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GUILHERME DE FIGUEIREDO FURTADO

PRODUCTION OF HETEROAGGREGATES OF LIPID DROPLETS AND GEL-LIKE EMULSIONS IN DIFFERENT EMULSIFICATION CONDITIONS

PRODUÇÃO DE HETEROAGREGADOS DE GOTAS LIPÍDICAS E EMULSÕES *GEL-LIKE* EM DIFERENTES CONDIÇÕES DE EMULSIFICAÇÃO

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RESUMO

Dentre as estratégias de redução de gordura em alimentos, destacam-se a heteroagregação de gotas lipídicas e a gelificação de emulsões. Dessa forma, este trabalho teve como objetivo produzir heteroagregados de gotas lipídicas e emulsões *gel-like* utilizando proteínas lácteas como agentes emulsificantes e diferentes métodos de emulsificação. Inicialmente emulsões foram produzidas com o intuito de caracterizar estruturalmente os sistemas obtidos e avaliar o efeito do ultrassom sobre as propriedades estruturais e tecnológicas das proteínas. O caseinato de sódio sofreu redução significativa de tamanho em sua estrutura molecular, o que não foi observado para a lactoferrina. A hidrofobicidade superficial foi afetada positivamente pelo aumento do tempo de ultrassom e foram observadas diferenças mínimas na eletroforese em gel da lactoferrina. Não foram detectadas diferenças no sinal dicróico para o caseinato de sódio, mas uma leve mudança conformacional foi observada para a lactoferrina. Emulsões com tamanho de gotas reduzido foram obtidas com maiores tempos de sonicação para ambas as proteínas, no entanto, a lactoferrina levou à formação de emulsões mais estáveis. O tratamento ultrassônico prévio das proteínas melhorou suas propriedades emulsificantes, mas este tratamento ultrassônico em conjunto com o processo de formação de gotas resultou em emulsões mais estáveis, facilitando a deposição dos emulsificantes na interface óleo/água. A partir desses resultados, as emulsões foram misturadas em diferentes razões de volume, permitindo a formação de heteroagregados. As características desses heteroagregados dependeram fortemente da razão de volume das emulsões misturadas. Além disso, variações de força iônica não influenciaram a estabilidade dos heteroagregados. Na etapa seguinte os heteroagregados foram caracterizados frente às condições do trato gastrointestinal. A digestão lipídica dependeu do tipo de emulsificante que recobria as gotas, bem como da ação das enzimas digestivas e dos sais biliares. Os heteroagregados apresentaram o menor percentual de digestão lipídica, possivelmente devido a interação dos lipídeos com os peptídeos que foram digeridos. Na última etapa, foram produzidas emulsões *gel-like* através de uma técnica a frio. Para isso, dispersões de lactoferrina foram previamente aquecidas por aquecimento ôhmico ou convencional. O efeito do aquecimento na agregação da lactoferrina foi avaliado a fim de fornecer conhecimento sobre os mecanismos que podem influenciar nas propriedades das emulsões gelificadas. A formação das emulsões foi atribuída à capacidade emulsificante da lactoferrina, combinada com o método de emulsificação e o tratamento térmico da proteína. O aquecimento ôhmico influenciou no desdobramento e agregação das moléculas de lactoferrina.

Comparado ao aquecimento convencional, foi possível obter moléculas menos agregadas, o que foi confirmado pelo menor aumento de tamanho, turbidez e fluorescência, refletindo diretamente nas emulsões obtidas. As propriedades reológicas e microestruturais dependeram do tipo de aquecimento, onde o aquecimento convencional possibilitou a formação de emulsões *gel-like* com uma estrutura mais forte. De uma forma geral, foi possível avaliar o efeito do ultrassom e do aquecimento nas propriedades estruturais e tecnológicas das proteínas, bem como propor estratégias de estruturação de emulsões visando a substituição e redução de gorduras em produtos alimentícios.

Palavras-chave: emulsões, proteínas, estabilidade, digestibilidade.

ABSTRACT

Among the fat reduction strategies in foods, the heteroaggregation of lipid droplets and the gelation of emulsions stand out. Thus, the aim of this work was to produce heteroaggregates of lipid droplets and gel-like emulsions using milk proteins as emulsifying agents and different emulsification methods. Initially, emulsions were produced with the purpose of characterizing structurally the obtained systems and evaluate the effect of ultrasound on the structural and technological properties of proteins. Sodium caseinate molecular structure showed a significantly reduce in size, which was not observed for lactoferrin. Surface hydrophobicity was positively affected by the increase in the duration of ultrasonic treatment and minimal differences were observed in lactoferrin gel electrophoresis. No differences were detected in the dichroic signal for sodium caseinate, but a slight conformational change was observed for lactoferrin. Emulsions with reduced droplet size were obtained with higher sonication times for both proteins, however, lactoferrin led to the formation of more stable emulsions. Previous ultrasonic treatment of the proteins improved their emulsifying properties, but this ultrasonic treatment associated to the droplet formation process resulted in more stable emulsions, facilitating the deposition of the emulsifiers at the oil/water interface. From these results, the emulsions were mixed in different volume ratios, allowing the formation of heteroaggregates. The characteristics of these heteroaggregates strongly depended on the volume ratio of the mixed emulsions. In addition, variations in ionic strength did not influence the stability of heteroaggregates. In the next step the heteroaggregates were characterized against conditions of the gastrointestinal tract. Lipid digestion depended on the type of emulsifier that covered the drops, as well as the action of digestive enzymes and bile salts. Heteroaggregates presented the lowest percentage of lipid digestion, possibly due to the interaction of the lipids with the peptides that were digested. In the latter step, gel-like emulsions were produced by a cold technique. For this, lactoferrin dispersions were preheated by ohmic or conventional heating. The effect of heating on aggregation of lactoferrin was evaluated in order to provide knowledge about the mechanisms that may influence the properties of the gelled emulsions. The formation of the emulsions was attributed to the emulsifying ability of lactoferrin, combined with the emulsification method and the heat treatment of the protein. The ohmic heating influenced the unfolding and aggregation of the lactoferrin molecules. Compared to conventional heating, it was possible to obtain less aggregate molecules, which was confirmed by the smaller increase in size, turbidity and fluorescence, directly reflecting the obtained emulsions. The rheological

and microstructural properties depended on the type of heating, since conventional heating allowed the formation of gel-like emulsions with a stronger structure. In general, it was possible to evaluate the effect of ultrasound and heating on the structural and technological properties of proteins, as well as to propose strategies for structuring emulsions aiming at the substitution and reduction of fats in food products.

Keywords: emulsions, proteins, stability, digestibility.

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1.1 INTRODUÇÃO

A obesidade é um problema de saúde em muitos países, pois está associada ao grande risco de ocorrência de algumas enfermidades, tais como doenças cardiovasculares, diabetes, acidente vascular cerebral e câncer (ARANCETA *et al.*, 2009). O aumento do número de obesos tem sido atribuído à ampla oferta de alimentos de baixo custo e altamente calóricos, e também pelo aumento do estilo de vida sedentário (MAO e MCCLEMENTS, 2012a). No entanto, as gorduras presentes nos alimentos desempenham um papel fundamental na determinação da aparência, textura e sabor, e quando são removidas dos alimentos muitas das qualidades desejáveis são perdidas, afetando negativamente atributos sensoriais de qualidade (MCCLEMENTS e DEMETRIADES, 1998). Dessa forma, várias estratégias de redução de gordura em alimentos têm sido desenvolvidas, incluindo o uso de gorduras de baixa absorção, gorduras de calorias reduzidas, espessantes e partículas coloidais (WILLIAMS e BUTTRISS, 2006).

Uma ampla variedade de produtos alimentícios é constituída parcial ou integralmente por emulsões como o leite, o iogurte, molho para saladas, maionese, sorvete e muitos outros. Emulsões óleo em água (O/A) convencionais são sistemas termodinamicamente instáveis e, para manterem-se estáveis por períodos consideráveis de tempo, devem ser adicionadas substâncias com atividade de superfície, denominados emulsificantes (MCCLEMENTS, DECKER e WEISS, 2007). Também podem ser adicionados estabilizantes que aumentam a viscosidade da fase contínua (DICKINSON, 2003). Dentre os agentes emulsificantes encontram-se as proteínas que são de grande interesse pois são naturais e de característica anfifílica, reduzindo a tensão na interface óleo/água (LAM e NICKERSON, 2013).

Outro fator muito importante que se encontra diretamente relacionado à estabilidade cinética das emulsões é a escolha do método de emulsificação a ser utilizado (SANTANA, PERRECHIL e CUNHA, 2013). A emulsificação através de homogeneização a altas pressões é uma das técnicas mais utilizadas na indústria de alimentos (SANTANA, PERRECHIL e CUNHA, 2013). O homogeneizador de alta pressão consiste essencialmente de uma bomba de alta pressão e uma válvula de homogeneização, sendo utilizado para produzir emulsões com diâmetros de gotas menores que 1,0 µm (até 0,1 µm) e com baixo índice de polidispersidade (STANG, SCHUCHMANN e SCHUBERT, 2001). A técnica de ultrassom tem sido bastante utilizada na produção de emulsões (BECHER, 1965; BROWN e

GOODMAN, 1965; ABISMAïL *et al.*, 1999; ABBAS *et al.*, 2013; SILVA, ROSA e MEIRELES, 2015) com diversos benefícios em relação aos métodos convencionais de emulsificação, dentre eles: a formação de gotas com tamanho reduzido e estreita distribuição de tamanho, maior estabilidade cinética, baixo custo de produção, facilidade de operação, limpeza e controle do dispositivo (ABBAS *et al.*, 2013). Esta técnica baseia-se na aplicação de um campo acústico que em conjunto com o fenômeno da cavitação provocam a formação de gotas (LI e FOGLER, 1978a; b). Além disso, o ultrassom também tem sido utilizado para melhorar as propriedades funcionais e tecnológicas de proteínas (SHANMUGAM, CHANDRAPALA e ASHOKKUMAR, 2012; O'SULLIVAN *et al.*, 2014; SHANMUGAM e ASHOKKUMAR, 2014; YANJUN *et al.*, 2014).

Emulsões com diferentes estruturas, propriedades físico-químicas, e atributos funcionais podem ser preparadas através do controle das características das partículas coloidais (como tamanho, carga, forma, concentração), das condições ambientais (como pH, força iônica, temperatura) e do método de preparação (como a ordem de adição dos ingredientes e condições de mistura). Estudos recentes têm relatado que a heteroagregação controlada de gotas lipídicas carregadas com cargas opostas pode ser usada para manipular as características dos produtos à base de emulsões (MAO e MCCLEMENTS, 2012a; b). Esta técnica tem refletido em uma série de vantagens para a criação de produtos com teor de gordura reduzido, contendo quantidades apreciáveis de proteína, que podem induzir a sensação de saciedade (WESTERTERP-PLANTENGA *et al.*, 2009). Emulsões *gel-like*, as quais são tratadas termicamente, acidificadas ou adicionadas de sais (CHEN *et al.*, 2000; BOUTIN *et al.*, 2007; YE e TAYLOR, 2009), também têm mostrado ser promissoras para a substituição e redução de gorduras em produtos alimentícios (PINTADO *et al.*, 2015; PINTADO *et al.*, 2016).

Desta forma, acredita-se que o uso de proteínas emulsificantes em conjunto com métodos de emulsificação de alta eficiência podem ser adequados para a produção de novas características desejáveis em produtos com teor de gordura reduzido através do processo de heteroagregação ou de gelificação de emulsão.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

O objetivo geral desta tese foi estudar a técnica de heteroagregação de gotas a partir de emulsões obtidas por ultrassom usando caseinato de sódio e lactoferrina como agentes emulsificantes, bem como estudar a produção de emulsões *gel-like* a partir do aquecimento ôhmico e convencional de dispersões de lactoferrina.

1.2.2 Objetivos específicos

=> Produzir emulsões utilizando caseinato de sódio e lactoferrina através do processo de emulsificação por ultrassom; caracterizar estruturalmente os sistemas obtidos e avaliar o efeito do ultrassom sobre as propriedades estruturais e tecnológicas das proteínas.

=> Estudar a formação, estabilização e caracterização de heteroagregados a partir de emulsões obtidas em diferentes condições de emulsificação e força iônica.

=> Caracterizar os heteroagregados frente às condições *in vitro* do trato gastrointestinal.

=> Produzir emulsões *gel-like* a partir de dispersões de lactoferrina aquecidas por um sistema ôhmico e um sistema convencional de aquecimento; caracterizar fisicamente e estruturalmente os sistemas obtidos e avaliar o efeito do aquecimento sobre as propriedades estruturais da lactoferrina.

1.3 ESTRUTURA DA TESE

A apresentação desta tese foi organizada em nove capítulos como descrito a seguir. No **Capítulo 1** são expostos uma introdução geral do estudo, o objetivo geral e os objetivos específicos envolvidos na realização desta tese. No **Capítulo 2** são abordados aspectos teóricos dos sistemas estudados, bem como uma revisão bibliográfica relatando a literatura recente e mais relevante sobre o tema deste trabalho. Os **Capítulos 3**, **4**, **5 e 6** consistem em artigos publicados (FURTADO *et al.*, 2016; FURTADO *et al.*, 2017a; b) ou submetidos para publicação em periódicos internacionais. No **Capítulo 3** são apresentados os resultados experimentais sobre a influência do ultrassom nas propriedades estruturais e emulsificantes do caseinato de sódio e da lactoferrina. Posteriormente, com base nos resultados obtidos de tempo de ultrassom, no **Capítulo 4**, a formação, estabilização e caracterização de heteroagregados a partir de emulsões obtidas em diferentes condições de emulsificação e força iônica foi avaliada. Com base nos resultados obtidos de formação e estabilidade dos heteroagregados, no **Capítulo 5**, a digestibilidade *in vitro* dos heteroagregados foi avaliada. No **Capítulo 6** são apresentados os resultados de um estudo complementar desenvolvido em estágio sanduíche na Universidade do Minho (Portugal) onde foi avaliado o efeito do aquecimento ôhmico nas propriedades estruturais da lactoferrina e na subsequente produção de emulsões *gel-like*. Por fim, no **Capítulo 7**, uma discussão geral é realizada e no **Capítulo 8** são apresentadas as principais conclusões do desenvolvimento da tese. O **Capítulo 9** apresenta as referências bibliográficas e os **Anexos** apresentam as licenças para utilização dos artigos publicados.

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CAPÍTULO 2 - REVISÃO BIBLIOGRÁFICA

2.1 REVISÃO BIBLIOGRÁFICA

2.1.1 Emulsões

As emulsões compreendem uma mistura de óleo e água, contendo gotas dispersas e uma fase contínua. O diâmetro das gotas é tipicamente da ordem de 0,1 a 100 µm, no entanto, podem variar de poucos nanômetros a centenas de micrômetros. Os dois tipos mais comuns de emulsões são do tipo água em óleo A/O e óleo em água O/A, no entanto, podem existir emulsões múltiplas como água em óleo em água A/O/A, óleo em água em óleo O/A/O e sistemas ainda mais complexos (MIKULA, 1992).

Uma emulsão é formada a partir de três requisitos fundamentais, sendo eles: a existência de dois líquidos imiscíveis; agitação suficiente para que um dos líquidos esteja disperso na forma de gotas no outro líquido; e um emulsificante para estabilizar as gotas dispersas (ARNOLD e SMITH, 1992). São sistemas termodinamicamente instáveis em virtude da grande tensão interfacial existente entre a água e o óleo, acompanhada pelo aumento da área interfacial no processo de formação das gotas, implicando em uma elevada energia livre de Gibbs ($\Delta G_f > 0$) (CASTELLAN, 1986). Nas emulsões, o termo de energia interfacial ($\gamma \Delta A$) da Eq. 2.1 geralmente é mais elevado que a entropia de formação das gotas (ΔS_f), devido ao processo de redução do tamanho das gotas e consequente aumento de área superficial. Desta forma, a emulsão tende ao equilíbrio termodinâmico com a diminuição da área interfacial entre as duas fases, promovendo a coalescência das gotas e favorecendo a separação das fases (ANTON, BENOIT e SAULNIER, 2008).

$$\Delta G_f = \gamma \Delta A - T \Delta S_f \qquad \text{(Eq. 2.1)}$$

onde:

ΔG_f é a energia livre de formação, [J/kg];

γ é a tensão interfacial, [J/kg.m^2];

ΔA representa o aumento da área superficial total das gotas, [m^2];

T a temperatura, [K];

ΔS_f a entropia de formação, [J/kg.K].

A separação das fases de uma emulsão está relacionada com sua propriedade mais importante que é a estabilidade cinética, ou o tempo em que a emulsão permanece sem mostrar mudanças significativas na distribuição e tamanho das gotas, bem como no estado de agregação e arranjo espacial (ADAMSON e GAST, 1987). A desestabilização das emulsões pode ocorrer devido aos seguintes processos: cremeação, sedimentação, floculação e coalescência das gotas (Figura 2.1). A cremeação ocorre se as gotas dispersas possuem densidade menor que a fase contínua, e estas migram para a parte superior da emulsão. No caso da densidade das gotas ser maior que a densidade da fase contínua ocorre o processo de sedimentação. Já quando duas ou mais gotas se agregam mantendo sua integridade é denominado floculação e na coalescência, duas ou mais gotas se fundem formando uma gota maior (MCCLEMENTS, 2005). Há ainda o mecanismo de amadurecimento de Ostwald onde o aumento no tamanho das gotas se dá pelo transporte de massa através da fase contínua (TAYLOR, 2003).
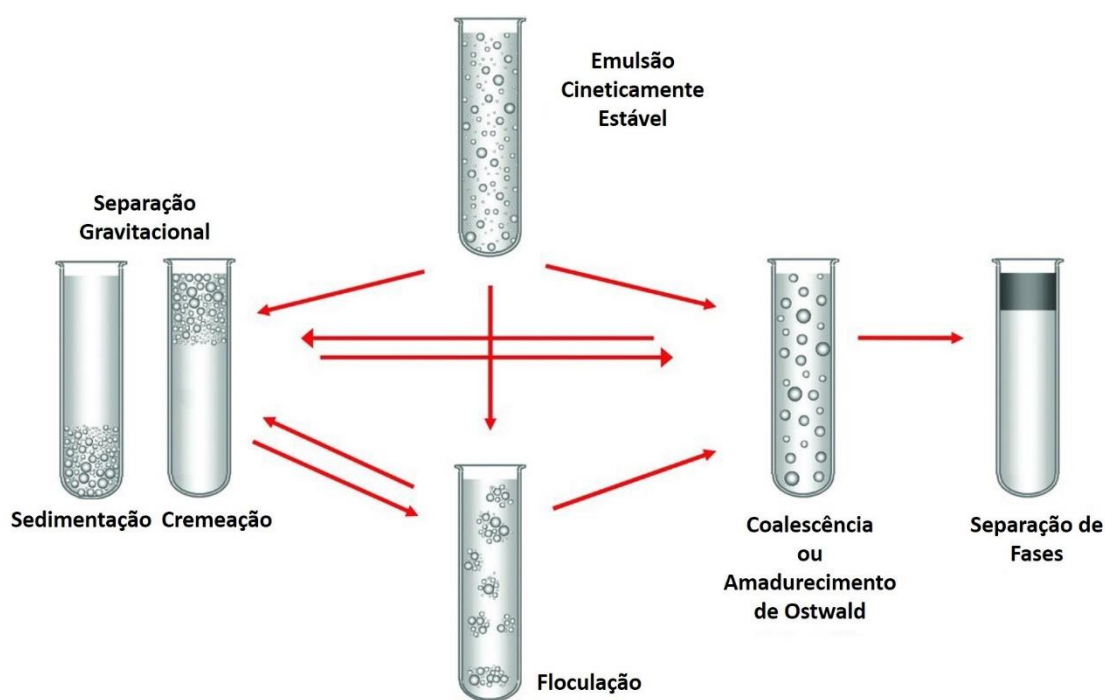
Figura 2.1 **-** Mecanismos de instabilidade em emulsões O/A. Fonte: (MCCLEMENTS e RAO, 2011)

2.1.2 Agentes Emulsificantes e Estabilizantes

A separação das fases de uma emulsão pode ser evitada ou retardada pela adição de agentes emulsificantes e/ou estabilizantes. Os emulsificantes se adsorvem na interface existente

entre o óleo e a água, fornecendo uma camada protetora à floculação ou coalescência das gotas. Estas moléculas atuam na redução da tensão interfacial durante a formação da emulsão, levando à formação de um filme viscoelástico na interface que pode ser rígido (barreira mecânica para a coalescência), formar uma dupla camada elétrica (barreira contra a aproximação das gotas) ou uma camada flexível (barreira estérica). Já os estabilizantes são definidos como componentes que conferem estabilidade às emulsões via modificação das propriedades reológicas da fase contínua, com o aumento da sua viscosidade ou mesmo sua gelificação (DICKINSON, 2003).

Os agentes emulsificantes são constituídos por uma parte hidrofílica e outra lipofílica, sendo denominados de anfifílicos. Na presença de água e óleo, a porção hidrofílica é atraída pela água, enquanto a lipofílica é atraída para o óleo (CAPEK, 2004). Existem duas grandes classes de agentes emulsificantes usadas no processamento de alimentos: surfactantes de baixa massa molecular (monoglicerídeos, polissorbatos, lecitina, etc.) e emulsificantes macromoleculares (geralmente proteínas, como as do leite e do ovo) (DICKINSON, 2003).

As proteínas são comumente usadas como agentes emulsificantes em emulsões óleo em água presentes em produtos alimentares, tais como bebidas, iogurte, maionese e sorvetes. Dentre as proteínas mais utilizadas estão as proteínas do leite, do ovo e da soja (MCCLEMENTS, 2005; DICKINSON, 2009). As propriedades estruturais e interfaciais das proteínas definem sua capacidade de adsorção na interface de modo a proporcionar uma combinação de repulsão eletrostática e estérica entre as gotas de óleo, obtendo-se então uma emulsão cineticamente estável (WILDE *et al.*, 2004; MCCLEMENTS, 2005). No entanto, as gotas das emulsões estabilizadas por proteínas tendem a se agregar, flocular ou coalescer quando expostas a ambientes desfavoráveis, como é o caso de sistemas alimentícios ácidos, principalmente em pH próximo ao ponto isoelétrico da proteína, na presença de eletrólitos, ou calor, devido à neutralização de carga eletrostática ou desnaturação das proteínas (DIFTIS e KIOSSEOGLOU, 2006; DAY *et al.*, 2009).

2.1.2.1 Caseinato de Sódio

As proteínas do leite são muito utilizadas como emulsificantes/estabilizantes (GUZEY e MCCLEMENTS, 2006), além de possuírem alto valor nutricional e serem consideradas seguras (GRAS) (CHEN, REMONDETTO e SUBIRADE, 2006). As caseínas representam cerca de 75 a 85% das proteínas do leite (DAMODARAN, PARKIN e FENNEMA, 2007), são

partículas coloidais, aproximadamente esféricas e altamente hidratadas ($\approx$ 4 g água / g proteína). Essas proteínas são consideradas fosfoproteínas compostas por diferentes frações (α_{s1}-, α_{s2}-, β- e κ-caseína) susceptíveis à associação em micelas devido às regiões de alta hidrofobicidade e distribuição de cargas dos aminoácidos. A distribuição de tamanho da estrutura supramolecular da micela de caseína tem sido reportada em uma extensa faixa entre 20 e 600 nm de diâmetro, com tamanho médio entre 100 e 200 nm, e o número de moléculas de proteínas que constituem a supramolécula de caseína é cerca de 10^4 para uma partícula coloidal de $\approx$ 150 nm (MCMAHON e OOMEN, 2008; DALGLEISH e CORREDIG, 2012; MCSWEENEY e FOX, 2013). Além disso, as caseínas são proteínas ricas em prolina e por isso, apresentam estrutura altamente desorganizada, formam pouca estrutura secundária e terciária e não formam estruturas globulares (WALSTRA, WOUTERS e GEURTS, 2006), permitindo-as serem flexíveis e não sofrerem alterações conformacionais induzidas pelo calor (MCCLEMENTS, 2015).

No processo de obtenção do caseinato de sódio, a caseína é precipitada com ácido e dissolvida em álcali (hidróxido de sódio), e essa soluço é então seca por atomização (WALSTRA, WOUTERS e GEURTS, 2006). No entanto, existem diferenças consideráveis entre o caseinato de sódio e as micelas de caseína. Em termos de composição, os dois materiais têm diferentes teores de fosfato de cálcio, e funcionalmente o caseinato é mais solúvel do que as micelas de caseínas. Hidrodinamicamente, o caseinato de sódio é menor (10 – 100 nm) do que as caseínas na forma micelar (PAN, ZHONG e BAEK, 2013). Em pH neutro o caseinato de sódio é carregado negativamente (MA *et al.*, 2009), e tem sido muito utilizado como ingrediente na indústria de alimentos, devido sua elevada capacidade gelificante e emulsificante (DICKINSON e GOLDING, 1997; DICKINSON, 2006).

2.1.2.2 Lactoferrina

As proteínas do soro do leite (α-lacotalbumina, β-lactoglobulina e albumina sérica que são as principais) representam 15 a 22% das proteínas do leite (DAMODARAN, PARKIN e FENNEMA, 2007). Existem ainda proteínas minoritárias no soro do leite, como a lactoferrina, uma glicoproteína globular da família das transferrinas (carreadores de ferro). A lactoferrina ocorre em muitos fluidos de secreção de mamíferos, e possuiu várias funções biológicas, como por exemplo, capacidade antioxidante, atividade antimicrobiana, antiviral e anticancerígena (WAKABAYASHI, YAMAUCHI e TAKASE, 2006). Esta proteína é composta por

polipeptídios de cadeia simples de cerca de 80 kDa contendo de uma a quatro glicanas (SPIK *et al.*, 1994). Além de apresentar efeitos benéficos contra doenças, o fato de ser segura para a saúde, amplia seu potencial de aplicação como aditivo alimentar para humanos e animais (WAKABAYASHI, YAMAUCHI e TAKASE, 2006). Estudos têm mostrado que a lactoferrina pode ser utilizada como agente emulsificante para estabilizar emulsões (SARKAR, GOH e SINGH, 2009; SARKAR, HORNE e SINGH, 2010; TOKLE e MCCLEMENTS, 2011; PINHEIRO, COIMBRA e VICENTE, 2016).

A lactoferrina apresenta ponto isoelétrico entre 8,6 e 8,9, e, portanto, é uma proteína básica, sendo catiônica em pH neutro enquanto que a maioria das proteínas globulares lácteas são aniônicas (STEIJNS e VAN HOOIJDONK, 2000). O ponto isoelétrico elevado da lactoferrina é devido ao alto teor de aminoácidos básicos (como lisina e arginina) (SGARBIERI, 1996; BAKER e BAKER, 2005), e em virtude de sua natureza básica, a lactoferrina pode ser purificada através de cromatografia de troca catiônica (CONESA *et al.*, 2008). Como a lactoferrina apresenta carga positiva sob uma ampla faixa de pH, comparada as demais proteínas lácteas, esta proteína estabiliza emulsões em um intervalo de pH muito mais amplo (YE e SINGH, 2006).

2.1.2.3 Estrutura e propriedades funcionais das proteínas

Uma proteína pode se apresentar em diferentes graus de estruturação (estrutura primária, secundária, terciária, quaternária) que são mantidos por vários tipos de ligação e/ou interações entre vários grupos funcionais dos aminoácidos que a compõem. As proteínas têm como base de sua estrutura os polipeptídeos, formados por ligações peptídicas entre os grupos ($-NH_2$) de um aminoácido e carboxílico (-COOH) de outro. A estrutura primária se caracteriza por apresentar apenas ligações peptídicas entre os aminoácidos formando polímeros de cadeia distendidas (*random coil*). Proteínas do tipo *random coil* existem em um estado desdobrado e de completa desordem estrutural. A estrutura secundária é mantida por ligações de hidrogênio que podem ser intramoleculares (estrutura helicoidal, α-hélice) ou intermoleculares (estrutura foliar, folha preguedada). As estruturas terciária e quaternária se referem ao arranjo espacial da cadeia polipeptídica (dobramento ou formação de laços), já dotada ou não de estrutura secundária. Na estabilização destas estruturas e na determinação da conformação de uma proteína entram forças de natureza diversas, tais como: ligações dissulfeto, ligações salinas ou

interações eletrostáticas, ligações de hidrogênio, interações dipolares, hidrofóbicas e de Van der Waals (SGARBIERI, 1996).

O tipo, número e sequência dos aminoácidos de uma proteína determina as suas características moleculares (como massa molecular, conformação, carga elétrica, flexibilidade e hidrofobicidade). Por sua vez, estas características moleculares determinam sua funcionalidade, como por exemplo, a sua capacidade para espessar soluções, formar géis, reter água, adsorver em interfaces, estabilizar emulsões e espumas, catalisar reações enzimáticas e ligar-se a moléculas (MCCLEMENTS *et al.*, 2009). Frequentemente, a funcionalidade das proteínas é associada a transições estruturais, como seu desdobramento em função da desnaturação (FOEGEDING e DAVIS, 2011) e subsequente exposição de grupos hidrofóbicos (RAFFAELE e PETER, 2013).

2.1.3 Métodos de emulsificação

Diferentes técnicas de emulsificação visam produzir emulsões com diâmetro de gotas reduzido e com baixo índice de polidispersidade. Para isso, faz-se necessário uma grande quantidade de energia, além da adição de um emulsificante (JAFARI *et al.*, 2008). As técnicas de emulsificação que empregam alta energia podem ser realizadas por dispositivos de alta pressão, ultrassônicos e tipo rotor-estator. Existem ainda técnicas que empregam baixa energia como emulsificação espontânea e temperatura de inversão de fases, no entanto os emulsificantes utilizados para produzir estas emulsões geralmente não são de grau alimentício ou são necessárias grandes concentrações do mesmo (SANTANA, PERRECHIL e CUNHA, 2013; SHANMUGAM e ASHOKKUMAR, 2014).

2.1.3.1 Emulsificação a altas pressões

A homogeneização a alta pressão visa a redução do tamanho das gotas de uma macroemulsão pré-formada por misturadores do tipo rotor-estator (MCCLEMENTS, 2005). O processo de homogeneização em alta pressão consiste na passagem da macroemulsão por um estreito orifício sob uma pressão elevada (Figura 2.2), sendo que o fluido acelera rapidamente, alcançando uma velocidade de até 300 m/s. A energia fornecida pelo processo como resultado do cisalhamento, impacto e cavitação é representada como a energia livre adicional necessária para a redução do tamanho de gotas que leva à criação de uma grande área interfacial (ANTON,

BENOIT e SAULNIER, 2008), permitindo produzir emulsões com gotas dispersas de diâmetros menores que 500 nm (WEISS, TAKHISTOV e MCCLEMENTS, 2006). Em geral, emulsões com reduzido tamanho de gota da fase dispersa, possuem maior estabilidade e textura mais fina (MCCLEMENTS, 2005). No entanto, a distribuição de tamanho também afeta diretamente a textura e estabilidade das emulsões (FLOURY, DESRUMAUX e LEGRAND, 2002) e dependendo da taxa de adsorção interfacial do emulsificante, a distribuição do tamanho de gotas pode ser mono ou polidispersa (JAFARI *et al.*, 2008).
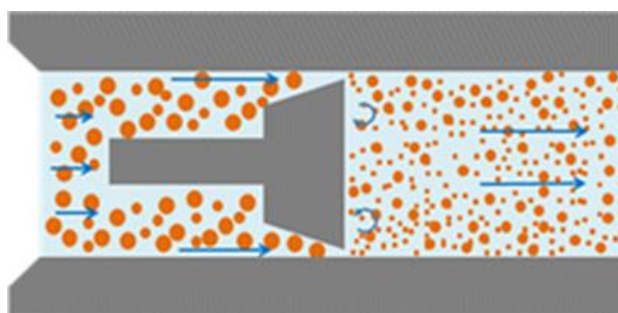
Figura 2.2 - Representação esquemática de um homogeneizador a alta pressão. Adaptado de SANTANA, PERRECHIL e CUNHA (2013)

O decréscimo no tamanho de gota pode levar a um aumento na viscosidade das emulsões estabilizadas por proteínas (KUHN e CUNHA, 2012; MANTOVANI, CAVALLIERI e CUNHA, 2016), o que pode ser atribuído a uma maior interação entre as gotas (PAL, 2000) devido a uma maior quantidade de proteína adsorvida ou a um maior empacotamento das proteínas na interface óleo-água (INNOCENTE *et al.*, 2009). No entanto, o decréscimo no tamanho de gota também pode levar a uma diminuição na viscosidade das emulsões (DESRUMAUX e MARCAND, 2002; SANTANA *et al.*, 2011), o que pode ser atribuído à redução da funcionalidade da proteína provocada pela pressão de homogeneização (FLOURY, DESRUMAUX e LEGRAND, 2002).

2.1.3.2 Emulsificação por ultrassom

O ultrassom é definido como ondas sonoras com frequências além do alcance da audição humana. Em frequências entre 20 e 100 kHz, ondas de ultrassom interagem com a matéria, produzindo mudanças físicas e químicas (ABBAS *et al.*, 2013). O ultrassom pode ser dividido em diferentes faixas de frequência usando sondas de alta e baixa frequência. Altas (100

kHz a 1 MHz) e baixas frequências (16-100 kHz) aplicam níveis de intensidade de 1 W.cm^{-2} e 10-1000 W.cm^{-2}, respectivamente (SORIA e VILLAMIEL, 2010).

