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CARACTERIZAÇÃO E AVALIAÇÃO DA QUALIDADE NUTRICIONAL E BIOLÓGICA DAS PROTEÍNAS DO SORO DE LEITE

TESE DE DOUTORADO

Sabrina Vieira da Silva

Santa Maria, RS, Brasil

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CARACTERIZAÇÃO E AVALIAÇÃO DA QUALIDADE NUTRICIONAL E BIOLÓGICA DAS PROTEÍNAS DO SORO DE LEITE

Sabrina Vieira da Silva

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Ciência e Tecnologia dos Alimentos

Orientadora: Profa. Dra. Neila Silvia Pereira dos Santos Richards

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Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos

A Comissão Examinadora, abaixo assinada,
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CARACTERIZAÇÃO E AVALIAÇÃO DA QUALIDADE NUTRICIONAL E BIOLÓGICA DAS PROTEÍNAS DO SORO DE LEITE

elaborada por
Sabrina Vieira da Silva

como requisito parcial para obtenção do grau de
Doutor em Ciência e Tecnologia dos Alimentos

COMISSÃO EXAMINADORA:

Neila Silvia Pereira dos Santos Richards, Dra. (UFSM)
(Presidente/Orientadora)

Renata Cristina de Souza Ramos, Dra. (UNISINOS)

Viviani Ruffo de Oliveira, Dra. (UFRGS)

Liliane de Freitas Bauermann, Dra. (UFSM)

Rosa Cristina Prestes, Dra. (UFSM)

Santa Maria, 30 de setembro de 2014.

Dedico este trabalho à minha família,

em especial aos meus pais,

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos
Universidade Federal de Santa Maria

CARACTERIZAÇÃO E AVALIAÇÃO DA QUALIDADE NUTRICIONAL E BIOLÓGICA DAS PROTEÍNAS DO SORO DE LEITE

Autora: Sabrina Vieira da Silva
Orientadora: Neila Silvia Pereira dos Santos Richards
Co-orientadora: Gilberti Helena Hübscher Lopes
Santa Maria, 30 de setembro de 2014.

O objetivo do estudo foi avaliar o valor nutricional e propriedades funcionais das proteínas do soro de leite bovino – concentrado proteico (WPC35 e WPC80), hidrolisado proteico (WPH) e isolado proteico (WPI), além de uma proteína do leite bovino, a caseína, utilizada como controle. Para tanto, foram realizados ensaios físico-químicos (composição centesimal, aminoácidos essenciais e não-essenciais, minerais) e biológicos para avaliar a qualidade proteica e a resposta metabólica das fontes proteicas. Após determinação de aminoácidos essenciais e não-essenciais, foi verificado que todas as fontes proteicas atendem as recomendações estabelecidas pela FAO/WHO (2007), com exceção do WPC35 provavelmente em função do teor reduzido de proteínas. A determinação de macro e microelementos revelaram que as proteínas do leite bovino podem ser consideradas não apenas uma fonte de aminoácidos essenciais e não-essenciais, mas também de minerais (Ca, Mg e K). Para iodo a concentração foi cerca de uma ordem de magnitude superior para as proteínas do soro do leite e sódio foi encontrado em grande quantidade nas proteínas do soro do leite, podendo atingir até 70% e 59% das DRIs, respectivamente. O ensaio biológico permitiu acompanhar o ganho de peso, consumo alimentar, quociente de eficácia alimentar (QEA) e proteína ingerida dos animais, avaliar a qualidade proteica e a resposta metabólica das fontes proteicas, além da integridade de alguns órgãos e histomorfometria do intestino delgado dos animais. Estes estudos revelaram que todas as fontes proteicas são de alto valor nutricional (com exceção do WPC35), mas que maiores efeitos biológicos benéficos foram observados para WPC80, WPH e WPI. Devido ao sabor amargo do WPH, foi feita a microencapsulação por *spray-drying*, que melhorou de forma significativa o sabor na análise sensorial (Teste Triangular). Considerando-se os resultados apresentados, conclui-se que, o WPH contempla características favoráveis para ser utilizado com ingrediente em alimentos para fins especiais e que o processo de microencapsulação proporcionou uma melhoria significativa no sabor, ampliando as possibilidades de desenvolvimento de alimentos adicionados de proteínas hidrolisadas, tais como fórmulas infantis. Algumas formulações comerciais para lactentes foram avaliadas com relação ao teor de minerais indicando que a quantidade de Ca, K e Zn encontradas foram consideradas fora dos padrões da legislação brasileira, enquanto que os teores de Cu, Mn e Zn determinados foram muito diferentes daqueles descritos no rótulo.

Palavras-chave: Caseína. Proteínas do soro de leite bovino. Propriedades físico-químicas. Avaliação nutricional.

ABSTRACT

Doctor of Philosophy Thesis
Post-Graduate Program in Food Science and Technology
Federal University of Santa Maria, RS, Brazil

CHARACTERIZATION AND EVALUATION OF NUTRITIONAL AND BIOLOGICAL QUALITY OF WHEY PROTEIN

Author: Sabrina Vieira da Silva
Adviser: Neila Silvia Pereira dos Santos Richards
Co-adviser: Gilberti Helena Hübscher Lopes
Santa Maria, September 30th, 2014.

The aim of the study was to evaluate the nutritional value and functional properties of several bovine whey proteins – concentrate (WPC35 and WPC80), hydrolysate (WPH) and isolate (WPI), casein was used as control. Physicochemical (chemical composition, essential and non-essential aminoacids, minerals) and biological assays were performed to assess the biological quality of proteins and their metabolic responses. After determination of essential and non-essential amino acids, it was found that all protein sources meet the recommendations of FAO/WHO (2007), with the exception of WPC35, probably due to its reduced protein content. The determination of macro and microelements revealed that proteins from bovine milk can be considered not only a source of essential and non-essential amino acids, but also minerals (Ca, Mg and K). Iodine concentration was about one order of magnitude greater for whey proteins and sodium was found in large quantities in these proteins, which can reach 70% and 59% of DRIs, respectively. The biological assay allowed the monitoring of weight gain, food consumption, feed efficiency ratio (FER) and protein intake of animals, evaluate the quality and the metabolic response of protein sources, and the integrity of certain organs and the morphology of the small intestine animals. These studies revealed that all protein sources are of high nutritional value (except WPC35), but larger beneficial biological effects were observed for WPC80, WPH and WPI. Because of the bitter taste of WPH, microencapsulation was made by spray-drying, which significantly improved the taste of panel test (Triangular Test). Considering the presented results, it is concluded that the WPH offers favorable characteristics for use with ingredient in foods for special purposes and that the microencapsulation process yields a significant improvement in flavor, expanding the possibilities for development of foods purposes added hydrolysed proteins, such as infant formula. Some commercial formulations for infants were evaluated with respect to mineral content indicating that the amount of Ca, K and Zn found were considered out of standard of Brazilian law, while the Cu, Mn and Zn levels were very different from those described in label.

Keywords: Casein. Whey proteins of bovine milk. Physicochemical properties. Nutritional assessment.

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1 INTRODUÇÃO

O avanço dos conhecimentos científicos, a inter-relação entre dieta e o processo saúde/doença, os custos da saúde pública e interesses econômicos, favorecem o surgimento de novos produtos, cujas funções pretendem ir além da importância nutricional e sensorial dos alimentos. Hoje, se admite que certos componentes da dieta além de evitar uma deficiência nutricional, ajudam na redução de riscos de futuras doenças (BORGES et al., 2001; THAMER & PENNA, 2006).

A literatura relata vários ingredientes alimentares com alegação funcional, os quais são utilizados no desenvolvimento de novos produtos para o mercado consumidor. Dentre eles pode-se citar os prebióticos, probióticos, oligossacarídios, fibras dietéticas, vitaminas, minerais, fitoquímicos, antioxidantes, peptídios e proteínas do soro de leite (PSZCZOLA, 2001).

O soro é a fração do leite obtida após a retirada das caseínas e contém mais da metade dos nutrientes do leite, os quais são representados por proteínas, lactose, sais minerais (cálcio, fósforo, magnésio e zinco), vitaminas, traços de gordura e enzimas. As proteínas do soro de leite em razão de sua composição rica em aminoácidos essenciais apresentam alta biodisponibilidade e valor nutritivo (HOFFMAN & FALVO, 2004).

Além das excelentes propriedades nutritivas e funcionais associadas às proteínas do soro de leite, algumas propriedades tecnológicas, tais como solubilidade, capacidade de retenção de água e gordura, capacidade de emulsificação e geleificação, formação de espuma e filmes comestíveis, permitem agregar aos produtos que as contêm características de aparência e sensoriais tornando os mesmos atraentes para elaboração de suplementos alimentares (SINGH et al., 2000).

A utilização de soro de leite bovino e seus derivados proteicos como ingrediente adicionado em alimentos para fins especiais agrega valor nutricional e de mercado a este resíduo da indústria de laticínios, além de minimizar os problemas ambientais gerados pelo descarte nos rios e mananciais (RICHARDS, 2002).

Os resultados desta tese encontram-se sob a forma de cinco artigos científicos, os quais serão apresentados no decorrer deste documento. O ARTIGO 5 foi um estudo inicial onde foram obtidos resultados preliminares para nortear a pesquisa.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a qualidade nutricional e biológica das proteínas do soro de leite.

2.2 Objetivos específicos

- Determinar a composição centesimal, aminoácidos essenciais e aminoácidos não essenciais das fontes proteicas.
- Quantificar os macro e microelementos, bem como bromo e iodo das proteínas do leite bovino.
- Avaliar a qualidade proteica, resposta metabólica e histomorfometria do intestino delgado através do ensaio biológico com animais alimentados com diferentes fontes proteicas.
- Analisar o grau de hidrólise da proteína parcialmente hidrolisada do soro de leite.
- Avaliar qualitativamente proteínas do soro de leite através da eletroforese em gel de poliacrilamida para identificar as proteínas de baixo peso molecular.
- Caracterizar o hidrolisado proteico do soro de leite bovino (WPH) microencapsulado.
- Avaliar o atributo sabor do hidrolisado proteico do soro de leite bovino (WPH) microencapsulado.

3 REVISÃO BIBLIOGRÁFICA

3.1 Soro de leite bovino

O Regulamento Técnico de Identidade e Qualidade de Bebida Láctea define soro de leite como o líquido residual obtido a partir da coagulação do leite destinado à fabricação de queijos ou de caseína (BRASIL, 2005). O soro é composto de, aproximadamente, 93% de água, 5% de lactose, 0,7 a 0,9% de proteínas, 0,3 a 0,5% de gordura, 0,2% de ácido láctico e pequenas quantidades de vitaminas (TEIXEIRA et al., 2005).

O soro de leite pode ser obtido em laboratório ou na indústria por três processos principais (BORGES et al., 2001):

a) Precipitação pela acidificação com ácido orgânico ou mineral em pH 4,6 (ponto isoelétrico) a 20 ºC, seguida de centrifugação para obtenção da caseína isoelétrica (que pode ser transformada em caseinatos) e de soro ácido;

b) Pelo processo de coagulação enzimática (enzima quimosina). Como produtos deste processo têm-se o coágulo de caseínas, matéria-prima para produção de queijos, e o “soro doce”, assim chamado para se diferenciar do soro ácido, obtido no processo anterior;

c) Separação física das micelas intactas de caseína por membranas, obtendo-se como produto a caseína na forma micelar e o soro natural, sem nenhuma alteração por agentes químicos ou enzimáticos.

Em relação à composição, o “soro doce” contém cerca de 6,4% de extrato seco, isto é, representa a metade da matéria seca do leite, 4,8% de lactose, 0,73% de proteínas, 0,05% de gordura, 0,5% de minerais, 0,05% de ácido láctico e densidade de 1,024 g/L à 15 ºC. Para o “soro ácido”, tem-se: 6,5% de extrato seco, 4,9% de lactose, 0,7% de proteínas, 0,04% de gordura, 0,8% de minerais, 0,40% de ácido láctico e densidade de 1,028 g/L à 15 ºC (RANHOTRA et al., 1997).

O soro é um ingrediente nutritivo, portanto indicado para dietas incompletas e inadequadas. Contém aminoácidos essenciais que são facilmente digeridos e possui, também, concentrações adequadas de potássio, cálcio, fósforo, sódio e magnésio, além de vitaminas hidrossolúveis como ácido pantotênico, riboflavina, tiamina, piridoxina, ácido ascórbico e cianocobalamina (MACHADO et al., 2002).

Cerca de 50% de todo o soro produzido no Brasil não é aproveitado, sendo descartado sem nenhum tratamento (SANTANA et al., 2005). Quando lançado em cursos de água

provoca efeito poluidor devido ao consumo de oxigênio da água pelo desenvolvimento de bactérias e outros organismos que utilizam seus componentes. O soro, do ponto de vista biológico, é um dos resíduos mais poluentes, tendo uma demanda bioquímica de oxigênio entre 30.000 e 60.000 ppm (MACHADO et al., 2002). Uma fábrica com eliminação diária média de 10.000 litros de soro polui diariamente o equivalente a uma população de 5.000 habitantes (SANTOS & FERREIRA, 2001).

A crescente demanda por alimentos cada vez mais nutritivos, acessíveis e com menor custo de produção, tornam o soro uma importante fonte de nutrientes nobres, passíveis de serem recuperados e empregados na elaboração de uma grande variedade de produtos alimentícios (MAROULIS & SARAVACOS, 2007).

Dentre as opções para o aproveitamento do soro pode-se citar o uso em bebidas para alimentação humana, fabricação de ricota, concentração e produção de soro em pó e soro desmineralizado em pó, além da separação das proteínas e de lactose com posterior secagem (GIROTO & PAWLOWSKY, 2004). O soro também é encontrado em produtos de panificação, confeitos, chocolates, molhos, sopas, produtos desidratados, barras de cereais, bebidas lácteas, biscoitos, isotônicos e até mesmo em produtos cárneos, como presuntos e hambúrgueres (MARCHIORI, 2006).

A adição de soro de leite e seus derivados em diferentes produtos alimentícios revela sua ampla aplicação na indústria de alimentos, resultando no aumento de sua demanda e na contribuição positiva para o estado nutricional da população, além de proporcionar diminuição dos custos destes produtos, em relação aos seus concorrentes (SEVERO, 1995).

3.2 Proteínas do leite bovino

O leite é considerado uma das principais fontes proteicas alimentares, sendo um dos mais importantes e um dos mais antigos produtos funcionais disponíveis para os seres humanos. Por esse motivo, cientistas estão desenvolvendo estudos dos componentes do leite, incluindo o soro, e o impacto que os mesmos podem proporcionar na saúde e na doença (MARSHALL, 2004). O leite bovino possui aproximadamente 3,25% de proteínas que compreendem duas frações principais: caseínas, que se apresentam principalmente no estado de partículas coloidais (micelas) e proteínas do soro, que estão em solução (ROMAN & SGARBIERI, 2005). A caseína representa 80% das proteínas do leite de vaca, sendo que os 20% restantes se encontram no soro (BORGES et al., 2001), como demonstrado na Figura 1.

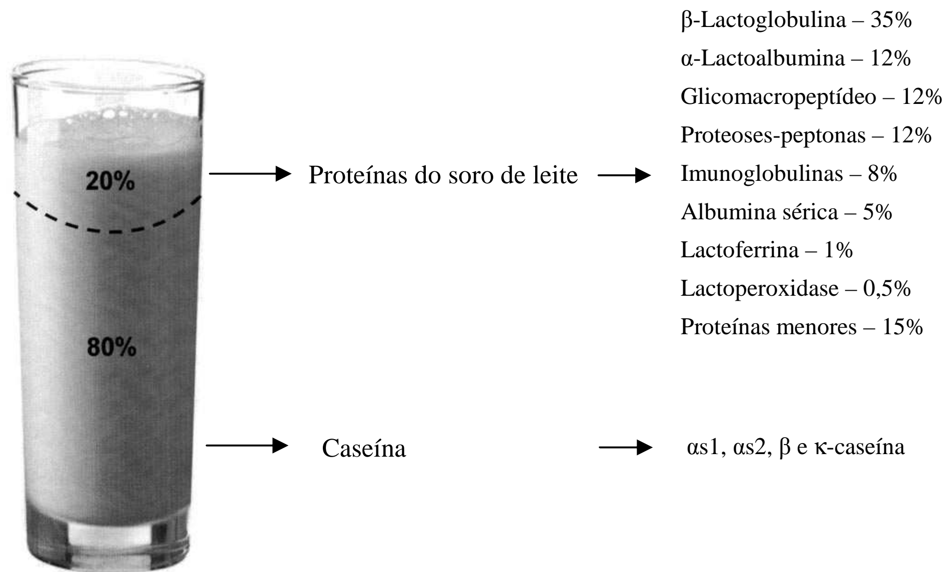

Figura 1. Distribuição das proteínas do leite bovino: caseína, proteínas do soro de leite e respectivas frações.

Fonte: Adaptado de Krissansen (2007).

O desenvolvimento das tecnologias de separação das proteínas do leite permitiu agregar valor ao leite, por meio da produção de ingredientes proteicos para usos específicos na indústria de alimentos. A introdução de modificações de processamento proporcionou a obtenção de ingredientes com amplas características funcionais, incluindo os concentrados e isolados proteicos de leite, caseínas, caseinatos, concentrados, isolados proteicos do soro, hidrolisados e outros. Em cada uma dessas categorias de ingredientes existe ainda maior diferenciação, relativa às necessidades nutricionais e funcionais desejadas ao produto final (HUFFMAN & HARPER, 1999).

3.2.1 Caseína

A caseína pode ser definida, de maneira simplificada, como sendo a proteína precipitada por acidificação do leite desnatado em pH 4,6 a 20 °C. As proteínas que permanecem em solução, nestas condições, podem ser obtidas por precipitação com sulfato de amônio. Essas proteínas encontram-se em forte associação devido à combinação de fatores como regiões hidrofóbicas, pontes de hidrogênio, ligações dissulfeto e hidrostáticas (SGARBIERI, 1996).

Consistem em um grupo heterogêneo de fosfoproteínas que podem ser isoladas por procedimentos físicos e químicos, como centrifugação do leite para separar as micelas de caseína, filtração em gel, hidrólise enzimática limitada da κ-caseína e o método clássico de precipitação isoelétrica, em pH 4,6, entre 20 e 35 °C, seguido de centrifugação para recolher o precipitado de caseínas. As caseínas do leite bovino compreendem quatro frações: αs1, αs2, β e κ-caseína, nas proporções de 44, 13, 33 e 10%, respectivamente (XU, 1998).

A caseína é considerada um dos derivados mais importantes do leite, utilizada na formulação de bebidas, queijos processados, produtos para confeitarias, indústria de chocolates, produtos fermentados, sobremesas, pastas, sopas, cereais matinais e produtos cárneos. Além do alto valor nutritivo, as caseínas conferem aos produtos formulados melhor aparência e melhores propriedades sensoriais, em virtude de suas propriedades funcionais e tecnológicas (MODLER, 2000).

As caseínas são de natureza anfotérica, possibilitando reações desejáveis em sistemas alimentícios, nas interfaces óleo-água e ar-água como componente de sistemas funcionais ou pelo aumento do valor proteico do produto final (MODLER, 2000).

3.2.2 Proteínas do soro de leite bovino

O soro contém uma mistura rica e heterogênea de proteínas com atributos funcionais podendo ser utilizadas para fins nutricionais, biológicos e em alimentos. O soro de leite bovino contém de 4 a 7 g de proteína/litro, sendo que a concentração pode variar dependendo do tipo de soro (ácido ou doce), estágio de lactação e condições de processamento usadas na produção de queijo ou da caseína (DE WIT, 1998).

O termo “proteínas do soro” refere-se às proteínas do leite que permanecem no soro após a separação da caseína ou da produção de queijo. As principais proteínas do soro são a β-lactoglobulina e a α-lactoalbumina, duas proteínas globulares de baixo peso molecular que representam de 70 a 80% das proteínas do soro, seguidas da albumina sérica, lactoferrina, lactoperoxidase, glicomacropeptídios e das imunoglobulinas. As imunoglobulinas incluem IgG, IgA e IgM, sendo que a IgG corresponde a 80% do total das imunoglobulinas de leite bovino (SGARBIERI, 2005).

Do ponto de vista nutricional, as proteínas do soro de leite apresentam excelente composição em aminoácidos, alta digestibilidade e biodisponibilidade de aminoácidos essenciais. Quanto à composição aminoacídica, as proteínas do soro apresentam os aminoácidos essenciais que atendem às recomendações para todas as idades, destacando-se o

triptofano, a cistina, a leucina, a isoleucina e a lisina, exceto os aminoácidos aromáticos (fenilalanina e tirosina), que não se encontram em quantidades suficientes para atender às recomendações para todas as idades (BORGES et al., 2001; SGARBIERI, 2005).

As proteínas do soro de leite e caseína apresentam diferentes propriedades digestivas. A ingestão de proteínas do soro de leite leva ao aumento rápido no nível de aminoácidos do plasma sanguíneo e concomitante estimulam a síntese de proteínas sanguíneas e teciduais, adequadas para situações de estresse metabólico. São altamente digeríveis e rapidamente absorvidas pelo organismo apresentando, portanto, um esvaziamento gástrico mais rápido em relação à caseína, conhecida por formar coágulos no estômago e demorar mais tempo para ser digerida e entrar no duodeno. É por essa razão que a caseína é denominada de proteína *slow*, enquanto que as proteínas do soro leite, de proteínas *fast metabolizing protein* (SÉVERIN & WENSHUI, 2005).

A funcionalidade das proteínas é um fator complementar à qualidade nutricional. Do ponto de vista tecnológico, as propriedades funcionais importantes são aquelas que melhoram o comportamento tecnológico da proteína, melhorando as características sensoriais e de aparência dos produtos que as contêm. Dentre essas propriedades, as de maior interesse são: solubilidade, capacidade de retenção de água e gordura, capacidade de emulsificação e estabilidade de emulsões, capacidade de formação de espuma e estabilidade da espuma, capacidade de geleificação, formação de filmes comestíveis e/ou biodegradáveis, que podem ser explicadas pelas características estruturais e físico-químicas (SINGH et al., 2000).

As ações fisiológicas específicas das proteínas do soro estão sendo associadas a vários efeitos biológicos observados em estudos com animais e humanos, que variam desde atividade antimicrobiana e antiviral, devido a seu conteúdo de lactoferrina, lactoperoxidase, α-lactoalbuminas e imunoglobulinas; atividade antiúlcera, por conter grande quantidade de aminoácidos sulfurados, particularmente a cistina, que promove o aumento da síntese de glutationa, que, por sua vez, é capaz de proteger tecidos epiteliais; atividade protetora do sistema cardiovascular; atividade anticancerígena; absorção de minerais, ação antioxidante; melhora do desempenho físico e promove a longevidade (TASSI et al., 1998; SGARBIERI, 2004; KITTS & NAKAMURA, 2006; PACHECO et al., 2006).

A Tabela 1 descreve os principais componentes do soro de leite, quantidade de proteínas do soro, peso molecular e os principais benefícios.

Tabela 1. Componentes do soro de leite bovino e principais benefícios.

Componentes do soro	**Quantidade (%) de proteínas do soro**	**Peso molecular (kDa*)**	**Principais benefícios**
β-Lactoglobulina	50 - 55	18,3	Fonte de AA** essenciais e de cadeia ramificada, antioxidante, efeito hipocolesterolêmico
α-Lactoalbumina	20 - 25	14,1	Fonte de AA essenciais e de cadeia ramificada, imunomodulação, ação anticancerígena, efeito hipocolesterolêmico
Imunoglobulinas	10 - 15	160 a 900	Modulação do sistema imunológico
Lactoferrina	1 - 2	80	Ação antimicrobiana, antiviral, antifúngica e antioxidante. Promove o crescimento de bactérias benéficas, atividade anticancerígena, imunomodulação e absorção de ferro
Lactoperoxidase	0,5	77	Ação antimicrobiana
Albumina do soro bovino (BSA)	5 - 10	66,2	Fonte de AA essenciais, transporte de nutrientes
Glicomacropeptídeo (GMP)	10 - 15	-	Fonte de AA de cadeia ramificada, ação antimicrobiana e antiviral

Fonte: Adaptado de Shah (2000); Marshall (2004). kDa* = kilo daltons. AA** = aminoácidos.

