LAISE TRINDADE PAES

FOAMING PROPERTIES OF MILK PROTEINS CROSSLINKED BY TRANSGLUTAMINASE AT DIFFERENT PH VALUES

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

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Co-Advisors: Evandro Martins
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" *Failure is simply the opportunity to begin again,*
this time more intelligently."
Henry Ford

ABSTRACT

PAES, Laise Trindade, M.Sc., Universidade Federal de Viçosa, February, 2020. **Foaming Properties of Milk Proteins Crosslinked by Transglutaminase at Different pH Values.** Advisor: Antônio Fernandes de Carvalho. Co-advisors: Evandro Martins and Naaman Francisco Nogueira Silva.

This study investigated the foaming properties of the suspension of milk proteins with a concentration of 40 g/L after treatment with the enzyme transglutaminase (TGase) and acidification at pH 5.1, 5.2, 5.4 and 5.6. Samples without (MP) and with the enzyme (MP-TG) were analyzed to find their viscosity, hydrodynamic diameter, interfacial tension, forming capacity (FC), stability (FS) and foam rheology. The results of hydrodynamic diameter were 256.18 and 245.18 nm for samples MP 5.1 and MP-TG 5.1, respectively, but there was no significant difference between treatments. The viscosity showed higher value in the MP 5.1 samples with 4.093 mPa.s and MP-TG 5.1 with 7.945 mPa.s, showing a significant difference from the other samples. Among all samples, the surface tension showed a maximum and minimum of 44.1 to 45.76 mN / m for samples MP 5.6 and MP 5.2, respectively, but with no significant difference. As for the foaming properties, the foaming capacity (FC) did not show variations in all samples and treatments. The foam stability (FS) was higher at pH 5.1 over the 180 minutes evaluated. When comparing the application of Tgase and pH variation, greater foam stability was observed with a decrease in the pH value. In the rheological analyzes of the foam, the MP-TG 5.4 samples showed a lower elastic limit with 6.27% of the shear stress. On the other hand, samples MP 5.1 and MP-TG 5.1 showed greater resistance to shear deformation, with shear stress above 50% of the measured interval. In conclusion, the decrease in pH allowed greater FS and elasticity of the foam, whereas the treatment with Tgase did not affect these parameters. The response obtained from higher FS to lower pH values allows investigating the foaming properties of new dairy products with pH values that are more acidic than milk.

Keywords: Micelles. Caseins. Whey proteins. Foams.

RESUMO

PAES, Laise Trindade, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Propriedades espumantes das proteínas do leite reticuladas por transglutaminase em diferentes valores de pH.** Orientador: Antônio Fernandes de Carvalho. Coorientadores: Evandro Martins e Naaman Francisco Nogueira Silva.

Esse estudo investigou as propriedades espumantes da suspensão de proteínas do leite com concentração de 40g/L após tratamento com a enzima transglutaminase (TGase) e acidificação a pH 5,1, 5,2, 5,4 e 5,6. Amostras sem (MP) e com a enzima (MP-TG) foram analisadas quanto à viscosidade, diâmetro hidrodinâmico, tensão interfacial, capacidade de formação (FC), estabilidade (FS) e reologia da espuma. Os resultados de diâmetro hidrodinâmico foram de 256,18 e 245,18 nm para as amostras MP 5.1 e MP-TG 5.1, respectivamente, mas não houve diferença significativa entre os tratamentos. A viscosidade apresentou maior valor nas amostras MP 5.1 com 4,093 mPa.s e MP-TG 5.1 com 7,945 mPa.s, apresentando diferença significativa das demais amostras. Entre todas as amostras, a tensão superficial apresentou máximo e mínimo de 44,1 a 45.76 mN/m para as amostras MP 5.6 e MP 5.2, respectivamente, mas sem diferença significativa. Quanto às propriedades espumantes, a capacidade de formação de espuma (FC) não mostrou variações em todas as amostras e tratamentos. A estabilidade da espuma (FS) foi maior em pH 5,1 ao longo dos 180 minutos avaliados. Ao comparar a aplicação da TGase e variação de pH observou-se maior estabilidade da espuma com a diminuição do valor de pH. Nas análises reológicas da espuma, as amostras MP-TG 5.4 apresentou menor limite elástico com 6,27% da tensão de cisalhamento. Por outro lado, as amostras MP 5.1 e MP-TG 5.1 apresentaram maior resistência à deformação por cisalhamento, com tensão de cisalhamento acima de 50% do intervalo medido. Em conclusão, a diminuição do pH permitiu maior FS e elasticidade da espuma, ao passo que o tratamento com TGase não afetou estes parâmetros. A resposta obtida de maior FS para menores valores de pH permite investigar propriedades espumantes de novos produtos lácteos com valores de pH mais ácidos que o do leite.

Palavras-chave: Micelas. Caseína. Proteínas do soro do leite. Espumas.

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SUMMARY

1. INTRODUCTION

Foams are widely used in cosmetics, flotation, fire extinguisher, and food products. Campbell & Mougeot (1999) discuss the possible results generated by the aeration of food as being a lower density product, a sensory difference in the mouth due to the change in rheology, an increase in surface area, a change in digestibility, an increase in oxidation and consequently shorter shelf life, and less intensity of flavors. Foams are easily found in well-known commercial dairy products, such as cappuccino, whipped cream and milkshake, but they can also be unwanted, as in the reconstitution of dairy powders (Huppertz, 2010).

It is the smooth sensation in the mouth that make foams conquer a consumer market, driving industries and laboratories to seek the science behind these products. (Green et al., 2013). Liquid foams are thermodynamically unstable with a lifetime of only few minutes, leading several studies to seek to increase the stability properties of the foam (Weaire & Hutzler, 1999; Fameau & Salonen, 2014; Rio et al., 2014; Lazidis et al., 2018). In food foams, the system is mostly stabilized by proteins, and therefore milk proteins are responsible for stabilizing bubbles in dairy foams. Among the researched strategies to improve the foaming properties, there is application of transglutaminase, an enzyme capable of creating cross-links in the protein, leading to higher stability of proteins against destabilizing factors, such as temperature and pH (Smiddy et al., 2006; Huppertz, 2014; Nogueira et al., 2019).

Milk proteins are divided into two groups with very different characteristics: caseins and whey proteins. As well as other food proteins, milk proteins have different functionalities, emulsifying capacity, nutritional properties and surface activity (Dombrowski et al., 2016; Narsimhan & Xiang, 2018). To increase the stability of dairy products, many manufacturers add food additives in order to also reduce costs, which can worry consumers about a “non-clean-label" product (Guyomarc’h et al., 2015). Most foods have an acidic pH, but milk is one of the few foods with a pH around neutral (6.7). Thus, if milk proteins are more stable at pH variations without additives (especially at lower pH), they can be more widely applied as foam stabilizers and well accepted by consumers.

Previous studies investigated the stability and foaming properties of casein micelles over a wide pH range (from 7.0 to 2.0) and found a more stable foam at pH around 5.0. The aim of this study was to investigate further whether the crosslinking of milk proteins by

transglutaminase changes the foaming properties of samples in a restricted pH values of 5.1, 5.2, 5.4 and 5.6.