A energia ultrassônica tem sido descrita como um eficiente modo para melhorar o desempenho de diferentes processos, como extração de compostos orgânicos e inorgânicos, homogeneização, dispersão de suspensões, dentre outros (NASCENTES *et al.*, 2001). Um aparato ultrassônico comum (Figura 2.3) usado na produção de emulsões é constituído de um gerador, um transdutor, um amplificador e uma sonda. O gerador produz energia elétrica que é convertida pelo transdutor em vibrações mecânicas em uma frequência similar à da corrente elétrica. Estas vibrações mecânicas são amplificadas e propagadas através de uma sonda, na forma de ondas acústicas. O processo de emulsificação por ultrassom ocorre em dois estágios. No primeiro estágio ocorre a geração de gotas primárias, devido a um campo acústico que produz ondas interfaciais, gerando uma instabilidade que causa a erupção da fase oleosa na fase aquosa na forma de gotas. O segundo estágio envolve a quebra das gotas primárias através da cavitação acústica, que exerce uma alta turbulência e força cisalhante local, produzindo violentas e assimétricas implosões das bolhas, que por consequência formam microjatos que também auxiliam na quebra das gotas primárias produzindo gotas de tamanho nanométrico (LI e FOGLER, 1978a; b).
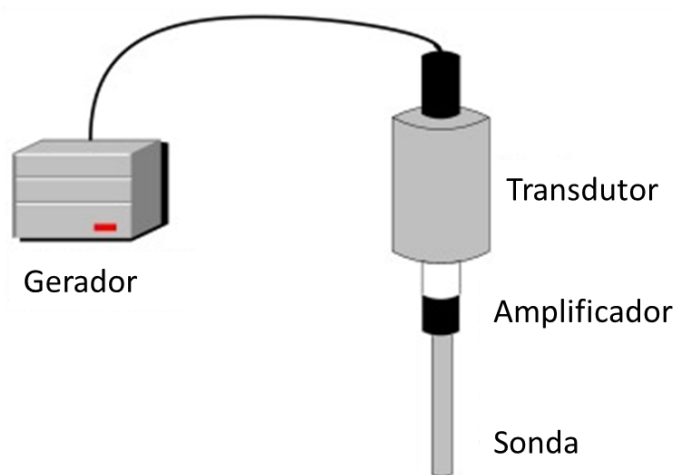
Figura 2.3 - Componentes de um ultrassom. Adaptado de SILVA, ROSA e MEIRELES (2015)

A eficácia do processo de emulsificação por ultrassom depende de fatores como o tempo e potência de ultrassom, a proporção óleo/água e as propriedades físico-químicas do óleo. Em geral, um aumento da potência e do tempo de ultrassom resulta em um maior

fracionamento da fase dispersa com uma diminuição no tamanho da gota (GAIKWAD e PANDIT, 2008). Estudos têm mostrado que dentre as técnicas de emulsificação o uso de ultrassom tem se estabelecido como uma ferramenta superior em comparação ao uso de rotor-estator em termos de obtenção de gotas de tamanho reduzido e alta eficiência energética (ABISMAïL *et al.*, 1999; KENTISH *et al.*, 2008).

2.1.3.2.1 Alterações estruturais causadas pelo ultrassom às proteínas

O uso do ultrassom tem demonstrado possuir uma capacidade de reduzir o tamanho das proteínas em solução aquosa e aumentar a sua dissolução. A sonicação de proteínas lácteas, como proteínas do soro e caseínas, resultou em uma redução de tamanho (CHANDRAPALA *et al.*, 2011; ARZENI *et al.*, 2012; SHANMUGAM, CHANDRAPALA e ASHOKKUMAR, 2012; MCCARTHY *et al.*, 2014; YANJUN *et al.*, 2014) associado às altas forças cisalhantes devido a cavitação ultrassônica (TRUJILLO e KNOERZER, 2011). No entanto, tempos prolongados de sonicação podem levar a um aumento de tamanho devido a desnaturação das proteínas, diminuição da solubilidade e agregação em virtude das elevadas temperaturas do tratamento ultrassônico (GÜLSEREN *et al.*, 2007; SHANMUGAM, CHANDRAPALA e ASHOKKUMAR, 2012; MCCARTHY *et al.*, 2014). O uso do ultrassom também tem mostrado reduzir a viscosidade das dispersões de proteína (ZISU *et al.*, 2010; ARZENI *et al.*, 2012; YANJUN *et al.*, 2014) em virtude da redução no tamanho como consequência da cavitação.

Apesar da redução de tamanho, muitos autores relataram que o uso do ultrassom não provocou mudanças na estrutura primária das proteínas (MARTINI, POTTER e WALSH, 2010; JIANG *et al.*, 2014; YANJUN *et al.*, 2014; O'SULLIVAN *et al.*, 2016), pois o tratamento ultrassônico fornece uma energia insuficiente para causar ruptura das ligações peptídicas (O'SULLIVAN *et al.*, 2017). No entanto, alguns autores reportaram a diminuição da massa molecular das proteínas após o tratamento ultrassônico (JAMBRAK *et al.*, 2010; JAMBRAK *et al.*, 2014).

Estudos também relatam a melhora nas propriedades emulsificantes das proteínas tratadas por ultrassom (O'SULLIVAN *et al.*, 2014; YANJUN *et al.*, 2014). A melhora na formação e estabilidade das emulsões pode ser atribuída às forças de cisalhamento geradas durante a cavitação acústica que levaram à desnaturação parcial das proteínas levando ao aumento da hidrofobicidade destas moléculas (SHANMUGAM, CHANDRAPALA e

ASHOKKUMAR, 2012; SHANMUGAM e ASHOKKUMAR, 2014), contribuindo para a melhor adsorção interfacial.

2.1.4 Heteroagregação de gotas lipídicas

A técnica de heteroagregação de gotas lipídicas carregadas com cargas opostas tem sido utilizada na produção de materiais altamente viscosos com reduzido teor de gordura (MAO e MCCLEMENTS, 2012a; b). O fenômeno da heteroagregação se dá pela agregação de partículas similares, as quais podem diferir em tamanho, forma, carga, composição química ou outras propriedades (YATES *et al.*, 2005; LOPEZ-LOPEZ *et al.*, 2006).

As gotas carregadas com cargas opostas interagem umas com as outras através de atração eletrostática (Figura 2.4), levando à formação de *microclusters* que formam uma rede tridimensional de agregados que apresentam comportamento elástico. Assim, a microestrutura e as propriedades reológicas destes sistemas dependem da concentração de gotas, da razão entre cargas positivas/negativas e das propriedades da fase aquosa que influenciam nas interações eletrostáticas, como pH e força iônica (MAO e MCCLEMENTS, 2012a; b). Controlar as propriedades interfaciais para induzir o fenômeno de agregação é complexo devido ao número limitado de emulsificantes que possuem carga superficial que sejam de grau alimentício, e ainda que possam ser utilizados dentro de uma ampla faixa de pH (MAIER, ZEEB e WEISS, 2014).
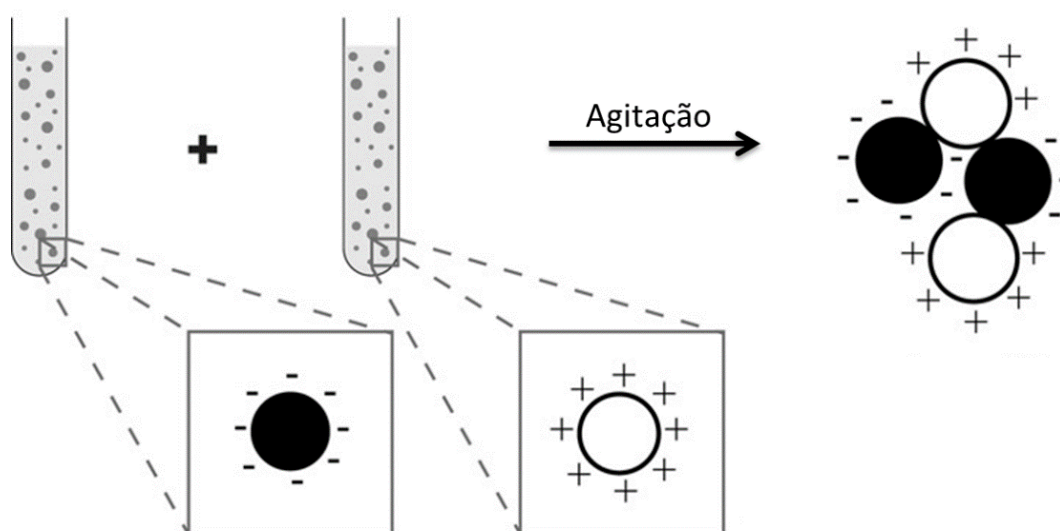
Figura 2.4 - Diagrama esquemático do processo de formação de heteroagregados de gotas recobertas com proteínas de cargas opostas. Adaptado de MAIER, ZEEB e WEISS (2014).

Um dos modelos mais utilizados para descrever as interações entre partículas coloidais carregadas é a teoria DLVO (ISLAM, CHOWDHRY e SNOWDEN, 1995; MAO e MCCLEMENTS, 2013). Nesta teoria, a interação entre as partículas coloidais é representada pelo somatório de três contribuições (Eq. 2.2):

$$V_T = V_V + V_E + V_S \qquad\qquad\qquad \text{(Eq. 2.2)}$$

onde:

V_T é a energia de interação total;

V_V é a energia resultante das forças de van der Waals;

V_E é a energia associada com as interações eletrostáticas;

V_S é a energia associada com as forças de repulsão estérica.

As interações eletrostáticas podem ser atrativas ou repulsivas, o que leva a diferentes perfis de interação coloidal (MAO e MCCLEMENTS, 2013).

2.1.5 Emulsões *gel-like*

Emulsões estabilizadas por proteínas lácteas, como proteínas do soro, podem ser transformadas em emulsões gelificadas (*gel-like*) através de técnicas tradicionais como tratamento térmico (CHEN *et al.*, 2000; LIU e TANG, 2011), acidificação com glucona-delta-lactona (BOUTIN *et al.*, 2007; YE e TAYLOR, 2009) ou adição de sais (SOK LINE, REMONDETTO e SUBIRADE, 2005; YE e TAYLOR, 2009).

O tratamento térmico necessário para produzir as emulsões *gel-like* limita suas aplicações em formulações contendo ingredientes sensíveis ao calor, como bioativos, enquanto que sistemas obtidos por técnicas a frio (sem tratamento térmico) são mais favoráveis para incorporar tais ingredientes (SOK LINE, REMONDETTO e SUBIRADE, 2005; LIU e TANG, 2011), mostrando melhores características funcionais, como maior proteção dos bioativos e uma melhor estabilidade oxidativa dos lipídeos (LEE, CHOI e MOON, 2006). No entanto, nas técnicas a frio, um tratamento térmico prévio das dispersões de proteína é necessário para garantir a formação dos agregados (SOK LINE, REMONDETTO e SUBIRADE, 2005; BOUTIN *et al.*, 2007) através da desnaturação parcial da proteína nativa e a subsequente

agregação das moléculas desnoveladas (NIELSEN, SINGH e LATHAM, 1996). A adição de íons divalentes pode ser feita de modo a melhorar a reticulação das proteínas e promover sua gelificação (BRYANT e MCCLEMENTS, 2000).

O tratamento térmico pode ser realizado de maneira convencional ou ainda pode-se utilizar o aquecimento ôhmico. Esta tecnologia tem recebido grande atenção devido ao seu rápido aquecimento, onde altas temperaturas são aplicadas em um curto espaço de tempo, permitindo obter produtos com qualidade superior àqueles processados com aquecimento convencional (CASTRO *et al.*, 2003; MACHADO *et al.*, 2010; RODRIGUES *et al.*, 2015). Durante o aquecimento ôhmico, uma corrente elétrica passa pelo alimento que se comporta como um resistor em um circuito elétrico, gerando calor internamente, de acordo com a lei de Joule (DE ALWIS e FRYER, 1990). A presença de variáveis elétricas inerentes ao aquecimento ôhmico (como campo elétrico, frequência elétrica e densidade da corrente) podem levar a mudanças conformacionais e comportamentos de gelificação distintos em proteínas lácteas, como as proteínas do soro do leite (PEREIRA *et al.*, 2010; PEREIRA, TEIXEIRA e VICENTE, 2011; RODRIGUES *et al.*, 2015).

2.1.6 Digestibilidade *in vitro*

Apesar das dificuldades de simular com precisão os eventos físico-químicos e fisiológicos que ocorrem no trato gastrointestinal humano, o uso de modelos de digestão *in vitro* com o intuito de estudar alterações estruturais, digestibilidade, biodisponibilidade e liberação de compostos alimentícios nas condições gastrointestinais tem sido de grande interesse (HUR, DECKER e MCCLEMENTS, 2009). Os estudos de digestão *in vitro* são bastante utilizados com o objetivo de avaliar a lipólise de emulsões alimentícias no trato gastrointestinal por apresentarem menor custo e tempo, quando comparados aos estudos *in vivo* e também por não possuírem restrições éticas. Existem dois tipos de modelos gastrointestinais: os estáticos e os dinâmicos. Os modelos estáticos são aqueles nos quais a agitação da amostra é realizada através de movimento orbital e não reproduzem os movimentos peristálticos. Já os modelos dinâmicos incluem os processos físicos e mecânicos além de promover a simulação das mudanças das condições físico-químicas de maneira contínua para se aproximar mais às condições *in vivo* (HOEBLER et al., 2002; PARADA e AGUILERA, 2007; KONG e SINGH, 2008). No entanto, grande parte dos estudos *in vitro* são realizados em modelos estáticos onde a digestão na boca, no estômago e intestino delgado são reproduzidas em etapas consecutivas, levando em conta a

presença e concentrações de enzimas digestivas, pH, tempo de digestão, concentrações de sais e temperatura. O modelo estático utilizado neste estudo foi um protocolo padronizado definido recentemente com o objetivo de uniformizar os protocolos existentes na literatura e produzir resultados mais comparáveis (MINEKUS *et al.*, 2014).

O estudo da digestão em sistemas emulsionados está relacionado principalmente a hidrólise lipídica que envolve a adsorção dos sais biliares para a posterior adsorção da lipase na superfície das gotas de óleo, o que faz com que a enzima tenha acesso aos triacilgliceróis (WILDE e CHU, 2011). Portanto, a natureza do emulsificante impacta diretamente na susceptibilidade das gotas de óleo quanto à coalescência e quebra dentro do trato gastrointestinal, alterando a área total superficial exposta à ação da lipase. Assim, as características da camada interfacial influenciam na adsorção e atividade da lipase na interface óleo/água (HUR, DECKER e MCCLEMENTS, 2009). Os produtos resultantes da lipólise são então incorporados nas micelas de sais biliares/fosfolipídeos para serem transportados no meio aquoso e absorvidos através da mucosa do intestino delgado, se estiverem em um sistema *in vivo* (DAMODARAN, PARKIN e FENNEMA, 2007).

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CAPÍTULO 3 - INFLUÊNCIA DO ULTRASSOM NAS PROPRIEDADES ESTRUTURAIS E EMULSIFICANTES DO CASEINATO DE SÓDIO E DA LACTOFERRINA

STRUCTURAL AND EMULSIFYING PROPERTIES OF SODIUM CASEINATE AND LACTOFERRIN INFLUENCED BY ULTRASOUND PROCESS

STRUCTURAL AND EMULSIFYING PROPERTIES OF SODIUM CASEINATE AND LACTOFERRIN INFLUENCED BY ULTRASOUND PROCESS

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Highlights

The ultrasound effect on proteins structure was investigated.

Ultrasound promoted conformational changes only for lactoferrin.

Higher proteins hydrophobicity was observed with increasing of sonication time.

Higher sonication time produced smaller droplets and more stable emulsions.

Graphical Abstract
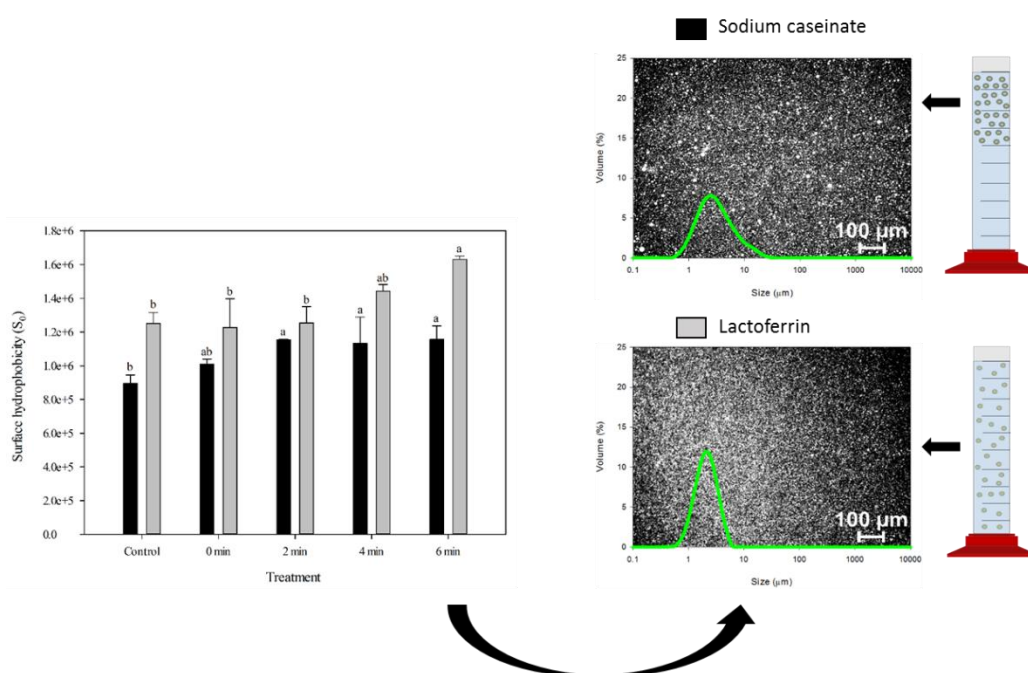
ABSTRACT

Structural, physical and emulsifying properties of sodium caseinate and lactoferrin were investigated after these proteins were subjected to ultrasound treatment. Aqueous sodium caseinate or lactoferrin solutions were sonicated for 2 to 6 minutes using a power of 300 W. Protein properties as size, surface charge, molecular weight distribution, intrinsic viscosity, surface hydrophobicity and structural conformation from circular dichroism were evaluated. Sodium caseinate size was significantly reduced after ultrasound treatment while an opposite effect was observed for lactoferrin. Slight differences in molecular weight after ultrasound treatment were observed only for lactoferrin. Intrinsic viscosity and surface hydrophobicity was positively affected by the increase of sonication time. Circular dichroism spectra revealed no differences for sodium caseinate structure but slight changes were observed for lactoferrin. In addition, a fixed amount (1 wt.%) of this ultrasound-treated protein was employed as an emulsifier to prepare oil in water emulsions (o/w). Emulsions were also produced using the same ultrasound conditions that aqueous protein solutions were subjected. They were evaluated in terms of droplet size, emulsifying activity, creaming index and emulsion stability. Emulsions showed reduced droplet size and improved stability with higher sonication times. Coarse emulsions stabilized by ultrasound-treated proteins showed a slightly higher stability when compared to coarse emulsions stabilized by non-treated proteins. However, completely stable emulsions were produced only by ultrasound emulsification of coarse emulsions, suggesting that the protein changes occurring simultaneously to the droplets size reduction contributed to the enhancement of emulsifying properties.

Keywords: emulsion, hydrophobicity, ultrasound.

3.1 INTRODUCTION

A wide variety of food products consists at least partially by emulsions such as milk, yogurt, salad dressing, mayonnaise and ice cream. Oil in water emulsions are thermodynamically unstable systems but a kinetic stability for considerable periods of time can be reached with the addition of emulsifiers that act onto the interface (McClements, Decker, & Weiss, 2007). Proteins can act as emulsifiers due to their amphiphilic nature, reducing the interfacial tension between oil and water (Lam & Nickerson, 2013). Moreover the protein adsorption onto the interface provides a combination of electrostatic and steric repulsion between the oil droplets which allows the formation of a kinetically stable emulsion (Wilde, Mackie, Husband, Gunning, & Morris, 2004). Milk proteins are commonly used as emulsifiers showing high nutritional value and can be considered as safe (GRAS) (Chen, Remondetto, & Subirade, 2006; Guzey & McClements, 2006). Caseins are approximately 75 to 85% of milk protein and these phosphoproteins are composed by four different fractions: α_{s1}-, α_{s2}-, β- and κ-caseins (McSweeney & Fox, 2013). In aqueous solution at neutral pH or in foods such as milk, casein is a mixture of small aggregates called casein micelles attached to calcium salts. They are prone to association in micelles due to regions of high hydrophobicity and the charge distribution arising from the amino acid sequence (O'Regan, Ennis, & Mulvihill, 2009). Calcium salts when replaced by sodium salts leads to the production of sodium caseinate, which is an ingredient widely used in food industry with high emulsifying capacity (Dickinson, 2006; McSweeney, et al., 2013).

Whey proteins represent 15 to 22% of milk proteins. The major fractions are α-lactalbumin, β-lactoglobulin and serum albumin with other minor proteins as lactoferrin (Damodaran, Parkin, & Fennema, 2007). Lactoferrin occurs in mammalian secretory fluids showing a number of biological functions such as antioxidant activity, antimicrobial activity, antiviral and anticancer (Wakabayashi, Yamauchi, & Takase, 2006). This protein is composed by a single polypeptide chain of about 80 kDa, containing one to four glycans (Spik, et al., 1994). Besides of their beneficial effects, lactoferrin is safe for health and shows potential application as food additive for human and animal (Wakabayashi, et al., 2006). Some studies have shown that lactoferrin can be used as an emulsifier to stabilize emulsions (Sarkar, Goh, & Singh, 2009; Sarkar, Horne, & Singh, 2010).

Another important factor that is directly related to the kinetic stability of emulsions is the emulsifying method (Jafari, He, & Bhandari, 2007; Santana, Perrechil, & Cunha, 2013).

Ultrasound can be used in the production of emulsions and is based on the application of an acoustic field that results in cavitation phenomena causing the formation of droplets (Abismaïl, Canselier, Wilhelm, Delmas, & Gourdon, 1999; Li & Fogler, 1978a, 1978b). The use of this technique presents a number of advantages as production of smaller droplets size (less than 1 μm) and narrow size distribution resulting in more stable emulsions; minimal emulsifier content requirements depending on the emulsifier used; easy operation, control and cleaning; and low production costs (Abbas, Hayat, Karangwa, Bashari, & Zhang, 2013). Changes on structural and technological properties of milk proteins has been associated to the application of ultrasound which usually improved their emulsifying properties due to structural changes (Arzeni, et al., 2012; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014; O'Sullivan, Arellano, Pichot, & Norton, 2014). However, a deeper investigation about the effects of ultrasound on the structural and functional properties of sodium caseinate (a protein with random coil structure negatively charged at pH 7.0) and lactoferrin (a globular protein positively charged at pH 7.0) is necessary in order to understand the influence of process conditions on the emulsifying properties of these proteins showing unlike conformational structure.

The objective of this research was to understand the effects of ultrasound treatment on the structural and physical properties of sodium caseinate and lactoferrin. Changes in the structural and physical properties of the proteins were measured in terms of protein size and surface charge, molecular structure, intrinsic viscosity, surface hydrophobicity and circular dichroism. Furthermore, we investigated the ultrasound effect on the proteins capacity to increase the stability of oil in water emulsions against coalescence and decrease droplets size.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Ultrapure water from a Millipore Milli-Q system (resistivity 18.2 MΩ/cm) was used. Sodium caseinate (protein content 87 wt. %) and lactoferrin (protein content 92.1 wt. %) were kindly provided by Allibra Ingredientes Ltd (Campinas, Brazil) and Synlait Milk Ltd (Canterbury, New Zeland), respectively. Sunflower oil (Bunge Alimentos S.A., Gaspar, Brazil) was purchased in the local market. The other reagents were of analytical grade.

3.2.2 Methods

3.2.2.1 Protein solutions preparation

Sodium caseinate or lactoferrin were dispersed in ultrapure water (0.25 – 1.429 wt. %) using magnetic stirring at room temperature overnight, ensuring complete dissolution of the protein. The pH of protein solutions was adjusted to pH 7.0 using sodium hydroxide (1 M) or hydrochloric acid (1 M).

3.2.2.2 Ultrasound treatment of protein solutions

Protein solutions (100 ml) were homogenized using a rotor-stator device (SilentCrusher M, Heidolph, Schwabach, Germany) at 5,000 rpm for 3 minutes prior to sonication. An ultrasonic processor (QR 750 W, Ultronique, Campinas, Brazil) attached with a titanium probe (13 mm diameter) was used to sonicate sodium caseinate and lactoferrin aqueous solutions at fixed concentration of 1.0 wt. %. Sonication time was 2, 4 or 6 minutes, while the power and the frequency were fixed in 300 W and 20 kHz, respectively. The temperature of the protein solutions was measured before and after sonication and did not exceed 30 °C. The pH of the protein solutions was measured before and after sonication using a pH meter (Metrohm 827, Metrohm, Herisau, Switzerland). The "control" is the protein solution and "0 min" is the protein solution dispersed by rotor-stator before the ultrasound treatment.

3.2.2.3 Characterization of untreated and ultrasound-treated proteins

3.2.2.3.1 Particles size distribution and zeta potential

The proteins size distribution and zeta potential were measured using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Mean protein size was reported as average hydrodynamic diameter ($\overline{D}$), which was calculated according to Eq. 3.1.

$$\overline{D} = \sum x_i D_i \qquad (3.1)$$

where x_i is the fraction of a given particle i with a given scattering intensity and D_i is the diameter of the particle i. The polydispersity index (PDI) was calculated from cumulant analysis of the measured dynamic light scattering intensity autocorrelation function. Zeta potential was determined at a fixed pH value (7.0).

3.2.2.3.2 Polyacrylamide gel electrophoresis

Molecular weight distribution of untreated and ultrasound-treated proteins was evaluated by Native-PAGE and SDS–PAGE under non-reducing conditions, according to Laemmli (1970). A vertical slab Mini-Protean electrophoresis system (Bio-Rad Laboratories, Hercules, USA) was used. For SDS-PAGE the resolving and stacking gels contained 15% and 5 wt. % of acrylamide, respectively. Untreated and ultrasound-treated protein solutions (1 wt. %) were diluted in deionized water (2 mg protein/mL). These solutions were diluted in a sample buffer containing SDS (1:1) to obtain non-reducing conditions. The gels were run at 120 V with a running buffer containing SDS (pH 8.3). For Native-PAGE a buffer without SDS (pH 8.3) and a 10 wt. % acrylamide gel was used for sodium caseinate while for lactoferrin a buffer without SDS (pH 10.2) and a 6 wt. % acrylamide gel was used. The gels were then stained with 0.25 wt. % Coomassie Brilliant Blue in ethanol:acetic acid:water (45:10:45 vol. %), and diffusion-destained by repeated washing in an ethanol:acetic acid:water solution (10:5:85 vol. %). Commercial molecular weight markers (Broad Range Protein Molecular Weight Markers, Promega Corporation, Madison, USA and BenchMark™ Pre-stained Protein Ladder, Carlsbad, CANADA) were used to evaluate molecular weight of proteins.

3.2.2.3.3 Intrinsic viscosity

The protein solutions viscosity was measured in a very dilute concentration range of 0.25 – 0.45 wt. % using a rheometer (AR1500ex, TA Instruments, New Castle, USA) equipped with a double gap concentric cylinders (31.85 mm inner diameter, 35.01 mm outer diameter, 42.07 mm height). Viscosity values of protein solutions and solvent (ultrapure water) were obtained from flow curves (0 – 1000 s^{-1}) which were subsequently used to determine the relative and intrinsic viscosity.

Intrinsic viscosity of untreated and treated protein solutions was obtained from the slope of relative viscosity-concentration relationship using equations 3.2 to 3.4 (Higiro, Herald, & Alavi, 2006; Tanglertpaibul & Rao, 1987).

$$\eta_{rel} = \frac{\eta}{n_s} = 1 + [\eta].C \tag{3.2}$$

$$\eta_{rel} = \frac{\eta}{n_s} = e^{[\eta].C} \tag{3.3}$$

$$\eta_{rel} = \frac{\eta}{n_s} = \frac{1}{1-[\eta].C} \tag{3.4}$$

where η_{rel} is the relative viscosity, η is the protein solution viscosity, n_s is the solvent viscosity, $[\eta]$ is the intrinsic viscosity and C is the protein concentration wt. %.

3.2.2.3.4 Surface hydrophobicity (S_0)

Protein surface hydrophobicity was determined according to Alizadeh-Pasdar and Li-Chan (2000) with minor modifications using the anionic fluorescence probe 1-anililo-naphthalene-8-sulfonate (ANS). Protein solution was prepared in 0.1 M phosphate buffer (pH 7.0) and the protein concentration ranged from 0.005 – 0.025 wt. %, while ANS was 8 mM. Samples containing 4 mL of diluted proteins was mixed by vortexing with 20 μL of ANS solution. After 15 minutes in the dark, the relative fluorescence intensity (RFI) was measured using a multiphase fluorometer (K2, ISS, Champaign, USA). The excitation/emission slits and wavelengths were set at 0.5 mm/0.5 mm and 390/470 nm respectively. S_0 was determined from the initial slope of the linear regression analysis of the plot of RFI against protein concentration (wt. %).

3.2.2.3.5 Far-UV circular dichroism (CD)

Far-UV circular dichroism was used to investigate the secondary structure of untreated and ultrasound-treated proteins. Protein solutions (0.1 mg/mL) were evaluated at 25±0.1 °C in the spectral range from 190 to 260 nm with a spectropolarimeter (Jasco J-810, Jasco Corp., Japan), using a quartz cuvette with an optical path of 0.1 cm. The spectral

resolution was 0.5 nm, and the scan speed was 100 nm/min, with a response time of 0.125 s at a bandwidth of 1 nm. Twenty scans were accumulated and averaged, and the spectra were corrected using a protein-free sample. Results were expressed as molar ellipicity (θ) using Eq. (3.5) (Barreto, et al., 2003):

$$\theta = \frac{3300.\Delta Abs}{c.l} \tag{3.5}$$

where ΔAbs is the observed difference in absorbance for the left and right circular components of the incident light, l is the pathlength (in cm), and c is the protein concentration in mol.l^{-1}.

3.2.2.4 Oil in water emulsions preparation

Oil in water emulsions were prepared according to the scheme presented in Figure 3.1. Coarse emulsions were formed by homogenizing 70 mL of untreated protein solutions (1.429 wt. %, pH 7.0, which were prepared according to section 3.2.2.1) and 30 mL of sunflower oil using a rotor-stator homogenizer (SilentCrusher M, Heidolph, Schwabach, Germany) at 5,000 rpm for 3 minutes. Fine emulsions were prepared by subjecting the coarse emulsion in an ultrasonic processor (QR 750W, Ultronique, Campinas, Brazil) with a 13 mm diameter titanium probe immersed 3 mm depth. A magnetic stirrer was also used in combination with ultrasound process to homogenize the mixture. Sonication time varied between 2 and 6 minutes, while the power and the frequency were fixed in 300 W and 20 kHz, respectively. The temperature of preparation did not exceed 30 °C during the homogenization process. Coarse emulsions were also prepared with the ultrasound-treated protein solutions in the same conditions of untreated protein solutions in order to evaluate only the effect of ultrasound on the emulsifying properties of the proteins.
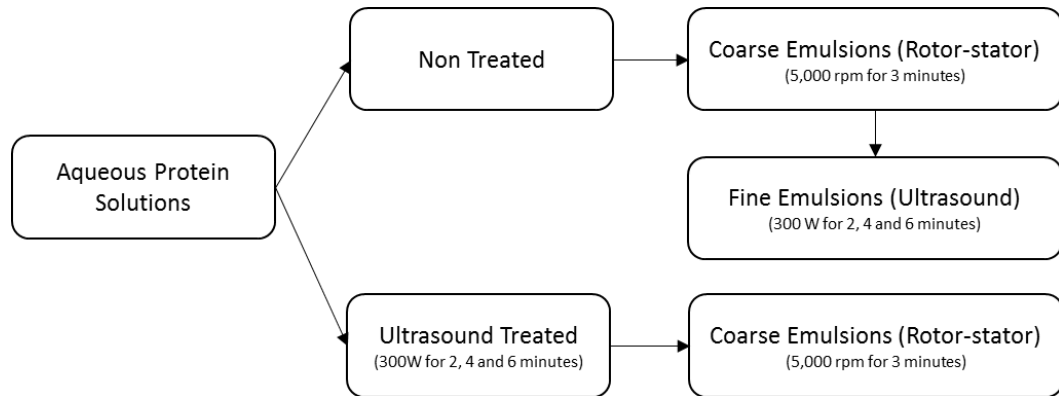
Figure 3.1 - Schematic representation of the procedure used for emulsions preparation.

3.2.2.5 Emulsion characterization

3.2.2.5.1 Emulsion stability

Immediately after preparation, 25 mL of each emulsion was poured into a cylindrical glass tube (internal diameter = 17 mm, height = 105 mm), sealed with a plastic cap and stored at 25 °C for 7 days. The change in height of the top phase (H) was measured during storage time and the creaming index (CI) determined according to Eq. (3.6). Emulsifying activity (EA) of the fresh emulsions was also determined from centrifugation. Samples (15 ml) were centrifuged at 1200g for 10 minutes, and the remaining emulsion height (H_{EA}) was measured and compared with the initial height of emulsion (Eq. 3.7). In addition, emulsion stability (ES) (Eq. 3.8) was determined by emulsion heating to 80°C for 30 minutes, cooling down to room temperature and centrifuging at 1200g for 5 minutes. ES was expressed as the percentage of the emulsified layer height (H_{ES}) remaining in the original emulsion volume (Chau, Cheung, & Wong, 1997).

$$CI\ (\%) = \frac{H}{H_0} 100 \qquad\qquad (3.6)$$

$$EA\ (\%) = \frac{H_{EA}}{H_0} 100 \qquad\qquad (3.7)$$

$$ES\ (\%) = \frac{H_{ES}}{H_0} 100 \qquad\qquad (3.8)$$

where H_0 represents the initial height of the emulsion.