As proteínas do soro do leite podem ser utilizadas em aplicações nutricionais, como fórmulas enterais e infantis, na forma de proteínas íntegras ou pré-digeridas, visando ganho de peso e recuperação do estado nutricional, para pacientes pós-cirúrgicos, geriátricos e imobilizados (DE WIT, 1998).

Devido às propriedades tecnológicas, funcionais e biológicas específicas de cada uma das proteínas do soro, há um crescente interesse no fracionamento dessas proteínas, pois muitas vezes estas características não se fazem notar nos concentrados proteicos devido às interações com os componentes presentes (BRAMAUD, AIRNAIR & DAUFIN, 1997).

A adoção de técnicas como ultrafiltração, cromatografia, extração líquido-líquido, entre outras, resultou em um alto grau de separação e purificação das proteínas do soro,

levando ao desenvolvimento de produtos de alto valor nutricional, como concentrados de proteína do soro e isolados de proteína do soro, adequados para o uso em alimentos e ingredientes alimentares (FOEGEDING et al., 2002).

Os concentrados proteicos de soro estão disponíveis em níveis de proteína entre 34 a 85%, os teores médios de cinzas, lactose e gordura no concentrado variam, significativamente, dependendo do conteúdo de proteína (EL-SALAM, EL-SHIBINY & SALEM, 2009). O concentrado é obtido por ultrafiltração do soro ácido ou doce e seco em "*spray dryer*" para manter a funcionalidade das proteínas. É possível obter-se um concentrado com teor maior de 80% de proteínas por meio de ultrafiltração e diafiltração seguido de secagem por atomização. A desnaturação promovida nas proteínas de soro varia de 14,3 a 21,7%. O concentrado proteico de soro com 34% de proteínas apresenta uma composição semelhante à do leite desnatado em pó, diferenciando-se no perfil de minerais e no tipo de proteína (RELKIN et al., 2007).

Os isolados proteicos de soro contêm, no mínimo, 90% de proteína em base seca, menos de 1% de gordura e lactose e cerca de 2% de cinzas. Os isolados compreendem as fontes mais puras de proteínas disponíveis e podem ser preparados por troca iônica ou por processo de ultrafiltração com utilização de membranas. Durante o processamento dos isolados proteicos há uma remoção significativa de gordura e lactose. Como resultado, indivíduos que são intolerantes à lactose podem ingerir com segurança esses produtos. Embora a concentração de proteína dos isolados seja maior, algumas vezes pode haver desnaturação proteica devido aos processos de obtenção utilizados. Essa desnaturação acarreta redução nas propriedades funcionais biológicas das proteínas (YEE et al., 2007; PHILIPINA & RIZVI, 2008; LANDS et al., 2010).

A utilização de hidrolisados proteicos do soro de leite tem recebido atenção especial da indústria alimentícia e em estudos relacionados à fisiologia animal, devido suas propriedades funcionais. Com a hidrólise de proteínas são formados agregados de peso molecular variável, como peptonas, misturas de peptídios e aminoácidos livres (LAHL & BRAUN, 1994).

Os peptídios bioativos são definidos como fragmentos específicos de proteína que tem um impacto positivo nas funções corporais, podendo influenciar na saúde, entretanto, para isso é necessário que sejam resistentes à ação de proteínas digestivas (KITTS, 2005). Os peptídios bioativos dos componentes das proteínas do soro de leite, como as imunoglobulinas, lactoferrina, lactoperoxidase e glicomacropeptídio, são disponíveis comercialmente de forma

isolada, com aplicações específicas em fórmulas infantis, dietas clínicas e componentes das pastas de dentes e agentes conservantes contra micro-organismos (STEIJNS, 2001).

A hidrólise de proteínas pode ser executada por enzimas, ácido ou base forte, mas o uso de enzimas, como por exemplo, as do sistema digestivo (pancreatina, pepsina, α-quimotripsina), algumas de origem vegetal como papaína e várias enzimas de origem microbiana, é o método preferido para a obtenção de hidrolisados para aplicações nutricionais, sendo utilizados em formulações especificas incluindo fórmulas infantis hipoalergênicas, preparações imunoestimulantes, produtos geriátricos, dietas terapêuticas e bebidas esportivas (CLEMENTE, 2000).

As características de composição, propriedades físico-químicas, funcionais e sensoriais de hidrolisados proteicos enzimáticos dependem de vários fatores, sendo os mais importantes à natureza química e estrutural da proteína, natureza e intensidade dos processos a que tenham sido submetidos, condições da hidrólise e especificidade da enzima proteolítica e finalmente o grau de hidrólise. Os critérios mais importantes a serem considerados na produção de hidrolisados proteicos incluem valor nutricional, custo, sabor, antigenicidade e funcionalidade (OBEN et al., 2008).

As alegações de atividades biológicas benéficas do soro do leite, combinadas ao grande crescimento do mercado de alimentos funcionais e ao aumento da demanda de consumidores por proteínas de alta qualidade, são um grande atrativo à utilização da proteína do soro e de suas frações como ingredientes para alimentos funcionais, e isto atualmente vem despertando o interesse da indústria de alimentos (MCINTOSH et al., 1998).

3.3 Microencapsulação

A tecnologia de microencapsulação foi definida por Todd (1970) como o empacotamento com finas coberturas poliméricas de sólidos, líquidos ou material gasoso, dando origem a microcápsulas que podem liberar seus conteúdos a taxas controladas sob influência de condições específicas (FÁVARO-TRINDADE, DE PINHO & ROCHA, 2008). Arshady (1993) descreveu as microcápsulas como embalagens extremamente pequenas, compostas por um polímero como material de parede e um material ativo chamado de núcleo. O diâmetro e a forma podem variar de acordo com o agente encapsulante e método utilizados (FÁVARO-TRINDADE, DE PINHO & ROCHA, 2008).

De acordo com Shahidi & Han (1993), as microcápsulas têm a capacidade de modificar e melhorar a aparência e as propriedades de uma substância. Esses autores

compilaram os seguintes motivos para o uso da microencapsulação na indústria alimentícia: (i) reduzir a reatividade do material de núcleo com o ambiente; (ii) diminuir a velocidade de evaporação ou de transferência do material de núcleo para o meio; (iii) facilitar a manipulação do material encapsulado; (iv) promover liberação controlada; (v) mascarar sabor e odor desagradáveis; e (vi) promover a diluição homogênea do material encapsulado em uma formulação alimentícia.

Para cada método de microencapsulação, as etapas fundamentais são: incorporação dos compostos bioativos; formação das gotículas; remoção do solvente; coleta das microcápsulas e secagem (DALMORO et al., 2012).

Dependendo das propriedades físico-químicas do núcleo, da composição do material de parede (agente encapsulante) e da técnica de microencapsulação utilizada, diferentes tipos de partículas podem ser obtidas: esfera simples circundada por uma camada de espessura uniforme; partícula contendo um núcleo de formato irregular; várias partículas de núcleos em uma matriz contínua de material de parede; vários núcleos distintos dentro da mesma cápsula; e microcápsulas multi-parede (GHARSALLAOUI et al., 2007). Dentre essas partículas, a esfera simples é a mais comum de ser fabricada e utilizada (ZHAO & ZHANG, 2011).

3.3.1 Métodos de microencapsulação

Entre as técnicas utilizadas para a microencapsulação, estão as de: i) *Spray-drying* ou atomização, em que geralmente são utilizados como materiais de revestimento polímeros solúveis em água; ii) *Spray-congealing*, que utiliza ceras, ácidos graxos, polímeros solúveis e insolúveis em água, além de outros monômeros como material de revestimento; iii) *Fluidizedbed coating/air-suspension*, que utiliza polímeros solúveis e insolúveis em água, lipídeos e ceras como material de revestimento; iv) Extrusão, que utiliza como revestimento da cápsula polímeros solúveis e insolúveis em água; v) Coacervação ou técnica de separação de fases, que utiliza como material encapsulante polímeros solúveis em água; e vi) Método eletrostático, que utiliza como material de revestimento polímeros e outros compostos com cargas opostas (MENEZES et al., 2013).

3.3.2 Agentes Encapsulantes

A escolha do agente encapsulante depende de uma série de fatores, entre eles a não reatividade com o material a ser encapsulado, o processo utilizado para a formação da

microcápsula e o mecanismo de liberação ideal (FÁVARO-TRINDADE, DE PINHO & ROCHA, 2008).

Muitos materiais podem ser utilizados como cobertura para as microcápsulas, dentre eles: goma arábica, ágar, alginato e carragena; os carboidratos amido, amidos modificados, dextrinas e sacarose; as celuloses carboximetilcelulose, acetilcelulose, nitrocelulose; os lipídios parafina, mono e diacilgliceróis, óleos e gorduras; os materiais inorgânicos sulfato de cálcio e silicatos; as proteínas do glúten, caseína, gelatina e albumina (FÁVARO-TRINDADE, DE PINHO & ROCHA, 2008).

4 ARTIGOS CIENTÍFICOS

ARTIGO 1

Elemental (Macro- and Microelements) and Amino Acid Characterization of Milk Proteins and Their Nutritional Value

(Artigo submetido para o *Journal of Food Composition and Analysis*)

Sabrina Vieira da Silva[1], Rochele Sogari Picolotto[2], Roger Wagner[1], Neila Silvia Peireira dos Santos Richards[1], Juliano Smanioto Barin[1]*

[1]*Departamento de Tecnologia e Ciência dos Alimentos, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil*

[2]*Departamento de Química, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil*

*Corresponding author: Tel/Fax: +55-55-3220-8254

E-mail address: juliano@ufsm.br

1 **ABSTRACT**

2 Whey protein concentrate (WPC), hydrolysate (WPH), isolate (WPI) and casein were
3 evaluated regarding to their proximal composition, essential and non-essential aminoacids and
4 macro- and microelement content. Casein and WPI presented the highest protein contents
5 (82.78 and 85.82%, respectively) and the lowest amount of lipids (1.70 and 1.15%,
6 respectively) and ashes (1.91 and 2.39, respectively). The highest ash contents were observed
7 for WPC35 and WPH and could be directly related to the amount of Ca, K, Mg, Na and P
8 presented in each sample. In general, WPC35, WPC80 and WPH were the products with the
9 highest concentrations of macroelements (Ca, K, Mg, Na and P) whereas for microelements
10 (Cu, Fe, Mn and Zn) higher amounts were observed for casein. Considering the Dietary
11 Reference Intakes (DRIs) for each element, WPC35, WPC80 and WPH showed high contents
12 of calcium (between 46.9 and 83.8% of DRIs) and could be considered important calcium
13 sources. Reasonable concentrations of Mg and K were observed in the WPC35 sample (26.9
14 and 30.6% of the DRIs of Mg and K, respectively). For microelements, only casein could be
15 considered a source of Zn and Fe, with concentrations of 40.1 and 93.0% of the DRIs,
16 respectively. For WPI values lower than 3.2% of the DRIs of Ca, Cu, Fe, K, Mg, Mn and Zn
17 were observed and the nutritional value of this product could not be considered with respect
18 to mineral content. However, WPI was a source of sulfur-containing and branched-chain
19 AAs, lysine, threonine and total EAA whereas casein could be considered a source of
20 aromatic AAs with contents that reached the DRIs values. The sodium content (up to 59.3%
21 of DRIs) was determined to be high for all whey proteins. Despite the limited number of
22 products evaluated, the protein source and/or method of manufacture appears to be related to
23 the differences observed in both the amino acid and mineral contents among milk proteins.

24 ***Keywords*:** Essential and non-essential amino acids; Macro- and microelements; Composition;
25 Dairy products.

1. INTRODUCTION

The high nutritional value of milk proteins is widely recognized, and dairy products contribute significantly to daily protein intake from the diet (Guigoz, 2011). The whey fraction of milk contains a great variety of proteins, and it can be considered the principal by-product of cheese and casein manufacture. Taking into account the presence of proteins of high nutritional value and the versatile functional properties of whey, the intensive use of this by-product by the food and pharmaceutical industries is expected (El-Sayed & Chase, 2011). However, the problem associated with whey utilization is that a very large volume of whey is produced worldwide each year, which contains only dilute concentrations of these valuable proteins. Therefore, whey protein concentrates (WPC), whey protein isolates (WPI) and the enzymatic hydrolysates of milk proteins (WPH) are manufactured industrially and have found extensive use in a wide range of foodstuffs, such as infant formulas, dietary supplements, and clinical and sports diet formulations (de Wit, 1998; Korhonen & Pihlanto, 2007).

The composition of milk could be affected by the environmental and nutritional conditions to which an animal is conditioned, as well as by post-milking handling, transportation and processing, which could affect the composition of milk proteins. However, whey protein preparations are also largely affected by the method used to process them (El-Sayed & Chase, 2011; Korhonen & Pihlanto, 2007). Membrane-separation processes are now industrially applied in the manufacture of ordinary whey powder (Akpinar-Bayizit, Ozcan, & Yilmaz-Ersan, 2009), and WPC with a protein content between 30 and 80% can be obtained commercially (Korhonen & Pihlanto, 2007). Gel filtration and ion-exchange chromatography techniques can be employed in the manufacture of whey protein isolates (WPI) with a protein content that could reach 90-95% (Korhonen & Pihlanto, 2007). Fractions of whey protein can also be obtained by nanofiltration, which allows for the selective separation of salts and ions from whey and has made it possible to manufacture industrially demineralized whey protein

(El-Sayed & Chase, 2011; Korhonen & Pihlanto, 2007). Therefore, depending on the origin of the milk used and the manufacturing process applied, variations in whey protein composition can be expected. However, the literature offers little information regarding the comparison of different whey preparations commercialized as ingredients for food manufacture. Thus, in this study, several chemical analyses were performed (proximate composition, mineral and amino acids determinations) to evaluate WPC (35 and 80% of protein), WPH and WPI commercially available in Brazil. Casein was also evaluated for comparison of results. A principal component analysis (PCA) was applied to demonstrate the similarities and differences among milk proteins. The nutritional value of each milk protein is discussed, particularly in relation to the presence of macro- and micromineral elements.

2. MATERIAL AND METHODS

2.1 Samples

Whey protein samples were supplied by Doremus Alimentos, Guarulhos, SP, Brazil with labeled protein contents of 35 and 80% for whey protein concentrate (WPC35 and WPC80, respectively), 80% for WPH and 90% for WPI. Commercial casein (Vetec Química Fina Ltda, Rio de Janeiro, RJ, Brazil) was used in this study.

2.2. Instrumentation

An oven (model 400/2ND, Nova Ética, Vargem Grande Paulista, SP, Brazil) was used for moisture determination and for drying samples before the determination of amino acids and mineral elements. Samples were weighed on an analytical balance (model AY 220, max. 220 g, 0.1 mg of resolution, Shimadzu, Kyoto, Japan). A microwave oven (Multiwave 3000 microwave sample preparation system, Anton Paar, Graz, Austria) equipped with eight high-

pressure quartz vessels (internal volume of 80 mL, maximum operational temperature and pressure of 280°C and 80 bar, respectively) was used in the experiments. Elements were determined using an inductively coupled plasma optical emission spectrometer (ICP OES, Optima 4300 DV, PerkinElmer, Shelton, USA) with an axial view configuration. A concentric nebulizer and cyclonic spray chamber were used. Argon (99.996%, White Martins, São Paulo, Brazil) was used for plasma generation, nebulization and as an auxiliary gas. A high-performance liquid chromatography instrument (HPLC, Shimadzu, Tokyo, Japan) equipped with a photodiode array (SPD-20A) and a Luna C-18 column (100 Å, 5 µm, 250 × 4.6 mm, 00G-4252-EQ, Phenomenex, Torrance, CA) heated to 50°C in an oven (CTO-20A) was used for total amino acid determination.

2.3 Reagents and Standards

Distilled, deionized water was purified (Milli-Q, 18.2 MΩ cm, Millipore, Billerica, MA, USA) before use. Analytical grade nitric acid (Merck, Darmstadt, Germany) was used to prepare samples and standards for element determination. A multi-element stock solution (SCP33 MS, SCP Science, Quebec, Canada) was used to prepare reference solutions for ICP OES determination. Argon (99.996%, White Martins-Praxair, São Paulo, SP, Brazil) was used for plasma generation and nebulization in ICP OES determination and as an auxiliary gas. For amino acid determination, dl-2-aminobutyric acid was used as an internal standard (Sigma-Aldrisch Corp., St Louis, MO, United States). The solvents used for the mobile phase of HPLC determination were of chromatography grade, and all other reagents were of analytical grade.

2.4. Proximate composition determination

1 The evaluation of proximate composition was performed using the methods described
2 by the AOAC (Association of Official Analytical Chemists) (AOAC, 1995). Moisture was
3 determined by loss on drying in an oven at 105 °C; ash content was determined at 550 °C, and
4 protein content (N x 6.38) was determined by the micro-Kjeldahl procedure. Total lipid
5 content was determine using 3.5 g of sample and chloroform, methanol and water (10, 20 and
6 8 mL, respectively) as solvents, as described previously (Bligh & Dyer, 1959). Carbohydrates
7 were calculated by the difference method. All determinations were performed in triplicate.
8

9 ***2.5. Microwave-assisted digestion and ICP OES determination of elements***

10 Samples were digested according to a procedure described previously (Bizzi, et al.,
11 2011; da Silva, Mattanna, Bizzi, Peireira dos Santos Richards, & Barin, 2013). Diluted nitric
12 acid (6 mL of 3 mol L^{-1} HNO_3) was used for the digestion of 400 mg of samples placed in
13 high-pressure quartz vessels. After closing and capping the rotor, the vessels were pressurized
14 with 7.5 bar of oxygen and placed inside the oven. The microwave-heating program was
15 initiated by applying a power of (i) 1000 W with a ramp of 5 min, (ii) 1000 W for 10 min, and
16 (iii) 0 W for 20 min (cooling step). After digestion, the pressure in each vessel was carefully
17 released. The digests were transferred to 50 mL polypropylene vials and diluted up to the
18 mark with water. After digestion, all vessels were cleaned with 6 mL of concentrated HNO_3
19 in the microwave oven at 1000 W for 10 min and 0 W for 20 min for cooling. The ICP OES
20 was calibrated using analytical solutions of concentrations ranging from 1.0 to 100 µg L^{-1} that
21 were prepared in 0.7 mol L^{-1} HNO_3 by appropriate dilution of the multi-element stock
22 solution. The plasma operating conditions and selected wavelengths were used as
23 recommended by the instrument manufacturer and according to previous work (Pereira, et al.,
24 2013). Glass and quartz material were soaked in 1.4 mol L^{-1} HNO_3 for 24 h and further
25 washed with water before use.

2.6. Determination of amino acids

Amino acid determination was performed as previously described by (Lollo, et al., 2013). For total amino acid determination, preliminary hydrolysis was performed using 6 M HCl (24 h, 110 °C). After centrifugation, the supernatant was filtered through a 0.22 µm membrane, and neutralization was performed (with a 4:4:2 solution of 0.2 N trihydrate sodium acetate, methanol and triethylamine). A 40 µL aliquot was derivatized with phenylisothiocyanate for further injection of 20 µL into the liquid chromatograph. An internal standard (dl-2-aminobutyric acid acid) was added before derivatization.

2.7. Statistical analysis

The obtained data were statistically evaluated by analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) using the Statistica 7.0 software (Tulsa, USA, 2004). Additionally, an exploratory analysis of the data via PCA was performed to evaluate the correlation between the variables and the possible groupings among the samples in the Pirouette 3.11 statistical program (Woodinville, USA, 2003). The data were autoscaled so that each variable could contribute the same weight in the analysis.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

Casein and WPI presented the highest protein contents (82.78 and 85.82%, respectively) but the lowest contents of lipids, carbohydrates and ashes (Table 1). The moisture content of casein was the highest (11.12%), whereas other milk proteins presented values between 4.73 and 8.10%. Casein and WPI showed the lowest total lipids contents (1.69 and 1.15%, respectively), and values between 3.48 and 5.02% were observed for WPC35,

WPC80 and WPH. It is important to note that the presence of lipids could affect the functional properties of whey and promotes the development of oxidation reactions, which impart off-flavors (Morr & Ha, 1991); therefore, methods for reducing the fat content in whey protein products have been developed (Shee, Angers, & Bazinet, 2007). Hence, this parameter could be considered important for the determination of the quality of whey protein.

The highest ash contents were observed for WPC35 and WPH, while for casein and WPI, lowest values were observed. The ash content could be directly related to the amount of Ca, K, Mg, Na and P presented in each sample (Table 2). By combining the concentrations of these elements and considering a ratio relative to the concentrations observed for WPC35 (the sample that presented the highest concentrations of these elements) for each sample, values of 0.28, 0.43, 0.71 and 0.27 for casein, WPC80, WPH and WPI, respectively, could be observed. Similar ratios were observed for the ash content by making the same comparison with WPC35 (the sample that presented the highest ash content) (0.27, 0.40, 0.70 and 0.33 for casein, WPC35, WPC80, WPH and WPI, respectively), demonstrating that the same proportion is maintained. Therefore, the ash content appears to be correlated to the amount of Ca, K, Mg, Na and P of milk proteins evaluated.

3.2. Mineral elements

Mineral salts affect the functionality and the value of whey products, and their determination could be used as an important quality parameter. The elements observed in the highest concentration for the evaluated milk proteins were Ca, K, Mg, Na, P and S. As previously mentioned, these macroelements, with the exception of sulfur, could be directly related to the ash content of samples. The total sulfur content could be related only to the total amount of sulfur-containing amino acids, such as cysteine (determined as cystine) and methionine (Fig. 1). By considering the total amount of sulfur-containing amino acids to be

1 the sum of the total cysteine and methionine concentrations determined by HPLC as well as
2 the estimate based on the data regarding the total sulfur content determined by ICP OES
3 (considering the same proportion between cysteine and methionine observed by HPLC), the
4 results obtained by ICP OES presented a level of agreement between 85 and 102% with
5 respect to the HPLC values obtained for casein, WPH and WPI. Thus, the total sulfur content
6 of these samples obtained by ICP OES could be associated with the samples' concentration of
7 sulfur-containing amino acids. However, for the WPC35 and WPC80 samples, the levels of
8 agreement observed were 127 and 167%, respectively, which could indicate that other sulfur-
9 containing amino acids or other sulfur species (e.g., sulfates) might be concentrated together
10 during the manufacture of whey protein concentrates.

11 It is well known that the mineral content of milk is not constant but varies according to
12 several different factors, such as stage of lactation, feed, and genetic variance (Vegarud,
13 Langsrud, & Svenning, 2000), and thus, the concentration of elements could change during
14 the production of different milk proteins. In this work, the samples used were not produced
15 from the same raw material, and the processes applied were different for each milk protein.
16 Nevertheless, even when considering these factors, important differences among the samples
17 evaluated could be established. In general, WPC35, WPC80 and WPH were the products with
18 the highest concentrations of macroelements (Ca, K, Mg, Na and P), as shown in Table 2.
19 With the exception of the P and Na content of casein and WPI, respectively, the
20 concentrations of macroelements were lower for these products. However, the highest
21 concentration of microelements (Cu, Fe, Mn and Zn) was observed for casein.

22 Casein showed a high content of phosphorus that could be related to phosphoseryl-
23 containing peptides found in the composition of α_{s1}-casein, α_{s2}-casein and β-casein. These
24 peptides have a high concentration of negative charges and could be related to the efficient
25 binding of microelements by casein (Vegarud, et al., 2000). For iron, the concentration in

casein was at least six times higher than that observed in the other milk proteins studied. For zinc, similar behavior was observed, with its concentration in casein at least five times as high. In contrast to casein, WPI presented the lowest concentration of phosphorus and was the poorest milk protein with respect to both macro- and microelement content. However, sodium was the only element whose concentration was observed to be higher in WPI than in the other milk proteins. For WPC35, a high concentration of macroelements (Ca, K, Mg, Na and P) was observed, and the concentration of these elements was always higher than that of WPC80. On the other hand, the microelement content (Cu, Fe, Mn and Zn) was always higher in WPC80 than in WPC35. Therefore, the mineral content of milk protein could be affected by protein type as well as by corresponding manufacturing process. In addition, the effects of these factor appear to vary for macro- and microelements.

Despite the importance of Table 2 in comparing the differences among milk proteins, there is no way to directly evaluate the nutritional significance of elements observed in these samples because the requirements are different for each element. Therefore, to provide this information, the data were compared to the Dietary Reference Intakes (DRIs) (Institute of Medicine, 2002-2005) of the elements considering the ingestion of 100 g of each sample per day (Table 3).