2. LITERATURE REVIEW

2.1. Foam

Foam is a colloidal system composed by a gas phase dispersed in a liquid (Walstra, 1989; Ho et al., 2019) and stabilized by particles or molecules with hydrophobic and hydrophilic fractions to act at the air/water interface to lessen surface tension (Lazidis et al., 2018). According to Patel (2018), foams can be classified as dry or wet depending on the amount of liquid in the thin film, therefore dry foam is assumed to be less than 10% (v/v) of liquid fraction. The thin film between two bubbles containing the liquid fraction, called lamellar liquid, connects to other films forming the Plateau borders (Pb) (Fameau and Salonen, 2014). Thereafter, nodes are formed by the encounter of Pb at the vertices of the bubbles, which assume a polyhedral form due to the contact to other bubbles (Cantat et al., 2013). The foam structure can be better visualized in Figure 1.

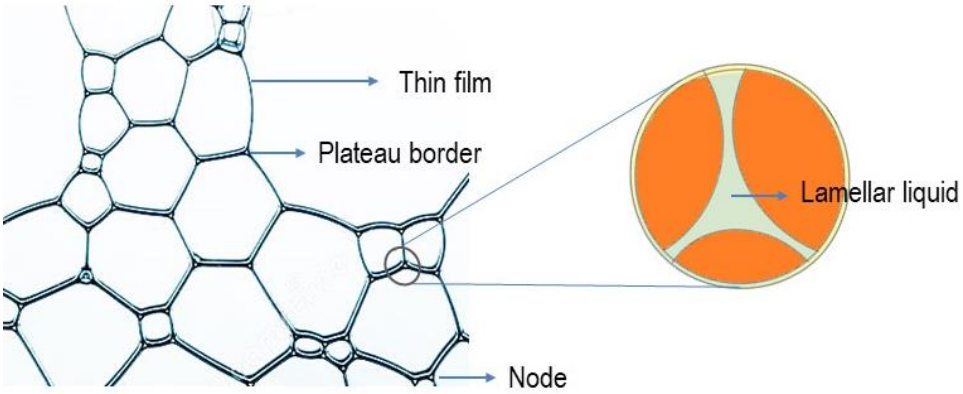

Figure 1. Structure of a liquid foam.

The foaming properties mostly researched are foaming capacity, here named FC, (or foamability) and foam stability (FS). Foaming capacity is defined as the amount (volume) of foam formed under fixed time and temperature conditions or the time required to form a certain volume of foam (Marinova et al., 2009). Narsimhan & Xiang (2018) defines foam stability as the ability to hold a volume of gas for a specific period of time. Foamability is related to the dynamics of adsorption, how fast proteins can stabilize the air/water interface, and foam

stability depends on the capacity of proteins to create a viscoelastic interface (Damodaran, 1997; Kamath et al., 2011).

As stated by Wang et al. (2016), the surface tension is directly proportional to the surface energy *E*, therefore a low surface tension can contribute to increase the foaming capacity. Although a fast adsorption and a low surface tension are both essential for foaming, they are not sufficient to make stable foams. Additionally, proteins must form a viscoelastic surface in order to resist the forces of compression and expansion that occur on the bubble surface (Fameau & Salonen, 2014). This is mainly because the interface is not static, but under dilational forces. Thus, the measurement of this parameter would be just a prediction of what actually happens in the foams (Bos & van Vliet, 2001).

The three mechanisms that affect foam stability and shorten the foam lifetime are drainage, coalescence and coarsening (Hutzler & Weaire, 2000; Koehler et al., 2000; Stevenson, 2012; Audebert et al., 2019). Firstly, drainage is the flow of the liquid inside the lamella downward as a result of the gravitational force (Cantat et al., 2013). As the film thinning happens, it becomes more susceptible to rupture. The joining of two bubbles due to this rupture is called coalescence (Fameau & Salonen, 2014). Lastly, coarsening is the gas diffusion between bubbles of different sizes, flowing from smaller to the larger bubbles, because of the difference in Laplace pressure (Rio et al., 2014). Despite having different principles, these mechanisms are not independent. Drainage and/or coarsening can contribute to coalescence, for example. Also, both coalescence and coarsening increase bubble size (Audebert et al., 2019), which leads to increased susceptibility to rupture.

2.1.1 Foam Stability

Due to the short life of the foams, many methods have been studied to delay the destabilization mechanisms by altering the properties of the liquid, such as viscosity. There are chemical (Park et al., 2000; Barbosa et al., 2014; Casanova et al., 2017), physical (Silva et al., 2013; Oetjen et al., 2014; Xiong, et al., 2016) and enzymatic methods (Thalmann & Lötzbeyer, 2002; Nogueira et al., 2019) that have been applied in order to modify the structure of the protein or add compounds to increase the stability of the foam.

In their review, Fameau & Salonen (2014) state that one way to lower drainage rate is by increasing the viscosity of the liquid phase, which was concluded experimentally by Safouane et al. (2006) and Martinez-Padilla et al. (2014). In addition, it is also known that the solution viscosity influences the average bubble size (Marinova et al., 2009), evidencing its importance for the stabilization of foams, and leading research to assess the relation between viscosity and bubble stability (Balerin et al., 2007; Chávez-Montes et al., 2007). This is mainly because drainage of the lamellar liquid leads to thinning of the film between two bubbles, resulting in coalescence and shorter foam life (Horozov, 2008).

Even the type of gas used to form foam influences its stability. If the gas applied as a dispersed phase has a low solubility in the continuous phase, the transfer of its molecules through the liquid will be slowed down; consequently, coarsening will also be delayed (Fameau & Salonen, 2014). When using a mixture of different gases, the tendency is for the air bubbles to group according to the solubility, thus, more soluble gases will form larger bubbles leading to a difference in osmotic pressure, easing the Laplace pressure (Webster & Cates, 2001).

The presence of protein aggregates in the liquid phase of the foam can be a cause for greater stability of the foams, by stabilizing thin films (Chen et al., 2016). These aggregate structures in addition to increasing the viscosity of the aqueous phase in the films, block the passage of liquid channels, decreasing the drainage rate (Fameau & Salonen, 2014). These particles or aggregates can be formed naturally or through physical or chemical methods. However, not every aggregate or particle is beneficial for foam stability. Fameau and Salomen (2014) point out that certain aggregates or particles are also used as defoaming agents.

There is a wide variety of macromolecules with different structures, conformations and concentrations that quickly adsorb at the air/water interface and are applied to stabilize foams (Fameau & Salonen, 2014). Proteins are active surface agents that act in the stabilization of food foams (Fains et al., 1997; Ewert et al., 2016) due to its amphiphilic character. For this, proteins form an adsorbed layer of molecules at the air/water interface (Wierenga & Gruppen, 2010). According to Audebert et al. (2019), proteins not only decrease the tension at the air/water interface, but also provide foam stability due to the repulsive forces between two bubble surfaces.

