubilization of the colloidal calcium phosphate (Liu & Guo, 2008). The higher absorbance for MCS-CMs-Tgase points out to a greater amount of small aggregates and/or CMs in suspension, as

visual precipitation was not detected. For native MCS-CMs, this result suggests that although precipitation happens, small aggregates can keep in suspension.

In the pH range from 3.0 - 2.0 absorbance decreases for both samples, indicating precipitation of CMs. However, the dispersed CMs indicted by absorbance in MCS-CMs-Tgase were 65% higher than in the native ones, what is in accordance with the higher pH stability found in this study and reported in literature (Smiddy et al., 2006; Nogueira et al., 2019).

11.4 Surface tension

The ability to efficiently adsorb at interfaces and decrease surface tension is a physicochemical factor usually related with good foaming behavior for a protein (Marinova, Basheva, Nenova, Temelska, Mirarefi, & Campbell, 2009). In this study, Tgase crosslinking significantly raised the surface tension of MCS from pH 4.0 to 2.0 (Figure 12).

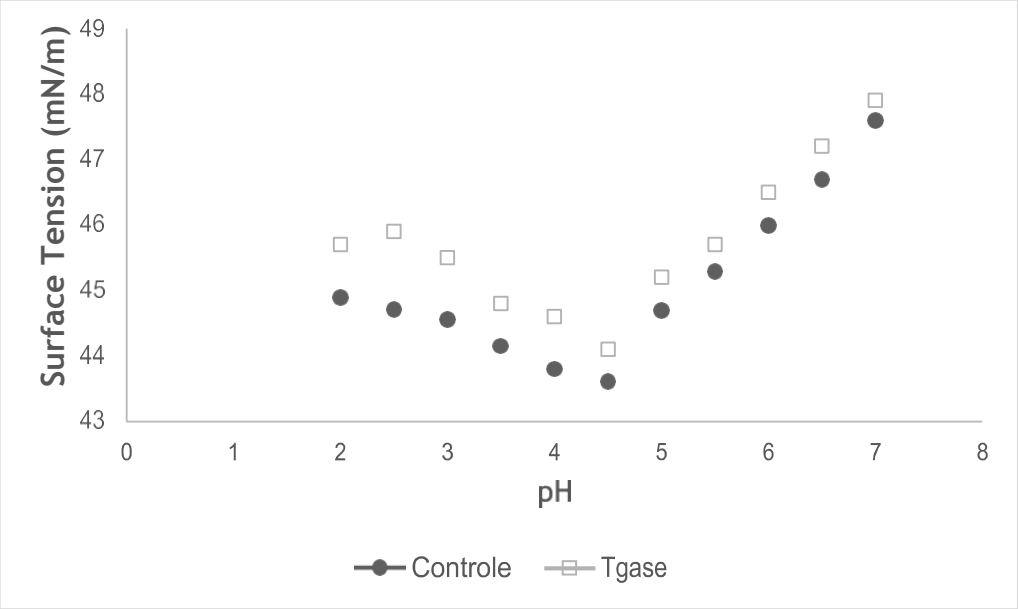

Figure 12. Surface tension as a function of pH of native CMs (●) and CMs-Tgase (○).

In the pH range from 7.0 to 4.5 there was no significant difference between MCS-CMs and MCS-CM-Tgase. The MCS-CMs presented a surface tension between 47.6 ± 0.3 mN/m and 43.6 ± 0.7 mN/m while the MCS-CMs-Tgase presented a surface tension between 47.9 ± 0.5 mN/m and 44.1 ± 0.2 mN/m at this pH range. Below pH 4.5 the difference between control and Tgase suspensions surface tension is significant. Tgase acts by crosslinking the CMs both internally and on the surface. Thus, it is possible to infer that such crosslinking would impair the unfolding of the CMs at the air-water interfaces, thus increasing surface tension. This effect is mainly noticed at low pH values when Tgase activity impacts on stability of CMs.

11.5 Foam stability

The stability of food foams is highly important in the food industry due to its relation to the sensorial characteristics and patterns sought by consumers of foamed products. Besides, high stability indicates a longer shelf life, which is of economic importance to the food industry.