For mineral elements determined in WPI, only a reasonable content of P (21.4% of DRIs) and a high content of Na were observed, whereas values lower than 3.2% of the DRIs of Ca, Cu, Fe, K, Mg, Mn and Zn were observed. Thus, WPI could not be considered nutritionally important with respect to mineral content. The concentration of Na was observed to be high in all whey proteins, ranging from 11.7 (WPC80) to 59.3% (WPI) of the DRIs, and negligible in casein (lower than 2.6% of DRIs). All proteins presented a high content of P ranging from 21.4 (WPI) to 110.7% (casein) of the DRIs. Whey protein concentrates and hydrolysate showed high contents of calcium (between 46.9 and 83.8% of DRIs) and could be

1 considered important calcium sources. Reasonable concentrations of Mg and K were observed
2 in the WPC35 sample (26.9 and 30.6% of the DRIs of Mg and K, respectively). For
3 microelements, only casein could be considered a source of Zn and Fe, with concentrations of
4 40.1 and 93.0% of the DRIs, respectively.

5 It is important to note that the content of whey salts, especially NaCl derived from the
6 production of cheese, could cause a drop in the quality of whey protein, limiting its use as a
7 food ingredient. Thus, to avoid changes in flavor and to allow for the use of whey proteins in
8 products for low-sodium diets, several whey protein demineralization processes are used
9 (Akpinar-Bayizit, et al., 2009; El-Sayed & Chase, 2011). However, a high sodium content
10 was observed for all commercially available whey proteins evaluated, demonstrating that this
11 element could not be efficiently separated in the manufacture process, and thus, casein was
12 the milk protein with lowest concentration of this element.

13

14 ***3.3. Amino acids***

15 Previously dried samples were evaluated with respect to their total contents of
16 essential and non-essential amino acids (EAA and NEAA, Fig. 1 and Fig. 2, respectively).
17 The sum of the concentrations of the amino acids could be related to the total protein content
18 of samples, with differences ranging from 1 to 9%. The content of NEAA was between 45.0
19 and 49.8% of the total amino acid value for all milk proteins evaluated, indicating a slight
20 predominance of EAA. WPC35 clearly presented the lowest values of EAA and NEAA and
21 was not used for the quantitative comparison of these amino acids. In contrast, WPI showed
22 the highest total amount of EAA, with similar results obtained for WPH, casein and WPC80.
23 Leucine and lysine were the EAA observed in the highest concentrations in milk proteins,
24 whereas histidine and tryptophan were observed in the lowest concentrations.

1 WPI was rich in branched-chain EAA (leucine, isoleucine, and valine), with
2 concentrations thereof ranging between 14.5 and 18.9%, higher than those observed in other
3 milk proteins. These amino acids are believe to play a role as metabolic regulators in protein
4 and glucose homoeostasis and in lipid metabolism, and as such, the amino acids may play a
5 role in weight control (Smilowitz, Dillard, & German, 2005; Smithers, 2008). With the
6 exception of WPC35, all milk proteins presented concentrations higher than the their
7 respective DRIs, and concentrations 2.5, 2.0 and 1.8 times the DRIs values (Institute of
8 Medicine, 2002-2005) were observed for isoleucine, leucine and valine, respectively, in WPI.
9 Casein, WPC80 and WPH presented similar concentrations of these amino acids (from 1.5 to
10 2.1 times the DRIs values).

11 WPI was also a source of sulfur-containing EAA (methionine and cysteine -
12 determined as cystine), as was WPH, though to a lower extent. These amino acids serve a
13 critical role as anti-oxidants, as precursors to the potent intracellular anti-oxidant glutathione,
14 and in one-carbon metabolism (Shoveller, Stoll, Ball, & Burrin, 2005; Smithers, 2008). The
15 amount of sulfur-containing EAA in casein and WPC80 was approximately two times lower
16 than that in WPI and could not be explained solely by the total protein values (82.78, 72.89
17 and 85.82% for casein, WPC80 and WPI, respectively), demonstrating that the source of
18 protein and/or the method of manufacture could affect the content of these amino acids. It is
19 important to note that all milk proteins evaluated could be considered a source of sulfur EAA,
20 because all concentrations were greater than the DRIs values, but the highest concentrations
21 of these amino acids were observed in WPH and WPI, which reached 2.56 and 3.12 times the
22 DRIs values, respectively.

23 The concentration of aromatic essential amino acids (phenylalanine, tyrosine, histidine
24 and tryptophan) was very similar among WPC80, WPH and WPI, with values slightly higher
25 than the DRIs values, with the exception of tryptophan. Phenylalanine and tyrosine were

1 observed in equal proportions in all milk proteins (approximately 50% of each one), and their
2 highest concentration was observed in casein, which was twice the DRIs value (Institute of
3 Medicine, 2002-2005). For WPC35, WPC80, WPH and WPI, concentrations equivalent to
4 approximately 47.7, 112.8, 113.7 and 112.9% of the DRIs values were observed for these
5 amino acids, respectively. Casein also presented the highest concentration of histidine, which
6 reached 1.4 times the DRIs value; thus, this milk protein could be considered a source of
7 aromatic amino acids. Clearly, the use of casein should be carefully considered when
8 preparing low-phenylalanine food, and WPC, WPH and WPI are better options for preparing
9 food containing milk protein in this case.

10 Lysine and threonine were observed in high concentration in WPI, but all milk
11 proteins (with exception of WPC35) exceeded the DRIs values for these amino acids. For
12 lysine, a similar concentration was observed in casein, WPC80 and WPH. However, for
13 threonine, casein presented a lower concentration than did WPC80 and WPH.

14 The NEAA were also evaluated, and their concentrations in milk proteins are shown in
15 Fig. 2. Casein and WPI presented the highest amounts of NEAA. Glutamic acid was observed
16 in high concentration in all milk proteins, followed by aspartic acid, with exception of casein,
17 which presented a high content of proline. In contrast, arginine and glycine were the NEAA
18 observed in the lowest concentration. The concentrations of aspartic acid, arginine, glycine,
19 proline and serine were similar among WPC80, WPH and WPI. Casein showed the highest
20 amounts of glutamic acid, arginine and proline. According to Wu et al. (2013), it is assumed
21 that all NEAA are synthesized in sufficient amounts in the body to meet the requirements for
22 maximal growth and health, but there has been no compelling experimental evidence to
23 support this assumption (Wu, et al., 2013). Some NEAA (e.g., glutamine, glutamate, proline,
24 glycine and arginine) are involved in the regulation of gene expression, cell signaling,
25 antioxidative responses, neurotransmission, and immunity. Moreover, glutamate, glutamine

1 and aspartate are major metabolic fuels that help the small intestine maintain its digestive
2 function and protect its mucosal integrity (Wu, et al., 2013). Thus, NEAA should be taken
3 into consideration in balanced diets to improve protein accretion, food efficiency and health,
4 and milk proteins could be considered a good source of these compounds, particularly casein
5 and WPI.

6

7 ***3.4. Exploratory analysis***

8 In this analysis, it was possible to extract relevant information regarding the
9 correlation among different variables to characterize milk proteins. With respect to the first
10 two principal components of PCA, 77.4% of the total variance of the data was obtained (Fig.
11 3). WPC35 was singled out mainly due to its high contents of ash and carbohydrates and also
12 due to its lowest content of proteins (and amino acids), as expected. However, the other whey
13 proteins (WPC80, WPH and WPI) formed a group of proteins, distinct from casein,
14 demonstrating the similarity among them. The high contents of phosphorus and
15 microelements, as well as phenylalanine, tyrosine, arginine and proline, in casein were
16 responsible for the differences observed in relation to the other whey proteins. The lowest
17 amount of sodium observed in casein was also a parameter that was responsible for its
18 distinction from whey proteins.

19

20 **4. CONCLUSION**

21

22 The mineral and amino acid profile of commercialized milk proteins presented
23 important information regarding their nutritional importance. All proteins could be considered
24 sources of both EAA and NEAA, with the exception of WPC35 due to its lower content of
25 protein compared to that of other milk proteins. Casein could be considered a source of

aromatic AAs and WPI a source of sulfur-containing and branched-chain AAs, lysine, threonine and total EAA. Microelements (mainly Fe and Zn) were observed in high concentration in casein, whereas macroelements (mainly Ca, Mg, K) were observed in high concentration in all whey proteins except for WPI, which was the poorest protein in terms of mineral element content. The sodium content (up to 59.3% of DRIs) was determined to be high for all whey proteins and should be carefully considered in the elaboration of low-sodium products. Despite the limited number of products evaluated, the protein source and/or method of manufacture appears to be related to the differences observed in both the amino acid and mineral contents among milk proteins.

1 **COMPLIANCE WITH ETHICS REQUIREMENTS**

2 This article does not contain any studies with human or animal subjects.

3 Sabrina Vieira da Silva declares that she has no conflict of interest.

4 Rochele Sogari Picolotto declares that she has no conflict of interest.

5 Roger Wagner declares that he has no conflict of interest.

6 Neila Silvia Pereira dos Santos Richards declares that she has no conflict of interest.

7 Roger Wagner declares that he has no conflict of interest.

8 Juliano Smanioto Barin declares that he has no conflict of interest.

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Table 1. Proximate composition of casein and whey proteins (mean ± standard deviation, n = 3).

Parameters (%)	Casein	WPC35	WPC80	WPH	WPI
Moisture	11.12 ± 0.02[a]	4.73 ± 0.02[e*]	8.10 ± 0.08[b]	7.31 ± 0.07[c]	6.73 ± 0.03[d]
Ashes	1.91 ± 0.13[e]	7.14 ± 0.03[a]	2.87 ± 0.05[c]	5.00 ± 0.02[b]	2.39 ± 0.02[d]
Protein	82.78 ± 0.33[b]	33.80 ± 0.10[d]	72.89 ± 0.75[c]	74.21 ± 0.30[c]	85.82 ± 0.30[a]
Total lipids	1.70 ± 0.11[d]	3.48 ± 0.09[c]	4.34 ± 0.44[b]	5.02 ± 0.08[a]	1.15 ± 0.11[d]
Carbohydrates	2.49 ± 0.35[e]	50.85 ± 0.14[a]	11.80 ± 0.87[b]	8.46 ± 0.32[c]	3.91 ± 0.32[d]

Same letters in the same line indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

Table 2. Mineral content of milk proteins (mean ± standard deviation, μg g^{-1}, n = 3).

Elements	Casein	WPC35	WPC80	WPH	WPI
Al	8.3 ± 0.4^{a}	$< 1.5^{*c}$	$< 1.5^{*c}$	$< 1.5^{*c}$	3.9 ± 0.1^{b}
B	1.47 ± 0.14^{b}	7.72 ± 0.33^{a}	0.91 ± 0.17^{b}	1.67 ± 0.60^{b}	1.69 ± 0.24^{b}
Ba	0.485 ± 0.029^{c}	0.365 ± 0.006^{d}	1.01 ± 0.03^{a}	0.786 ± 0.009^{b}	$< 0.10^{*e}$
Ca	1697 ± 15^{d}	8377 ± 145^{a}	5252 ± 386^{b}	4693 ± 227^{c}	200 ± 9^{e}
Cu	0.956 ± 0.015^{a}	0.215 ± 0.004^{c}	0.76 ± 0.06^{b}	0.998 ± 0.031^{a}	0.285 ± 0.041^{c}
Fe	74.4 ± 6.6^{a}	4.14 ± 0.31^{bc}	8.77 ± 0.75^{bc}	12.5 ± 0.2^{b}	1.99 ± 0.22^{c}
K	395 ± 4^{d}	14023 ± 285^{a}	4456 ± 363^{c}	11819 ± 526^{b}	516 ± 26^{d}
Mg	132 ± 2^{c}	1128 ± 17^{a}	703 ± 59^{b}	665 ± 31^{b}	20 ± 2^{d}
Mn	1.04 ± 0.07^{a}	$< 0.05^{*d}$	0.12 ± 0.00^{c}	0.550 ± 0.018^{b}	$< 0.05^{*d}$
Na	342 ± 12^{e}	6422 ± 340^{b}	1748 ± 169^{d}	5277 ± 226^{c}	7719 ± 374^{a}
P	7754 ± 146^{a}	6757 ± 84^{b}	3597 ± 268^{c}	3428 ± 126^{c}	1496 ± 138^{d}
S	6491 ± 160^{b}	4810 ± 94^{b}	9914 ± 749^{a}	9632 ± 337^{a}	10772 ± 1341^{a}
Sr	2.07 ± 0.05^{d}	8.68 ± 0.32^{a}	5.11 ± 0.03^{b}	3.17 ± 0.08^{c}	$< 0.01^{*e}$
Zn	44.1 ± 0.9^{a}	3.42 ± 0.01^{c}	8.38 ± 0.33^{b}	7.19 ± 0.42^{b}	$< 1.0^{*d}$

Same letters in the same line indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

* limit of quantification

Table 3. Concentration of elements in 100 g of each milk protein and their Dietary Reference Intakes (DRIs).

Elements	DRIs (per day)	Casein	WPC35	WPC80	WPH	WPI
Ca (mg)	1000*	170	838	525	469	20.0
Cu (μg)	900*	95.6	21.5	76.0	99.8	28.5
Fe (mg)	8*	7.44	0.414	0.877	1.25	0.199
K (g)	4.7**	0.040	1.40	0.446	1.18	0.052
Mg (mg)	400-420*	13.2	113	70.3	66.5	2.00
Mn (mg)	2.3**	0.104	<0.005	0.012	0.055	<0.005
Na (g)	1.3-1.5**	0.034	0.642	0.175	0.528	0.772
P (mg)	700*	775	676	360	343	150
Zn (mg)	11*	4.41	0.342	0.838	0.719	<0.100

*Recommended Dietary Allowances (RDAs)
**Adequate Intakes (AIs)

Figure Captions

Figure 1. Concentration of total essential amino acids (EAA) in different milk proteins and respective Dietary Reference Intakes (DRIs).

Figure 2. Concentration of total non-essential amino acids (NEAA) in different milk proteins and respective Dietary Reference Intakes (DRIs).

Figure 3. Principal component analysis of proximate, mineral and aminoacid composition of the milk proteins. a - Score plots (samples), WPC35 - whey protein concentrate 35%, WPC80 - whey protein concentrate 80%, WPH - whey protein hydrolysate, WPI - whey protein isolate; b - weight plots (variables).

Figure 1

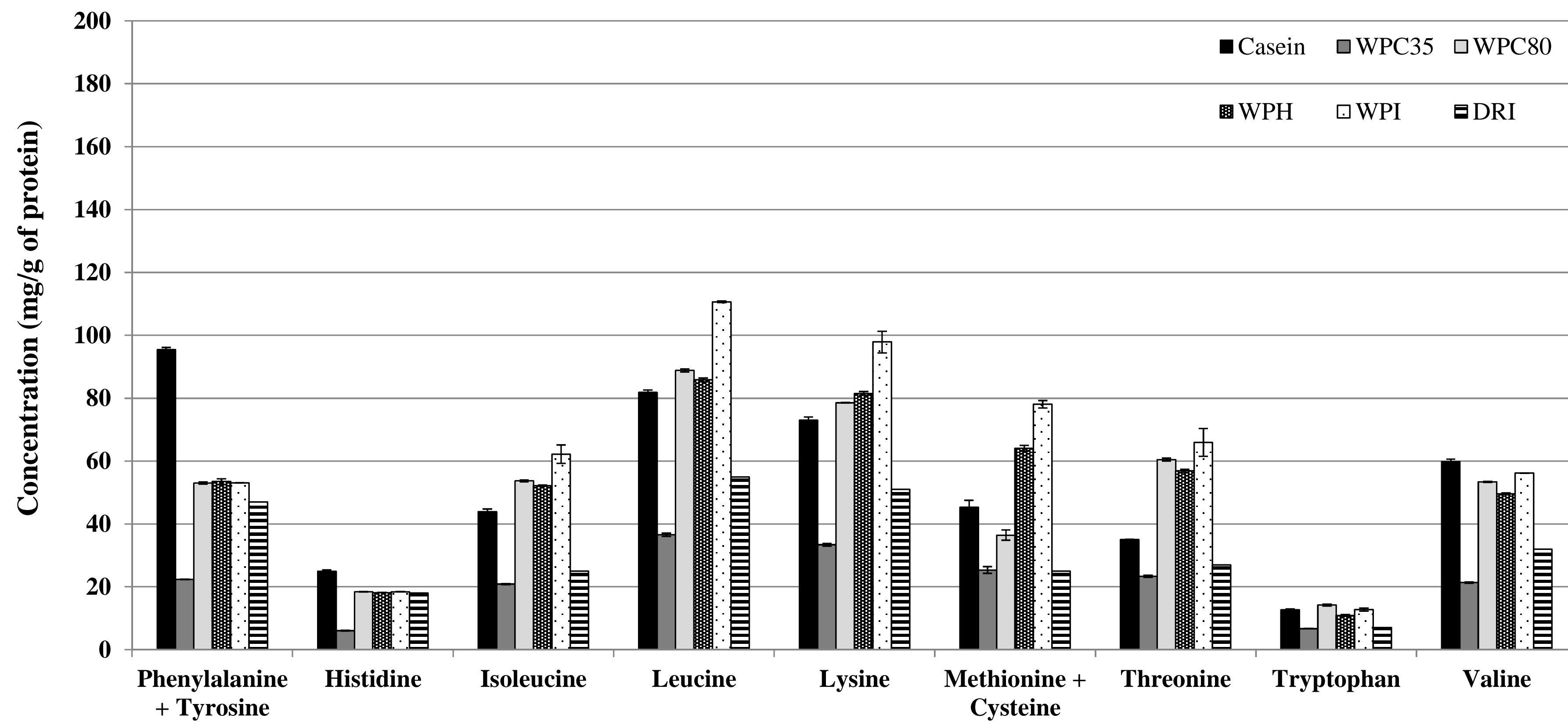


Figure 2

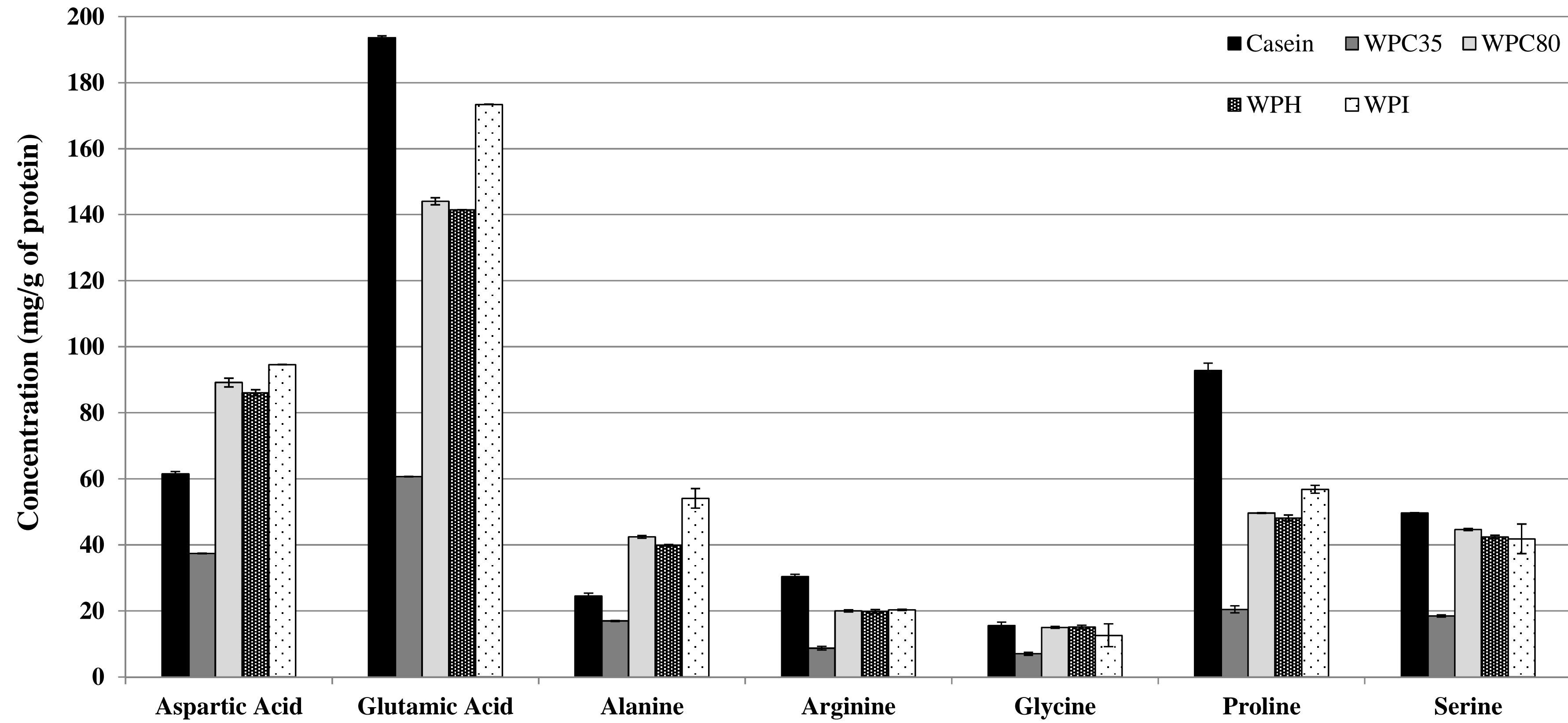
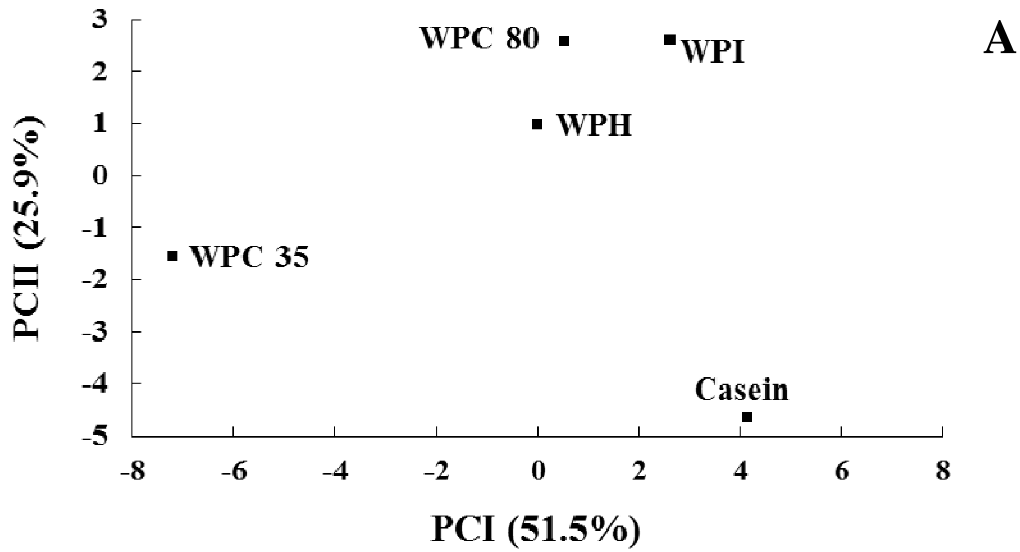
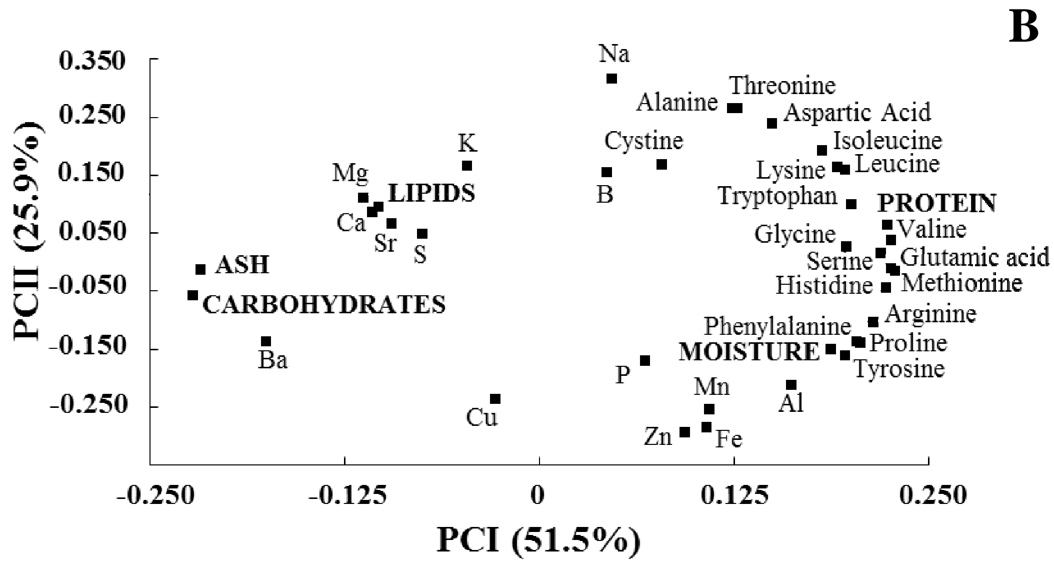


Figure 3





ARTIGO 2

Evaluation of Bromine and Iodine Content of Milk Whey Proteins Combining Digestion by Microwave-Induced Combustion and ICP-MS Determination

(Artigo submetido para *Food Control*)

Sabrina Vieira da Silva[1], Rochele Sogari Picoloto[2], Erico Marlon Moraes Flores[2], Roger Wagner[1], Neila Silvia Peireira dos Santos Richards[1], Juliano Smanioto Barin[1]*

[1]*Departamento de Tecnologia e Ciência dos Alimentos, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil*

[2]*Departamento de Química, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil*

*Corresponding author: Tel/Fax: +55-55-3220-8254

E-mail address: juliano@ufsm.br

1 **Abstract**

2 The use of whey proteins has been increased in food industries and depending on the
3 production processes used, their composition could be changed. In this way, some trace
4 elements, such as Br and I, could be enriched during whey production and should be
5 evaluated to assure intakes within safe levels. In this work the content of Br and I in whey
6 protein concentrate (WPC, 35% and 80% of protein), hydrolysate (WPH) and isolate (WPI)
7 was evaluated combining microwave-induced combustion (MIC) digestion with inductively
8 coupled plasma mass spectrometry (ICP-MS) determination. Casein was also evaluated for
9 comparison. MIC digestion allowed an efficient decomposition of up to 500 mg of samples
10 (residual carbon content lower than 1%) using diluted NH_4OH solution (25 mmol L^{-1}) as a
11 suitable medium for absorption of analytes assuring the compatibility with ICP-MS
12 determination. Accuracy was evaluated using milk powder certified reference material (NIST
13 8415) with good agreements for Br and I (102 and 105%, respectively) an also by comparison
14 with ion chromatographic determination presenting no statistical difference. For Br and I, the
15 limit of quantification obtained by ICP-MS was 7 and 281 times lower in comparison to ion
16 chromatography determination, respectively, showing the suitability of proposed method for
17 evaluation of Br and I in whey proteins. Iodine was found in higher content in whey proteins
18 in comparison to casein showing the possibility of enrichment during whey protein
19 production. For WPC 80, around 70% of the tolerable upper intake level of I could be reached
20 showing the need of monitoring of this element in whey proteins. On the other hand, the
21 concentration of Br was by far bellow its acceptable daily intake and for evaluated samples no
22 risks were found for Br content of whey proteins.