In dairy foams or aerated dairy products, milk proteins are mostly responsible for the stability of the air/water interface. Their physical and chemical properties give them remarkable activity on the surface to act as stabilizers. Both the foaming properties of milk (Ho et al., 2019) and fractions of isolated milk proteins (Martínez-Padilla et al., 2014; Dombrowski et al., 2016; Ewert et al., 2016; Chen M. et al., 2018) have been investigated. However, studies of the foaming properties of milk are more complicated because it is a more complex matrix (Dombrowski et al., 2016). The foaming properties can vary according to the milk protein, be it whey proteins or caseins, but it can also vary for individual fractions of these protein groups, such as α_{s1}-, α_{s2}, β- and κ-caseins or β-lactoglobulin and α-lactalbumin.

2.2. Dairy proteins

Dairy proteins are broadly applied in many food products due to their functionality, since they present an interesting emulsifying, gelling and foaming capacities (Singh, 2011). Milk proteins are divided into two groups: caseins, comprising 80% of total proteins; whey proteins (WP) representing the remaining 20%. These proteins are quite different in terms of conformation, structure and physicochemical properties.

Caseins are composed of four fractions, named α_{s1}-, α_{s2}, β- and κ-caseins, containing the approximate proportions of 40%, 10%, 35% and 15%, respectively (Dalgleish & Corredig, 2012). However, caseins are not disposed in milk in individual fractions, instead they are arranged in milk in the form of flexible, porous, highly hydrated and phosphorylated structures called micelles, with an average diameter of 200 nm (Holt et al., 2013; Silva et al., 2019). On a dry basis, 94% of the micelle are proteins of the casein group and 6% are inorganic material, which consists mainly of calcium and phosphate (Smiddy et al., 2006), called colloidal calcium phosphate (CCP) or micellar casein phosphate.

The clear tendency of caseins to aggregate into micelles has an important biological function. Casein micelles (CM) make milk secretion or internal storage possible without calcification in the mammary tissue, due to the transportation of calcium phosphate in their structure. Thus, CM works as a packaging system to transport high amounts of phosphate and calcium that, without the presence of caseins, would precipitate in the mammary gland (Horne, 2016). However, Holt et al. (2013) points out that non-casein structures can also carry out this

transport, as is the case of non-casein phosphoproteins that present degrees of phosphorylation, unfolded conformation and type of flexibility similar to that of caseins. The relationship between caseins and calcium is crucial to be understood since it involves issues such as differences in sensitivity to calcium by different fractions of caseins, the role of calcium in maintaining the micelle structure, in addition to nutritional importance (Huppertz et al., 2018).

The casein fractions form the micellar structure through hydrogen bonds, hydrophobic and electrostatic interactions, in addition to calcium bridging (Fox & Brodkorb, 2008). Although there is still a debate around the internal structure of the casein micelles, it is known that, α_{s1}-, α_{s2} and β-caseins are predominantly located in the inner part, whereas κ-caseins with its c-terminal part are arranged in the outer surface due to its hydrophilicity (Singh, 2011; Dalgleish & Corredig, 2012). The caseins located in the core of the micelle present centers of phosphorylation, which allows them to bind to the CCP (De Kruif & Holt, 2003; Smiddy et al., 2006). Among caseins, beta-casein is considered the most surface-active protein due to its high amount of hydrophobic residues (Zhang et al., 2004).

Regarding the stability of the CM, κ-casein is one of the responsible for maintaining steric stabilization through a layer (also commonly called "hairy" layer) on the micelle surface (Horne, 2016). Many factors can cause the destabilization of CM, among them there are urea addition, pH and temperature variation, and removal of CCP from the CM (Horne, 2020), in addition to high pressure, ethanol, and attack of κ-casein by proteinases (Fox & Brodkorb, 2008). The precipitation of individual caseins by adding calcium chloride occurs mainly in α_{s1}-, α_{s2} and β-caseins, due to the presence of phosphoserine residues these caseins have a high capacity to bind calcium. As κ-caseins have very few phosphoserine residues, they do not easily precipitate in the presence of calcium chloride (Horne, 2016), but depending on its concentration.

Milk pH is around neutral (6.7-6.8) and in this condition, CM behave like a hard sphere, that means well-defined structures without intermolecular connections, but when pH decreases to near CM isoelectric point (pI~4.6) this type of configuration changes favoring the attraction between the micelles (Tuinier & de Kruif, 2002). By definition of some authors

(Wong et al., 1996; Pelegrine & Gasparetto, 2005), pI is the pH value where proteins have the lowest solubility with the medium, increasing interactions with each other through the loss of electrostatic forces of repulsion, whereas interactions with water decrease. With the decrease in pH, changes occur in the outer layer of the CM (κ-caseins), leading them to lose their "brushes"; and in the interior as well, where calcium, phosphate, magnesium and citrate ions are progressively released (Dalgleish & Corredig, 2012).

The other dairy protein group, whey proteins, is also able to form foam. Whey proteins are composed majoritarily by β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), proteins with tertiary structure, disulphide bridges and a globular shape (Marinova et al., 2009). β-LG represents 50%, has an amphiphilic character, quickly adsorbing on interfaces, and contains a free thiol group (Vasbinder & de Kruif, 2003; Singh, 2011). These proteins are 1.8 nm and 6 nm in size for α-LA and β-LG, respectively (De Wit, 1998). Unlike CM, whey proteins are quite sensitive to temperature rise. Heating leads to changes in the conformation of these globular proteins leading to their unfolding. Vasbinder & de Kruif (2003) explains that the free thiol group of β-LG starts the reaction of denaturation by polymerization and so β-LG aggregation. β-LG also can link to CM by disulfide bridges.

The effect of temperature also depends on pH, as proved by previous studies (Pelegrine & Gasparetto, 2005; Lam & Nickerson, 2015; Cao et al., 2018) which tested the correlation of pH and temperature. Isoelectric points of whey proteins are 5.2 for β-LG and between 4.5-4.2 for α-LA, according to Guyomarc'h et al. (2015), who add that below pH 5.0 α-LA lose their calcium binding affinity, while β-LG changes from stable dimer to octamer as pH decreases until around its pI.

Among the different methods applied to modify dairy proteins enhancing their foaming properties, crosslink has been an alternative to chemical modifications. Crosslinking of milk proteins has been a method applied to increase stability against external factors. Casanova et al. (2017) studied the stability of CM crosslinked by genipin and found out a greater stability at low pH of crosslinked samples in comparison to native CM. Crosslinking by the enzyme transglutaminase is also an alternative to increase stability that has been investigated (Smiddy et al., 2006; Nogueira et al., 2019).