The stability of the foams formed for all suspensions was evaluated as a function of pH and expressed in half-life time, which was the time taken for the initial total volume of foam formed to reach half of its original volume. All 22 suspensions tested in duplicate can form an initial volume of 8 mL of foam. Both MCS-CMs and MCS-CMs-Tgase showed peaks in the curves at pH values between
5.5 and 5.0 and at pH 3.5.

In the pH 7.0 to 6.0 quick coalescence of bubbles were observed for foams formed with both MCS-CMs and MCS-CMs-Tgase, resulting in half-life time shorter than 500 s. While the foams from MCS-CMs measurements were between 440 ± 11 s and 472 ± 12 s, the ones from MCS-CMs-Tgase presented half-life time within an interval of 378 ± 11s and 272 ± 28 s. At these pH values, the amount of protein available for adsorption at the interface is high, which explains the rapid formation of the foam. However, the particles are small, and have no ability to act to preventing other important mechanisms of destabilization, such as drainage (Chen et al., 2016). The even lower foam half-life time found for CMs-Tgase suspensions could be predicted since Tgase crosslinking decreases the amount of CMs able to unfold the at the air-water interfaces, thus increasing surface tension (Marinova et al., 2009). This results in a fragile interfacial film between bubbles, explaining the faster coalescence observed for foams formed with MCS-CMs-Tgase in relation to the foams formed with native ones.

In the pH 5.5, both foams formed with MCS-CMs and MCS-CMs-Tgase start forming more stable foams whereas. However, while MCS-CMs presented visible aggregates at this pH values, it was not observed in MCS-CMs-Tgase. As particle size is strongly correlated with foam stability, this behavior could be associated to the fact that in native CMs suspensions small CMs aggregates would be available to stay trapped in the lamellar phase and plateau borders, due to their bigger size and low surface activity, forming a gel network (Chen et al., 2017).

Lowering the pH until reach the value of 5.0, the CMs-Tgase suspensions presented an interesting foam stability behavior (Figure 13). The foams formed took 24 hours to achieve the half-life time and kept stable for over 48 hours until complete destabilization. It was seen in the particle size and ζ-potential analyzes, that at this

pH value the CMs-Tgase are still dispersed and away from their pI. This was confirmed by the absorbance analysis, which suggested a high concentration of particles at pH 5.0. However, in the surface tension analysis, no evidence was found to explain this CMs-Tgase behavior at this pH value. The foams formed had a peculiar appearance compared to all the other samples, making clear that a gel network was formed. It could be investigated if the hydrophilicity of CMs-Tgase at this pH is different from the other pH values, explaining the formation of a gel network.

