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Faculdade de Engenharia de Alimentos

DÉBORA PARRA BAPTISTA

PEPTÍDEOS BIOATIVOS EM QUEIJO PRATO: ESTRATÉGIAS PARA OBTENÇÃO, POTENCIAL BIOATIVO E BIOACESSIBILIDADE

BIOACTIVE PEPTIDES IN PRATO CHEESE: STRATEGIES FOR OBTAINING, BIOACTIVE POTENTIAL AND BIOACCESSIBILITY

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Tecnologia de Alimentos.

Thesis presented to the School of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in the area of Food Technology.

Orientadora: Profa. Dra. Mirna Lúcia Gigante

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA DÉBORA PARRA BAPTISTA, E ORIENTADA PELA PROFA. DRA. MIRNA LÚCIA GIGANTE.

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RESUMO GERAL

As proteínas lácteas são consideradas as principais fontes de peptídeos bioativos que podem apresentar diversos efeitos benéficos à saúde tais como atividades antimicrobiana, antioxidante, imunomodulatória, opióide, carreadora de minerais e anti-hipertensiva. Esses peptídeos estão presentes nas proteínas precursoras em sua forma latente e podem apresentar atividade biológica se liberados pela ação enzimática durante o processamento ou digestão de leite e produtos lácteos. No caso do queijo, esses peptídeos podem ser formados pela proteólise que ocorre nos processos de fermentação, fabricação e maturação. De forma geral, todos os fatores que afetam a proteólise em queijos podem também afetar a liberação de peptídeos bioativos. Esses fatores incluem as culturas láticas utilizadas no processamento, o teor de sal e o tempo de maturação. Nesse contexto, o presente trabalho teve como objetivos avaliar o efeito da adição de uma cultura adjunta proteolítica, da redução no teor de sal e do tempo de maturação sobre perfil de peptídeos e potencial anti-hipertensivo de queijo Prato, bem como avaliar o efeito da digestão gastrointestinal sobre o perfil de peptídeos formado durante fabricação e maturação do queijo. Para alcançar esses objetivos, as atividades do projeto foram divididas em três etapas. Na primeira etapa, o potencial da cultura adjunta *Lactobacillus helveticus* LH-B02 para modificação do perfil de peptídeos do queijo Prato foi avaliado durante 60 dias de maturação. Na segunda etapa do projeto, foram avaliados os efeitos da adição da cultura adjunta *Lactobacillus helveticus* LH-B02, da redução de 25% no teor de sal e do tempo de maturação (120 dias) sobre o perfil de peptídeos e atividade inibidora da enzima conversora de angiotensina (ECA) do queijo Prato. Na terceira e última etapa do projeto, o perfil de peptídeos de queijos Prato com e sem a adição de *Lactobacillus helveticus* LH-B02 foi acompanhado durante a simulação *in vitro* da digestão gastrointestinal, utilizando dois modelos de digestão, o modelo estático proposto pela rede de cooperação internacional *INFOGEST* e o simulador do ecossistema microbiano humano (SEMH®), um modelo dinâmico de simulação da digestão. O perfil de peptídeos dos queijos e das amostras obtidas nas etapas das simulações *in vitro* da digestão gastrointestinal foram avaliadas por espectrometria de massas por ionização e dessorção a laser assistida por matriz (MALDI-MS). Os resultados revelaram que tanto a adição de *Lactobacillus helveticus* LH-B02 quanto a redução de sal modificaram o perfil de peptídeos do queijo Prato. A adição de *Lactobacillus helveticus* como cultura adjunta na fabricação de queijo Prato resultou em maior atividade inibidora da ECA, sendo, portanto, uma estratégia eficaz para aumentar o potencial bioativo do queijo. As simulações *in vitro* da digestão gastrointestinal em modelos estático e dinâmico demonstraram que a hidrólise enzimática que

ocorre durante a digestão aproximou o perfil de peptídeos de queijos produzidos com e sem a adição da cultura adjunta.

Palavras-chave: queijo Prato, peptídeos bioativos, digestão *in vitro*, proteólise.

ABSTRACT

Dairy proteins are considered the main sources of bioactive peptides that may have several beneficial health effects such as antimicrobial, antioxidant, immunomodulatory, opioid, mineral carrier, and antihypertensive activities. These peptides are present in the precursor proteins in their latent form and may present biological activity if released through enzymatic hydrolysis that occurs during the processing or digestion of milk and dairy products. In cheeses, these peptides can be released by the proteolysis that occurs in the fermentation, manufacturing and ripening processes. In general, every factor that affects proteolysis in cheese may also affect the release of bioactive peptides. These factors include the lactic cultures used in the cheesemaking process, the salt content and the ripening time. In this context, the present study aimed to evaluate the effect of the addition of an adjunct proteolytic lactic culture, the reduction in salt content and the ripening time on the peptide profile and antihypertensive potential of Prato cheese, as well as to evaluate the effect of the gastrointestinal digestion on the peptide profile formed during cheese manufacturing and ripening. To achieve these objectives, the project activities were divided into three phases. In the first phase, the potential of the adjunct culture *Lactobacillus helveticus* LH-B02 to modify the peptide profile of Prato cheese was evaluated during 60 days of ripening. In the second phase of the project, the effects of the addition of the adjunct culture *Lactobacillus helveticus* LH-B02, the reduction of 25% in the salt content and the ripening time (120 days) on the peptide profile and the angiotensin-converting enzyme inhibitory activity (ACE) of Prato cheese were evaluated. In the third and last phase of the project, the peptide profiles of Prato cheese produced with and without the addition of *Lactobacillus helveticus* LH-B02 were tracked during the *in vitro* simulation of gastrointestinal digestion using two digestion models, the static model proposed by the INFOGEST international cooperation network and the simulator of the human microbial ecosystem (SHIME®), a dynamic digestion simulation model. The peptide profile of cheeses and digests from different stages of the *in vitro* simulations of gastrointestinal digestion were evaluated by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The results showed that the addition of *Lactobacillus helveticus* LH-B02 and the reduction of salt content modified the peptide profile of Prato cheese. The addition of *Lactobacillus helveticus* as an adjunct culture in the manufacture of Prato cheese resulted in a higher ACE-inhibitory activity, thus being an effective strategy to increase the bioactive potential of the cheese. The *in vitro* simulations of gastrointestinal digestion in static and dynamic models showed that

enzymatic hydrolysis that occurs during digestion approximated the peptide profile of cheeses produced with and without the addition of the adjunct culture.

Keywords: Prato cheese, bioactive peptides, *in vitro* digestion, proteolysis.

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CAPÍTULO 1 - INTRODUÇÃO GERAL

1.1. Introdução

Dentre as tendências observadas na indústria de alimentos, a produção de alimentos com apelos saudáveis é sem dúvida uma das mais exploradas. Os alimentos funcionais surgiram como uma alternativa para melhorar a nutrição e a saúde em um ambiente no qual doenças relacionadas ao estilo de vida associadas ao envelhecimento da população são consideradas uma ameaça ao bem-estar da sociedade (KORHONEN, 2009).

Seguindo a tendência de saudabilidade, explicitando ou não uma alegação funcional, a indústria láctea comercializa uma série de produtos que remetem à alimentação saudável, tais como produtos lácteos fermentados adicionados de microrganismos probióticos, adicionados de fibra solúvel, com alto teor proteico, com redução dos teores de açúcar ou de sal, dentre outros. Adicionalmente, as proteínas lácteas têm sido consideradas as principais fontes de peptídeos bioativos, o que justifica a recente recomendação da Sociedade Brasileira de Cardiologia de ingestão de produtos lácteos como tratamento não medicamentoso da hipertensão arterial por conterem cálcio, potássio e peptídeos bioativos (KORHONEN, 2009; MALACHIAS et al., 2016).

A hipertensão é uma doença que atinge cerca de 32,5% da população adulta no Brasil, representando um risco potencial à saúde por contribuir direta ou indiretamente para 50% das mortes por doença cardiovascular. Se por um lado os produtos lácteos são recomendados para o tratamento não medicamentoso da hipertensão por conterem, entre outros compostos de interesse, peptídeos bioativos que podem atuar no controle da pressão arterial, por outro, o teor de sal de queijos ainda representa um aspecto negativo do consumo dessa classe de produtos por hipertensos, pois o sódio é considerado um dos principais fatores de risco para a hipertensão arterial (MALACHIAS et al., 2016). Dessa forma, o desenvolvimento de queijos que tragam os benefícios da redução no teor de sal e atividade anti-hipertensiva devido à presença de peptídeos bioativos pode atender à demanda por produtos adequados à hipertensos.

As proteínas do leite apresentam em sua estrutura primária diversas sequências de aminoácidos com atividades biológicas latentes, sem efeito fisiológico quando na estrutura da proteína. No entanto, as hidrólises enzimáticas que ocorrem nos processos de fermentação, maturação e digestão gastrointestinal, podem resultar na liberação dessas sequências de aminoácidos com atividades bioativas reconhecidas (FITZGERALD e MURRAY, 2006).

Embora os peptídeos bioativos estejam naturalmente presentes em queijos como um resultado do seu processo de fabricação (BÜTIKOFER et al., 2007; GÓMEZ-RUIZ et al., 2006; LU et al., 2016; PRITCHARD et al., 2010), diversos fatores podem afetar o perfil de peptídeos e, consequentemente a bioatividade desses produtos. O uso de culturas adjuntas proteolíticas, por exemplo, é uma estratégia promissora para a modificação do perfil peptídico e, consequentemente, desenvolvimento de bioatividades em queijos (ONG e SHAH, 2008; TORRES-LLANEZ et al., 2011).

Em estudo recente, nosso grupo de pesquisa verificou que a redução de 25% do teor de sal na fabricação do queijo Prato é possível sem comprometer o desenvolvimento da maturação e, consequentemente, sua qualidade físico-química e sensorial, viabilizando a fabricação de um produto mais saudável (BAPTISTA et al., 2017). A associação dessa estratégia com uso de uma cultura adjunta proteolítica pode viabilizar a produção de queijos com benefício duplo: redução no teor de sódio e presença de peptídeos bioativos.

Nesse contexto, os objetivos desse trabalho foram avaliar o efeito da adição de uma cultura adjunta proteolítica e da redução de sal no perfil de peptídeos e atividade anti-hipertensiva avaliada *in vitro* de queijo Prato, bem como avaliar a bioacessibilidade dos biopeptídeos formados durante sua maturação. Para atingir esses objetivos, o trabalho foi dividido em etapas apresentadas em 6 capítulos. No Capítulo 2 apresenta-se uma revisão bibliográfica focada na presença de peptídeos bioativos em queijos maturados, fatores que afetam a sua liberação durante a fabricação e maturação de queijos, efeito da digestão gastrointestinal sobre o perfil de peptídeos do queijo e metodologias disponíveis para a avaliação *in vitro* da digestão de proteínas e peptídeos lácteos. O Capítulo 3 apresenta um estudo acerca do efeito da adição da cultura adjunta *Lactobacillus helveticus* LH-B02 no perfil de peptídeos do queijo Prato, avaliado por 60 dias de maturação. No estudo apresentado no Capítulo 4, foram avaliados os efeitos da adição da cultura adjunta *Lactobacillus helveticus* LH-B02 e da redução de 25% do teor de sal sobre o perfil de peptídeos e atividade anti-hipertensiva avaliada *in vitro* do queijo Prato durante 120 dias de maturação. O quinto capítulo apresenta a avaliação da bioacessibilidade de peptídeos presentes no queijo Prato controle e adicionado de cultura adjunta *Lactobacillus helveticus* LH-B02 através de modelos estático e dinâmico de simulação *in vitro* da digestão gastrointestinal. Por fim, os Capítulos 6 e 7 apresentam a discussão geral dos resultados e a conclusão geral da tese, respectivamente.

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CAPÍTULO 2 - REVISÃO BIBLIOGRÁFICA

2.1. Peptídeos bioativos em queijos maturados

Muitas proteínas apresentam em sua estrutura primária sequências peptídicas que podem exercer um papel benéfico na saúde humana quando liberadas pela ação enzimática (HERNÁNDEZ-LEDESMA et al., 2011). Essas sequências peptídicas são conhecidas como peptídeos bioativos, que são definidos como fragmentos específicos de proteínas que apresentam efeitos positivos nas funções ou condições corpóreas, podendo apresentar impacto na saúde (KITTS e WEILER, 2003). Os peptídeos biologicamente ativos provenientes das proteínas do leite contêm de 2 a 20 aminoácidos em sua estrutura e podem apresentar diversas funcionalidades biológicas tais como atividades antimicrobiana, anti-hipertensiva, antioxidante, imunomodulatória, opióide e atividade ligante de minerais (PARK, 2009; KORHONEN, 2009). Dessa forma, peptídeos bioativos provenientes das caseínas e proteínas do soro podem atuar nos sistemas gastrointestinal, cardiovascular, endócrino, imune e nervoso central (KORHONEN, 2009).

Os peptídeos bioativos provenientes das proteínas do leite estão presente na sua forma inativa ou latente na estrutura da proteína precursora podendo ser liberados através da fermentação do leite por culturas lácticas proteolíticas, hidrólise enzimática durante a fabricação e maturação de queijos ou, ainda, pela hidrólise de proteínas e peptídeos durante a digestão gastrointestinal (SAITO et al., 2000; SÁNCHEZ-RIVERA et al., 2014; PARK, 2009).

No processo de fabricação de queijos, culturas láticas apresentam a função primária de produzir ácido lático a partir da lactose presente no leite. A acidificação é essencial no processo de fabricação pois favorece a atividade do coagulante, contribui para a expulsão de soro da massa e previne o desenvolvimento de bactérias indesejáveis (FOX e McSWEENEY, 2017; PARENTE et al., 2017). Durante o processo de fermentação, as bactérias ácido láticas hidrolisam as proteínas do leite, principalmente as caseínas, à peptídeos e aminoácidos que são utilizados como fontes de nitrogênio necessárias para o seu crescimento, podendo levar à formação de peptídeos bioativos (HERNÁNDES-LEDESMA et al., 2011).

O processo de fabricação de queijos por coagulação enzimática requer a desestabilização da dispersão coloidal estável na qual as micelas caseínas se encontram no leite. A coagulação do leite se inicia pela fase enzimática da coagulação que consiste na proteólise das moléculas de κ-caseína na ligação Phe_{105}-Met_{106} pela ação do coagulante, liberando a fração C-terminal, κ-CN f106-169, para o meio aquoso. A fração N-terminal da κ-caseína, κ-CN f1-105, é denominada para-κ-caseína e permanece ligada à micela após a hidrólise. Em seguida,

há uma etapa não enzimática da coagulação na qual ocorre a agregação das caseínas, na presença de Ca^{2+}, levando à formação de um gel (HORNE e LUCEY, 2017; CRABBE, 2004). Após a coagulação, o coágulo é cortado e seguem-se as demais etapas de fabricação, que podem variar de acordo com o protocolo de fabricação de cada variedade de queijo, mas, em geral, incluem as etapas de agitação, cozimento no caso de queijos de massa cozida e semi-cozida, dessoragem, enformagem, prensagem e salga (FOX e McSWEENEY, 2017).

A maioria dos queijos, sobretudo os produzidos por coagulação enzimática, são maturados, o que leva a formação das características de sabor, aroma e textura de cada variedade de queijo. Isso ocorre devido a diversas reações bioquímicas que ocorrem durante a maturação tais como glicólise da lactose residual e catabolismo do lactato, lipólise e catabolismo dos ácidos graxos livres, proteólise e catabolismo de amino ácidos e, ainda, catabolismo do citrato em algumas variedades (McSWEENEY, 2017).

A proteólise é considerada a mudança bioquímica mais complexa e, na maioria dos queijos, o principal evento que ocorre na maturação. Os principais agentes proteolíticos que atuam na maturação dos queijos são o coagulante residual, enzimas naturais do leite, como a plasmina, enzimas provenientes do fermento lático, culturas secundárias ou contaminantes e, ainda, proteases exógenas adicionadas para acelerar a maturação (McSWEENEY, 2017; ARDÖ et al., 2017; WALSTRA et al.; 2006). De forma geral, as caseínas são hidrolisadas inicialmente pela ação do coagulante residual e pela plasmina, formando peptídeos de tamanhos grande e intermediário que são posteriormente hidrolisados pelas proteases e peptidases provenientes das culturas láticas ou microflora secundária a pequenos peptídeos e aminoácidos (Figura 2.1). A proteólise provoca mudanças na textura do queijo por meio da hidrólise da matriz proteica, aumento da capacidade de retenção de água decorrente da quebra das ligações peptídicas e consequente liberação de grupos carboxílico e amino. Além de contribuir para o desenvolvimento das características desejáveis em queijos maturados, a proteólise resulta na liberação de diversos peptídeos bioativos durante a maturação dos queijos (ARDÖ et al., 2017).

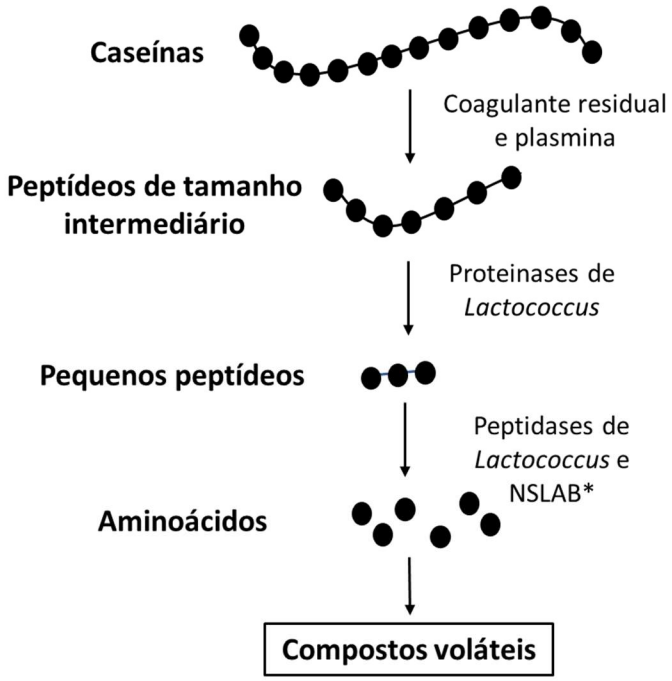

Figura 2.1. Representação esquemática da proteólise durante a maturação de queijos.
*NSLAB (do inglês *non starter lactic acid bacteria*).
FONTE: Adaptado de McSWEENEY, 2017.

Durante os diferentes estágios da digestão, proteínas e peptídeos são submetidos à hidrólise pela ação de enzimas presentes no trato gastrointestinal tais como pepsina, tripsina, quimotripsina e peptidases resultando na liberação de peptídeos de tamanhos de cadeia variados (HERNÁNDES-LEDESMA et al., 2011).

Dentre os peptídeos bioativos, os peptídeos anti-hipertensivos são os mais estudados e bem caracterizados na literatura (GOBBETTI et al., 2002; HERNÁNDEZ-LEDESMA et al., 2011). Atividade anti-hipertensiva associada à presença biopeptídeos foi reportada em queijos comerciais de diversas variedades tais como Gouda (SAITO et al., 2000; BÜTIKOFER et al., 2007), Cheddar (LU et al., 2016; PRITCHARD et al., 2010), Cabrales (GÓMEZ-RUIZ et al., 2006), Idiazábal (GÓMEZ-RUIZ et al., 2006), Roncal (GÓMEZ-RUIZ et al., 2006), Mahón (GÓMEZ-RUIZ et al., 2006), Manchego (GÓMEZ-RUIZ et al., 2006; BÜTIKOFER et al., 2007), Gruyère (BÜTIKOFER et al., 2007) e Emmental (BÜTIKOFER et al., 2007).

A atividade anti-hipertensiva de peptídeos bioativos está associada à habilidade de peptídeos específicos de inibir a ação da enzima conversora de angiotensina (ECA) (HERNÁNDEZ-LEDESMA et al., 2011; AKUZAWA et al., 2009). A ECA faz parte do sistema renina-angiotensina, a principal via de regulação da pressão arterial. Atua como catalizador da

conversão de angiotensina I a angiotensina II, um potente vasoconstritor e, catalisa a hidrólise de bradicinina, um peptídeo vasodilatador, a fragmentos inativos (UDENIGWE e MOHAN, 2019; SIEBER et al., 2010). Essas duas reações resultam em contração dos vasos sanguíneos e no consequente aumento da pressão arterial. Portanto, inibição da ação da ECA pela ação de peptídeos bioativos reduz tanto a formação de angiotensina II quanto a inibição da bradicinina, resultando em redução da pressão arterial (SIEBER et al., 2010; HERNÁNDEZ-LEDESMA et al., 2011).

A avaliação da atividade inibidora de ECA *in vitro* é uma ferramenta muito utilizada para avaliar o potencial bioativo dos queijos (LU et al., 2016; SAITO et al., 2000; GÓMEZ-RUIZ et al., 2006; ONG e SHAH, 2008). Essa ampla utilização ocorre, possivelmente, devido à boa correlação entre os resultados obtidos *in vitro* e a real atividade anti-hipertensiva do produto, demostrada através de testes *in vivo*, com animais (SAITO et al., 2000) e humanos (SEPPO et al., 2003; MIZUSHIMA et al., 2004).

2.2. Fatores que afetam a liberação de peptídeos bioativos durante a fabricação e maturação de queijos

Como a formação de peptídeos ocorre através da proteólise, é possível afirmar que todos os fatores que afetam o perfil enzimático podem também afetar a formação de peptídeos bioativos durante a fabricação e maturação de queijos. Estes fatores incluem o tratamento térmico do leite, o tipo de cultura láctica utilizada, o teor de sal e o tempo de maturação dos queijos (SIEBER et al., 2010).

Em relação ao tratamento térmico do leite, estudos sugerem que a fabricação de queijos com leite cru favorece a formação de peptídeos bioativos, e, em especial, peptídeos inibidores da ECA, devido à presença de enzimas naturais do leite e da maior diversidade de microrganismos que produzem uma variedade de enzimas proteolíticas (BÜTIKOFER et al., 2007; GÓMEZ-RUIZ et al., 2002). Em queijos obtidos a partir de leite pasteurizado, o uso culturas adjuntas proteolíticas que produzem enzimas com especificidade de hidrólise para liberação de peptídeos bioativos tem sido considerado uma estratégia para obtenção de queijos funcionais. Essa estratégia foi utilizada na fabricação de queijo Cheddar adicionado de *Lactobacillus casei* LAFTI® L26 ou *Lactobacillus acidophilus* LAFTI® L10 (ONG e SHAH, 2008) e na fabricação de *queso Fresco* mexicano adicionado de *Lactococcus lactis* ssp. *lactis*, *Enterococcus faecium*, *Lactobacillus casei*, e misturas de *Lactococcus lactis* ssp. *lactis*–*Lactobacillus casei* e *Lactococcus lactis* ssp. *lactis*–*Enterococcus faecium* (TORRES-

LLANEZ et al., 2011). Ambos os estudos reportaram maior atividade inibidora da ECA nos queijos adicionados de cultura adjunta.

A escolha da cultura adjunta a ser utilizada como modificadora do perfil enzimático de queijos deve considerar tanto seu potencial para liberação de peptídeos bioativos quanto a sua adequação ao processo de fabricação do queijo em questão. Cepas de *Lactobacillus helveticus* têm se mostrado mais capazes de produzir peptídeos anti-hipertensivos do que culturas de *Lactococcus lactis*, que constituem as culturas láctica comumente utilizadas em queijo Prato, devido à atividade acentuada de suas proteases associadas à membrana celular (FUGLSANG et al., 2003; TAVERNITI e GUGLIELMETTI, 2012). Diferentes cepas de *Lactobacillus helveticus* são reconhecidas pela capacidade de produzirem enzimas com especificidade para hidrolisar as proteínas do leite levando à liberação de peptídeos biativos (GRIFFITHS e TELLEZ, 2013). Leites fermentados produzidos com *Lactobacillus helveticus* CP790, *Lactobacillus helveticus* CP611, *Lactobacillus helveticus* CP615 e *Lactobacillus helveticus* JCM 1004 apresentaram efeito anti-hipertensivo quando avaliados *in vivo* em modelo animal (YAMAMOTO et al., 1994).

O *Lactobacillus helveticus* é uma cultura ácido lática termofílica, com temperatura ótima de crescimento na faixa de 42 a 45°C, amplamente utilizada na fabricação de produtos lácteos fermentados devido à sua capacidade de acidificar o leite e produzir peptídeos e compostos aromáticos (SADAT-MEKMENE et al., 2011; SLATTERY et al., 2010). Devido à sua intensa atividade proteolítica e consequente capacidade de melhorar o sabor de queijos através da hidrólise de peptídeos amargos, é comumente utilizado como cultura adjunta na fabricação de queijos duros e semi-duros (SOERYAPRANATA et al., 2004; SOERYAPRANATA et al., 2002; SLATTERY et al., 2010; SADAT-MEKMENE et al., 2011). Dessa forma, por ser uma cultura lática proteolítica reconhecida pelo seu potencial para liberação de peptídeos bioativos e com faixa de temperatura ótima adequada para incorporação na fabricação do queijo Prato, um queijo de massa semi-cozida, com temperaturas de processo que variam entre 35 a 42°C, a adição de *Lactobacillus helveticus* como cultura adjunta consiste em uma interessante estratégia para modificação do perfil peptídico e desenvolvimento de bioatividade nessa variedade de queijo.

Em relação ao teor de sal, estudos relacionam sua redução em queijos à maior atividade proteolítica durante a maturação (RULIKOWSKA et al., 2013; MISTRY e KAPERSON, 1998). No entanto, a relação entre o teor de sal e liberação de peptídeos bioativos e bioatividade de queijos ainda não foi estabelecida. Estudo *in vitro* demonstrou que o teor de sal afeta a estabilidade de β-casomorfinas, peptídeos opióides derivados da β-caseína

(MUEHLENKAMP e WARTHESEN, 1996). De acordo com os autores, a redução no teor de sal favoreceu a hidrólise enzimática dos peptídeos bioativos.

Em estudo conduzido pelo nosso grupo de pesquisa, a redução no teor de sal do queijo Prato (25 e 50%) resultou em redução da intensidade relativa de um peptídeo reconhecido como anti-hipertensivo (α_{s1}-CN f1-9) durante a maturação (BAPTISTA, 2016). Possivelmente, da mesma forma que observado por Muehlenkamp e Warthesen (1996), a redução no teor de sal favoreceu a hidrólise enzimática do peptídeo com atividade anti-hipertensiva durante a maturação.

O tempo de maturação também constitui um fator determinante para a atividade bioativa dos queijos, uma vez que a evolução da maturação está relacionada à liberação e à posterior hidrólise de peptídeos. Os estudos que avaliaram o potencial bioativo dos queijos são, no geral, pontuais, e identificam biopeptídeos originários das diferentes frações caseicas em queijos comerciais, em um único tempo de maturação (SAITO et al., 2000; SIEBER et al., 2010; BÜTIKOFER et al., 2007; SIENKIEWICS-SZŁAPKA et al., 2009; SILVA et al., 2012). Raros estudos acompanharam o processo de maturação avaliando o potencial bioativo do produto ao longo do tempo. Este foi o caso dos estudos conduzidos por Gómez-Ruiz et al. (2002) para queijo Manchego, por um período de 12 meses, e por Ong e Shah (2008) para queijo Cheddar por um período de 6 meses. Para o queijo Manchego, os autores observaram que a atividade anti-hipertensiva, avaliada *in vitro*, aumentou com a evolução da proteólise, mas foi reduzida após 8 meses de maturação, sugerindo a existência de um pico de atividade bioativa devido ao acúmulo de peptídeos em um determinado estágio da maturação. No caso do queijo Cheddar, os autores observaram o aumento da atividade bioativa ao longo do período avaliado (6 meses) e não observaram a redução da atividade neste período. Para o queijo Cheddar, é possível que um tempo de maturação mais elevado fosse necessário para identificar um pico de atividade bioativa.

Dessa forma, o desenvolvimento de queijos maturados com atividade bioativa deve considerar o efeito do tempo de maturação sobre a bioatividade de forma a otimizar esse parâmetro de processo já que o acompanhamento dos peptídeos e da bioatividade desses peptídeos ao longo da maturação permite identificar o período no qual é possível obter a máxima atividade biológica.