2.3. Transglutaminase

Transglutaminase (glutaminyl-peptide:amine γ-glutamyltransferase) is an enzyme from transferase class, broadly found in nature from various sources, such as from animal, vegetable and microbiological sources (Kuraishi et al., 2001; Kieliszek & Misiewicz, 2014). Transglutaminase (TGase) creates covalent bonds between the γ-carboxyamide group of glutamine residue and primary ε-amines (Shleikin et al., 2011; Calvarro et al., 2016). This enzyme can act in a wide pH range, from 4.0 to 7.0, presents an optimum temperature around 50 ºC, with activity between 40 ºC and 70 ºC (Zhu et al., 1995; Motoki & Seguro, 1998; Yokoyama et al., 2004; Macedo & Sato, 2009; Gaspar & Góes-Favoni, 2015)., TGase is a GRAS ingredient (Generally Recognized As Safe) by FDA, and it has been widely used in the dairy (Lauber et al., 2000; Moon et al., 2009; Romeih & Walker, 2017), fish (Téllez-Luis et al., 2002; Yi et al., 2006; Li et al., 2018), and meat products (Barreiro & Seselovsky, 2003; Ferreira et al., 2012; Gaspar & Góes-Favoni, 2015). The wide application of TGase in food products is due to the ability to modify rheological, texture, and gelation properties, in addition to increasing stability without changing nutritional properties, color or flavor.

The porous and flexible structure of caseins, along with a low degree of terciary structure make caseins more susceptible to TGase than whey proteins, which present globular structure (Romeih & Walker, 2017). Among caseins, κ-casein is the most susceptible to TGase action, followed by β- and α_s-caseins, which is related to which of the casein fractions are most accessible in CM (Sharma et al., 2001; Tang et al., 2006; Smiddy et al., 2006; Hinz et al., 2012; Romeih & Walker, 2017). Although various studies indicated a preliminary modification in whey proteins to expose potencial sites to TGase, such as denaturation (O'Sullivan et al., 2002; Eissa & Khan, 2006; Damodaran & Agyire, 2013), there are other studies which reported crosslinking reaction of β-LG and α-LA by TGase without the necessity of a previous treatment (Sharma et al., 2002; Góes-Favoni & Bueno, 2014).

Smiddy et al. (2006) investigated the stability of CM crosslinked by TGase against urea, sodium dodecyl sulfate and heating with ethanol. The results showed that treatment with TGase increased CM stability in all methods of destabilization applied. Similarly, Nogueira et al. (2019) evaluated stability of MP-TGase at different pH values (from 2.0 to 7.0) against urea, sodium citrate and high temperature and ethanol. While native CM precipitated at pH below

5.5, MP-TGase precipitated at pH between 4.5 and 3.5, being stable in pH ranges between 2.0 – 3.0 and 4.5 – 7.0. Also, MP-TGase samples were stable at pH 2.0 and against all destabilization methods. The use of TGase is constantly associated with changes in the rheology of dairy products (Lauber et al., 2000; Farnsworth et al., 2006; Guyot & Kulozik, 2011; Pakseresht et al., 2017; Romeih & Walker, 2017; Gharibzahedi & Chronakis, 2018; Gharibzahedi et al., 2018). However, studies on the foaming properties of TGase-treated milk proteins are still lacking.

3. RESEARCH QUESTIONS

There are a few questions which this dissertation aims to answer:

- Does crosslinking of milk proteins by transglutaminase increase foam stability?
- At what pH value is the foam stability the highest?
- What is the influence of crosslinking and acidification on the rheological properties of milk protein foams?

4. MATERIALS AND METHODS

4.1. Suspension preparation

Milk Protein Concentrate (Danone, Brazil) powder at 80% (w/w) concentration of protein, in which the average whey protein content was 22.39 ± 0.51% (w/w) determined by the Kjeldahl method, with no fat content. Dispersions were prepared rehydrating MPC powder, at concentration of 40 g/L of milk proteins, with ultrapure type 1 water (Thermo Scientific, USA), and 2 mMol of calcium chloride at pH 6.7. 0.3 g/L of sodium azide (Synth, Brazil) was added to prevent microbial growth. The solutions were stirred at 900 rpm in a magnetic stirrer during 42 hours at room temperature (25 ºC ± 2 ºC).

4.2. Application of transglutaminase

Microbial Transglutaminase (Activa®, Ajinomoto, France) containing 100 U/g was resuspended in ultrapure type 1 water in a concentration of 10% (w/w). Tgase solution was then added to half of the suspensions in 3 U/g of proteins, according to the recommendation of the producer, and the suspensions were left stirring for 15 minutes to better incorporate the enzyme into the medium. Samples were placed in a water bath (Thermomix, B. Braun Biotech

International, Germany) at 45 ºC for 1 hour. The enzyme was inactivated by heating the samples in a water bath until reaching 85 ºC and maintaining the temperature for 1 minute. The samples were submitted to an ice bath immediately afterwards to the temperature of 4 ºC to avoid denaturation of proteins. For sample identification, suspension of MPC with and without enzymatic treatment were named MP and MP-TG, respectively.

4.3. Sample acidification

To perform acidification, HCl (Synth, Brazil) solutions in different concentrations (0.5, 0.25 and 0.125 M) were prepared with distilled water. MP and MP-TG samples were acidified with HCl to the pH values of 5.1, 5.2, 5.4 and 5.6, in an ice bath to keep the samples at 4 ºC and under stirring, in order to avoid casein micelles destabilization. The minimum pH was 5.1 because below this value the samples showed visible precipitation. The measurement was done by a pH meter (Kasvi, Brazil) at room temperature (25 ºC ± 2 ºC). The samples were named according to the treatment received and pH value, as shown in Table 1.

Table 1. Nomenclature of the samples.

pH value	Sample name	
	Without TGase	**With TGase**
5.1	MP 5.1	MP-TG 5.1
5.2	MP 5.2	MP-TG 5.2
5.4	MP 5.4	MP-TG 5.4
5.6	MP 5.6	MP-TG 5.6

4.4. Colloidal aspects after rehydration and crosslinking

4.4.1. Hydrodynamic size

Hydrodynamic diameter (Dh) was determined by the Dynamic Light Scattering (Brookhaven, Holtsville, USA) equipment. MP and MP-TG suspensions were diluted by adding a 50 µL aliquot of the sample in 10 mL of ultrapure water at the same pH as the samples, at room temperature (25 ± 2 ºC). Measurements were performed over a period of 2 minutes on average.

4.4.2. Viscosity

The viscosity of the MPC suspensions were performed on the rheometer (MCR 702, Anton Paar, Germany) with cone and plate geometry, at a controlled temperature of 20 ºC. For each measurement, samples (approximately 3 mL) were placed into the equipment plate, in a steady state flow, with a shear rate from 0 to 300 s^{-1} and carried out in duplicate.

4.4.3. Interfacial tension

A drop tensiometer (Easy drop, DAS 100, Germany) equipped with a CCD (charged-coupled device) camera was used to investigate surface tension for all treatments, using the pendant drop method. The dispersions were placed in a Hamilton syringe with a stainless-steel needle which is kept in a controlled temperature environment (20 ºC). At the needle tip one drop (15 µL) was formed and surface tension measurements were performed for about 1000 seconds to stabilization. The mean value of the variation in interfacial tension during this time period was used. To determine the drop profile the captured images were used. The calculation of the interfacial tension is given by the Laplace equation (or Young-Laplace equation) (Equation 1), where the difference in pressure and curvature between the interfaces are taken into account (Ravera et al., 2010). This equation is demonstrated at below:

$$\Delta P = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad (1)$$

where ΔP is the pressure difference between the two sides of the interfaces, γ is the surface tension, and R_1 and R_2 are the radii of interface curvature.