3.2.2.5.2 Droplet size

The droplets size distribution of the cream phase was determined based on the static light scattering method using a Multi-Angle Static Light-Scattering Mastersizer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The emulsions were analyzed immediately after their preparation and after 7 days of storage. The mean diameter of the oil droplets was expressed as the volume-surface mean diameter ($D_{3,2}$) (Eq. 3.9) and volume mean diameter ($D_{4,3}$) (Eq. 3.10). $Span$ was used as a polydispersity index (Eq. 3.11).

$$D_{3,2} = \frac{\sum n_i D_i^3}{\sum n_i D_i^2} \tag{3.9}$$

$$D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \tag{3.10}$$

$$Span = \frac{(D_{90} - D_{10})}{D_{50}} \tag{3.11}$$

where n_i is the number of droplets with diameter D_i, and D_{10}, D_{50} and D_{90} are diameters at 10, 50 and 90% of cumulative volume, respectively.

3.2.2.5.3 Microstructure

Microstructure of the emulsions was evaluated after 7 days of storage. The samples were poured onto microscopes slides, covered with glass cover slips and observed using a Carl Zeiss Axio Scope A1 microscope (Zeiss, Oberkochen, Germany) with x40 objective lenses.

3.2.3 Statistical analysis

The results were reported as the average and the standard deviation of three replicates and evaluated by one way analysis of variance (ANOVA), and significant differences ($p < 0.05$) between the treatments were evaluated by the Tukey procedure. The statistical

analyses were carried out using the trial version software Minitab 16.1.0 (Minitab Inc., State College, PA, USA).

3.3 RESULTS AND DISCUSSION

3.1 Changes on structural and physical properties of proteins in aqueous medium induced by ultrasound

Zeta potential, size and pH measurements of protein as a function of sonication time are shown in Table 3.1. A significant reduction of pH values was observed for both proteins indicating a higher exposure of acidic amino acid residues (Bermudez-Aguirre, Mawson, & Barbosa-Canovas, 2008; O'Sullivan, et al., 2014; Sakurai, Konuma, Yagi, & Goto, 2009). On the other hand, zeta potential values did not vary significantly, but at pH 7.0 sodium caseinate is negatively charged while lactoferrin is positively charged. Sodium caseinate presented a monomodal size distribution and a significant reduction in average hydrodynamic diameter with the increase in the sonication time as a consequence of the cavitational forces, micro-streaming and turbulent forces of the ultrasonic treatment exerted by the probe, which could lead to changes in electrostatic and hydrophobic interactions (Jambrak, et al., 2014; O'Brien, 2007; O'Sullivan, et al., 2014; Yanjun, et al., 2014). Furthermore, caseins present completely flexible chains and a certain number of susceptible residues that are more easily broken (Swaisgood, 1993). However, lactoferrin presented a multimodal size distribution with three peaks and a significant increase in average hydrodynamic diameter (peak 1) with the increase in the sonication time. Such result was also observed for sonicated bovine serum albumin suggesting that small aggregates may have been formed (Gülseren, Güzey, Bruce, & Weiss, 2007). Commercial lactoferrin is not completely purified and non-covalent molecular interactions such as electrostatic and hydrophobic interactions between this protein and other residual whey proteins can be occurring. In addition, by presenting a low concentration of iron (iron saturation 9.9 %, according to the manufacturer), lactoferrin may be in the *apo* form which shows a more open conformation (Andersen, Baker, Morris, Rumball, & Baker, 1990) and treatment by ultrasound may have triggered a wider opening chain. The polydispersity index can vary from 0 to 1 (Table 3.1), where higher values indicate a less homogeneous particle size distribution. PDI values remained below 0.2 and tended to increase at higher sonication times.

Table 3.1 - Zeta potential, pH, protein diameter and PDI of sodium caseinate and lactoferrin solutions subjected to different sonication times.

Protein	Treatment	pH	Zeta potential (mV)	$\bar{D}$ (nm)			PDI		
				Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
Sodium Caseinate	Control	7.05±0.00[a]	- 29.17±1.55[a]	345±12[a]	-	-	0.14±0.09	-	-
	0 min	7.03±0.00[b]	- 29.60±0.78[a]	328±10[ab]	-	-	0.06±0.04	-	-
	2 min	7.03±0.00[bc]	- 27.57±0.64[a]	307±17[bc]	-	-	0.11±0.08	-	-
	4 min	7.02±0.01[bc]	- 28.8±0.80[a]	286±20[c]	-	-	0.13±0.06	-	-
	6 min	7.02±0.01[c]	- 28.53±0.58[a]	276±2[c]	-	-	0.20±0.02	-	-
Lactoferrin	Control	7.04±0.00[a]	17.43±0.12[a]	332±7[b]	48±11[a]	7±2[ab]	0.06±0.01	0.08±0.01	0.05±0.05
	0 min	6.99±0.00[b]	17.97±0.42[a]	316±20[b]	50±11[a]	13±0[a]	0.03±0.01	0.06±0.01	0.05±0.01
	2 min	6.96±0.00[d]	17.40±0.52[a]	386±4[ab]	52±7[a]	11±6[ab]	0.06±0.04	0.12±0.04	0.06±0.01
	4 min	6.97±0.00[c]	17.27±0.76[a]	421±38[ab]	41±4[a]	6±1[b]	0.05±0.04	0.07±0.06	0.03±0.01
	6 min	6.87±0.00[e]	18.10±0.40[a]	487±79[a]	47±2[a]	7±0[ab]	0.04±0.01	0.14±0.05	0.03±0.05

Identical letters in the same column for each protein indicate that there are no differences between the measurements ($p > 0.05$)

Native-PAGE electrophoretic profiles showed no differences for sodium caseinate treated in different conditions (Fig. 3.2a). The band near 26-37 kDa refers to the major fractions of sodium caseinate (β- and $α_s$-casein) (O'Regan & Mulvihill, 2009). However, bands between 49 and 64 kDa and above 115 kDa refer to aggregates formed by non-covalent interactions such as electrostatic and hydrophobic interactions since they disappeared after SDS addition (Fig. 3.2c). Lactoferrin native-PAGE electrophoretic profile (Fig. 3.2b) showed a band near 82 kDa and higher than 180 kDa that refers to the lactoferrin (Raei, Rajabzadeh, Zibaei, Jafari, & Sani, 2015; Spik, et al., 1994) and protein aggregates, respectively. Furthermore, some aggregates between 82-115 kDa were observed for ultrasound-treated lactoferrin, which corroborates the particles size distribution presented in Table 3.1. These aggregates also disappeared after SDS addition, confirming that these aggregates are formed by non-covalent interactions. However, a band near 15 kDa appeared and possibly refers to α-lactalbumin (14.2 kDa) (Jambrak, et al., 2014) which was forming aggregates observed in native-PAGE. Electrophoretic profiles obtained by SDS-PAGE for untreated and ultrasound treated proteins did not show differences in protein fractions in agreement with those reported for other milk proteins like bovine serum albumin (Gülseren, et al., 2007), milk protein concentrate (Yanjun, et al., 2014), sodium caseinate and whey protein isolate (O'Sullivan, et al., 2014). The absence of differences in the electrophoretic profile of the untreated and ultrasound treated proteins in SDS-PAGE confirms that the structural changes induced by ultrasound process are of non-covalent nature.
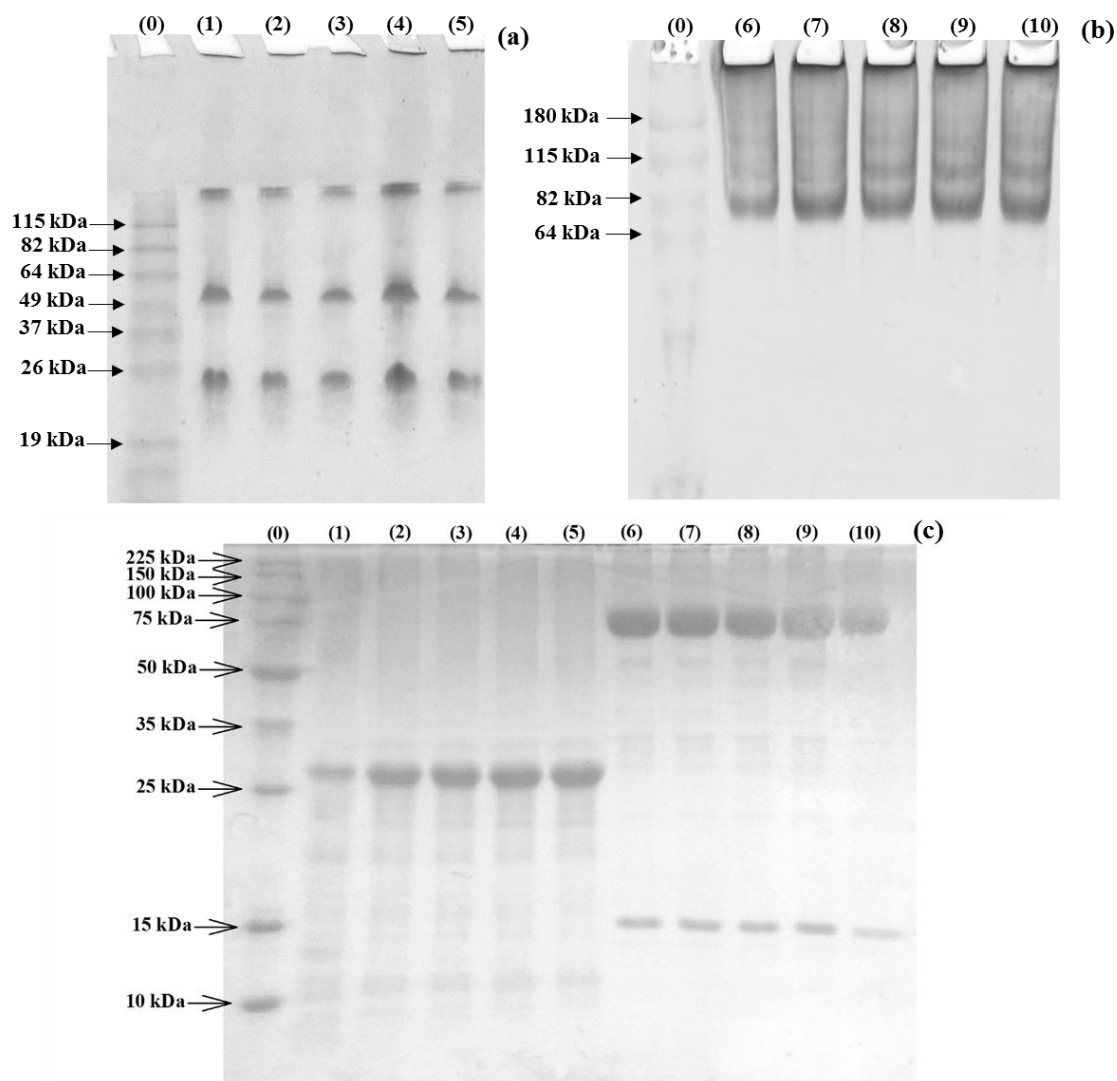
Figure 3.2 - Native-Page (a and b) and SDS-PAGE (c) eletrophoretic profiles of protein solutions: (0) Molecular weight standard, (1) Untreated sodium caseinate, (2) Rotor-stator stirred sodium caseinate, (3) Ultrasound treated sodium caseinate for 2 minutes, (4) Ultrasound treated sodium caseinate for 4 minutes, (5) Ultrasound treated sodium caseinate for 6 minutes, (6) Untreated lactoferrin, (7) Rotor-stator stirred lactoferrin, (8) Ultrasound treated lactoferrin for 2 minutes, (9) Ultrasound treated lactoferrin for 4 minutes, (10) Ultrasound treated lactoferrin for 6 minutes.

Intrinsic viscosity [η] values of protein solutions were determined from fitting Eqs. (3.2), (3.3) and (3.4) as can be observed in Table 3.2. A tendency of the intrinsic viscosity decreasing with the increase in sonication time was observed, but such a decrease was not statistically significant. Intrinsic viscosity reflects the ability of a solvent to hydrate proteins and provides information about the molecular hydrodynamic volume, which is related to the

chain conformation of the proteins in solution (Behrouzian, Razavi, & Karazhiyan, 2014). Despite of the non significant differences, these results are in agreement with the reduction in size measured by dynamic light scattering (Table 3.1) for sodium caseinate, but for lactoferrin occurred the opposite behavior. However, the protein conformation is also a consequence of the hydrophobic interactions. Therefore, the decrease of proteins intrinsic viscosity after ultrasound treatment could indicate an increase of the hydrophobicity degree of the proteins. Since the most of the hydrophobic side chains are buried in the interior of proteins modifying protein intrinsic viscosity (Kauzmann, 1959; Tanner & Rha, 1980). Such assumption can be confirmed by the surface hydrophobicity (S_0) measurements (Fig. 3.3), since the S_0 for all proteins increased with the increase of sonication time. Increasing hydrophobicity is positive for the adsorption of amphiphilic biopolymers at the oil water interface (Khan, Bibi, Pervaiz, Mahmood, & Siddiq, 2012). Ultrasound treatment induced a partial molecular unfolding of the protein molecules leading to exposure of more hydrophobic groups which made easier the access of ANS to previously hindered hydrophobic sites (Arzeni, et al., 2012; Chandrapala, et al., 2011; Gülseren, et al., 2007). However, as the ANS is anionic, electrostatic interactions may have occurred with lactoferrin because it presents positive zeta potential value giving a higher value of hydrophobicity. Although ultrasound caused the exposure of hydrophobic groups with a consequent increase in the hydrophobicity value, these groups did not change the zeta potential values.

Table 3.2 - Intrinsic viscosity values for sodium caseinate and lactoferrin solutions treated at different sonication times.

Protein	Treatment	Tanglertpaibul-Rao (Eq. 3.2)		Higiro 1 (Eq. 3.3)		Higiro 2 (Eq. 3.4)	
		$[\eta]$ (dl/gr)	R^2	$[\eta]$ (dl/gr)	R^2	$[\eta]$ (dl/gr)	R^2
Sodium Caseinate	Control	0.15 ± 0.02^{aA}	0.985	0.15 ± 0.02^{aA}	0.975	0.14 ± 0.02^{aA}	0.975
	0 min	0.16 ± 0.03^{aA}	0.915	0.16 ± 0.03^{aA}	0.925	0.15 ± 0.03^{aA}	0.920
	2 min	0.14 ± 0.02^{aA}	0.940	0.14 ± 0.02^{aA}	0.950	0.13 ± 0.02^{aA}	0.935
	4 min	0.14 ± 0.01^{aA}	0.960	0.13 ± 0.01^{aA}	0.970	0.13 ± 0.02^{aA}	0.965
	6 min	0.12 ± 0.01^{aA}	0.980	0.12 ± 0.00^{aA}	0.975	0.12 ± 0.01^{aA}	0.985
Lactoferrin	Control	0.15 ± 0.01^{aA}	0.999	0.14 ± 0.01^{aA}	0.999	0.14 ± 0.01^{aA}	0.999
	0 min	0.14 ± 0.03^{aA}	0.915	0.14 ± 0.03^{aA}	0.925	0.14 ± 0.03^{aA}	0.999
	2 min	0.14 ± 0.00^{aA}	0.999	0.14 ± 0.00^{aA}	0.999	0.14 ± 0.00^{aA}	0.999
	4 min	0.13 ± 0.01^{aA}	0.925	0.13 ± 0.01^{aA}	0.925	0.13 ± 0.01^{aA}	0.915
	6 min	0.10 ± 0.01^{aA}	0.999	0.10 ± 0.01^{aA}	0.999	0.10 ± 0.01^{aA}	0.999

Identical capital letters in the same raw for each protein indicate that there are no significant differences between results ($p > 0.05$)

Identical small letters in the same column for each protein indicate that there are no significant differences between results ($p > 0.05$)
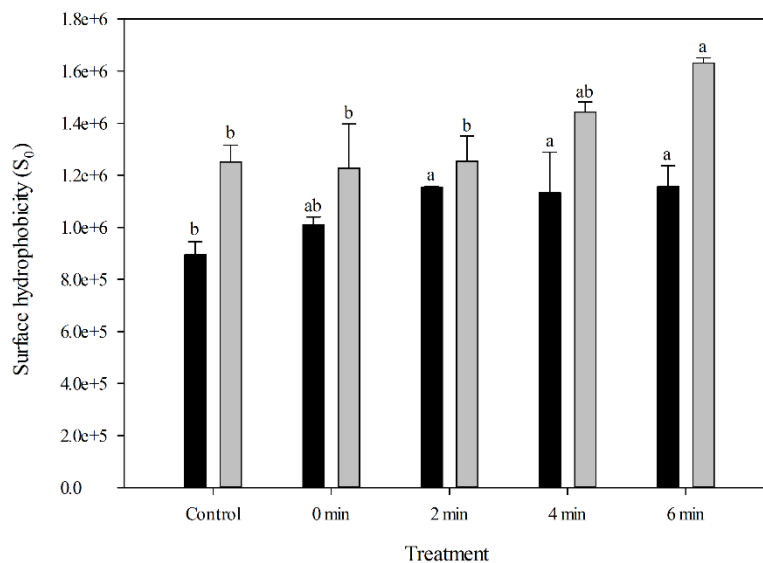
Figure 3.3 - Surface hydrophobicity values of sodium caseinate (■) and lactoferrin (▬) after different sonication times. Identical letters for each protein indicate that there are no significant differences between the values ($p > 0.05$).

Circular dichroism (CD) spectroscopy was used to determine the changes in the secondary structure of the proteins, considering that the major elements, α-helix, β-sheet and coil have a characteristic CD spectra. The α-helix configuration shows an intense and positive band at 190 nm and negative peaks at 208 and 220 nm. A negative dichroic peak with a minimum in the 215 nm region is typical of β-sheet configuration and random coil structures generally have a positive CD peak near 215 nm and a negative one near to 200 nm (Barreto, et al., 2003; Kasinos, et al., 2013). Fig. 3.4 shows the far UV-CD spectrum of sodium caseinate and lactoferrin. CD spectra of sodium caseinate did not change with ultrasound treatment and the shape of the curve and the minimum ellipticity at 203 nm was attributed to random coil structure, representing the pattern of typical unfolding proteins (Kato, Miyazaki, Kawamoto, & Kobayashi, 1987). Despite the high content of hydrophobic amino acids (proline residues), casein presents an open and hydrated random structure with only small amount of secondary structure (Barreto, et al., 2003) which can not be affected by ultrasound. However, lactoferrin presented a minimum near 210 nm suggesting that its structure is partially based on a β-sheet conformation (Daidone, et al., 2010). Furthermore, CD spectra differed slightly in intensity and shape with the increase in sonication time, indicating that ultrasound treatment promoted conformational changes in the protein structure. This conformational change was also observed in bovine serum albumin treated with ultrasound (Gülseren, et al., 2007) and the increase in the

magnitude of ellipticity may indicate the formation of less ordered structures and a possible unfolding of the protein, which favors hydrophobic interactions, aggregation and size increase (Barreto, et al., 2003; Munialo, Martin, van der Linden, & de Jongh, 2014).
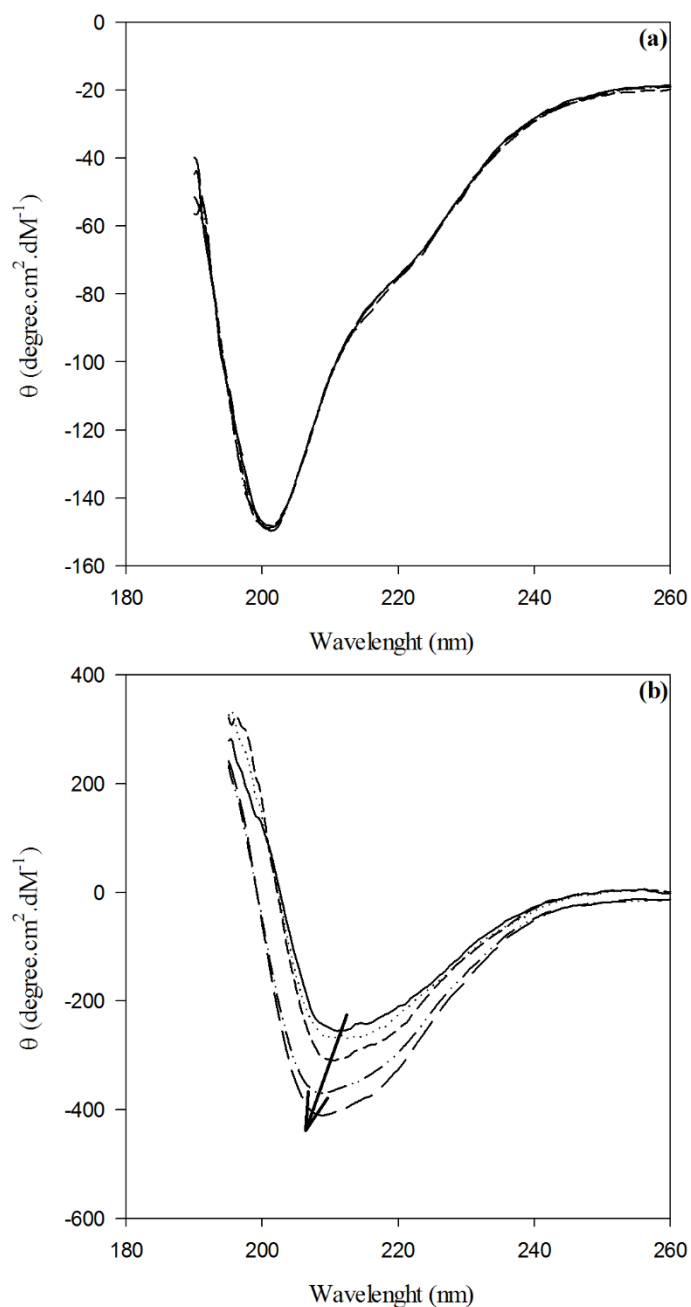
Figure 3.4 - Far UV-CD spectra of sodium caseinate solutions (a) and lactoferrin solutions (b) under different treatments: Untreated protein solution (solid line), Rotor-stator stirred protein solution (dotted line), Ultrasound treated protein solution for 2 minutes (dashed line), Ultrasound treated protein solution for 4 minutes (dash-dotted line) and Ultrasound treated protein solution for 6 minutes (double dashed line).

3.3.2 Effect of sonication time on the emulsion properties

The influence of different sonication times on the structure and volume particles size distribution of the emulsions stabilized by non-treated proteins can be observed in Figure 3.5. Whole emulsions were evaluated at day 0 while the cream (top) phase of the emulsions was analyzed at day 7 (except for lactoferrin emulsions produced with higher sonication times) since most of the emulsions showed phase separation during storage. The microscopy images of cream phase showed that increasing the sonication time decreased the droplet size. In addition, particles size distribution pattern changed since a shorter sonication time led to a bimodal distribution. A longer sonication time led to a unimodal size distribution and lower droplet size (Table 3.3), in agreement with results presented by Shanmugam & Ashokkumar, (2014) and Shamsara, et al. (2015).

Depending on the mean droplet size of the dispersed phase, emulsions are generally classified as nano (0.01-0.1 μm), mini (0.1-1 μm) and macroemulsions (1-100 μm) (Windhab, Dressler, Feigl, Fischer, & Megias-Alguacil, 2005). Only macroemulsions were obtained in this work since the mean droplet sizes ($D_{3,2}$) ranged from 1.89±0.06 to 14.68±3.98 μm, immediately after preparation (Table 3.3). The $D_{3,2}$ and $D_{4,3}$ values showed a 6 ($D_{3,2}$) and 13 ($D_{4,3}$) fold reduction for sodium caseinate and 7 ($D_{3,2}$) and 14 ($D_{4,3}$) fold reduction for lactoferrin between 0 and 6 min of ultrasound treatment. These values were significantly different for cream phase stabilized by for lactoferrin after 7 days of storage at 25 °C since higher droplets were observed. However, for sodium caseinate mean droplets size of cream phase remained similar to whole emulsions. The span values were used to express the width of the size distribution and the degree of polydispersity. A high span value of the emulsion implies in a wide size distribution and high droplets polydispersity. Despite the rotor-stator homogenization have resulted in lower span values (0 min) (Table 3.3), the mean droplets size was much higher than the ultrasound treated emulsions and the span value was similar to the higher sonication time, immediately after preparation of emulsions. Span values of cream phase (7 day) for sodium caseinate increased comparing to fresh emulsions (Day 0) while for lactoferrin they decreased with storage time. The homogenization process is one of the factors that determines the droplet size (Henry, Fryer, Frith, & Norton, 2009).
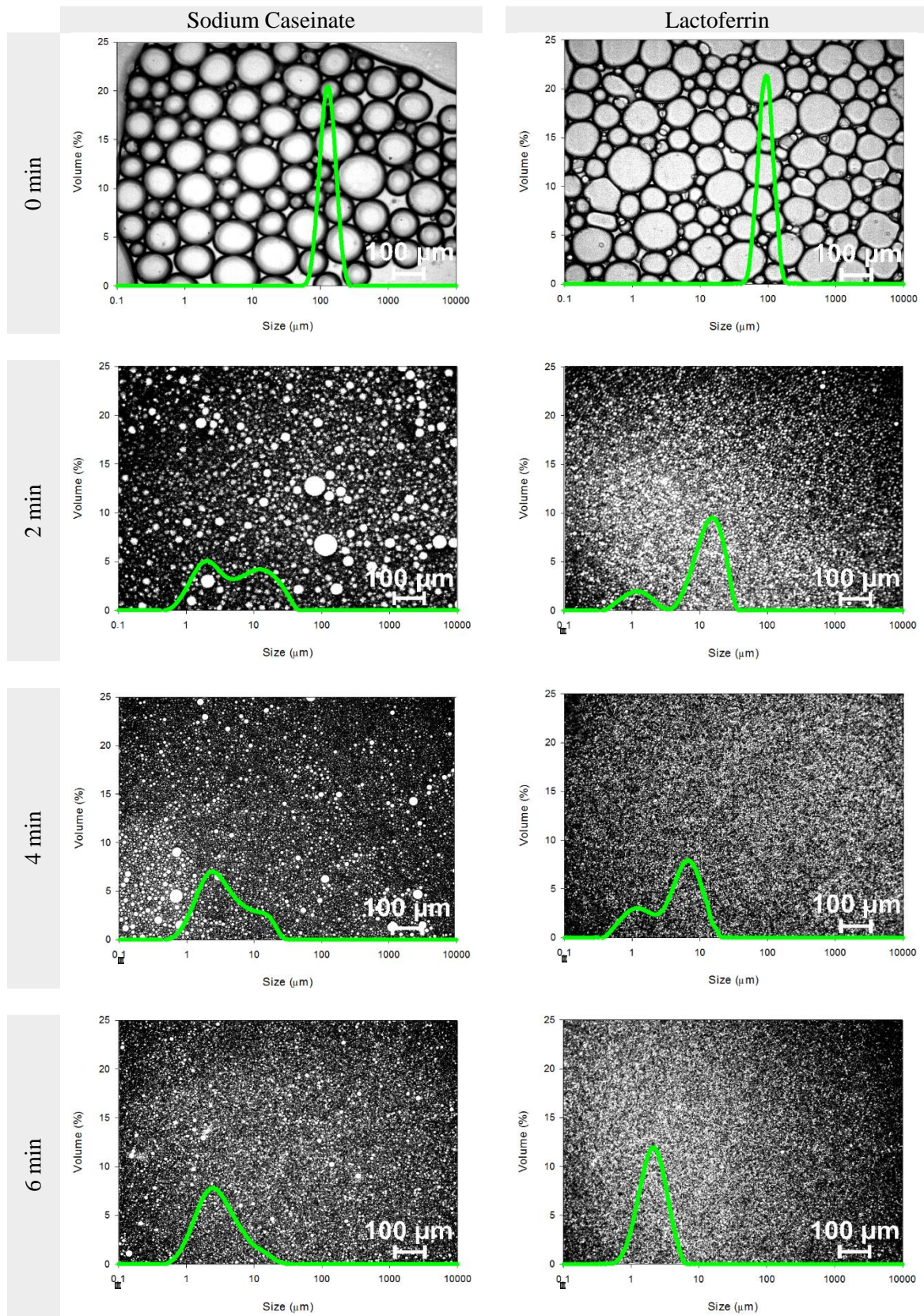
Figure 3.5 - Optical micrographs and droplets size distribution of the cream phase of the oil in water emulsions stabilized by sodium caseinate and lactoferrin under different sonication times after 7 days of storage at 25 °C.

Table 3.3 - Mean droplet size and polydispersity of the oil in water emulsions stabilized by sodium caseinate or lactoferrin under different sonication times.

Protein	Treatment	Day 0			Day 7		
		$D_{3,2}$ (µm)	$D_{4,3}$ (µm)	*Span*	$D_{3,2}$ (µm)	$D_{4,3}$ (µm)	*Span*
Sodium Caseinate	0 min	12.10±0.34[aB]	34.53±0.09[aB]	1.90±0.24[aA]	116.22±1.80[aA]	124.197±1.26[aA]	0.68±0.03[aB]
	2 min	2.90±0.08[bA]	7.17±0.58[bA]	3.73±0.11[bB]	2.87±0.00[bA]	7.70±0.41[bA]	4.01±0.01[bA]
	4 min	2.21±0.03[cA]	3.57±0.12[cA]	2.36±0.14[cB]	2.53±0.38[bA]	4.82±1.21[cA]	3.18±0.58[cA]
	6 min	1.89±0.03[dA]	2.57±0.09[dA]	1.63±0.11[aA]	1.35±1.57[bB]	4.91±0.94[cB]	1.36±1.58[cB]
Lactoferrin	0 min	14.68±3.98[aB]	42.02±10.67[aB]	1.86±0.30[aA]	96.05±0.60[aA]	102.06±0.0.74[aA]	0.65±0.01[aB]
	2 min	2.98±0.03[bB]	7.07±0.73[bB]	3.33±0.22[bA]	4.61±0.36[bA]	13.46±0.71[bA]	1.74±0.05[bB]
	4 min	2.39±0.05[bB]	4.11±0.21[bB]	2.54±0.11[cA]	2.75±0.17[cA]	5.87±0.64[cA]	1.95±0.13[cB]
	6 min	2.04±0.06[bA]	3.10±0.05[bA]	2.08±0.11[aA]	2.00±0.02[dA]	2.42±0.05[dB]	1.19±0.04[dB]

Identical capital letters in the same raw for each protein between Day 0 and Day 7 indicate that there are no significant differences between the values ($p > 0.05$)

Identical small letters in the same column for each protein indicate that there are no significant differences between the values (p>0.05)

However, these differences in the effectiveness of the studied proteins may also be explained by their adsorption rate to the oil water interface and effectiveness at generating repulsive interactions between droplets (Jafari, Assadpoor, He, & Bhandari, 2008). Sodium caseinate molecules have a flexible structure and relatively low molecular weight ($\approx$ 20 kDa), whereas lactoferrin molecules have a globular structure and relatively high molecular weight ($\approx$ 80 kDa). Thus, these differences in molecular characteristics may account for their different relative affinities for the droplet surfaces (Lesmes, Baudot, & McClements, 2010).

Table 3.4 shows the stability parameters (EA, CI, ES) of the prepared emulsions. Stable emulsions show EA and ES parameters values equal to 100% and CI equal to 0%. Emulsions prepared with lactoferrin under 6 minutes of sonication time showed 100% of emulsifying activity and stability against creaming until 7 days of storage, while those prepared with shorter sonication times presented lower values of emulsifying activity and stability against creaming. For sodium caseinate emulsions the highest values of EA, CI and ES were 40.8±1.0, 40.5±1.9 and 42.4±0.5%, respectively and these values corresponded to the highest sonication time. At pH 7.0, sodium caseinate is negatively charged (Ma, et al., 2009) and lactoferrin is positively charged (Lonnerdal & Iyer, 1995) suggesting that electrostatic repulsion contributed to the emulsions stability (see zeta potential values, Table 3.1). Steric repulsion may also be involved in the lactoferrin emulsion stabilization because it is a glycoprotein that has some sugar groups covalently attached to its peptide backbone (Oliver, Melton, & Stanley, 2006). In addition, this protein showed aggregates of high molecular weight (Figure 3.2) formed by non-covalent interactions (probably hydrophobic) (Figure 3.3) favoring their deposition onto interface and contributing to both electrostatic and steric stabilization. Thus, formation of stable emulsions was considered to be the effect of the ultrasound process and the emulsifying properties of the proteins. These results are also in agreement with the intrinsic viscosity (Table 3.2) analysis demonstrating that emulsifying properties of proteins improved during the emulsification provided by ultrasound and this enhancement was favored with increasing of sonication time.

Table 3.4 - Stability parameters for fine emulsions stabilized by sodium caseinate or lactoferrin under different sonication times. Protein was not previously ultrasound-treated.

Protein	Treatment	EA	CI	ES
		[%]	[%]	[%]
Sodium Caseinate	0 min	30.0 ± 7.1^b	16.0 ± 2.8^c	19.5 ± 0.7^c
	2 min	36.7 ± 2.5^{ab}	34.1 ± 1.4^b	37.8 ± 2.1^b
	4 min	40.4 ± 1.4^a	39.5 ± 1.0^a	37.8 ± 1.6^b
	6 min	40.8 ± 1.0^a	40.5 ± 1.9^a	42.4 ± 0.5^a
Lactoferrin	0 min	27.5 ± 3.5^c	40.4 ± 0.6^c	42.0 ± 0.0^b
	2 min	40.0 ± 0.0^b	39.1 ± 1.0^d	42.3 ± 2.1^b
	4 min	43.8 ± 2.5^b	98.0 ± 0.0^b	100.0 ± 0.0^a
	6 min	100.0 ± 0.0^a	0.0 ± 0.0^a	100.0 ± 0.0^a

Identical letters in the same column for each protein indicate that there are no significant differences between the values ($p > 0.05$)

3.3.2.1 Coarse emulsions produced with ultrasound-treated protein solutions

Coarse emulsions produced with ultrasound-treated protein solutions showed bimodal size distributions and mean droplets size varying between 19.6-26.3 μm ($D_{3,2}$), 62.3-74.5 μm ($D_{4,3}$) and polydispersity between 1.4-1.7 for sodium caseinate, while for lactoferrin mean droplets size varied between 18.8-21.4 μm ($D_{3,2}$), 56.7-66.9 μm ($D_{4,3}$) and polydispersity between 1.7-1.8 (Fig. 3.6). These mean droplets size were much higher than those obtained for coarse emulsions produced with untreated protein solutions (0 min), but span values were similar. When these coarse emulsions are compared to the fine emulsions produced by ultrasound, it is clear that the ultrasound emulsification process is much more effective than rotor stator homogenization producing smaller droplets (near 2 μm (Table 3.3)) due to the cavitation effects.