2.3. Resistência dos peptídeos bioativos ao trato gastrointestinal

Durante a passagem pelo trato gastrointestinal, proteínas são hidrolisadas pela ação de uma série de enzimas digestivas levando à liberação de peptídeos e aminoácidos

(PICARIELLO et al., 2013). A digestão gastrointestinal de queijos pode afetar significativamente a atividade biológica associada à presença de peptídeos bioativos tanto pela possível liberação de novos biopeptídeos a partir de proteínas e peptídeos presentes no alimento quanto pela hidrólise dos peptídeos bioativos levando a formação de fragmentos sem atividade biológica (Figura 2.2) (SÁNCHEZ-RIVERA et al., 2014). Dessa forma, a presença de peptídeos bioativos no queijo não garante sua atividade biológica quando o produto é ingerido. A bioatividade depende da bioacessibilidade, que é a capacidade do composto bioativo de resistir à ação das enzimas digestivas e atingir o lúmen intestinal (QUIRÓS et al., 2009). Uma vez bioacessíveis, os peptídeos bioativos precisam ser absorvidos no intestino para que se tornem biodisponíveis para o uso em uma função fisiológica (FERNÁNDEZ-GARCÍA et al., 2009).

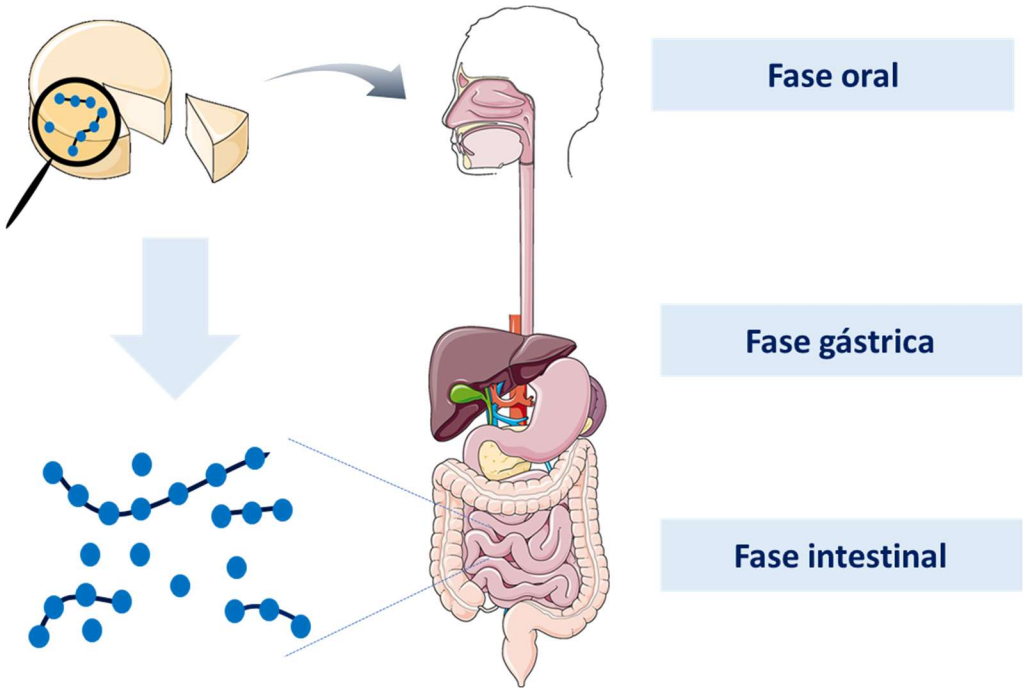

Figura 2.2. Representação esquemática da hidrólise enzimática de peptídeos nas etapas de digestão gastrointestinal.
FONTE: Essa figura foi criada usando imagens fornecidas pelo *Servier Medical Art* (http://smart.servier.com/), sob a licença *Commons Attribution 3.0 License*.

No intuito de melhor compreender as mudanças que ocorrem no perfil de peptídeos bioativos provenientes das caseínas quando submetidos à digestão gastrointestinal, diversos estudos foram realizados utilizando modelos de digestão *in vitro* estáticos e dinâmicos (EGGER

et al., 2019; JIN et al., 2016; MÉNARD et al., 2014; SÁNCHEZ-RIVERA et al., 2014; QUIRÓS et al., 2009) e digestão *in vivo* (BARBÉ et al., 2014; EGGER et al., 2019).

A cinética de digestão de proteínas no trato gastrointestinal e a consequente formação de peptídeos durante o processo digestivo depende de um conjunto de fatores que incluem estrutura da matriz alimentícia, o pH em que a proteína se encontra no alimento e a resistência das proteínas à ação das enzimas digestivas, já que esses fatores afetam a estrutura do quimo formado no estômago, o tempo necessário para atingir o pH ótimo de ação da pepsina e o acesso das enzimas digestivas aos sítios de hidrólise nas estruturas proteicas (BARBÉ et al., 2014).

O efeito de diferentes matrizes lácteas (leite, gel ácido e gel enzimático obtido pela ação de renina) na digestão de proteínas do leite e liberação de peptídeos no trato gastrointestinal foi avaliado por Barbé et al. (2014). Os autores verificaram que apesar da estrutura da matriz proteica apresentar pouca influência na localização dos sítios de hidrólise das enzimas digestivas nas sequências proteicas, afetou o número de peptídeos identificados durante a digestão. Géis ácidos apresentaram maior número de peptídeos identificados durante a digestão quando comparados ao leite e ao gel enzimático, que apresentou o menor número de peptídeos liberados durante a digestão. Possivelmente, o caráter ácido do gel favoreceu a ação de pepsina durante a digestão do gel ácido, enquanto o leite, por ter pH próximo à neutralidade, teve maior efeito tamponante no estomago e retardou a atividade da pepsina devido ao tempo necessário para atingir o pH ótimo da ação da enzima. No mesmo estudo, o gel enzimático apresentou menor número de peptídeos identificados quando comparado ao leite e gel ácido, o que sugere maior resistência do gel enzimático à hidrólise.

A importância da matriz láctea na cinética da digestão proteica e liberação de peptídeos durante o trato gastrointestinal, sugere a necessidade de avaliação da bioacessibilidade de peptídeos bioativos em cada matriz láctea e, em especial, em diferentes variedades de queijo. Devido à diferença de estrutura da matriz proteica em diferentes variedades de queijo, diferentes padrões de modificações estruturais e hidrólise proteica são observados durante a digestão (DO et al., 2018; ASENSIO-GRAU et al., 2019).

Estudo recente descreveu a cinética da proteólise durante a simulação da digestão gastrointestinal de queijo Gouda, um queijo semi-duro. Os autores observaram redução do tamanho de glóbulos de gordura na matriz proteica após a fase oral, a liberação de gordura da matriz proteica durante a fase gástrica da digestão acompanhada de proteólise pela ação da pepsina e degradação da estrutura proteica mais pronunciada durante a fase intestinal (ŽOLNERE et al., 2019).

A avaliação da bioacessibilidade de peptídeos de queijo maturado foi conduzida por Sánchez-Rivera et al. (2014) em estudo peptidômico no qual foram avaliados os perfis de peptídeos de queijo *Valdeón* e de leite em pó desnatado antes e após a simulação de digestão *in vitro* estática com fluidos gástrico e duodenal. Os autores observaram a presença de 121 peptídeos no queijo e 139 peptídeos na solução obtida após a digestão, sendo que 28 peptídeos presentes no queijo demonstraram resistência ao trato digestivo, sendo identificados após a digestão simulada. O queijo apresentou peptídeos com atividades inibidora da ECA e opióide enquanto a solução obtida após a digestão gastrointestinal simulada apresentou peptídeos com atividade anti-hipertensiva, antioxidante e antibacteriana. Os resultados obtidos após a digestão do leite em pó, no entanto, demonstram que nem todas as sequencias bioativas identificadas após a digestão do queijo foram observadas para o leite em pó digerido, sugerindo que as diferenças nos perfis de peptídeos obtidos se devem aos diferentes precursores presentes nas amostras.

A modificação do perfil peptídico ao longo da simulação da digestão gastrointestinal de produtos lácteos fermentados sugere a necessidade de inclusão de experimentos de digestão *in vitro* ou *in vivo* em estudos que objetivem desenvolver bioatividades associadas à presença de peptídeos bioativos em queijos.

2.4. Digestômica e os modelos de digestão *in vitro*

Acompanhando a tendência de estudos "ômicos", o termo "digestômica de proteínas" foi proposto por Picariello et al. (2013) como referência ao estudo global da digestão de proteínas alimentares e a liberação de peptídeos funcionais e ativos. Esse estudo envolve a maior compreensão da bioacessibilidade dos compostos bioativos presentes em alimentos.

O estudo da bioacessibilidade de compostos bioativos envolve a identificação dos componentes liberados no trato gastrointestinal que efetivamente chegam ao lúmen intestinal antes de serem absorvidos ou excretados (PICARIELLO et al., 2013). Diferentes métodos analíticos podem ser empregados para avaliar a bioacessibilidade de compostos bioativos *in vivo* ou *in vitro* (FERNÁNDEZ-GARCÍA et al. 2009).

Experimentos *in vivo* são caros e menos viáveis do que os experimentos *in vitro* devido às dificuldades técnicas e preocupações éticas envolvidas no uso de animais e humanos em estudos de digestão (PICARIELLO et al., 2013). Por esse motivo, modelos de digestão *in vitro* têm sido aperfeiçoados e cada vez mais utilizados como uma alternativa aos estudos *in vivo* (GUERRA et al., 2012).

Os modelos de digestão *in vitro* foram desenvolvidos para simular as condições fisiológicas do trato gastrointestinal humano à partir da mimetização das condições físico-químicas da digestão humana que levam à transformações mecânicas e enzimáticas, considerando três áreas do sistema digestivo: boca, estômago e intestino (FERNÁNDEZ-GARCÍA et al. 2009; GUERRA et al., 2012). A desintegração mecânica dos alimentos ocorre principalmente nas etapas oral e gástrica, enquanto atividades enzimáticas são predominantes nas fases gástrica e intestinal da digestão. Modelos de digestão *in vitro* podem variar de modelos constituídos de um único reator estático a sistemas dinâmicos que incluem processos físico-químicos e mecânicos, bem como em mudanças nas condições do lúmen como as que ocorrem *in vivo* (GUERRA et al., 2012).

Os modelos estáticos são os métodos de digestão *in vitro* mais empregados (GIROMINI et al., 2019). Nesses modelos, as proteínas são sequencialmente expostas às condições que simulam boca, estômago e intestino, cuja seleção de etapas varia de acordo com o modelo utilizado sendo os modelos que simulam o estômago e o duodeno os mais empregados no estudo da digestão proteica (PICARIELLO et al., 2013). Diversos protocolos de digestão estática foram propostos e utilizados ao longo dos anos (SÁNCHEZ-RIVERA et al., 2014; QUIRÓS et al., 2009; HERNÁNDEZ-LEDESMA et al., 2004, JIN et al., 2016; LIU e PISCHETSRIEDER, 2017). No entanto, diferenças nos protocolos disponíveis na literatura dificultam a comparação de dados obtidos. Em 2014, um protocolo harmonizado de simulação estática *in vitro* da digestão gastrointestinal foi proposto pelo *INFOGEST,* uma rede de cooperação internacional composta por mais de 200 cientistas de 32 países com objetivo de consolidar condições de digestão *in vitro* de alimentos através de um modelo consensual (MINEKUS et al., 2014). O protocolo proposto por Minekus et al. (2014), baseado em condições fisiológicas do organismo humano, é um método composto por três fases estáticas e sequenciais (fase oral, fase gástrica e fase intestinal), com parâmetros padronizados como concentração de eletrólitos, enzimas, bile, fatores de diluição, pH e tempos de digestão.

Em estudo comparativo entre o método *in vitro* proposto pela rede *INFOGEST* e a digestão *in vivo* usando modelo animal para avaliação da digestão de leite em pó desnatado, resultados similares foram observados para níveis de proteínas, peptídeos e aminoácidos livres no fim das etapas gástrica e intestinal em ambos os sistemas (EGGER et al., 2017). Recentemente, uma atualização desse protocolo foi publicada, o *INFOGEST* 2.0 (BRODKORB et al., 2019). A principal modificação em relação ao protocolo original foi a inclusão da lipase gástrica, que embora apresente importância na digestão de alimentos de base lipídica, não foi

considerada na publicação original devido à indisponibilidade de enzimas comerciais, na época, que reproduzissem a atividade da lipase gástrica humana (MINEKUS et al., 2014).

A despeito da harmonização de modelos de digestão *in vitro* estáticos seguindo as condições fisiológicas conhecidas, modelos de digestão estáticos são simplificações de um processo *in vivo* complexo já que é impossível mimetizar a cinética de digestão em modelos estáticos. A hidrólise enzimática que ocorre durante a simulação estática da digestão é especialmente diferente do observado *in vivo* na digestão gástrica de matrizes alimentícias sólidas e semi-sólidas. Nessa etapa da digestão, o pH é gradualmente reduzido e o quimo alimentar é dinamicamente hidrolisado e gradualmente liberado no duodeno, condições que não são mimetizadas em modelos estáticos (EGGER et al., 2016). Nesse contexto, os modelos de digestão dinâmicos apresentam vantagens em relação aos modelos estáticos por considerarem o trânsito gradual dos compostos ingeridos com esvaziamento dinâmico dos compartimentos digestivos e mistura apropriada em cada estágio devido aos movimentos peristálticos (PICARIELLO et al., 2013).

Diversos modelos de digestão dinâmicos já foram propostos e podem apresentar um, dois ou múltiplos compartimentos e utilizam controles de temperaturas, pHs, esvaziamento gástrico, adições de pepsina, suco pancreático e bile, de forma a simular as condições que ocorrem *in vivo* (GUERRA et al., 2012).

Os sistemas TIM (do inglês *TNO intestinal model*), modelos de digestão gastrointestinal do TNO, organização holandesa de pesquisa em ciência aplicada, são modelos multi-compartimentais que simulam as condições dinâmicas do trato gastrointestinal com controle de parâmetros fisiológicos como agitação, trânsito do alimento, pHs, secreção de fluidos digestivos e remoção de compostos e água. O sistema TIM-1, configuração mais utilizada da plataforma TIM, é composto por quatro compartimentos que simulam as condições do estômago, duodeno, jejuno e íleo. Esses compartimentos são conectados por bombas peristálticas que viabilizam a transferência controlada e dinâmica dos fluidos de um compartimento ao outro (MINEKUS, 2015).

O DIDGI® é um modelo de digestão dinâmica *in vitro* simples, composto por 2 compartimentos consecutivos simulando as condições do estômago e duodeno. O sistema foi validado pela comparação da cinética da proteólise no sistema *in vitro* com dados coletados *in vivo* utilizando modelo animal. A relevância fisiológica do sistema proposto foi demonstrada pela boa correlação entre dados obtidos *in vitro* e *in vivo* (MÉNARD et al., 2015).

O modelo de digestão *in vitro* SHIME® (do inglês *simulator of the human intestinal microbial ecosystem*) é um exemplo de modelo de digestão dinâmico colônico que simula o

ecossistema microbiano humano com a utilização de reatores que mimetizam diferentes etapas do trato gastrointestinal (MOLLY et al., 1993). O modelo é composto por 5 reatores que simulam o estômago, o duodeno e os cólons ascendente, transversal e descendente em condições controladas (pH, tempo de residência e temperatura) (BIANCHI et al., 2014). O SHIME®, por ser um modelo de digestão dinâmico colônico, é frequentemente utilizado para avaliação do efeito da alimentação da modulação da microbiota intestinal (DUQUE et al., 2016; BIANCHI et al., 2014).

Independentemente do modelo empregado, estudos que tenham como objetivo o acompanhamento da digestão de peptídeos e proteínas requerem a identificação do perfil de peptídeos formados. A espectrometria de massas é considerada a melhor ferramenta analítica para o acompanhamento dos peptídeos formados durante a digestão de proteínas e peptídeos, por permitir a aquisição de dados confiáveis sobre a cinética de liberação dos peptídeos provenientes dos alimentos no corpo humano (DUPONT e TOME, 2014; PICARIELLO et al., 2013). O uso do MALDI-MS (Espectrometria de massas por ionização e dessorção a laser assistida por matriz) para a análise proteômica e peptidômica das etapas da digestão gastrointestinal apresentam como vantagem a especificidade e a sensibilidade da resposta analítica para identificação de proteínas e peptídeos. O uso de espectrômetros de massas, que permitem a separação das moléculas ionizadas de acordo com sua razão massa-carga (*m/z*), tem sido fundamental para o avanço do estudo proteômico e peptidômico da digestão proteica (PICARIELLO et al., 2013).

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CAPÍTULO 3 - ARTIGO 1. *Lactobacillus helveticus* LH-B02 favours the release of bioactive peptide during Prato cheese ripening

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ABSTRACT

Adjunct cultures are traditionally used in cheesemaking with the aim of improving the flavor development of ripened cheeses. Additionally, different adjunct cultures are recognized for the ability to produce enzymes with specificity to hydrolyze caseins, leading to the release of bioactive peptides. The objective of this study was to evaluate the effect of the addition of the adjunct culture *Lactobacillus helveticus* LH-B02 on the proteolysis and peptide profile of Prato cheese during ripening. The addition of the adjunct culture affected the Prato cheese peptide profile and favored the release of peptides from β-casein and, in particular, the Angiotensin-converting enzyme (ACE)-inhibitory peptide β-CN (f194-209) (*m/z* 1718), which was the most important variable for samples discrimination after 60 d of ripening.

3.1. Introduction

In the functional foods era, great prominence has been given to dairy proteins as the main source of biologically active peptides. Milk proteins have several amino acid sequences with latent biological activities encrypted in their primary structure (Korhonen, 2009). Hydrolysis of these proteins through fermentation and ripening processes, and even during gastrointestinal digestion may result in the release of peptides with different bioactivities such as antimicrobial, antioxidant, immunomodulatory, opioid, mineral carrier, and antihypertensive activities (Fitzgerald & Murray, 2006; Park, 2009).

In cheeses, the bioactive peptides are released through the enzymatic hydrolysis of the milk proteins in the fermentation and ripening processes (Sánchez-Rivera et al., 2014; Park, 2009). During ripening, caseins are hydrolyzed by the action of the residual coagulant, natural milk enzymes, enzymes from starter culture, secondary cultures, and non-starter lactic acid bacteria leading to the release of peptides, including the bioactive peptides (McSweeney, 2004; Park, 2009).

Prato cheese, one of the most popular Brazilian cheeses, is the third most produced cheese in the country (Datamark, 2018). It is classified as a semi-hard, high-fat cheese, obtained by enzymatic coagulation of milk and ripened for at least 25 days (Brasil, 1997). Prato cheese proteolysis is mainly characterized by the initial hydrolysis of α_{s1}-casein by the action of the residual chymosin at the Phe_{23}-Phe_{24} bond and the slower degradation of β-casein during ripening (Alves, Merheb-Dini, Gomes, Da Silva, & Gigante, 2013; Chaves & Gigante, 2016; Baptista, Araújo, Eberlin, & Gigante, 2017b).

Even though the biopeptides can be found in cheeses as a result of the natural ripening process (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; Bütikofer, Meyer, Sieber,

& Wechsler, 2007; Lu, Govindasamy-Lucey, & Lucey, 2016; Pritchard, Philipps, & Kailasapathy, 2010; Gómez-Ruiz, Taborda, Amigo, Recio, & Ramos, 2006; Baptista, Araújo, Eberlin, & Gigante, 2017a), from the industrial point of view, it is interesting to understand the effect of different process variables on the peptide profile formed during cheese ripening. This information may allow the establishment of the conditions that lead to maximum bioactivity. Based on the general principles of proteolysis, it is possible to state that the parameters that affect the enzymatic activity, such as the type of culture used and ripening time (Sieber et al., 2010), also affect the release of bioactive peptides. Therefore, the formation of these peptides could be intensified by the appropriate choice of lactic culture and ripening time.

The use of adjunct cultures in cheesemaking has been explored due to their ability to produce enzymes with specificity to hydrolyze caseins leading to the release of peptides with different bioactivities (Torres-Llanez, González-Córdova, Hernandez-Mendoza, & Garcia, 2011; Ong & Shah, 2008). The choice of the adjunct culture to be used in cheesemaking should consider the manufacturing process of each cheese variety and the desirable characteristics of the final product. The use of different strains of *Lactobacillus helveticus*, a thermophilic culture with optimum growth temperature between 42 and 45°C, was initially proposed for the manufacture of hard and semi-hard cheeses with the aim of favoring the development of the flavor of ripened cheeses through hydrolysis of bitter-tasting peptides (Slattery, O´Callaghan, Fitzgerald, Beresford, & Ross, 2010; Sadat-Mekmene, Genay, Atlan, Lortal, & Gagnaire, 2011; Soeryapranata, Powers, Weller, Hill, & Siems, 2004, Soeryapranata et al., 2002b). Moreover, this lactic culture has also been recognized by its ability to favor the release of bioactive peptides in fermented dairy products (Griffiths & Tellez, 2013; Yamamoto, Akino & Takano, 1994). The potential of *Lactobacillus helveticus* strains to produce bioactive peptides is due to the complex proteolytic system generally composed of three types of components: cell-envelope proteinases that act in the hydrolysis of caseins to oligopeptides; oligopeptide transport system through bacterial membrane and intracellular peptidases (Sadat-Mekmene et al., 2011).

Our hypothesis for this study was that the use of an adjunct culture that favors the formation of bioactive peptides might allow the design of a functional Prato cheese with defined intensities of bioactive peptides. To our knowledge, the use of *Lactobacillus helveticus* as an adjunct culture in Prato cheese production has not previously been studied at the molecular level through the identification of peptides that are released during cheese ripening. Therefore, the objective of this study was to evaluate the effect of the addition of the adjunct culture

Lactobacillus helveticus LH-B02 on the proteolysis and peptide profile of Prato cheese during ripening.

3.2. Materials and Methods

3.2.1. Preparation of culture

Lyophilized starter cultures of *Lactococcus lactis* spp *lactis* and *Lactococcus lactis* spp *cremoris* (R 704; Chr. Hansen, Valinhos, Brazil) were activated in 10% (w/v) sterile reconstituted skim milk and incubated at 30 °C for 8 h. The lyphilized adjunct culture of *Lactobacillus helveticus* (LH-B02; Chr. Hansen, Valinhos, Brazil) was activated in 10% (w/v) sterile reconstituted skim milk and incubated at 40 °C for 18 h. The incubation time required for the activation of the culture was previously established by the growth curve of the microorganism.

3.2.2. Cheese manufacture

The cheeses were produced by the traditional manufacturing method as described by Mazal, Vianna, Santos, & Gigante (2007) with modifications, using 50 l whole cows' milk. After the heat treatment (68 °C for 2 min) sufficient to inactivate alkaline phosphatase (Alves et al., 2013; Chaves & Gigante, 2016; Baptista et al., 2017b), milk was cooled to 35 °C and divided into two portions. One portion was used for the manufacture of control Prato cheese, with the addition of starter culture of *Lactococcus lactis* spp *lactis* and *Lactococcus lactis* spp *cremoris* (R 704 - Chr. Hansen, Valinhos, Brazil). The second portion was used for the manufacture of Prato cheese with the addition of starter culture of *Lactococcus lactis* spp *lactis* and *Lactococcus lactis* spp *cremoris* (R 704 - Chr. Hansen, Valinhos, Brazil) and the adjunct culture of *Lactobacillus helveticus* LH-B02. In both cases, calcium chloride (250 ppm), starter culture (1%, v/v) and adjunct culture (1%, v/v) when applied, urucum dye (80 ppm) and coagulant (Ha La 1175, Chr. Hansen, Valinhos - SP, Brazil) sufficient to coagulate the milk within 35 min were added. After coagulation, the curd was cut in 1 cm cubes and submitted to slow continuous mixing for 15 min, which was followed by indirect heating to increase the temperature of the curd by 1 °C every 3 min until 42 °C. After stirring for 40 min at 42 °C, the whey was drained off, and the curd was placed in rectangular cheese molds (13 cm x 8 cm x 7 cm) with cheesecloth, pressed with sucessive turns (0.1 MPa for 15 min; 0.1 MPa for 15 min; 0.24 MPa for 30 min, and 0,31 MPa for 90 min) and kept at room temperature for 5 h. The cheeses were salted for 12 h in static brine [20% (w/v) NaCl, 0.5% (w/v) $CaCl_2$, pH 5,5]. The volume of the brine was 3.5 times greater than the volume of cheese, and it was kept at 4 °C

during salting period. After salting, cheeses were dried at 12 °C for 48 h, vacuum-packed into heat-shrinkable packaging (co-extruded multilayer film combining polyamide and ethylene-vinyl acetate) and stored at 12 °C for 60 d.

3.2.3. Evaluation of cheeses during storage

After 1, 15, 30 and 60 d of storage, the cheeses were evaluated for hydrolysis profile of caseins by capillary electrophoresis (CE) and peptide profile by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

3.2.3.1. Analysis of proteolysis by Capillary Electrophoresis

The electrophoretic profile of caseins was analyzed by CE according to Ortega, Albillos, & Busto (2003) and Otte, Zakora, Kristiansen, & Qvist (1997), with the modifications described by Alves et al. (2013). CE was carried out using a P/ACE MDQ system (Beckman Coulter, Santana de Parnaíba, SP, Brazil) controlled by Karat software (Beckman Coulter). The separation was performed using a 57 cm fused-silica capillary (50 cm effective length x 75 μm internal diameter). To dissociate caseins, cheese samples (20 mg) were dissolved in 1 mL of sample buffer containing 10 mM sodium phosphate, 8 M urea and 10 mM dithiothreitol (DTT) at pH 8.0 and kept at room temperature for 1 h. The samples were filtered (0.45 μm) and injected for 5 s at 3.45 x 10^3 Pa. The separation occurred at 18.5 kV, and 23 °C and the detection was performed at 214 nm for 70 min. Between runs, the capillary was conditioned by washing with NaOH (0.5 M) for 5 min, pure water for 5 min and running buffer (10 mM sodium phosphate, 6 M urea and 0.05% hydroxypropyl methylcellulose, pH 3.0) for 5 min. α_s-CN, β-CN and κ-CN standards (Sigma-Aldrich, St. Louis, MO, USA) were used for peak identification.

3.2.3.2. Fractionation of peptides by the solubility at pH 4.6 and 70% ethanol

Grated cheese samples (40g) were homogenized with 80 mL of water for 10 min in a Stomacher 400 (Seward Laboratory, Worthing, UK), as described by Kuchroo & Fox (1982). Peptides fractionation was performed according to the procedure of Piraino et al. (2007). The homogenate obtained was adjusted to pH 4.6 with 1.0 M HCl, maintained at room temperature for 30 min, and then the pH was readjusted to 4.6. The homogenate was kept at 40 °C for 1 hour and centrifuged at 3000g for 30 min at 4 °C in a Allegra R64 Centrifuge (Beckman Coulter, Indianápolis, IN, USA). The supernatant was filtered through glass wool and Whatman filter paper No. 113 and frozen at -80 °C. This supernatant was concentrated by freeze-drying. Duplicate aliquots of the freeze-dried pH 4.6-soluble extract (10 mg) were dissolved in 1 mL

of 70% ethanol, hold at room temperature for 30 min and centrifuged at 13000g for 10 min. The supernatant, pH 4.6 and 70% ethanol-soluble extract, was analyzed by MALDI-MS.

3.2.3.3. Peptide analysis by Matrix-assisted laser desorption/ionization mass spectrometry

Mass spectra were acquired with a using a MALDI-TOF/TOF Autoflex III instrument (Bruker Daltonics, Germany) equipped with a SmartBeam laser. The instrument was calibrated using peptide calibration standard II from Bruker Daltonics (Bremen, Germany), which is composed by 9 peptides (Bradykinin 1-7, M+H 757.3992, monoisotopic; Angiotensin II, M+H 1046.5418, monoisotopic; Angiotensin I, M+H 1296.6848, monoisotopic; Substance P, M+H 1347.7354, monoisotopic; Bombesin, M+H 1619.8223, monoisotopic; Renin Substrate, M+H 1758.9326, monoisotopic; ACTH clip 1-17, M+H 2093.0862, monoisotopic; ACTH clip 18-39, M+H 2465.1983, monoisotopic; Somatostatin 28, M+H 3147.4710, monoisotopic). Samples (1 µL) were applied on a steel target (MSP 96 polished-steel target, Bruker Daltonics, Bremen, Germany), dried at room temperature and covered by the matrix solution (CHCA dissolved in 70% acetonitrile and 0.25% trifluoroacetic acid). After the complete evaporation of the droplet at room temperature, the plate was inserted in the mass spectrometer. The equipment was operated in the positive ion reflector mode in the mass range of *m/z* 600 to 3500, controlled by Flex Control 3.3 software (Bruker Daltonics). The laser power was adjusted to 50% to 80% and ion source 1, ion source 2, lens, reflector, and reflector 2 were 20,00, 17,77, 7,90, 21,95 e 10,03 kV, respectively. The extraction time of the pulsed ions was 30 ns and the mass suppression threshold was set at *m/z* 600. Each duplicate sample was analyzed in quintuplicate (5 different spots).