23 ***Keywords*:** Dairy products; Quality control; Microelements; Halogens determination; Ion
24 chromatography; Dietary recommended intake.

25

1 **1. Introduction**

2 The whey fraction of milk can be considered the principal byproduct of cheese and
3 casein production and due to the presence of proteins of high nutritional value whey has been
4 largely used in food and pharmaceutical industries (El-Sayed & Chase, 2011). Whey contains
5 only diluted concentrations of proteins and many industrial processes have been used to
6 obtain protein-rich products, such as whey protein concentrates (WPC), whey protein isolates
7 (WPI) and the enzymatic hydrolysates of milk proteins (WPH). Depending on the kind of
8 process (e.g., membrane-separation, nanofiltration, gel filtration, ion-exchange
9 chromatography) as well as the composition of milk, variations in whey protein composition
10 can be expected (Akpinar-Bayizit, Ozcan, & Yilmaz-Ersan, 2009; Korhonen & Pihlanto,
11 2007; Roman, Wang, Csanadi, Hodur, & Vatai, 2009). However, few information regarding
12 to the amount of Br and I in whey proteins could be found despite the importance of these
13 elements for human health. Iodine is an essential element for growth, development and well-
14 being, as it is an important component of the thyroid hormones, but adverse reactions have
15 been associated with excessive intake (Gad, 2014). Bromine is not an essential element and
16 has been used in pharmaceuticals, disinfection byproducts and many polymers as flame
17 retardants, which are associated with endocrine disruption and subject to bioaccumulation in
18 food chains (van Paemel, 2010). Therefore, the determination of both Br and I is important to
19 assure the quality of food and the ingestion of safe levels of these elements from diet.

20 The lack of information about the content of Br and I in whey proteins could be
21 explained at least partially by the analytical challenges related to their determination at low
22 concentration (Mello, et al., 2013). In this way, several techniques as ion chromatography,
23 potentiometry with ion-selective electrodes, inductively coupled plasma optical emission
24 spectrometry and graphite furnace molecular absorption spectrometry present limitations due
25 to unsuitable limit of detection (LOD) for the determination of Br and I in whey proteins.

1 Inductively coupled plasma mass spectrometry (ICP-MS) could be the choice for this
2 purpose. However, ICP-MS often requires a previous digestion step in order to bring analytes
3 into solution, decomposing sample matrix and avoiding interferences in the determination
4 step. In general, microwave-assisted wet digestion in closed vessels with inorganic acids has
5 been recommended to milk powder samples (Bizzi, et al., 2011). However, limitations can
6 occur due to the formation of volatile species of Br and I, which could not remain in solution
7 due to losses even using closed vessels (Mesko, et al., 2010). Moreover, the high acidity of
8 digests could cause interferences on the determination by ICP-MS and a dilution or
9 neutralization of digests is often required (Todoli & Mermet, 1999). In this way, alkaline
10 media (e.g. KOH, TMAH, CFA-C) could be used to minimize losses, but in general these
11 procedures provide only a dissolution of sample and interferences of organic matrix
12 remaining in digests are expected in ICP-MS determination (Mello, et al., 2013; Sullivan &
13 Zywicki, 2012).

14 In order to avoid these drawbacks, combustion-based digestion systems have been
15 proposed for digestion of organic samples for subsequent determination of Br and I by ICP-
16 MS (Flores, et al., 2008; Mesko, et al., 2010; Pereira Barbosa, et al., 2013). Combustion
17 bombs and oxygen flask system could be used for this purpose, allowing digestion in closed
18 vessels and recovery of analytes in a diluted alkaline absorbing solution. Nevertheless, several
19 limitations related to their use could be pointed out, such as the low sample throughput,
20 impossibility of a reflux step to improve analyte absorption and problems related to safety
21 (pressure and temperature control is not allowed) (Mello, et al., 2013). In order to overcome
22 these limitations, the microwave-induced combustion (MIC) digestion could be applied
23 allowing the digestion of food samples in quartz vessels pressurized with oxygen (e.g. 20 bar)
24 (Mesko, et al., 2010; Pereira Barbosa, et al., 2013). The ignition is performed by microwave
25 irradiation in few seconds and in general 5 min are used for reflux of absorbing solution to

improve analyte recovery. Eight vessels could be used simultaneously and pressure and temperature control is allowed. This system has been successfully applied for organic samples but its feasibility for milk products has been not still demonstrated.

Thus, in this study, the combination of MIC digestion with ICP-MS determination of Br and I in WPC (35 and 80% of protein), WPH and WPI is proposed. Casein was also analysed for comparison of results. The concentration of absorbing solution was evaluated in order to achieve better the recoveries of analytes. For accuracy evaluation, the results obtained by ICP-MS were compared with those obtained by ion chromatography determination and also with values of certified reference material (CRM). Results were evaluated based on Dietary Reference Intakes (DRIs) for these elements (Institute of Medicine, 2002-2005).

2. Material and Methods

2.1. Samples

Whey protein samples were supplied by Doremus Alimentos, Guarulhos, SP, Brazil with labeled protein contents of 35 and 80% for whey protein concentrate (WPC35 and WPC80, respectively), 80% for WPH and 90% for WPI. Commercial casein (83% of protein, Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil) was also used in this study.

2.2. Instrumentation

An oven (model 400/2ND, Nova Ética, Vargem Grande Paulista, SP, Brazil) was used for drying WPC, WPH, WPI and casein samples before the determination of elements. Samples were weighed on an analytical balance (model AY 220, max. 220 g, 0.1 mg of resolution, Shimadzu, Kyoto, Japan). A microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) equipped with eight high-pressure quartz vessels (internal volume of 80 mL,

1 maximum operational temperature and pressure of 280 °C and 80 bar, respectively) was used
2 in the experiments. Br and I were determined by using an ICP-MS instrument (PerkinElmer
3 Sciex, Model Elan DRC II, Thornhill, Canada) equipped with a concentric nebulizer
4 (Meinhard Associates, Golden, USA), a baffled cyclonic spray chamber (Glass Expansion,
5 Inc., West Merbourne, Australia) and a quartz torch with a quartz injector tube (2 mm i.d.).
6 The following operational conditions were used: radiofrequency power of 1300 W and
7 plasma, auxiliary and nebulizer gas flow rate of 15, 1.2 and 1.08 L min^{-1}, respectively. The
8 isotopes monitored were 79 and 127 for Br and I, respectively.
9

10 ### *2.3 Reagents and Standards*

11 Distilled, deionized water was purified in a Milli-Q system (8.2 MΩ cm, Millipore,
12 Billerica, MA, USA) before use. All reagents used were from analytical grade (Merck,
13 Darmstadt, Germany). A multielement anion standard solution (Fluka, Buchs, Switzerland)
14 was used for the preparation of Br and I reference solutions by dilution in water. Argon
15 (99.996%, White Martins-Praxair, São Paulo, SP, Brazil) was used in ICP-MS determination
16 of elements.
17

18 ### *2.4. Microwave-induced combustion and ICP-MS determination of elements*

19 Samples of WPC, WPH, WPI and casein were digested following the operational
20 parameters described in a previous work (Pereira Barbosa, et al., 2013). Pellets of samples
21 were placed on a quartz holder with a small disc of filter paper. A 6 mol L^{-1} NH_4NO_3 solution
22 (50 µL) was added to the filter paper followed by the introduction of quartz holder in quartz
23 vessel previously charged with 6 mL of absorbing solution (10 to 100 mmol L^{-1} NH_4OH).
24 After closing and capping the rotor, vessels were pressurized with 20 bar of oxygen. The rotor
25 was placed inside the microwave cavity and the heating program was started using 1400 W

1 for 5 min and 0 W for 20 min for the cooling step. After digestion, the pressure of each vessel
2 was carefully released and the digests were transferred to a 25 mL volumetric flask and
3 diluted with water. All vessels were cleaned with 6 mL of concentrated HNO_3 under
4 microwave heating at 1000 W for 10 min and 0 W for 20 min for cooling. Glass and quartz
5 material were soaked in 1.4 mol L^{-1} HNO_3 for 24 h and further washed with water before use.
6

7 **3. Results and Discussion**

8

9 ***3.1. Evaluation of MIC digestion and ICP-MS determination of Br and I***

10 Initial experiments were carried out using relatively low sample amount (50 to 150
11 mg) of WPC 35 sample in order to evaluate the maximum pressure reached during
12 combustion process in order to assure the safety of experiment. Considering the low increase
13 in pressure (up to 27 bar) the sample mass was increased up to 500 mg reaching maximum
14 pressure of 39 bar, which is far from the maximum working pressure (80 bar). Therefore, the
15 subsequent digestions were performed with 500 mg as the maximum sample mass.
16 Afterwards, the concentration of NH_4OH absorbing solution was evaluated in order to assure
17 a complete retention of analytes (Fig. 1). For all solutions evaluated, suitable recoveries were
18 found and a 25 mmol L^{-1} NH_4OH solution was considered as suitable for further experiments
19 based on the good recoveries and relatively low relative standard deviation (<6%). The
20 residual carbon was determined in digests reaching values always lower than 1% due to high
21 efficiency of digestion observed during combustion process. These experimental parameters
22 were used for subsequent determination of elements in whey samples by ICP-MS.

23 The accuracy of proposed method was evaluated by using a milk powder CRM (NIST
24 8415) and results are shown in Table 1. Good agreement (between 102 and 105%) with
25 reference values were found. In addition, the determination of Br and I was also performed by

1 ion chromatography. For Br, the results for all samples evaluated presented no statistical
2 difference (Student t test, $p < 0.05$) in comparison to ICP-MS results, confirming the accuracy
3 of proposed method. However, for I determination by IC the respective concentration was
4 below the limit of quantification (LOQ) for this technique, with exception for WPC80, which
5 presented results above LOQ, but without statistical difference with ICP-MS. It is important
6 to mention the LOQ obtained by ICP-MS (0.317 and 0.027 for Br and I, respectively) were 7
7 and 281 times lower in comparison to ion chromatography, respectively.
8
9 ***3.2. Determination of Br and I in milk proteins***
10 As can be seen in Table 1, relatively higher concentration of I was found in whey
11 proteins (from 2.53 to 8.04 µg g^{-1}) in comparison to casein (0.371 µg g^{-1}), showing a
12 enrichment of this element during whey production. These results are in agreement with
13 literature data showing that I could be separated together with whey proteins (Martino,
14 Sanchez, & Medel, 2002).
15 The role of I is well known as an essential nutrient, but its excess could cause several
16 problems to health. Considering the I content of whole milk powder is at lower when
17 compared to whey proteins a comparison with the DRIs was performed to evaluate the
18 contribution of whey proteins on the intake of I (Institute of Medicine, 2002-2005). In this
19 way, 100 g of each protein was considered for intake per day. The estimated average
20 requirements (EAR) for I is estimated in 95 µg d^{-1} from diet and the recommended dietary
21 allowances (RDA) is 150 µg d^{-1} (Institute of Medicine, 2002-2005). For casein, the intake of
22 100 g reach only 37.1 µg d^{-1}, while for whey proteins the intake range from 240 to 804 µg d^{-1}.
23 Despite the limited number of whey proteins evaluated, the results indicated the I could be
24 enriched in these products and their use as ingredient of food should be carefully considered
25 because their intake exceed the RDA and for WPC80 around 70% of the tolerable upper

intake level (UL) of 1100 μg d^{-1} is reached. If the amount of I from diet is considered, the UL could be reached by the ingestion of WPC 80 leading to problems to health. Therefore, the monitoring of I content of whey proteins is an important task and should be considered for their quality control.

Bromine is not an essential trace element and scientific literature has not established its requirements (van Paemel et al., 2010). From studies with human volunteers a non observed adverse effect level value of 4 mg kg^{-1} of body weight was identified. Based upon this value a provisional acceptable daily intake (ADI) for bromine of 0.4 mg kg^{-1} of body weight could be derived (van Leeuwen & Sangster, 1987). Therefore, the concentration of Br in whey proteins and casein is far from ADI (e.g., 28 mg for a 70 kg man) ranging from 0.26 to 9.16 mg per 100 g of protein.

4. Conclusion

The combination of MIC digestion with ICP-MS determination allowed the determination of Br and I in trace levels, which is required for the determination of these elements in whey proteins. The LOQs for these elements were improved more than two hundred times in comparison to ion chromatography determination. Iodine was found in higher content in whey proteins in comparison to casein and whole milk powder showing the possibility of enrichment during whey protein production. For WPC 80, around 70% of the tolerable upper intake level of I could be reached by the ingestion of 100 g of product showing the need of continuous monitoring of this element. Therefore, the quality control issues for whey protein should recommend the determination of I. On the other hand, the concentration of Br was by far bellow its acceptable daily intake and for evaluated samples no risks were found for Br content of whey proteins.

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Table 1. Results for Br and I determination in whole milk powder and milk protein fractions after digestion using the MIC method (25 mmol L^{-1} NH_4OH as absorbing solution) and ICP-MS determination. Results are in µg g^{-1} (mean ± standard deviation, n = 3).

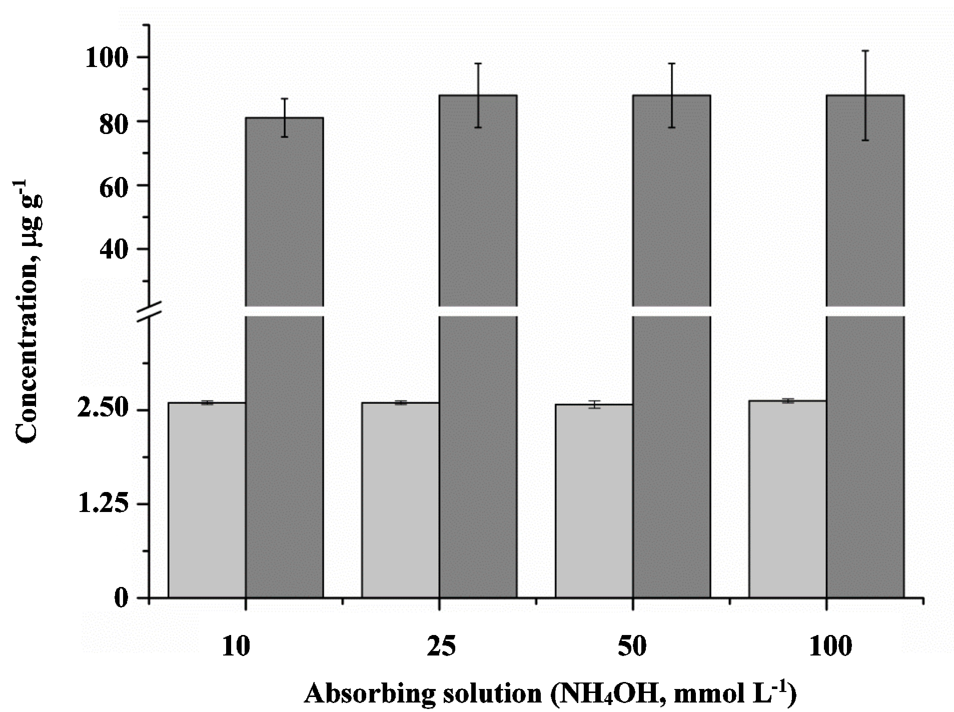
Samples	Br		I	
	ICP-MS	IC	ICP-MS	IC
WPC35	90.4 ± 0.7	91.6 ± 0.4	2.53 ± 0.09	< 7.6*
WPC80	2.76 ± 0.14	2.59 ± 0.04	8.04 ± 0.04	7.92 ± 0.25
WPH	3.20 ± 0.05	3.47 ± 0.08	3.51 ± 0.02	< 7.6
WPI	1.45 ± 0.10	< 2.3*	3.31 ± 0.16	< 7.6
Casein	6.39 ± 0.11	5.88 ± 0.12	0.371 ± 0.009	< 7.6
NIST 8435	20.5 ± 0.8	20.7 ± 1.1	2.4 ± 0.2	< 7.6

Reference values for NIST 8435 (Br: 20 ± 10 and I: 2.3 ± 0.4 µg g^{-1})

*Limit of quantification

Figure Caption

Figure 1. Influence of absorbing solutions on I and Br (□ and ■, respectively) determination in WPC35 sample, using 10, 25, 50 and 100 mmol L^{-1} NH_4OH. Determination by ICP-MS (mean ± standard deviation, n = 3).



100
80
60
40
2.50
1.25
0
Concentration, µg g-1
10
25
50
100
Absorbing solution (NH4OH, mmol L-1)

1 **ARTIGO 3**

2

3 Evaluation of the Composition and Nutritional Properties of Bovine Milk 4 Proteins

5

6 (Artigo submetido para o *Journal of Functional Foods*)

7

8 Sabrina Vieira da Silva[1]*, Tiffany Prokopp Hautrive[1], Angela Souza Rodrigues[1], Karine Inês 9 Bolson Moro[1], Marina de Souza Vencato[2], Marcelo Leite da Veiga[2], Gilberti Helena 10 Hübscher Lopes[1], Neila Silvia Peireira dos Santos Richards[1]

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12 [1]*Departamento de Tecnologia e Ciência dos Alimentos, Universidade Federal de Santa 13 Maria, 97105-900, Santa Maria, RS, Brasil*

14 [2]*Departamento de Morfologia, Universidade Federal de Santa Maria, 97105-900, Santa 15 Maria, RS, Brasil*

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18 *Corresponding author: Tel/Fax: +55-55-3220-8254

19 *E-mail address*: sabrinutri@yahoo.com.br

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21

1 **Abstract**

2 The objective of this study was to evaluate the physicochemical characteristics and
3 nutritional properties of bovine milk proteins. Five protein sources were investigated in this
4 study: four proteins from bovine whey, namely bovine whey protein concentrate (WPC35 and
5 WPC80), bovine whey protein hydrolysate (WPH) and bovine whey protein isolate (WPI),
6 and one bovine milk protein, casein, which was used as a control. The protein sources were
7 analyzed with regard to their physicochemical characteristics (moisture content, ash, proteins,
8 total lipids, carbohydrates and essential amino acids) and the degree of hydrolysis of the WPH
9 sample and gel electrophoresis to identify the low molecular weight proteins of the whey
10 proteins. We also conducted a 33 day biological assay that allowed us to monitor weight gain,
11 feed intake, feed efficiency ratio (FER) and the amount of protein ingested by animals, to
12 evaluate the protein quality of and metabolic responses to the protein sources, and to evaluate
13 organ integrity and small intestine histomorphometry in the experimental animals. The casein
14 and WPI samples had the highest protein concentration and the lowest ash and total lipid
15 contents, based on percent composition. All protein sources met the Food and Agriculture
16 Organization/Word Health Organization (FAO/WHO, 2007) ecommendations for essential
17 amino acids, with the exception of WPC35, most likely due to its reduced protein content.
18 The degree of hydrolysis of the WPH sample favors protein use. Gel electrophoresis
19 demonstrated the presence of functional proteins in the bovine whey protein samples. Weight
20 gain, feed intake and the level of ingested protein were higher in the animals fed with the
21 WPC80 diet, but the FER was higher in the animals fed with casein. Based on the protein
22 evaluation, all of the protein sources had high nutritional value. The animals fed with WPH
23 exhibited more favorable biochemical parameters. The weight of the organs showed that the
24 different experimental diets did not induce excessive weight gain. The animals that received a
25 diet formulated with WPC35 exhibited greater small-intestine integrity. Taken together, our

results suggest that the WPC80, WPH and WPI protein sources are functional ingredients that can be used to formulate special-purpose feed.

Keywords: Milk whey; Casein; Functional foods.

1 **1. Introduction**

2

3 Milk proteins comprise a complex mixture of various components that differ in
4 quantity and quality among mammal species. The two main classes of bovine milk proteins
5 are caseins, which are primarily in a colloidal state and account for 76 to 86% of the total
6 protein concentration, and whey proteins, which are in solution and represent approximately
7 20% of the milk protein (Schanbacher et al., 1998; Marshall, 2004; Sgarbieri, 2005; Rusu et
8 al., 2009).

9 Whey is a translucent liquid that is widely recognized for the value of its components,
10 especially proteins. The primary protein components in whey include β-lactoglobulins (β-Lg),
11 α-lactoalbumins (α-La), immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin,
12 enzymes, glycomacropeptide (GMP) and peptide subfractions. The nutritional and biological
13 properties of these proteins are particularly important for health promotion and disease
14 prevention (Marshall, 2004; Madureira et al., 2007). Studies performed in animals, humans
15 and *in vitro* have shown that whey proteins help to modulate metabolism; increase the ability
16 to combat infections and inflammatory processes; increase immunomodulatory capacity;
17 stimulate intestinal absorption and function; increase antibacterial activity, antiviral activity,
18 hormone synthesis, mineral absorption and anticancer activity; improve physical
19 performance; promote longevity; and have a cytoprotective effect due to the promotion of
20 glutathione synthesis (Sgarbieri, 2004; Pacheco et al., 2006).

21 Techniques such as ultrafiltration, chromatography, liquid-liquid extraction and others
22 have been used to separate and purify whey proteins to a high degree, leading to the
23 development of high-nutritional-value products, such as whey protein concentrates and whey
24 protein isolates that are appropriate for use in foods and food ingredients (Foegeding et al.,
25 2002).