Treatment	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)	Span
0 min	12.10±0.2	34.53±0.1	1.9±0.1
2 min	20.5±0.0	63.4±0.2	1.6±0.0
4 min	19.6±0.1	62.3±0.3	1.7±0.0
6 min	26.3±0.2	74.5±0.4	1.4±0.0

Treatment	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)	Span
0 min	14.7±4.0	42.0±10.7	1.9±0.3
2 min	18.8±0.1	56.7±0.7	1.7±0.0
4 min	19.7±0.1	62.3±0.4	1.8±0.0
6 min	21.4±0.1	66.9±0.4	1.7±0.0
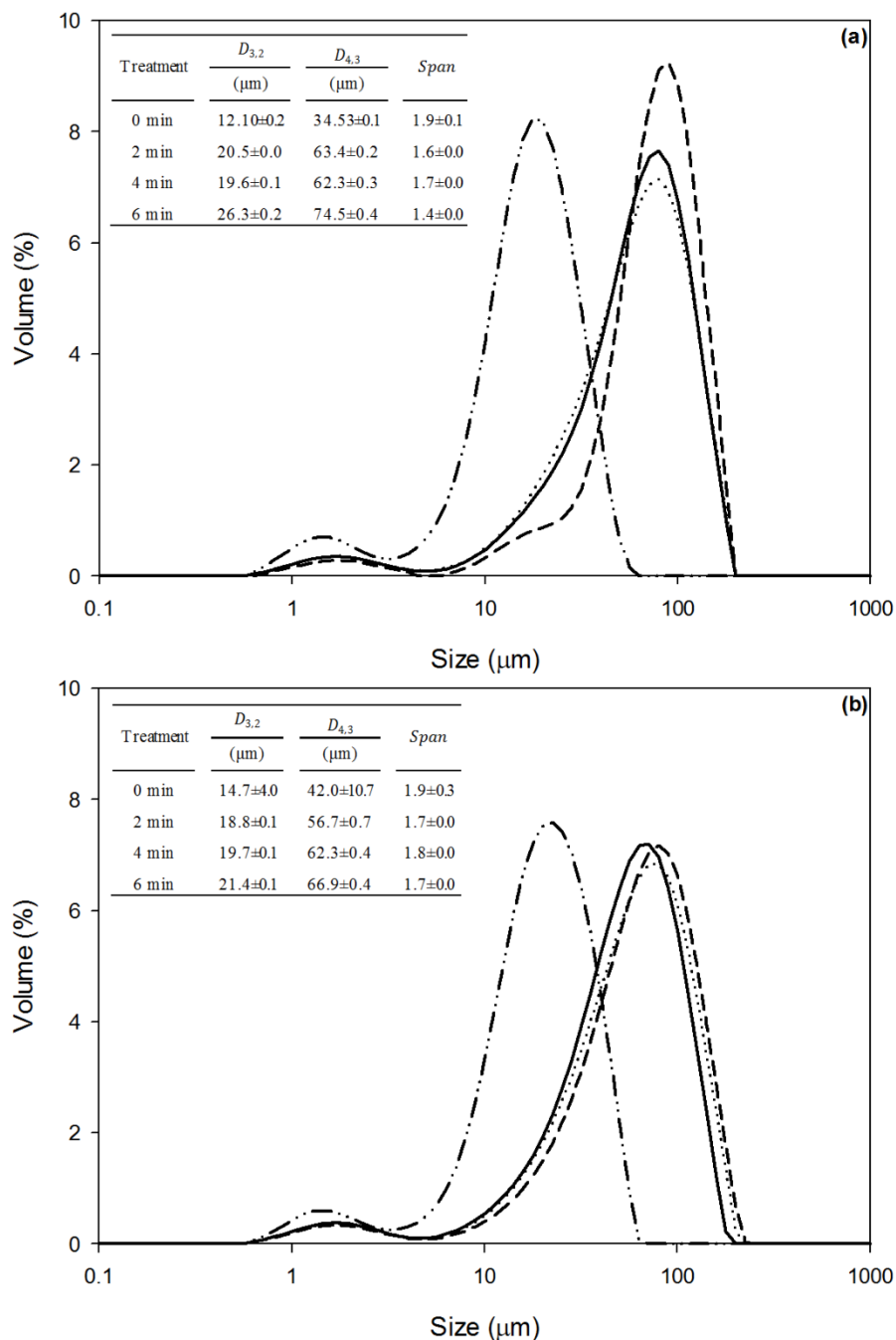
Figure 3.6 - Size distribution, mean droplet size and polydispersity of coarse emulsions stabilized by ultrasound-treated sodium caseinate (a) and lactoferrin (b) solutions under different times: 0 minutes (dashed double dotted line), 2 minutes (solid line), 4 minutes (dotted line) and 6 minutes (dashed line).

Table 3.5 shows the stability parameters (EA, CI, ES) of the coarse emulsions produced with ultrasound-treated protein solutions. It can be observed that these parameters increased when compared to those from emulsions produced with untreated protein solutions

(0 min), however ES from lactoferrin decreased and there was no significant difference between the sonication times used. Such slight increase in the stability parameters could be related to the increase in hydrophobicity (Fig. 3.3), but the effective reduction in droplets size and the complete stability (for lactoferrin) was only possible when ultrasound was used for droplets formation. This finding has also been related by other authors (Silva, Gomes, Hubinger, Cunha, & Meireles, 2015). Thus, we assumed that the interaction between hydrophobic groups and the oil phase during emulsification contributed positively to the decrease in the coalescence rate of the droplets and consequently to the emulsions stabilization, while in the absence of oil phase the proteins could be interacting with themselves hindering more efficient process of emulsification. Thus, ultrasound was suitable for change the surface properties (increasing hydrophobicity) of the proteins but its main contribution was reducing the droplets size at the same time during emulsification.

Table 3.5 - Stability parameters for coarse emulsions stabilized by ultrasound-treated sodium caseinate or lactoferrin solutions under different times.

Protein	Treatment	*EA*	*CI*	*ES*
		[%]	[%]	[%]
Sodium Caseinate	0 min	30.0 ± 7.1^a	16.0 ± 2.8^b	19.5 ± 0.7^b
	2 min	37.0 ± 1.7^a	40.0 ± 0.0^a	34.3 ± 0.6^a
	4 min	39.3 ± 1.2^a	40.8 ± 0.8^a	36.3 ± 2.9^a
	6 min	37.7 ± 2.5^a	40.5 ± 0.5^a	36.0 ± 2.0^a
Lactoferrin	0 min	27.5 ± 3.5^b	40.4 ± 0.6^b	42.0 ± 0.0^a
	2 min	41.7 ± 7.5^a	42.0 ± 0.0^{ab}	31.7 ± 2.9^a
	4 min	42.0 ± 6.9^a	43.3 ± 1.2^a	37.7 ± 7.5^a
	6 min	39.7 ± 0.6^{ab}	41.3 ± 1.2^{ab}	35.3 ± 0.6^a

Identical letters in the same column for each protein indicate that there are no significant differences between the values ($p > 0.05$)

3.4 CONCLUSIONS

The ultrasound treatment of sodium caseinate caused a significant reduction in the protein diameter while an opposite effect was observed for lactoferrin. This effect was attributed

to the unlike proteins conformation that showed opposite behavior when exposed to the strong mechanical forces resulting from ultrasonic cavitation. Surface hydrophobicity was also positively affected by the increase in sonication time and slight differences in molecular weight were observed between untreated and ultrasound treated lactoferrin attributed to non-covalent interactions between proteins. CD spectra revealed no differences for sodium caseinate but slight conformational changes were observed for lactoferrin since β-sheet structures decreased and random coil increased after ultrasound treatment. Emulsions stabilized by lactoferrin and prepared using ultrasound showed reduced droplet size and improved stability with the highest sonication times, while that it is not enough to stabilize emulsions containing sodium caseinate. However, both proteins showed a greater hydrophobicity with increasing sonication time but different emulsifying activity and stability against creaming. Therefore, a higher ability of lactoferrin to stabilize emulsions was attributed to electrostatic and steric repulsions caused by protein aggregates. Previous ultrasound treatment of aqueous protein solutions allowed to promote a slight improvement of emulsifying properties while treating aqueous protein solutions during emulsions or droplets formation resulted in a clear enhancement of emulsions stability, which means that the presence of oil (non-polar phase) and droplets formation occurring at the same time of emulsifiers deposition onto interface were essential to obtain stable emulsions using ultrasound treatment.

3.5 ACKNOWLEDGEMENTS

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CAPÍTULO 4 - PRODUÇÃO DE HETEROAGREGADOS DE GOTAS LIPÍDICAS RECOBERTAS COM CASEINATO DE SÓDIO E LACTOFERRINA

HETEROAGGREGATION OF LIPID DROPLETS COATED WITH SODIUM CASEINATE AND LACTOFERRIN

HETEROAGGREGATION OF LIPID DROPLETS COATED WITH SODIUM CASEINATE AND LACTOFERRIN

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Highlights

Heteroaggregates formation with oppositely charged lipid droplets was investigated.

Their properties depended on droplets volume ratio and protein properties.

Improved rheological properties can be obtained compared to a single emulsion.

Heteroaggregates with gel-like behavior were obtained depending on protein ratio.

Heteroaggregates were not affected to salt addition.

Graphical Abstract
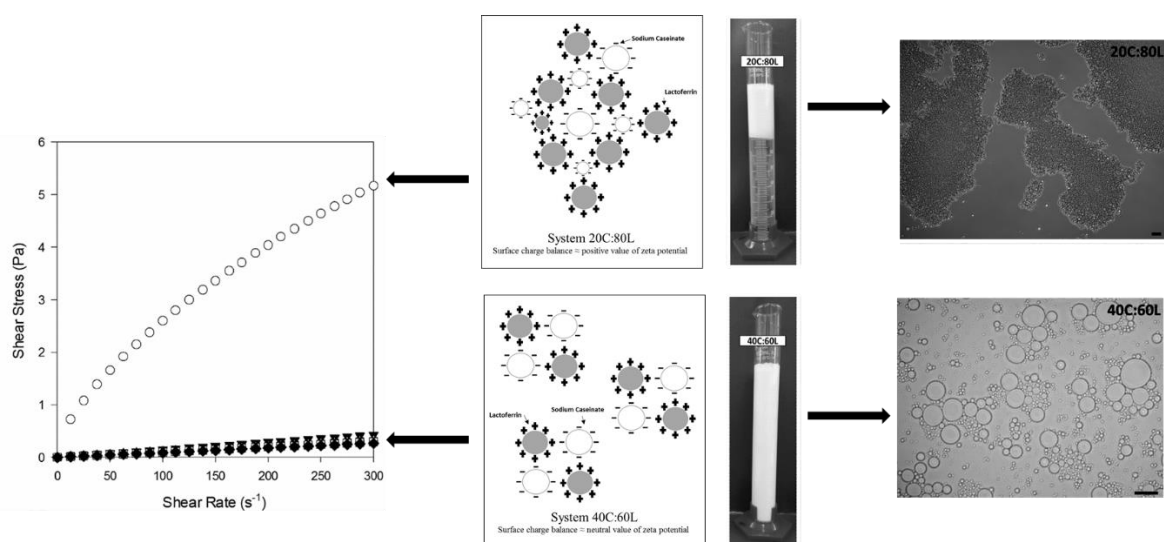
ABSTRACT

Formation and characterization of droplet heteroaggregates were investigated by mixing two emulsions previously stabilized by proteins oppositely charged. Emulsions were composed of 5 vol. % of sunflower oil and 95 vol. % of sodium caseinate or lactoferrin aqueous dispersions. They were produced using ultrasound with fixed power (300 W) and sonication time (6 min). Different volume ratios (0-100%) of sodium caseinate-stabilized emulsion (droplet diameter around 1.75 µm) to lactoferrin-stabilized emulsion (droplet diameter around 1.55 µm) were mixed under conditions that both proteins showed opposite charges (pH 7). Influence of ionic strength (0-400 mM NaCl) on the heteroaggregates stability was also evaluated. Creaming stability, zeta potential, microstructure, mean particle diameter and rheological properties of the heteroaggregates were measured. These properties depended on the volume ratio (0-100 %) of sodium caseinate to lactoferrin-stabilized emulsion (C:L) and the ionic strength. In the absence of salt, different zeta potential values were obtained, rheological properties (viscosity and elastic moduli) were improved and the largest heteroaggregates were formed at higher content of lactoferrin-stabilized emulsion (60-80%). The system containing 40 and 60 vol. % of sodium caseinate and lactoferrin stabilized emulsion, respectively, presented good stability against phase separation besides showing enhanced rheological and size properties due to extensive droplets aggregation. Phase separation was observed only in the absence of sodium caseinate, demonstrating the higher susceptibility of lactoferrin to NaCl. The heteroaggregates produced may be useful functional agents for texture modification and controlled release since different rheological properties and sizes can be achieved depending on protein concentrations.

Keywords: Emulsion, sodium caseinate, lactoferrin, heteroaggregation, electrostatic interactions.

4.1 INTRODUCTION

Structural design principles have been utilized to create food with improved or novel functional properties as high quality food products with reduced calorie content (McClements, Decker, Park & Weiss, 2009; Nehir El Simsek, 2012). However, fats plays a fundamental role in food products since they determine the appearance, texture and flavor thereof. Indeed, fat removal is associated to the lost of desirable qualities affecting adversely sensory quality attributes (McClements & Demetriades, 1998). Thus, a number of fat reduction strategies have been developed, including the use of non-absorbable fats, reduced calorie fats, thickeners and colloidal particles (Williams & Buttriss, 2006).

A wide variety of food products consists, at least partially, by emulsions such as milk, yogurt, salad dressing, mayonnaise and ice cream (McClements, 2004). Emulsions with different structures, physicochemical properties and functional attributes may be prepared by controlling the characteristics of the colloidal particles (such as size, surface charge, concentration), environmental conditions (such as pH, ionic strength, temperature) and the method of preparation (such as the order of addition of ingredients and mixing conditions) (Mao & McClements, 2011). Recent studies have reported that controlled heteroaggregation of lipid droplets may be used to manipulate the characteristics of the emulsion-based products (Mao & McClements, 2011, 2012b, 2012c, 2012d). Heteroaggregated emulsions are formed by mixing two single emulsions containing lipid droplets coated by electrically charged emulsifier molecules as proteins (Mao & McClements, 2011, 2012c). This technique allows creating products with reduced fat content but substantial amounts of protein, inducing a feeling of satiety that could be related to kinetics of amino acid profiles consumption (Westerterp-Plantenga, Nieuwenhuizen, Tome, Soenen, & Westerterp, 2009).

Proteins can act as emulsifiers providing a combination of electrostatic and steric repulsion between the oil droplets which allows the formation of a kinetically stable emulsion (Wilde, Mackie, Husband, Gunning, & Morris, 2004). Casein is a mixture of small aggregates in milk at neutral pH which is called casein micelles and they are attached to calcium salts. These calcium salts when replaced by sodium salts lead to the production of sodium caseinate. Sodium caseinate is a complex mixture of different casein variants (α, β, and κ casein), showing an average molecular weight around 24 kDa and isoelectric point around pH 4.5. At neutral pH sodium caseinate is negatively charged (Ma et al., 2009).

Lactoferrin is a minor milk protein composed by a single polypeptide chain of about 80 kDa, containing from one to four glycans (Spik et al., 1994). Due to the high levels of basic amino acids, it has high isoelectric point (pI > 8). Therefore, this protein is positively charged at neutral pH whereas most of other dairy proteins are anionic (Steijns & van Hooijdonk, 2000). Besides of their beneficial effects like antioxidant, antimicrobial, antiviral and anticancer activity (Actor, Hwang, & Kruzel, 2009; Huang, Satué-Gracia, Frankel, & German, 1999; Tomita et al., 2009), lactoferrin is safe for health and shows potential application as a food additive for human and animal (Wakabayashi, Yamauchi, & Takase, 2006). Many studies have shown that lactoferrin is an excellent emulsifier since it adsorbs to the oil water interface and produces a cationic emulsion (Tokle & McClements, 2011; Ye & Singh, 2006).

Many methods are available to produce emulsions and they are directly related to the kinetic stability of these emulsions (Santana, Perrechil, & Cunha, 2013). Ultrasound can be used in the production of emulsions and is based on the application of an acoustic field that results in cavitation phenomena causing the formation of droplets (Abismail, Canselier, Wilhelm, Delmas, & Gourdon, 1999). The use of this technique presents several advantages, such as smaller droplets size production and narrower size distribution resulting in more stable emulsions; minimal emulsifier content requirements depending on the emulsifier used; easy operation, control and cleaning; and low production costs (Abbas, Hayat, Karangwa, Bashari, & Zhang, 2013).

In the current study we investigated the droplets heteroaggregation by mixing two emulsions stabilized by proteins oppositely charged varying the emulsion volume ratio and ionic strength. Their properties were evaluated in terms of creaming stability, microstructure, mean particle size and rheological parameters. Valuable information about heteroaggregates formation and characteristics were provided to better understand about the mechanisms involved in their formation.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Ultrapure water from a Millipore Milli-Q system (resistivity 18.2 MΩ/cm) was used. Sodium caseinate (protein content 87 wt. %) and lactoferrin (protein content 92.1 wt. %) were kindly provided by Allibra Ingredientes Ltd (Campinas, Brazil) and Synlait Milk Ltd

(Canterbury, New Zeland), respectively. Sunflower oil (Bunge Alimentos S.A., Gaspar, Brazil) was purchased in the local market. The other reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Protein dispersions preparation

Sodium caseinate and lactoferrin were dispersed in ultrapure water (0.30 wt. %) using overnight magnetic stirring at room temperature, ensuring complete dissolution of the protein. The pH of protein solutions was adjusted to pH 7.0 using sodium hydroxide (1 M) or hydrochloric acid (1 M).

4.2.2.2 Oil in water emulsions preparation

Coarse emulsions were prepared by homogenizing 95 ml of protein dispersions (0.30 wt. %, pH 7.0) and 5 mL of sunflower oil using a rotor-stator homogenizer (SilentCrusher M, Heidolph, Schwabach, Germany) at 5,000 rpm for 3 minutes giving a final protein concentration of 0.29 wt. %. This protein concentration was based on the same protein:oil ratio used by Dickinson & Golding (1997) which resulted in good stability against creaming at nearly saturation coverage of droplets for sodium caseinate. The same approximation was used for lactoferrin since a protein:oil ratio closed to 0.05 is considered adequate to stabilize lactoferrin emulsions (Acero-Lopez, Schell, Corredig, & Alexander, 2010). Fine emulsions were prepared by subjecting the coarse emulsion in an ultrasonic processor (QR 750W, Ultronique, Campinas, Brazil) with a 13 mm diameter titanium probe immersed 3 mm depth, which was used in combination with a magnetic stirrer to enhance mixture homogenization. Sonication time, power and the frequency were fixed at 6 minutes, 300 W and 20 kHz, respectively. The time and power used were based on preliminary tests, considering a decrease of droplets without a significant heating of emulsions. The temperature of preparation did not exceed 30 °C during the homogenization process.

4.2.2.3 Heteroaggregate preparation

The heteroaggregates were prepared by mixing different volume ratios of the two fine emulsions under magnetic stirring at 250 rpm for 10 min (HEI-TEC, Heidolph, Schwabach, Germany). After that, this mixed material was stand for 24 hours prior to analysis. Mixed emulsions showed different amounts of sodium caseinate coated droplets (0 – 100 vol. %) and lactoferrin coated droplets (0 – 100 vol. %) in order to keep unlike volume ratios of negative-to-positive droplets. For convenience, we used the notation 30C:70L to refer to heteroaggregate containing 30 vol. % of sodium caseinate stabilized emulsion and 70 vol. % of lactoferrin stabilized emulsion. A similar notation was used to other formulations.

The influence of ionic strength was evaluated for three heteroaggregate conditions (0C:100L, 40C:60L and 100C:0L). Aliquots of these heteroaggregates (50 mL) were mixed with 50 mL of different sodium chloride solutions (NaCl) (0, 50, 100, 200, 400, and 800 mM) at pH 7.0. The final mixed emulsions contained 2.5% oil and 0–400 mM NaCl.

4.2.2.4 Emulsion and heteroaggregates characterization

4.2.2.4.1 Interfacial tension

The interfacial tension between the aqueous protein solutions (0.30 wt. %) and sunflower oil was measured by the pendant drop method using a TrackerS tensiometer (Teclis, Longessaigne, France). Assays were performed at 25 ± 0.1 °C with the formation of a drop of the aqueous phase in the oil phase. A syringe with diameter of 3 mm was used and the drop volume was 10 µL.

4.2.2.4.2 Particle size

The particle size distribution of emulsions and heteroaggregates was determined based on the static light scattering method using a Multi-Angle Static Light-Scattering Mastersizer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The emulsions and the heteroaggregates were analyzed at the first and the seventh day after their preparation. The samples were diluted in ultrapure water (pH 7) (refractive index 1.33). The mean diameter was

expressed as the volume mean diameter ($D_{4,3}$) (Eq. 4.1). The polidispersity ($Span$) was also evaluated (Eq. 4.2).

$$D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \tag{4.1}$$

$$Span = \frac{(D_{90} - D_{10})}{D_{50}} \tag{4.2}$$

where n_i is the number of droplets with diameter D_i, and D_{10}, D_{50} and D_{90} are diameters at 10, 50 and 90% of cumulative volume, respectively.

Mean particle size of the heteroaggregates should only be treated as an indicative of the dimensions. The theory used to interpret light scattering data assumes that the scattering particles are homogeneous spheres with well-defined refractive indices and flocculated systems are non-spherical and non-homogeneous particles. Furthermore, the stirring and dilution used for this measurement may alter the dimensions and structural organization of the heteroaggregates (Mao & McClements, 2012a).

4.2.2.4.3 Microstructure

Microstructure of the emulsions and the heteroaggregates were evaluated after 1 day of storage. Small quantities of sample were poured onto microscopes slides, covered with glass cover slips and observed using a Carl Zeiss Axio Scope A1 microscope (Zeiss, Oberkochen, Germany) with x40 and x100 objective lenses.

Confocal laser scanning microscopy was also used to investigate microstructure of the samples. Sunflower oil was stained with Nile Red (0.005 wt. %) and lactoferrin was stained with FITC (0.5 wt. %). Each material was stained separately and then emulsions and heteroaggregates were prepared as described previously (2.2.2 and 2.2.3). Samples were examined using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany) with a x100 objective lense. Images were collected using 551 and 498 nm laser lines for excitation of Nile Red and FITC fluorophores, respectively.

4.2.2.4.4 Creaming stability

Immediately after preparation, 100 mL of each emulsion were poured into a cylindrical glass tube (internal diameter = 26 mm, height = 173 mm), sealed with a plastic cap and stored at 25 °C for 7 days. Change in height of the top phase H (cm) was measured visually using a ruler during storage time and the creaming index (CI) determined according to Eq. (4.3) (Keowmaneechai & McClements, 2002).

$$CI\ (\%) = \frac{H}{H_0} 100 \tag{4.3}$$

where H_0 represents the initial height (cm) of the emulsion.

4.2.2.4.5 Zeta potential

Zeta potential was determined using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK) in a fixed pH value (7.0). The heteroaggregates were diluted in MilliQ water to a droplet concentration of approximately 0.001 vol. %. Samples were equilibrated for about 120 s into the instrument before particle charge data was collected over 10 continuous readings.

4.2.2.4.6 Rheology

Rheological measurements of the emulsions and the heteroaggregates were performed using a rheometer Physica MCR301 (Anton Paar, Graz, Austria) with a stainless steel plate geometry (75 mm) and a 0.05 – 0.1 mm gap. Flow curves were obtained by an up-down-up steps program with shear rate ranging from 0 to 300 s^{-1} and the models for Newtonian Eq. (4.4) and shear-thinning fluids (power law model) Eq. (4.5) were fitted to the data to obtain the consistency index (k) and the behavior index (n). The viscoelastic properties were evaluated by oscillatory measurements, using a frequency sweep between 0.1 and 10 Hz within the linear viscoelasticity domain. These measurements were done at 25 ± 0.1°C after one day of samples storage. The contribution of the elastic and viscous characteristics was evaluated from storage (G′) and loss (G″) moduli. Evaluation of viscoelastic properties can help understanding the

contribution of viscous and elastic components on emulsions stability since these measurements are performed at low deformation (Torres, Iturbe, Snowden, Chowdhry, & Leharne, 2007).

$$\sigma = \eta.\dot{\gamma} \tag{4.4}$$

$$\sigma = k.\dot{\gamma}^n \tag{4.5}$$

where σ is the shear stress (Pa) , η is the viscosity (Pa.s), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency index (Pa.s^n) and n is the flow behavior index (dimensionless).

4.2.3 Statistical analysis

The results were reported as the average and the standard deviation of three replicates and evaluated by one way analysis of variance (ANOVA), and significant differences (p <0.05) between the treatments were evaluated by the Tukey test. The statistical analyses were carried out using the trial version software Minitab 16.1.0 (Minitab Inc., State College, PA, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Interfacial tension

The interfacial tension between aqueous protein solutions and oil phase is shown in Fig. 4.1. The initial interfacial tension was around 15 mN/m for water and decreased from 10.3 to 9.3 mN/m for sodium caseinate and lactoferrin aqueous dispersions, respectively. For all the systems, the interfacial tension decreased with time and lactoferrin addition was more efficient to reduce the interfacial tension at water–sunflower oil interface suggesting a better surface activity compared to sodium caseinate. Lower interfacial tension means greater adsorption of surfactant molecules at the interface, decreasing thermodynamically unfavorable contacts between immiscible liquids (Furtado, Picone, Cuellar, & Cunha, 2015; Gülseren & Corredig, 2013; Spinelli, Mansur, González, & Lucas, 2010). Furthermore, sodium caseinate molecules show a flexible structure and relatively low molecular weight ($\approx$ 20 kDa), whereas lactoferrin is a globular protein with a relatively high molecular weight ($\approx$ 80 kDa). Thus, besides the

amino acidic composition, these differences may account for the different relative affinities for the droplet surfaces (Lesmes, Baudot, & McClements, 2010). Despite sodium caseinate was not more effective in reducing interfacial tension, due to its low molecular weight, the amino acid composition of lactoferrin could have a greater relevance in the interfacial tension reduction velocity.
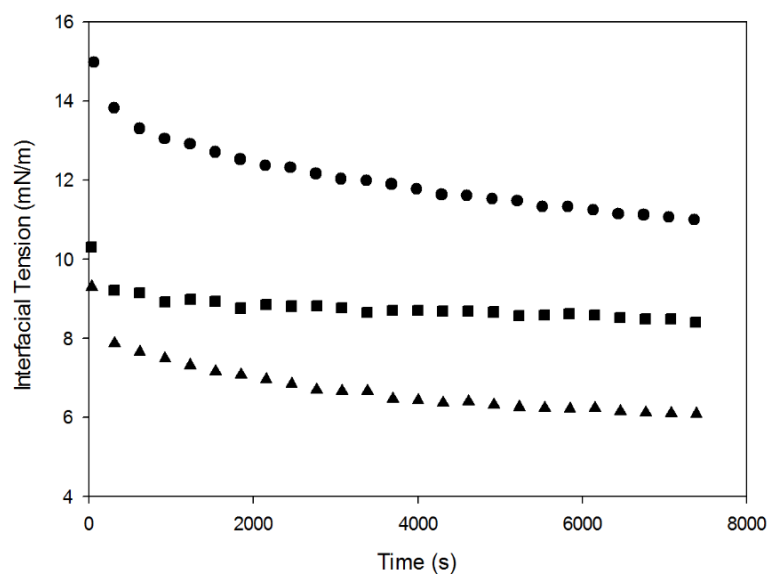
Figure 4.1 - Kinetics of interfacial tension between sunflower oil and water (●), sodium caseinate (■) or lactoferrin (▲) aqueous dispersions.

4.3.2 Formation of sodium caseinate and lactoferrin emulsions

The influence of protein type on the structure and volume particles size distribution of the emulsions can be observed in Figure 4.2. Emulsions stabilized by both proteins were kinetically stable during 7 days and the microscopy images showed that fine emulsions were obtained for both proteins. Furthermore, ultrasound process allowed to obtain a unimodal particle size distribution pattern, in agreement with results presented by some authors (Gaikwad & Pandit, 2008; Shanmugam & Ashokkumar, 2014).
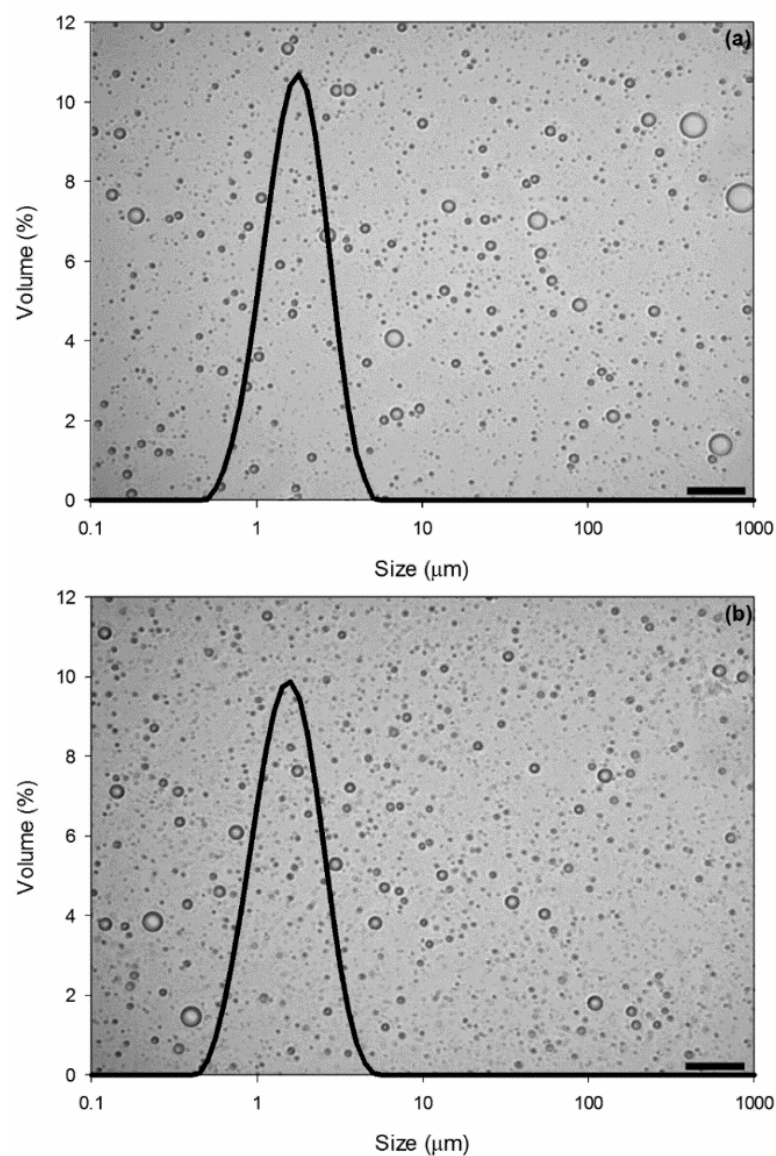
Figure 4.2 - Optical micrographs and droplets size distribution of the oil in water emulsions stabilized by sodium caseinate (a) and lactoferrin (b) after 1 day of storage at 25 ℃. Scale bar: 10 µm.

Mean droplet size was 1.75±0.01 µm for emulsions stabilized with sodium caseinate while for emulsions stabilized with lactoferrin it was slightly lower ($D_{4,3}$=1.55±0.01 µm). Low span values (around 1) were obtained and small droplet size values around 1-2 µm were also observed in another studies using ultrasound emulsification (Huck-Iriart, Pizones Ruiz-Henestrosa, Candal, & Herrera, 2012; Shanmugam & Ashokkumar, 2014). $D_{4,3}$ and span values did not vary statistically during storage, showing the good kinetic stability of the emulsions since phase separation was not observed during this period (7 days) (data not shown).

Emulsions are unflocculated using protein content around that required for monolayer saturation coverage and consequently they are very stable towards creaming and coalescence (Huck-Iriart, Álvarez-Cerimedo, Candal, & Herrera, 2011). At pH 7.0, sodium caseinate is negatively charged (Ma et al., 2009) and lactoferrin is positively charged (Lönnerdal & Iyer, 1995), suggesting that electrostatic repulsion contributed to the emulsions stability. Steric repulsion may also be involved in the lactoferrin emulsion stabilization because it is a glycoprotein that has some sugar groups covalently attached to its peptide backbone (Oliver, Melton, & Stanley, 2006). Furthermore, ultrasound treatment can induce protein aggregate formation, favoring their deposition onto interface and contributing to both electrostatic and steric stabilization (O'Sullivan et al., 2014).

4.3.3 Heteroaggregate formation

Different mixtures of negatively and positively charged droplets were evaluated to establish conditions of heteroaggregate formation, which was reached after evaluation of zeta potential, particle size, creaming index, microstructure and rheology.