3.2.3.4. Data processing and chemometric analysis

Mass spectra were processed using FlexAnalysis 3.4 software (Bruker Daltonics), which included baseline subtraction and normalization. For each spectrum, a mass list and corresponding relative intensities (%) were exported to Excel, where the data matrix was assembled. The online software MetaboAnalyt 3.0 was used for the chemometric analysis (Xia, Mandal, Sinelnikov, Broadhurst, & Wishar, 2012), in which data was normalized by sum and pretreated by the Pareto scaling method. The chemometric tool used was the partial least squares discriminant analysis (PLS-DA), a supervised method used to identify important variables with discrimination power, which was validated by multiple correlation coefficients

(R2) and cross-validation (Q2). The significance of the biomarkers was ranked using variable importance in projection score (VIP score> 1) from the PLS-DA model.

3.3. Results and Discussion

The evaluation of cheese proteolysis by CE (Figure 3.1) revealed a pattern of casein hydrolysis characteristic of cheese ripening, with the formation of para-κ-casein, the release of α_{s1}-I-casein due to α_{s1}-casein hydrolysis by chymosin at the Phe_{23}-Phe_{24} bond and the hydrolysis of β-casein, evidenced by the different peak intensities of this fraction over the 60 d of ripening. Control cheese and cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct showed similar casein degradation profile. However, after 60 d of ripening, an unidentified peak appeared only in the cheese with addition of *Lactobacillus helveticus*, possibly due to the greater proteolytic activity of this culture on the casein fractions and the consequent formation of degradation products (Figure 3.1B).

Casein peptides were identified throught the comparison of the mass-to-change ratio (*m/z)* values obtained in the mass spectra (Supplementary material: Figures 3.S1 and 3.S2) for protonated molecules and thoses from peptides previously identified in cheese samples (Addeo et al., 1992; Addeo et al., 1994; Alli, Okonieska, Gibbs, & Kunishi, 1998; Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Exterkate, Lagerwerf, Haverkamp, & Schalkwijk, 1997; Gagnaire, Mollé, Herrouin, & Léonil, 2001; Gouldsworthy, Leaver, & Banks, 1996; Pihlanto-Leppälä, Rokka, & Korhonen,1998; Soeryapranata, Powers, & Ünlü, 2008; Soeryapranata, et al. 2004). Among 63 ions detected, 13 were identified as peptides from α_{s1}-casein, 10 from β-casein, 1 from κ-casein and 1 from α_{s2}-casein (Table 3.1).

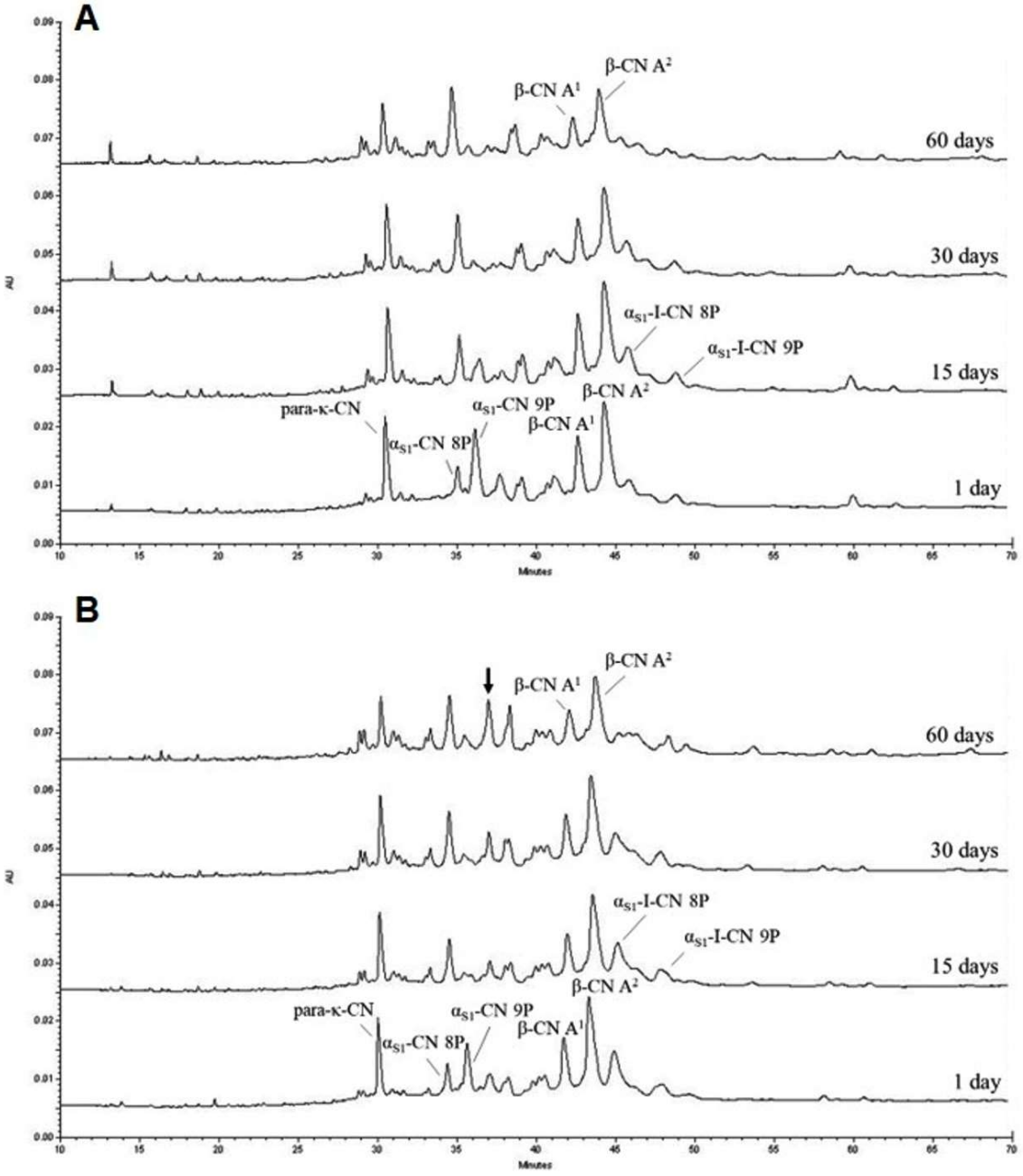

Figure 3.1. Capillary electropherograms of control Prato cheese (A) and functional Prato cheese (B), showing the hydrolysis profile of caseins during storage.

Identification of peaks according to αs-CN, β-CN and κ-CN standards (Sigma-Aldrich, St. Louis, MO, USA).

Table 3.1. Peptides detected by MALDI-TOF-MS in the pH 4.6 and 70% ethanol soluble fractions of control Prato cheese and Prato cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct during ripening.

		Control Cheese				Cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct			
m/z	**Suggested peptide**	**1 d**	**15 d**	**30 d**	**60 d**	**1 d**	**15 d**	**30 d**	**60 d**
748	α_{s1}-CN (f194-199)	-	-	-	-	3.93 ± 2.91	0.68 ± 0.90	-	-
789	*	-	-	-	-	1.12 ± 0.93	-	-	-
792	*	3.61 ± 3.65	2.76 ± 2.02	3.49 ± 2.41	2.13 ± 1.00	1.05 ± 1.00	0.14 ± 0.44	-	-
906	α_{s1}-CN (f17-23) /κ-CN (f161-169)	26.72 ± 13.93	15.85 ± 6.10	10.71 ± 6.14	3.81 ± 1.31	28.28 ± 15.32	11.05 ± 5.56	4.50 ± 2.29	0.57 ± 0.62
944	*	0.14 ± 0.45	-	-	-	0.79 ± 0.85	-	-	-
1019	α_{s1}-CN (f16-23)	-	-	-	-	4.43 ± 2.52	1.58 ± 1.45	0.76 ± 1.00	-
1118	α_{s1}-CN (f15-23)	5.69 ± 2.59	4.59 ± 1.44	3.27 ± 1.59	0.74 ± 0.79	15.77 ± 5.91	7.60 ± 3.16	2.60 ± 1.19	-
1127	*	1.63 ± 1.72	0.13 ± 0.41	0.42 ± 0.88	0.14 ± 0.43	0.21 ± 0.66	-	-	-
1141	α_{s1}-CN (f1-9)	9.80 ± 5.85	5.04 ± 2.91	6.58 ± 4.35	5.15 ± 1.88	2.00 ± 1.87	0.96 ± 1.05	-	-
1151	β-CN (f199-209)	1.04 ± 1.18	-	-	-	-	-	-	-
1152	β-CN (f197-207)	-	-	-	-	4.33 ± 0.70	4.06 ± 0.98	4.39 ± 1.23	5.10 ± 0.87
1199	*	2.76 ± 1.57	2.91 ± 1.61	4.70 ± 2.91	6.05 ± 1.80	1.38 ± 1.24	2.13 ± 1.93	4.65 ± 2.43	3.89 ± 1.54
1204	*	2.21 ± 1.01	-	-	-	-	-	-	-
1209	*	-	1.32 ± 0.92	0.93 ± 1.23	-	-	-	-	-
1247	α_{s1}-CN (f14-23)	12.82 ± 4.99	10.32 ± 3.05	5.05 ± 2.29	0.42 ± 0.67	6.77 ± 2.36	1.95 ± 1.55	0.39 ± 0.82	-
1284	α_{s1}-CN (f4-14)	-	0.46 ± 0.74	0.74 ± 1.21	1.52 ± 1.08	-	-	-	-
1326	*	4.73 ± 2.34	1.76 ± 1.23	1.72 ± 1.67	1.40 ± 0.99	-	-	-	-
1393	β-CN (f194-206)	-	-	-	-	3.66 ± 0.55	3.10 ± 0.77	4.09 ± 1.18	4.54 ± 0.78
1431	*	0.40 ± 0.87	0.28 ± 0.60	0.67 ± 1.09	1.07 ± 0.93	-	-	-	-
1439	*	1.85 ± 1.28	2.02 ± 1.42	3.23 ± 2.21	3.63 ± 0.74	1.00 ± 1.09	1.05 ± 1.40	2.39 ± 1.16	0.40 ± 0.65
1522	*	1.09 ± 1.57	0.90 ± 0.96	0.92 ± 1.49	0.68 ± 0.89	1.81 ± 1.65	0.49 ± 1.04	0.21 ± 0.66	-
1536	α_{s1}-CN (f1-13)	32.51 ± 18.07	27.51 ± 11.36	31.61 ± 18.06	25.65 ± 8.15	41.44 ± 21.77	28.87 ± 14.87	25.16 ± 13.03	12.99 ± 5.44
1556	β-CN (f193-206)	2.44 ± 0.97	0.82 ± 1.06	0.65 ± 1.07	0.94 ± 1.00	2.49 ± 0.91	3.18 ± 0.41	4.04 ± 0.81	5.30 ± 0.54
1558	*	1.16 ± 1.57	0.40 ± 0.84	1.55 ± 2.14	1.06 ± 1.41	1.94 ± 2.31	0.58 ± 1.24	-	-
1574	*	1.05 ± 1.39	0.64 ± 1.04	1.18 ± 1.92	0.53 ± 0.85	2.21 ± 2.07	0.67 ± 1.08	-	-

Table 3.1. continued.

		Control Cheese				Cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct			
m/z	**Suggested peptide**	**1 d**	**15 d**	**30 d**	**60 d**	**1 d**	**15 d**	**30 d**	**60 d**
1590	β-CN (f195-209)	-	-	-	-	2.86 ± 0.39	1.60 ± 1.13	3.26 ± 0.25	3.09 ± 0.21
1614	*	-	-	-	-	-	0.21 ± 0.67	-	1.00 ± 0.88
1642	α_{s1}-CN (f10-23)	-	-	-	-	4.67 ± 0.38	0.87 ± 1.15	-	-
1665	α_{s1}-CN (f1-14)	8.58 ± 4.72	10.24 ± 4.12	13.83 ± 7.55	13.77 ± 4.02	10.58 ± 5.12	8.32 ± 4.09	6.56 ± 3.40	2.25 ± 1.39
1682	*	-	3.30 ± 0.58	4.36 ± 1.00	4.70 ± 0.72	-	-	-	-
1701	β-CN (f12-24) / β-CN (f194-209)	-	-	0.14 ± 0.44	-	1.43 ± 1.55	1.74 ± 1.24	2.85 ± 0.37	2.74 ± 0.22
1718	β-CN (f194-209)	8.14 ± 0.62	4.03 ± 0.32	3.49 ± 0.32	1.65 ± 0.88	50.36 ± 8.40	45.49 ± 2.24	51.93 ± 1.95	45.06 ± 2.31
1740	*	-	-	-	-	1.25 ± 1.33	1.29 ± 1.39	1.61 ± 1.15	1.50 ± 0.82
1756	*	-	-	-	-	1.91 ± 1.39	1.12 ± 1.47	0.60 ± 0.97	1.31 ± 0.95
1764	*	-	-	-	-	3.27 ± 1.88	1.27 ± 1.67	-	-
1782	β-CN (f193-208)	0.16 ± 0.51	-	0.11 ± 0.33	1.20 ± 0.83	-	-	-	0.00 ± 1.00
1853	*	0.57 ± 0.93	0.35 ± 0.75	0.33 ± 0.69	0.32 ± 0.68	0.20 ± 0.64	0.17 ± 0.54	0.31 ± 0.66	1.37 ± 0.49
1863	*	5.53 ± 1.72	4.97 ± 1.61	2.37 ± 1.80	-	5.34 ± 1.45	2.68 ± 1.69	-	-
1877	α_{s1}-CN (f1-16)	88.44 ± 15.42	87.29 ± 23.65	48.47 ± 16.98	17.13 ± 3.30	88.87 ± 19.20	55.55 ± 16.25	23.47 ± 8.65	3.72 ± 0.84
1881	β-CN (f193-209)	97.53 ± 5.41	94.11 ± 7.96	100.00 ± 0.00	100.00 ± 0.00	84.54 ± 14.05	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1899	*	3.49 ± 2.14	3.00 ± 1.73	1.68 ± 2.33	-	3.56 ± 2.84	1.04 ± 1.37	-	-
1903	*	4.18 ± 1.07	3.76 ± 0.83	4.65 ± 1.49	5.32 ± 1.32	3.17 ± 0.67	4.38 ± 1.23	3.79 ± 0.74	3.40 ± 0.52
1907	*	-	-	-	-	1.09 ± 1.15	-	-	-
1915	*	3.20 ± 1.67	3.00 ± 1.70	1.20 ± 1.99	-	4.12 ± 2.40	1.60 ± 1.70	-	-
1919	*	3.90 ± 1.11	4.04 ± 0.69	4.54 ± 1.49	4.15 ± 0.74	3.87 ± 1.58	4.72 ± 1.52	3.39 ± 0.58	3.56 ± 0.83
1977	*	2.76 ± 1.51	2.52 ± 1.78	2.49 ± 1.91	1.66 ± 1.20	-	-	-	-
1991	α_{s1}-CN (f1-17)	19.59 ± 8.49	24.42 ± 7.58	23.36 ± 8.76	17.72 ± 3.78	4.09 ± 1.02	2.90 ± 1.76	2.46 ± 1.03	-
2006	*	-	-	-	-	0.18 ± 0.56	1.11 ± 1.20	1.91 ± 0.72	1.07 ± 0.58
2035	α_{s1}-CN (f7-23)	-	-	-	-	4.79 ± 0.45	0.75 ± 0.97	-	-
2120	*	4.40 ± 1.84	3.98 ± 2.48	3.81 ± 1.30	1.38 ± 1.02	5.23 ± 1.01	4.05 ± 2.39	2.41 ± 0.97	-
2234	*	-	0.97 ± 1.03	0.74 ± 0.96	-	-	-	-	-
2348	*	4.56 ± 3.94	9.90 ± 7.05	5.62 ± 4.34	2.78 ± 1.54	12.45 ± 4.54	11.52 ± 1.78	3.73 ± 1.03	-

Table 3.1. continued.

		Control Cheese				Cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct			
m/z	**Suggested peptide**	**1 d**	**15 d**	**30 d**	**60 d**	**1 d**	**15 d**	**30 d**	**60 d**
2463	*	-	0.25 ± 0.52	0.62 ± 0.68	1.54 ± 0.58	-	0.54 ± 0.69	0.12 ± 0.37	0.39 ± 0.41
2678	*	-	0.82 ± 0.58	0.78 ± 0.69	1.85 ± 0.23	0.24 ± 0.51	1.15 ± 0.62	0.98 ± 0.55	1.03 ± 0.12
2728	*	-	-	-	-	1.05 ± 0.74	-	-	-
2749	*	2.42 ± 0.26	2.19 ± 0.27	-	-	4.81 ± 0.76	1.25 ± 0.88	-	-
2764	α_{s1}-CN (f1-23)	12.67 ± 6.77	14.78 ± 1.00	4.22 ± 0.45	1.00 ± 0.36	28.95 ± 4.49	12.88 ± 0.86	3.95 ± 0.38	0.98 ± 0.10
2786	*	-	-	-	-	0.87 ± 0.62	-	-	-
2802	*	-	-	-	-	0.70 ± 0.74	-	-	-
2836	*	-	-	0.06 ± 0.20	-	0.10 ± 0.30	0.08 ± 0.26	0.39 ± 0.50	0.58 ± 0.31
3051	*	-	0.16 ± 0.34	0.16 ± 0.34	0.43 ± 0.46	0.27 ± 0.46	1.05 ± 0.38	1.14 ± 0.41	1.13 ± 0.08
3398	*	-	-	-	-	-	0.38 ± 0.40	0.73 ± 0.39	-
3497	*	-	0.05 ± 0.17	0.17 ± 0.28	0.38 ± 0.27	-	-	0.05 ± 0.16	-

Table 3.1 shows that the peptide associated with the ion of *m/z* 1881 [β-CN (f193-209)] was the most intense signal in the control cheese at all periods evaluated, and it was, therefore, used as a reference for calculating the relative intensity of the other peaks. In the cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct, the α_{s1}-CN (f1-16) peptide (*m/z* 1877) showed the highest average relative intensity at the beginning of ripening (day 1). However, after 15, 30 and 60 d of ripening, the same most intense peptide in control Prato cheese [β-CN (f193-209) (*m/z* 1881)] was also the most abundant in Prato cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct. The β-CN (f193-209) peptide is formed by the cleavage of the β-casein at the Leu_{192}-Tyr_{193} bond by the action of the residual chymosin (Upadhyay, McSweeney, Magboul, & Fox, 2004) and its high relative intensity was previously observed by our research group in Prato cheese with different salt contents (Baptista et al., 2017b). The accumulation of this peptide, composed of 13 hydrophobic amino acids (AAs) in a total of 17 AAs, has been associated with bitterness in ripened cheeses (Soeryapranata et al. 2002a). The high relative abundance of this peptide [β-CN (f193-209) (*m/z* 1881)] in both cheeses shows that the proteolytic system of *Lactobacillus helveticus* LH-B02 was not able to reduce the relative intensity of this peptide during the 60 d of ripening.

Chemometric analysis of the mass spectral data allowed the evaluation of the effect of the addition of the adjunct culture on the peptide profile of Prato cheese after 1, 30 and 60 d of ripening (Figure 3.2). Partial least squares discriminant analysis (PLS-DA) of control cheese and cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct revealed that they could be distinguished from each other based on the first two PCs, explaining 55.3%, 87.8, and 91.0% of the total variance after 1, 30 and 60 d of storage, respectively.

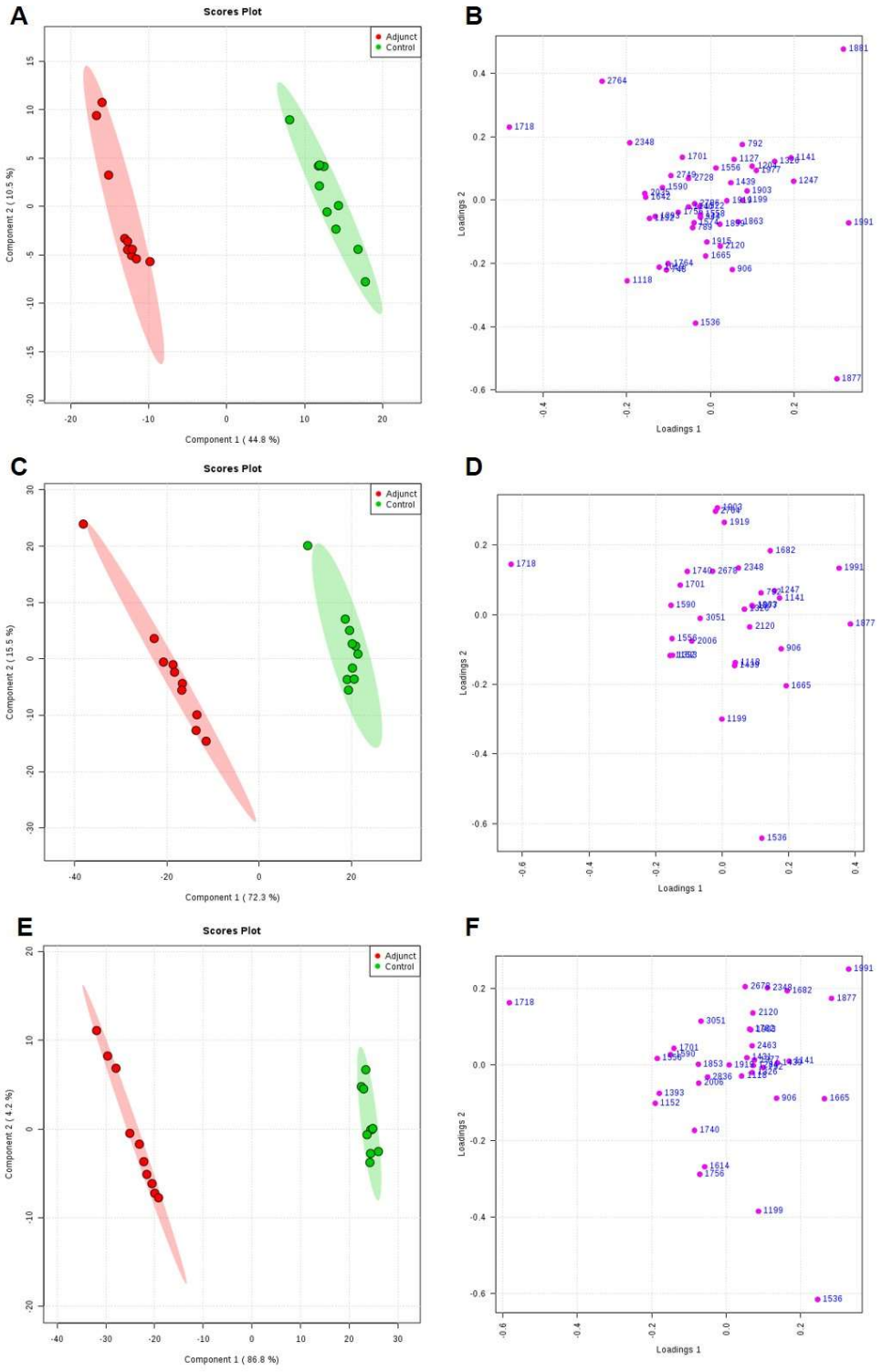

Figure 3.2. Partial least square discriminant analysis (PLS-DA) of mass spectra data of pH 4.6 and 70% ethanol soluble fractions. Score plots (A, C, E) and loading plots (B, D, F) of cheeses after 1 day (A, B), 30 days (C, D) and 60 days (E, F) of storage.

PLS-DA cross validation details were: R2 = 0.99498, Q2 = 0.98222 (A, B); R2 = 0.99098, Q2 = 0.97503 (C, D) and R2 = 0.99812, Q2 = 0.99686 (E, F).

The addition of the adjunct culture affected the peptide profile of Prato cheese, evidenced by the separation of cheeses at all stages of ripening (Figure 3.2). The increase in the total variance explanation over the storage time (55.3% on the first day of ripening, and 91% after 60 d of ripening) revealed that the development of ripening resulted in greater differentiation of cheeses peptide profiles. After 60 d of ripening, the PLS-DA allowed the selection of 9 potential peptides for cheeses discrimination (VIP *score* > 1) as showed in Figure 3.3. The potential peptides α_{s1}-CN (f1-17) (*m/z* 1991), α_{s1}-CN(f1-16) (*m/z* 1877), α_{s1}-CN(f1-14) (*m/z* 1665), α_{s1}-CN (f1-13) (*m/z* 1536), and α_{s1}-CN (f1-9) (*m/z* 1141), were more abundant in the control cheese, and the peptides β-CN (f194-209) (*m/z* 1718), β-CN (f197-207) (*m/z* 1152), β-CN (f193-206) (*m/z* 1556), and β-CN (f194-206) (*m/z* 1393), were more abundant in the cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct (Figure 3.3). Figure 3.3 shows that the peptide β-CN (f194-209) (*m/z* 1718), more abundant in the cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct, was the most important variable for cheese discrimination (VIP *score* = 3.4155).

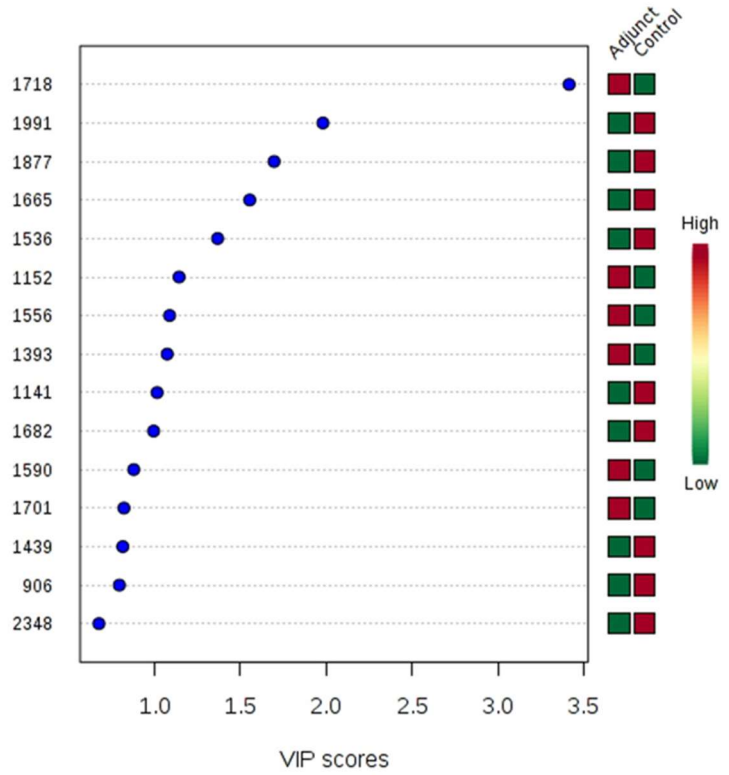

Figure 3.3. Important characteristics identified by PLS-DA for control cheese and cheese with addition of adjunct culture after 60 d of ripening.

Colored boxes on the right indicate the relative concentrations of the corresponding metabolites in each group. VIP score > 1 is considered statistically significant. Potential peptides for cheeses discrimination (VIP score > 1): β-CN (f194-209) (*m/z* 1718), α_{s1}-CN (f1-17) (*m/z* 1991), α_{s1}-CN(f1-16) (*m/z* 1877), α_{s1}-CN(f1-14) (*m/z* 1665), α_{s1}-CN (f1-13) (m/z 1536), β-CN (f197-207) (*m/z* 1152), β-CN (f193-206) (*m/z* 1556), β-CN (f194-206) (*m/z* 1393), α_{s1}-CN (f1-9) (*m/z* 1141).