Whey protein concentrates are available at protein levels between 34 and 85%, and the average content of ash, lactose and fat in the concentrates varies significantly depending on the protein content (El-Salam, El-Shibiny, & Salem, 2009). The concentrates are obtained through ultrafiltration of the acid or sweet whey and drying in a spray dryer to maintain the functionality of the proteins. Concentrates with a protein concentration greater than 80% can be obtained through ultrafiltration and diafiltration followed by atomization drying. The denaturation induced in the whey proteins varies from 14.3% to 21.7%. Whey protein concentrate containing 34% protein has a composition similar to that of skimmed powdered milk, although the mineral profile and the type of proteins differ (Relkin et al., 2007).

Whey protein isolates contain at least 90% protein (based on dry weight), less than 1% fat and lactose and approximately 2% ash. Isolates represent the purest sources of proteins available and can be prepared by ion exchange or through an ultrafiltration process using membranes. During protein isolate processing, a significant amount of fat and lactose is removed. As a result, lactose intolerant individuals can safely ingest these products (Yee et al., 2007; Philipina & Rizvi, 2008; Lands et al., 2010).

The use of whey protein hydrolysate has received special attention from the food industry and in animal physiology studies, due to its functional properties. Protein hydrolysis leads to the formation of aggregates with variable molecular weight, such as peptones, mixtures of peptides and free amino acids (Lahl & Braun, 1994).

Protein hydrolysis can be performed using enzymes, a strong acid or a strong base, but using enzymes, such as digestive system enzymes (pancreatin, pepsin, α-chymotrypsin), some plant enzymes (such as papain) or various microbial enzymes, is the preferred method of obtaining hydrolysates for nutritional applications. Protein hydrolysates are used in specific formulations including hypoallergenic infant formulas, immunostimulatory preparations, geriatric products, therapeutic diets and sports drinks (Clemente, 2000).

The composition, physicochemical properties, functional properties and sensory properties of enzymatic protein hydrolysates depend on various factors. The most important factors are the chemical and structural nature of the protein, the nature and intensity of the processes to which they have been submitted, the hydrolysis conditions, the specificity of the proteolytic enzyme and the degree of hydrolysis (DH) (Oben et al., 2008).

The reported biological benefits of whey, combined with a growing market for functional foods and increasing consumer demand for high quality proteins, have made the use of whey protein and its fractions as ingredients in functional foods particularly attractive to the food industry (Mcintosh et al., 1998).

The objective of this study was to evaluate the physicochemical characteristics and nutritional properties of bovine milk proteins.

2. Materials and Methods

2.1 Materials

The bovine whey proteins, including WPC35 with a protein concentration of 35%, WPC80 with a protein concentration of 80%, WPH with a protein concentration of 80% and WPI with a protein concentration of at least 90%, were provided by Doremus Alimentos Ltda, Guarulhos, SP, Brazil. The casein used as a standard for comparison was acquired from Vetec Química Fina Ltda, Rio de Janeiro, RJ, Brazil.

2.2 Methods

2.2.1 Proximate composition determination

1 The analysis of the chemical composition was performed using the methods described
2 by the AOAC (Association of Official Analytical Chemists) (AOAC, 1995). The moisture
3 content was determined by loss on dryng in an oven dryer at 105 °C; ash content was
4 determined at 550 °C; and protein content was determined using the micro-Kjeldahl method
5 (N x 6.38). The total lipid content was determined using 3.5 g of sample and a mixture of
6 chloroform, methanol and water (10, 20 and 8 mL, respectively), as described previously
7 (Bligh & Dyer, 1959). The carbohydrate content was obtained by subtraction. All of the
8 analyses were performed in triplicate.
9
10 ***2.2.2 Determination of essential amino acid content***
11 The amino acid content was determined according to the procedure described by Lollo
12 et al. (2013). One hydrolysis stage was performed with 6 mol L^{-1} HCl (24 h, 110 °C). After
13 centrifugation, the supernatant was filtered through a 0.22 µm membrane and neutralized with
14 a 4:4:2 solution of 0.2 N sodium acetate trihydrate, methanol and triethylamine. A 40 µL
15 aliquot was derivatized with phenylisothiocyanate for further analysis by liquid
16 chromatograph. DL-2-aminobutyric acid was used as an internal standard and was added
17 before the derivatization process.
18
19 ***2.2.3 Analysis of the degree of hydrolysis (DH)***
20 The DH of the bovine WPH was determined according to the method described by
21 Nielsen et al. (2001) using an OPA reagent (3.81 g of sodium tetraborate decahydrate, 100 mg
22 of sodium dodecyl sulfate, 80 mg of o-phthaldehyde (OPA) in 2 mL of ethanol, 88 mg of
23 dithiothreitol (DTT) dissolved in 100 mL of deionized water). This method is based on the
24 formation of a complex between the free amine groups and the reactive OPA in the presence

of DTT or β-mercaptoethanol, which is detected at a wavelength of 340 nm. A 0.05% solution of the sample was prepared, and the analysis was performed in triplicate.

2.2.4 Polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight profile was determined by electrophoresis. The bovine whey protein concentrate (WPC35 and WPC80) and bovine whey protein isolate (WPI) samples were prepared at a concentration of 4 mg/mL, and the bovine WPH sample was prepared at a concentration of 10 mg/mL in a buffer containing SDS and β-mercaptoethanol.

A 12.0% acrylamide resolving gel (12% T; 2.67% C) with a 4% acrylamide stacking gel was used to resolve the protein fractions, according to Laemmli (1970). The 0.75 mm-thick gel was run at 120 V. We included a Bio-Rad standard 161-0304 (Bio-Rad Laboratories, Inc., Hercules, California, USA) that contained the following proteins: phosphorylase b (97,400 Da), albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da) and α-lactalbumin (14,400 Da). In Figure 2, the standard is identified as S.

2.2.5 Biological assay

2.2.5.1 Preparation of the experimental diets

The experimental diets were prepared based on the AIN-93G diet, according to the American Institute of Nutrition for Rodents (Reeves et al., 1993).

A completely randomized experimental design with six treatments was used (Figure 1). Each treatment consisted of one experimental feed containing an equivalent amount of fat, vitamins and minerals. The treatments differed with regard to the protein source to evaluate the physiological and metabolic consequences of consuming different types of bovine whey proteins.

A no-protein diet (treatment 1) was included to allow us to determine the loss of body weight over the duration of the biological assay. This data point was necessary for calculating the net protein efficiency ratio (NPR), as well as for calculating the endogenous fecal nitrogen (of body origin), which was used to determine true digestibility (D_T)

The experimental diets were adjusted to contain the same concentration of protein, with the exception of the no-protein diet.

The other ingredients used in the preparation of the experimental diets, including corn starch, sucrose and soybean oil, were acquired in the local market. The cellulose, mineral mix, vitamin mix, L-cysteine and choline bitartrate were purchased from Rhoster Indústria e Comércio Ltda, Araçoiaba da Serra, SP, Brazil. The ingredients were weighed on an analytical balance and further mixed and sieved three times for perfect homogenization.

After preparation, the diets were labeled and stored in a freezer (-18 °C) until use. The experimental diets were prepared weekly to avoid lipid oxidation. The diets were offered to the animals in powdered form.

2.2.5.2 Animals and experimental protocol

Twenty-four-day old male Wistar specific pathogen-free (SPF) rats were used. The rats were recently weaned and weighed an average of 45 g. The rats were obtained from the Central Animal Facility of the Rural Sciences Center of the Federal University of Santa Maria (Universidade Federal de Santa Maria - UFSM), Santa Maria, state of Rio Grande do Sul (RS), Brazil.

The 42 animals were randomly distributed into six groups of seven animals each. The animals were placed in individual metabolic cages in a controlled temperature environment (22 ± 1 °C) with a 12 hour light/dark cycle. The rats were allowed to adapt to the new environment for a period of five days, during which time they received water and commercial

feed. After this adaptation period, the animals received water and their respective diets *ad libitum* for 28 days, for a total experimental duration of 33 days.

The animals were weighed at the beginning of the experiment to select those that showed the least variation in weight. The animals with the highest and lowest weights (extremes) were excluded.

The body weight of the animals was then recorded every five days during the experimental period to generate a growth curve.

To measure the feed intake, each feeder was weighed, and the scale was then zeroed prior to the addition of the experimental diet. The feeders were removed from the cages and weighed daily, after which more experimental diet was placed in the feeders. Additionally, the metabolic cages were cleaned every day, and the leftovers were evaluated by measuring the losses in the trays for later deduction in the calculations, thus allowing us to determine the true consumption of the experimental diet by the animals.

The feed efficiency ratio (FER) was derived from the relationship between the total weight gain of the animals (g) and the total dietary intake (g). The FER determines how much of the diet the animal needs to ingest to increase their body weight. Thus, the greater the FER, the less food the animal needs to ingest to gain an equivalent weight, indicating an increase in feed efficiency (Donato Junior et al., 2007).

The feces were carefully collected for a period of seven days (corresponding to the last week of the biological assay) and separated from possible contaminants (food particles and hair, among others). The fecal samples were refrigerated (4 ºC) until the end of the experimental period, when they were dried in an oven dryer at 105 °C for later analysis of dry and wet feces excretion, evaluation of the protein concentration (micro-Kjeldahl method) and pH analysis (measured on a previously calibrated digital pH meter – DMPH – 2 Digimed).

The study began after approval was obtained from the Animal Welfare and Ethics Committee of the UFSM. All of the procedures conducted were in accordance with those recommended by the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal - COBEA), in compliance with the State constitution under Law no. 11,915, article 82, item IV from May 21, 2003.

2.2.5.3 Evaluation of protein quality

2.2.5.3.1 Essential amino acid score (EAAS)

The EAAS was determined by comparing the concentration of each essential amino acid from the protein fractions analyzed to the standard reference amino acids established by FAO/WHO (1997). The values were calculated according to Henley & Kuster (1994), using the following expression:

$$\text{EAAS} = \frac{\text{mg amino acids / g test protein}}{\text{mg amino acids / g standard or reference protein}}$$

The EAAS values allowed us to determine the limiting amino acids in each protein source. A protein with a chemical score equal to or greater than 1.0 for all the amino acids is considered to have high nutritional value. An amino acid with a chemical score less than 1.0 is considered to be a limiting amino acid (Sgarbieri, 1996).

2.2.5.3.2 Protein digestibility-corrected amino acid score (PDCAAS)

The PDCAAS index described by Henley & Kuster (1994) evaluates the quality of a protein by multiplying the chemical score of the most limiting essential amino acid from the different protein sources evaluated by the true D_T, according to the following formula:

$$\text{PDCAAS} = \text{EAAS} \times D_T$$

1

2 ***2.2.5.3.3 Protein efficiency ratio (PER)***

3 The PER was determined using the following formula (AOAC, 1975):

4

$$\text{PER} = \frac{\text{weight gain (g) of the test group}}{\text{protein consumed (g) by the test group}}$$

5

6

7 The relative protein efficiency ratio (RPER) was calculated using the following

8 formula:

9

$$\text{RPER} = \frac{\text{PER result for the test group}}{\text{PER result for the casein group}} \times 100$$

10

11

12 ***2.2.5.3.4 Net Protein Efficiency Ratio (NPR)***

13 The NPR was determined using the following formula (Bender & Doell, 1957):

14

$$\text{NPR} = \frac{\text{weight gain (g) of the test group + weight loss (g) of the no-protein group}}{\text{protein consumed (g) by the test group}}$$

15

16

17 The relative net protein efficiency ratio (RNPR) was calculated using the following

18 formula:

$$\text{RNPR} = \frac{\text{NPR result for the test group}}{\text{NPR result for the casein group}} \times 100$$

19

20 ***2.2.5.3.5 True digestibility (DT)***

To determine D_T, feces were collected from each animal and refrigerated. The feces were later dried in a forced-air oven dryer at 105 °C for 24 hours. The feces were then cooled, weighed and ground, and the nitrogen concentration was determined using the micro-Kjeldahl method (AOAC, 1995).

The D_T was calculated by measuring the quantity of nitrogen ingested in the diet (I), the quantity excreted in the feces (F) and the metabolic loss in the feces. This last value was estimated by the quantity of nitrogen excreted by the rats fed with a nitrogen-free (no-protein) diet (FK). The D_T was calculated using the following formula (AOAC, 1975):

$$D_T = \frac{I - (F - FK)}{I} \times 100$$

2.2.5.3.6 Nitrogen balance (NB)

The NB measures the quantity of nitrogen ingested in the diet and the quantity of nitrogen excreted in the feces and urine (Pellet & Young, 1980). The amount of nitrogen excreted in the urine is negligible; thus, the apparent nitrogen balance (NBap) was determined using the following formula:

$$NBap = \text{nitrogen ingested} - \text{nitrogen excreted in the feces}$$

2.2.5.4 Biochemical parameter measurement and organ removal

Once the experimental stage was completed and after 12 hours of fasting, the animals were anesthetized with 150 µg Ketamine and 200 µg Xylazine per kilogram of body weight and then euthanased. Incisions were made in the abdominal and thoracic cavities, and the blood was collected via cardiac puncture, using a separate disposable syringe for each animal. The epididymal fat, kidneys, liver and cecum were removed, and each organ was weighed (both the full cecum and empty cecum weights were recorded). Each animal's intestine was

also collected for histomorphometry to identify potential alterations in the morphology of the intestinal villi.

Aliquots of blood were transferred to test tubes containing heparin to measure the total hemoglobin, and the remaining blood was transferred to another test tube that was centrifuged to separate the serum for analysis. The serum was frozen for use in later biochemical analyses.

The serum levels of total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides, glucose, serum albumin, total proteins and hemoglobin were measured using colorimetric-enzymatic kits provided by Doles Reagentes, Goiânia, GO, Brazil, and the readings were performed using a spectrophotometer.

2.2.5.5 Histomorphometry of the small intestine

Samples of the small intestine were fixed in a 10% formaldehyde solution and embedded in paraffin using routine histological processes. Next, the tissues were sliced into 6 mm histological sections and stained using hematoxylin and eosin. The histomorphometry analysis was performed using image analysis software (Image J, NHI, Bethesda, Maryland, USA). For each slide, two villi were analyzed from two random fields. The degree of preservation and the presence of leukocytic infiltrate were determined qualitatively. The area, height and width of the villus, the thickness from the base of the villus to the serosa, and the epithelium thickness were measured.

2.2.6 Statistical analysis

A completely randomized block experimental design was used. The results were analyzed using analysis of variance (ANOVA), and the means of pairs were compared using Tukey's test (at a significance level of 5%), using the *Statistical Analysis System* (SAS, 1997).

1 The qualitative results from the histological evaluation of the small intestine samples
2 were subjected to Pearson chi-squared statistical analysis (IBM SPSS®), while the quantitative
3 results were analyzed using the ANOVA test followed by Tukey's test (Graphpad Prism®) at
4 a significance level of $p < 0.05$.
5
6 **3. Results and Discussion**
7
8 ***3.1. Percent composition***
9 As shown in Table 1, there were statistically significant differences in the moisture,
10 ash and carbohydrate contents of all of the samples. The protein concentrations were close to
11 the protein concentrations indicated on the product labels (casein - 85%, WPC35 - 35%,
12 WPC80 - 80%, WPH - 80% and WPI - 90%). The protein sources used in the study were
13 obtained from commercial sources; thus, the reduced protein content relative to the labeled
14 value was likely due to the processes used to generate the proteins. The total lipid
15 concentrations of the WPC35, WP80 and WPI samples showed statistically significant
16 differences. Lower concentrations of lipids (1.70% and 1.15%) were found in casein and WPI
17 due to their higher protein content.
18 The carbohydrate content (50.85%) was greater in WPC35 due to the lower protein
19 concentration.
20
21 ***3.2. Quantification of essential amino acids***
22 Table 2 shows the quantity of essential amino acids in the different protein sources
23 studied. Casein, WPC80, WPH and WPI provided all of the essential amino acids
24 recommended by FAO/WHO (1997). The essential amino acid values for the WPC35 sample
25 did not meet the recommendations established by FAO/WHO (1997), which is most likely

due to the reduced protein concentration (33.80%) compared to the higher protein content (≥ 72.89%) of the other samples.

WPC80, WPH and WPI contained large amounts of the amino acids isoleucine, leucine and valine (branched-chain amino acids), methionine + cysteine (sulfur amino acids) and lysine and threonine. The levels of aromatic amino acids (phenylalanine + tyrosine) were not excessive, but instead met the recommendations established by FAO/WHO (1997) (Table 2).

The whey proteins contained more than the recommended levels of almost all of the essential amino acids, particularly tryptophan, leucine, isoleucine and lysine. Phenylalanine and tyrosine were present at lower quantities, but met the recommendations for all age groups (Sgarbieri, 2004).

As shown in Table 2, the casein, WPC80, WPH and WPI samples exhibited EAAS values equal to or greater than 1.0 for all of the essential amino acids, meaning that these samples can be classified as high nutritional value protein sources. For the WPC35 sample, all of the EAAS values were less than 1.0.

The PDCAAS indicates the proportion of the specific protein source that is used by the organism, according to the FAO/WHO standards (1997). As shown in Table 2, the PDCAAS value of the WPC35 sample (27.55%) was lower than that of the other samples, due to the low EAAS of this sample. The PDCAAS values for casein, WPC80, WPH and WPI were equivalent to the D_T values, as the EAAS values for these four samples met the standards established by FAO/WHO (1997).

3.3. Analysis of the degree of hydrolysis (DH)

The WPH sample had a DH value of 18.24% ± 0.31. According to Vioque et al. (2001), hydrolysates with a DH greater than 10% are used in food and for special purposes.

1 Ramos (2001), working with WPH with a DH (30%), did not observe any benefits
2 from using hydrolysates compared to the intact protein, most likely due to the very high
3 concentrations of free amino acids. This suggests that high quantity of free amino acids
4 saturated the absorption sites and had greater difficulty crossing the intestinal mucosa than the
5 peptides, therefore exhibiting a lower protein efficiency. Our unpublished results indicate that
6 the effect of the pre-hydrolyzed proteins may depend on the DH.
7

8 ***3.4. Polyacrylamide gel electrophoresis (SDS-PAGE)***

9 We used SDS-PAGE to determine the distribution of low molecular weight proteins in
10 the bovine whey proteins, as shown in Figure 2. In columns 2, 3 and 4, which correspond to
11 the WPI, WPC80 and WPC35 samples, the presence of β-Lg and α-La is clearly detectable.

12 β-Lg accounts for half of the total whey proteins (45-57%), representing
13 approximately 3.2 g/L in bovine milk. It has an average molecular weight of 18.4-36.8 kDa,
14 which makes it resistant to the acids and proteolytic enzymes present in the stomach, so it is
15 absorbed in the small intestine. α-La, which accounts for 15-25% of the total whey proteins, is
16 rich in tryptophan and is heavily used in the production of infant formulas due to its structural
17 similarity to proteins present in maternal milk. α-La can bind to certain minerals such as
18 calcium and zinc, which can positively affect absorption. Additionally, it shows antimicrobial
19 activity against pathogenic bacteria. The molecular weight of α-La is 14.2 kDa, and the
20 protein is easily and rapidly digested. β-Lg and α-La are considered to be sources of essential
21 amino acids, including the branched-chain amino acids (Markus, Olivier, & De Haan, 2002;
22 Marshall, 2004; Haraguchi et al., 2006).

23 BSA was also present in the WPI sample, as well as in the WPC80 and WPC35
24 samples, although at lower resolution for the latter two. BSA accounts for approximately 10%
25 of the whey proteins. It has a high molecular weight (66 kDa) and is an important precursor of

glutathione. It has affinity for free fatty acids and other lipids, favoring transport in the bloodstream (De Wit, 1998).

Only faint bands were visible in the WPH sample (column 5), most likely due to hydrolysis, which resulted in peptides with molecular weights less than 14.000 Da.

3.4. Biological assay

As shown in Table 3, the animals fed with the diet containing WPC80 exhibited greater total weight gain, total feed intake and ingested protein compared to the animals in the other treatment groups. However, the experimental diet prepared with casein showed a higher FER.

The animals that received experimental diets prepared with WPH and WPI exhibited higher FER values than the animals fed with the diets that contained WPC35 and WPC80.

The animals fed with WPC35 and WPC80 had greater wet feces weight (12.03 g and 10.98 g, respectively) and dry feces weight (3.69 g and 3.54 g, respectively) than the other groups, as shown in Table 4. This may be related to the lower FER value for WPC35 and WPC80 (0.31, Table 3), which indicates lower feed efficiency because the feces represent the material remaining from the digestion and absorption of the food by the digestive tract. Additionally, two of the seven animals from the WPC35 group exhibited persistent diarrhea throughout the entire experimental period. The lactose concentration (45.10%) of WPC35 is the probable cause of this diarrhea, which was classified as osmotic, as this type of diarrhea occurs when osmotically active solutes, such as lactose, are present in the intestinal tract but are poorly absorbed (Mahan & Escott-Stump, 2005).

The weight of the full cecum and empty cecum (Table 4) of the animals fed with WPC35 differed statistically from the cecum weights of the animals in the other treatment groups. This may be related to a likely intestinal alteration that resulted in diarrheic feces and

1 a lower fecal pH value (5.97) (Table 4). A fecal pH value of less than 5.3 can indicate poor
2 absorption of carbohydrates (Mahan & Escott-Stump, 2005).

3 The quantity of epididymal fat was higher in the animals that consumed casein and
4 WPC80. This is most likely because the total feed intake was higher in these groups. The
5 weights of the livers and kidneys did not differ significantly between the treatment groups
6 (Table 4).

7 As shown in Table 5, the PER and the NPR, which are related to total weight gain and
8 ingested protein, were higher in the casein treatment group compared to the other treatment
9 groups. The casein and bovine whey proteins samples are classified as high-nutritional-value
10 proteins. According to Friedman (1996), a PER below 1.5 indicates a low quality protein, a
11 PER between 1.5 to 2.0 indicates a medium quality protein and a PER greater than 2.0
12 indicates a high-nutritional-value protein.

13 The NBap (Table 5), which indicates protein use, was higher in the diets containing
14 casein and WPC80, most likely due to the higher total feed intake. The NBap value of the diet
15 formulated with WPH may be related to the increased absorption of peptides in the intestine.

16 According to Boza et al. (1996), weaned and undernourished rats fed with diets
17 prepared with whey protein and hydrolyzed whey protein recovered adequately. The
18 hydrolyzed protein diet appears to be a better source of nitrogen, as it resulted in increased
19 peptide absorption in the intestine, increased protein content and low antigenicity.

20 Digestibility is the percentage of the proteins that are hydrolyzed by the digestive
21 enzymes and absorbed in the form of amino acids, peptides or any other nitrogen compounds.
22 The diet containing WPC35 exhibited the lowest D_T value (81.03%) of all the diets (Table 5).

23 The total cholesterol, LDL cholesterol, VLDL cholesterol, triglyceride and glucose
24 values were higher in the groups fed with casein and WPC35 (Table 6). The higher
25 concentrations of HDL cholesterol and lower concentrations of triglycerides and glucose

provided by the WPH and WPI protein sources may have induced hypocholesterolemic effects and helped control the glycemic index.

Haraguchi et al. (2010) conducted a study in rats comparing the biological quality of a protein from commercial whey with casein and its effect on biochemical parameters. The authors concluded that whey protein and casein have similar biological qualities, as they both exhibit a positive influence on fasting glycaemia homeostasis and on the lipid profile, without affecting renal and hepatic function.

The group fed with the diet containing WPH exhibited a higher concentration of serum albumin and total proteins (3.70 g/dL and 8.90 g/dL, respectively) (Table 6), which suggests that the hydrolysis process favored the improved use of dietary protein. The animals that were fed with casein exhibited higher hemoglobin levels, most likely due to the higher iron concentration (74.4 mg/g) identified in the sample.

Previous studies of diets containing bovine milk proteins found that the animals fed with whey proteins exhibited higher serum glucose and albumin levels, as well as an improved capacity to preserve muscle glycogen, compared to animals that received a diet containing casein as the only protein source (Tassi et al., 1998; Pimenta et al., 2006). Pimenta et al. (2006) also showed that the consumption of whey proteolysate as the only protein source for young rats resulted in superior performance in physical exercise compared to animals fed with the intact protein, based on lactate, albumin and total serum proteins. This demonstrates the advantage of partially hydrolyzed proteins compared to intact proteins.