4.5. Foam characterization

A graduated cylinder with capacity of 25 mL was filled to its half capacity (12.5 mL) with MPC suspensions. The foam was prepared by homogenizing the suspension by using Ultraturrax (Colonial Scientific, DI 25 basic yellow line, Richmond, USA) at 9,500 rpm for 1 min, and then sealed with parafilm. After that, foams were analyzed regarding to the FC, FS and foam rheology.

4.5.1. Foam capacity

Foaming properties of samples were measured according to Jarpa-Parra et al. (2014) and Jarpa-Parra et al. (2016) with slight modifications. Foaming capacity (*Fc*) was measured in duplicate immediately after stirring (t_0) and calculated by Equation 2 below:

$$Fc = \frac{V_{foam} - V_{liquid}}{V_{liquid}} \times 100 \quad (2)$$

where V_{foam} is the volume of the foam formed right after stirring and V_{liquid} is the volume of the liquid not incorporated into the foam.

4.5.2. Foam stability

Foam volume reduction with time was used to visually evaluate foam stability. Foam volume was successively measured in duplicate after 1, 5, 10, 15, 30, 60, 120 and 180 minutes, at room temperature (25 ºC ± 2 ºC). Foaming stability (FS) was calculated according to Equation 3:

$$FS = \frac{V_{t\ time}}{V_0}\ x\ 100\ \% \quad (3)$$

where $V_{t\ time}$ represent the foam volume at the specific time (1, 5, 10, 15, 30, 60, 120 and 180 min) and V_0 represents the initial foam volume.

4.5.3. Foam rheology

The rheology of foam was performed according to Audebert et al. (2019). After 1 minute of foam formation, rheology measurements were executed by taking a foam aliquot and placing it on the rheometer MCR 301 (Anton Paar, Germany) with a 75 mm cone-plate geometry. The viscoelastic shear moduli G' and G" (storage and loss moduli, respectively) and the yield strain γ_c, also called elastic limit, (Cantat et al., 2013) were measured. The experiments were carried out with an oscillatory amplitude sweep from 1 to 50% strain and a frequency of 1 Hz. G'_0 and G''_0 (moduli at initial time) were established as the moduli when the strain is equal to 1%. The results had the shear strain data changed to the logarithmic scale.

4.5.4. Statistical analysis

The experiments were performed in four repetitions and in duplicate. The experimental data were subjected to analysis of variance (ANOVA) at significance level of $p < 0.05$. Tuckey test was performed to verify significant differences $p < 0.05$.

5. RESULTS AND DISCUSSION

5.1. Hydrodynamic size (Dh)

Table 2 shows the results of the average hydrodynamic size of particles present in the suspension. The average size of casein micelles is reported in literature as been around 200 nm (Dalgleish & Corredig, 2012; Silva et al., 2019). There was no significant difference ($p < 0.05$) between treatments. Xiong et al. (2020) reported an average particle size of MPC dispersions at pH 6.7 - 6.8 of 178.4 nm, concluding that the difference in fractions of different milk proteins (caseins and WP) did not significantly influence the particle size distribution of the samples. As whey proteins are barely available in their natural form to TGase action due to their globular structure (Romeih & Walker, 2017), it is believed that they did not influence Dh of samples with or without TGase.

Table 2. Hydrodynamic diameter results of MP and MP-TG samples.

Sample	Mean D_h (nm)	Std deviation (nm)	Std Error (nm)
MP 5.1	256.18[a]	66.36	33.18
MP 5.2	217.93[a]	34.84	17.42
MP 5.4	182.38[a]	31.50	15.75
MP 5.6	171.80[a]	17.49	8.75
MP-TG 5.1	245.18[a]	85.69	42.84
MP-TG 5.2	211.20[a]	22.34	11.17
MP-TG 5.4	219.30[a]	45.41	22.70
MP-TG 5.6	216.90[a]	54.64	27.32

[a]Same superscript letters indicate there is no statistically significant differences ($p < 0.05$).

All samples but those at pH 5.1 showed D_h values near the average casein micelle size. Dalgleish & Corredig (2012) explain that the reason for caseins to organize in micelles but not to form aggregates is due to κ-casein, which is located predominantly on the micelle surface

where it creates steric stabilization. Under acidification, as the pH decreases the net charge of CM also decreases (Gonzalez-Jordan et al., 2015) leading to aggregation. This slight variation in samples at pH 5.1 can be explained by the proximity of the isoelectric point (4.6), which causes the casein micelles to lose repulsion between them (Casanova et al., 2017) and start to form larger particles.

Regarding the application of the transglutaminase enzyme, with the exception of samples acidified to pH 5.2, samples without enzymatic treatment had lower average D_h than those treated with TGase. This result of a small size variation between treatments was also found by Mounsey et al. (2005) with sizes of 195 ± 3 nm and 213 ± 3 nm for casein micelles and casein micelles treated with TGase, respectively. The authors considered, in agreement with Faergemand and Qvist (1997), that the crosslinking took place intramolecularly, not significantly affecting the average size of the casein micelles. Similarly, Silva et al. (2018) found the suchlike results: an average diameter of 211 nm for casein micelles and 225 nm for CM treated with TGase.

5.2. Viscosity

Viscosity is directly related to foam stability, since a high viscosity will slow down drainage rate of the liquid (Huppertz, 2010). The mean viscosity data of the samples are shown in Table 3. Samples at pH 5.1 presented a significant higher viscosity than at others pH values, which may be related to the presence of protein particles or aggregates in the suspension. According to the Krieger-Dougherty equation, viscosity is proportional to the volumetric fraction and Dh of the particles. This means that the greater the volume occupied by a molecule of the solute or aggregates, the greater the viscosity. Thus, the explanation for the MP 5.1 and MP-TG 5.1 samples to have higher viscosity is directly related to these samples also present larger particle size, therefore a larger occupied volume fraction.

Table 3. Apparent viscosity of MP and MP-TG samples at shear rate of 300 s^{-1}.[a]

Sample	Viscosity (mPa.s)
MP 5.1	4.043^{a}
MP 5.2	1.642^{bc}
MP 5.4	1.493^{b}

MP 5.6	1.534[b]
MP-TG 5.1	7.945[d]
MP-TG 5.2	2.612[c]
MP-TG 5.4	1.499[b]
MP-TG 5.6	1.512[bc]

[a]Same superscript letters indicate there is no statistically significant differences ($p < 0.05$).

Xiong et al. (2020) found an apparent viscosity value of a MPC dispersion with the same protein concentration and shear rate, measured at 20 ºC, of 2.70 ± 0.07 mPa.s. Silva et al. (2014) cross-linked casein micelles using genipin and found lower viscosity values as the genipin concentration increased. The decrease in viscosity was attributed to the extent of the crosslinking and consequently to the smaller particle size. However, pH was not varied in these studies, and this factor influenced strongly the results.