Although the β-CN (f193-209) peptide (*m/z* 1881) has maintained the high relative intensity until the end of ripening (60 d), all the potential peptides more abundant in the cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct are possible degradation products of this peptide. It demonstrates that the *Lactobacillus helveticus* LH-B02 produces enzymes with specificity to hydrolyze this peptide at the peptide bonds Tyr_{193}-Gln_{194}, Pro_{196}-Val_{197}, Pro_{206}-Ile_{207} and Ile_{207}-Ile_{208}, as shown in Figure 3.4. These peptide bonds were previously identified as hydrolysis sites from different strains of *Lactobacillus helveticus*. The peptides bonds Tyr_{193}-Gln_{194}, Pro_{206}-Ile_{207} and Ile_{207}-Ile_{208} were identified as hydrolysis sites of intracellular enzymes from the strain *Lactobacillus helveticus* W900R. The peptide bonds Tyr_{193}-Gln_{194}, Pro_{196}-Val_{197}, Pro_{206}-Ile_{207} e Ile_{207}-Ile_{208} were identified as hydrolysis sites of intracelular enzymes of

the strain *Lactobacillus helveticus* WSU-19 (Soeryapranata et al., 2004) and the bond Pro_{206}-Ile_{207} was identified was a hydrolysis site of a cell-wall-associated proteinase from *Lactobacillus helveticus* CP790 (Yamamoto, Akino & Takano, 1993).

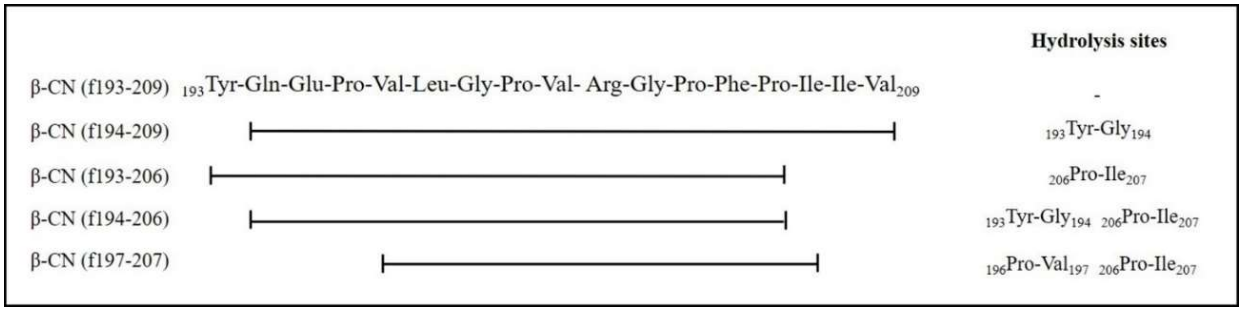

Figure 3.4. Amino acid sequence of the peptide β-CN (f193-209) indicating the identified hydrolysis sites and degradation products.

Among the 9 peptides identified as potential peptides for cheeses discrimination after 60 d of ripening, three are recognized for their bioactive potential: the antihypertensive and ACE-inhibitory peptide α_{s1}-CN (f1-9) (*m/z* 1141) (Saito et al., 2000), the ACE-inhibitory peptide β-CN (f194-209) (*m/z* 1718) (Stepaniak, Jedrychowski, Wróblewska, & Sørhaug, 2001), and the potential antihypertensive peptide β-CN (f193-206) (*m/z* 1556) (Ha et al., 2015). Figure 3.5 shows that the addition of the adjunct culture *Lactobacillus helveticus* LH-B02 resulted in the reduction of the average relative intensity of the ACE-inhibitory and antihypertensive peptide α_{s1}-CN (f1-9) (*m/z* 1141) and in the accumulation of the peptides β-CN (f194-209) (*m/z* 1718) and β-CN (f193-206) (*m/z* 1556), recognized by ACE-inhibitory and potential antihypertensive activities, respectively. The ACE-inhibitory peptide β-CN (f194-209) (*m/z* 1718), which was the most important variable for samples discrimination (Figure 3.3), showed an average relative intensity of 1.65% and 45.06% in the control cheese and in the cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct, respectively, after 60 d of ripening (Table 3.1).

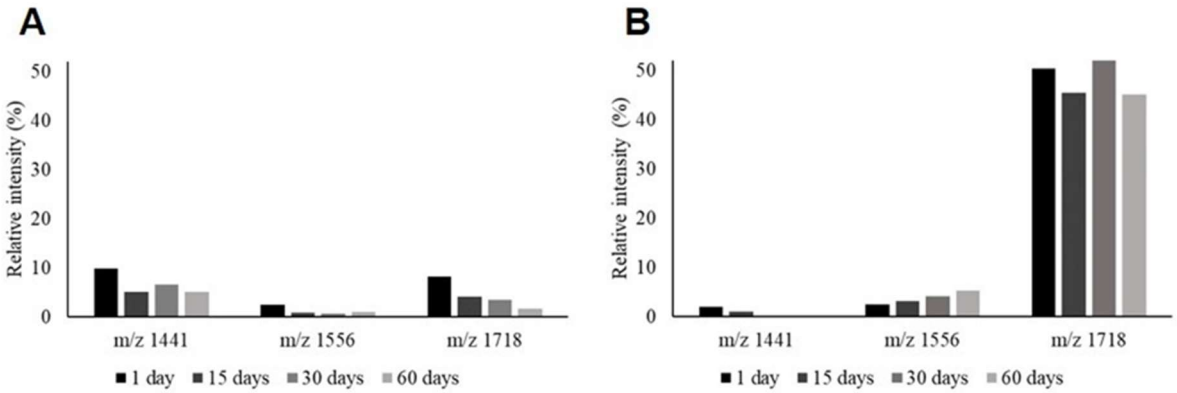

Figure 3.5. Relative intensities (%) of the bioactive peptides αs1-CN (f1-9) (*m/z* 1141), β-CN (f193-206) (*m/z* 1556) and β-CN (f194-209) (*m/z* 1718) during 60 d of storage. (A) control Prato cheese, (B) functional Prato cheese.

The increase in the relative intensity of the hydrophobic peptide β-CN (f194-209) due to the addition of the adjunct culture may be associated with sensory changes, such as the development of bitterness. However, other authors, who did not evaluate the cheese peptide profile, reported higher sensory acceptance of traditional (Azambuja et al., 2017) and reduced fat (Barros, Cunha, Gallina, Viotto, & Viotto, 2006) Prato cheeses produced with *Lactobacillus helveticus* LH-B02 as an adjunct culture when compared to control cheeses without the addition of the adjunct. This results suggest that further studies should be designed to correlate the sensory attributes and the peptide profile of Prato cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct through the association of a complete sensory evaluation with descriptive techniques and mass spectrometry.

3.4. Conclusion

The addition of the adjunct culture of *Lactobacillus helveticus* LH-B02 resulted in changes in the relative intensities of potentially bioactive peptides, favoring the release of β-CN (f193-206) (*m/z* 1556) and β-CN (β194-209) (*m/z* 1718) and reducing the relative intensity of the α_{s1}-CN (f1-9) peptide (*m/z* 1141). Future *in vitro* and *in vivo* studies using animal and human models, however, are necessary to establish the relationship between the addition of the adjunct culture of *Lactobacillus helveticus* LH-B02 in the manufacture of Prato cheese and possible physiological effects associated with its consumption.

Acknowledgments

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Supplementary material

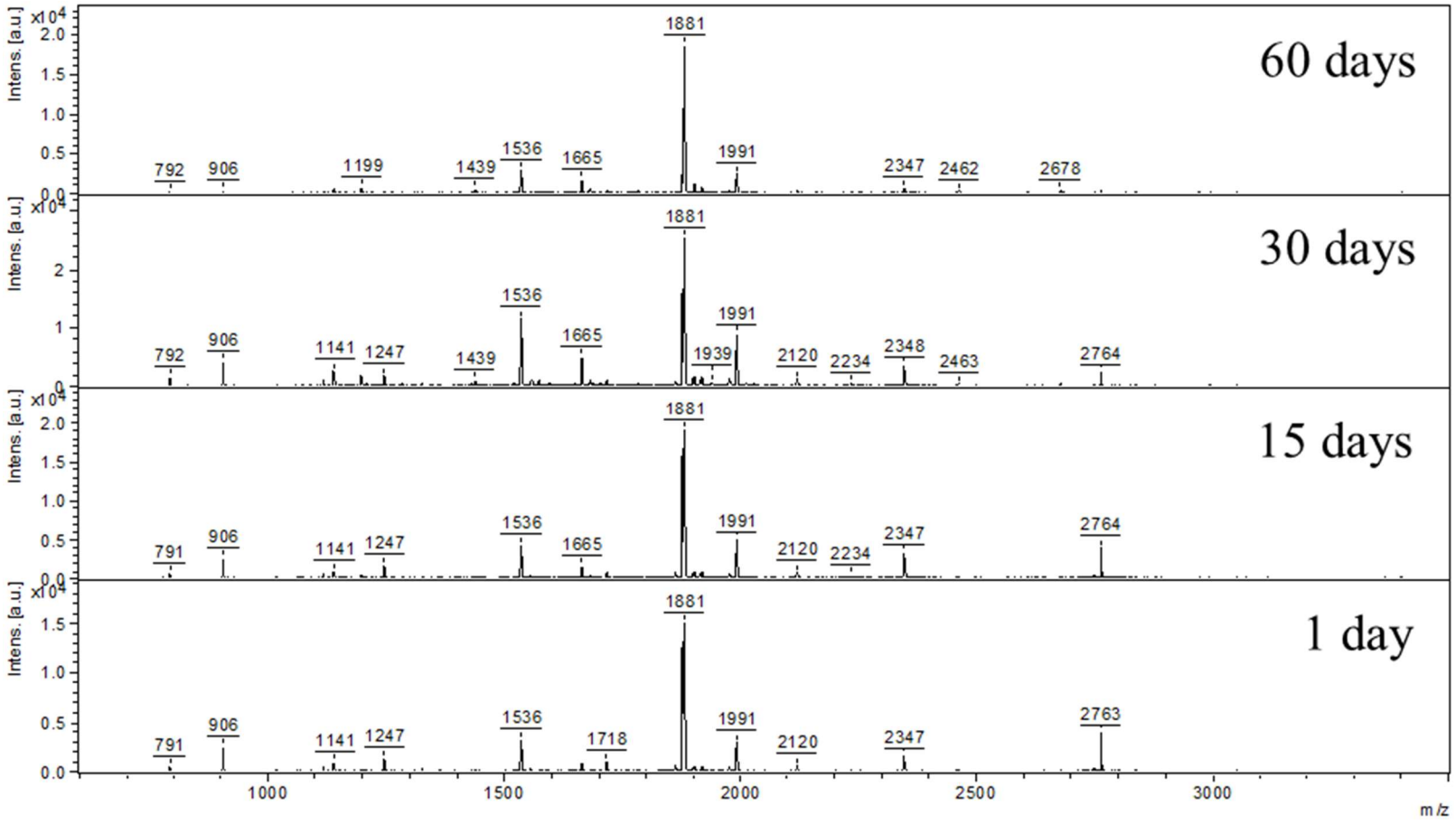

Figure 3.S1. Mass spectra of pH 4.6 and 70% ethanol soluble fractions of the control Prato cheese by MALDI-MS. [a.u.] = arbitrary units; *m/z* = mass-to-charge ratio.

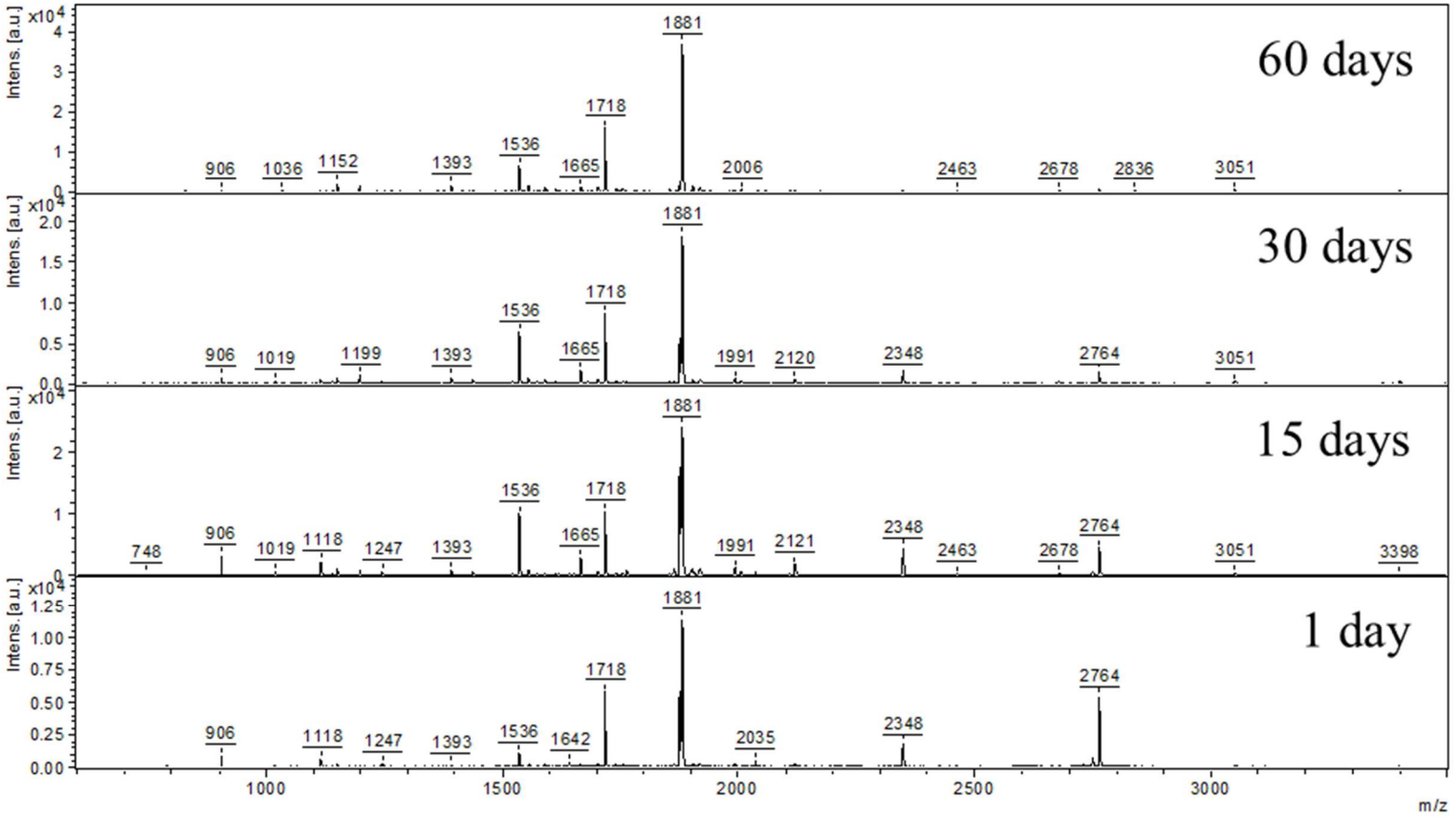

Figure 3.S2. Mass spectra of pH 4.6 and 70% ethanol soluble fractions of the cheese cheese made with *Lactobacillus helveticus* LH-B02 as an adjunct by MALDI-MS. [a.u.] = arbitrary units; *m/z* = mass-to-charge ratio.

CAPÍTULO 4 - ARTIGO 2. Peptide profile and angiotensin-converting enzyme inhibitory activity of Prato cheese with salt reduction and *Lactobacillus helveticus* as an adjunct culture

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ABSTRACT

Among strategies to improve the health-related aspects of dairy products, great prominence has been given to salt reduction and the use of adjunct cultures that can favor the release of bioactive peptides during cheese ripening. This study aimed to evaluate the effect of the salt reduction, the addition of *Lactobacillus helveticus* LH-B02 and the ripening time of Prato cheese on the casein hydrolysis profile by capillary electrophoresis, peptide profile by mass spectrometry, and antihypertensive potential evaluated *in vitro* through the inhibitory activity of the angiotensin-converting enzyme (ACE). Both the salt reduction and the addition of adjunct culture favored the accumulation of the bioactive peptide β-CN (f193-209) (m/z 1881). The adjunct culture led to a higher ACE inhibitory activity during the ripening of Prato cheese, thus proving to be an effective strategy for the development of potentially bioactive cheese.

Keywords: proteolysis, ripening, MALDI-MS, bioactive potential.

4.1. Introduction

The dairy industry has been prominent in the healthy products segment since milk is considered a product of excellent nutritional profile (Walstra, Woulter, & Geurts, 2006). Milk is recognized as nutritious food, and fermented dairy products have been used as vehicles for probiotics (Chaves & Gigante, 2016; Albenzio et al., 2013; Ribeiro et al., 2014) and prebiotics (Martínez-Villaluenga, Frías, Gómez, Vidal-Valverde, 2006), while milk proteins have been considered the main sources of bioactive peptides (Korhonen, 2009). However, in an attempt to meet society's demand for healthy foods, the dairy industry has faced the challenge of reducing the salt content of cheese due to the association between excessive sodium intake and the prevalence of hypertension (Doyle & Glass, 2010). Salt plays an important role in the development of cheese ripening as it affects microbial and enzymatic activities (Guinee & Fox, 2004). Despite the salt reduction has been associated with increased proteolytic activity during ripening (Rulikowska et al., 2013; Mistry & Kaperson, 1998), its relationship with the formation of bioactive peptides has not been established. Muehlenkamp & Warthesen (1996) demonstrated that salt content is determinant for the stability of β-casomorphins, β-casein-derived opioid peptides, and the lower salt content favored the enzymatic activity and degradation of β-casomorphins. A similar result was observed in a previous study of our research group, that found that salt reduction (25% and 50%) led to a lower relative intensity of a peptide with antihypertensive activity (α_{s1}-CN f1-9) in Prato cheese (unpublished results). As reported by Muehlenkamp and Warthesen (1996), the lower salt content may have favored

the enzymatic activity, resulting in hydrolysis of the biopeptide initially produced during ripening.

In cheese, bioactive peptides are released through the enzymatic hydrolysis of milk proteins during fermentation and ripening (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; Sánchez-Rivera et al., 2014; Park, 2009), and antihypertensive peptides are the most studied and well-characterized in the literature among the bioactive peptides.

Peptides with antihypertensive activity act to lower blood pressure by inhibiting the action of angiotensin-converting enzyme (ACE), a major regulator of blood pressure (Hernández-Ledesma, Contreras, & Recio, 2011; Akuzawa, Miura, & Kawakami, 2009). ACE is part of the renin-angiotensin system, which regulates peripheral blood pressure. It acts as a catalyst for the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and simultaneously catalyzes the degradation of bradykinin, a vasodilator peptide. These two reactions result in blood vessel contraction and consequent rise in blood pressure. Thus, the bioactive peptides may play an important role in lowering blood pressure by inhibiting the ACE activity (Sieber et al., 2010; Hernández-Ledesma et al., 2011).

Peptides with antihypertensive activity have been reported in several cheese varieties such as Gouda (α_{s1}-CN f1-9, β-CN f60-68), Manchego (α_{s1}-CN f102-109, α_{s2}-CN f205-208, β -CN f199-204), Crescenza (β-CN f58-72) and Prato (α_{s1}-CN f1-9) (Saito et al., 2000; Gómez-Ruiz, Taborda, Amigo, Recio, & Ramos, 2006; Smacchi & Gobbetti, 1998; Baptista, Araújo, Eberlin, & Gigante, 2017).

Prato cheese is a typical Brazilian ripened cheese, classified as fatty (45 to 59.9% FDM) and medium moisture cheese (36 to 45.9%) (Brasil, 1997). It is the most produced ripened cheese in Brazil (Datamark, 2018), thus studies aiming to improve its functional characteristics have economic and public health interests.

Sodium reduction in Prato cheese had already been explored in the recent literature using different approaches such as the replacement of sodium chloride by other salts and the association of salt reduction with the use of flavor enhancers (Costa et al. 2018; Silva et al., 2018). A recent study by our research group used the simple reduction of salt content as a strategy to reduce sodium content in Prato cheese and demonstrated that it is possible to reduce 25% salt in this type of cheese without compromising the development of ripening and, consequently, the physicochemical and sensory quality of cheese, making it possible to manufacture a healthy product (Baptista et al., 2017). In another study, our research team demonstrated that the use of *Lactobacillus helveticus* LH-B02 as an adjunct culture in the

manufacture of Prato cheese favored the formation of β-casein peptides, in particular the peptide β-CN (f194-209), recognized for its potential angiotensin-converting enzyme (ACE) inhibitor (Baptista et al., 2018).

Our hypothesis for the present study is that the combined effect of the salt reduction and the addition of adjunct culture that favors the release of bioactive peptides can result in a double benefit product with reduced salt and antihypertensive potential. Thus, this study aimed to evaluate the effect of salt reduction, the addition of adjunct culture *Lactobacillus helveticus* LH-B02, and the ripening period (120 days) on proteolysis, peptide profile, and ACE inhibitory activity of Prato cheese.

4.2. Materials and methods

4.2.1. Preparation of lactic cultures

The freeze-dried culture consisting of *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris* (R 704 - Chr. Hansen, Hoersholm, Denmark) was activated in 10% sterile reconstituted milk and incubated at 30 °C for 8 h. One package (50U) of the freeze-dried adjunct culture of *Lactobacillus helveticus* LH-B02 (Chr. Hansen, Hoersholm, Denmark) was rehydrated in 1 L of 10% sterile reconstituted milk for 1 h at 4 °C to avoid culture activation during the rehydration process. The rehydrated culture was then divided into portions for inoculation in the cheese tank, according to the manufacturer's instructions (50U/500 liters of milk), and frozen until cheese processing. Before the use in the cheese manufacturing process, the culture was defrosted and pre-activated for 15 min at 37°C. This condition was defined by preliminary tests performed to establish the best way to introduce the adjunct culture in Prato cheese manufacture without compromising cheese processing and composition (Baptista & Gigante, 2019).

4.2.2. Cheese manufacture

The cheeses were made on a pilot scale in a stainless-steel tank for cheese production using 200 liters of heat-treated whole milk (68 °C/2min), cooled and stored at 4 °C. For each processing, milk was divided into two 100-liter portions: one portion for the manufacture of control Prato cheese, using a traditional O-type starter culture consisting of *Lactococcus lactis* spp *lactis* and *Lactococcus lactis* spp *cremoris* (R 704 - Chr. Hansen, Hoersholm, Denmark) and the other portion used for the manufacture of Prato cheese with the addition of traditional lactic culture and adjunct culture of *Lactobacillus helveticus* LH-B02

(Chr. Hansen, Hoersholm, Denmark). Prato cheeses were processed as described by Mazal, Vianna, Santos, & Gigante (2007), with modifications described by Baptista et al. (2017), except for the type of culture used. First, milk was heated to 35 °C and calcium chloride (250 ppm), O-type starter culture (1%, v/v, 9-10 log CFU/g) or O-type starter culture (1%, 9 -10 log CFU/g) plus adjunct culture (0.2%, v/v, 8-9 log CFU/g), annatto dye (80 ppm) were added. Then, coagulant (CHYMAX, Chr. Hansen, Hoersholm, Denmark) was added for milk coagulation within 35 minutes. After cutting, the curd was submitted to slow continuous mixing for 15 min and indirect heating (1 °C every 3 minutes) was performed until the temperature of 42 °C was reached. This temperature was maintained until reaching the curd mass point. The whey was drained off, and curd was placed in rectangular plastic molds (0.5 kg). The cheeses were then pressed (15 min at 0.1 MPa; 15 min at 0.1 MPa; 30 min at 0.24 MPa, and 90 min at 0.31 MPa) and fermented for 5 hours at room temperature (~ 25 °C).

After pressing, the control and the cheeses made with adjunct culture were divided into two portions, which were salted in static brine for 12 and 6 hours (20% NaCl; 0.5% $CaCl_2$; pH 5.5; 4 °C; volume 3.5 times greater than cheese volume) to obtain the control and reduced-salt cheese, respectively. After drying, the cheeses were vacuum packed and ripened for 120 days at 12 °C. At the end of each processing, four cheese formulations were obtained: control Prato cheese; Prato cheese with salt reduction; Prato cheese with adjunct culture; and Prato cheese with adjunct culture and salt reduction.

4.2.3. Physicochemical composition of milk and cheese

Heat-treated milk was evaluated for the following parameters: pH in a calibrated potentiometer; fat by Gerber method (AOAC, 2006; method 2000.18); acidity (AOAC, 2006; method 947.05); total solids (AOAC, 2006; method 990.19); ash (AOAC, 2006; method 945.46); and total nitrogen (AOAC, 2006; method 991.20). The protein content was determined by multiplying the total nitrogen by 6.38. Milk pasteurization efficiency was evaluated by the activity of the enzymes alkaline phosphatase (AOAC, 2006; method 979.13) and peroxidase (LANARA, 1981).

After one day of manufacture, the cheeses were analyzed for pH and titratable acidity (AOAC, 2006; method 920.124). The physicochemical characterization of the cheeses was performed after 15 days of manufacture for fat by Gerber method (British Standard Institution, 1989), total solids (AOAC, 2006; method 926.08), ash (AOAC, 2006; method 935.42), salt by the Volhard method (Richardson, 1985) and total nitrogen (AOAC, 2006;

method 2001.14). The protein content was calculated by multiplying the total nitrogen content by the conversion factor 6.38. Sodium content was determined by a flame photometer (Digimed DM62, São Paulo, SP, Brazil) after incineration in a muffle (550 °C for 16 hours) and ash dilution in 3% HNO_3 solution.

4.2.4. Evaluation of cheese during ripening

Cheeses were evaluated for the casein hydrolysis profile by capillary electrophoresis (CE), peptide profile by matrix-assisted laser ionization mass spectrometry (MALDI-MS) during ripening, after 1, 15, 30, 60, 90, and 120 days of storage. ACE inhibitory activity was determined after 1, 30, 60, 90, and 120 days of ripening.

4.2.4.1. Fractionation of peptides by pH 4.6-soluble and 70% ethanol-soluble fractions

For the pH 4.6-soluble extract, cheese samples were homogenized in Stomacher 400 (Seward Laboratory, UK) using twice as much water for 10 minutes, as described by Kuchroo & Fox (1982). The fractionation of peptides was performed as described by Piraino et al. (2007). The homogenized mixture was acidified to pH 4.6 with 1.0 M HCl, kept at room temperature for 30 minutes, and then the pH was readjusted to 4.6. The solution was maintained at 40 ° C for 1 hour and centrifuged at 3000 g for 30 minutes at 4 °C in a centrifuge R64 Allegra (Beckman Coulter, Indianapolis, IN, USA). The supernatant was filtered through glass wool and Whatman filter paper n.113 and frozen at -80 °C. The resulting pellet (pH 4.6-insoluble fraction) was analyzed by CE, and the supernatant (pH 4.6-soluble fraction) was subjected to freeze-drying (Edwards Super Modulyo 12k Lyophilizer) for 24 h under the following conditions: condenser temperature -40 °C to -60 °C and pressure 10^{-2} mbar. Duplicate aliquots of the freeze-dried samples (10 mg) were dissolved in 1 mL of 70% ethanol, kept at room temperature for 30 minutes and centrifuged at 13000 g for 10 minutes. The resulting supernatants of the pH 4.6-soluble and 70% ethanol-soluble fractions were separated for MALDI-MS analysis.

4.2.4.2. Analysis of pH 4.6-insoluble fraction by CE

The pH 4.6-insoluble fraction was analyzed by CE according to Ortega, Albillos, & Busto (2003) and Otte, Zakora, Kristiansen, & Qvist (1997) with modifications described by Alves, Merheb-Dini, Gomes, Da Silva, & Gigante (2013). The separation was performed on a

fused silica capillary of total length 57 cm (50 cm effective length x 75 μm) in the P/ACE MDQ system (Beckman Coulter, Santana de Parnaiba, SP, Brazil) and Karat software (Beckman Coulter). For that, 20 mg of the pH 4.6-insoluble fraction was dissolved in 1 mL buffer solution containing 10 mM sodium phosphate, 8 M urea, and 10 mM dithiothreitol (DTT) at pH 8.0, and allowed to stand for 1 hour. The solutions were filtered (0.45 μm) and injected for 5 seconds at 3.45×10^3 Pa. The separation occurred at 18.5 kV and 23 °C and the detection was performed at 214 nm for 70 minutes. Between runs, the capillary was washed with NaOH (0.5 M) for 5 minutes, water for 5 minutes and running buffer (10 mM sodium phosphate, 6 M urea, 0.05% hydroxypropyl methylcellulose, pH 3.0) for 5 minutes.