3.5 Histomorphometry of the small intestine

As shown in Figure 3, the heights of the intestinal villi were similar in the animals fed with experimental diets formulated with all of the milk proteins. Regarding the area and width of the villus and the epithelial thickness, the animals fed with WPC35 showed higher

1 morphological preservation. The intestines of the animals fed with casein and WPI exhibited
2 greater thickness from the base of the villus to the serosa.

3 Alterations were observed in the villi of the animals in the treatment groups compared
4 to the animals on the no-protein diet, which demonstrates that the absence of nutrients in the
5 intestinal lumen can induce atrophy of the mucosa. The smaller size of the villi results in the
6 reduced transport of nutrients through the surface of the enterocyte, which also decreases the
7 enzymatic content of the intestinal mucosa cells (Caruso & Demonte, 2005).

8 Recent studies have demonstrated that whey proteins have some advantages compared
9 to caseins. There are fundamental differences in the metabolism and physiological action of
10 caseins and whey proteins, due to the fact that whey proteins do not suffer conformational
11 alterations due to the action of the stomach acids. When whey proteins reach the small
12 intestine, they are rapidly digested and their amino acids are absorbed, rapidly increasing the
13 amino acid concentration in the plasma and stimulating the protein synthesis in the tissues
14 (Pacheco et al., 2005).

15 Our results show that the morphology of the small intestine of animals that received
16 bovine whey proteins as a protein source did not differ significantly from the casein group.
17 Greater morphological preservation was expected in the WPH group compared to the other
18 treatment groups because, although whey proteins are more digestible and are rapidly
19 absorbed into the bloodstream, protein hydrolysates containing small peptides (di- and tri-
20 peptides) are absorbed at a higher rate.

21

22 **4. Conclusions**

23

24 In general, casein, WPC80 and WPI had similar compositions and contained adequate
25 concentrations of essential amino acids according to the standards established by FAO/WHO

(1997). In addition, according to the EAAS and the PDCAAS, casein, WPC80, WPH and WPI are high nutritional value protein sources, unlike WPC35.

Based on qualitative gel electrophoresis, we detected the presence of β-Lg, α-La and BSA in the WPI, WPC80 and WPC35 samples.

The FER, PER and NPR results suggested that casein and whey proteins have similar biological effects. The WPC80, WPH and WPI protein sources exhibited more favorable biochemical parameters, including total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, glucose, serum albumin and total proteins.

We observed a significant difference between the animals that received a no-protein diet and the other treatment groups concerning to histomorphometry of the animal small intestine. In the group with WPC35, we observed increased morphological preservation, particularly in the area and width of the villi and the epithelial thickness, which indicates differences in the absorption of bovine milk proteins.

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Table 1. Proximate composition of casein and whey proteins (mean ± standard deviation, n = 3).

Parameters (%)	Casein	WPC35	WPC80	WPH	WPI
Moisture	11.12 ± 0.02^{a}	4.73 ± 0.02^{e}	8.10 ± 0.08^{b}	7.31 ± 0.07^{c}	6.73 ± 0.03^{d}
Ashes	1.91 ± 0.13^{e}	$7.14 \pm 0,03^{a}$	2.87 ± 0.05^{c}	5.00 ± 0.02^{b}	2.39 ± 0.02^{d}
Protein	82.78 ± 0.33^{b}	33.80 ± 0.10^{d}	72.89 ± 0.75^{c}	$74,21 \pm 0.30^{c}$	85.82 ± 0.30^{a}
Total lipids	1.70 ± 0.11^{d}	3.48 ± 0.09^{c}	4.34 ± 0.44^{b}	5.02 ± 0.08^{a}	1.15 ± 0.11^{d}
Carbohydrates	2.49 ± 0.35^{e}	50.85 ± 0.14^{a}	11.80 ± 0.87^{b}	8.46 ± 0.32^{c}	3.91 ± 0.32^{d}

Same letters in the same column indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

Table 2. Essential amino acid composition, essential amino acid score (EAAS) and protein digestibility-corrected amino acid score (PDCAAS) of casein and bovine whey proteins and reference standard of FAO/WHO for 2-5 years children (2007).

Essential amino acid (mg/g of protein)	Protein sources					
	Casein	WPC35	WPC80	WPH	WPI	FAO
Phenylalanie & Tyrosine	95.40 ± 0.71	22.40 ± 0.85	53.00 ± 0.28	53.45 ± 0.92	53.05 ± 0.92	46
Histidine	24.95 ± 0.49	6.05 ± 0.07	18.45 ± 0.07	18.10 ± 0.00	18.40 ± 0.00	18
Isoleucine	43.90 ± 0.85	20.90 ± 0.14	53.70 ± 0.28	52.15 ± 0.21	62.20 ± 2.97	31
Leucine	81.90 ± 0.71	36.55 ± 0.49	88.85 ± 0.49	85.80 ± 0.57	110.65 ± 0.21	63
Lysine	72.95 ± 1.06	33.40 ± 0.42	78.55 ± 0.07	81.50 ± 0.57	100.25 ± 0.07	52
Methionine + Cysteine	45.35 ± 2.19	25.35 ± 1.06	36.45 ± 1.63	64.05 ± 0.92	78.05 ± 1.20	26
Treonine	35.00 ± 0.14	23.35 ± 0.35	60.45 ± 0.49	56.85 ± 0.49	65.95 ± 4.45	27
Tryptophano	12.75 ± 0.21	6.70 ± 0.00	14.20 ± 0.28	10.90 ± 0.28	12.75 ± 0.49	7.4
Valine	59.85 ± 0.78	21.35 ± 0.21	53.40 ± 0.14	49.60 ± 0.28	56.20 ± 0.00	42

Essential amino acid (mg/g of protein)	Protein sources				
	EAAS = mg/g protein sample ÷ mg/g protein standard FAO/WHO				
	Casein	WPC35	WPC80	WPH	WPI
Phenylalanie + Tyrosine	2.07	0.49*	1.15	1.16	1.15
Histidine	1.39	0.34**	1.03	1.01	1.02
Isoleucine	1.42	0.67*	1.73	1.68	2.01
Leucine	1.30	0.58*	1.41	1.36	1.76
Lysine	1.40	0.64*	1.51	1.57	1.93
Methionine + Cysteine	1.74	0.98*	1.40	2.46	3.00
Treonine	1.30	0.86*	2.24	2.11	2.44
Tryptophano	1.72	0.91*	1.92	1.47	1.72
Valine	1.43	0.51*	1.27	1.18	1.34
PDCAAS (%)	96.16	27.55	99.69	91.27	98.39

Results are expressed as mean ± standard deviation (n = 2).
*Limiting amino acid. PDCAAS = EAAS most limiting ** x protein true digestibility (D_T) obtained from rats evaluation. D_T: casein = 96.16%; WPC35 = 81.03%; WPC80 = 99.69%; WPH = 91.27%; WPI = 98.39%.

Table 3. Total weight gain, total feed intake, feed efficiency ratio (FER) and ingested protein of animals feed with experimental diets containing casein and bovine whey proteins.

Parameters	Protein sources				
	Casein	WPC35	WPC80	WPH	WPI
Total weight gain (g)	156.73 ± 12.98[a]	125.21 ± 12.46[b]	159.68 ± 20.19[a]	137.56 ± 9.90[ab]	126.98 ± 18.35[b]
Total feed intake (g)	450.00 ± 42.63[b]	410.00 ± 27.99[b]	522.32 ± 39.68[a]	422.06 ± 39.57[b]	388.74 ± 50.33[b]
Feed efficiency ratio (FER)	0.35 ± 0,03[a]	0.31 ± 0.03[a]	0.31 ± 0.04[a]	0.33 ± 0.02[a]	0.33 ± 0.02[a]
Ingested protein (g)	67.50 ± 6.39[b]	61.50 ± 4.20[b]	78.35 ± 5.95[a]	63.31 ± 5.93[b]	58.31 ± 7.55[b]

Results are expressed as mean ± standard deviation (n = 7).
Same letters in the same column indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

Table 4. Production of wet feces, dry feces and pH values as well as weight of full cecum, empty cecum, epididymal fat, livers and kidneys of animals feed with experimental diets containing casein and bovine whey proteins.

Parameters	Protein sources				
	Casein	WPC35	WPC80	WPH	WPI
Wet feces (g)	8.71 ± 1.22^{b}	12.03 ± 3.77^{a}	10.98 ± 0.76^{ab}	9.07 ± 1.33^{ab}	8.61 ± 1.31^{b}
Dry feces (g)	1.46 ± 0.63^{c}	3.69 ± 2.35^{a}	3.54 ± 0.59^{ab}	1.89 ± 0.92^{ab}	1.93 ± 0.83^{ab}
pH	6.11 ± 0.10^{c}	5.97 ± 0.10^{c}	6.43 ± 0.26^{bc}	6.99 ± 0.38^{a}	6.63 ± 0.51^{ab}
Full cecum (g)	0.97 ± 0.22^{b}	2.50 ± 0.35^{a}	1.08 ± 0.13^{b}	1.23 ± 0.32^{b}	1.15 ± 0.29^{b}
Empty cecum (g)	0.28 ± 0.09^{c}	0.71 ± 0.20^{a}	0.52 ± 0.10^{ab}	$0.37 \pm 0,11^{bc}$	0.36 ± 0.13^{bc}
Epididymal fat (g)	1.10 ± 0.32^{ab}	0.81 ± 0.19^{b}	1.32 ± 0.23^{a}	1.17 ± 0.19^{a}	1.03 ± 0.24^{ab}
Livers (g)	3.79 ± 0.37^{a}	3.86 ± 0.34^{a}	3.66 ± 0.33^{a}	4.06 ± 0.32^{a}	4.07 ± 0.45^{a}
kidneys (g)	0.81 ± 0.05^{a}	0.92 ± 0.11^{a}	0.83 ± 0.07^{a}	0.88 ± 0.15^{a}	0.82 ± 0.06^{a}

Results are expressed as mean ± standard deviation (n = 7).
Same letters in the same column indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

Table 5. Protein efficiency ratio (PER), relative protein efficiency ratio (RPER), net protein efficiency ratio (NPR), relative net protein efficiency ratio (RNPR), nitrogen ingested, nitrogen fecal, true digestibility (D_T) and apparent nitrogen balance (NBap) of animals feed with experimental diets containing casein and bovine whey proteins.

Parameters	Protein sources				
	Casein	WPC35	WPC80	WPH	WPI
PER	2.33 ± 0.17^{a}	2.04 ± 0.20^{a}	2.04 ± 0.26^{a}	2.18 ± 0.11^{a}	2.18 ± 0.12^{a}
RPER (%)	100.00 ± 0.00^{a}	87.56 ± 8.79^{b}	87.63 ± 11.30^{b}	93.50 ± 4.51^{ab}	93.45 ± 5.16^{ab}
NPR	2.65 ± 0.19^{a}	2.39 ± 0.21^{a}	2.32 ± 0.27^{a}	2.52 ± 0.13^{a}	2.55 ± 0.13^{a}
RNPR (%)	100.00 ± 0.00^{a}	90.18 ± 8.07^{ab}	87.50 ± 4.69^{b}	95.07 ± 4.93^{ab}	96.22 ± 4.98^{ab}
Nitrogen ingested (g)	10.80 ± 1.02^{b}	9.84 ± 0.67^{b}	13.22 ± 1.29^{a}	10.13 ± 0.95^{b}	9.33 ± 1.21^{b}
Nitrogen fecal (g)	2.08 ± 0.14^{c}	3.52 ± 0.39^{a}	1.71 ± 0.25^{d}	2.56 ± 0.27^{b}	1.82 ± 0.13^{cd}
D_T (%)	96.16 ± 1.25^{a}	81.03 ± 4.57^{c}	99.69 ± 1.97^{a}	91.27 ± 2.31^{b}	98.39 ± 1.39^{a}
NBap (g)	8.72 ± 1.01^{b}	6.32 ± 0.89^{c}	11.51 ± 1.34^{a}	7.57 ± 0.86^{bc}	7.52 ± 1.25^{bc}

Results are expressed as mean ± standard deviation (n = 7).
Same letters in the same column indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

Table 6. Biochemical parameters of animals feed with experimental diets containing casein and bovine whey proteins.

Biochemical parameters (mg/dL)	Protein sources				
	Casein	WPC35	WPC80	WPH	WPI
Total cholesterol	190.42 ± 16.02[a]	184.38 ± 15.33[a]	129.53 ± 10.62[c]	147.65 ± 20.65[bc]	169.44 ± 26.67[ab]
HDL cholesterol	69.21 ± 15.24[a]	49.68 ± 14.04[a]	56.05 ± 8.99[c]	68.05 ± 22.51[a]	62.08 ± 21.73[a]
LDL cholesterol	94.50 ± 16.02[a]	109.20 ± 15.33[a]	59.79 ± 10.62[c]	63.04 ± 20.65[bc]	88.38 ± 26.67[ab]
VLDL cholesterol	26.71 ± 2.91[a]	25.49 ± 3.75[ab]	13.69 ± 5.32[c]	16.56 ± 5.55[c]	18.98 ± 3.95[bc]
Triglycerides	133.53 ± 14.55[a]	127.45 ± 18.73[ab]	68.43 ± 26.58[c]	82.82 ± 27.76[c]	94.90 ± 19.73[bc]
Glucose	92.02 ± 13.43[ab]	97.90 ± 11.86[a]	95.27 ± 12.77[ab]	72.21 ± 29.04[b]	87.61 ± 15.76[ab]
Biochemical parameters (g/dL)	**Casein**	**WPC35**	**WPC80**	**WPH**	**WPI**
Serum albumin	3.11 ± 0.56[ab]	3.03 ± 0.23[b]	3.26 ± 0.26[ab]	3.70 ± 0.53[a]	3.51 ± 0.43[ab]
Total proteins	6.60 ± 0.72[a]	6.96 ± 0.67[a]	7.13 ± 1.40[a]	8.90 ± 2.63[a]	8.89 ± 2.35[a]
Hemoglobin	13.47 ± 2.82[a]	7.38 ± 2.64[b]	8.44 ± 1.63[b]	10.63 ± 1.77[ab]	8.58 ± 2.05[b]

Results are expressed as mean ± standard deviation (n = 7).
Same letters in the same column indicate that the data do not differ statistically among them (Tukey's test; $p \leq 0.05$).

Figure Captions

Figure 1. [1]Diet prepared without protein (no-protein), where casein used in AIN 93G was substituted by corn starch; [2]diet prepared with casein according to recommendations of AIN 93G and [3]diets where casein used in AIN 93G was substituted by bovine whey proteins.

Figure 2. Polyacrylamide gel electrophoresis (SDS-PAGE) of bovine whey proteins.

Figure 3. Histomorphometry of the animal small intestine feed with no-protein and bovine milk proteins diets (casein, WPC35, WPI and WPH).

Figure 1

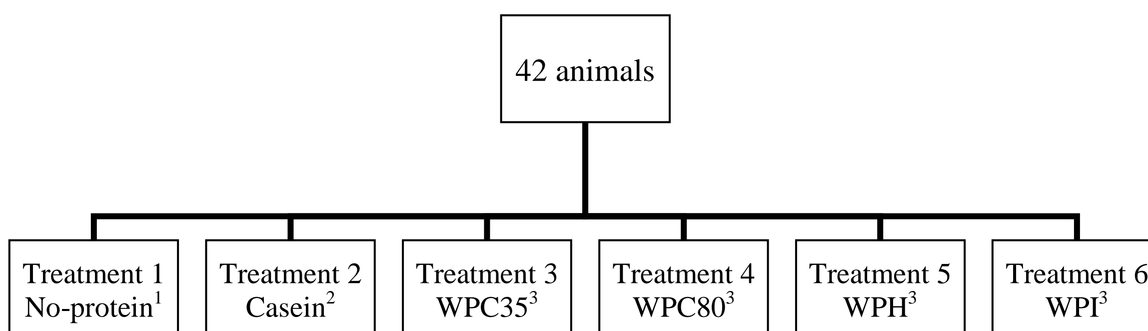


Figure 2

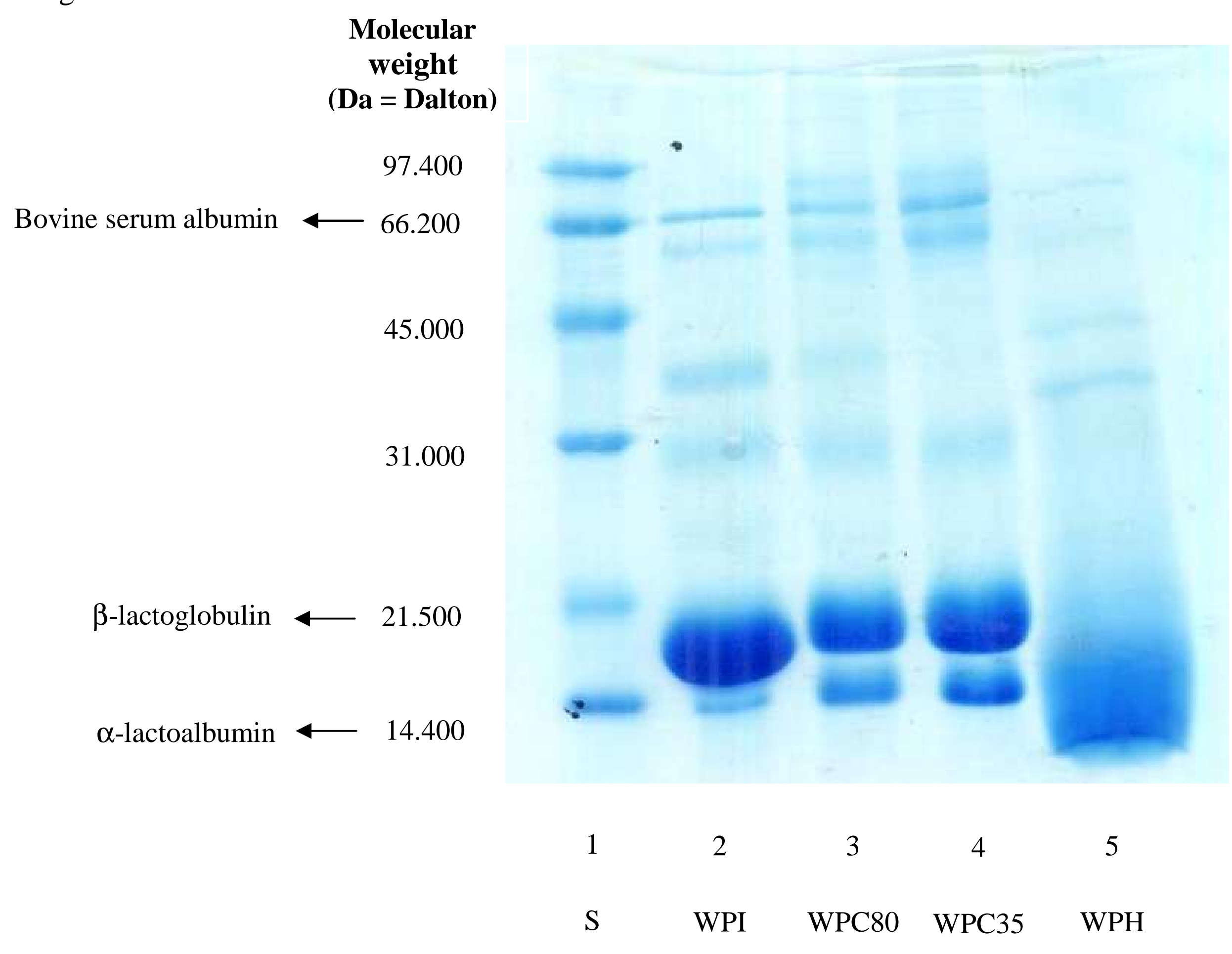
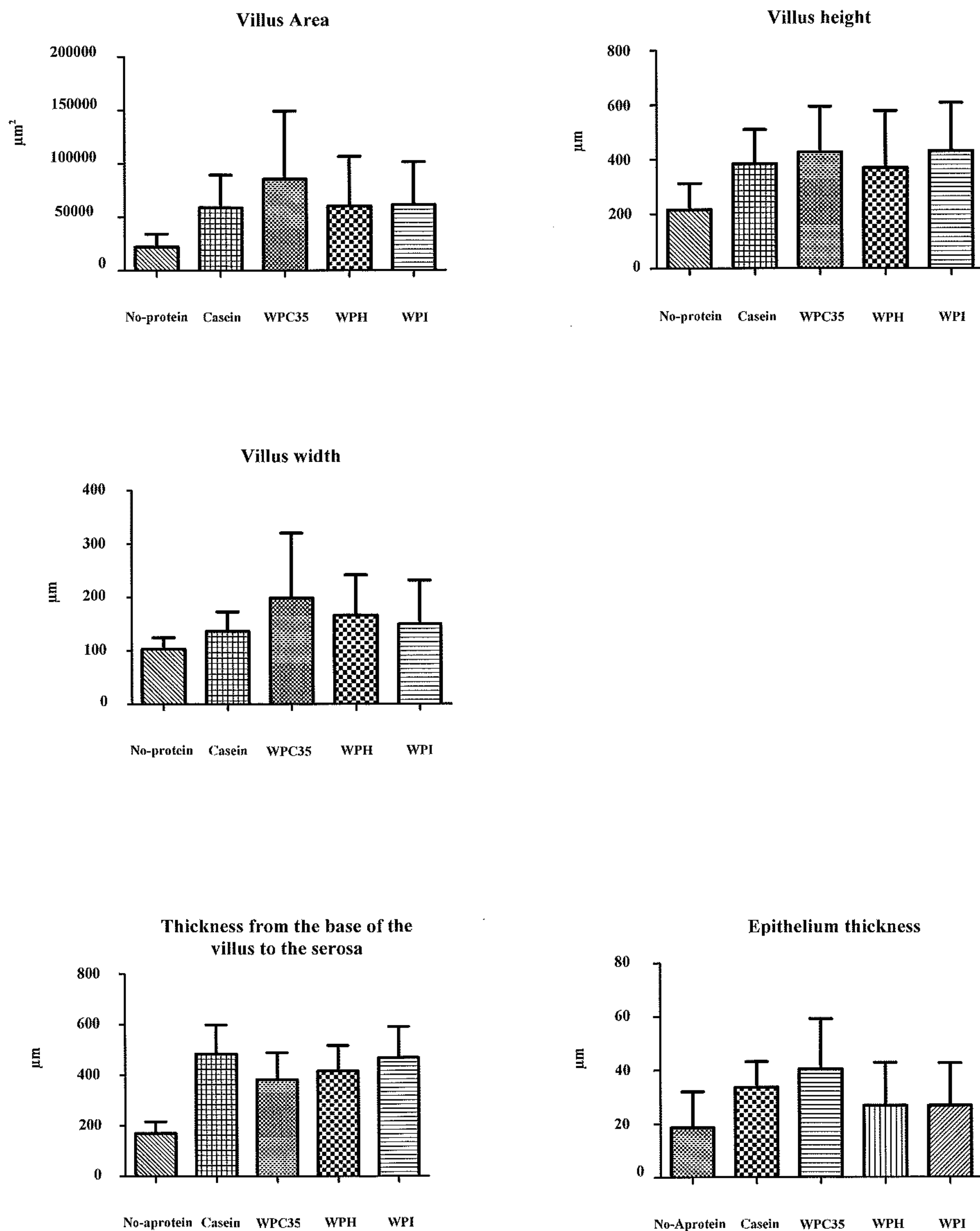


Figure 3



ARTIGO 4

Artigo em fase de revisão

Efeito da microencapsulação nas propriedades sensoriais e biológicas do hidrolisado proteico do soro de leite bovino

Sabrina Vieira da Silva[1]*, Tiffany Prokopp Hautrive[1], Angela Souza Rodrigues[1], Karine Inês Bolson Moro[1], Gilberti Helena Hübscher Lopes[1], Neila Silvia Peireira dos Santos Richards[1]

Departamento de Tecnologia e Ciência dos Alimentos, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brasil

*Endereço para correspondência: Tel/Fax: +55-55-3220-8254

E-mail: sabrinutri@yahoo.com.br

RESUMO

A proteína hidrolisada do soro de leite bovino está na forma de dipeptídeo e tripeptídeo sendo facilmente digerida, proporcionando efeitos fisiológicos benéficos. Porém, o sabor amargo limita a utilização como ingrediente alimentar. O objetivo deste estudo foi avaliar o efeito da microencapsulação nas propriedades sensoriais e biológicas do WPH. A microcápsula foi obtida pelo processo de *spray drying* utilizando como agentes encapsulantes os materiais: gordura vegetal, pectina genu, benzoato de sódio, sorbato de potássio, fosfato tricálcico, lactose, tocoferol e ascorbil palmitato. O Microscópio Eletrônico ZEISS, modelo EVO LS 15 foi utilizado para análise morfológica das microcápsulas. As fontes proteicas (caseína, WPH e WPH microencapsulado) foram analisadas quanto às características físico-químicas – teor de umidade, cinzas, proteínas, lipídios totais e carboidratos. O ensaio biológico permitiu acompanhar o ganho de peso, consumo alimentar, quociente de eficácia alimentar (QEA) e proteína ingerida dos animais, avaliar a qualidade proteica e a resposta metabólica das fontes proteicas, além da integridade de alguns órgãos. Na análise sensorial utilizou-se o Teste Triangular para avaliar a diferença de sabor entre WPH e WPH microencapsulado. Na composição centesimal verificou-se que o processo de microencapsulação resultou em alteração dos parâmetros avaliados na amostra WPH microencapsulado. As imagens obtidas microscopia eletrônica de varredura mostraram microcápsulas com formato esférico, presença de achatamentos e diâmetro em torno de 20 μm. No ensaio biológico os valores referentes ao consumo alimentar e proteína ingerida foram superiores no tratamento da dieta formulada com WPH microencapsulado, porém o ganho de peso total e o QEA foram superiores no tratamento caseína. A avaliação proteica mostrou que a caseína e a WPH são consideradas de alto valor nutritivo, porém a WPH microencapsulado classificou-se como uma proteína de qualidade média tendo em vista o resultado do PER (1,63). Não houve diferença significativa entre os animais alimentados com

a dieta formulada com WPH e WPH microencapsulado em relação aos parâmetros bioquímicos. O peso dos órgãos permitiu constar que as diferentes dietas experimentais não geraram sobrecargas. Na análise sensorial verificou-se que o processo de microencapsulação melhorou de forma significativa o sabor da amostra WPH. Diante do exposto, concluiu-se que a microencapsulação não alterou as propriedades biológicas e atenuou o sabor amargo do WPH.