Zeta potential values of the heteroaggregates are presented in Fig. 4.3, showing that heteroaggregates with different surface charge characteristics can be created. Zeta potential varied from highly positive to highly negative values, depending on the volume ratio of sodium caseinate to lactoferrin stabilized emulsions. Droplets coated only with sodium caseinate presented high negative charge (≈ - 39 mV) and droplets coated only with lactoferrin showed high positive charge (≈ + 32 mV). These opposite charge values are a consequence of pH 7, since the isoelectric point of lactoferrin is around 8.5 and sodium caseinate is close to 4.5 (Lönnerdal & Iyer, 1995; Ma et al., 2009). Zero surface charge was observed with 40 vol. % of sodium caseinate and 60 vol. % of lactoferrin stabilized emulsions, indicating that more positively charged protein was necessary to coat droplets and reach neutral charge. Similar results were obtained in the production of mixtures of beta lactoglobulin and lactoferrin coated droplets (Mao & McClements, 2012a).
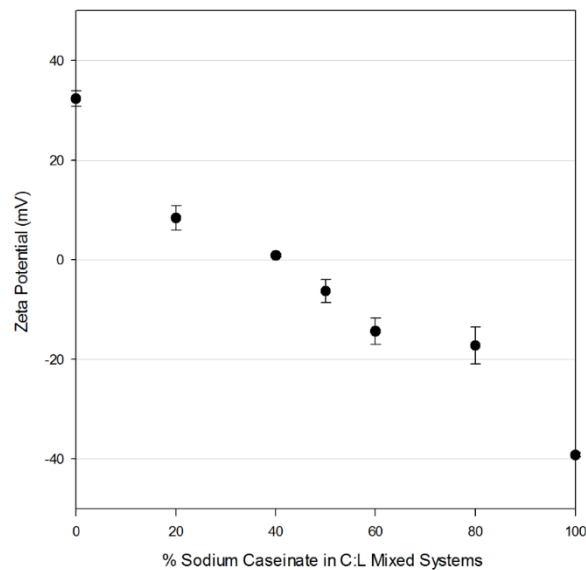
Figure 4.3 - Zeta potential values of the heteroaggregates produced at pH 7.0 using different volume ratio of sodium caseinate and lactoferrin stabilized emulsions after 1 day of storage at 25 °C.

Most of heteroaggregates were stable during 7 days including 40C:60L that showed zero surface charge. However, 20C:80L showed phase separation (Fig. 4.4) and its cream phase was gel-like. The other conditions showed the formation of extensive aggregated droplets (Fig. 4.5), suggesting that the main driving force is the electrostatic interaction between droplets coated with oppositely charged proteins. Furthermore, some partial droplet coalescence was observed, mainly at conditions that zeta potential was close to zero, due to the strong attraction between the droplets coated with oppositely charged proteins, as also observed for other authors (Mao & McClements, 2011, 2012a; Ye, Hemar, & Singh, 2004).

Figure 4.4 - Visual aspect of the heteroaggregates produced under different volume ratio of sodium caseinate:lactoferrin stabilized emulsions after 1 day of storage at 25 °C.
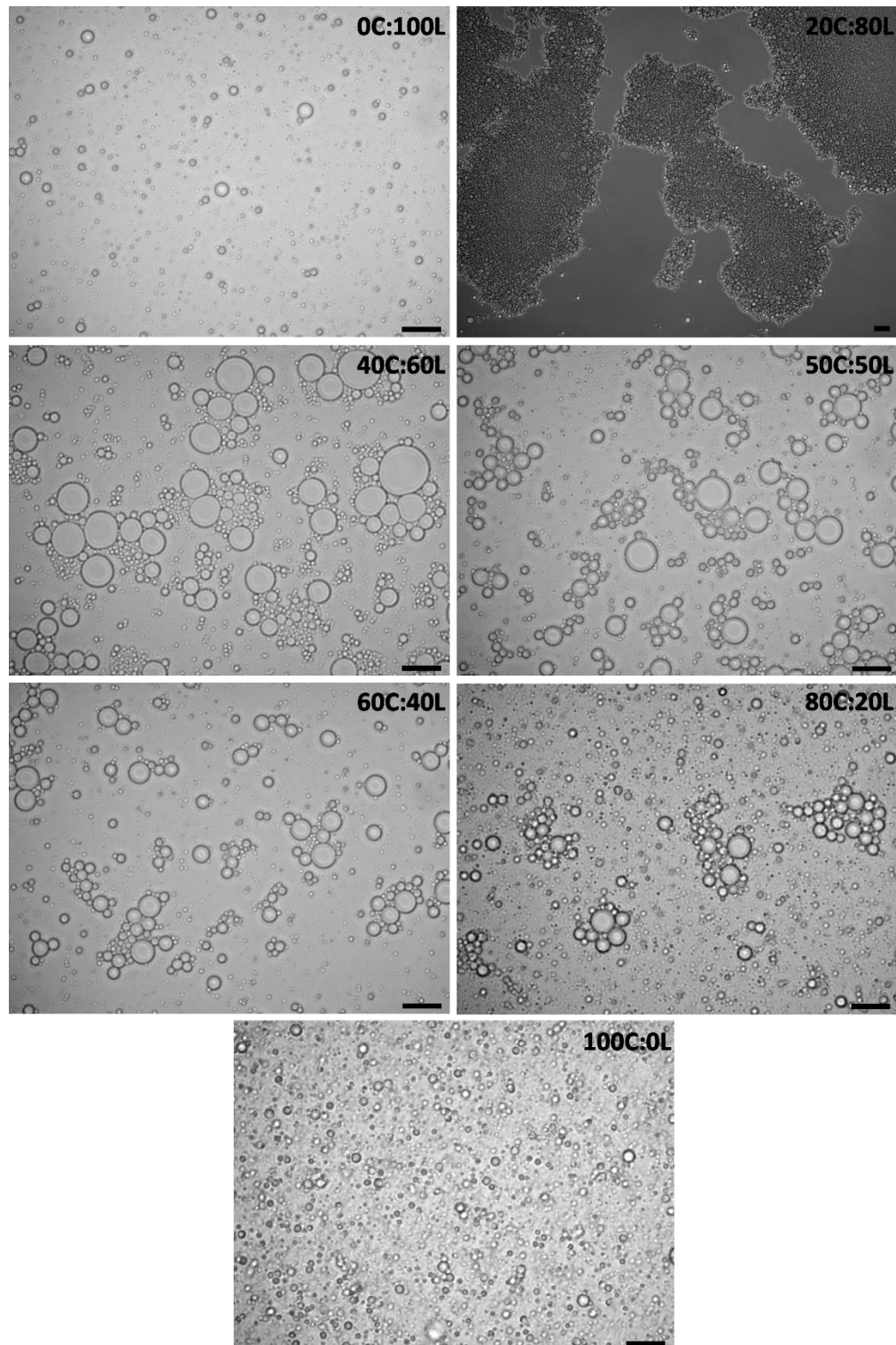
Figure 4.5 - Optical micrographs of the heteroaggregates produced under different volume ratio of sodium caseinate:lactoferrin stabilized emulsions after 1 day of storage at 25 °C. Scale bar: 10 µm.

The heteroaggregates presented a unimodal particles size distribution pattern (Fig. 4.6). A broad peak was observed for heteroaggregates formed with higher content of lactoferrin stabilized emulsion (20C:80L and 40C:60L), while the other conditions presented a narrow peak, differing slightly from the control emulsions 0C:100L and 100C:0L. Figures 4.5 and 4.6 suggest that besides the partial droplets coalescence, a marked droplets flocclulation occurred in the presence of sodium caseinate and high lactoferrin content while small heteroaggregates were formed at low or intermediate lactoferrin content (higher sodium caseinate content). Aggregation between sodium caseinate and lactoferrin stabilized droplets can be visualized from the confocal micrograph presented in Figure 4.7. Lactoferrin stabilized droplets (oil stained with Nile Red) are surrounded by a green color interface (lactoferrin stained with FITC) while oil droplets with a non-stained interface should be stabilized by sodium caseinate.
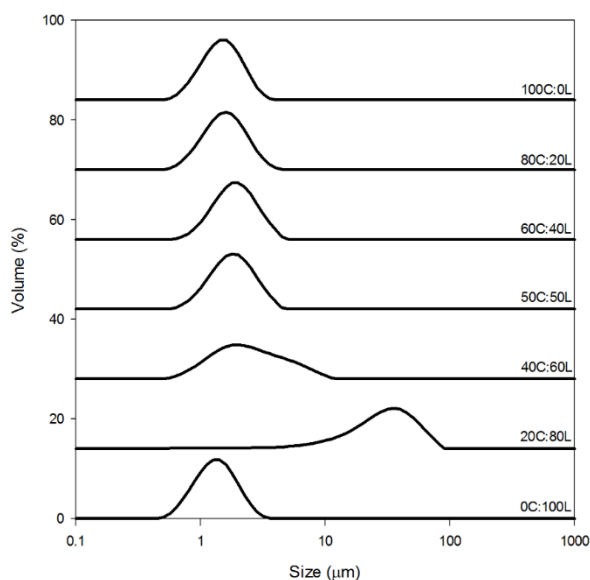
Figure 4.6 - Particles size distribution of the heteroaggregates produced under different volume ratio of sodium caseinate:lactoferrin stabilized emulsions after 1 day of storage at 25 ºC.
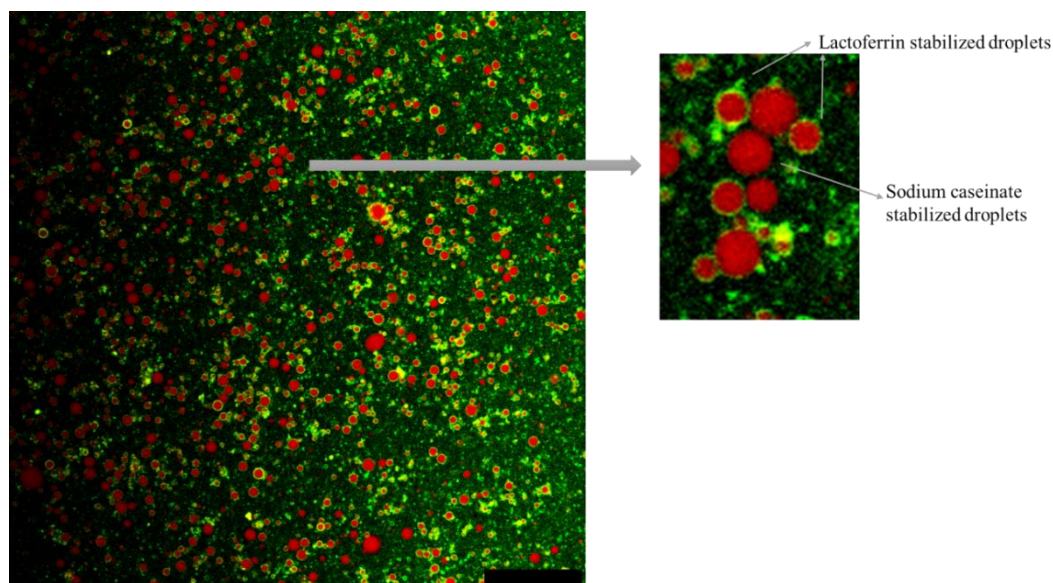
Figure 4.7 - Confocal micrograph of the 40C:60L heteroaggregate after 1 day of storage at 25 ºC. Scale bar: 25 µm.

The mean particle diameter and span of the heteroaggregates are presented in Table 4.1. The values increased with the increase in volume ratio of lactoferrin reaching $D_{4,3} = 69.13\pm2.15$ µm to the 20C:80L heteroaggregates since they formed a gel network (Fig. 4.5). After seven days $D_{4,3}$ values of the 20C:80L and 40C:60L heteroaggregates increased while $D_{4,3}$ values from the other systems remained approximately constant. Although the 40C:60L heteroaggregate showed zero charge and both 20C:80L and 40C:60L heteroaggregates exhibited size increase (Fig. 4.3), only the 20C:80L heteroaggregate presented creaming index around 27% (Table 4.1).

Low amount of droplets was stabilized by sodium caseinate in the 20C: 80L system which allowed a strong aggregation and the higher exposition of lactoferrin stabilized droplets, resulting in a net positive zeta potential value (Fig. 4.8). We can assume that such intense aggregation led to the formation of larger heteroaggregates influencing negatively the stability against gravitational separation. On the other hand, the higher amount of sodium caseinate stabilized droplets in the 40C: 60L system allowed the formation of heteroaggregates with a more uniform ratio of positive and negatively charged droplets, implying that the balance between surface charges remained close to neutrality (Fig. 4.8). Furthermore, the size of the heteroaggregates was relatively lower, contributing to the good stability against gravitational separation.

Table 4.1 - Mean droplets size, polydispersity and creaming index of the heteroaggregates produced under different volume ratio of sodium caseinate:lactoferrin stabilized emulsions after 1 day of storage at 25 ºC.

Heteroaggregate	$D_{4,3}$ (µm)		Span		CI (%)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
0C:100L	1.56±0.01[cA]	1.58±0.01[cA]	1.22±0.05[cdA]	1.24±0.05[cA]	0.0±0.0	0.0±0.0
20C:80L	69.13±2.15[aB]	87.19±3.86[aA]	1.8±0.04[bA]	1.75±0.03[bA]	30.0±2.0	27.0±2.0
40C:60L	3.83±0.05[bB]	7.88±0.34[bA]	2.57±0.04[aA]	2.64±0.17[aA]	0.0±0.0	0.0±0.0
50C:50L	2.25±0.01[bcA]	2.19±0.09[cA]	1.29±0.00[dB]	1.54±0.04[bA]	0.0±0.0	0.0±0.0
60C:40L	2.37±0.01[bcA]	2.39±0.01[cA]	1.25±0.01[cdA]	0.99±0.00[dB]	0.0±0.0	0.0±0.0
80C:20L	1.91±0.00[cA]	1.92±0.01[cA]	1.31±0.06[dA]	1.19±0.06[cdB]	0.0±0.0	0.0±0.0
100C:0L	1.76±0.00[cB]	1.80±0.00[cA]	1.15±0.04[cA]	1.17±0.04[cdA]	0.0±0.0	0.0±0.0

Identical capital letters in the same raw between Day 1 and Day 7 results indicate that there are no significant differences ($p < 0.05$)

Identical small letters in the same column indicate that there are no significant differences ($p < 0.05$)
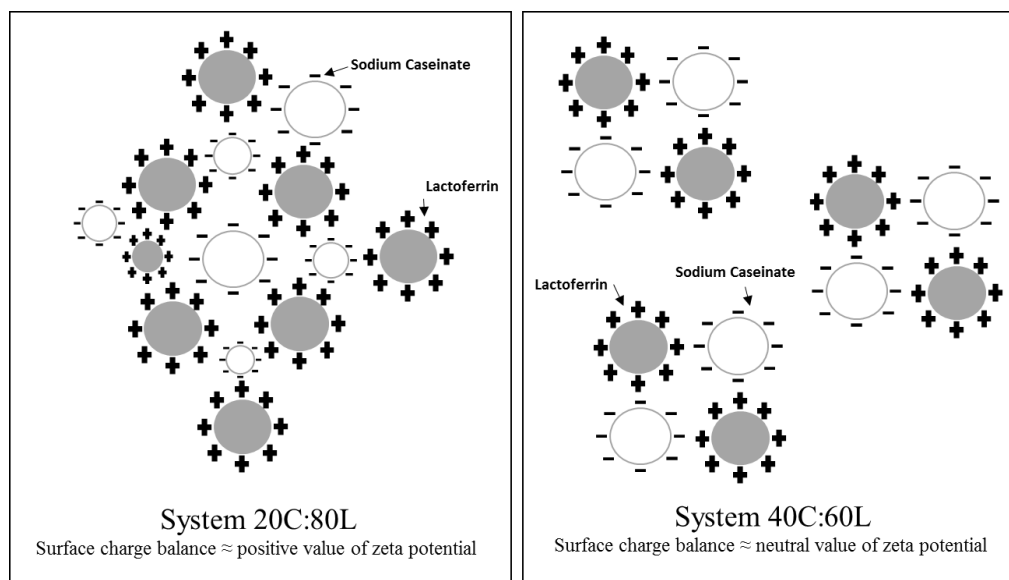
Figure 4.8 - Illustration of a model demonstrating the proposed behavior of the
heteroaggregates.

Emulsions containing heteroaggregates showed Newtonian behavior, except for 20C:80L heteroaggregate that showed shear-thinning behavior (Table 4.2). The behavior index (n) obtained from power law equation indicates the degree of deviation from the Newtonian linear rheological behavior. Newtonian fluids show *n* equals to the unit whilst shear-thinning samples present *n* < 1. The shear-thinning behavior is typically observed in concentrated suspensions of solid particles or liquid droplets in emulsions interacting with each other (McClements, 2004). Values of viscosity (η) (Table 4.2) showed a statistical significant increase for heteroaggregates with higher lactoferrin content (20C:80L and 40C:60L), confirming the increase of emulsion structuration due to a probable higher interaction between the droplets. Therefore, the most of the heteroaggregates were dispersed in a stable emulsion showing a unimodal particle size distribution and a Newtonian rheological behavior, which was also observed by Eliot and Dickinson (2003).

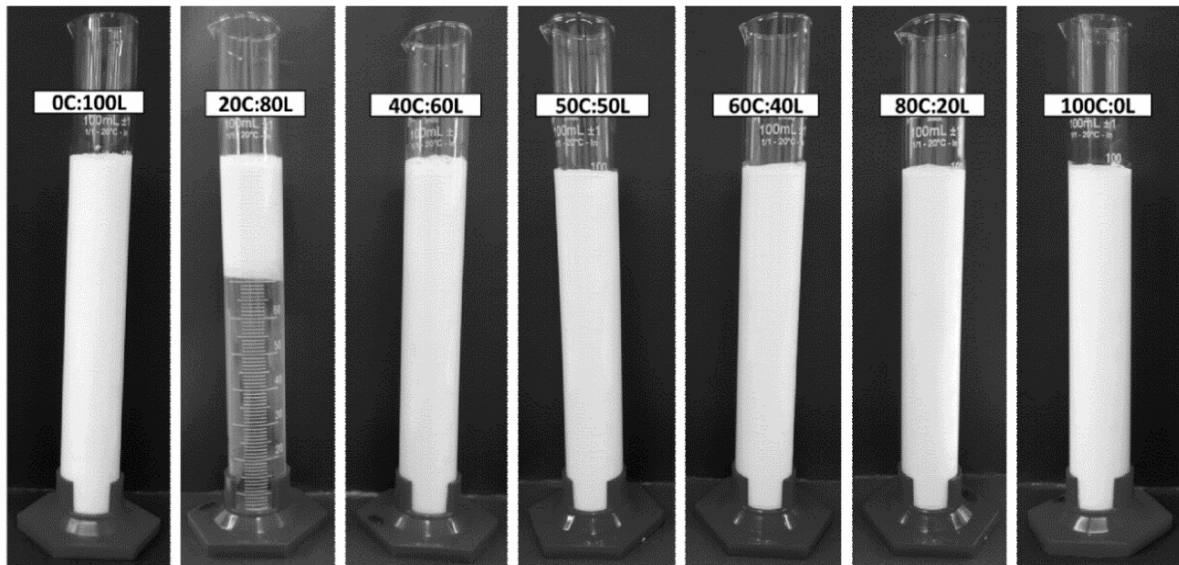Table 4.2 - Rheological parameters of the heteroaggregates produced under different volume ratio of sodium caseinate and lactoferrin stabilized emulsions after 1 day of storage at 25 °C.

Heteroaggregates	η or $*\eta_{100}$ (mPa.s)	k (mPa.s^n)	n	R^2
0C:100L	0.97±0.03[cd]	-	-	0.9992
20C:80L	*784.58±0.00[a]	142.77±0.00	0.63±0.00	0.9999
40C:60L	1.46±0.01[b]	-	-	0.9996
50C:50L	1.06±0.05[c]	-	-	0.9999
60C:40L	1.02±0.00[cd]	-	-	0.9999
80C:20L	0.98±0.00[cd]	-	-	0.9999
100C:0L	0.91±0.00[d]	-	-	0.9995

Identical small letters in the same column indicate that there are no significant differences ($p < 0.05$)

$*\eta_{100}$ is the apparent viscosity at 100 s^{-1}.

Preliminary measurements were done to identify the linear viscoelastic region by measuring the shear moduli at different strain values at a fixed frequency (1 Hz). The shear modulus remained constant until 5% strain, decreasing appreciably at higher strain values. Thus, a constant strain of 1% was used for the subsequent measurements. Storage modulus (G′) and loss modulus (G″) can indicate whether the emulsion system is strongly or weakly associated (Torres et al., 2007). Mechanical spectra of the heteroaggregates (Fig. 4.9) indicates that all systems presented gel-like behavior (G′ higher than G″ throughout the frequency) and the higher content of lactoferrin led to higher moduli values or stronger gel structure. The same behavior was observed in the heteroaggregates formation with beta lactoglobulin and lactoferrin coated droplets (Mao & McClements, 2012e). Elastic modulus (G′) of the heteroaggregates showed a frequency-dependence, which is associated to a weaker gel or a less stable emulsion. Such increase of the elastic moduli could be associated to the formation of an aggregated droplet network with a weak elastic network structure. However, the elastic modulus (G′) was close and typical of strong gels for the 20C:80L heteroaggregate (Torres et al., 2007). Furthermore, due to the phase separation a more packed phase was obtained increasing particle-particle interactions, which led to an increase of the gel strength (Quemada & Berli, 2002).

Thus, this increase in viscosity and elastic moduli at higher content of lactoferrin stabilized emulsion allowed to produce heteroaggregated systems with much more structured rheological behavior using the same oil volume fraction than that used in a single emulsion.
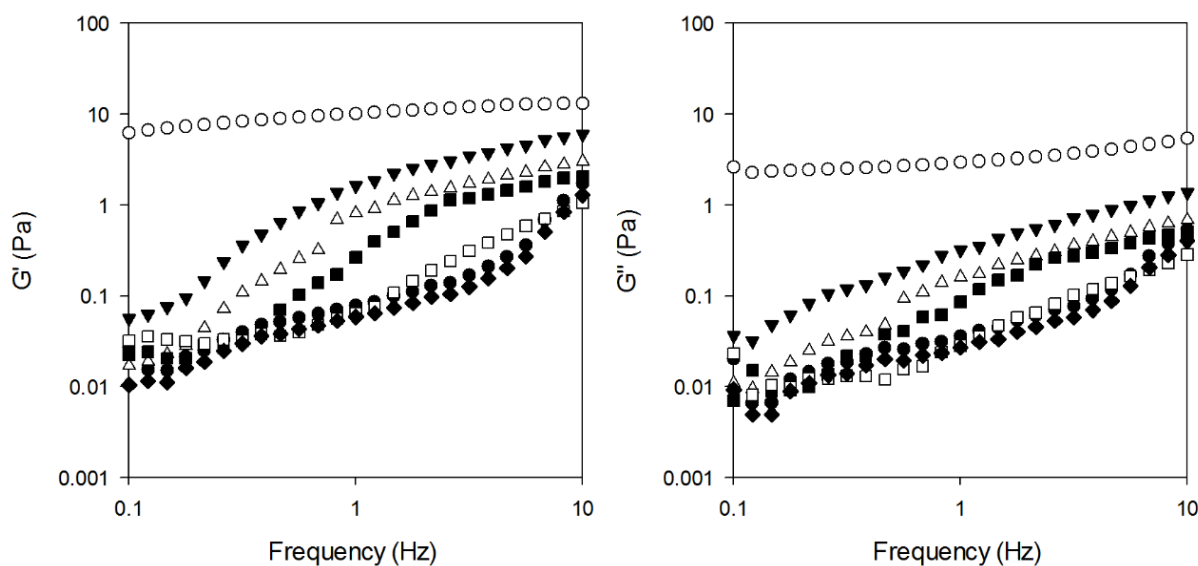
Figure 4.9 - Storage (G') and loss moduli (G'') of the heteroaggregates produced under different volume ratio of sodium caseinate:lactoferrin stabilized emulsions (●) 0C:100L, (○) 20C:80L, (▼) 40C:60L, (△) 50C:50L, (■) 60C:40L, (□) 80C:20L, (◆) 100C:0L after 1 day of storage at 25 ℃.

4.3.4 Influence of ionic strength on heteroaggregates formation

We investigated the influence of ionic strength on heteroaggregates formation, since salt addition is common in a great variety of commercial products besides of changing the magnitude and range of electrostatic interactions between droplets (Mao & McClements, 2012a; McClements, 2004; Srinivasan, Singh, & Munro, 2000). Three systems were investigated: controls (0C:100L and 100C:0L) and 40C:60L. The later system was chosen because it did not present phase separation (keeping its liquid form), but showed improved rheological properties. We observed that 40C:60L and 100C:0L systems were stable against creaming independent of NaCl concentration, but 0C:100L system showed phase separation after 24 hours (Fig. 4.10). A pronounced creaming occurred for all NaCl concentration, but phase separation was more evident at intermediate concentrations (100 to 200 mM NaCl).
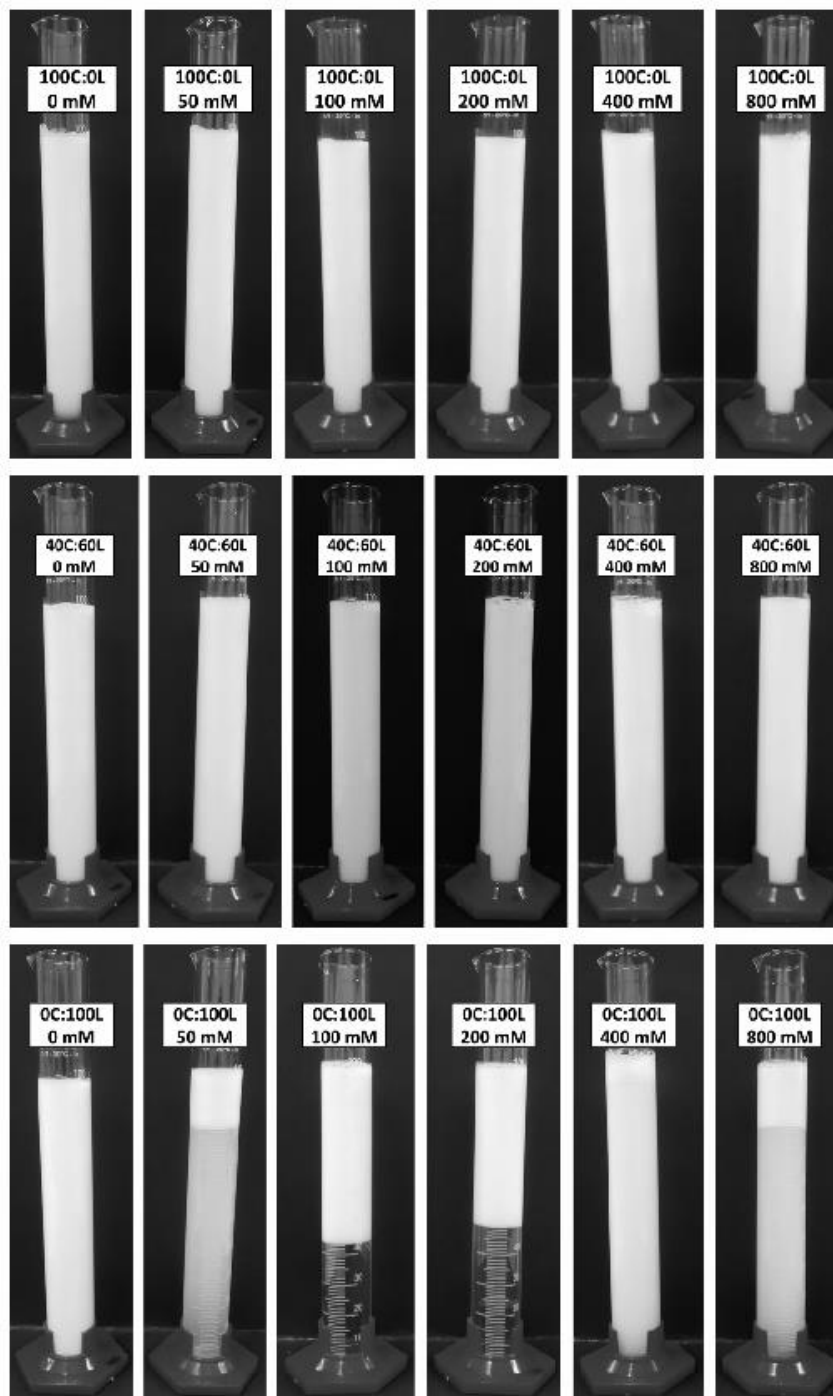
Figure 4.10 - Visual aspect of the heteroaggregates under different concentration of NaCl after 1 day of storage at 25 ºC.

The influence of NaCl concentration on particles size distribution and mean droplet size depended on the volume ratio of the heteroaggregates and proteins composition (Fig. 4.11). The 100C:0L control presented almost no difference on particle size distribution with NaCl addition, but a broad peak was observed at higher ionic strength (400 to 800 mM NaCl) and

mean size values ($D_{4,3}$) were slightly higher (span values increased from 1.10 to 1.48 for the highest salt content). The 0C:100L control tended to exhibit a higher mode and mean size value with salt addition, probably due to intense droplets aggregation. Interestingly, the system 40C:60L was stable to salt addition but exhibited an increase in mean particle diameter. These results suggest that some partial destabilization and coalescence of lactoferrin stabilized droplets occurred due to its susceptibility to NaCl addition as observed in the control 0C:100L.
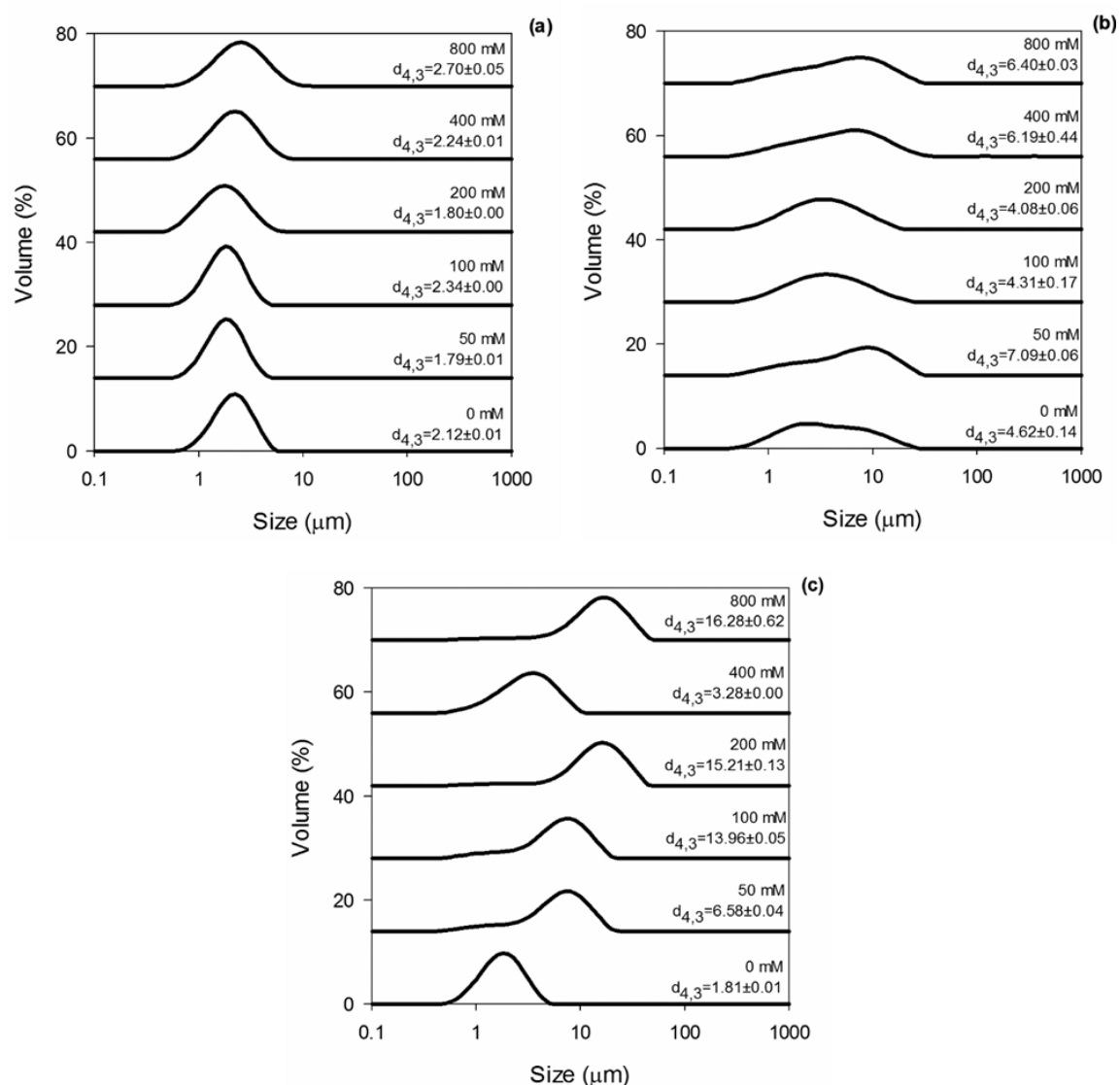
Figure 4.11 - Particles size distribution and mean size of the heteroaggregates 100C:0L (a), 40C:60L (b) and 0C:100L (c) under different concentration of sodium chloride after 1 day of storage at 25 ºC.

Zeta potential of the 0C:100L system decreased as the NaCl concentration increased (Fig. 4.12), which could be attributed to the high amount of Cl^- ions closed to the droplet surfaces promoting a change of electrostatic interactions and also a salting out effect. As ionic strength is increased, the electrostatic repulsion between droplets decreases because counter-ions in the aqueous phase shield the charges on droplet surfaces (Demetriades, Coupland, & McClements, 1997; Mao & McClements, 2012a; Srinivasan et al., 2000) increasing the protein solubility which is attributed to a salting-in effect. But when the ion concentration increases reaching a critical level, the protein-protein interactions prevail, which is named salting-out effect. However, for 100C:0L system the magnitude of zeta potential values only slightly increased with NaCl addition and remained constant with salt concentration higher than 200 mM. Since the zeta potential of the 40C:60L system remained close to zero across the entire range of NaCl concentration used.
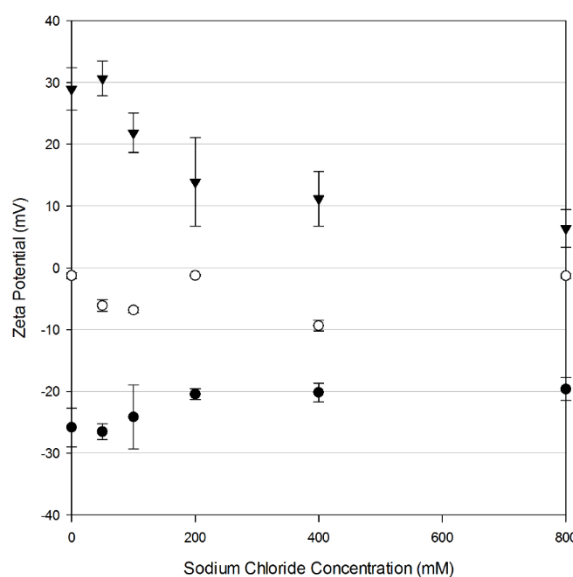
Figure 4.12 - Zeta potential values of the heteroaggregates (●) 100C:0L, (○) 40C:60L and (▼) 0C:100L under different concentration of sodium chloride after 1 day of storage at 25ºC.