4.2.4.3. Analysis of pH 4.6-soluble and 70% ethanol-soluble fraction by MALDI-MS

The mass spectra were acquired in a MALDI-TOF instrument Autoflex III (Bruker Daltonics, Germany) equipped with a Smart Beam laser. Samples (1 μL) were applied on a steel target plate (MSP 96 polished-steel target, Bruker Daltonics, Bremen, Germany), dried at room temperature, and covered with matrix solution (1 μL) previously prepared from CHCA (α-cyano-4-hydroxycinnamic acid) dissolved in 70% acetonitrile and 0.25% trifluoroacetic acid. After complete droplet evaporation, the plate was inserted into the mass spectrometer. The equipment was operated in the positive ion reflector mode in the mass range of *m/z* 600-3500, controlled by FlexControl 3.3 software (Bruker Daltonics). The laser power was adjusted to 50-80%, and the voltages of ion source 1, ion source 2, lens, reflector and reflector 2 were 20.00, 17.77, 7.90, 21.95, and 10.03 kV, respectively. The pulsed ion extraction time was 30 ns, and the mass deletion threshold was set at *m/z* 600. The external calibration of the equipment was performed with Bruker Daltonics peptide calibration standard II (Bremen, Germany) composed of 9 peptides (Bradykinin 1-7, M+H 757.3992, monoisotopic; Angiotensin II, M+H 1046.5418, monoisotopic; Angiotensin I, M+H 1296.6848, monoisotopic; Substance P, M+H 1347.7354, monoisotopic; Bombesin, M+H 1619.8223, monoisotopic; Renin Substrate, M+H 1758.9326, monoisotopic; ACTH clip 1-17, M+H 2093.0862, monoisotopic; ACTH clip 18-39, M+H 2465.1983, monoisotopic; Somatostatin 28, M+H 3147.4710, monoisotopic). Each duplicate was analyzed in quintuplicate (5 spots), totaling 10 spots per sample.

4.2.4.4. Determination of ACE inhibitory activity

To determine the ACE inhibitory activity of the samples, a freeze-dried aqueous extract was obtained according to Ong and Shah (2008). For that, 10 g of cheese was

homogenized with 20 mL of MilliQ water in an Ultraturrax dispenser tube for 2 minutes. The homogenate was centrifuged at 4000 g for 20 minutes at 4 °C in a centrifuge R64 Allegra (Beckman Coulter, Indianapolis, IN, USA). The water-soluble fraction was filtered on Whatman n. 41, centrifuged at 4000 g for 20 minutes at 4 °C and filtered on glass wool. The resulting soluble extract was frozen (-80 °C) and concentrated by freeze-drying (Edwards Super Modulyo 12k Lyophilizer) for 24 h under the following conditions: condenser temperature -40 to -60 °C and pressure 10^{-2} mbar. The ACE inhibitory activity of the freeze-dried extracts was determined by the Cushman and Cheung (1971) spectrophotometric method with modifications proposed by Ong and Shah (2008), with further adaptations. The methodology is based on the reaction of hippuric acid release from the substrate hypuril-histidil-leucine (HHL) catalyzed by the action of ACE. For each assay, 3.8 mM HHL (Sigma-Aldrich, St. Louis, MO, USA) was diluted in 200 µL of 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl. Then, 35 µl of the sample solution (10 mg of freeze-dried extract in 1 mL of MilliQ water) was added and the tube was incubated at 37 °C for 5 minutes. The reaction was initiated by the addition of 20µL of ACE solution (0.1U/mL in borate buffer). After incubation at 37 °C for 30 minutes, the reaction was stopped by the addition of 250µL of 1M HCL. The hippuric acid formed in the reaction was extracted by the addition of 1.5 mL of ethyl acetate, followed by vortexing for 15 seconds and centrifugation for 10 minutes at 700g at 20 °C in a centrifuge R64 Allegra (Beckman Coulter, Indianapolis, IN, USA). After centrifugation, 1 mL of the organic phase (ethyl acetate) was transferred to another tube and evaporated in a water bath for 30 minutes at 100 °C. The remaining residue was dissolved in 1 mL of MilliQ water and the absorbance of the solution was measured at 228 nm in a spectrophotometer (Orion AquaMate 8000 UV-Vis, Thermo Scientific) using MilliQ water as a blank. All samples were analyzed in triplicate. The ACE inhibitory capacity was calculated according to the following equation: ACE inhibition (%) = [1- (A-C) / (B-D)] x100, where A is the absorbance with ACE, HHL, and sample; B is absorbance with ACE and HHL, without sample; C is the absorbance with HHL and sample; and D is the absorbance with HHL without ACE and sample.

4.2.5. Experimental design and data analysis

A 2 x 2 x 5 factorial experimental design in completely randomized blocks was used, with three replicates. The effect of the treatments on cheese composition was evaluated by one-way analysis of variance (ANOVA). The effect of the salt content, type of culture, ripening period (1, 30, 60, 90, and 120 days), as well as the interaction between these factors

on the ACE inhibitory activity was evaluated by analysis of variance (ANOVA). For both cases, in case of difference, the averages were compared by Tukey's test considering a significance level of 5%. The results were analyzed using the Statistica 7.0 software.

MALDI-MS spectra were analyzed using FlexAnalysis 3.4 software (Bruker Daltonics), which included baseline subtraction and data normalization. The MetaboAnalyst 4.0 online software was used for chemometric analysis (Chong et al., 2018), assessing data filtering, normalization by summing, and Pareto scaling. Partial least squares-discriminant analysis (PLS-DA) was used as the chemometric tool, consisting of a supervised method to identify important variables with discriminating power, which was validated by the multiple correlation (R^2) and cross validation (Q^2) coefficients. The significance of the biomarkers was ranked using the PLS-DA variable importance in projection scores (VIP score> 1).

4.3. Results and discussion

4.3.1. Physicochemical characterization of milk and cheese

The heat-treated milk used in the cheese manufacture presented 11.98 ± 0.20% total solids; 3.39 ± 0.08% fat; 3.42 ± 0.11% protein; 0.69 ± 0.01% ash; and pH 6.77 ± 0.02. The milk presented positive results for the peroxidase activity and inactivation of alkaline phosphatase, revealing the efficiency of the heat treatment.

No significant differences were observed among the treatments for pH and moisture, fat, and protein content on a dry matter. In contrast, the treatments significantly affected the salt content, salt-in-moisture ratio (S/M), and sodium levels of the cheeses (Table 4.1). Regardless of the type of lactic culture used, the reduction of salting time from 12 to 6 h resulted in cheeses with lower salt and sodium levels. The reduction of salt in moisture was 29.04 and 29.03% for Prato cheese made with O-type or O-type + adjunct culture, respectively. Concerning the sodium levels, the reduction was 28.63% and 25.46%, respectively (Table 4.1). No significant differences were observed for salt, salt-in-moisture ratio, and sodium levels of cheeses submitted to the same salting time (12h or 6h).

Table 4.1. Physicochemical composition of cheeses after 15 days of ripening (n=3).

	Control Prato cheese	Prato cheese with salt reduction	Prato cheese with adjunct culture	Prato cheese with adjunct culture and salt reduction	*P*-Value
pH[1]	5.14 ± 0.01[a]	5.15 ± 0.01[a]	5.16 ± 0.02[a]	5.16 ± 0.02[a]	0.3410
Acidity[1] (%)	0.63 ± 0.11[a]	0.72 ± 0.15[a]	0.62 ± 0.15[a]	0.60 ± 0.03[a]	0.6683
Moisture (%)	39.01 ± 0.60[a]	39.57 ± 0.24[a]	40.02 ± 0.46[a]	39.96 ± 0.13[a]	0.0532
FDM[2] (%)	54.38 ± 2.14[a]	54.70 ± 2.29[a]	55.50 ± 4.04[a]	54.17 ± 2.82[a]	0.8752
PDM[3] (%)	41.15 ± 2.52[a]	40.91 ± 2.52[a]	41.64 ± 3.58[a]	42.30 ± 3.28[a]	0.9429
Salt[4] (%)	1.73 ± 0.13[a]	1.24 ± 0.07[b]	1.63 ± 0.20[a]	1.16 ± 0.08[b]	0.0018
S/M[5] (%)	4.43 ± 0.35[a]	3.14 ± 0.20[b]	4.08 ± 0.52[a]	2.89 ± 0.21[b]	0.0017
Sodium (mg/100g)	653.57 ± 63.65[a]	466.43 ± 20.85[b]	594.73 ± 69.03[a]	443.30 ± 7.94[b]	0.0018

[1]pH and acidity were determined after 1 day of storage; [2]Fat in dry matter; [3]Protein in dry matter; [4]NaCl content as determined by chloride content analysis using Volhard method; [5]Salt in moisture.

Means with different superscript letters in the same line are significantly different by the Tukeys's test (*P*-value < 0,05).

4.3.2. Protein hydrolysis by CE

The electropherograms showed that all cheeses presented similar casein degradation profiles (Figure 4.1), with the formation of para-κ-casein by cleavage of κ-casein at Phe_{105}-Met_{106} during milk coagulation. A classic Prato cheese casein hydrolysis profile was observed, with the hydrolysis of α_{s1}-casein at Phe_{23}-Phe_{24} by the residual coagulant in the early stages of ripening, evidenced by the formation of peptides α_{s1}-CN (f24-199) (α_{s1}-I-CN), and a slower degradation of β-casein and para-κ-casein evidenced by the lower peak intensities of para-κ-casein and the β-CN A1 and β-CN A2 variants at 120 days of ripening.

The present research is the first study to assess this data for more than 60 days. Little changes were observed in the casein profile after 60 days of ripening, with progressive hydrolysis of α_{s1}-I-CN, formed in the early ripening stages, which was practically completely hydrolyzed after 120 days of storage.

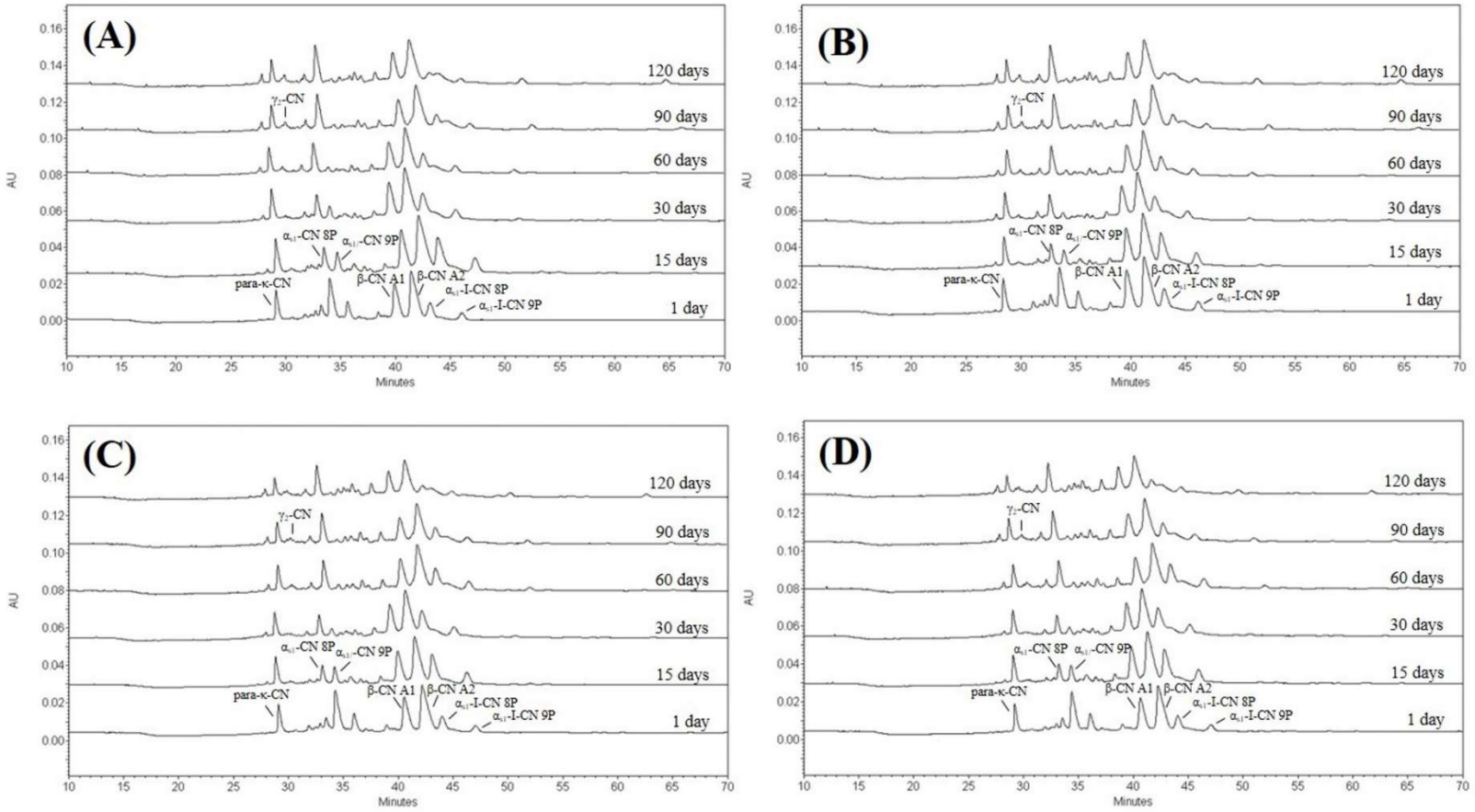

Figure 4.1. Capillary electropherograms of control Prato cheese (A), Prato cheese with salt reduction (B), Prato cheese with adjunct culture (C), Prato cheese with adjunct culture, and salt reduction (D), showing the hydrolysis profile of caseins during storage. Identification of peaks according to α-CN, β-CN and κ-CN standards (Sigma-Aldrich, St. Louis, MO, USA) and previous findings (Otte et al., 1997; Rehn et al., 2010; Alves et al., 2013). AU: arbitrary unit.

4.3.3. Peptide Profile by MALDI-MS

The mass spectra of the pH 4.6-soluble and 70% ethanol-soluble fractions measured by MALDI-TOF-MS are shown in Table 4.2. Of the 31 peaks detected, 12 referred to peptides identified by the comparison of the mass-charge ratios (*m/z*) obtained in the mass spectra with peptides previously identified in cheese (Alli, Okonieska, Gibbs, & Kunishi, 1998; Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Exterkate, Lagerwerf, Haverkamp, & Schalkwijk, 1997; Gagnaire, Mollé, Herrouin, & Leonon, 2001; Gouldsworthy, Leaver, & Banks, 1996; Soeryapranata, Powers, & Ünlü, 2008).

Table 4.2. Peptides detected by MALDI-TOF-MS in the 4.6 and 70% ethanol soluble fraction of Prato cheeses, reported in mean relative intensities (%).

m/z	Suggested peptide	Control Prato cheese						Prato cheese with salt reduction						Prato cheese with adjunct culture						Prato cheese with adjunct culture and salt reduction					
		d1	d15	d30	d60	d90	d120	d1	d15	d30	d60	d90	d120	d1	d15	d30	d60	d90	d120	d1	d15	d30	d60	d90	d120
792	*	<1	2.16	4.39	3.27	2.56	1.51	<1	<1	2.05	2.83	2.31	<1	<1	1.02	1.75	<1	-	-	<1	<1	<1	<1	-	-
906	α_{s1}-CN (f17-23) /κ-CN (f161-169)	21.36	30.14	33.11	11.26	3.13	1.04	18.00	17.83	21.73	12.65	3.97	1.30	22.18	26.13	29.72	5.45	1.33	-	19.77	16.81	16.85	7.26	1.38	-
1118	α_{s1}-CN (f15-23)	<1	1.36	1.96	<1	-	-	<1	<1	<1	<1	-	-	<1	1.35	2.05	<1	-	-	<1	<1	<1	<1	-	-
1141	α_{s1}-CN (f1-9)	1.55	1.47	4.09	6.52	8.48	7.33	<1	<1	1.28	5.68	9.22	5.96	<1	<1	3.14	5.71	5.01	2.37	<1	<1	1.97	6.24	4.94	4.16
1199	*	-	-	<1	3.49	6.40	7.69	-	-	<1	6.00	9.99	13.39	-	-	<1	6.23	13.13	12.53	-	-	<1	9.15	13.78	17.12
1247	α_{s1}-CN (f14-23)	1.32	3.62	3.14	<1	-	-	1.03	1.29	<1	-	-	-	1.20	2.52	1.89	-	-	-	<1	1.07	<1	-	-	-
1522	*	-	-	<1	<1	<1	<1	-	-	-	-	<1	<1	-	<1	<1	<1	<1	<1	-	-	<1	<1	<1	<1
1536	α_{s1}-CN (f1-13)	12.23	30.81	67.81	98.84	98.88	90.91	5.43	10.13	20.76	76.62	85.88	71.04	7.66	23.00	64.75	99.41	98.92	59.46	5.28	8.66	29.31	81.14	73.12	62.64
1558	*	2.05	5.96	10.24	6.70	7.65	3.56	<1	<1	1.02	3.02	4.13	2.38	<1	2.86	7.60	6.68	4.55	2.14	<1	<1	2.02	3.48	2.74	1.83
1574	*	<1	1.28	2.76	1.72	1.94	<1	<1	<1	<1	<1	1.30	<1	<1	<1	1.66	1.74	1.21	<1	-	<1	<1	1.10	<1	<1
1665	α_{s1}-CN (f1-14)	<1	1.53	4.89	10.03	10.28	8.93	<1	1.06	2.85	15.95	16.04	17.46	<1	1.31	4.44	8.46	6.88	3.28	<1	<1	3.90	14.24	10.83	8.32
1682	*	-	-	-	<1	<1	<1	-	-	<1	<1	<1	<1	-	-	-	<1	<1	<1	-	-	-	<1	<1	<1
1718	β-CN (f194-209)	1.80	<1	<1	-	-	-	1.40	<1	<1	<1	-	-	3.16	<1	<1	-	-	<1	2.49	<1	<1	<1	-	-
1863	*	1.20	1.01	<1	-	-	-	1.87	1.70	1.07	<1	-	-	1.40	1.17	<1	-	-	-	1.66	1.69	1.12	-	-	-
1876	*	-	-	-	-	<1	<1	-	-	-	-	<1	<1	-	-	-	-	<1	-	-	-	-	-	<1	<1
1877	α_{s1}-CN (f1-16)	96.12	93.33	69.70	24.59	4.50	<1	70.00	76.67	86.67	82.37	7.78	3.21	96.67	89.17	56.16	9.23	-	-	96.67	93.33	76.67	29.44	3.98	<1
1881	β-CN (f193-209)	34.54	15.18	15.48	38.65	45.30	49.67	25.45	11.27	29.28	75.66	56.33	68.11	43.75	25.33	22.53	36.68	64.07	77.52	30.49	23.55	17.73	61.93	83.06	75.69
1899	*	24.95	23.21	11.67	<1	-	-	17.74	12.18	9.73	2.51	<1	-	21.02	15.17	7.69	<1	-	-	16.55	11.32	11.68	<1	-	-
1903	*	2.63	<1	<1	1.64	1.28	1.02	2.05	<1	1.27	2.25	2.46	2.51	3.30	1.23	<1	1.11	1.65	1.53	<1	1.05	<1	1.90	2.82	2.42
1904	*	<1	<1	<1	<1	-	<1	<1	<1	<1	-	<1	<1	<1	<1	<1	-	-	<1	<1	<1	<1	<1	-	<1
1915	*	9.82	5.93	3.42	-	-	-	7.80	4.31	2.94	<1	-	-	8.57	4.24	1.43	-	-	-	7.09	4.05	3.29	-	-	-
1919	*	<1	<1	<1	1.02	<1	<1	1.09	<1	1.01	1.52	1.50	1.46	1.83	1.01	<1	<1	<1	<1	<1	<1	<1	1.36	1.74	1.67
1920	*	1.64	<1	<1	<1	-	-	<1	<1	<1	-	<1	<1	<1	<1	<1	-	-	<1	1.12	<1	<1	<1	-	<1

Table 4.2. Continued.

m/z	Suggested peptide	Control Prato cheese						Prato cheese with salt reduction						Prato cheese with adjunct culture						Prato cheese with adjunct culture and salt reduction					
		d1	d15	d30	d60	d90	d120	d1	d15	d30	d60	d90	d120	d1	d15	d30	d60	d90	d120	d1	d15	d30	d60	d90	d120
1937	*	<1	<1	<1	-	-	-	<1	<1	-	-	-	-	<1	<1	-	-	-	-	<1	<1	-	-	-	-
1977	*	<1	-	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	-	-	<1	<1	-	-	-	-	<1	<1	<1	<1
1991	α_{s1}-CN (f1-17)	5.56	8.21	12.84	25.55	23.99	12.57	4.71	6.48	10.80	45.08	25.06	21.39	3.33	6.92	7.20	16.95	12.66	3.92	3.17	4.91	12.89	24.75	13.35	6.94
1992	*	2.45	5.04	16.09	18.16	4.90	10.62	3.53	6.09	10.90	11.94	17.06	15.22	2.06	3.28	19.54	6.00	4.92	3.67	1.83	4.60	6.74	13.21	8.11	6.36
2013	*	<1	<1	<1	<1	<1	-	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	-	<1	<1	<1	<1	-	-
2014	*	<1	<1	1.17	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	1.08	<1	<1	-	-	<1	<1	<1	<1	-
2348	*	-	<1	1.28	<1	-	-	<1	1.54	1.72	<1	<1	-	<1	<1	<1	-	-	-	<1	1.38	<1	<1	-	-
2764	α_{s1}-CN (f1-23)	<1	<1	<1	-	-	-	<1	<1	<1	-	-	-	<1	<1	<1	-	-	-	<1	<1	-	-	-	-

* Unidentified.

The effect of ripening time on the development of the peptide profile of cheeses was observed by the partial least squares-discriminant analysis (PLS-DA) of the spectral data of control Prato cheese (Figures 4.2A and 4.2B), Prato cheese with salt reduction (Figures 4.2C and 4.2D), Prato cheese with adjunct culture (Figures 4.3A and 4.3B), and Prato cheese with adjunct culture and salt reduction (Figures 4.3C and 4.3D). In all cases, the PLS-DA revealed that cheeses subjected to different ripening times were distinguished from each other in the first two PCs, with 71.2%, 70.3%, 73.4%, and 74.5% of total variance for the control Prato cheese, Prato cheese with salt reduction, Prato cheese with adjunct culture, and Prato cheese with adjunct culture and salt reduction, respectively. Figures 4.2 and 4.3 show a clear separation of the samples into two groups: cheeses with up to 30 days and after 60 days of ripening. At the beginning of ripening, samples were essentially overlapped up to 30 days after the manufacture. However, a separation of the previous clusters was observed with the increase in ripening time, especially from 60 days, with similar clustering for the different treatments after 60, 90, and 120 days of ripening. Regardless of the evolution of the peptide profile, for all cheeses, the peptide α_{s1}-CN (f1-16) (*m/z* 1887) [RPKHPIKHQGLPQEVL] was the variable with the highest negative weight for sample separation and, therefore, the most important peptide for the separation of samples at the left of the score plots, which corresponded to the cheese at the beginning of ripening. With the evolution of the ripening period, an accumulation of the peptide α_{s1}-CN (f1-13) (*m/z* 1536) [RPKHPIKHQGLPQ] was observed, corresponding to a variable with a higher positive weight for sample separation, which was more intense in the right side of the score plots, corresponding to the samples with greater ripening time. The Gln_{13}-Glu_{14} and Val_{15}-Leu_{16} bonds of α_{s1}-casein are the preferred cleavage sites of endopeptidases from *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (Upadhyay, McSweeney, Magboul, & Fox, 2004), which is the starter culture used in the cheese manufacture, and possibly responsible for the release of peptides that characterize the evolution of proteolysis of Prato cheese made with O-type culture.

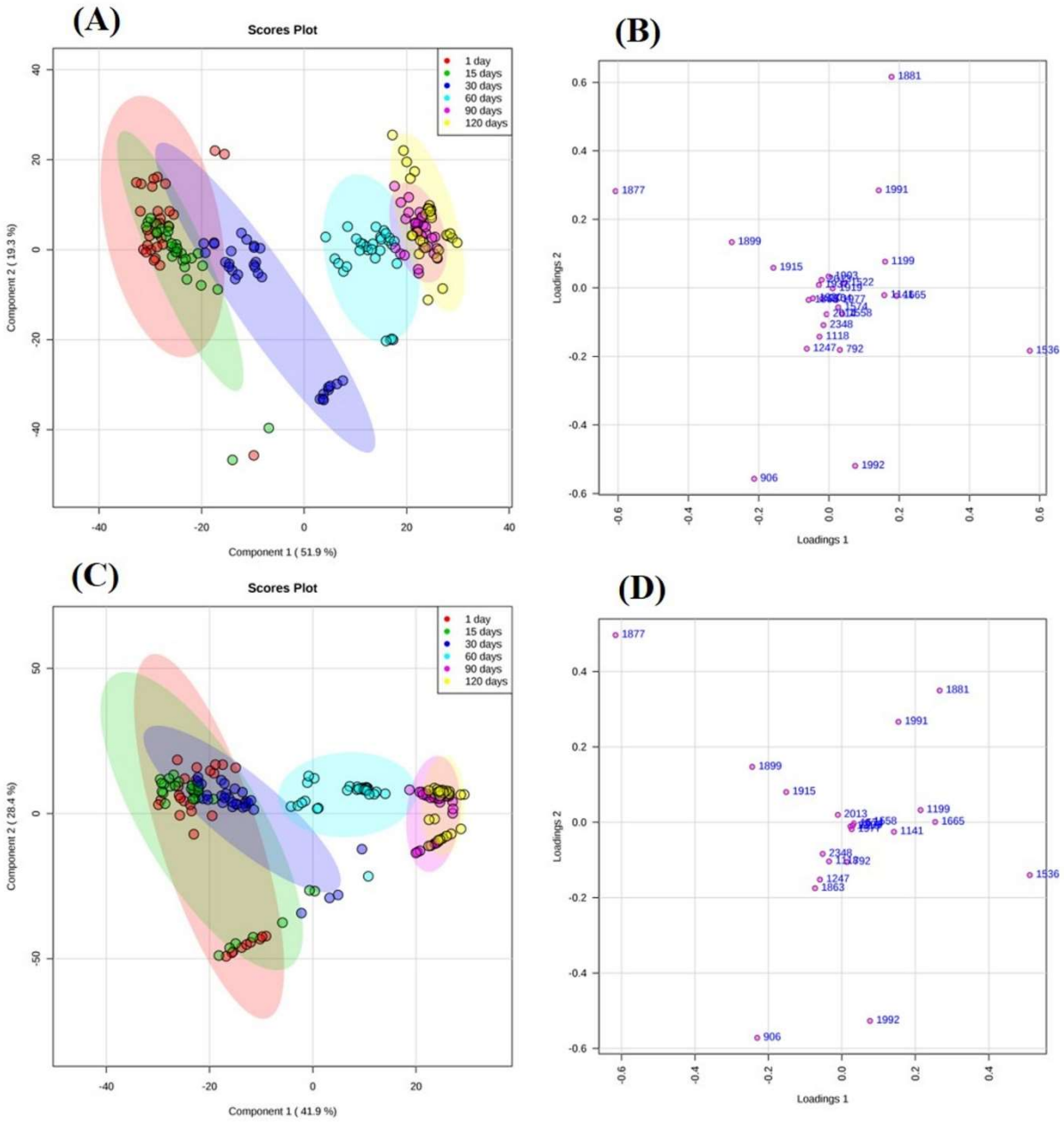

Figure 4.2. Partial least square discriminant analysis (PLS-DA) of mass spectra data of pH 4.6 and 70% ethanol soluble fractions. Score plots (A, C) and loading plots (B, D) of control Prato cheese (A, B) and Prato cheese with salt reduction (C, D) during 120 days of storage.