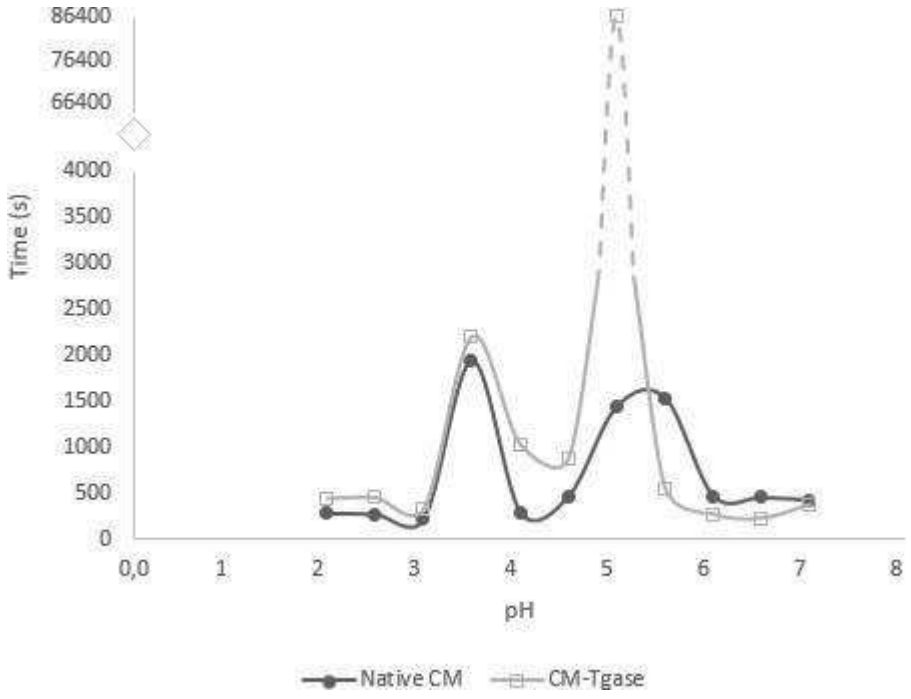

Figure 13. Foaming stability as a function of pH of native CMs (●) and CMs-Tgase (○).

At the pH values of 4.5 and 4.0 the half –life time decreases sharply for both native CMs and CMs-Tgase. High protein precipitation was observed during foam destabilization. This means that most of CMs were no available to adsorb at the

interface due to aggregated state. The half-life time becomes similar to the ones found in pH 7.0 to 6.0. This indicates that even with precipitation, small aggregates and CMs may be sufficient to allow foam formation and brief stabilization.

Moving away from the pI towards lower pH values, a new stability peak is observed, this time coincident for native CMs and CMs-Tgase suspensions. At pH 3.5, once more, the absorbance analysis indicates that dispersed CMs and small aggregates in suspension can contribute for better foam stability due to the effect of particle size in extending interfacial films life-time (Chen et al., 2016; Chen et al., 2017).

Lowering the pH to values bellow 3.5 leads to decrease in half-life time for all the samples tested, but the CMs-Tgase suspensions had better resistance to destabilization when compared to native CMs suspensions.

In all cases, below the pI, it seems that the higher surface tension caused by CMs crosslinking was compensated for by other phenomena involved, such as particle size and higher protein concentration, which resulted in greater stability of foams using cross-linked CMs.

12. CONCLUSION

In this study, the foam stability of native CMs and CMs cross-linked by Tgase was evaluated as a function of pH. CMs-Tgase showed higher foam stability than native CMs in pH range from 5.0 to 2.0, and formed foams stable for over 48 hours at pH 5.0. These same suspensions can be useful to study relationships between CMs-Tgase and foaming properties considering the effect of varying the temperature of foam formation and the rate of CMs crosslinking. Besides, the hydrophilicity of CMs- Tgase could be evaluated. An interesting possibility is the study of the spray-drying of the CMs-Tgase and subsequent rehydration and foam formation, evaluating the spray drying effect on crosslinking and the stability of the formed foams. The results show the potential application of CMs-Tgase as foam stabilizers in food industry, mainly in products such as fruit ice cream, frozen yogurt, and other acid desserts. The possibility to produce “clean-label” dairy products, without synthetic foam stabilizers, and low-fat products, keeping desirable texture characteristics, can make CMs-Tgase of great importance for food industry (Nielsen, 1995; Gauche, Tomazi & Bordignon-Luiz, 2008).

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14. GENERAL CONCLUSIONS AND PERSPECTIVES

In this study, after reviewing the foam structure, the main factors influencing the CMs structure and its relation to foam stability and how Tgase enzymatic crosslinking increase CMs stability against pH collapse, we evaluated the stability of foams formed with CMs-Tgase in the pH range from 7.0 to 2.0 and compared it with foam formed using native CMs at same conditions. We observed higher stability in foams formed with CMs-Tgase in pH values from 5.0 to 2.0, highlighting foams at pH 5.0 that took over 48 hours to destabilize. Lower foam stability was found in pH values from 7.0 to 5.5. This first result opened the possibility to use CMs-Tgase as an alternative for improving CMs performance as foam stabilizers. The findings of this studyshow the potential application of CMs- Tgase in many dairy products such as fruit ice cream, frozen yogurt, and other acid desserts. Besides, the use of CMs-Tgase opens the possibility to produce “clean- label” dairy products, without synthetic foam stabilizers, and low-fat products, keeping desirable texture characteristics.