Palavras-chaves: Microencapsulação; Hidrolisado proteico do soro de leite bovino; Avaliação sensorial.

1 INTRODUÇÃO

A utilização de hidrolisados proteicos do soro de leite tem recebido atenção especial da indústria alimentícia e em estudos relacionados à fisiologia animal, devido suas propriedades funcionais. Com a hidrólise de proteínas são formados agregados de peso molecular variável, como peptonas, misturas de peptídios e aminoácidos livres (LAHL & BRAUN, 1994).

Os peptídeos bioativos são definidos como fragmentos específicos de proteína que tem um impacto positivo nas funções corporais, podendo influenciar na saúde, entretanto, para isso é necessário que sejam resistentes à ação de proteínas digestivas (KITTS, 2005). Dentre as propriedades fisiológico-funcionais que esses peptídeos podem desempenhar destacam-se: atividade anti-hipertensiva, ação estimulante do sistema imunológico, atividade opióide, aumento da biodisponibilidade de minerais, atividade anti-úlcera, antioxidante, anti-carcinogênica, anti-microbiana e atividade hipocolesterolêmica (WALZEM et al., 2002).

Entre os peptídios bioativos os componentes das proteínas do soro de leite, como as imunoglobulinas, lactoferrina, lactoperoxidase e glicomacropeptídio, são disponíveis comercialmente de forma isolada, com aplicações específicas em fórmulas infantis, dietas clínicas e componentes das pastas de dentes e agentes conservantes contra micro-organismos (STEIJNS, 2001).

A hidrólise de proteínas pode ser executada por enzimas, ácido ou base forte, mas o uso de enzimas, como por exemplo, as do sistema digestivo (pancreatina, pepsina, α-quimotripsina), algumas de origem vegetal como papaína e várias enzimas de origem microbiana, é o método preferido para a obtenção de hidrolisados para aplicações nutricionais, sendo utilizados em formulações específicas incluindo fórmulas infantis hipoalergênicas, preparações imunoestimulantes, produtos geriátricos, dietas terapêuticas e bebidas esportivas (CLEMENTE, 2000).

As características de composição, propriedades físico-químicas, funcionais e sensoriais de hidrolisados proteicos enzimáticos dependem de vários fatores, sendo os mais importantes à natureza química e estrutural da proteína, natureza e intensidade dos processos a que tenha sido submetida, condições da hidrólise e especificidade da enzima proteolítica e finalmente o grau de hidrólise. Os critérios mais importantes a serem considerados na produção de hidrolisados proteicos incluem valor nutricional, custo, sabor, antigenicidade e funcionalidade (OBEN et al., 2008).

As fontes proteicas na nossa alimentação são geralmente consumidas na sua forma intacta para serem posteriormente digeridas ou hidrolisadas no trato gastrointestinal e absorvidas na forma de peptídeos de diversos tamanhos. Os possíveis efeitos de consumir as proteínas na forma pré-hidrolisada tem sido objeto de pesquisas que atualmente apontam para algumas vantagens, incluindo aquelas relacionadas ao metabolismo proteico (TASSI et al., Z1998; PIMENTA et al., 2006).

A tecnologia de microencapsulação é definida como o processo de empacotamento de materiais sólidos, líquidos ou gasosos em cápsulas extremamente pequenas, as quais podem liberar seus conteúdos de maneira controlada e sob a influência de condições específicas. As microcápsulas são constituídas por uma membrana semipermeável, esférica, fina e resistente que envolve um centro sólido/líquido (ANAL & SINGH, 2007). Essa tecnologia vem sendo empregada com êxito na indústria de cosméticos, farmacêutica e alimentícia (FÁVARO-TRINDADE, DE PINHO & ROCHA, 2008).

Na indústria de alimentos, a microencapsulação pode ser empregada com diversas finalidades, entre elas, transformar um líquido em um sólido, a fim de facilitar a manipulação; separar materiais reativos; promover liberação controlada do material ativo encapsulado; controlar reações de oxidação; mascarar sabores, cores e odores de determinados componentes; estender a vida útil; e proteger contra a luz, umidade e perda nutricional dos

componentes (ANAL & SINGH, 2007; FÁVARO-TRINDADE, DE PINHO & ROCHA, 2008).

Entre as técnicas utilizadas para a microencapsulação, estão as de: i) *Spray-drying* ou atomização, em que geralmente são utilizados como materiais de revestimento polímeros solúveis em água; ii) *Spray-congealing*, que utiliza ceras, ácidos graxos, polímeros solúveis e insolúveis em água, além de outros monômeros como material de revestimento; iii) *Fluidizedbed coating/air-suspension*, que utiliza polímeros solúveis e insolúveis em água, lipídeos e ceras como material de revestimento; iv) Extrusão, que utiliza como revestimento da cápsula polímeros solúveis e insolúveis em água; v) Coacervação ou técnica de separação de fases, que utiliza como material encapsulante polímeros solúveis em água; e vi) Método eletrostático, que utiliza como material de revestimento polímeros e outros compostos com cargas opostas (MENEZES et al., 2013).

O material microencapsulante geralmente é de natureza semipermeável, apresentando morfologia esférica, envolta por uma resistente membrana sólida ou sólida/líquida, com um diâmetro variando de poucos mícrons a 1mm. Esse material de revestimento deve ser capaz de resistir a condições ácidas no estômago, permitindo que os ingredientes ativos possam atravessar o estômago de maneira intacta (MENEZES et al., 2013).

Tendo em vista que o emprego da microencapsulação tem sido intensificado devido às novas necessidades demonstradas nas formulações de produtos alimentícios o objetivo desse estudo foi avaliar as propriedades sensoriais e biológicas do hidrolisado proteico do soro de leite bovino microencapsulado.

2 MATERIAL E MÉTODOS

2.1 Material

As proteínas do soro de leite bovino - hidrolisado proteico do soro de leite bovino (WPH) com teor de proteína de 80% e hidrolisado proteico do soro de leite bovino (WPH) que foi submetido ao processo de microencapsulação foram fornecidos pela empresa Doremus Alimentos Ltda, Guarulhos, SP, Brasil. A caseína utilizada como padrão para comparação foi adquirida na empresa Vetec Química Fina Ltda, Rio de Janeiro, RJ, Brasil.

2.2 Métodos

2.2.1 Microencapsulação do hidrolisado proteico do soro de leite bovino

A amostra WPH foi submetida ao processo de microencapsulação por *spray-drying*. Porém, como o processo foi realizado na empresa Ultrapan Indústria e Comércio Ltda, Divisão Funcional *Mikron*, Valinhos, SP, Brasil, a metodologia com a descrição completa é confidencial, e por este motivo, a empresa está avaliando a possibilidade de liberação na publicação de artigo científico.

Os materiais utilizados com agente encapsulante foram: gordura vegetal, pectina genu, benzoato de sódio, sorbato de potássio, fosfato tricálcico, lactose, tocoferol e ascorbil palmitato.

2.2.2 Análise morfológica por microscopia eletrônica de varredura (SEM)

A avaliação microscópica da amostra WPH e a aparência externa da microcápsula do WPH microencapsulado foi realizada no Microscópio Eletrônico ZEISS, modelo EVO LS 15, no modo de vácuo ambiental. A energia utilizada foi de 15 kV, sendo a aquisição das imagens feitas em detector de elétrons secundários.

As amostras foram fixadas a um *stub* (porta amostra) de alumínio com uma fita de carbono dupla-face. Em seguida foi depositada uma fina camada de ouro, a fim de aumentar a condutividade das amostras, melhorando a resolução das imagens.

Para calcular o diâmetro médio das microcápsulas foram realizadas medidas no microscópio, de acordo com metodologia proposta por Krasaekoopt, Bhandari & Deeth (2004).

2.2.3 Determinação da composição centesimal

A análise da composição centesimal foi efetuada empregando os métodos descritos pela AOAC (*Association of Official Analytical Chemists*) (AOAC, 1995). A umidade foi determinada por perda por dessecação em estufa a 105 °C; cinzas foi realizada em 550 °C e proteínas pelo método micro-Kjekdahl (N x 6,38). A quantidade de lipídios totais foi determinada empregando 3,5 g de amostra e uma mistura de clorofórmio, metanol e água (10, 20 e 8 mL, respectivamente) (BLIGH & DYER, 1959). Carboidratos foram obtidos por diferença. Todas as determinações foram efetuadas em triplicata.

2.2.4 Ensaio biológico

2.2.4.1 Preparo das dietas experimentais

As dietas experimentais foram elaboradas baseadas nas dietas AIN 93G, segundo o *American Institute of Nutrition for Rodents* (REEVES et al., 1993).

O delineamento estatístico utilizado foi inteiramente casualizado, com quatro tratamentos. Cada tratamento foi composto por uma ração experimental contendo quantidade equivalente de gordura, vitaminas e minerais, mas diferenciadas quanto à fonte proteica de

modo a avaliar os eventos fisiológicos e metabólicos decorrentes do consumo de diferentes tipos de proteínas do soro de leite bovino.

As dietas experimentais elaboradas foram divididas nos seguintes tratamentos:

- Tratamento 1: dieta formulada sem proteína (aproteica), onde a caseína, usada na AIN 93G foi substituída por amido de milho.
- Tratamento 2: dieta formulada com caseína, de acordo com as recomendações do AIN 93G.
- Tratamento 3: dieta onde a caseína, usada na AIN 93G foi substituída por hidrolisado proteico do soro de leite com teor de proteína de 80%.
- Tratamento 4: dieta onde a caseína, usada na AIN 93G foi substituída por hidrolisado proteico do soro de leite microencapsulado.

O tratamento 1 dieta sem proteína (aproteica) serviu para determinação da perda de peso corporal durante todo o período do ensaio biológico, dado necessário para o cálculo do quociente de eficiência líquida da proteína (NPR), assim como para o cálculo de nitrogênio fecal endógeno (origem corporal) utilizado na determinação da digestibilidade verdadeira (Dv).

As dietas experimentais foram ajustadas de modo a apresentarem os mesmos teores de proteína, com exceção da dieta experimental aproteica.

Os demais ingredientes utilizados na elaboração das dietas experimentais, amido de milho, sacarose e óleo de soja foram adquiridos no comércio local. Celulose, mistura de minerais, mistura de vitaminas, L cistina e bitartarato de colina foram adquiridos do fornecedor Rhoster Indústria e Comércio Ltda, Araçoiaba da Serra, SP, Brasil. Os ingredientes foram pesados em balança analítica e posteriormente, misturados e peneirados por três vezes, para perfeita homogeneização.

Após o preparo, as dietas foram identificadas e armazenadas no *freezer* (-18 °C) até o momento do consumo pelos animais. As dietas experimentais foram elaboradas semanalmente para evitar que ocorresse a oxidação lipídica. As dietas foram oferecidas na forma de pó aos animais.

2.2.4.2 Animais e protocolo experimental

Foram utilizados ratos machos, da linhagem *Wistar*, livres de patógenos específicos (SPF), recém-desmamados, com de peso médio de 45 g e 24 dias de idade, provenientes do Biotério Central do Centro de Ciências Rurais da Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brasil.

Os animais foram distribuídos aleatoriamente em quatro grupos, sendo que cada grupo foi composto de sete animais, totalizando 28 animais. Os animais foram alocados em gaiolas metabólicas individuais, em ambiente de temperatura controlada a 22 ± 1 °C e ciclo de 12 horas claro/escuro. O período de cinco dias foi utilizado para adaptação, no qual os mesmos receberam água e ração comercial. Após, os animais receberam água e suas respectivas dietas experimentais *ad libitum* durante 28 dias, totalizando um período de 33 dias.

Os animais foram pesados no início do experimento com o objetivo de selecionar os que apresentarem menor variação de peso, sendo que os animais de maior e menor peso (extremos) foram excluídos.

O controle do peso corporal dos animais foi realizado de cinco em cinco dias durante o período experimental previsto, para posterior elaboração da curva de crescimento.

Em relação ao consumo alimentar, inicialmente todos os comedouros foram pesados e tarados com posterior adição de dieta experimental. Diariamente, os comedouros foram retirados das gaiolas, pesados e foi recolocada mais dieta experimental. Além disso, as gaiolas metabólicas foram higienizadas todos os dias e as sobras avaliadas pelas perdas nas bandejas

também foram monitoradas para posterior desconto e verificação do consumo real de dieta experimental pelos animais.

O quociente de eficiência alimentar (QEA) foi obtido a partir da relação do ganho de peso total dos animais (g) pelo consumo total de dieta (g). O QEA permitiu a avaliação do quanto de dieta o animal necessitou ingerir para aumentar seu peso corporal. Dessa forma, quanto maior for o QEA, menos alimento o animal precisou ingerir para ganhar um peso equivalente, indicando aumento da eficácia alimentar (DONATO JUNIOR et al., 2007).

As fezes foram coletadas cuidadosamente por um período de sete dias, correspondente a última semana do ensaio biológico, separando-se os possíveis contaminantes (partículas de alimentos, pêlos entre outros), foram mantidas sob refrigeração (4 ºC) até o final do período experimental e posteriormente foram secas em estufa à temperatura de 105 °C para moagem e posterior análise de excreção de fezes secas, fezes úmidas, avaliação do teor de proteínas (método micro-Kjekdahl) e análise de pH (mensurado em pHmetro digita – DMPH – 2 Digimed, previamente calibrado).

O estudo foi iniciado após a aprovação pelo Comitê de Ética e Bem Estar Animal da UFSM, onde todos os procedimentos estavam de acordo com o que o preconiza Colégio Brasileiro de Experimentação Animal (COBEA), cumprindo a constituição do Estado sob a Lei n.°11.915, artigo 82, inciso IV de 21 de maio de 2003.

2.2.4.3 Avaliação da qualidade proteica

2.2.4.3.1 Quociente de eficiência proteica (PER)

O PER foi determinado pela expressão que relaciona o ganho de peso (g) do grupo teste em relação à proteína ingerida por este, através da seguinte fórmula (AOAC, 1975):

$$\text{PER} = \frac{\text{ganho de peso (g) do grupo teste}}{\text{proteína consumida (g) pelo grupo teste}}$$

O quociente de eficácia proteica relativo (PERR) foi calculado de acordo com a seguinte fórmula:

$$\text{PERR} = \frac{\text{resultado PER do grupo teste}}{\text{resultado PER do grupo caseína}} \times 100$$

2.2.4.3.2 Quociente de eficiência líquida da proteína (NPR)

O NPR foi determinado através do ganho de peso do grupo-teste mais a perda de peso do grupo de dieta aproteica, em relação ao consumo de proteína do grupo-teste, de acordo com a fórmula (BENDER & DOELL, 1957):

$$\text{NPR} = \frac{\text{ganho de peso (g) do grupo teste + perda de peso (g) do grupo aproteico}}{\text{proteína consumida (g) pelo grupo teste}}$$

O quociente de eficiência líquida da proteína relativo (NPRR) foi calculado de acordo com a seguinte fórmula:

$$\text{NPRR} = \frac{\text{resultado NPR do grupo teste}}{\text{resultado NPR do grupo da caseína}} \times 100$$

2.2.4.3.3 Digestibilidade verdadeira (Dv)

Para determinação da Dv, as fezes dos animais foram coletadas em recipientes individuais para cada animal, e mantida sob refrigeração, sendo, posteriormente secas em estufa com circulação de ar a 105 °C, por 24 horas. As fezes foram resfriadas, pesadas e trituradas, para determinação do teor de nitrogênio, de acordo com método de micro-Kjeldahl (AOAC, 1995).

A Dv foi calculada medindo-se a quantidade de nitrogênio ingerido na dieta, a quantidade excretada nas fezes e a perda metabólica nas fezes. Esta última foi estimada pela quantidade de nitrogênio excretada pelos ratos alimentados com a dieta livre de nitrogênio (aproteica). O cálculo da Dv foi feito de acordo com fórmula (AOAC, 1975):

$$D_V = \frac{I - (F - FK)}{I} \times 100$$

Onde:

D_V = digestibilidade verdadeira;

I = nitrogênio ingerido pelo grupo com dieta teste;

F = nitrogênio fecal do grupo com dieta teste e

FK = nitrogênio fecal do grupo com dieta aproteica.

2.2.4.3.4 Balanço nitrogenado (BN)

O BN mede a quantidade de nitrogênio ingerida na dieta e a quantidade de nitrogênio excretada nas fezes e urina (PELLET & YOUNG, 1980). A mensuração do nitrogênio excretado na urina pode ser desprezado, determinando-se assim apenas o balanço nitrogenado aparente (BNap), pela fórmula:

BNap = nitrogênio ingerido – nitrogênio excretados nas fezes

2.2.4.4 Dosagem dos parâmetros bioquímicos e retirada de órgãos

Finalizada a etapa experimental, os animais após jejum de 12 horas foram insensibilizados com os anestésicos na dose de 150 µg de Quetamina e 200 µg de Xilazina por kilo de peso corporal, para a eutanásia. Foram realizadas a incisão das cavidades abdominal e torácica, e a coleta de sangue foi realizada através de punção cardíaca, utilizando seringas descartáveis para cada animal. Posteriormente, foram removidos e pesados a gordura epididimal, rins, fígado, ceco cheio e ceco vazio.

Alíquotas de sangue foram separadas em tubos de ensaio contendo heparina para a dosagem de hemoglobina total e o restante do sangue foi acondicionado em outro tubo de ensaio que foi centrifugado, para separação do soro a ser analisado, o qual foi conservado sob congelamento até as análises bioquímicas.

As dosagens sorológicas de colesterol total, colesterol HDL, colesterol LDL, colesterol VLDL, triglicerídeos, glicose, albumina sérica, proteínas totais e hemoglobina foram realizadas utilizando *kits* enzimáticos-colorimétricos fornecidos pela empresa Doles Reagentes, Goiânia, GO, Brasil e a leitura foi realizada em espectrofotômetro.

2.2.5 Análise sensorial

Optou-se por utilizar o Teste Triangular por ser o método mais comumente utilizado em testes discriminatórios. Neste teste foram apresentadas aos julgadores treinados três amostras, sendo duas idênticas (WPH) e uma diferente (WPH microencapsulado), com o objetivo do julgador identificar qual das amostras é a diferente.

As amostras foram preparadas em uma concentração de 5% anteriormente à análise sensorial. Os 15 provadores treinados receberam as três amostras diluídas em água na

proporção 1:1 em copos descartáveis de 50 mL, codificadas com três digitos aleatórios, sendo duas iguais e uma diferente. Os provadores avaliaram as amostras da esquerda para a direita e foram orientados a fazer um circulo no código da amostra diferente.

A análise de resultados do Teste Triangular foi realizada pela soma das respostas corretas. Anotou-se o número total de respostas e verificou-se o número de respostas corretas foi maior ou igual ao da Tabela para análise de resultados do Teste Triangular, se positivo, conclui-se que existe diferença significativa entre as duas amostras em nível de significância de 5% (MEILGAARD et al., 1987).

2.2.6 Análise estatística

O delineamento experimental utilizado foi de blocos inteiramente casualizados. Os resultados foram analisados estatisticamente pela análise de variância (ANOVA) e comparação das médias de pares de amostras pelo teste de Tukey ao nível de significância de 5%, utilizando o aplicativo *Statistical Analysis System* (SAS, 1997).

3 RESULTADOS E DISCUSSÃO

3.1 Microencapsulação do hidrolisado proteico do soro de leite bovino

Para discussão deste item são necessários os dados referentes à metodologia empregada na obtenção das microcápsulas.

3.2 Análise morfológica por microscopia eletrônica de varredura (SEM)

A microcápsula da amostra WPH microencapsulado apresentou formato esférico, com a presença de achatamentos (Figura 1).

De acordo com Fávaro-Trindade et al. (2010), as concavidades são típicas de produtos atomizados, no entanto Rodríguez-Huezo et al. (2007) relataram que a formação destas depende da temperatura de secagem, sendo que temperaturas de secagem moderadas (como temperatura de entrada de 140 °C e de saída de 60 °C) ocasionam estas concavidades na superfície, as quais dão aos pós obtidos características como resistência à fratura mecânica e à difusão do soluto.

As microcápsulas apresentaram um diâmetro médio em torno de 20 µm. Fang & Bhandari (2010) relatam que o diâmetro de microcápsulas obtidas por *spray drying* podem variar de 10 a 100 µm. De acordo com Champagne & Fustier (2007), microcápsulas muito grandes podem afetar a textura do alimento no qual será incorporada. Diâmetros menores que 100 µm são preferidos para a maioria das aplicações (ANNAN et al., 2008).

3.3 Composição Centesimal

Em relação aos parâmetros umidade, cinzas e proteínas observou-se que houve diferença estatisticamente significativa entre todas as amostras avaliadas (Tabela 1). Sendo observado, uma maior concentração de umidade e proteínas na amostra de caseína. Verificou-se uma redução significativa dos teores de proteína (74,21 e 55,97) e aumento nos valores de lipídios totais (5,02 e 18,04) em entre as amostras WPH e WPH microencapsulado, respectivamente. A provável justificativa na alteração da composição centesimal do WPH microencapsulado foram os agentes encapsulantes utilizados na elaboração da microcápsula.

3.4 Ensaio Biológico

Em relação ao ganho de peso observou-se diferença significativa entre os três tratamentos. O tratamento da dieta experimental elaborada com WPH microencapsulado

apresentou diferença significativa em relação aos demais nas variáveis consumo alimentar, QEA e proteína ingerida (Tabela 2).

Os animais alimentados com o WPH microencapsulado obtiveram maior peso de fezes úmidas (12,18 g) e peso de fezes secas (3,76 g), conforme Tabela 3. Esse fato pode estar relacionado com o maior consumo alimentar total (Tabela 2). O valor de pH fecal dos animais que receberam WPH foi maior.