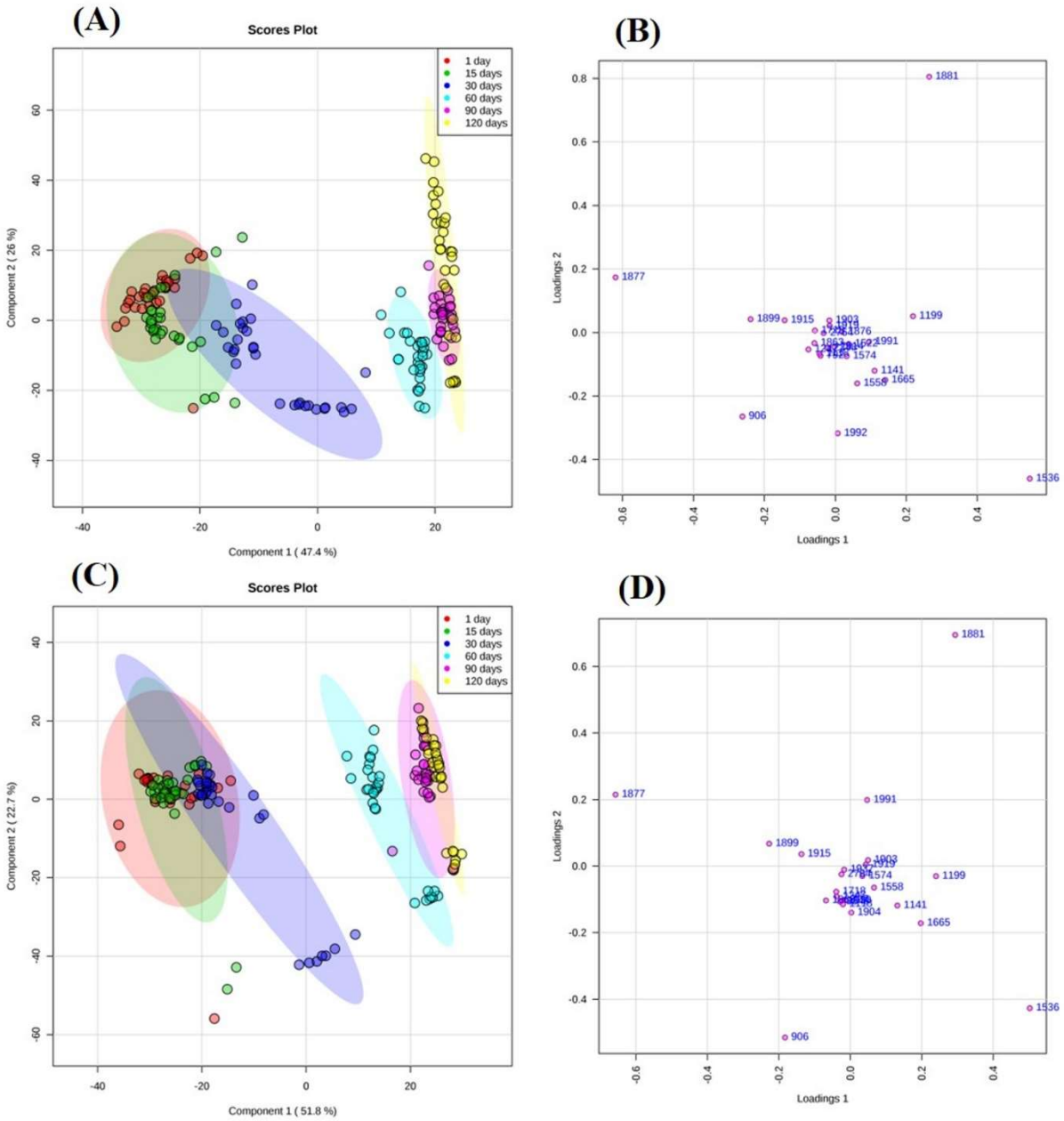

Figure 4.3. Partial least square discriminant analysis (PLS-DA) of mass spectra data of pH 4.6 and 70% ethanol soluble fractions. Score plots (A, C) and loading plots (B, D) of Prato cheese with adjunct culture (A, B) and Prato cheese with adjunct culture and salt reduction (C, D) during 120 days of storage.

For a better understanding and visualization of the samples evaluated, chemometric analyses were used to predict the isolated effect of the factors on the peptide profile, i.e., the salt reduction and the addition of adjunct culture. The PLS-DA results showed that the control cheese and cheese made with the addition of the adjunct culture *Lactobacillus helveticus* LH-B02 can be distinguished from each other after 90 days of ripening in the first two PCs, with

71.2% of the total variance (Figures 4.4A and 4.4B). The potential peptides for the separation of the samples in the first two PC (VIP score> 1) were peptide β-CN (f193-209) (*m/z* 1881), more intense in cheese made with the adjunct culture, and the peptides α_{s1}-CN (f1-16) (*m/z* 1877), α_{s1}-CN (f1-17) (*m/z* 1991), and the unidentified peptides *m/z* 792, 1199, and 1558 that were more intense in the control cheese. Among the important peptides for sample separation, the peptide β-CN (f193-209) (*m/z* 1881), which was more intense in the cheese made with the adjunct culture, is recognized as a bioactive peptide with immunomodulatory antimicrobial and ACE inhibitory properties (Sandré et al., 2001; Birkemo, O'Sullivan, Ross, & Hill, 2007; Torres-Llanez, González-Córdova, Hernandez-Mendoza, Garcia & Vallejo-Cordoba, 2011). In addition, the PLS-DA results showed that the control cheese and the cheeses with salt reduction were distinguished from each other after 120 days of ripening in the first two PCs, with 85.9% of the total variance (Figure 4.4C and 4.4D). The potential peptides for the samples separation (VIP score> 1) were α_{s1}-CN (f1-14) (*m/z* 1665), β-CN (f193-209) (*m/z* 1881) and an unidentified peak with *m/z* 1199, which were more abundant in the salt-reduced cheeses, and the peptide α_{s1}-CN (f1-13) (*m/z* 1536), which was more abundant in the control cheese.

Thus, both the addition of the adjunct culture and the salt reduction favored the relative abundance of the bioactive peptide β-CN (f193-209) (*m/z* 1881). This peptide sequence (YQEPVLGPVRGPFPIIV) has three hydrophobic amino acids in its C-terminal tripeptide sequence, which is a determining factor for ACE binding and consequent inhibitory activity (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002).

Additionally, the hydrophobic peptide β-CN (f193-209) is recognized as a bitter-tasting peptide whose accumulation in ripened cheeses may lead to a taste defect (Soeryapranata et al., 2002). Previous studies reported that the addition of *Lactobacillus helveticus* LH-B02 as an adjunct culture increased the sensory acceptance of Prato cheeses (Azambuja et al., 2017; Barros, Cunha, Gallina, Viotto, & Viotto, 2006), and the salt reduction level used in the present study was previously tested by our research group (Baptista et al., 2017) and it did not affect Prato cheese sensory acceptance after 30 and 60 days of ripening. Considering the modification of the peptide profile of cheeses after 90 days of ripening due to the addition of the adjunct culture and after 120 days of ripening due to the salt reduction, further studies are recommended to correlate the peptide profile of the cheeses studied and their sensory attributes.

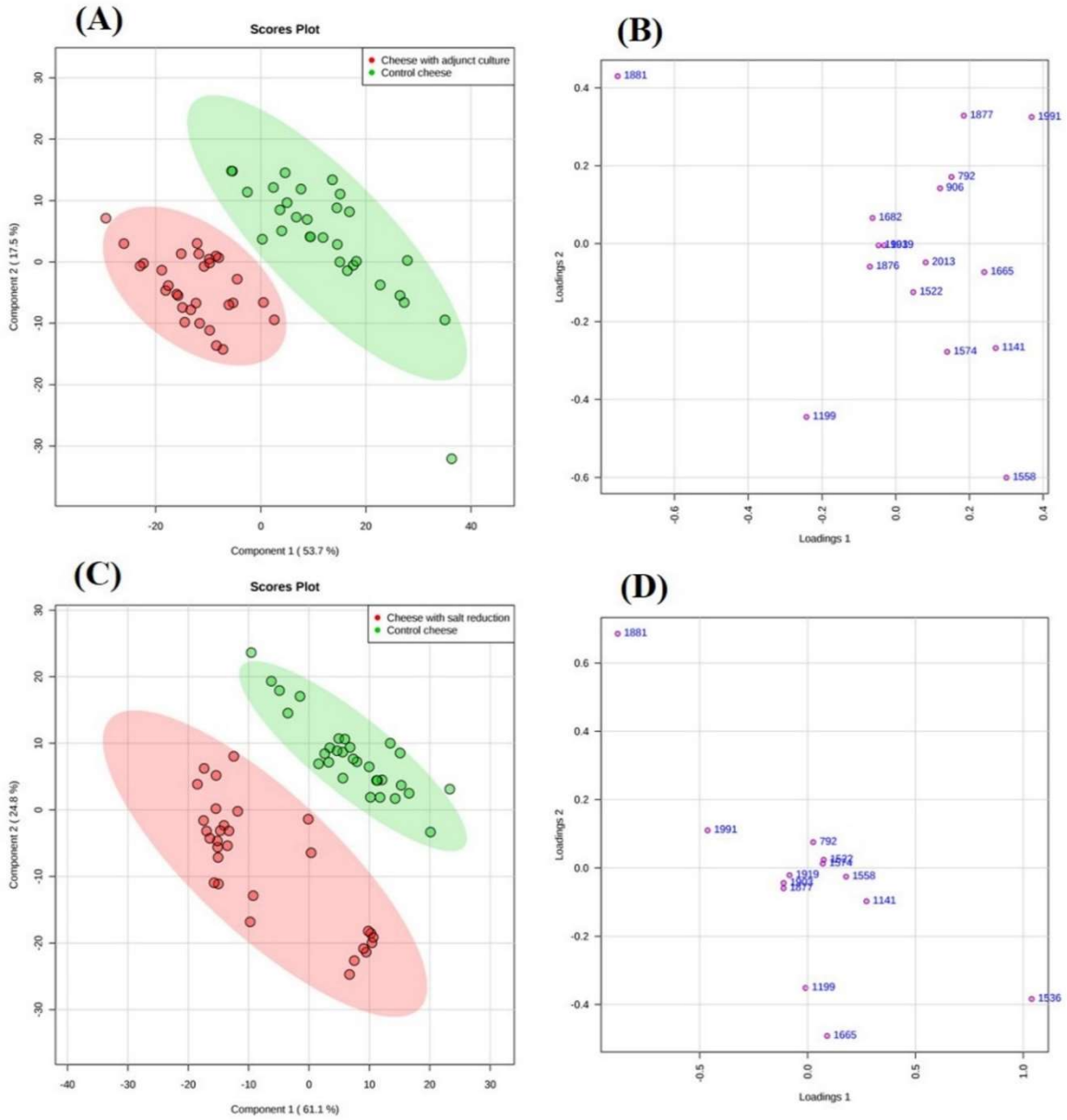

Figure 4.4. Partial least square discriminant analysis (PLS-DA) of mass spectra data of pH 4.6 and 70% ethanol soluble fractions. Score plots (A, C) and loading plots (B, D) of control Prato cheese and Prato cheese with adjunct culture (A, B) after 90 days of storage, and control Prato cheese and Prato cheese with salt reduction (C, D) during 120 days of storage.

4.3.4. ACE inhibitory activity

The type of culture (*P*-value = 0.0491) and the ripening time (*P*-value < 0.0001) significantly affected the ACE inhibitory activity, which was not affected by the salt levels (*P*-value > 0.05) and the interaction between all factors (*P*-value > 0.05). The use of *Lactobacillus helveticus* LH-B02 in the manufacture of Prato cheese resulted in a significant increase in ACE inhibitory activity, which ranged from 75.48% in control cheese to 79.67% in cheese made with the addition of adjunct culture, regardless of the salt content. The higher ACE inhibitory activity in cheeses made with *Lactobacillus helveticus* LH-B02 is probably associated with the modification of the peptide profile induced by the adjunct culture addition. This bioactivity is enhanced by the presence of biopeptides capable of inhibiting the ACE action, such as the peptide β-CN (f193-209) (*m/z* 1881), which was significantly (VIP score > 1) more intense in cheese with the addition of adjunct culture after 90 days of ripening. This result suggests that the use of *Lactobacillus helveticus* LH-B02 is an effective strategy to increase the antihypertensive potential of Prato cheese. It is worth noting that regardless of the type of culture or salt content of the cheeses, the ACE inhibitory activity increased during the ripening period (Figure 4.5) and the higher inhibition increase was observed in the first 30 days of ripening, which increased from 54.77 to 76.24%. After 30 days of ripening, no significant increase was observed until 120 days, with a mean ACE inhibitory activity of 90.22%.

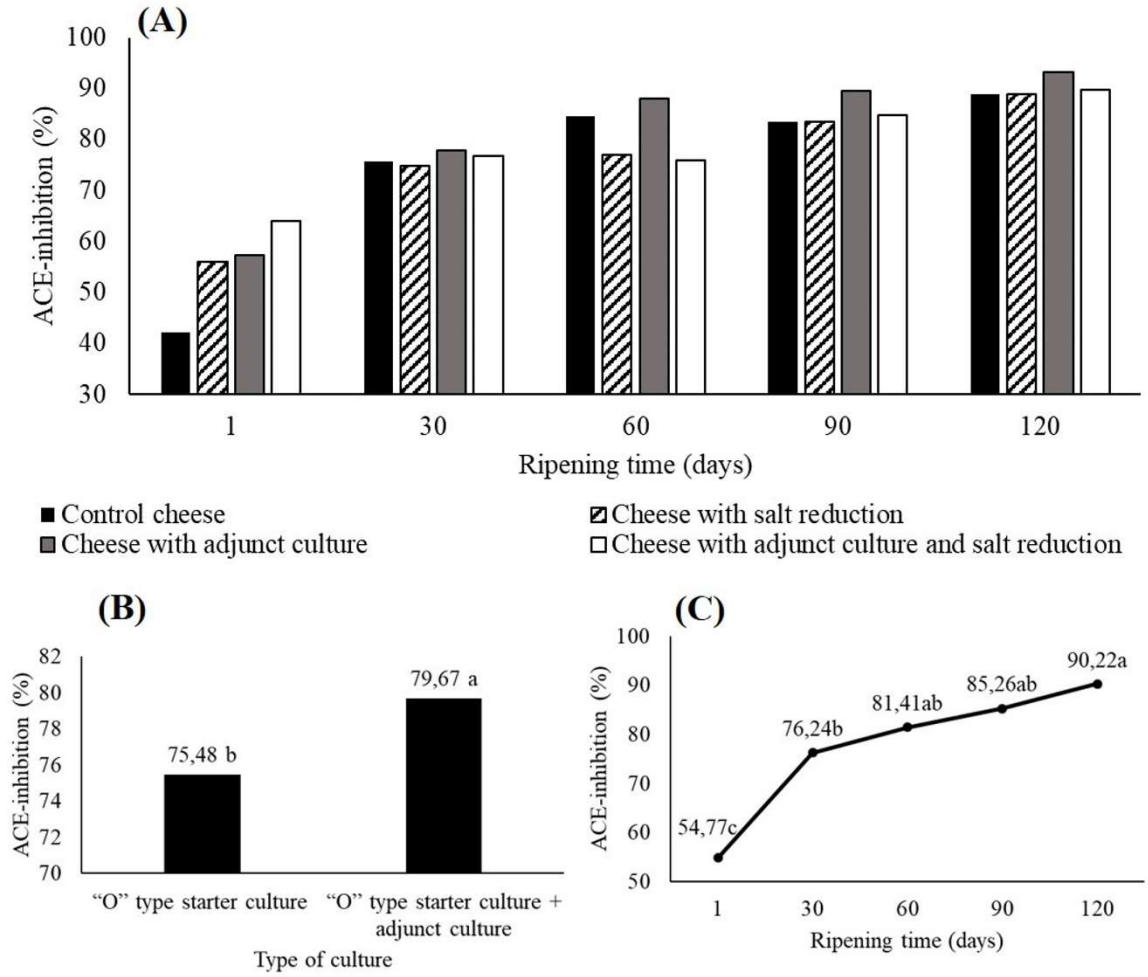

Figure 4.5. Angiotensin-converting enzyme (ACE)-inhibitory activities of control Prato cheese, Prato cheese with salt reduction, Prato cheese with adjunct culture, and Prato cheese with adjunct culture and salt reduction during ripening presented as percentage of inhibition of cheese water-soluble extract (wse) (A). Effect of the type of culture used on cheese production on ACE-inhibitory activity (B). Effect of ripening time on ACE-inhibitory activity (C).

4.4. Conclusions

The use of the adjunct culture *Lactobacillus helveticus* LH-B02 in the manufacture of Prato cheese affected the relative intensity of a peptide recognized for its bioactive potential and favored the ACE inhibitory activity during ripening. Considering the null effect of the salt reduction on the ACE inhibitory activity, *in vivo* studies with human and animal models are recommended to evaluate the complementary effect of the salt reduction and the use of the adjunct culture *Lactobacillus helveticus* in Prato cheese on hypertension. Further studies on the

evaluation of the peptides present on pH 4.6 soluble and 70% ethanol insoluble fractions are needed.

Acknowledgments

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CAPÍTULO 5 - ARTIGO 3. Use of static and dynamic *in vitro* models to simulate Prato cheese gastrointestinal digestion: effect of *Lactobacillus helveticus* LH-B02 addition on peptides bioacessibility

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Abstract

The use of adjunct proteolytic cultures in the manufacture of ripened cheese is a strategy used to favor the release of bioactive peptides during ripening. However, the physiological relevance of bioactive peptides depends on their stability during gastrointestinal digestion. This study aimed to evaluate the effect of the gastrointestinal digestion on the peptide profile of Prato cheese with and without the addition of *Lactobacillus helveticus* LH-B02 as an adjunct culture, using *in vitro* static and dynamic simulated digestion models. In both models studied, proteolysis during the simulation of gastrointestinal digestion resulted in an approximation of the peptide profile of the evaluated cheeses suggesting that the modulation of the peptide profile of ripened cheeses may not represent a physiological advantage since cheeses with different peptide profiles showed essentially the same peptide profile after digestion.

5.1. Introduction

Adjunct cultures are traditionally used to favor the development of flavour and texture characteristics in ripened cheeses (Irlinger, Helinck, & Jany, 2017). Moreover, adjunct proteolytic cultures have recently been used to induce casein hydrolysis and the release of peptides with biological activities during cheesemaking and ripening (Baptista et al., 2020; Baptista et al., 2018; Galli et al., 2019; Ong & Shah, 2008).

In a recent study, our research group demonstrated the ability of the adjunct culture *Lactobacillus helveticus* LH-B02 to modify the peptide profile and, specifically, to alter the relative intensity of peptides recognized by their bioactive potential in Prato cheese, the most produced ripened cheese in Brazil (Baptista et al., 2018; Datamark, 2020). In a subsequent study, we found that the strain *Lactobacillus helveticus* LH-B02 also increased the inhibitory activity of the angiotensin-converting enzyme (ACE) in Prato cheese, revealing the potential antihypertensive effect of cheeses produced with the addition of this adjunct culture (Baptista et al., 2020).

However, bioactive activity associated with the presence of bioactive peptides in cheese does not guarantee its bioactivity when the cheese is ingested. During gastrointestinal digestion, proteins and peptides present in the cheese will be submitted to the action of several enzymes leading to the release of new bioactive peptides not initially present in the cheese, as well as the hydrolysis of bioactive peptides and the consequent release of fragments without biological activity (Sánchez-Rivera et al., 2014). Thus, the study of bioaccessibility, which is the fraction of a component that is released from the food matrix, effectively reaches the

intestinal lumen and is therefore available for absorption in the intestine, is essential for understanding the possible physiological effects of bioactive products (Guerra et al., 2012; Picariello, Mamone, Nitride, Addeo, & Ferranti, 2013). The determination of bioaccessibility is usually performed by *in vitro* studies that enable the identification of compounds released into the gastrointestinal tract (Guerra et al., 2012; Picariello et al., 2013).

Several static and dynamic *in vitro* digestion models have already been proposed and applied in the simulation of gastrointestinal digestion of dairy proteins (Egger et al., 2019; Havenaar et al., 2013; Jin et al., 2016; Sánchez-Rivera et al., 2014). The harmonized *in vitro* protocol for simulation of the gastrointestinal digestion proposed by INFOGEST, an international cooperation network, presents the standardization of parameters such as electrolytes, enzymes, and bile concentrations, dilution factors, pH and digestion times (Minekus et al., 2014).

However, despite the standardization of a protocol that enables a better comparison of inter-laboratory results, static digestion models are considered simplifications of a dynamic physiological process. Dynamic *in vitro* digestion models include the physicochemical and mechanical processes as well as the gradual changes that occur in gastrointestinal conditions *in vivo* (Guerra et al., 2012). The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) is a colonic dynamic *in vitro* digestion model that simulates the human microbial ecosystem with 5 reactors representing the stomach, duodenum and ascending, transverse and descending colon under controlled conditions of pH, residence time, and temperature (Molly, Woestyne, Smet, & Verstraete, 1994). As a modular system, it can be adapted for the use in protein and peptide digestion studies using only the first two reactors that simulate the stomach and the duodenum.

In this context, the present study aimed to use two *in vitro* simulation models of gastrointestinal digestion, one static and one dynamic, to evaluate the effect of the gastrointestinal digestion on the peptide profile of Prato cheeses produced with and without the addition of the adjunct culture *Lactobacillus helveticus* LH-B02.

5.2.Material and Methods

5.2.1. Preparation of lactic cultures

The freeze-dried lactic mesophilic culture of *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris* (R 704 - Chr. Hansen, Hoersholm, Denmark) was activated in 10% sterile reconstituted milk and incubated at 30 °C for 8 h. For the preparation of the adjunct

culture, one package (50U) of the freeze-dried thermophilic lactic culture of *Lactobacillus helveticus* LH-B02 (Chr. Hansen, Hoersholm, Denmark) was rehydrated in 1L of 10% sterile reconstituted milk and kept for 1 h at 4 °C. The rehydrated culture was then divided into portions for inoculation in the cheese vat, according to the manufacturer's instructions (50U/500 liters of milk), and kept at -20°C until cheese processing.

5.2.2. Cheese manufacturing

The cheeses were produced on a pilot scale using 200 liters of heat-treated milk (68°C/2min), which was divided into two 100 liters portions. One portion was used to produce Control Prato cheese, with addition of the starter lactic culture of *Lactococcus lactis* spp *lactis* and *Lactococcus lactis* spp *cremoris* (R 704 - Chr. Hansen, Hoersholm, Denmark) and the second portion was used for the manufacture of Prato cheese with addition of starter lactic culture and the adjunct culture *Lactobacillus helveticus* (LH-B02 - Chr. Hansen, Hoersholm, Denmark). The cheeses were processed in a stainless-steel vat as described by Mazal, Vianna, Santos, & Gigante (2007), with the modifications described by Baptista, Araújo, Eberlin, & Gigante (2017). In both cases, the milk was heated to 35°C and calcium chloride (250 ppm), starter culture (1 %, 9-10 log UFC/g) and adjunct culture (0,2 %, 8-9 log UFC/g) when applied, urucum dye (80 ppm) and coagulant (CHY-MAX, Chr. Hansen, Hoersholm, Denmark) sufficient to coagulate the milk within 35 min were added. The curd was cut into 1 cm cubes and submitted to slow continuous mixing for 15 min. Then, the mixture of curd and whey was submitted to indirect heating to increase the temperature of the curd by 1 °C every 3 min until 42 °C. This temperature was kept for 40 minutes. After cooking, the whey was drained off, and the curd was placed in rectangular plastic molds (13 cm x 8 cm x 7 cm). Then, the cheeses were pressed (0.1 MPa for 15 min; 0.1 MPa for 15 min; 0.24 MPa for 30 min, and 0,31 MPa for 90 min) and fermented for 5 hours at room temperature (~ 25 °C). After fermentation, cheeses were salted in brine for 12 hours (20% NaCl; 0.5% $CaCl_2$; pH 5.5; 4°C; brine volume 3.5 times greater than the cheese volume). Finally, the cheeses were dried for 48h, vacuum packed, and ripened for 90 days at 12°C. The cheeses were produced in independent triplicates, which were submitted to simulated digestion, totaling 3 digestion experiments for each cheese evaluated in each digestion model.

5.2.3. *In vitro* simulations of gastrointestinal digestion

In vitro digestion simulations of both cheeses (control cheese and cheese with the addition of the adjunct culture) were performed according to the INFOGEST static protocol and the SHIME® dynamic model. In both cases, an experiment was performed using MilliQ water as the sample to be digested to obtain blanks of all digestion phases which were also analyzed by mass spectrometry.

5.2.3.1. Static model: INFOGEST

Cheese digestion was simulated *in vitro* according to the harmonized protocol proposed by the INFOGEST international scientific cooperation network (Minekus et al., 2014), using a Dubnoff-type metabolic bath for incubation (Marconi, Piracicaba, Brazil). 5 g of ground cheese sample or 5 mL MilliQ water as a blank control were homogenized with 4 mL of simulated salivary stock solution (15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2$, 0.06 mM $(NH_4)_2CO_3$), 25 µL of 0.3 M $CaCl_2$ and 975 µL of ultrapure water in a Stomacher 400 (Seward Laboratory, Worthing, UK). The mixture was incubated for 2 min at 37 °C. Then, 7.5 mL of simulated gastric stock solution (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2$, 0.5 mM $(NH_4)_2CO_3$), 1.6 mL of pepsin solution (Sigma Aldrich, P7000, 25000 U/mL), 5 µL of 0.3 M $CaCl_2$, 0.2 mL of 1M HCl and 0.695 mL of ultrapure water were added. The pH was adjusted to 3 and the mixture was incubated at 37 °C for 2 h to simulate the gastric digestion. Finally, 11 mL of simulated intestinal stock solution (6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2$), 5 mL of pancreatin solution (Sigma Aldrich, P1750, 800 U/mL), 2.5 mL of 160 mM bile solution (Sigma Aldrich, B8631, 160 mM), 40 µL of 0.3 M $CaCl_2$, 0.15 mL of 1 M NaOH, and 1.31 mL of ultrapure water were added to the gastric chyme. The pH was adjusted to 7 and the mixture was incubated at 37 °C for 2 h to simulate the intestinal digestion. The complete experiment was conducted in parallel with 3 incubation flasks. After the oral phase, one flask, corresponding to the oral digest, was separated and immediately refrigerated in an ice bath and frozen. After gastric phase, the second flask, corresponding to the gastric digest, was separated and the pH was adjusted to 7 with 1 M NaOH to inactivate the pepsin and digest was immediately refrigerated in an ice bath and frozen. After the intestinal digestion, the last flask, corresponding to the intestinal digest, had the pH adjusted to 11 with 1M NaOH to inactivate the pancreatin and then, the digest was immediately refrigerated in an ice bath and frozen.

5.2.3.2. Dynamic model: SHIME®

Dynamic digestion simulation was performed in the SHIME®, a multicompartment human microbial ecosystem simulator, which consists of five connected reactors representing the different parts of the human gastrointestinal tract (Molly et al., 1994). The experiment was performed using only the first two reactors, which simulate the stomach and duodenum, in this study generically cited as intestine, were used in the experiment. 30 g of cheese was homogenized with 163.2 mL of simulated salivary solution (Minekus et al., 2014) and transferred to the system's first reactor, which simulates the stomach. The pH was adjusted to 2.3-2.5, and 16.8 mL pepsin solution (25000 U/mL; P7000, Sigma-Aldrich, St. Louis, MO, USA) to reach 2000 U/mL gastric content (Minekus et al., 2014), was transferred to the reactor with a flow rate of 1mL/min. One hour and 45 minutes after the beginning of the gastric phase, the second reactor, which simulates intestinal digestion, was fed with 60 mL of pancreatic juice composed of 12.5 g/L $NaHCO_3$, 6 g/L bovine bile (ox-bile 70168, Sigma-Aldrich, St. Louis, MO, USA), 0.9 g/L porcine pancreatic pancreatin (P1625, Sigma-Aldrich, St. Louis, MO, USA) at a flow rate of 4 mL/min. Two hours after the beginning of the gastric phase, the gastric content started to be transferred to the second reactor of the system, which simulates intestinal digestion, at a flow rate of 4 mL/min. Intestinal digestion lasted 4 hours and the pH was monitored during this period. An aliquot was taken from the first reactor at the end of the gastric phase to monitor the peptide profile and its pH adjusted to 7 with NaOH for pepsin inactivation. At the end of the intestinal digestion stage, the pH of the entire reactor content corresponding to the duodenum was adjusted to 11 with 2M NaOH to inactivate pancreatin (Jin et al., 2016). Both samples were immediately refrigerated in an ice bath and frozen.