Mao and McClements (2012a) suggest that cationic patches of the lactoferrin stabilized droplets could be highly bounded to anionic counter-ions (e.g., Cl^-). Thus, these emulsions could be highly unstable to gravitational separation even at low salt concentrations. Susceptibility of lactoferrin to salt addition was also observed for other authors and could be attributed to the ions ability to screen the electrostatic repulsion between droplets leading to the salting-out effect, which causes collapse of the protein structures around the droplets. As a

consequence, stabilizing effects of steric repulsion between the droplets decrease (Acero-Lopez et al., 2010; Lesmes et al., 2010). Furthermore, salt interaction with aqueous medium could inhibit the steric stabilization action of lactoferrin sugar groups. On the other hand, addition of NaCl to sodium caseinate stabilized emulsions seems to enhance creaming stability. Other authors also reported improved emulsifying properties of casein with the addition of salt (Mohanty, Mulvihill, & Fox, 1988; Pearce & Kinsella, 1978). According to Srinivasan et al. (2000), addition of NaCl to sodium caseinate solution may cause α_s-casein to form dimers, tetramers, hexamers etc., which may subsequently become adsorbed, resulting in a high concentration of this casein fraction at the interface. Moreover α_s-casein can act as a molecular chaperone avoiding protein aggregation which could kept a intact structure independent of salt addition (Morgan, Treweek, Lindner, Price, & Carver, 2005).

4.4 CONCLUSIONS

The formation of heteroaggregates by mixing two emulsions stabilized by proteins with opposite charges, varying the emulsion volume ratio and ionic strength allowed to produce systems with distinct properties. Biggest heteroaggregates with good stability were formed with 60 vol. % and 40 vol. % of lactoferrin and sodium caseinate emulsions, respectively. Mixed emulsions with higher concentration of lactoferrin produced strong aggregation that resulted in phase separation and gel formation besides increasing viscosity and elastic moduli, thus producing heteroaggregated systems with much more structured rheological behavior using the same oil volume fraction than that used in a single emulsion. The lactoferrin stabilized emulsion was susceptible to NaCl addition showing phase separation and changes in zeta potential and size values, while sodium caseinate stabilized emulsion and the heteroaggregate (40C:60L) were stable to salt addition. The 40C:60L heteroaggregate showed an adequate combination of sodium caseinate and lactoferrin coated droplets improving rheological properties, but the presence of sodium caseinate provided the stabilization to salt addition while lactoferrin was necessary to provide the steric stabilization. Thus, valuable information about heteroaggregate formation and characteristics were provided showing that the choice of protein concentration and properties are fundamental to obtain heteroaggregates to be used as functional agents for texture/viscosity modification and controlled release.

4.5 ACKNOWLEDGEMENTS

4.6 REFERENCES

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CAPÍTULO 5 - DIGESTIBILIDADE DOS HETEROAGREGADOS

IN VITRO DIGESTIBILITY OF HETEROAGGREGATED DROPLETS COATED WITH SODIUM CASEINATE AND LACTOFERRIN

IN VITRO DIGESTIBILITY OF HETEROAGGREGATED DROPLETS COATED WITH SODIUM CASEINATE AND LACTOFERRIN

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Highlights

In vitro digestibility of heteroaggregated lipid droplets was evaluated.

After digestion proteins were hydrolyzed forming small peptides.

Heteroaggregated droplets presented the lowest extent of lipid digestion.

ABSTRACT

Aggregation of droplets coated with oppositely charged proteins could be affected during emulsion digestion. Thus, emulsions stabilized by sodium caseinate, lactoferrin or heteroaggregated droplets formed from the mixture of both emulsions were evaluated by in vitro digestibility. Emulsions properties were analyzed in terms of stability, microstructure, particle size, surface charge and free fatty acids. Changes in physical properties at the different digestion steps depended on the emulsifier properties and were probably attributed to physico-chemical environment conditions and protein hydrolysis. The heteroaggregates undid in simple emulsions at the gastric phase due to the electrostatic repulsion between the proteins at low pH. Free fatty acids release depended on emulsifier properties, in addition to the presence of bile salts. Heteroaggregates presented the lowest extent of lipid digestion followed by droplets coated with lactoferrin. These results may be useful for the design of food matrices with a decreased digestibility.

Keywords: emulsions, heteroaggregation, lipid digestion.

5.1 INTRODUCTION

Emulsions with different structures, physicochemical properties, and functional attributes can be prepared by controlling the characteristics of the colloidal particles (such as size, surface charge, concentration), environmental conditions (such as pH, ionic strength, temperature) and the method of preparation (such as the order of ingredients addition and mixing conditions) (Mao and McClements, 2011). Recent studies have reported that controlled heteroaggregation of lipid droplets can be also used to manipulate the characteristics of emulsion-based products (Furtado et al., 2016; Mao and McClements, 2011, 2012b, c, d). Heteroaggregated emulsions are formed by mixing two simple emulsions with lipid droplets coated by electrically charged emulsifier molecules, as proteins (Mao and McClements, 2011, 2012c). This technique allows the creation of products with reduced fat content but substantial amounts of protein, inducing a feeling of satiety (Westerterp-Plantenga et al., 2009).

Generally, researches are focused on the influence of emulsifiers on the stability of emulsions prior to consumption, but more recently there has been increasing interest in the fate of emulsions after ingestion (Hur et al., 2009; Malaki Nik et al., 2011; McClements and Li, 2010a, b; Mun et al., 2007; Zhang et al., 2015a). Understanding the role of emulsion composition and structure on the gastrointestinal fate is a big challenge, but this knowledge is useful for the design of foods and beverages with improved nutritional quality (Zhang et al., 2015a). In addition, the interfacial composition of the droplets can become quite complex, depending on the concentration and surface activities of the gastric and intestinal components during digestion. Therefore, model systems are used to understand such complex conditions because the digestion behavior and the effect of interactions between individual physiological components of an emulsion can be investigated separately (Li et al., 2012). Consequently, emulsions can be designed to increase the bioavailability of encapsulated substances, to control satiety, or to deliver components to specific regions of the gastrointestinal tract (Marze, 2015; McClements et al., 2008).

Previous researches evaluated the stability and digestion of sodium caseinate stabilized emulsions (Li et al., 2012; Mun et al., 2007; Zhang et al., 2015a), lactoferrin stabilized emulsions (Lesmes et al., 2010; Sarkar et al., 2009a; Zhang et al., 2015a) and heteroaggregates (Mao and McClements, 2012c; Simo et al., 2012). However, to our knowledge there are no previous studies about the behavior of heteroaggregated droplets coated with sodium caseinate

(a cheaper protein with random coil structure) and lactoferrin (a globular protein) under gastric and intestinal conditions using a simulated gastrointestinal tract system.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Ultrapure water from a Millipore Milli-Q system (resistivity 18.2 MΩ/cm) was used. Sodium caseinate (protein content 87 wt. %) and lactoferrin (protein content 92 wt. %) were kindly provided by Allibra Ingredientes Ltd (Campinas, Brazil) and Synlait Milk Ltd (Canterbury, New Zeland), respectively. Sunflower oil (Bunge Alimentos S.A., Gaspar, Brazil) was purchased in the local market. Bile extract porcine (B8631), pancreatin from porcine (P7545) and pepsin from porcine gastric mucosa (P6887) were purchased from Sigma-Aldrich (St. Louis, USA). The other reagents used in this study were of analytical grade.

5.2.2 Methods

5.2.2.1 Protein dispersions preparation

Sodium caseinate and lactoferrin were dispersed separately in ultrapure water (0.30 wt. %) using magnetic stirring at room temperature overnight, ensuring complete dissolution of the protein. The pH of protein dispersions was adjusted to pH 7.0 using 1 M NaOH or 1 M HCl.

5.2.2.2 Oil in water emulsions preparation

Coarse emulsions were prepared by homogenizing 95 mL of protein dispersions (0.30 wt. %, pH 7.0) and 5 mL of sunflower oil using a rotor-stator homogenizer (SilentCrusher M, Heidolph, Schwabach, Germany) at 5,000 rpm for 3 minutes, giving a final protein concentration of 0.29 wt. %. Fine emulsions were prepared by subjecting the coarse emulsions in an ultrasonic processor (QR 750W, Ultronique, Campinas, Brazil) with a 13 mm diameter titanium probe immersed to 3 mm depth, which was used in combination with a magnetic stirrer to enhance mixture homogenization. Sonication time, power and frequency were fixed at 6 minutes, 300 W and 20 kHz, respectively, since these conditions resulted in emulsions with

good stability against creaming (Furtado et al., 2016). The temperature of preparation did not exceed 30 °C during the homogenization process.

5.2.2.3 Heteroaggregates preparation

The heteroaggregates were prepared by mixing 40 vol. % of sodium caseinate stabilized emulsion and 60 vol. % of lactoferrin stabilized emulsion under magnetic stirring at 250 rpm for 10 min (HEI-TEC, Heidolph, Schwabach, Germany). Emulsions volume fraction (%) was based on previous results (Furtado et al., 2016), since the heteroaggregates obtained using this condition showed a relatively big size ($\approx$ 4 µm), compared to single emulsions ($\approx$ 1.6 µm), but without presenting phase separation. For convenience, we used the notation 40C:60L to refer to heteroaggregates containing 40 vol. % of sodium caseinate stabilized emulsion and 60 vol. % of lactoferrin stabilized emulsion. A similar notation was used in other formulations (0C:100L or 100C:0L). Simple emulsions stabilized by sodium caseinate or lactoferrin stirred at 250 rpm for 10 min were used as control samples. Heteroaggregates and simple emulsions were evaluated after one day of storage.

5.2.2.4 *In vitro* digestion of emulsions and fatty acid release

Emulsions/heteroaggregates were digested by subjecting them to sequential incubation in simulated gastric fluid (SGF) and then simulated intestinal fluid (SIF) using the slight modified *in vitro* digestion protocol of Minekus et al. (2014), where according to the authors the mouth step can be eliminated for liquid samples. The samples were placed in a stirred (100 rpm) double jacketed reaction vessel maintained at 37±1°C (Mun et al., 2016). 60 mL of each sample was incubated for 2 hours with 60 mL of simulated gastric fluid (SGF) at pH 3 (SGF contained 6.9 mmol L^{-1} of KCl, 0.9 mmol L^{-1} KH_2PO_4, 25.0 mmol L^{-1} $NaHCO_3$, 47.2 mmol L^{-1} NaCl, 0.1 mmol L^{-1} $MgCl_2(H_2O)_6$, 0.5 mmol L^{-1} $(NH_4)_2CO_3$, 0.15 mmol L^{-1} $CaCl_2(H_2O)_2$ and 9.6 mL of fresh pepsin dispersion (25,000 U mL^{-1}). After 2 hours of incubation in SGF, 20 mL of sample was collected for immediate characterization (section 5.2.2.5). Then sample+SGF was mixed (1:1) with SIF. The temperature was adjusted to 37±1°C and pH was adjusted to 7 with 1M NaOH. The SIF contained 6.8 mmol L^{-1} KCl, 0.8 mmol L^{-1} KH_2PO_4, 85.0 mmol L^{-1} $NaHCO_3$, 38.42 mmol L^{-1} NaCl, 0.33 mmol L^{-1} $MgCl_2(H_2O)_6$, 0.6

mmol L^{-1} $CaCl_2(H_2O)_2$, 70.72 g L^{-1} of bile salts and 25 mL of fresh pancreatin dispersion (800 U mL^{-1} based on trypsin activity).

During intestinal digestion, the pH was maintained at 7.0 by the addition of 1 M NaOH, through a burette, under continuous magnetic stirring (100 rpm) using a pH meter (Metrohm 827, Metrohm, Herisau, Switzerland). The measurements were taken every 15 minutes. The volume of NaOH added to the samples was used to calculate the concentration of free fatty acids (FFA) released in the reaction vessel. FFAs released were calculated using Eq. 1, taking into account the number of moles of NaOH required to neutralize the FFA that could be produced from the triacylglycerols if they were completely digested (assuming the generation of 2 FFAs per triacylglycerol molecule by the action of lipase) (Li and McClements, 2010).

$$\% \, FFA = 100x \frac{V_{NaOH} x M_{NaOH} x MW_{lipid}}{2x W_{lipid}} \tag{5.1}$$

where V_{NaOH} is the volume of NaOH, M_{NaOH} is the molarity of NaOH, MW_{lipid} is the average molecular weight of sunflower oil and W_{lipid} is the weight of lipid initially present in the reaction vessel.

5.2.2.5 Emulsions/heteroaggregates characterization

5.2.2.5.1 Particle size

Particle size distribution of the emulsions/heteroaggregates was determined based on the static light scattering method using a Multi-Angle Static Light-Scattering Mastersizer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The mean diameter was expressed as the volume mean diameter ($D_{4,3}$) (Eq. 2).

$$D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \tag{5.2}$$

where n_i is the number of droplets with diameter D_i.

Mean particle size of the heteroaggregates should only be treated as an indicator of their dimensions. The theory used to interpret light scattering data assumes that the scattering particles are homogeneous spheres with well-defined refractive indices, although flocculated systems are non-spherical and non-homogeneous particles. Furthermore, stirring and dilution used for this measurement may alter the dimensions and structural organization of the heteroaggregates (Mao and McClements, 2012a).

5.2.2.5.2 Microstructure

Emulsions/heteroaggregates were poured onto microscope slides, covered with glass cover slips and observed using a Carl Zeiss Axio Scope A1 microscope (Zeiss, Oberkochen, Germany) with x100 objective lenses. At least six pictures of each slide were taken, with three slides per sample. The AxioVision Rel. 4.8 (Zeiss, Oberkochen, Germany) imaging software was used.

5.2.2.5.3 Zeta potential

Zeta potential of the emulsions/heteroaggregates was determined using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Samples were diluted in MilliQ water (0.001 vol. %) and then equilibrated for 120 s into the instrument before particle charge data was collected over 10 continuous readings. In addition, the zeta potential of the aqueous protein dispersions under different pH values was also determined.

5.2.2.5.4 Polyacrylamide gel electrophoresis

Molecular weight distribution of the proteins used to stabilize emulsions/heteroaggregates was evaluated by Tricine-SDS–PAGE under reducing conditions, according to Schägger and von Jagow (1987). The 1.5 mm thickness gels consisted of a resolving gel (16.5 %T, 3 %C), spacing gel (10 %T, 3 %C) and stacking gel (4 %T, 3 %C). Emulsions/heteroaggregates were diluted in deionized water to 0.75 mg protein/mL, mixed with sample buffer (62.5 mM Tris–HCl pH 6.8, 20% glycerol, 2% SDS, 0.1% Coomassie Blue G250 and 5% β-mercaptoethanol, pH 6.8) (1:1) and heated at 40 °C for 30 min. Aliquots of 20 μL were loaded and the electrophoresis was performed at 80 V in a vertical slab Mini-Protean

electrophoresis system (Bio-Rad Laboratories, Hercules, USA). The gels were then stained with 0.25 wt. % Coomassie Brilliant Blue in ethanol:acetic acid:water (45:10:45 vol. %), and diffusion-destained by repeated washing in an ethanol:acetic acid:water solution (10:5:85 vol. %). BenchMark™ Pre-stained Protein Ladder (Carlsbad, Canada) was used as molecular weight marker (from 6 to 180 kDa).

5.2.3 Statistical analysis

All experiments were performed in triplicate, with at least three measurements being made per sample. The results were reported as the average and the standard deviation of these measurements.

5.3 RESULTS AND DISCUSSION

5.3.1 Emulsions/heteroaggregates characterization in SGF

Physical stability of the emulsions/heteroaggregates was evaluated after their passage through the *in vitro* digestion steps. The mean particle size, particle size distribution, microstructure, and macroscopic appearance of the emulsions were determined at each stage of the *in vitro* digestion (Figs. 5.1 to 5.4).

Mean particle diameter ($D_{4,3}$) was relatively small for both emulsions stabilized by sodium caseinate or lactoferrin, showing values near 2 μm, but it was considerably higher for the 40C:60L heteroaggregates ($\approx$ 4 μm) (Fig. 5.1). They also showed a broader particle size distribution while the sodium caseinate and lactoferrin stabilized emulsions showed narrower particle size distribution (Fig. 5.2). These results corroborate with microscopy images (Fig. 5.3), since oil droplets from the fine emulsions stabilized by the single proteins were uniformly distributed throughout the emulsions. However, an extensive aggregation of droplets coated by sodium caseinate or lactoferrin was observed for the 40C:60L heteroaggregates, since the main driving force is the electrostatic interaction between droplets coated with oppositely charged proteins (Furtado et al., 2016).

Initially, all the samples presented stability against creaming and phase separation (Fig. 5.4), however, after exposure to gastric conditions, particle size distribution became slightly broader for simple emulsions and slightly thinner for heteroaggregates (Fig. 5.2). For

sodium caseinate stabilized emulsions there was evidence of flocculation (Fig. 5.3) and creaming (Fig. 5.4), while these properties did not show clear changes for lactoferrin stabilized emulsions. Emulsion stability against creaming depends on the strength of interaction between droplets, which is determined by the electrostatic interaction and steric hindrance co-existing on their interface. In emulsions stabilized by sodium caseinate, the droplets are negatively charged when the pH is above the isoelectric point (pI), while for pH < pI they are positively charged (Perrechil and Cunha, 2010). If the pH is adjusted to values close to the pI, the repulsive forces may no longer be strong enough to prevent the droplets from aggregating leading to phase separation (McClements, 2004). Previous studies also reported that the most part of protein-stabilized emulsions is prone to aggregation under gastric conditions due to hydrolysis of adsorbed proteins (Singh et al., 2009; Zhang et al., 2015a, b). Pepsin addition could promote some kind of surface hydrolysis diminishing the protective effect of protein, and possibly predisposing the droplets towards flocculation (Sarkar et al., 2009b). Furthermore, protein surface adsorption can affect pepsin hydrolysis depending fundamentally on the specific conformation of the protein (del Castillo-Santaella et al., 2014; Macierzanka et al., 2009; Maldonado-Valderrama et al., 2013; Maldonado-Valderrama et al., 2012). Besides that, caseins in their native state are very susceptible to proteolysis by pepsin due to their flexible random coil structure (Li et al., 2012). Maldonado-Valderrama et al. (2013) also reported that hydrolysis of adsorbed β-caseins by pepsin increases the elasticity interfacial network, which is indicative of the presence of irreversibly adsorbed peptides. Lactoferrin is also unable to resist to pepsin proteolysis (Onishi, 2011; Wang et al., 2017), besides that, the pH range of the lactoferrin stabilized emulsions remained below the pI favoring electrostatic repulsion, contributing to the good stability of the emulsions. Furthermore, lactoferrin is a glycoprotein that shows some sugar groups covalently attached to its peptide backbone contributing to a better steric repulsion effect (Oliver et al., 2006). The 40C:60L heteroaggregates undid after gastric conditions (Fig. 5.2 and 5.3) probably because both proteins present positive zeta potential value at pH 3.0 (Fig. 5.5) and electrostatic repulsion between them occurred, undoing the heteroaggregates in individual droplets.
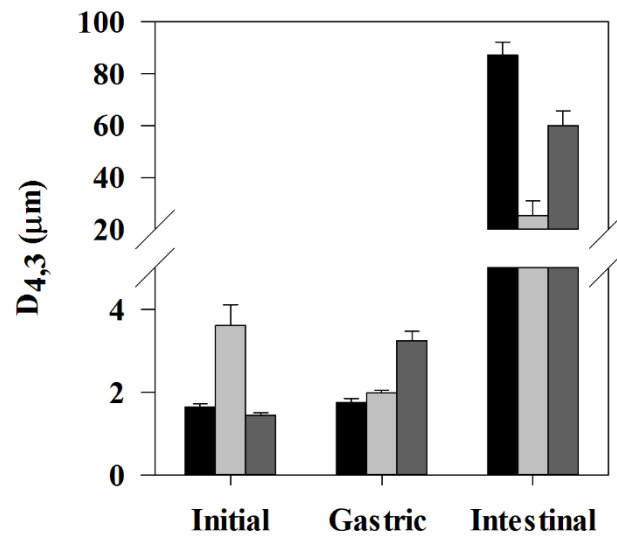
Figure 5.1 – Droplet size ($D_{4,3}$) of the emulsions/heteroaggregates 0C:100L (black), 40C:60L (light gray) and 100C:0L (dark gray) before (initial) and after gastric and intestinal phases.
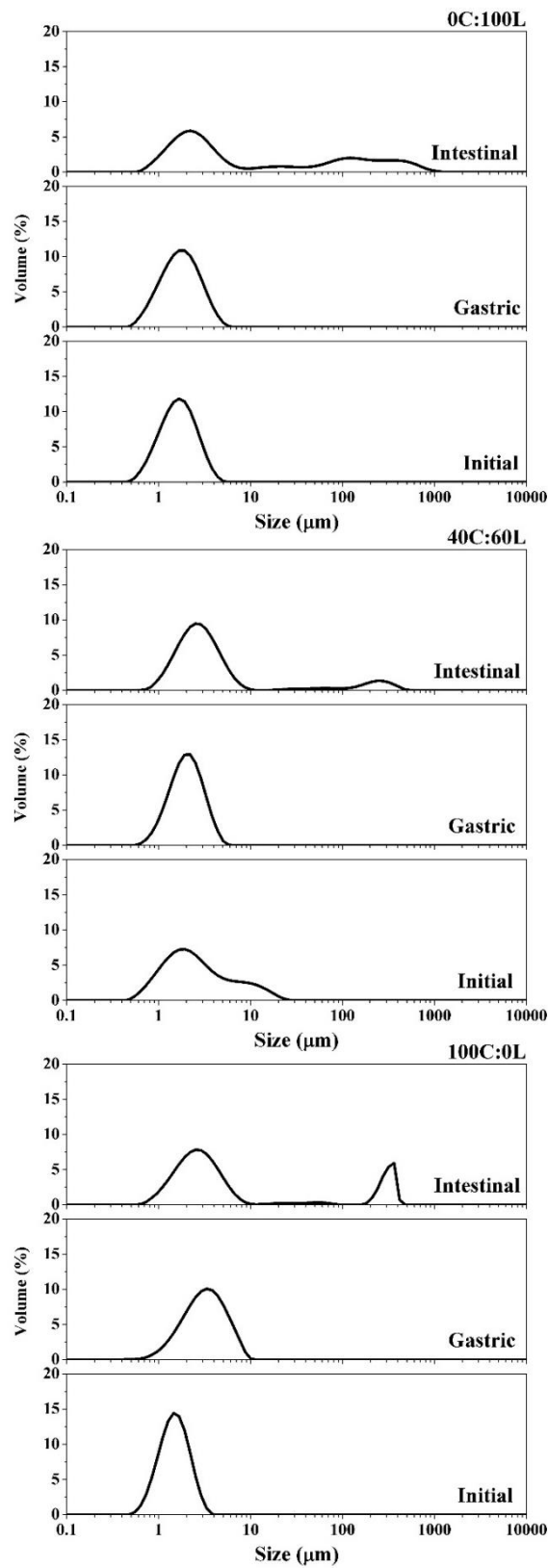
Figure 5.2 – Volume size distribution of the emulsions/heteroaggregates before (initial) and after gastric and intestinal phases.
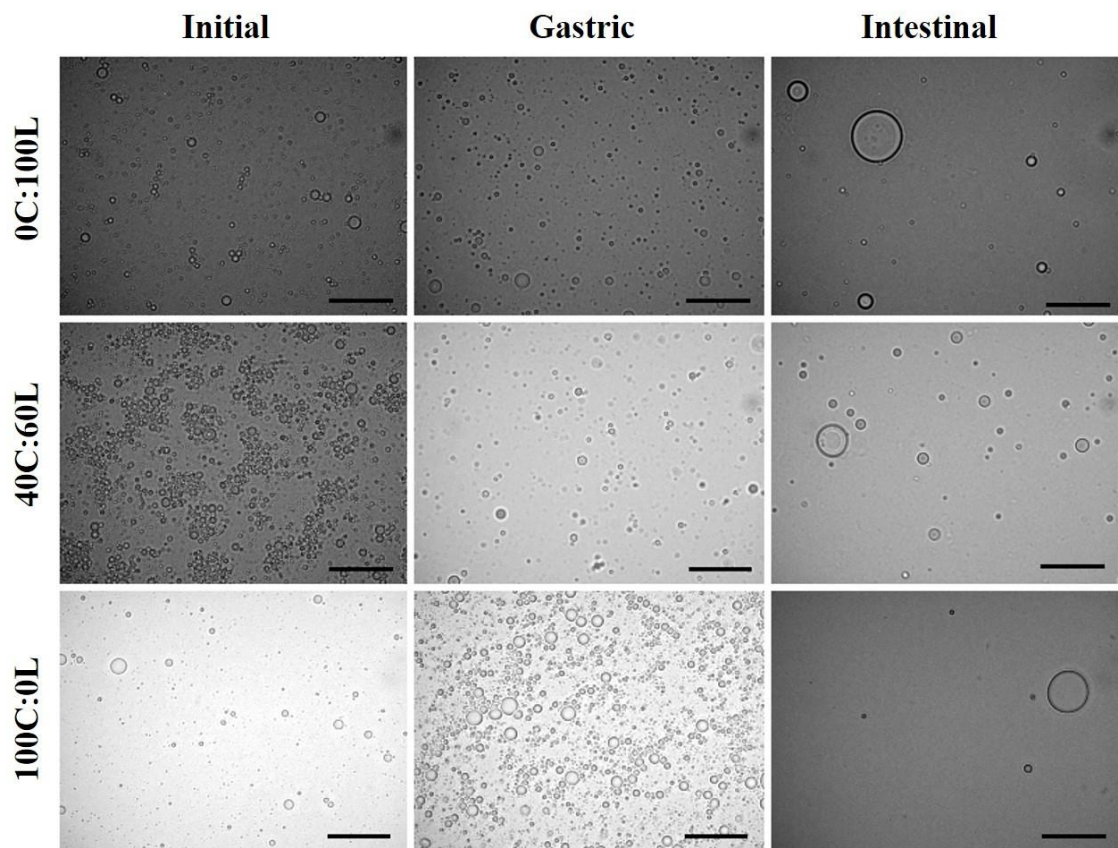
Figure 5.3 – Microscopy of the emulsions/heteroaggregates before (initial) and after gastric and intestinal phases. Scale bar: 20 μm.
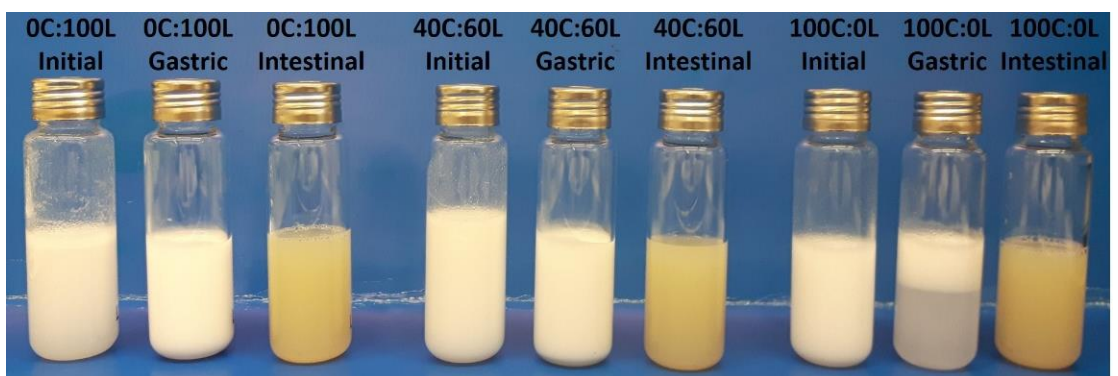
Figure 5.4 – Visual aspect of the emulsions/heteroaggregates before (initial) and after gastric and intestinal phases.
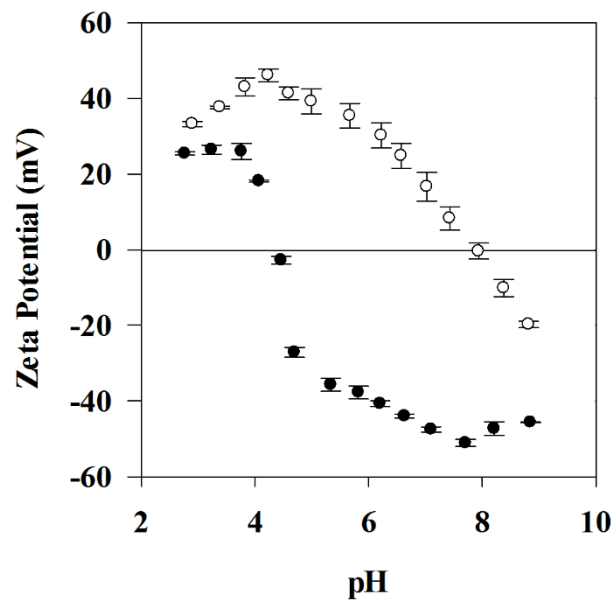
Figure 5.5 – Zeta potential values of sodium caseinate (full symbols) and lactoferrin dispersions (empty symbols) under different pH values.

Initially, sodium caseinate stabilized droplets presented highly negative zeta potential values, while the 40C:60L heteroaggregates remained close to zero and lactoferrin stabilized droplets showed a positive value of zeta potential. There was an increase in the magnitude of positive charge for all the samples after passing through the gastric phase (Fig. 5.6), since proteins are quite below their isoelectric point at this pH (3.0) (Fig. 5.5).
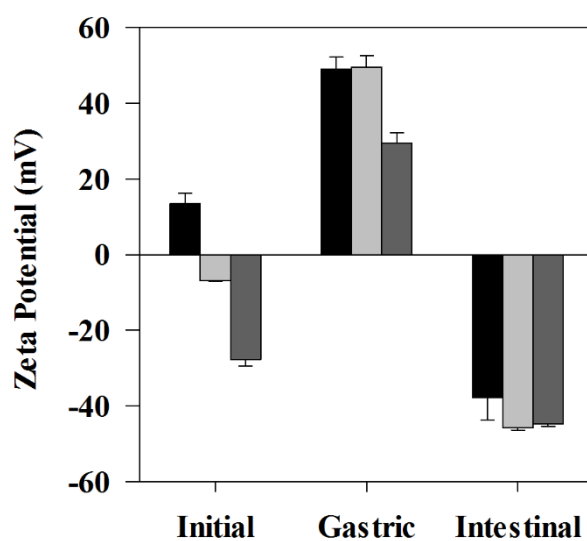
Figure 5.6 – Zeta potential values of emulsions/heteroaggregates (0C:100L (black), 40C:60L (light gray) and 100C:0L (dark gray)) before (initial) and after gastric and intestinal phases.

Tricine-SDS-PAGE was used to determine the hydrolysis of proteins adsorbed onto the interface of the droplets during gastric and intestinal digestion (Fig. 5.7). Electrophoretic profiles of initial sodium caseinate stabilized emulsion showed a band between 20-30 kDa that refers to the major fractions of sodium caseinate (β- and α_s-casein) (O'Regan and Mulvihill, 2009) and bands above 64 kDa that could refer to aggregates. The lactoferrin stabilized emulsion electrophoretic profile showed a band near 82 kDa and higher than 180 kDa that refers to the lactoferrin (Spik et al., 1994) and protein aggregates, respectively (Fig. 5.7).