When evaluating the effect of TGase in viscosity of samples, samples at pH 5.2, 5.4 and 5.6 did not showed major difference. The sample MP-TG 5.1 presented a higher viscosity if compared to MP 5.1 and the other samples, showing that the enzymatic treatment and lower pH value significantly influenced this property. The results of the TGase effect on viscosity differed from Chen, L. et al. (2018), who investigated TGase action on acid-induced MPC suspensions observed changes in properties, including viscosity. Their results showed that crosslinking improved the texture of the MPC suspensions and increased viscosity.

5.3. Interfacial tension

Surface tension is related to adsorption of surface-active agents lowering the energy at the interface (Bos & van Vliet, 2001). It is important to keep in mind that the interfacial tension is not a measure of static conditions, therefore it is not reached instantly, depending on the surfactant adsorption time and its diffusion over the surface (Drenckhan & Saint-Jalmes, 2015; Wang et al., 2016). Results of surface tension experiments (Table 4) showed a very little variation, meaning that neither the enzymatic treatment nor the pH difference caused significant changes ($p < 0.05$) in the value.

Table 4. Surface tension of acidified samples with and without enzymatic treatment during 1000 s.

Sample	Surface tension (mN/m)

MP 5.1	44.95[a] ± 0.59
MP 5.2	45.76[a] ± 0.80
MP 5.4	44.72[a] ± 1.30
MP 5.6	44.1[a] ± 1.30
MP-TG 5.1	45.06[a] ± 2.11
MP-TG 5.2	45.43[a] ± 0.92
MP-TG 5.4	45.33[a] ± 1.01
MP-TG 5.6	45.11[a] ± 0.35

[a]Same superscript letters indicate there is no statistically significant differences ($p < 0.05$).

According to Bos and van Vliet (2001), the closer to the isoelectric point of the protein, the greater the amount adsorbed at the interface. Previous research (Devaud, 2019) has proved it by investigating surface tension in casein micelles, comparing samples with and without TGase, but in a much larger pH range (from 2.0 to 8.0). Surface tension of casein micelles suspensions reached the minimum value when the pH was close to 4.6 for both control (43.6 ± 0.7 mN/m) and TGase samples (44.1 ± 0.2 mN/m). In a wider pH range, there was a greater variation in interfacial tension than what was found between pH 5.1 and 5.6.

MPC is a complex matrix (including CM and whey proteins) and instantaneous adsorption and saturation probably occurs at the air/water interface. Caseins have activity at interface level, showing a tendency to migrate to hydrophobic surface, but when competing with denatured whey proteins caseins can be rapidly removed by them (Holt et al., 2013). Marinova et al. (2009) and Martínez-Padilla et al. (2014) state that adsorption rate of caseins are faster than whey proteins, because caseins present higher surface activity and flexible structure. Zhang et al. (2004) also reported a preferential adsorption of casein micelles over whey proteins. This was also confirmed by Xiong et al. (2020) who concluded, varying CM and whey protein ratios, that surface tension was lower when whey protein content was higher. However, the competitive adsorption between CM and whey proteins could not be identified in this experiment to clearly explain FC results.

5.4. Foam characterization

5.4.1. Foam Capacity

Cantat et al. (2013) state that this is a property related to the adsorption dynamics, in other words, how and how fast the proteins stabilize the air/water interface, decreasing the interfacial tension. Drenckhan & Saint-Jalmes (2015) also add that the method chosen to generate energy for foaming influences the foaming capacity. The lowest values found were at pH 5.6 for MP and MP-TG (Table 5), however, there was no significant difference ($p < 0.05$) between any treatment. Comparing it with the other pH values, a direct relationship such as the lower pH, the greater foam capacity was not found.

Table 5. Foam capacity of suspensions.

Sample	Foam capacity (%)	Std deviation (%)	Std error (%)
MP 5.1	64	17.44	10.07
MP 5.2	69	9.24	5.33
MP 5.4	65	11.55	6.67
MP 5.6	61	15.14	8.74
MP-TG 5.1	64	10.58	6.11
MP-TG 5.2	63	6.11	3.53
MP-TG 5.4	65	9.24	5.33
MP-TG 5.6	60	10.58	6.11

Martínez-Padilla et al. (2014) have shown that increasing the concentration of sodium caseinate increased the foaming capacity (642 - 1422%) more than a higher concentration of whey proteins (380 - 534%). This may be due to the casein fractions present higher FC than casein micelles structures (Zhang et al., 2004). Controversially, Xiong et al. (2020) found lower FC when the caseins:WP ratio was 80:20 and higher FC in the 40:60 ratio.

Analyzing whether application of TGase had an effect on the foam capacity, overall, FC results were very similar. Additionally, the lack of difference between FC of the samples may rely on the foaming method applied in this experiment. Although foam generation by stirring is a valid method, it may not have generated a satisfactory amount of foam, affecting FC results.

However, this could only be tested if another method was performed for comparison, i.e. as by double syringe.

5.4.2. Foam Stability

The other property investigated was the stability of the foam, which is the central issue of this study, since the biggest difficulty when working with foams is its short lifetime, leading several researches (Kamath et al., 2008; Marinova et al., 2009; Oetjen et al., 2014; Chen et al., 2017; Chen, M. et al., 2018; Hatakeyama, 2019; Ho et al., 2019) to look for methods to prolong this time. FS results were divided into treatments without and with TGase for better visualization (Figure 2 and 3).

In the first 30 minutes, the non-crosslinked samples showed similar foam stability and, after that, there was a marked variation, while the crosslinked samples presented such variation just after a few minutes. The MP 5.1 sample had FS of 50.52% at t_{60}, while MP 5.4 showed a FS of 26.09% at the same time. In those first minutes, the destabilizing mechanism that acts most on the foam is the draining of the lamellar liquid, due to gravitational forces. As the liquid in the channels flows, the thickness of the film decreases until it breaks and forms a single bubble, which is the coalescence (Lazidis et al., 2017; Narsimhan & Xiang, 2018). Concomitantly, the transfer of gas from smaller to larger bubbles is also happening. The difference in FS after 30 minutes can be explained by the interdependence of drainage, coalescence and coarsening (Rio et al., 2014), which causes one mechanism to affect the other, accelerating the destabilization of the foam.

At t_{30}, MP 5.1 and MP-TG 5.1 showed stability of 57.73% and 65.91%, respectively. Unlike the others, MP-TG 5.1 was the only sample with TGase showed more stability than the control sample at the same pH over the evaluated time. MP-TG 5.6 had higher stability (74.65%) than MP 5.6 (67.09%) only until t_{15}, and MP samples at pH 5.2 and 5.4 had a higher stability than MP-TG samples.