5.2.4. Peptide extraction from cheeses and digests

Samples were defrosted and had their volumes adjusted with ultrapure water to standardize the cheese: digestive fluid ratio to that found at the end of the digestion experiment. For comparison of the peptide profile in the cheese sample and digests, for each digestion model, cheese was homogenized with ultrapure water in the same proportions used in the digestion experiment. For INFOGEST, 5 g of cheese was homogenized with 35 mL of ultrapure water and, for SHIME®, 30 g of cheese was homogenized with 240 mL of ultrapure water. The samples were than deffated by centrifugation for 30 min at 4°C in a R64 Allegra centrifuge (Beckman Coulter, Indianápolis, IN, USA). The soluble phase was ultrafiltered using ultrafiltration centrifugal units with 10 kDa cutoff (Merck Millipore, Cork, Ireland) and the

permeate pH was adjusted to 7. The peptides were extracted from the permeates by stop-and-go-extraction tips (StageTip) microextraction according to the method described by Rappsilber, Mann, & Ishihama (2007), modified by Baum, Ebner, & Pischetsrieder (2013) and Ebner, Baum, & Pischetsrieder (2016) with further modifications described as follows. For StageTips preparation, three layers of 1 mm of diameter were punched from a C18 AttractSPE™ Disks (Affinesep, Petit-Couronne, France) with a biopsy punch (Kai Industries Co., Japan). The layers were sequentially placed into a 200 µL pipet tip and the tip was inserted into a perforated cap of a 2 mL microtube. For the peptide extraction, the StageTip was first equilibrated with 100 µL of acetonitrile (ACN), followed by centrifugation at 5000 rpm for 1 min, and 100 µL of 0.1% trifluoracetic acid (TFA), followed by centrifugation at 5000 rpm for 1 min. Then, 20 µL of the ultrafiltrate was loaded into the StageTip, followed by centrifugation at 7000 rpm for 5 min. The tip was then washed with 50 µL of 0.1% TFA by centrifugation at 5000 rpm for 3 min. Finally, peptides were eluted with 10 µL of 60% ACN in 0.1% TFA followed by centrifugation at 5000 rpm for 3 min. The eluates were stored at −20 °C until MALDI-MS analysis.

5.2.5. Peptide analysis by MALDI-MS

The eluates from StageTip microextraction were diluted with 15 µL of 60% ACN in 0.1% TFA and aliquots (1 µL) of the diluted extract were applied on a steel target plate (MSP 96 polished-steel target, Bruker Daltonics, Bremen, Germany), dried at room temperature, and covered with 1 µL matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in 70% ACN and 0.25% TFA). For each extract, 10 spots were applied on the plate. After the complete droplet evaporation, the plate was inserted into an MALDI-TOF/TOF Autoflex III instrument (Bruker Daltonics, Alemanha) equipped with a SmartBeam laser. The equipment was operated in the positive ion reflector mode in the mass range of *m/z* 600-3500, using the FlexControl 3.3 software (Bruker Daltonics). The laser power was adjusted to 50-80%, and the voltages of ion source 1, ion source 2, lens, reflector and reflector 2 were 20.00, 17.77, 7.90, 21.95, and 10.03 kV, respectively. The external calibration of the equipment was performed with Bruker Daltonics peptide calibration standard II (Bremen, Germany) composed of 9 peptides (Bradykinin 1-7, M+H 757.3992, monoisotopic; Angiotensin II, M+H 1046.5418, monoisotopic; Angiotensin I, M+H 1296.6848, monoisotopic; Substance P, M+H 1347.7354, monoisotopic; Bombesin, M+H 1619.8223, monoisotopic; Renin Substrate, M+H 1758.9326,

monoisotopic; ACTH clip 1-17, M+H 2093.0862, monoisotopic; ACTH clip 18-39, M+H 2465.1983, monoisotopic; Somatostatin 28, M+H 3147.4710, monoisotopic).

The identification of peptides from the mass spectra was performed by comparing the mass-charge ratios (*m/z*) observed in the mass spectra obtained with those previously identified in cheese or dairy protein digestion samples (Addeo et al., 1992; Addeo et al., 1994; Alli, Okoniewska, Gibbs, & Konishi, 1998; Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Gagnaire, Mollé, Herrouin, & Léonil, 2001; Gouldsworthy, Leaver, & Banks, 1996; Jin et al., 2016; Ong & Shah, 2008; Soeryapranata, Powers, & Ünlü, 2008; Soeryapranata, Powers, Weller, Hill, & Siems, 2004)

5.2.6. Data processing and chemometric analysis

Mass spectral data obtained by MALDI-MS were preprocessed by baseline subtraction and normalization using FlexAnalysis 3.4 software (Bruker Daltonics). Chemometric analysis of the data was performed using the MetaboAnalyt 4.0 online software (Chong et al., 2018), assessing data filtering, normalization by summing, and scaling by Pareto method. Peaks detected in the ultrapure water digests (blanks) were removed from the spectra directly in the software. The chemometric tool used was the partial least squares discriminant analysis (PLS-DA), a supervised method applied to identify important variables with discriminant power. Important variables for sample discrimination were selected using the PLS-DA projected variable importance score (VIP score> 1).

5.3. Results e discussion

The mass spectra obtained from the cheeses samples (control cheese and cheese with adjunct culture) and oral, gastric and intestinal digests obtained by gastrointestinal digestion simulation in the INFOGEST and SHIME® digestion models are presented in Figures 5.1 and 5.2, the tables with all the peaks detected in the cheeses and digests are presented in the supplementary material (Tables 5.S1 e 5.S2). The cheeses and their oral digestion phases presented essentially the same peptide profile (Figures 5.1 and 5.2). This occurs because the oral phase of digestion consists of a mechanical process in which chewing promotes the food fragmentation (Minekus et al., 2014). In the present study, due to the cheese composition, amylase was not included in the oral phases of digestion in both models, thus, this digestion step did not include enzymatic hydrolysis.

In the gastric phase of digestion, however, an important modification in the peptide profile is observed, regardless of the model used. The gastric phase of digestion is characterized by the mechanical and enzymatic processing of the oral bolus. At this phase of digestion, a decrease in pH occurs and there is the action of pepsin, the only proteolytic enzyme that acts in the stomach (Guerra et al., 2012; Minekus et al., 2014). The optimal pH for pepsin action is 2.5, which may explain the important difference in the peptide profile observed in the static and dynamic systems (Minekus et al., 2014). In the INFOGEST protocol, the stomach pH is adjusted to 3, while in the SHIME® protocol, the pH is kept in the range of 2.3-2.5. Thus, possibly, the pH closest to the ideal range of pepsin action may have favored the enzymatic activity in the gastric phase of SHIME® digestion system, where a reduction in the relative intensity of the peak of *m/z* 1881 (β-CN f193-209) was observed. The same ion of *m/z* 1881 remained the most intense peak after gastric phase of simulated digestion in the INFOGEST protocol. Despite the clear difference in the hydrolysis of the β-CN f193-209 peptide (*m/z* 1881), in general, it is possible to observe a similarity in the peptide profile of the gastric phase in both methods used with the high relative intensity of peaks of *m/z* 1152, 1461, 1782, 2877.

The highest difference in peptide profiles obtained with the two different simulate digestion methods used was observed in intestinal digestion (Figures 5.1 and 5.2). At the end of intestinal digestion, complete hydrolysis of the β-CN f193-209 peptide (*m/z* 1881) was observed in the SHIME® digestion model, while the same peptide remained the most intense signal among all the ions detected in the spectra when the INFOGEST protocol was used. Additionally, the peptide profile observed in the intestinal phase of the SHIME® system indicates more intense proteolysis in this system, with a higher release of peptides in the range of 1000 to 1600 Da. Indeed, the protocols differed greatly at this stage of digestion. In the static method proposed by Minekus et al. (2014), the intestinal digestion lasts 2 hours with pH maintained at 7. In the dynamic protocol used for digestion in the SHIME® system, the same step lasts 4 hours and there is no pH adjustment after the addition of $NaHCO_3$, bile, and pancreatin. Thus, the pH of this phase of cheese digestion ranged from 3.7 to 4, which probably affected the enzymatic activity of pepsin and pancreatin enzymes resulting in the observed differences.

The β-CN f193-209 peptide (*m/z* 1881) is recognized as a bioactive peptide with antithrombotic, antimicrobial, angiotensin-converting enzyme (ACE) inhibitory and immunomodulatory activities (Birkemo, O'Sullivan, Ross, & Hill, 2009; Rojas-Ronquillo et al., 2012; Sandré et al., 2001). Its resistance to the gastrointestinal tract has already been

reported by *in vitro* studies that simulated cheese (Sánchez-Rivera et al., 2014), yogurt (Jin et al., 2016) and kefir (Liu & Pischetsrieder, 2017) digestions. Additionally, this peptide has already been detected in the blood plasma of individuals who consumed 100 g of Parmegiano Reggiano cheese per day for one week, suggesting the resistance of this peptide to the gastrointestinal tract and its effective absorption in the human body (Caira et al., 2016).

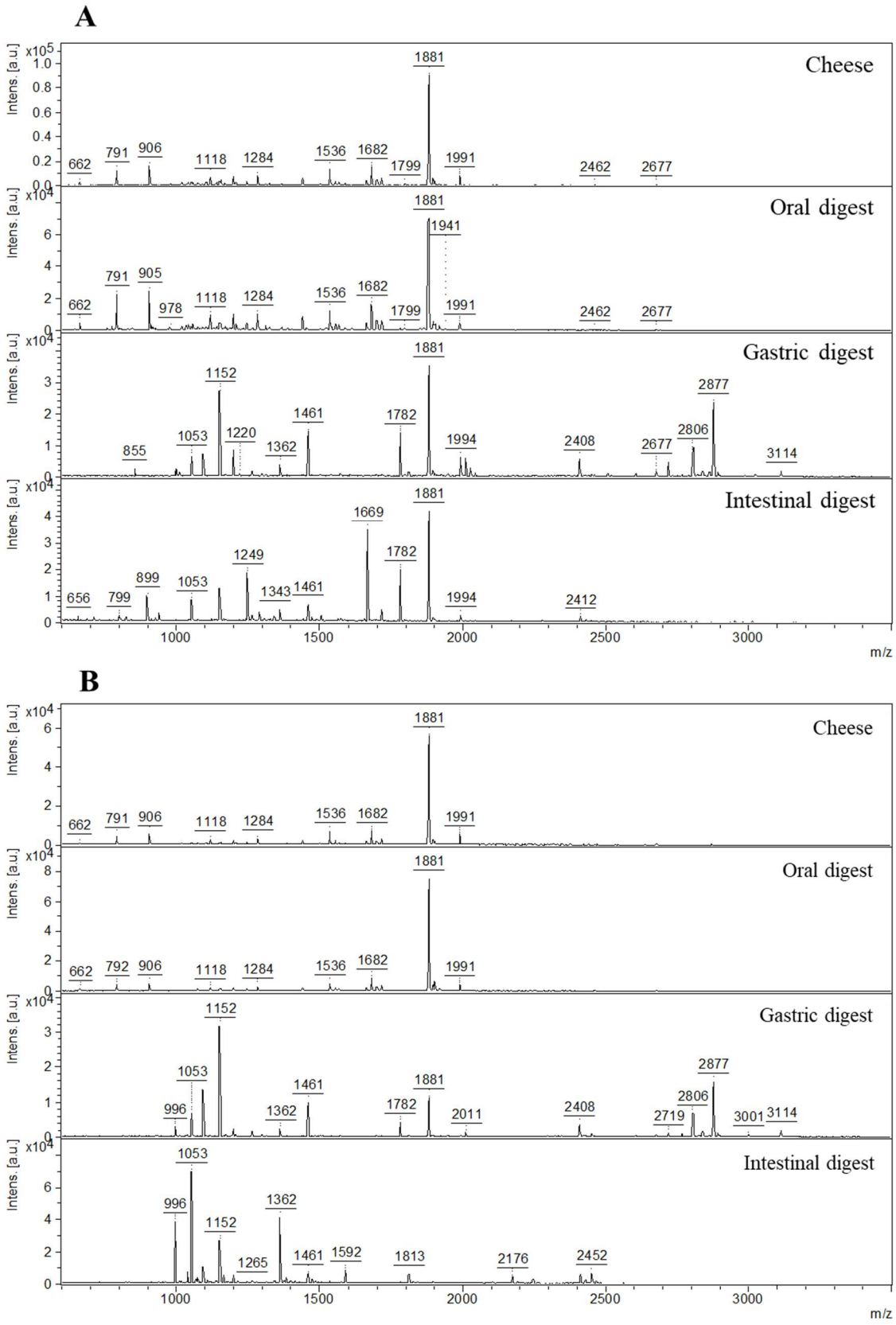

Figure 5.1. MALDI-MS spectra of control cheese, oral, gastric, and intestinal digests obtained by INFOGEST (A) and SHIME® (B) protocols. [a.u.] = arbitrary units; *m/z* = mass-to-charge ratio.

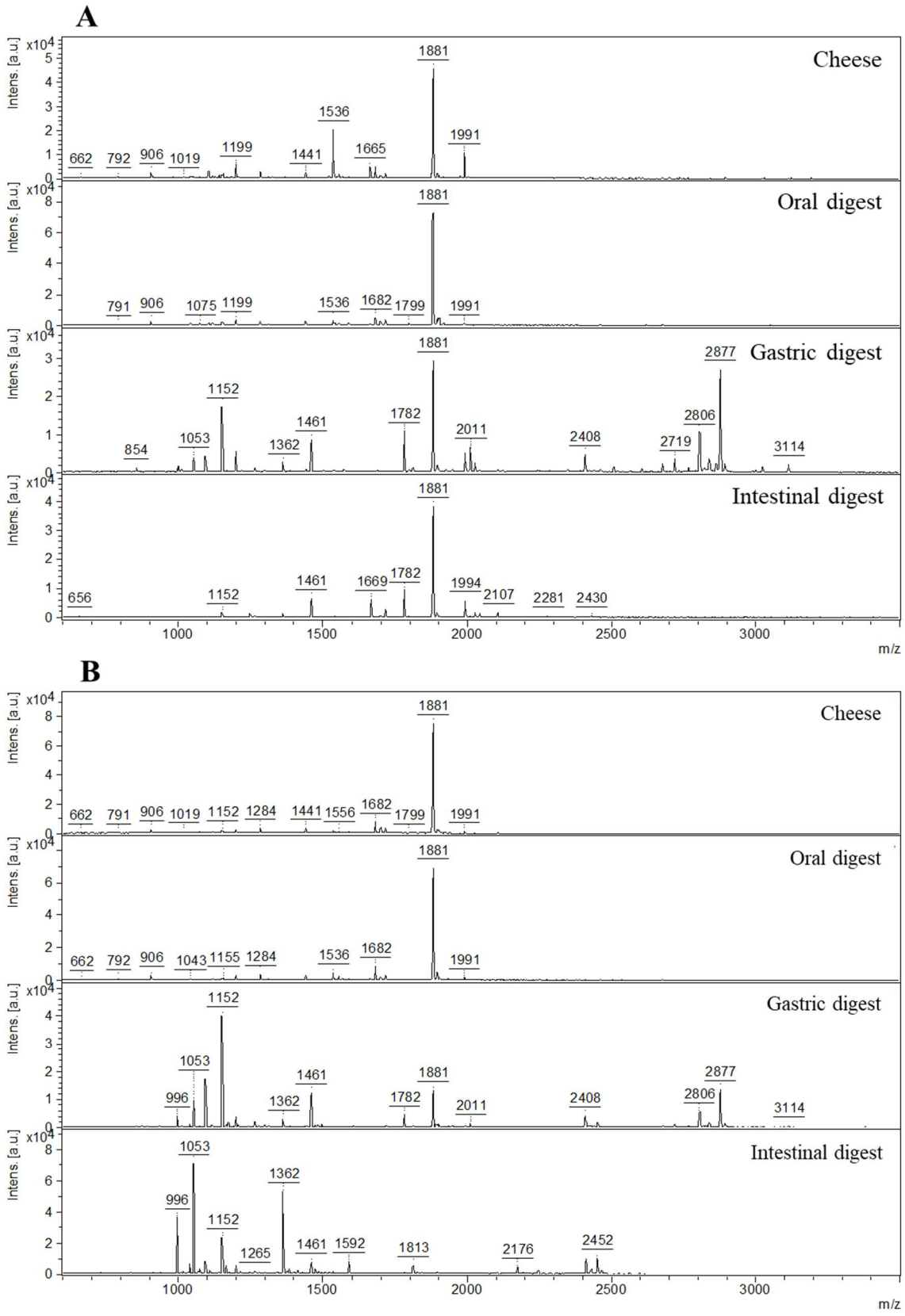

Figure 5.2. MALDI-MS spectra of cheese with adjunct culture, oral, gastric, and intestinal digests obtained by INFOGEST (A) and SHIME® (B) protocols. [a.u.] = arbitrary units; *m/z* = mass-to-charge ratio.

The effect of the addition of the adjunct culture *Lactobacillus helveticus* on the peptide profile of cheeses and digests was evaluated by the partial least squares-discriminant analysis (PLS-DA) of the mass spectral data of cheeses and digests. The chemometric analysis performed for the cheeses and digests obtained using the INFOGEST and SHIME® protocols are shown in Figures 5.3 and 5.4, respectively. As there was no modification in the cheese peptide profile in the oral phase of gastrointestinal digestion, regardless of the protocol used, this step was not considered for chemometric analysis. Figures 5.3 and 5.4 show that despite the separation of the peptide profiles of cheeses with and without the addition of adjunct culture (Figures 5.3A, 5.3B, 5.4A, and 5.4B), the hydrolysis by the action of pepsin and pancreatin enzymes during the gastric and intestinal digestion, respectively, led to an approximation of the cheeses peptide profile, that cannot be distinguished from each other based on the first two components after the gastric (Figures 5.3C, 5.3D, 5.4C and 5.4D) and intestinal (Figures 5.3E, 5.3F, 5.4E and 5.4F) phases of simulated digestion. The greater sample overlap observed in the SHIME® system after the intestinal phase of digestion (Figures 5.4E and 5.4F) compared to the INFOGEST protocol (Figures 5.3E and 5.3F) is probably associated with a more intense proteolysis in the dynamic system, as previously discussed.

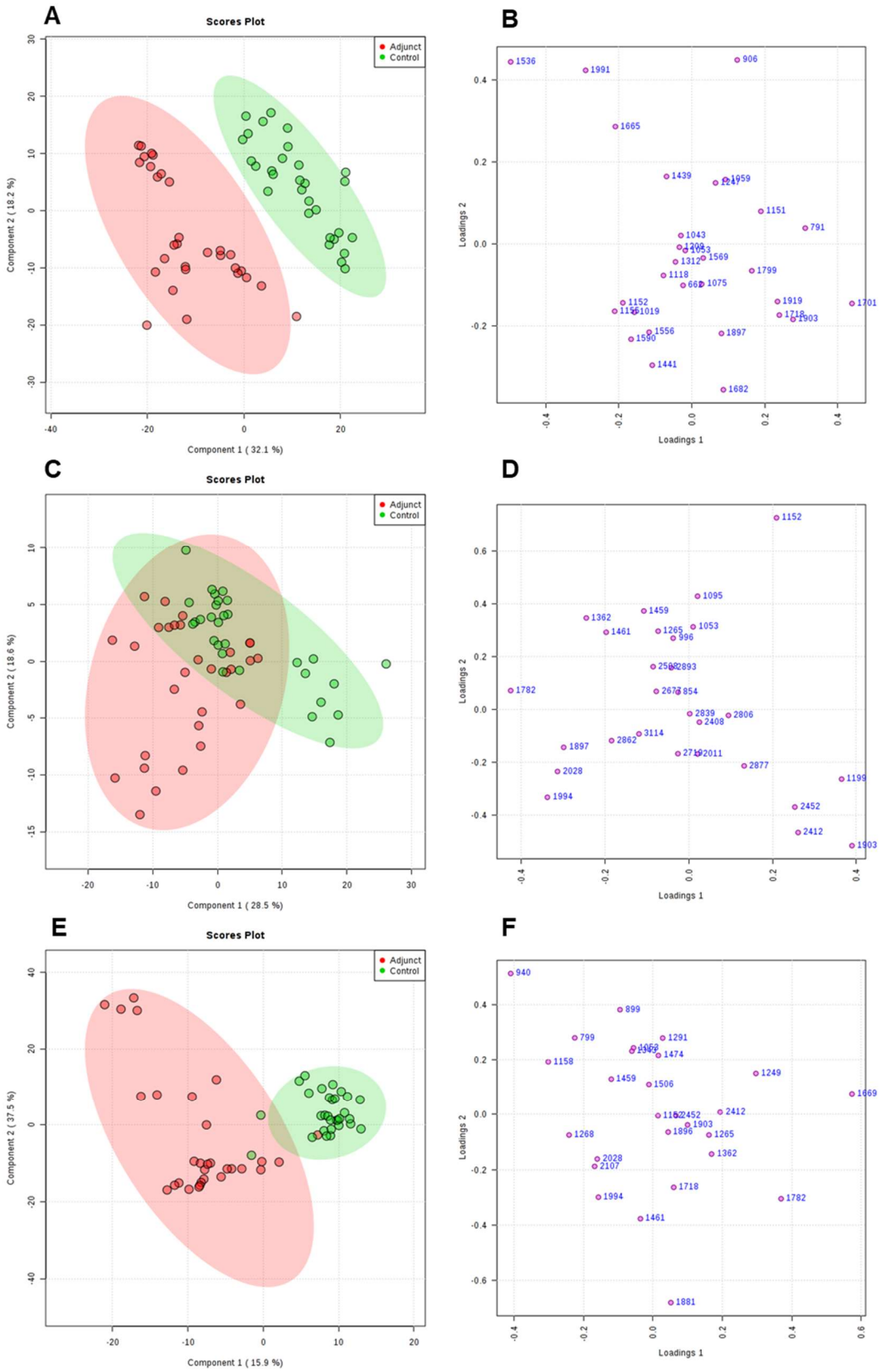

Figure 5.3. Partial least square discriminant analysis (PLS-DA) of mass spectra data of cheese and digests obtained by INFOGEST protocol. Score plots (A, C, E) and loading plots (B, D, F) of cheeses (A, B), gastric digests (C, D), and intestinal digests (E, F).

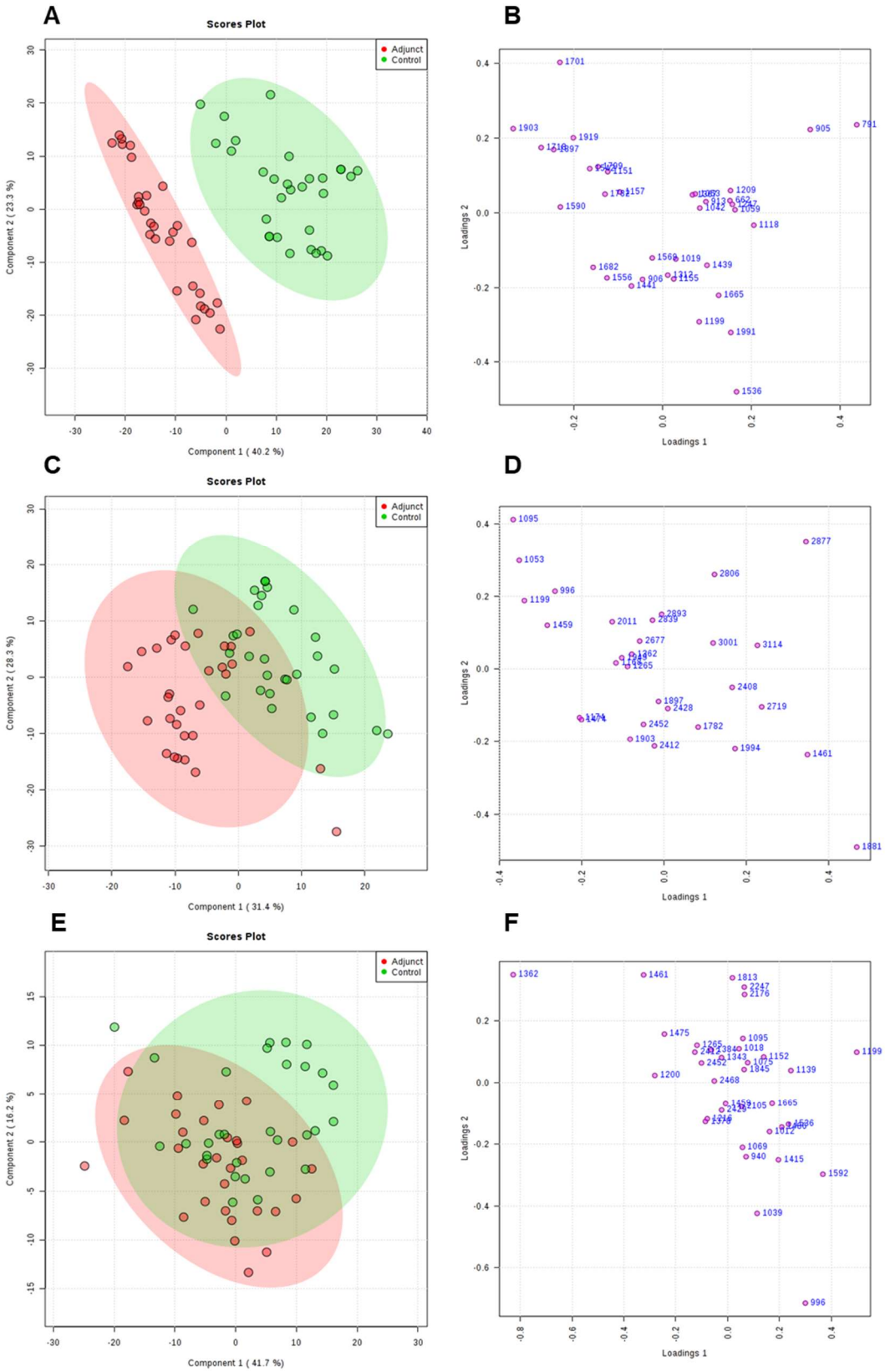

Figure 5.4. Partial least square discriminant analysis (PLS-DA) of mass spectra data of cheese and digests obtained by SHIME® protocol. Score plots (A, C, E) and loading plots (B, D, F) of cheeses (A, B), gastric digests (C, D), and intestinal digests (E, F).

5.4. Conclusion

The results showed that although it is possible to modulate the peptide profile of ripened cheese by the addition of an adjunct proteolytic culture, possibly, this modification in the peptide profile will not have a physiological effect *in vivo* since enzymatic hydrolysis that occurs during gastrointestinal digestion approximated the peptide profile of cheeses produced with or without the addition of the adjunct culture *Lactobacillus helveticus*. Further studies evaluating bioactive activities after gastrointestinal digestion are needed to support this hypothesis.