A band near 15 kDa appeared and possibly refers to α-lactalbumin (14.2 kDa) (Jambrak et al., 2014). Initial electrophoretic profiles of the 40C:60L heteroaggregates (lane 10) showed bands of caseins and lactoferrin. After gastric phase, both adsorbed proteins were hydrolyzed to produce small peptides, and the band near 37 kDa in gastric condition observed for all samples probably refers to pepsin (Macierzanka et al., 2009). Smearing bands (related to the peptides around 6 kDa) were also observed due to the high salt concentration in simulated intestinal fluid. The band near 49 kDa observed for all samples after the intestinal phase probably refers to lipase (Iizuka et al., 1991). Lactoferrin is highly susceptible to gastric proteolysis and can generate bioactive peptides in the human stomach (Kuwata et al., 1998; Troost et al., 2001). No intact casein or lactoferrin remained after gastric digestion. Therefore, we can assume that the surfaces of the droplets after the gastric phase were covered by small peptides.
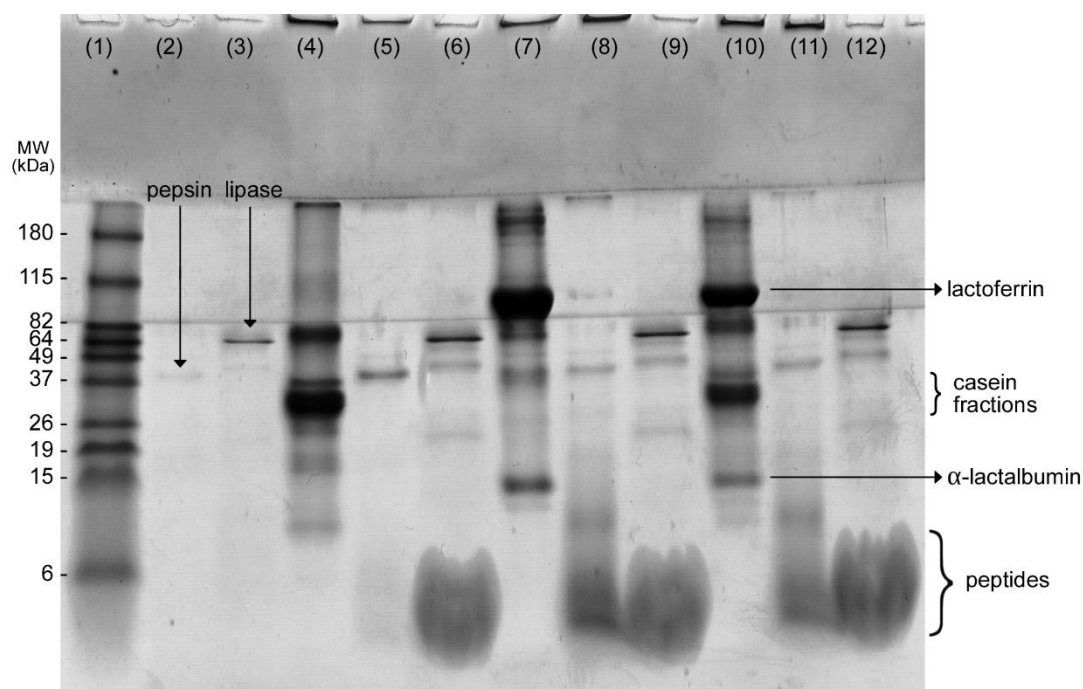
Figure 5.7 – Tricine-SDS-PAGE electrophoretic profiles of the emulsions/heteroaggregates: (1) molecular weight standard (MW), (2) gastric digestion control, (3) intestinal digestion control, (4) 100C:0L initial, (5) 100C:0L after gastric phase, (6) 100C:0L after intestinal phase, (7) 0C:100L initial, (8) 0C:100L after gastric phase, (9) 0C:100L after intestinal phase, (10) 40C:60L initial, (11) 40C:60L after gastric phase and (12) 40C:60L after intestinal phase.

5.3.2 Emulsions/heteroaggregates characterization in SIF

After passing through the intestinal condition, the particle size distributions of all emulsions became multimodal (Fig. 5.2), with a corresponding increase in mean particle diameter ($D_{4,3}$) (Fig. 1) also observed in microscopy images (Fig. 5.3). Most of the big particles could be undigested lipid droplets, since the coalescence occurring after intestinal phase could be attributed to protein digestion (Qian et al., 2012) as also observed in the electrophoretic profile (Fig. 5.7). However, micelles, vesicles, and insoluble calcium salts could be also present (Sarkar et al., 2009b), leading to the multimodal particle size distribution.

Samples showed no phase separation (Fig. 5.4) and highly negative zeta potential values (Fig. 5.6) after incubation in the intestinal phase, which could be attributed to the presence of different anionic particles arising from the intestinal fluids (as bile salts) or from

the lipid digestion products, such as free fatty acids (Sarkar et al., 2010; Zhang et al., 2015a). Moreover, the intense yellow color from the bile salts made difficult the observation of any indicative of separation.

5.3.3 In vitro lipid digestion in SIF

The extent of lipid digestion of emulsion samples that had been previously digested in SGF was quantified by determining the amount of free fatty acids released under SIF conditions (Fig. 5.8). There were clear differences in the extent of lipid digestion among the control emulsions stabilized by sodium caseinate or lactoferrin and the 40C:60L heteroaggregates. However, for all of them, there was a rapid increase in the FFA release during the first 15 min of digestion, followed by a more gradual increase at longer times, reaching a constant value. This same behavior was observed in the digestibility of heteroaggregates produced with β-lactoglobulin and pectin (Simo et al., 2012) and could be attributed to protein hydrolysis (Mao and McClements, 2012c), since pancreatin and bile extracts have protease activity (Singh and Sarkar, 2011). Sodium caseinate stabilized emulsion showed the highest final extent of lipid digestion, which could be related to the action of bile salts acting to displace the original emulsifiers from the interface facilitating the lipase adsorption/activity (Golding et al., 2011; Pilosof, 2017; Wilde and Chu, 2011). On the other hand, lactoferrin stabilized emulsion and the 40C:60L heteroaggregates presented a lower final extent of FFA, which is probably related to the interaction of lactoferrin peptides with bile salts. Small amounts of anionic bile salts can bind to the lactoferrin interfacial layer, shifting cationic lactoferrin molecules from the continuous phase to the emulsion interface, which could change the ability of lipase to interact with the lipids (Sarkar et al., 2010; Zhang et al., 2015b). Somehow, the 40C: 60L heteroaggregates presented the lowest final extent of FFA, possibly due to an interaction between the two proteins or their peptides forming a thicker barrier in the residual droplets interface and delaying lipid digestion. Furthermore, droplet size also importantly affected lipid digestion (Fig. 5.1), as larger droplets protect from lipolysis due to their low interfacial area available for the adsorption of lipase (Giang et al., 2015; Torcello-Gomez et al., 2011). However, the initial microstructure of the emulsions had lower influence on their subsequent lipolysis than the protein conformation type. Additionally, lipids may not have been fully digested because of the inhibition of lipase by the free fatty acids present at the droplet surface (Pafumi et al., 2002). Due to their surface activity, fatty acids compete for the surface

of lipid droplets displacing lipase molecules from the oil–water interface. Alternatively, a liquid crystalline phase around the lipid droplets could be formed, which prevents the lipase from accessing the undigested lipid inside (Patton and Carey, 1979).
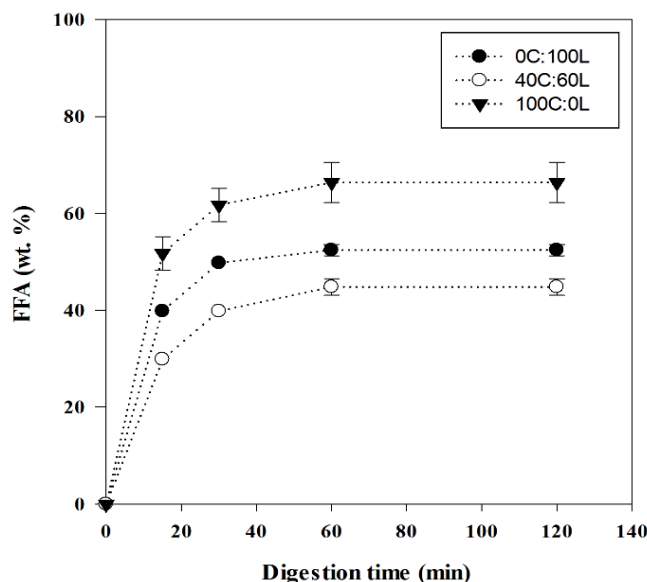
Figure 5.8 - FFAs released under simulated intestinal conditions as a function of time.

5.4 CONCLUSIONS

Lipid digestion depended on the emulsifier type used to coat droplets since zeta potential changes and other interactions occurring in the different stages of simulated gastrointestinal tract affected the aggregation of lipid droplets. These changes can be attributed to physico-chemical conditions of the environment and hydrolysis of the interfacial proteins due to pepsin action under gastric conditions. The heteroaggregates undid in simple emulsions at the gastric phase due to the electrostatic repulsion between the sodium caseinate and lactoferrin in gastric pH. The extent of lipid digestion within the intestinal phase was lower in the presence of lactoferrin, which could be attributed to the interaction of peptides of this protein with bile salts. However, the heteroaggregates presented the lowest extent of lipid digestion, possibly due to an interaction between the peptides of both proteins suppressing lipid digestion. These results are relevant for the design of food matrices with improved functional properties such as decreased digestibility and/or controlled energy intake.

5.5 ACKNOWLEDGEMENTS

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CAPÍTULO 6 - EFEITO DO AQUECIMENTO ÔHMICO NAS PROPRIEDADES DA LACTOFERRINA E NA PRODUÇÃO DE EMULSÕES *GEL-LIKE*

COLD GEL LIKE EMULSIONS OF LACTOFERRIN SUBJECTED TO OHMIC HEATING

Os resultados desse capítulo foram submetidos ao periódico

"*Food Research International*"

COLD GEL LIKE EMULSIONS OF LACTOFERRIN SUBJECTED TO OHMIC HEATING

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Highlights

Ohmic or conventional heating were used to promote structural changes in lactoferrin.

Cold gel like emulsions were produced with heat treated lactoferrin.

Emulsion properties were intrinsically related to the heat treatment of the protein.

Graphical Abstract
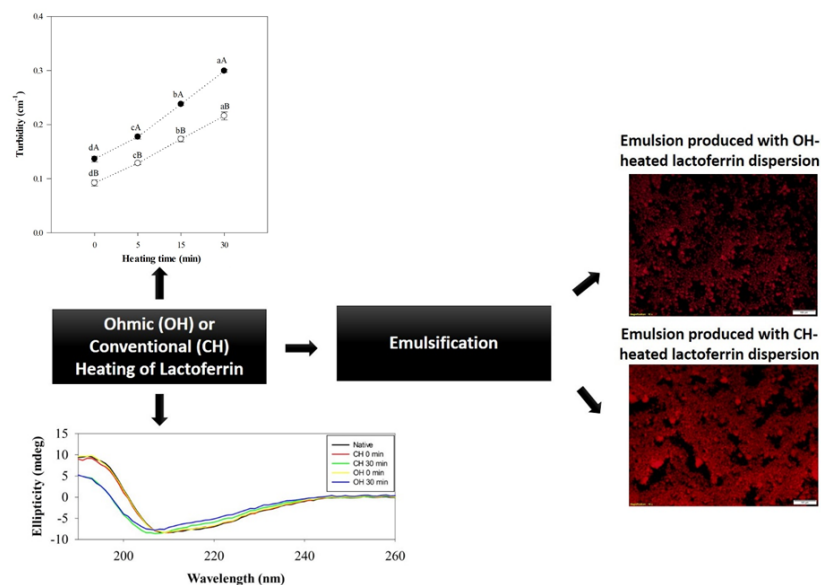
ABSTRACT

Ohmic heating is a technique that has gained increasing attention because of its capacity to produce uniform heating, and claimed electrical influence on the functional and technological properties of treated protein dispersions. The aim of this work was to evaluate the influence of ohmic heating on the properties of cold gel-like emulsions, comparing them with those obtained by conventional heating. The effect of ohmic and conventional heating on physical and structural properties of lactoferrin was also addressed. Ohmic heating treatment resulted in less pronounced aggregation of lactoferrin, when compared to conventional heating. An increase of particle size, turbidity, intrinsic and extrinsic fluorescence values and a decrease of dichroic signal after heat treatment indicated an increase of protein interactions. Emulsions produced from heat-treated lactoferrin showed gel-like behavior which was related to the emulsifying capacity of lactoferrin, combined with the emulsification method and the heat pre-treatment applied to the protein. Rheological and microstructural properties were intrinsically related to the heat treatment of the protein since ohmic heating produced gel-like emulsions with a less rigid structure. These emulsions could be interesting for food applications containing heat-sensitive ingredients.

Keywords: gel-like emulsions, lactoferrin, ohmic heating, rheological properties

6.1 INTRODUCTION

Emulsions tend to be destabilized by several mechanisms like aggregation, phase inversion, flocculation and coalescence, thus being considered as thermodynamically unstable systems (Dickinson, 1997), but a kinetic stability for a considerable time can be reached with the addition of emulsifiers (McClements, Decker, & Weiss, 2007). Milk proteins are widely used to form and stabilize oil-in-water emulsions, and this functionality is related to their capacity to be adsorbed onto the water-oil interface (Dalgleish, 1997; Dickinson, 1997). Recently, works have shown the potential of utilization of lactoferrin as an emulsifier (Furtado, Mantovani, Consoli, Hubinger, & Cunha, 2017; Pinheiro, Coimbra, & Vicente, 2016; Sarkar, Goh, & Singh, 2009; Sarkar, Horne, & Singh, 2010; Tokle & McClements, 2011). This protein presents a globular structure with a single polypeptide chain of about 80 kDa (containing one to four glycans) and high isoelectric point (pI $\approx$ 8.6 – 8.9) (Spik et al., 1994; Steijns & van Hooijdonk, 2000) attributed to the high content of basic aminoacids. Furthermore, lactoferrin shows a number of biological functions, which can include antioxidant, antimicrobial, antiviral and anticancer activity (Wakabayashi, Yamauchi, & Takase, 2006) making it a very desirable functional ingredient to be incorporated into food formulations.

Whey protein stabilized emulsions can be transformed into gelled emulsions by traditional techniques, such as heat treatment (Chen, Dickinson, Langton, & Hermansson, 2000; Liu & Tang, 2011), acidification with glucono-δ-lactone (GDL) (Boutin, Giroux, Paquin, & Britten, 2007; Ye & Taylor, 2009) and addition of salts of divalent ions (e.g. $CaCl_2$) (Sok Line, Remondetto, & Subirade, 2005; Ye & Taylor, 2009). As lactoferrin is a globular protein (Damodaran, Parkin, & Fennema, 2007) we hypothesize it could form gels. Previous studies investigated the formation of nanohydrogels (Bourbon et al., 2015) and viscoelastic gels from heated emulsion stabilized by lactoferrin (Tokle & McClements, 2011). However, to our knowledge there are no previous studies about the production of cold gel-like emulsions with lactoferrin.

The heat treatment necessary to produce gelled emulsions limits their application in formulations containing heat-sensitive ingredients, such as bioactives, while those obtained by cold-set techniques (without heat treatment) are more favorable (Liu & Tang, 2011; Sok Line et al., 2005) and exhibit some enhanced functional characteristics - e.g. controlled release of bioactives and improved oxidative stability of lipids (Lee, Choi, & Moon, 2006). However, in cold-set techniques, a heat treatment of protein dispersions before emulsification step is

needed to ensure denaturation of the protein native structure. This denaturation is characterized by the formation of soluble aggregates (Boutin et al., 2007; Sok Line et al., 2005) through the partial unfolding of the native protein, and a subsequent aggregation of unfolded molecules (Nielsen, Singh, & Latham, 1996). These soluble aggregates will act as "building blocks" necessary for the development of protein network systems, such as gels (Pereira et al., 2016). Subsequent addition of salts with divalent ions, such as $CaCl_2$, can be done, improving cross-linking of proteins and thus promoting gelation (Bryant & McClements, 2000; Kuhn, Cavallieri, & Da Cunha, 2010, 2011).

Ohmic heating (OH) has been receiving increased attention due to its volumetric heating and rapid heating rates that enable high temperatures to be applied in a short-time, thus allowing to obtain products of a superior quality to those processed by conventional heating technologies (Castro, Teixeira, Salengke, Sastry, & Vicente, 2003; Machado, Pereira, Martins, Teixeira, & Vicente, 2010; Rodrigues et al., 2015). During OH treatment the food product to be treated behaves as an electrical resistance, allowing the passage of an alternating electric current through it which results in generation of internal heat, according to Joule's law (De Alwis & Fryer, 1990). The presence of the inherent electric variables of OH (i.e. electric field, electric frequency and current density) lead to protein conformational disturbances and distinct gelation behavior during heat-induced denaturation of whey protein isolate (Pereira, Souza, Cerqueira, Teixeira, & Vicente, 2010; Pereira, Teixeira, & Vicente, 2011; Rodrigues et al., 2015). However, there are no particular studies about the influence of OH on lactoferrin properties.

The objective of this work was to produce cold gel-like emulsions from ohmic heated lactoferrin dispersions. These gelled emulsions were characterized in terms of their physical and structural properties and compared with emulsions produced using a conventional heating method. The effect of ohmic and conventional heating on the protein secondary structure and consequent thermal aggregation of lactoferrin were evaluated in order to provide insight to the mechanisms that may be influencing the observed changes in gelled emulsions properties.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Lactoferrin from bovine milk (protein content 92.1 wt. %, iron saturation 9.9 %) was kindly provided by Synlait Milk Ltd (Canterbury, New Zealand) and sunflower oil (Fula, Portugal) was purchased in a local market. The other reagents were of analytical grade.

6.2.2 Methods

6.2.2.1 Preparation of Lactoferrin Dispersion

Lactoferrin (3.0 wt. %) was dispersed in 20 mM sodium phosphate buffer (pH 6.0) using magnetic stirring overnight at room temperature, ensuring complete dissolution of the protein. Electrical conductivity of the prepared lactoferrin dispersion was approximately 1.5 mS.cm^{-1} (WTW LF 538 conductivity meter, Weilheim, Germany), which allowed the ohmic heating effect to take place.

6.2.2.2 Conventional Heating (CH) of Lactoferrin Dispersion

A double-walled water jacketed glass reactor vessel (30 mm of internal diameter and 100 mm in height) was used, as previously reported by Pereira et al. (2010). Temperature was controlled by circulating thermostabilized water from a bath. A magnetic stirrer was used to homogenize lactoferrin dispersion, improving heat transfer during the heating cycle. Temperature was measured online with a type K thermocouple (accuracy of ±1 °C; Omega Engineering, Inc., Stamford, CT, USA), placed at the center of the sample volume, and connected to an acquisition system (USB-9161, National Instruments Corporation, Austin, TX, USA), as described by Pereira et al. (2016).

6.2.2.3 Ohmic Heating (OH) of Lactoferrin Dispersion

Experiments were performed in a cylindrical glass tube of 30 cm total length and an inner diameter of 2.3 cm, with two stainless steel electrodes isolated at each edge with

polytetrafluoroethylene (PTFE) caps, as previously reported by Pereira et al. (2010). A gap between the electrodes of 4 cm was used, and the supplied voltage ranged from 36 to 86 V. The sample temperature was controlled through regulation of the supplied voltage using a function generator (1 Hz‑25 MHz and 1 to 10 V; Agilent 33220A, Penang, Malaysia) connected to an amplifier device (Peavey CS3000, Meridian, MS, USA). The voltage was adjusted to simulate the thermal history of samples treated by CH, allowing to discriminate the existence of additional non-thermal effects on denaturation of proteins due to the presence of electric variables (Pereira et al., 2017). To eliminate different shearing conditions between treatments, sample volume and stirring conditions were identical, as described previously for CH treatment.

6.2.2.4 Heating Conditions of Lactoferrin Dispersion

Lactoferrin dispersion (20 mL) was heated at 90 °C for 30 min through CH or OH. After a heating come-up time (time to raise temperature from 25 to 90 °C), the temperature was held constant at 90 °C for 30 min. The temperature profile was quite similar for CH and OH (Figure 6.1) which allowed to evaluate the non-thermal effects (or electric effects) of OH treatment. The temperature used was based on the thermal behavior of native bovine lactoferrin, that presents two denaturation peaks around 60 and 89 °C, respectively (Bengoechea, Peinado, & McClements, 2011; Bokkhim, Bansal, GrØndahl, & Bhandari, 2013).
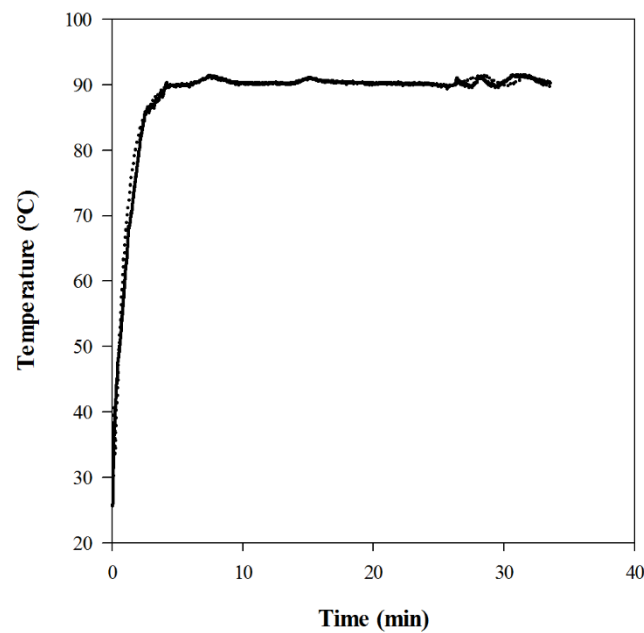
Figure 6.1 - Example of similar thermal histories at 90 °C for conventional (dotted line) and ohmic (solid line) heating treatments.

6.2.2.5 Characterization of Lactoferrin Dispersion

6.2.2.5.1 Turbidity

The turbidity of undiluted protein dispersions was analyzed using an UV/visible spectrophotometer at 600 nm (Synergy HT, Bio-Tek, Winooski, USA), according to Bengoechea et al. (2011). The sodium phosphate buffer described previously (20 mM at pH 6.0) was used as blank measurement.

6.2.2.5.2 Intrinsic Fluorescence

Intrinsic fluorescence spectra of the protein dispersions (0.01 wt. %) were obtained at 20 °C using a spectrofluorimeter (Horiba Scientific, USA) equipped with a standard thermostated cell holder and a 1.0 cm path length quartz cuvette. The excitation wavelength was 290 nm. Emission spectra were recorded between 300 and 400 nm with 1% attenuation, and fluorescence intensities were recorded every 0.5 nm.

6.2.2.5.3 Extrinsic Fluorescence

For the extrinsic fluorescence, lactoferrin dispersion (3 µM) was incubated at 20 °C with a 50-fold molar excess of freshly prepared 8-anilo-1-naphthalenesulfonate (ANS) (Sigma-Aldrich, St. Louis, EUA) for 60 min in the dark before the analysis. The excitation was fixed at 365 nm and the emission was collected between 400 and 600 nm at 20 °C, using a 1.0 cm path length quartz cuvette. Spectra of ANS fluorescence were acquired with a spectrofluorimeter (Horiba Scientific, USA) (Stroylova et al., 2011).

6.2.2.5.4 Free Sulfhydryl Groups

Free sulfhydryl (SH) groups on lactoferrin dispersion were determined on heat-treated samples using Ellman's 5,5-dithiobis-2-nitrobenzoicacid (DTNB) method, as described in previous literature (Pereira et al., 2010).

6.2.2.5.5 Hydrodynamic Diameter and Polydispersity Index

Particle size measurements were made by dynamic light scattering (DLS) using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Unheated and heat-treated samples (0.01 wt. %, 2 mL) were poured into sizing cuvettes and measurements were carried out, at least, in triplicate. The temperature of the cell was maintained at 20 °C. The method of cumulant fit was used to translate the average diffusion coefficients into average particle diameters (Z-Average value) using Stokes-Einstein relationship (Anema & Li, 2003). The polydispersity index (PDI) describes the width or the relative variance of the particle size distribution and was based on the measurements of dynamic light scattering intensity autocorrelation function.

6.2.2.5.6 Far-UV Circular Dichroism (CD)

Far-UV circular dichroism was used to investigate the secondary structure of untreated and heat-treated lactoferrin. Protein dispersions (0.01 wt. %) were evaluated at 20 °C in the spectral range from 190 to 260 nm with a spectropolarimeter (Jasco J-1500, Jasco Corp., Japan), using a quartz cuvette with an optical path of 0.1 cm. Scan speed was set to 50 nm/min

and three scans were accumulated and averaged. All the spectra were corrected using a protein-free sample.

6.2.2.6 Emulsion Preparation

Emulsions were prepared by homogenizing 10 % (v/v) of sunflower oil with 90 % (v/v) of preheated lactoferrin dispersion, using an Ultra-Turrax homogenizer (T 25, Ika, Germany) at 15,000 rpm for 2 min followed by passage through a high pressure homogenizer (NanoDeBee, Bee International, South Easton, Massachusetts, USA) at 137.9 MPa, according to Pinheiro et al. (2016). A divalent salt was used to induce cold-set gelation. Thus, emulsions were mixed with a concentrated $CaCl_2$ solution to obtain a final concentration of 60 mM by vortexing at room temperature, as previously described by Sok Line et al. (2005). The emulsions were stored at room temperature for 24 h prior to further analysis.

6.2.2.7 Emulsion characterization

6.2.2.7.1 Droplet size

Droplet size was measured by dynamic light scattering (DLS) using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Samples were diluted (0.01 wt. %, 2 mL) in the same buffer of lactoferrin dispersion (20 mM sodium phosphate buffer, pH 6.0). They were poured into sizing cuvettes and measurements were carried out, at least, in triplicate. The temperature of the cell was maintained at 20 °C.

6.2.2.7.2 Rheology

Rheological measurements of the emulsions were performed using a rheometer Discovery HR-1 (TA Instruments, New Castle, USA) with a stainless-steel cone plate geometry (60 mm, 2° angle, truncation 64 μm). Flow curves were obtained by an up-down-up steps program with shear rate ranging from 0 to 300 s^{-1}. Newtonian (Eq. (6.1)) and power law model (Eq. (6.2)) were fitted to the data to obtain the rheological properties. Viscoelastic behavior of samples was determined from oscillatory measurements using a frequency sweep between 0.1 and 10 Hz performed within the linear viscoelasticity domain. These measurements were done

at 20 °C after one day of samples storage. The contribution of the elastic and viscous characteristics was evaluated from storage (G') and loss (G'') moduli, respectively. Preliminary measurements were done in order to identify the linear viscoelastic region by measuring the shear moduli at different strain values at a fixed frequency (1 Hz), where a constant strain of 1% was used for the subsequent measurements.

$$\sigma = \eta.\dot{\gamma} \tag{6.1}$$

$$\sigma = k.\dot{\gamma}^n \tag{6.2}$$

where σ is the shear stress (Pa) , η is the viscosity (Pa.s), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency index ($Pa.s^n$) and n is the flow behavior index (dimensionless).

6.2.2.7.3 Microstructure

Emulsions were analyzed using an epifluorescence microscope (Olympus BX51) coupled with a DP71 digital camera and three sets of filters in the range of 360-370/420; 470-490/520; and 530-550/590 (Olympus Portugal SA, Porto, Portugal). All images were acquired using the Olympus cellSens software. Sunflower oil was stained with Nile Red (0.005 wt. %).

6.2.3 Statistical analysis

The experiments were run in triplicate, and all measured parameters are means of nine experimental points. The results were evaluated by one-way analysis of variance (ANOVA), and significant differences ($p < 0.05$) between the treatments were evaluated by the Tukey procedure. The statistical analyses were performed using a trial version of the software Minitab 16.1.0 (Minitab Inc., State College, PA, USA).

6.3 RESULTS AND DISCUSSION

6.3.1 Characterization of lactoferrin dispersion

Turbidity values of lactoferrin dispersion increased with heating time for both treatments. However, OH resulted in a lower turbidity value, indicating less protein aggregation than in conventional heating (Fig. 6.2). This effect may be attributed to the increase of more reactive (unfolded) globular protein molecules at higher temperatures, enhancing protein-protein collision frequency, thus favoring protein aggregation (Bengoechea et al., 2011).

The average hydrodynamic diameter (Z-Average) increased considerably in both treatments. However, OH produced smaller protein aggregates ($p < 0.05$) when compared with CH (Fig. 6.3a). Lactoferrin dispersions heated 30 min by CH presented Z-Average of 76.0 ± 0.7 nm while OH showed a value of 68.7 ± 0.4 nm. PDI values decreased after heating, reaching a value of 0.2 for both treatments (Fig 6.3b). Figure 6.3c shows the size distribution curves obtained for unheated lactoferrin dispersions and after heating at 90 °C. Size distribution curves of unheated lactoferrin dispersions showed a first population with a maximum located around 10 nm that refers to lactoferrin monomers (Tavares et al., 2015), a second population with a maximum between 10 and 100 nm, and a third peak between 100 and 1000 nm that could be some aggregates. When lactoferrin dispersions were heated at 90 °C, the first and third population disappeared with a concomitant increase of the middle peak. These results are in agreement with previous works that observed size increase after heating (Pereira et al., 2010; Rodrigues et al., 2015), and also corroborates turbidity results.

Figure 6.2 – Turbidity values of lactoferrin dispersions after different times of conventional (full symbols) or ohmic (empty symbols) heating. Different lowercase letters represent significant differences ($p < 0.05$) between heating time and different uppercase letters represent significant differences ($p < 0.05$) between heating treatments.
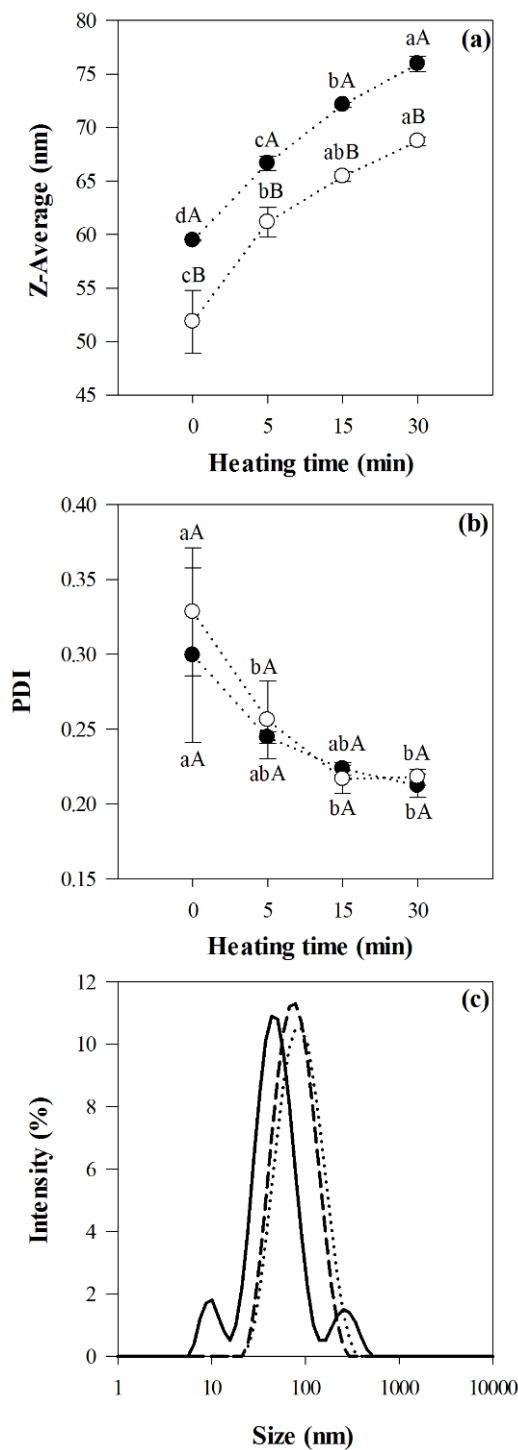
Figure 6.3 – Z-Average (a), PDI (b) and particle size distribution (c) of unheated and thermally heated lactoferrrin dispersions. Unheated lactoferrin (solid line), CH-heated lactoferrin (dotted line or full symbols) and OH-heated lactoferrin (dashed line or empty symbols). Different lowercase letters represent significant differences ($p < 0.05$) between heating time and different uppercase letters represent significant differences ($p < 0.05$) between heating treatments.

Local disturbances and changes on protein structure and dynamics are widely accessed through intrinsic tryptophan fluorescence measurements (Vivian & Callis, 2001). Then, this analysis was performed in order to investigate the effect of heating time on the conformational and structural changes of lactoferrin. An increase of fluorescence intensity was observed with increasing heating time of protein dispersions, but no significant differences were observed between both treatments after 30 min of heating (Fig. 6.4a). This increase in fluorescence intensity may be related to changes in the conformational structure of the protein molecule due to molecular local unfolding, allowing exposure of buried tryptophan residues (Royer, 2006; Stănciuc et al., 2013; Bourbon et al., 2015). The averaged emission fluorescence spectra for tryptophans, contained in different parts of lactoferrin, showed a maximum emission band near 336 nm (Fig. 6.4a), which is in agreement with previous works (Bourbon et al., 2015; Fang et al., 2014). The red shift of the wavelength of maximum fluorescence intensity indicates a considerable increase of the accessibility of these residues to the solvent (Uversky, Narizhneva, Kirschstein, Winter, & Löber, 1997).