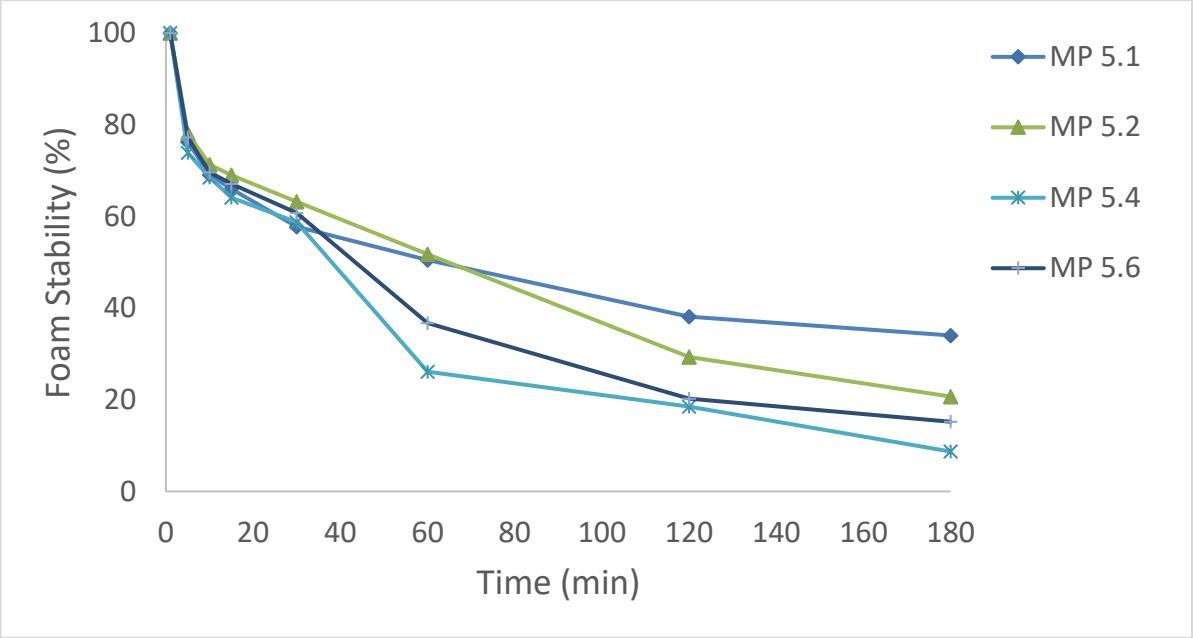

Figure 2. Foam stability of samples at different pH without transglutaminase treatment over 180 minutes.

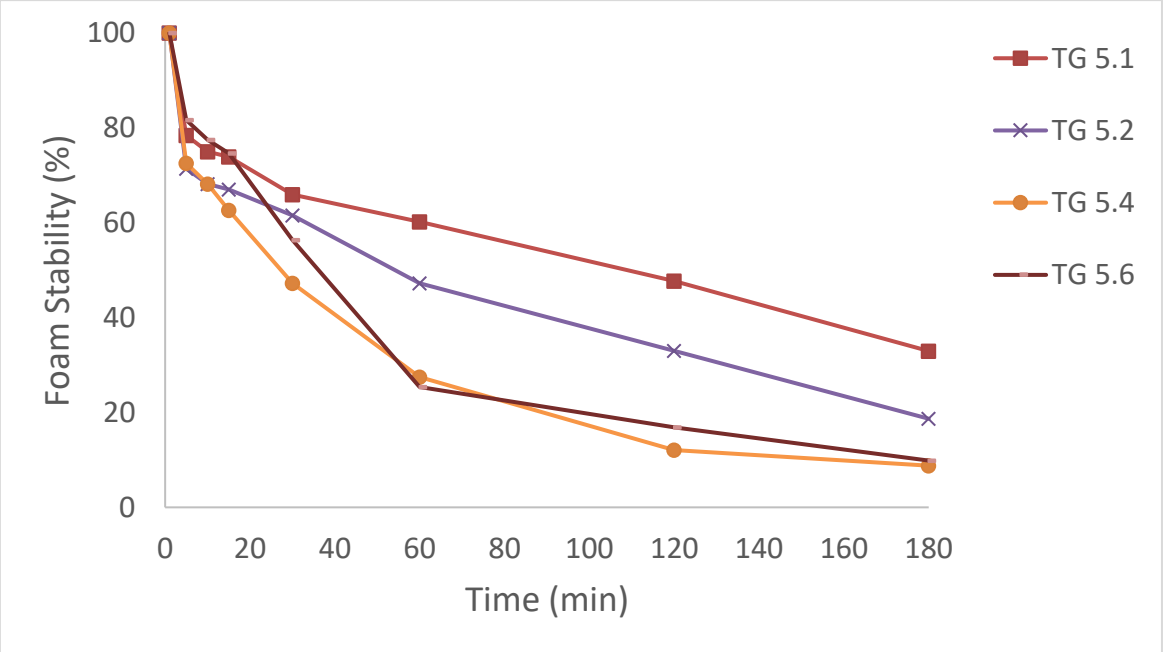

Figure 3. Foam stability of samples at different pH subjected to transglutaminase treatment over 180 minutes.

Overall, it can be seen that TGase did not have as much effect on FS as pH variation. In the pH range evaluated, the foam was more stable at a lower pH, as 5.1 and 5.2, in both treatments, which can be explained by the viscosity analyzes. The changes caused by the

proximity to the isoelectric point of the casein micelles most likely increase the viscoelastic properties of the proteins at the interface, making them withstand the forces of compression and expansion of the bubbles. Assuming that some aerated drinks, such as cappuccino, are consumed within a few minutes, any foam sample would be a great candidate for the formulation. For longer periods, the MP-TG 5.1 sample would be more appropriate, as it has remained less unstable over time.

5.4.3. Foam rheology

When a small strain is applied to a foam, only an elastic stress is generated, but if the applied strain is large enough to reach the yield strain, the bubbles remodel initiating a flow of foam (Audebert & Saint-Jalmes, 2019). G' is the storage modulus that is related to the solid structure of a foam, whereas G" is the loss modulus related to a more liquid structure. Thus, the greater the strain applied to the foam, the more it loses its structure and behaves like a liquid. The turning point where this happens is called yield strain, shown in Figures 7 and 8 as the intersection of the two curves G' and G". A higher yield strain of a foam means that the foam sample took longer to lose their structure, as it is the case of the sample MP 5.1 (Figure 4), which did not reach yield strain in the measuring range of 50% shear strain. In the samples MP 5.2, MP 5.4 and MP 5.6, G" exceeded G' after a shear strain of 23.9, 11.5 and 13.5%, respectively.