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Supplementary Material

Table 5.S1. Peptide profile of control Prato cheese and digests obtained by static and dynamic *in vitro* simulated digestion.

m/z	**Suggested peptide**	**Cheese**	**INFOGEST**		**SHIME**	
			Gastric digest	**Intestinal digest**	**Gastric digest**	**Intestinal digest**
662	α_{s1}-CN (f19-23)	✓				
773		✓				
788		✓				
791	α_{s1}-CN (f18-23)	✓				
792		✓				
799				✓		
854			✓		✓	
899				✓		
905	α_{s1}-CN (f17-23)	✓				
906	α_{s1}-CN (f17-23)/κ-CN (f161-169)	✓				
913		✓				
940				✓		✓
996			✓		✓	✓
1012			✓		✓	✓
1018	α_{s1}-CN (f16-23)	✓				✓
1019	α_{s1}-CN (f16-23)	✓				
1039					✓	✓
1042		✓				
1043		✓				✓
1053	α_{s1}-CN (f24-32)	✓	✓	✓	✓	✓
1059		✓				
1069						✓

Table 5.S1. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
1075		✓				✓
1095			✓		✓	✓
1109						✓
1118	α_{s1}-CN (f15-23)	✓				
1139						✓
1141	α_{s1}-CN (f1-9)	✓				
1151	β-CN (f199-209)	✓				
1152	β-CN (f197-207)	✓	✓	✓	✓	✓
1155	α_{s1}-CN (f4-13)	✓				
1157		✓				
1158				✓		
1168					✓	
1174			✓		✓	✓
1199		✓	✓		✓	✓
1200						✓
1209		✓				
1216						✓
1247	α_{s1}-CN (f14-23)	✓				
1249				✓		
1265	β-CN (f195–206)		✓	✓	✓	✓
1268						
1291				✓		
1312		✓				✓

Table 5.S1. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
1343			✓	✓		✓
1362			✓	✓	✓	✓
1367		✓				
1378						✓
1384						✓
1415						✓
1439		✓	✓		✓	
1441		✓				
1459			✓	✓	✓	✓
1461			✓	✓	✓	✓
1474			✓	✓	✓	✓
1475						✓
1486						✓
1506				✓		
1536	α_{s1}-CN (f1-13)	✓				✓
1542		✓				
1556	β-CN (f193-206)	✓				
1569		✓				
1590	β-CN (f195-209)	✓				✓
1592						✓
1665	α_{s1}-CN (f1-14)	✓				✓
1669				✓		
1682		✓				
1701	β-CN (f12-24)	✓				
1718	β-CN (f194-209)	✓		✓		
1782	β-CN (f193-208)	✓	✓	✓	✓	✓
1799		✓				

Table 5.S1. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
1813			✓		✓	✓
1845						✓
1877	α_{s1}-CN (f1-16)	✓	✓		✓	✓
1881	β-CN (f193-209)	✓	✓	✓	✓	
1894		✓				
1895		✓		✓		
1896				✓		✓
1897		✓	✓	✓	✓	✓
1903		✓	✓	✓	✓	
1919		✓			✓	
1949			✓		✓	
1991	α_{s1}-CN (f1-17)	✓	✓		✓	✓
1994		✓	✓	✓	✓	
2011			✓		✓	
2028			✓	✓	✓	
2044			✓	✓		
2105						✓
2107		✓		✓		
2176						✓
2247						✓
2408			✓		✓	
2412			✓	✓	✓	✓
2428				✓	✓	✓
2429				✓	✓	✓
2452			✓	✓	✓	✓
2468					✓	✓
2508			✓			
2677		✓	✓		✓	
2719			✓		✓	

Table 5.S1. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
2763				✓		
2806			✓		✓	
2822			✓		✓	
2839			✓		✓	
2862			✓			
2877			✓		✓	
2893			✓		✓	
3001					✓	
3024			✓			
3114			✓		✓	

Table 5.S2. Peptide profile of Prato cheese with adjunct culture and digests obtained by static and dynamic *in vitro* simulated digestion.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
662	α_{s1}-CN (f19-23)	✓				
788		✓				
791	α_{s1}-CN (f18-23)	✓				
792		✓				
799				✓		
854			✓		✓	
899				✓		
905	α_{s1}-CN (f17-23)	✓				
906	α_{s1}-CN (f17-23)/κ-CN (f161-169)	✓				
913		✓				
940				✓		✓
996			✓		✓	✓
1012			✓	✓	✓	✓
1018	α_{s1}-CN (f16-23)	✓				✓
1019	α_{s1}-CN (f16-23)	✓				
1039					✓	✓
1042		✓				
1043		✓				✓
1053	α_{s1}-CN (f24-32)	✓	✓	✓	✓	✓
1059		✓				
1069						✓
1075		✓				✓
1095			✓	✓	✓	✓
1109						✓
1118	α_{s1}-CN (f15-23)	✓				
1139						✓

Table 5.S2. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
1141	α_{s1}-CN (f1-9)	✓				
1151	β-CN (f199-209)	✓	✓		✓	
1152	β-CN (f197-207)	✓	✓	✓	✓	✓
1155	α_{s1}-CN (f4-13)	✓				
1157		✓				
1158				✓		
1168					✓	
1174			✓		✓	✓
1199		✓	✓		✓	✓
1200						✓
1209		✓				
1216						✓
1247	α_{s1}-CN (f14-23)	✓				
1249				✓		✓
1265	β-CN (f195–206)		✓	✓	✓	✓
1268				✓		✓
1291				✓		
1312		✓	✓			✓
1343				✓		✓
1362			✓	✓	✓	✓
1367		✓				
1378						✓
1384				✓		✓
1415						✓
1439		✓	✓		✓	
1441		✓				

Table 5.S2. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
1459			✓	✓	✓	✓
1461			✓	✓	✓	✓
1474			✓	✓	✓	
1475						✓
1486						✓
1506				✓		
1536	α_{s1}-CN (f1-13)	✓				✓
1542		✓	✓			
1556	β-CN (f193-206)	✓				
1569		✓				
1590	β-CN (f195-209)	✓				✓
1592						✓
1665	α_{s1}-CN (f1-14)	✓				✓
1669				✓		
1682		✓				
1701	β-CN (f12-24)	✓		✓		
1718	β-CN (f194-209)	✓		✓		
1782	β-CN (f193-208)	✓	✓	✓	✓	✓
1799		✓				
1813			✓		✓	✓
1845						✓
1877	α_{s1}-CN (f1-16)	✓	✓		✓	✓
1881	β-CN (f193-209)	✓	✓	✓	✓	
1894		✓		✓		
1895		✓	✓	✓		
1896				✓	✓	

Table 5.S2. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
1897		✓	✓	✓	✓	✓
1903		✓	✓	✓	✓	
1919		✓	✓		✓	
1949			✓		✓	
1991	α_{s1}-CN (f1-17)	✓	✓			✓
1994		✓	✓	✓	✓	
2011			✓		✓	
2028			✓	✓	✓	
2044			✓	✓	✓	
2105						✓
2107		✓		✓	✓	
2176						✓
2247						✓
2408			✓		✓	
2412			✓	✓	✓	✓
2428			✓		✓	✓
2429				✓	✓	✓
2452			✓	✓	✓	✓
2468					✓	✓
2508			✓			
2677		✓	✓		✓	
2719			✓		✓	
2763				✓		
2806			✓		✓	
2822			✓		✓	
2839			✓		✓	

Table 5.S2. continued.

m/z	Suggested peptide	Cheese	INFOGEST		SHIME	
			Gastric digest	Intestinal digest	Gastric digest	Intestinal digest
2862			✓		✓	
2877			✓		✓	
2893			✓		✓	
3001			✓		✓	
3024			✓		✓	
3114			✓		✓	

CAPÍTULO 6 - DISCUSSÃO GERAL

6.1. Discussão

O presente estudo apresentou a avaliação do efeito da adição de *Lactobacillus helveticus* e da redução de 25% de sal no perfil de peptídeos e atividade anti-hipertensiva *in vitro* de queijo Prato, bem como a avaliação do efeito da simulação *in vitro* da digestão gastrointestinal sobre o perfil de peptídeos de queijos Prato produzidos com a sem a adição da referida cultura adjunta.

A primeira etapa experimental do trabalho (Capítulo 3) apresentou a avaliação do efeito da adição de *Lactobacillus helveticus* LH-B02 na hidrólise caseica avaliada por eletroforese capilar e perfil de peptídeos avaliado por MALDI-MS. A adição da cultura adjunta de *Lactobacillus helveticus* LH-B02 afetou o perfil de proteólise do queijo Prato e a intensidade relativa de peptídeos reconhecidos como potencialmente bioativos, favorecendo a liberação dos peptídeos β-CN (f193-206) (*m/z* 1556) e β-CN (f194-209) (*m/z* 1718), provenientes da β-caseína e reduzindo a intensidade relativa do peptídeo α_{s1}-CN (f1-9) (*m/z* 1141). Esses resultados demonstraram a eficácia da cultura adjunta escolhida para a modificação do perfil de peptídeos bioativos e reforçaram a necessidade de avaliar o potencial bioativo dos queijos, uma vez que foi observado tanto o aumento como a redução de intensidade relativa de peptídeos potencialmente bioativos.

Nessa etapa do trabalho, o queijo controle foi fabricado com culturas tradicional para fabricação de queijo Prato (*Lactococcus lactis* spp *lactis* e *Lactococcus lactis* spp *cremoris*) e o queijo com cultura adjunta foi produzido com a cultura tradicional e *Lactobacillus helveticus* LH-B02. Ambas as culturas foram ativadas em leite em pó reconstituído estéril 10%, sendo a cultura tradicional incubada a 30°C por 8 h e a de *Lactobacillus helveticus* LH-B02 a 40°C por 18 h. O tempo de incubação necessário para a ativação da cultura foi previamente estabelecido através da curva de crescimento do microrganismo.

O comportamento de acidificação, controlado pelo pH durante o processo de fabricação dos queijos indicou maior acidificação no processamento de queijo com adição da cultura adjunta (Figura 6.1). A variação no comportamento de acidificação durante o processo de fabricação não é desejável, uma vez que pode afetar a composição dos queijos por afetar a sinérese da massa (FOX et al., 2017).

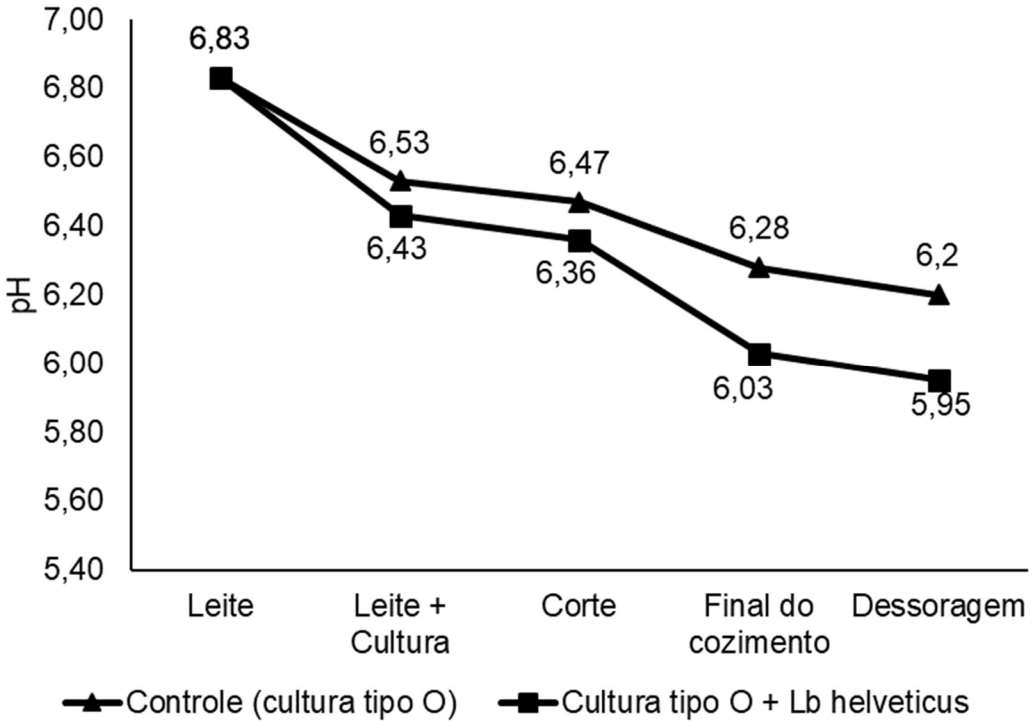

Figura 6.1.pH do leite e comportamento do pH durante a fabricação do Queijo Prato controle (▲) e com adição de cultura adjunta *Lactobacillus helveticus* LH-B02 (■).

A diferença de pH no processo de fabricação dos queijos (Figura 6.1) nos motivou à realização de experimentos adicionais com o objetivo de padronizar esse comportamento, a fim de evitar diferença nos teores de umidade, de sal e de minerais dos queijos (FOX et al., 2017) e, dessa forma, viabilizar o isolamento do fator adição de cultura adjunta. Nossa hipótese foi que a diferença de pH observada no processamento dos queijos poderia ser evitada se a cultura de *Lactobacillus helveticus* LH-B02 não fosse ativada antes da sua adição ao leite, conforme recomendação do fabricante para culturas liofilizadas tipo DVS (do inglês *direct vat set*). Assim, um novo experimento foi delineado a fim de avaliar o efeito da ativação da cultura sobre o comportamento do pH durante o processamento dos queijos.

Neste caso, o processamento do queijo Prato foi mimetizado utilizando-se 1,5 L de leite tratado termicamente (68°C/2 min), dividido em 3 porções de 500 mL. Uma porção foi adicionada de cultura acidificante constituída de *Lactococcus lactis* spp *lactis* e *Lactococcus lactis* spp *cremoris*, a segunda porção foi adicionada de cultura acidificante e cultura adjunta *Lactobacillus helveticus* LH-B02 ativada por 18h a 40°C e a terceira porção foi adicionada de cultura acidificante e cultura adjunta *Lactobacillus helveticus* LH-B02 pré-ativada por 15 min a 37°C após descongelamento. Após a adição das culturas, seguiu-se o protocolo de fabricação

de queijo Prato. O processamento foi repetido 3 vezes e durante cada processamento o pH foi avaliado no leite, no leite após adição da cultura, no ponto de corte, após o aquecimento da massa e após a dessoragem. O efeito dos tratamentos, das etapas do processamento, bem como a interação desses fatores sobre o pH foi avaliado por Análise de Variância (ANOVA). As médias foram comparadas pelo teste de Tukey considerando-se nível de significância de 5%. Os resultados foram analisados utilizando-se o programa Statistica 7.0.

O tratamento (<0,0001), tempo (<0,0001) e a interação desses fatores (<0,0001) afetaram significativamente o pH. Observa-se na Figura 6.2 que a ativação da cultura lática adjunta por 18 h a 40°C resultou em menor pH em todas as etapas do processamento. Em contrapartida, quando a cultura de *Lactobacillus helveticus* LH-B02 foi apenas pré-ativada, ou seja, mantida por 15 min a 37°C, seu comportamento de acidificação no tanque de fabricação foi essencialmente igual ao comportamento da cultura utilizada para a fabricação do queijo Prato controle (*Lactococcus lactis* spp *lactis* e *Lactococcus lactis* spp *cremoris*). Esta condição de processamento padronizada foi utilizada para a realização dos experimentos do Capítulo 4.

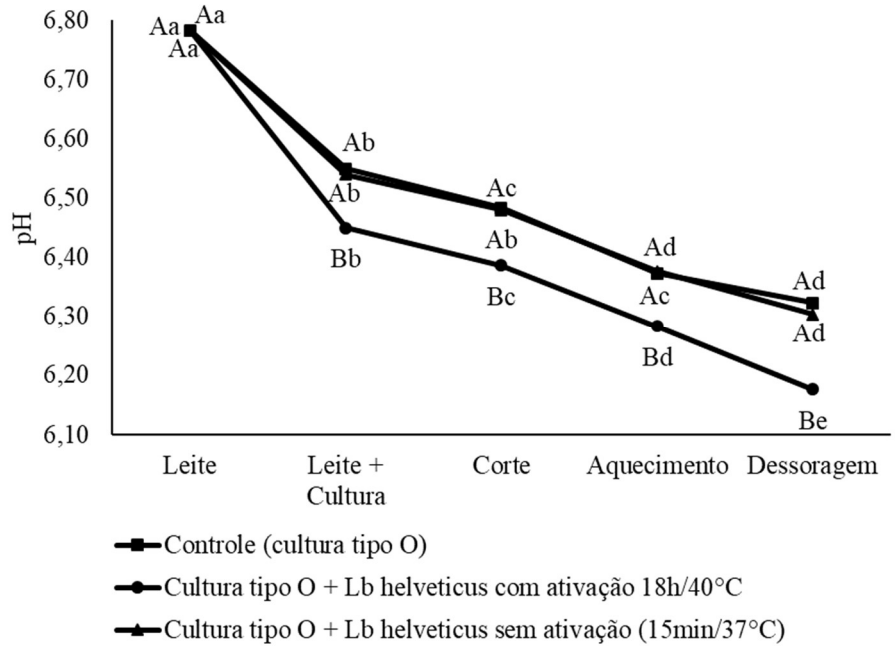

Figura 6.2. Efeito da interação entre os tratamentos e o tempo sobre pH durante a fabricação do queijo Prato. (n=3).

[A,B] Letras maiúsculas diferentes entre os tratamentos apresentam diferença significativa pelo teste de Tukey ($p < 0,05$).

[a,b] Letras minúsculas diferentes ao longo do tempo apresentam diferença significativa pelo teste de Tukey ($p < 0,05$).

Na segunda etapa experimental do trabalho (Capítulo 4), avaliamos o efeito da adição da cultura adjunta *Lactobacillus helveticus* LH-B02, da redução no teor de sal e do tempo de maturação (120 dias) na proteólise, perfil de peptídeos e atividade inibidora da enzima conversora de angiotensina (ECA) avaliada *in vitro*. Os resultados obtidos demonstraram que a adição da cultura adjunta afetou o perfil peptídico do queijo Prato e favoreceu a inibição da atividade da ECA durante a maturação do queijo.

Com relação à composição dos queijos, a adaptação na forma de adição da cultura adjunta atendeu ao objetivo proposto e resultou na produção de queijos com a mesma composição físico-química que os queijos sem a adição da cultura. No entanto, a separação dos queijos com e sem a adição de cultura adjunta na avaliação do perfil peptídico através de análises quimiométricas foi possível apenas após 90 dias de maturação, resultado muito diferente do observado no Capítulo 3, no qual os queijos com e sem a adição de cultura adjunta apresentaram diferença no perfil peptídico desde o início da maturação. A partir desse resultado, uma hipótese foi levantada: possivelmente a ativação da cultura também afeta o perfil de peptídeos do queijo. A fim de comprovar essa hipótese, um novo processamento foi realizado, a partir de 75 L de leite tratado termicamente (68°C/2 min), dividido em 3 porções de 25 L. Uma porção foi utilizada para fabricação de queijo Prato controle adicionado de cultura tradicional constituída de *Lactococcus lactis* spp *lactis* e *Lactococcus lactis* spp *cremoris*; a segunda porção foi utilizada para fabricação de queijo Prato com cultura adjunta sem ativação, adicionado de cultura tradicional e cultura adjunta *Lactobacillus helveticus* LH-B02 pré-ativada por 15 min a 37°C após descongelamento; e a terceira e última porção foi utilizada para fabricação de queijo Prato com cultura adjunta ativada, adicionado de cultura tradicional e cultura adjunta *Lactobacillus helveticus* LH-B02 ativada por 18h a 40°C. Os queijos foram fabricados de acordo com o protocolo tradicional de fabricação de queijo Prato e maturados a 12°C. Após 30 dias de maturação, foram avaliados quanto ao perfil de peptídeos por MALDI-MS. Os espectros de massa obtidos foram processados utilizando o software FlexAnalysis 3.4 (Bruker Daltonics), com subtração da linha de base e normalização. Análises quimiométrica foram realizadas utilizando o software online MetaboAnalyt 4.0 (CHONG et al., 2018), no qual foi desenvolvido filtro de dados, normalização pela soma e escalamento dos dados pelo método de Pareto. A ferramenta quimiométrica utilizada foi a análise discriminante pelo método de mínimos quadrados parciais (PLS-DA). A significância dos biomarcadores foi ranqueada usando o escore de importância de variável em projeção (VIP *score* > 1) do PLS-DA.

Os resultados obtidos comprovaram a hipótese de que a ativação prévia da cultura adjunta *Lactobacillus helveticus* LH-B02 por 18h a 40°C afeta o perfil de peptídeos dos queijos (Figura 6.3), levando à formação de um perfil de peptídeos diferenciado nos queijos com a adição de cultura adjunta ativada ou sem ativação prévia. Esse resultado está associado ao metabolismo da cultura lática, que apresenta temperatura ótima de crescimento na faixa de 42 a 45°C (SLATTERY et al., 2010). No processamento do queijo Prato, a massa é aquecida lentamente até atingir a temperatura de 42°C e é mantida nessa temperatura por cerca de 40 minutos até atingir o ponto de massa. Dessa forma, caso a cultura não seja ativada previamente, essa é mantida por pouco tempo na sua faixa ótima de temperatura de crescimento, o que possivelmente resulta em contagens baixas após o processo de fabricação do queijo. Embora o objetivo do trabalho não seja a obtenção da cultura ativa no produto final, a menor contagem certamente afeta o perfil enzimático e, consequentemente a proteólise no queijo.

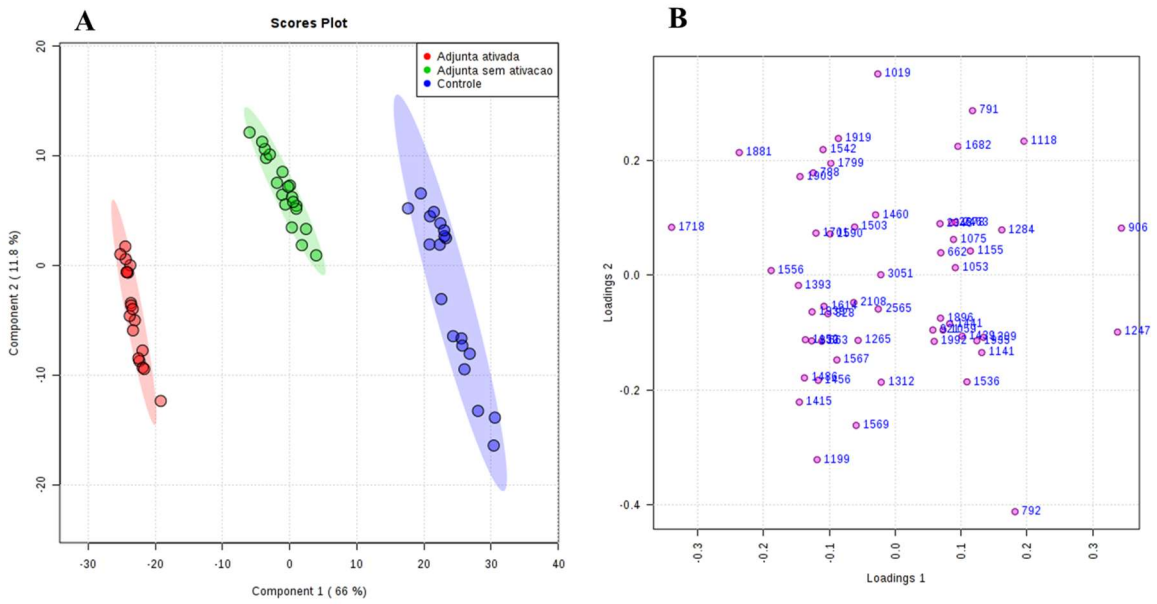

Figura 6.3. Análise discriminante pelo método dos mínimos quadrados parciais (PLS-DA) dos queijos controle, com cultura adjunta sem ativação e com cultura adjunta com ativação. Gráfico de escores (A) e gráfico de pesos (B).

A partir dos resultados obtidos no Capítulo 4, selecionamos dois queijos para serem submetidos à simulação da digestão gastrointestinal para avaliação da modificação de perfil de peptídeos pela ação das enzimas digestivas. Os queijos controle, produzido apenas com adição de cultura lática acidificante, e com cultura adjunta, com adição de cultura lática controle e *Lactobacillus helveticus* LH-B02, ambos com 90 dias de maturação, foram selecionados por

terem a maior diferença no perfil peptídico dentre os queijos avaliado nessa etapa do projeto, ou seja, a melhor separação de amostras na análise quimiométrica dos dados de massa espectrais por PLS-DA.

Dessa forma, no experimento apresentado no Capítulo 5, os queijos foram submetidos à simulação *in vitro* da digestão gastrointestinal utilizando o protocolo estático desenvolvido pela rede de cooperação internacional *INFOGEST* (MINEKUS et al., 2014), e o SHIME®, um modelo dinâmico de digestão gastrointestinal (MOLLY et al., 1993). Para realização dessa etapa do trabalho, foi necessário estabelecer um novo protocolo para o preparo de amostras provenientes da digestão simulada dos queijos para análise de peptídeos por espectrometria de massas. Esse protocolo foi desenvolvido durante estágio de doutorado sanduíche (PDSE – CAPES; Edital N° 47/2017) no *Department of Chemistry and Pharmacy* da Friedrich-Alexander-Universität Erlangen-Nürnberg – FAU, na Alemanha, sob supervisão da Profa. Dra. Monika Pischetsrieder. A técnica de microextração de peptídeos StageTip (do inglês *stop-and-go-extraction tips*) já havia sido empregada pelo grupo alemão para a extração de peptídeos de leite e kefir (MELTRETTER et al., 2008; BAUM et al., 2013a; BAUM et al., 2013b; MELTRETTER et al., 2014; EBNER et al., 2016; EBNER et al., 2015), bem como peptídeos liberados durante a digestão do kefir (LIU e PISCHETSRIEDER, 2017). Durante o estágio no exterior, a técnica foi adaptada para extração de peptídeos de queijo e amostras obtidas durante a simulação *in vitro* da digestão gastrointestinal de queijo. Após o estágio no exterior, o protocolo de microextração de peptídeos foi implementado no Laboratório de Leite e Derivados da Universidade Estadual de Campinas e utilizado para realização dos experimentos apresentados no Capítulo 5.

Os resultados obtidos na última etapa experimental do presente trabalho, apresentados no Capítulo 5, demonstraram que a despeito das diferenças no perfil de peptídeos dos queijos, a hidrólise enzimática que ocorre durante a digestão gastrointestinal é tão intensa que aproxima o perfil de peptídeos dos queijos produzidos com e sem a adição de cultura adjunta, que apresentaram essencialmente o mesmo perfil de peptídeos após a simulação da digestão com ambos os métodos de digestão utilizados.

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CAPÍTULO 7 - CONCLUSÃO GERAL

A modificação do perfil peptídico do queijo Prato é possível através da utilização da cultura adjunta *Lactobacillus helveticus* LH-B02. Os resultados demonstraram a possibilidade de modular a intensidade relativa de peptídeos bioativos e aumentar o potencial anti-hipertensivo do queijo através da adição da cultura adjunta. A ativação da cultura adjunta por 18h a 40°C afetou a acidificação durante o processo de fabricação e o perfil de peptídeos dos queijos. A utilização da cultura adjunta sem prévia ativação não afetou o perfil de acidificação no tanque de fabricação e resultou em queijos com a mesma composição que os queijos controle. No entanto, essa condição, embora permita o isolamento do fator adição de cultura adjunta, resultou também em menor diferenciação no perfil de peptídeos em relação ao controle se comparada à adição da cultura adjunta ativada por 18h a 40°C.

A redução de 25% no teor de sal modificou o perfil de peptídeos do queijo apenas após 120 dias de maturação e não afetou seu potencial anti-hipertensivo avaliado através da atividade inibidora da enzima conversora de angiotensina avaliada *in vitro*. A despeito do efeito nulo do teor de sal sobre o potencial anti-hipertensivo do queijo, sua redução em queijos atende a uma importante demanda da indústria de laticínios. Considerando a associação entre o consumo excessivo de sódio e o desenvolvimento da hipertensão, estudos *in vivo* são necessários para avaliar o efeito conjunto das duas estratégias, redução de sal e adição de cultura adjunta proteolítica em queijos, sobre a hipertensão arterial.

Estudos *in vitro* da digestão gastrointestinal de queijo Prato utilizando o modelo estático proposto pela *INFOGEST* e uma adaptação do protocolo do simulador do ecossistema microbiano humano (SEMH®) revelaram que o processo digestivo resulta em importante modificação do perfil de peptídeos do produto. Em ambos os modelos utilizados no estudo, a proteólise durante a digestão aproximou o perfil peptídico de queijos produzidos com cultura lática tradicional e com cultura lática tradicional e cultura adjunta *Lactobacillus helveticus* LH-B02. Os resultados reforçaram, portanto, a importância do estudo de digestão *in vitro* de queijos com potenciais atividades bioativas associadas à presença de peptídeos bioativos. As diferenças no perfil de peptídeos das amostras obtidas durante as etapas da simulação *in vitro* da digestão gastrointestinal com a utilização de diferentes modelos (estático e dinâmico) sugerem a necessidade de realização de estudos *in vivo* subsequentes aos estudos *in vitro* para confirmação do efeito da digestão gastrointestinal sobre o perfil de peptídeos do queijo e sua potencial bioatividade.

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ANEXO I. Autorização da editora para inclusão do artigo 1 na tese.

ANEXO 2. Autorização da editora para inclusão do artigo 2 na tese.

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Peptide profile and angiotensin-converting enzyme inhibitory activity of Prato cheese with salt reduction and Lactobacillus helveticus as an adjunct culture

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