ANS fluorescence measurements on the lactoferrin dispersions were performed in order to provide information about variation of the accessible hydrophobic areas. Figure 6.4b shows that an increase in heating time was accompanied by significant changes in the intensity of ANS fluorescence, probably due to progressive unfolding of the protein and increasing accessibility of ANS probe to the protein hydrophobic core. This increase in extrinsic fluorescence intensity corroborates with intrinsic fluorescence results. However, hydrophobic groups of lactoferrin treated by OH were less prone to react with ANS than conventional heated ones. As the ANS is anionic, electrostatic interactions may have occurred with lactoferrin due to its basic character. The interaction of ANS with exposed hydrophobic groups of the protein due to heating is accompanied by a considerable increase in the dye fluorescence intensity and a pronounced blue shift of the maximum fluorescence (Stănciuc et al., 2013). However, a slight blue shift in wavelength for ANS fluorescence was observed (Fig. 6.4b). Probably, the lower denaturation/aggregation of OH-induced lactoferrin led to changes at the conformational level making these hydrophobic groups less exposed to ANS.
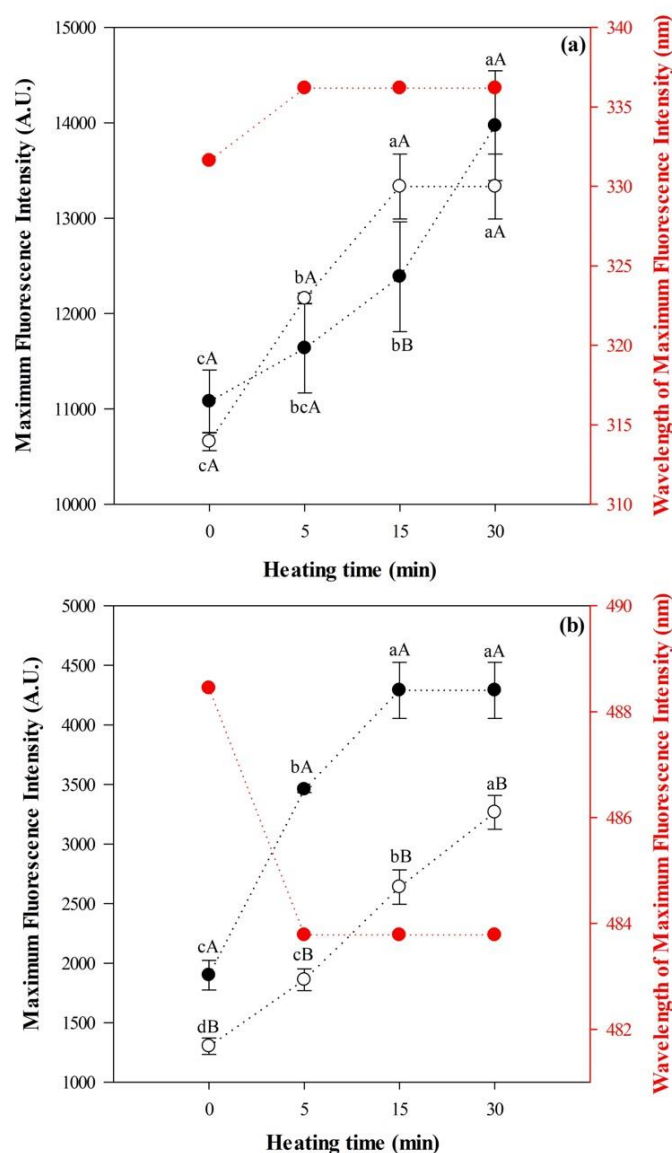
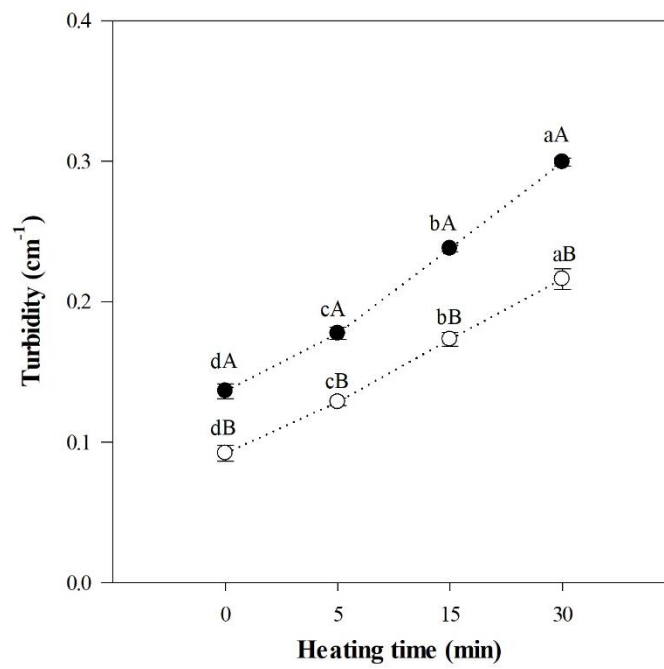Figure 6.4 – Maximum fluorescence intensity and wavelength of maximum fluorescence intensity of lactoferrin dispersions after different times of conventional (full symbols) or ohmic (empty symbols) heating for intrinsic (a) and ANS (b) fluorescence. Different lowercase letters represent significant differences ($p < 0.05$) between heating time and different uppercase letters represent significant differences ($p < 0.05$) between heating treatments.

The content of free SH groups was very low, ranging from ≈ 0.005 to 0.030 µmol/g (Fig. 6.5). This was somehow expected once lactoferrin in its native state contains intramolecular disulfide bonds but no free sulfhydryl groups (Lönnerdal & Suzuki, 2013). However, results from Figure 6.5 show that upon heating SH groups of lactoferrin become

available to react with DTNB reagent. This gives an indication that the lactoferrin unfolding occurred under the applied heating treatments, thus exposing their SH groups initially inaccessible in the native protein structure, which is in agreement with a previous work regarding heat-induced changes of lactoferrin (Brisson, Britten, & Pouliot, 2007). Unfolding of lactoferrin in respect to the SH groups was apparently very similar between OH and CH treatments. A statistically significant difference was observed for the OH and CH treatments after 30 min of heating despite these values being very close to each other. This may be linked with the development of protein aggregates.
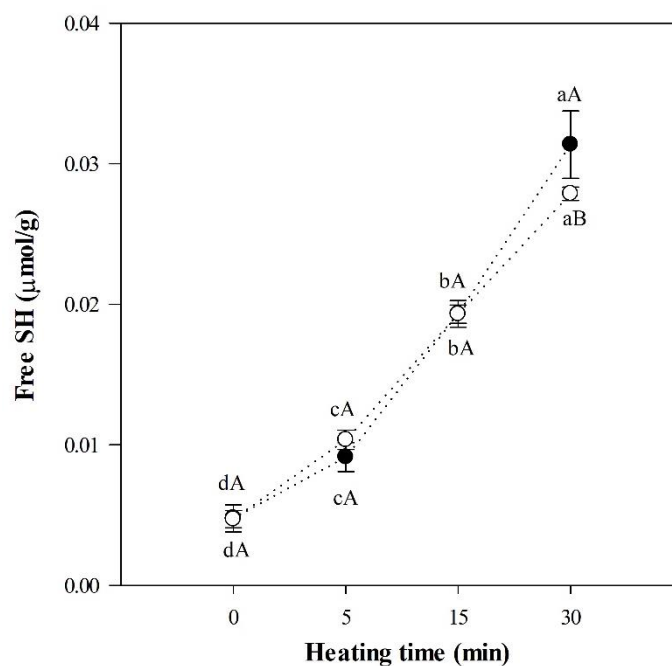
Figure 6.5 – Concentration of free sulfhydryl groups (SH) of lactoferrin dispersions after different times of conventional (full symbols) or ohmic (empty symbols) heating. Different lowercase letters represent significant differences ($p < 0.05$) between heating time and different uppercase letters represent significant differences ($p < 0.05$) between heating treatments.

Circular dichroism (CD) spectroscopy was used to investigate the changes in the secondary structure of the proteins, considering that the major elements (α-helix, β-sheet and coil) have characteristic CD spectra. The α-helix configuration presents an intense and positive band at 190 nm and negative peaks at 208 and 220 nm, while β-sheet configuration presents a

negative dichroic peak with a minimum in the 215 nm region. Random coil structures generally have a positive CD peak near 215 nm and a negative one near to 200 nm (Barreto et al., 2003; Kasinos et al., 2013). Unheated lactoferrin presented an ellipticity minimum near 210 nm (Fig. 6.6) suggesting that its structure is partially based on α-helix and β-sheet conformation (Shimazaki, Kawano, & Yung Choon, 1991; Stǎnciuc et al., 2013; Wang et al. 2013), which is in agreement with other works (Bokkhim et al., 2013; Furtado et al., 2017). CD profile of unheated lactoferrin and lactoferrin dispersions heated for 0 min (for both treatments) was similar, but after 30 min of heating, a decrease in the magnitude of ellipticity in the range of 190-210 nm and a slight increase in the range of 210-240 nm was observed for both treatments. The loss of magnitude in CD signal can be attributed to aggregation (Ioannou, Donald, & Tromp, 2015), which corroborates results presented above. Samples heated by OH showed a higher loss of magnitude in CD signal in the range of 210-240 nm when compared to CH. It has been suggested that the presence of moderate electric fields during heating may change not only the number of globular protein aggregates but also their shape or volume (Pereira et al., 2016), and this may have contributed to differences observed in dichroic signals among the heating treatments.
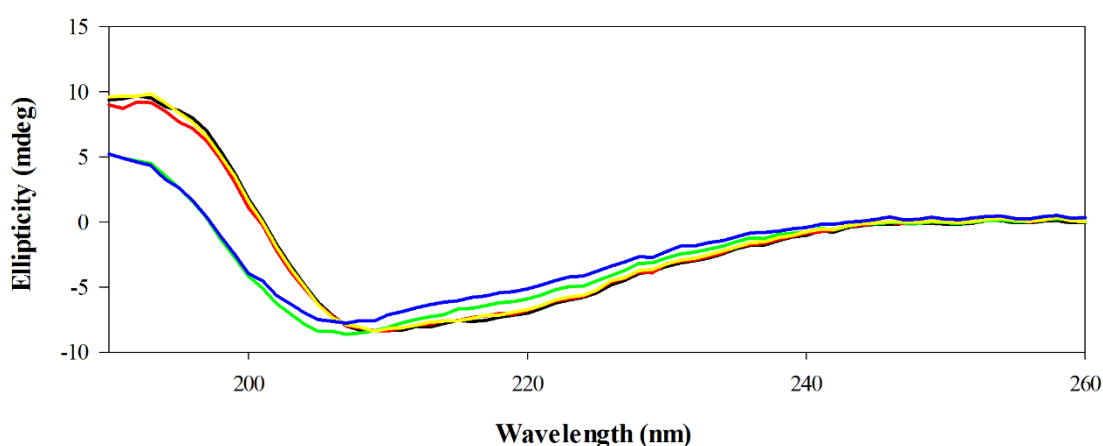
Figure 6.6 - Far UV-CD spectra of lactoferrin dispersions after different times of CH or OH: unheated lactoferrin (black line), lactoferrin in CH for 0 min (red line), lactoferrin in CH for 30 min (green line), lactoferrin in OH for 0 min (yellow line) and lactoferrin in OH for 30 min (blue line).

Based on results presented above, this lower aggregation observed for heat treated lactoferrin by the ohmic system when compared to conventional heating has been reported in

the literature for whey proteins, where it is suggested that the application of moderate electric fields may be linked to conformational disturbances on tertiary protein structure; reorientation of hydrophobic clusters occurring in the protein structure; modification of the molecular environment due to the increased number of ions, and their different distributions around the protein molecules; and splitting of large protein aggregates (Pereira et al., 2016; Pereira et al., 2010; Rodrigues et al., 2015). Furthermore, the electric field may also affect the ionic movement in the medium (Castro, Macedo, Teixeira, & Vicente, 2004), interfering with electrostatic interactions which play a fundamental role in folding, conformational stability and protein-protein interactions (Neves-Petersen & Petersen, 2003). Among these hypothesis, results show that the presence of an electric field during thermal denaturation seems to affect in a very distinctive way the hydrophobic core or local hydrophobic groups of lactoferrin. These local disturbances may have contributed to a distinct form of aggregation or interaction between unfolded molecules, once it is recognized that the way how hydrophobic groups are exposed during the initial stage of denaturation can have a crucial role on the mechanism of protein aggregation (Wijayanti, Bansal, & Deeth, 2014). These events may explain the apparent smaller or different type of protein aggregates, as well as the distribution of protein secondary structures found under the influence of OH.

6.3.2 Characterization of cold gel-like emulsions

Figure 6.7 shows the visual aspect of the emulsions produced with unheated lactoferrin dispersion and lactoferrin heated by OH and CH after one day of storage at room temperature. It was observed that for both heating treatments the emulsions showed a soft gel-like appearance, while the emulsion produced with unheated lactoferrin remained in a liquid state (Fig. 6.7). Similar appearance was observed in emulsions produced with whey proteins (Liu & Tang, 2011; Manoi & Rizvi, 2009), but the network structure seems to be denser for the later.
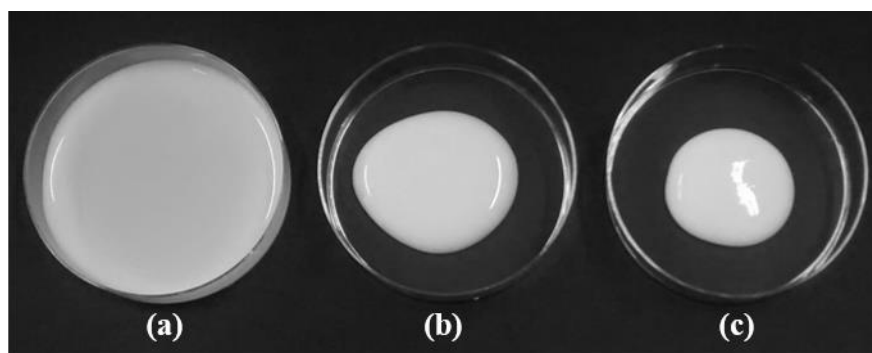
Figure 6.7 – Visual aspect of the emulsions produced with unheated (a), OH-heated (b) and
CH-heated (c) lactoferrin dispersions after one day of storage at room temperature.

When submitted to rheological analysis, emulsions produced with unheated lactoferrin dispersion showed Newtonian behavior, while emulsions produced with lactoferrin heated by OH or CH showed a shear-thinning behavior (Table 6.1). Such shear thinning behavior is related to flocculation of oil droplets forming a network of aggregated droplets due to protein matrix (Boutin et al., 2007; Liu & Tang, 2011; Manoi & Rizvi, 2009). Viscosity values (η) (Table 6.1) showed a statistically significant increase ($p < 0.05$) for heat treated emulsions, being this increase more pronounced for lactoferrin heated by the conventional system, which confirms the increase of emulsion structuration due to a probably higher interaction among the droplets (through the formation of new inter- and intramolecular disulfide bonds that will engage toward protein aggregation, besides electrostatic and hydrophobic interactions between them).

Table 6.1 – Rheological parameters of the emulsions produced with unheated (A), ohmic
heated (B) and conventionally heated (C) lactoferrin dispersion.

Emulsion	η (mPa.s)	k (Pa.s^n)	n	R^2
(A)	1.68 ± 0.12^c	-	-	0.98
(B)	$*8.06 \pm 0.71^b$	0.02 ± 0.00^b	$0.82 \pm .01^a$	0.99
(C)	$*29.51 \pm 3.47^a$	0.31 ± 0.1^a	0.49 ± 0.05^b	0.99

Identical small letters in the same column indicate that there are no statistically significant differences ($p > 0.05$).

* in this case the value corresponds to the apparent viscosity at 100 s^{-1} (η_{100}).

Viscoelastic properties were determined, once the ratio between storage (G') and loss (G'') moduli may indicate if the emulsion is strongly or weakly associated (Torres, Iturbe, Snowden, Chowdhry, & Leharne, 2007). The viscoelastic properties of the emulsions produced with unheated lactoferrin dispersion, lactoferrin heated by OH and CH after one day of storage at room temperature are presented in Figure 6.8. Mechanical spectra of the emulsions indicate that emulsions heated by ohmic or conventional systems presented a gel-like behavior (G' higher than G'' throughout the frequency range). Somehow, the emulsion produced with lactoferrin heated by the conventional system resulted in the strongest gel-like structure – i.e. G' values were 10 times greater than those obtained for emulsions produced with lactoferrin heated by the ohmic system and almost 1000 times greater than those obtained for emulsions produced with unheated lactoferrin (for low frequency values, 0.1 - 1). On the other hand, it was observed only a slight increase in the moduli of heat treated samples for increasing values of frequency, which has been attributed to a predominantly solid behavior of the gel-like emulsions, indicating that they may formed permanent interactions (Liu & Tang, 2011). However, the elastic modulus (G') of the emulsions produced with unheated lactoferrin showed a frequency-dependence, which is associated to a weaker gel or a less stable emulsion (Torres et al., 2007). Furthermore, the network entanglement between adsorbed and non-adsorbed protein molecules is the main factor that leads to high elastic modulus values and a gel-like structure (Dickinson & Hong, 1995).
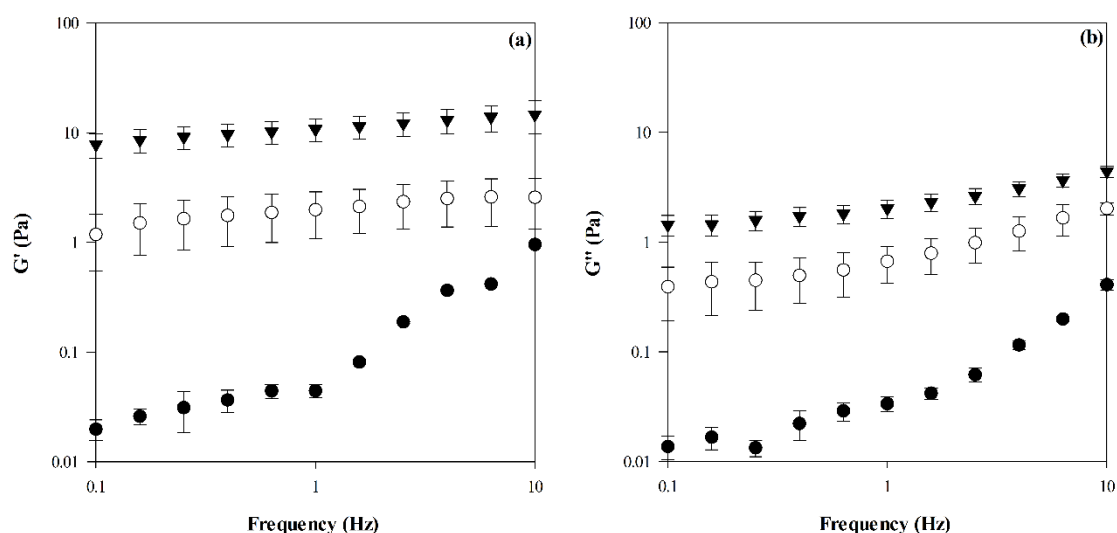
Figure 6.8 – Storage (G') (a) and loss (G'') (b) moduli of the emulsions produced with unheated (●), OH-heated (○) and CH-heated (▼) lactoferrin dispersions.

The emulsion prepared with unheated lactoferrin presented droplet size around 206±2 nm, but it was not possible to measure the droplet size of the other systems due to the intense aggregation observed. In order to confirm aggregation of oil droplets, the microstructure of the emulsions produced with unheated and heated lactoferrin dispersions was analyzed, and the corresponding pictures are shown in Figure 6.9. Emulsions presented remarkable differences depending on the heating treatment. The oil droplets of the emulsions produced with unheated lactoferrin were homogeneously distributed in the aqueous protein phase with no signs of flocculation/aggregation. However, oil droplets entrapped in an entangled protein network was observed for emulsions produced with heat treated protein. Besides the heat aggregation of the proteins, salt addition also contributed to the formation of this particulate structure composed of random aggregates (Sok Line et al., 2005). Microscopic images suggest a denser network for emulsions produced with lactoferrin dispersions heated by the conventional system. These images confirm rheology results and corroborate protein characterization results (section 6.3.1). Furthermore, the differences between the microstructures are reflecting the differences in interactive forces involved in the formation of the cold gel-like emulsions.
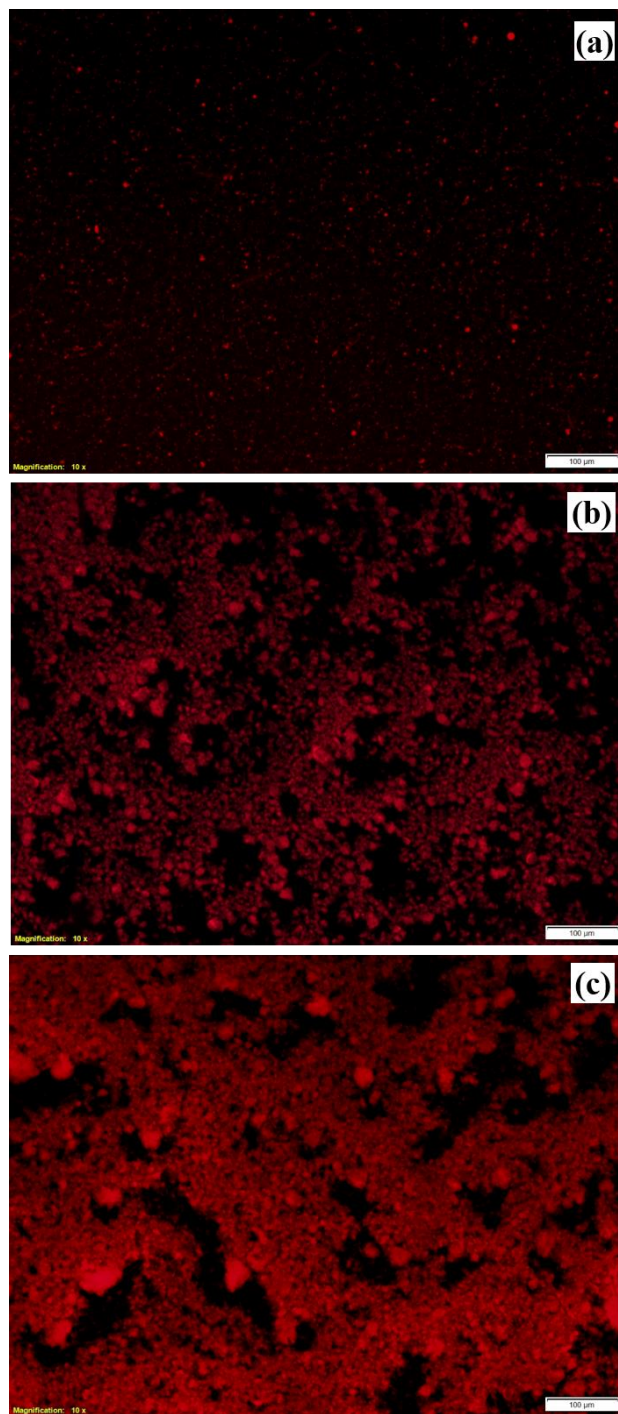
Figure 6.9 – Microstructure of the emulsions produced with unheated (a), OH-heated (b) and CH-heated (c) lactoferrin dispersions. Scale bar: 100 μm.

6.4 CONCLUSIONS

Cold gel-like emulsions were produced using lactoferrin dispersions subjected to a heat pretreatment in an ohmic or conventional system. Their formation was associated to the good emulsifying capacity of lactoferrin combined with heat treatment of the protein. Both OH and CH influenced thermal unfolding and aggregation of lactoferrin molecules. However, internal electrical heating and the existence of non-thermal effects - i.e. electric field - possibly affected the molecular flexibility or stability of hydrophobic groups of lactoferrin. OH led to less aggregated protein molecules, when compared to conventional heating. Such different aggregation pattern was confirmed by a lower increase in particle size, turbidity, intrinsic and extrinsic fluorescence and a distinct dichroic signal. These events had a direct impact on structural and mechanical properties of the prepared emulsions. Therefore, the rheological and microstructural properties depended on the heat treatment applied, since conventional heating produced stronger gel-like emulsions than OH treatment. OH appears to be an efficient heating technology that can be used to modulate lactoferrin thermal denaturation and lead to distinct gel-like emulsions. These emulsions could be interesting for several innovative food applications, i.e. as texturizers, fat replacers, carrier of heat-sensitive and lipid-soluble bioactives. The results from this study also suggest a further fundamental approach about the influence of electrical variables of ohmic heating technology on the stability and functionality of lactoferrin molecules. Ohmic heating seems to impose small structural changes at the nanoscale level that can have a major impact on the macrostructural and functional properties of globular proteins.

6.5 ACKNOWLEDGEMENTS

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CAPÍTULO 7 - DISCUSSÕES GERAIS

7.1 DISCUSSÕES GERAIS

Em uma primeira etapa foi avaliado o efeito do tratamento ultrassônico nas propriedades físicas e estruturais do caseinato de sódio e da lactoferrina. As mudanças nas propriedades das proteínas foram avaliadas em termos de tamanho, potencial zeta, estrutura molecular, viscosidade intrínseca, hidrofobicidade superficial e dicroísmo circular. Também foi avaliado o efeito do ultrassom na capacidade de melhorar a estabilização de emulsões e diminuir o tamanho de gota. O caseinato de sódio sofreu uma redução significativa de tamanho, enquanto que um comportamento oposto foi observado para a lactoferrina. Este efeito pode ser atribuído às características conformacionais distintas das proteínas quando submetidas às forças mecânicas resultantes da cavitação ultrassônica que podem levar à mudanças nas interações eletrostáticas e hidrofóbicas (JAMBRAK *et al*., 2014; O'BRIEN 2007; O'SULLIVAN *et al*., 2014; YANJUN *et al*., 2014). A hidrofobicidade superficial foi afetada positivamente pelo aumento no tempo de tratamento ultrassônico e foram observadas pequenas diferenças no perfil eletroforético em gel de poliacrilamida nativo da lactoferrina, atribuídas à interações não covalentes. O dicroísmo circular não detectou diferenças no sinal dicróico para o caseinato de sódio, mas uma leve mudança conformacional foi observada para a lactoferrina, com aumento de estruturas do tipo desordenadas (Barreto *et al*., 2003; Munialo *et al*., 2014). As emulsões produzidas com lactoferrina, utilizando o ultrassom por maiores tempos, apresentaram tamanho de gota reduzido e boa estabilidade, o que não foi observado para o caseinato de sódio. Assim, a melhor estabilidade da lactoferrina pode ser atribuída às repulsões eletrostáticas e estéricas causadas pelos agregados de proteína (JAFARI *et al*., 2008). O tratamento ultrassônico prévio das proteínas melhorou suas propriedades emulsificantes, mas este tratamento ultrassônico em conjunto com o processo simultâneo de formação de gotas resultou em emulsões mais estáveis, facilitando a deposição dos emulsificantes na interface óleo/água.

Uma vez definido o melhor tempo de ultrassom para se obter emulsões estáveis, o próximo passo foi avaliar a heteroagregação de gotas lipídicas através da mistura de duas emulsões estabilizadas por proteínas carregadas com cargas opostas, variando a razão de volume de emulsão e a força iônica. As propriedades foram avaliadas em termos de estabilidade à cremeação, microestrutura, diâmetro de gotas, e parâmetros reológicos. Foram produzidos heteroagregados com características distintas. Os maiores heteroagregados que apresentaram boa estabilidade à cremeação foram produzidos com uma proporção em volume de 60 % de emulsão estabilizada por lactoferrina e 40 % de emulsão estabilizada por caseinato de sódio.

Heteroagregados produzidos com uma maior proporção de emulsão estabilizada com lactoferrina apresentaram forte agregação, resultando em separação de fases e formação de gel, além de aumentar a viscosidade e o módulo elástico. Desta forma, foi possível produzir heteroagregados com comportamento reológico muito mais estruturado usando uma mesma fração volumétrica de óleo usada em uma emulsão simples. A lactoferrina demonstrou-se susceptível ao NaCl, o que pode ser atribuído a sua alta capacidade de ligação a ânions (como Cl^-) (MAO e MCCLEMENTS, 2012), no entanto, a presença de caseinato de sódio permitiu maior estabilidade frente à adição do sal, o que pode ser atribuído a uma maior adsorção interfacial após a interação com o NaCl (SRINIVASAN *et al*., 2000). Assim, estes sistemas podem ser usados como agentes funcionais para modificação de textura/viscosidade e liberação controlada.

Os maiores e mais estáveis heteroagregados foram selecionados para serem submetidos às condições simuladas do trato gastrointestinal, de acordo com o protocolo de MINEKUS *et al*. (2014). As propriedades dos heteroagregados foram avaliadas em termos de estabilidade, microestrutura, tamanho de gota, potencial zeta, eletroforese em gel de poliacrilamida e liberação de ácidos graxos livres. As mudanças nas propriedades físicas, nas diferentes etapas da digestão, dependeram das propriedades do emulsificante. Estas mudanças podem ser atribuídas às condições físico-químicas do ambiente e a hidrólise das proteínas aderidas na interface devido a ação da pepsina sob as condições gástricas, bem como a ação dos sais biliares e da lipase sob as condições da fase intestinal. Os heteroagregados se desfizeram na etapa gástrica devido a repulsão eletrostática entre o caseinato de sódio e a lactoferrina no pH gástrico. A liberação de ácidos graxos livres foi menor para a emulsão estabilizada com lactoferrina, sendo atribuído à maior interação desta proteína com os sais biliares (SARKAR *et al*., 2010; ZHANG *et al*., 2015). No entanto, os heteroagregados apresentaram a menor liberação de ácidos graxos livres, possivelmente devido a interação dos peptídeos que foram digeridos, suprimindo a digestão lipídica.

A produção de emulsões *gel-like* através da técnica de gelificação a frio também foi avaliada. Para isso, dispersões de lactoferrina foram previamente tratadas por aquecimento ôhmico ou convencional e caracterizadas em termos físicos e estruturais. Os efeitos do aquecimento na estrutura secundária e agregação térmica da lactoferrina foram avaliados a fim de fornecer conhecimento sobre os mecanismos que podem influenciar nas propriedades das emulsões gelificadas. Foi possível produzir emulsões *gel-like* com um pré-aquecimento das dispersões de lactoferrina por ambos os tratamentos térmicos. Sua formação foi atribuída ao

efeito combinado da capacidade emulsificante da lactoferrina, o método de emulsificação e o tratamento térmico da proteína. O aquecimento ôhmico influenciou no desdobramento e agregação das moléculas de lactoferrina, sendo que as variáveis elétricas inerentes a este tratamento possivelmente afetaram a flexibilidade molecular das proteínas (PEREIRA *et al*., 2010; PEREIRA *et al*., 2011; RODRIGUES *et al*., 2015). Comparado ao aquecimento convencional, foi possível obter moléculas menos agregadas, o que foi confirmado pelo menor aumento de tamanho, turbidez, fluorescência, grupos sulfidrilas livres e diferença no sinal dicroico, refletindo diretamente nas emulsões obtidas. As propriedades reológicas e microestruturais dependeram do tipo de aquecimento, sendo que o aquecimento convencional possibilitou a formação de emulsões *gel-like* com uma estrutura mais forte. O aquecimento ôhmico demonstrou ser uma tecnologia de aquecimento eficiente que pode ser usada para modular a desnaturação térmica da lactoferrina e levar à formação de emulsões *gel-like* com características distintas. Estas emulsões podem ser interessantes em diversas aplicações, como texturizantes, substitutos de gordura e carreadores de bioativos lipossolúveis e sensíveis ao calor.

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CAPÍTULO 8 - CONCLUSÃO GERAL E SUGESTÕES PARA TRABALHOS FUTUROS

8.1 CONCLUSÃO GERAL

Com base nos estudos realizados no decorrer deste projeto foi possível concluir que:

A caseína e a lactoferrina têm características conformacionais distintas e mostraram comportamento oposto quando submetidas a forças mecânicas resultantes da cavitação ultrassônica. O tratamento ultrassônico prévio das proteínas melhorou suas propriedades emulsificantes, mas este tratamento quando em conjunto com o processo simultâneo de formação de gotas resultou em emulsões mais estáveis, facilitando a deposição dos emulsificantes na interface óleo/água.

Heteroagregados com características distintas foram produzidos dependendo da composição das emulsões, sendo que os maiores heteroagregados que apresentaram boa estabilidade à cremeação foram produzidos com uma proporção em volume de 60 % de emulsão estabilizada por lactoferrina e 40 % de emulsão estabilizada por caseinato de sódio. Além disso, a adição de NaCl não influenciou a estabilidade dos heteroagregados. Os heteroagregados apresentaram comportamento reológico muito mais estruturado que uma emulsão simples mantendo-se a mesma fração volumétrica de óleo.

A digestão lipídica dependeu do tipo de emulsificante que recobria as gotas, bem como da ação das enzimas digestivas e dos sais biliares. Os heteroagregados se desfizeram na etapa gástrica devido à repulsão eletrostática entre o caseinato de sódio e a lactoferrina no pH gástrico. E na etapa intestinal estes heteroagregados apresentaram o menor percentual de liberação de ácidos graxos, possivelmente devido a interação dos peptídeos que foram digeridos, suprimindo a digestão lipídica.

A formação de emulsões *gel-like* foi atribuída à capacidade emulsificante da lactoferrina, combinada com o método de emulsificação e o tratamento térmico da proteína. O tratamento térmico influenciou no desdobramento, desnaturação e agregação das moléculas de lactoferrina. As propriedades reológicas e microestruturais das emulsões dependeram do tipo de aquecimento, sendo que o aquecimento convencional possibilitou a formação de emulsões *gel-like* com uma estrutura mais forte. No entanto, o aquecimento ôhmico demonstrou ser uma

tecnologia de aquecimento eficiente que pode ser usada para modular a desnaturação térmica da lactoferrina e levar à formação de emulsões *gel-like* com características diferenciadas.

De uma forma geral, foi possível avaliar o efeito do ultrassom e do aquecimento nas propriedades estruturais e tecnológicas das proteínas, bem como propor estratégias de estruturação de emulsões (heteroagregados e emulsões *gel-like*) visando a substituição e redução de gorduras em produtos alimentícios. Através dos ensaios de digestibilidade *in vitro* foi possível confirmar um menor percentual de liberação de ácidos graxos dos heteroagregados, mostrando assim um potencial uso como moduladores de saciedade.

8.2 SUGESTÕES PARA TRABALHOS FUTUROS

São sugestões para trabalhos futuros:

Substituir a lactoferrina na formação dos heteroagregados por polissacarídeos ou proteínas de menor valor agregado, bem como avaliar a estabilidade destes sistemas em diferentes condições de pH.

Estudar maiores frações volumétricas de óleo ou maiores concentrações de emulsificantes na formação do heteroagregados, de forma a obter sistemas com maior viscosidade.

Encapsular bioativos nas emulsões *gel-like* de forma a fazer um estudo comparativo com as emulsões controle quanto à estabilidade e/ou bioacessibilidade após a digestão *in vitro*.

Fazer um estudo adicional sobre a influência das variáveis elétricas da tecnologia de aquecimento ôhmico na estabilidade e funcionalidade das moléculas de lactoferrina.

CAPÍTULO 9 - REFERÊNCIAS BIBLIOGRÁFICAS

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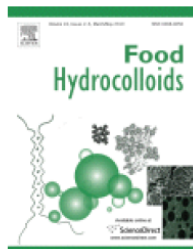
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Author: Guilherme de Figueiredo Furtado,Raphaela Araújo Mantovani,Larissa Consoli,Miriam Dupas Hubinger,Rosiane Lopes da Cunha

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Author: Guilherme de Figueiredo Furtado,Mariano Michelon,Davi Rocha Bernardes de Oliveira,Rosiane Lopes da Cunha

Publication: Food Research International

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Title: In vitro digestibility of heteroaggregated droplets coated with sodium caseinate and lactoferrin

Author: Guilherme de Figueiredo Furtado,Karen Cristina Guedes Silva,Cristiane Conte Paim de Andrade,Rosiane Lopes Cunha

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