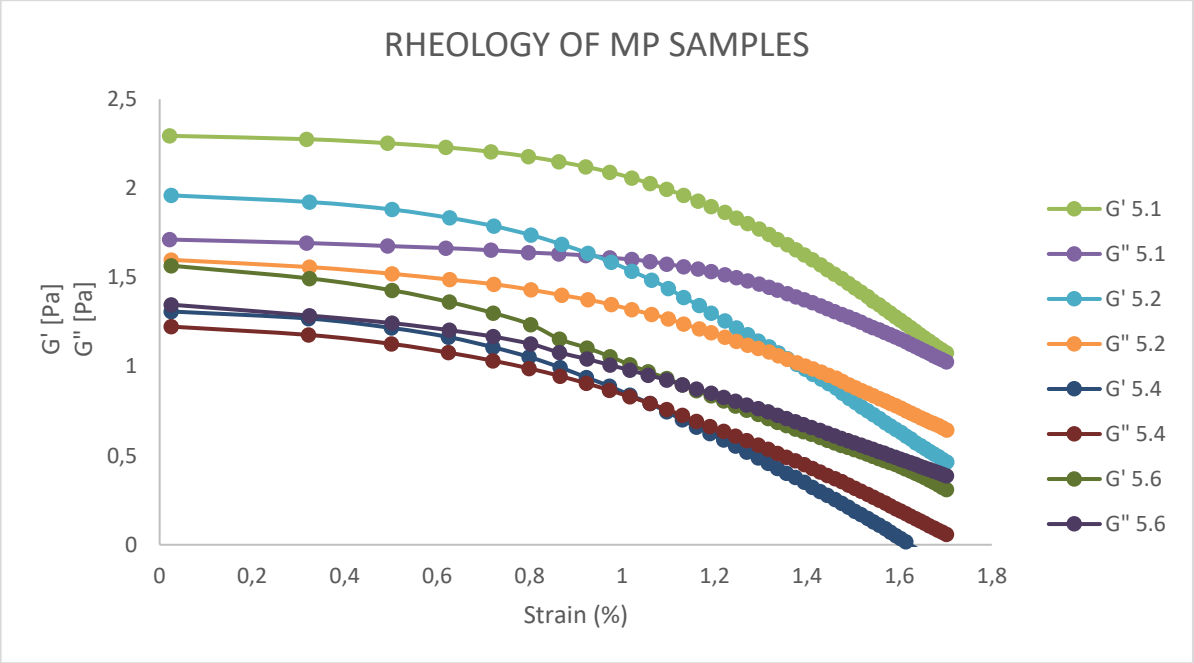

Figure 4. Foam rheology of suspension without enzymatic treatment.

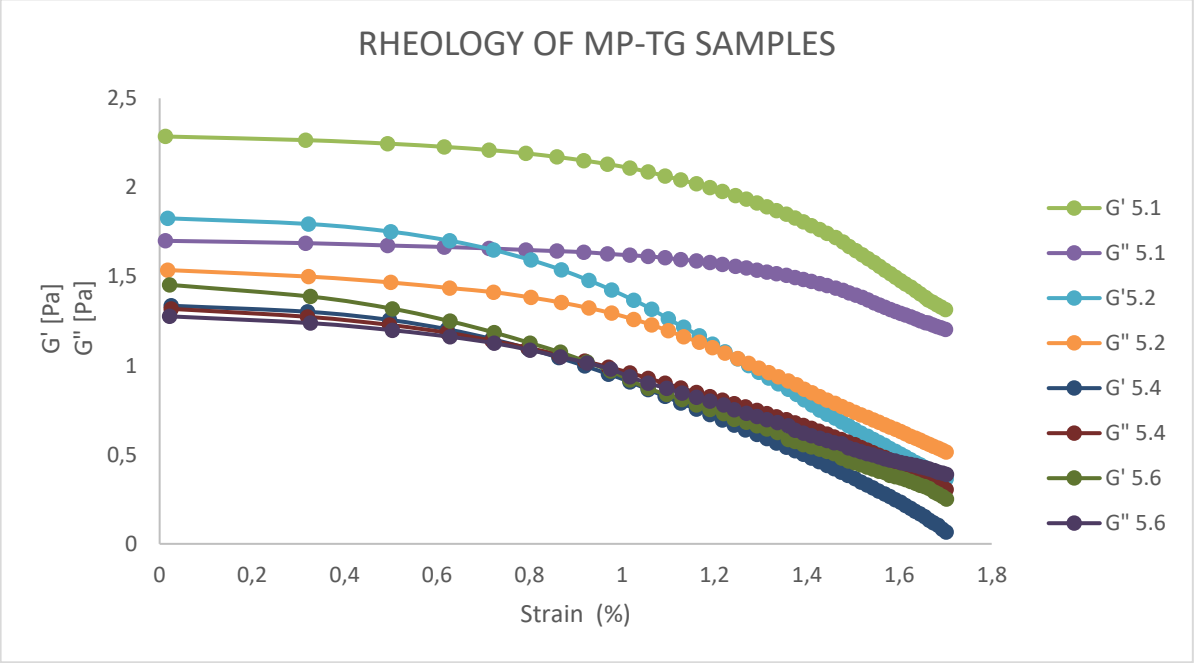

Figure 5. Foam rheology of suspension with enzymatic treatment.

Regarding the samples treated by TGase, an elastic behavior of the MP-TG 5.1 sample was observed (Figure 5), which, like the MP 5.1 sample, also did not reach the elastic limit within the analyzed range of shear deformation. Among the other samples, MP-TG 5.4 showed the least elasticity against deformation, with a yield strain of 6.27%. In general, the treatment

with TGase did not improve the elastic properties against deformation in the foams, thus the biggest improvement happened in relation to the pH variation. Jimenez-Junca et al. (2011) evaluated the rheology of milk foams and found similar behavior, wherein the foams lost their structure in the yield point behaving like a shear thinning fluid.

6. CONCLUSION

Suspension and foam analysis were performed to verify how the application of Transglutaminase in acidified MPC samples (pH 5.1, 5.2, 5.4 and 5.6) influences the foaming properties. The results showed that the largest particle size was found in samples at pH close to the isoelectric point of the caseins, however, there was no statistically significant difference between the particle size of the samples. Viscosity was higher in samples at pH 5.1, which can be related to the presence of aggregates. Also, only in samples at pH 5.1 did samples with TGase show higher viscosity than without the enzyme. Regarding the adsorption of proteins at the interface, the interfacial tension did not vary significantly in relation to the pH variation or in relation to the enzymatic treatment. In general, it seems that the enzyme Transglutaminase does not improve the foaming properties of milk proteins at the pH values of 5.1, 5.2, 5.4, and 5.6. However, the pH decrease tends to improve slightly the foaming properties, e.g. the stability of the foams. The results of rheology corroborate this hypothesis, once the samples with lower pH tend to be more elastic. The viscosity and hydrodynamic diameter helped to understand why the foams at lower pH showed better stability. These results allow further investigation of foaming properties of dairy aerated products at pH lower than milk pH (6.7 – 6.8).

7. PERSPECTIVES

Subsequently, more analysis should be done to characterize the interface and thin film to a deeper understanding of the stabilization mechanisms at different pH. Among these analyzes, there is interfacial rheology to study the viscoelastic properties of proteins adsorbed at the air/water interface. With respect to adsorption, it is important to investigate the adsorption kinetics, amount adsorbed, and layer thickness at the interface, which are possible to determine through ellipsometry. Also, average bubble size, thin film balance, and Small Angle Neutron Scattering (SANS) are additional experiments that can be done to assess the evolution of size

over time, thickness and stability of the film, and the shape and presence of protein aggregates, respectively.

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