O peso de gordura epididimal e dos rins não apresentaram diferenças estaticamente significativas entre os tratamentos. O valor do peso do fígado dos animais alimentados com WPH microencapsulado diferiu-se significativamente dos demais tratamentos

Conforme os resultados da Tabela 4, o quociente de eficácia proteica (PER) e o quociente de eficiência líquida da proteína (NPR) foram maiores no tratamento da caseína quando comparado com os demais tratamentos, o que está relacionado com o ganho de peso total e proteína ingerida. As amostras de caseína e WPH classificam-se como proteínas de alto valor nutritivo, pois, Friedman (1996) considera um PER abaixo de 1,5 como uma proteína de baixa qualidade; PER entre 1,5 a 2,0 como uma proteína de qualidade média e PER acima de 2,0 como uma proteína de alto valor nutritivo. A amostra WPH microencapsulado classificou-se como uma proteína de qualidade média tendo em vista o resultado do PER (1,63). A provável justificativa deve-se a matéria-prima utilizada na elaboração da microcápsula que resultou na alteração da composição centesimal.

A digestibilidade é a medida da porcentagem das proteínas que são hidrolisadas pelas enzimas digestivas e absorvidas na forma de aminoácidos, peptídios ou qualquer outro composto nitrogenado. Considerando as variáveis utilizadas para o cálculo da digestibilidade verdadeira (Dv), nitrogênio ingerido pelo grupo com a dieta teste, nitrogênio fecal do grupo com dieta teste e o nitrogênio fecal do grupo com dieta aproteica, observou-se que o

tratamento que utilizou o WPH microencapsulado apresentou o maior valor para Dv (96,91%) (Tabela 4).

O balanço nitrogenado aparente (BNap) (Tabela 4) que indica o aproveitamento proteico foi maior nos tratamentos com caseína e WPH microencapsulado, provavelmente em função do maior consumo alimentar total. Em relação à dieta formulada com WPH o valor do BNap está relacionado possivelmente com a melhor absorção dos peptídios no intestino.

Os parâmetros bioquímicos colesterol total, HDL colesterol, LDL colesterol, VLDL colesterol, triglicerídeos e glicose não apresentaram diferenças significativas nos grupos tratados com o WPH e com o WPH microencapsulado (Tabela 5). Constatando-se com isso, que o processo de microencapsulação não resultados em efeitos bioquímicos desfavoráveis.

O mesmo verificou-se em relação à concentração de albumina sérica, proteínas totais e hemoglobina.

3.5 Análise sensorial

Na avaliação das fichas do Teste Triangular respondidas pelos julgadores treinados, observou-se que 100% dos indivíduos identificaram a amostra diferente, ou seja, o WPH microencapsulado. Diante deste resultado constatou-se que as amostras diferiram significativamente ao nível de 5% de probabilidade.

Dentre os comentários descritos pelos julgadores, foi detectada diferença sensorial quanto ao atributo sabor, indicando-se com isso, que o processo de microencapsulação proporcionou uma melhoria significativa em relação a este atributo.

Em estudos realizados com WPH microencapsulado, utilizando como agentes encapsulantes maltodextrina e maltodextrina β-ciclodextrina, observou-se uma redução significativa do sabor amargo quando comparado à amostra WPH não microencapsulado (YANG SHU et al. 2012).

4 CONCLUSÕES

No estudo realizado, constatou-se que os agentes encapsulantes utilizados no processo de microencapsulação do WPH por *spray drying* resultaram em microcápsulas com formato esférico, presença de achatamentos e diâmetro em torno de 20 μm .

Em relação à composição centesimal, verificou-se que a amostra WPH microencapsulado apresentou alteração em comparação com a amostra WPH, principalmente para os parâmetros proteínas e lipídios totais, provavelmente em função dos materiais utilizados como cobertura para as microcápsulas.

O decréscimo no teor de proteína pode estar relacionado ao valor do quociente de eficiência proteica (PER) que classificou a WPH microencaspulado como uma proteína de qualidade média.

Já os parâmetros bioquímicos colesterol total, HDL colesterol, LDL colesterol, VLDL colesterol, triglicerídeos, glicose, albumina séria, proteínas totais e hemoglobina indicaram que não houve diferenças significativas entre as fontes proteicas WPH e WPH microencapsulado.

Na avaliação sensorial que comparou as amostras WPH e WPH microencapsulado percebeu-se uma melhoria significativa na aceitação do atributo sabor. Esse resultado amplia as possibilidades de desenvolvimento de alimentos para fins especiais adicionados de proteínas hidrolisadas.

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Tabela 1. Composição centesimal da caseína, WPH e WPH microencapsulado.

Parâmetros (%)	Caseína	WPH	WPH microencapsulado
Umidade	11,12 ± 0,02[a]	7,31 ± 0,07[b]	6,78 ± 0,02[c]
Cinzas	1,91 ± 0,13[c]	5,00 ± 0,02[b]	5,51 ± 0,11[a]
Proteínas	82,78 ± 0,33[a]	74,21 ± 0,30[b]	55,97 ± 0,36[c]
Lipídios totais	1,70 ± 0,11[b]	5,02 ± 0,08[b]	18,04 ± 3,86[a]
Carboidratos	2,49 ± 0,35[b]	8,46 ± 0,32[ab]	13,70 ± 3,84[a]

Valores expressos como média ± desvio padrão (n = 3).
Médias seguidas de letras distintas, na linha, diferem significativamente entre si pelo teste de Tukey a 5% de significância.

Tabela 2. Ganho de peso total, consumo alimentar total, quociente de eficácia alimentar (QEA) e proteína ingerida dos animais alimentados com dietas experimentais contendo caseína, WPH e WPH microencapsulado.

Variáveis	Fontes proteicas		
	Caseína	WPH	WPH microencapsulado
Ganho de peso total (g)	156,73 ± 12,98[a]	137,56 ± 9,90[b]	120,33 ± 13,87[c]
Consumo alimentar total (g)	450,00 ± 42,63[b]	422,06 ± 39,57[b]	493,29 ± 49,30[a]
Quociente de eficácia alimentar	0,35 ± 0,03[a]	0,33 ± 0,02[a]	0,24 ± 0,02[b]
Proteína ingerida (g)	67,50 ± 6,39[b]	63,31 ± 5,93[b]	73,99 ± 7,40[a]

Valores expressos como média ± desvio padrão (n = 7).
Médias seguidas de letras distintas, na linha, diferem significativamente entre si pelo teste de Tukey a 5% de significância

Tabela 3. Produção de fezes úmidas (PFU), fezes secas (PFS), valores de pH, peso do ceco cheio, ceco vazio, gordura epididimal, fígado e rins dos animais alimentados com dietas experimentais contendo caseína, WPH e WPH microencapsulado.

Parâmetros	Fontes proteicas		
	Caseína	WPH	WPH microencapsulado
PFU (g)	8,71 ± 1,22[b]	9,07 ± 1,33[b]	12,18 ± 0,77[a]
PFS (g)	1,46 ± 0,63[b]	1,89 ± 0,92[b]	3,76 ± 0,50[a]
pH	6,11 ± 0,10[b]	6,99 ± 0,38[a]	6,04 ± 0,14[b]
Ceco cheio (g)	0,97 ± 0,22[b]	1,23 ± 0,32[ab]	1,43 ± 0,11[a]
Ceco vazio (g)	0,28 ± 0,09[b]	0,37 ± 0,11[b]	0,62 ± 0,06[a]
Gordura epididimal (g)	1,10 ± 0,32[a]	1,17 ± 0,19[a]	1,22 ± 0,25[a]
Fígado (g)	3,79 ± 0,37[a]	4,06 ± 0,32[a]	3,30 ± 0,22[b]
Rins (g)	0,81 ± 0,05[a]	0,88 ± 0,15[a]	0,84 ± 0,10[a]

Valores expressos como média ± desvio padrão (n = 7).
Médias seguidas de letras distintas, na linha, diferem significativamente entre si pelo teste de Tukey a 5% de significância.

Tabela 4. Quociente de eficácia proteica (PER), quociente de eficácia proteica relativo (PERR), quociente de eficiência líquida da proteína (NPR), quociente de eficiência líquida da proteína relativo (NPRR), nitrogênio ingerido, nitrogênio fecal, digestibilidade verdadeira (Dv) e balanço nitrogenado aparente (BNap) dos animais alimentados com dietas experimentais contendo caseína, WPH e WPH microencapsulado.

Parâmetros	Fontes proteicas		
	Caseína	WPH	WPH microencapsulado
PER	2,33 ± 0,17[a]	2,18 ± 0,11[a]	1,63 ± 0,11[b]
PERR (%)	100,00 ± 0,00[a]	93,50 ± 4,51[b]	69,77 ± 4,97[c]
NPR	2,65 ± 0,19[a]	2,52 ± 0,13[a]	1,92 ± 0,12[b]
NPRR (%)	100,00 ± 0,00[a]	95,07 ± 4,93[ab]	72,37 ± 4,41[b]
Nitrogênio ingerido (g)	10,80 ± 1,02[b]	10,13 ± 0,95[b]	13,22 ± 1,29[a]
Nitrogênio fecal (g)	2,08 ± 0,14[b]	2,56 ± 0,27[a]	2,03 ± 0,39[b]
Dv (%)	96,16 ± 1,25[a]	91,27 ± 2,31[b]	96,91 ± 3,06[a]
BNap (g)	8,72 ± 1,01[b]	7,57 ± 0,86[c]	9,81 ± 1,26[a]

Valores expressos como média ± desvio padrão (n = 7).
Médias seguidas de letras distintas, na linha, diferem significativamente entre si pelo teste de Tukey a 5% de significância.

Tabela 5. Parâmetros bioquímicos dos animais alimentados com dietas experimentais contendo caseína, WPH e WPH microencapsulado.

Parâmetros bioquímicos (mg/dL)	Fontes proteicas		
	Caseína	WPH	WPH microencapsulado
Colesterol total	190,42 ± 16,02[a]	147,65 ± 20,65[b]	135,20 ± 4,99[b]
HDL colesterol	69,21 ± 15,24[a]	68,05 ± 22,51[a]	69,22 ± 15,22[a]
LDL colesterol	94,50 ± 16,02[a]	63,04 ± 20,65[b]	48,99 ± 5,17[b]
VLDL colesterol	26,71 ± 2,91[a]	16,56 ± 5,55[b]	16,83 ± 4,42[b]
TG	133,53 ± 14,55[a]	82,82 ± 27,76[b]	84,15 ± 19,12[b]
Glicose	92,02 ± 13,43[a]	72,21 ± 29,04[a]	85,11 ± 28,03[a]
Parâmetros bioquímicos (g/dL)	**Caseína**	**WPH**	**WPH microencapsulado**
Albumina sérica	3,11 ± 0,56[a]	3,70 ± 0,53[a]	3,44 ± 0,38[a]
Proteínas totais	6,60 ± 0,72[a]	8,90 ± 2,63[a]	7,57 ± 3,75[a]
Hemoglobina	13,47 ± 2,82[a]	10,63 ± 1,77[b]	9,69 ± 1,28[b]

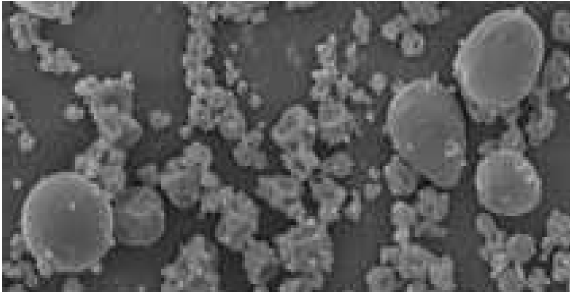
Valores expressos como média ± desvio padrão (n = 7).
Médias seguidas de letras distintas, na linha, diferem significativamente entre si pelo teste de Tukey a 5% de significância.

Legenda da figura

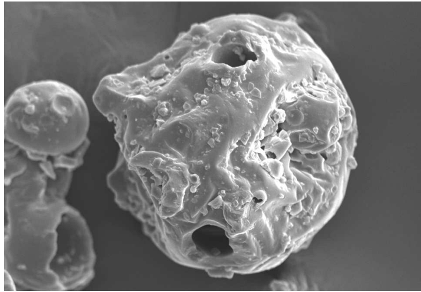
Figura 1. Imagem da morfologia das microcápsulas obtidas em microscópio eletrônico de varredura da amostra WPH microencapsulado nos aumentos de 500 x e 1000 x (A e B, respectivamente) e a imagem da amostra WPH não microencapsulado no aumento de 100 x (C).

Figura 1

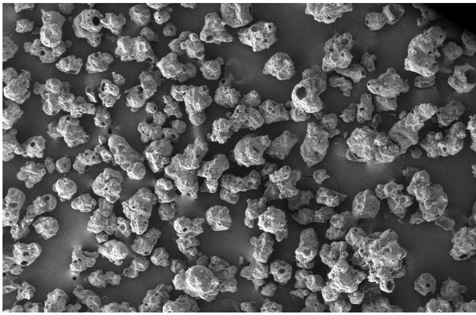
(A)



(B)



(C)



ARTIGO 5

Evaluation on the mineral content of infant formulas consumed in Brazil

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Evaluation of the mineral content of infant formulas consumed in Brazil

Sabrina Vieira da Silva,* Paula Mattanna,* Cezar Augusto Bizzi,† Neila Silvia Peireira dos Santos Richards,* and Juliano Smanioto Barin*[1]
*Departamento de Tecnologia e Ciência dos Alimentos, and
†Departamento de Química, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil

ABSTRACT

The mineral content of 10 commercially available milk-based infant formulas widely consumed in Brazil was investigated. The levels of elements (Ca, Cu, Fe, K, Mg, Mn, Na, and Zn) were determined by inductively coupled plasma optical emission spectrometry after microwave-assisted wet digestion. The results were compared with the producers' label declarations and levels proposed by the Codex Alimentarius and the Brazilian National Health Surveillance Agency. The obtained results were in good agreement with certified reference materials, and the relative standard deviation of the measurements was always below 10%, with the exception of Cu (up to 14%). A high degree of variation between the experimental and producers' data was found for trace elements among different brands, with differences as high as 41 and 68% for Cu and Zn, respectively. The content of minerals in the starting formulas was in accordance with levels established by the Codex Alimentarius, with the exception of 1 brand that had a Zn level that was not in agreement with the Codex minimum value. Among follow-up formulas, 2 brands contained levels of Ca and K that were higher than the maximum established by Brazilian standards, and 1 brand contained less than the minimum value recommended for Zn. The guidance upper levels have not been established in the Codex Alimentarius for follow-up formulas, and the maximum value has been established for only 2 of the analyzed minerals. This lack of a standard could be a problem; as shown in the present study, if the Brazilian standard is used, several formulas could be considered out of specification. Despite the limited number of samples evaluated in this work, the levels of some elements were out of specification and special attention by the Brazilian government on the content of minerals in infant formulas is needed.
Key words: microwave-assisted wet digestion, infant nutrition, trace element, milk

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[1]Corresponding author: juliano@ufsm.br

INTRODUCTION

Human milk is considered an optimal source of nutrients for infants and provides all of the nutritive elements for normal infant growth in a form that is suitable for digestion. When breast feeding is not possible, desirable, or sufficient, infant formulas are often used as substitutes for human milk and play an indispensable role in infant nutrition (Ruiz et al., 1996; Alles et al., 2004; Sola-Larrañaga and Navarro-Blasco, 2006). Infant formulas are described as breast-milk substitutes that are specially manufactured to satisfy the nutritional requirements of infants and are based on the composition of human milk, which is the ideal food for infants (Rodríguez Rodríguez et al., 2000; Alles et al., 2004). Among the nutrients available (e.g., protein, lipids, and carbohydrates) in infant formulas, trace and mineral elements are essential for biological processes and play a vital role in normal growth and development. Trace element requirements are critical during infancy and early childhood due to the very high growth rate of children, and insufficient mineral intake may lead to deficiencies that can impair body functioning (Brätter et al., 1998; Taylor et al., 2004; Zand et al., 2011). Industrially produced foods could represent an important part of the diet of many infants, and it is very important that such food contains sufficient amounts of minerals and trace elements.

The Codex Alimentarius (Codex) is a global food standards program that is jointly sponsored by the Food and Agricultural Organization (Rome, Italy) and World Health Organization (Geneva, Switzerland). Its goal is to establish standards for various foods that will ensure consumer safety and facilitate trade (MacLean et al., 2010). A revised standard (Codex standard 72-1981, revision 2007; Codex Alimentarius Commission, 2007) has been adopted for infant formula, which, in Brazil, is used during the first 6 mo of life (Ministry of Health, National Health Surveillance Agency, 2011a). The minimum and maximum nutrient levels for infant formulas established by Codex are important to ensure that the nutritional needs of all healthy infants will be met, while avoiding levels leading to high intake, which

could produce adverse effects (MacLean et al., 2010). In 1987, the Codex Committee on Foods for Special Dietary Uses adopted a standard for follow-up formula, which is used in Brazil for infants from 6 to 12 mo of age (Codex standard 156-1987; Codex Alimentarius Commission, 1987; Ministry of Health, National Health Surveillance Agency, 2011b). Follow-up milk formula ensures a balanced intake of nutrients and is undoubtedly more suitable for infants than cow milk because follow-up formula provides a lower intake of proteins and saturated fats and a higher concentration of PUFA, iron, and zinc than cow milk (Riva et al., 2007).

According to the Codex Alimentarius, the term infant formula is used for products designed for infants during the first months of life until the introduction of appropriate complementary feeding (at no later than 12 mo of age; Codex Alimentarius Commission, 2007). Follow-up formula is the term used for products for infants aged 6 mo and older and for young children (persons from the ages of 12 to 36 mo; Codex Alimentarius Commission, 1987). However, the Codex does not establish a specific term for the formulations used exclusively for infants less than 6 mo of age that are currently commercialized in Brazil. Thus, the terms starting and follow-up will be used for infant formulas used from 0 to 6 mo of age and from 6 to 12 mo of age, respectively, to avoid using different terms for the same formula.

Because starting and follow-up formulas are the major sources of nutrients during the first months of life, several authors have studied their quality to ensure adequate infant health (Hamill et al., 1989; Richmond et al., 1993; Guo et al., 1996; Ruiz et al. 1996; Bermejo et al., 2000; Hua et al., 2000; Krachler and Rossipal, 2000; Ikem et al., 2002; Sola-Larrañaga and Navarro-Blasco, 2006; Al Khalifa and Ahmad, 2010; Lesniewicz et al. 2010; MacLean et al., 2010). However, only one study (De Castro et al., 2010) related to the mineral content of infant formulas consumed in Brazil has been published. Moreover, that work included only results for Pb, Cd, Ca, and Zn. Thus, the purpose of the present study was to determine the levels of essential elements (Ca, Cu, Fe, K, Mg, Mn, Na, and Zn) in the 10 most-consumed starting and follow-up formulas in Brazil and to compare the results with producer label declaration as well as the levels proposed by the Codex Alimentarius (Codex Alimentarius Commission, 1987, 2007) and Brazilian standards (Ministry of Health, National Health Surveillance Agency, 2011a,b). In addition, microwave-assisted digestion with diluted acids was used as a green sample preparation procedure for further simultaneous determination of elements by inductively coupled plasma optical emission spectrometry (ICP OES).

MATERIALS AND METHODS

Samples

Ten commercial starting and follow-up formulas that are widely accepted and consumed in Brazil were investigated. Formulas in powdered form were purchased in local supermarkets between late 2010 and early 2011. The starting formulas purchased were Nan Pro 1 (Nestlé Brasil Ltda., São Paulo, Brazil), Nestogeno 1 (Nestlé Brasil Ltda.), Aptamil 1 (Danone Brasil, São Paulo, Brazil), Milupa 1 (Danone Brasil), and Similac Advance 1 (Abbott Laboratórios do Brasil Ltda., São Paulo, Brazil). The follow-up formulas purchased were Nan Pro 2 (Nestlé Brasil Ltda.), Nestogeno 2 (Nestlé Brasil Ltda.), Aptamil 2 (Danone Brasil), Milupa 2 (Danone Brasil), and Similac Advance 2 (Abbott Laboratórios do Brasil Ltda.). Samples were coded with different letters for each producer. The numbers 1 and 2 were used for starting and follow-up formulas, respectively. A detailed description of the characteristics of the analyzed infant formulas is presented in Table 1.

Instrumentation

Samples were dried at 60°C in an oven (model 400/2ND; Nova Ética, Vargem Grande Paulista, SP, Brazil) before digestion and were weighed with an analytical balance (model AY 220; maximum: 220 g, 0.1 mg of resolution; Shimadzu Corp., Kyoto, Japan). A microwave oven (Multiwave 3000 microwave sample preparation system; Anton Paar GmbH, Graz, Austria) equipped with 8 high-pressure quartz vessels (internal volume of 80 mL, maximum operational temperature and pressure of 280°C and 8 MPa, respectively) was used in the experiments. Elements were determined by ICP OES using an axial view configuration spectrometer (Spectro Ciros CCD; Spectro Analytical Instruments GmbH & Co. KG, Kleve, Germany) with a cross-flow nebulizer coupled to a Scott double-pass-type nebulization chamber. The plasma operating conditions and selected wavelengths were used as recommended by the instrument manufacturer (Spectro Analytical Instruments GmbH & Co. KG, 2003) and are listed in Table 2.

Reagents and Standards

Distilled-deionized water (Milli-Q; 18.2 MΩ·cm; Millipore Corp., Billerica, MA) and analytical-grade nitric acid (Merck KGaA, Darmstadt, Germany) were used to prepare samples and standards. Metal determination by ICP OES was performed with external calibration using analytical solutions ranging from 1.0 to 100 µg/L

Table 1. Characteristics of the investigated starting and follow-up formulas

Item	Characteristics[1]
Starting formula	
A1	70% whey protein and 30% casein; 97% vegetable oil and 3% milk fat; addition of LcPUFA, DHA, and ARA; 100% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development
B1	60% whey protein and 40% casein; 97% vegetable oil and 3% milk fat; provides recommended levels of n-6 and n-3; 30% maltodextrin and 70% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development
C1	60% whey protein and 40% casein; 98% vegetable oil and 2% milk fat; contains LcPUFA (DHA and ARA); 98% lactose and 2% maltodextrin; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development. Prebiotics were added at 0.8 g/100 mL (10% FOS and 90% GOS)
D1	60% whey protein and 40% casein; 80% vegetable oil and 20% milk fat; 100% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development
E1	48% whey protein and 52% casein; 100% vegetable oil; addition of LcPUFA (DHA and ARA) and essential FA (n-6 and n-3); 100% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development
Follow-up formula	
A2	40% whey protein and 60% casein; 97% vegetable oil and 3% milk fat; addition of LcPUFA (DHA); 100% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development. Probiotics (*Lactobacillus* and *Bifidobacterium* cultures) were added
B2	35% whey protein and 65% casein; 97% vegetable oil and 3% milk fat; provides recommended levels of essential FA (n-6 and n-3); 30% maltodextrin and 70% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development
C2	50% whey protein and 50% casein; 75% vegetable oil and 25% milk fat; contains LcPUFA (DHA and ARA); 98% lactose and 2% maltodextrin; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development. Prebiotics were added at 0.8 g/100 mL (10% FOS and 90% GOS)
D2	50% whey protein and 50% casein; 62% vegetable oil and 38% milk fat; 84% lactose and 16% maltodextrin; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development
E2	48% whey protein and 52% casein; 100% vegetable oil; addition of LcPUFA (DHA and ARA) and essential FA (n-6 and n-3); 100% lactose; vitamins, trace elements, and minerals were added in amounts necessary for proper infant development

[1]LcPUFA = long-chain PUFA; DHA = docosahexaenoic acid; ARA = arachidonic acid; n-6 = linoleic acid; n-3 = α-linolenic acid; FOS = fructooligosaccharide; GOS = galactooligosaccharide.

that were prepared in 0.7 mol/L HNO_3 by appropriate dilution of the multi-element stock solution (SCP33MS; SCP Science, Baie D'Urfé, QC, Canada). Glass and quartz material were soaked in 1.4 mol/L HNO_3 for 24 h and further washed with water before use. Argon (99.996%; White Martins Praxair, São Paulo, SP, Brazil) was used for ICP OES determination for plasma generation and nebulization and as an auxiliary gas. Oxygen (99.9991%; White Martins Praxair) was used as a reagent in digestions performed under oxygen pressure, using safety conditions, as recommended by the microwave oven manufacturer (Anton Paar GmbH, 2003). Accuracy was evaluated using certified reference materials (CRM) of skim milk powder (BCR 151; Community Bureau of Reference, Brussels, Belgium) and of nonfat milk powder (SRM NIST 1549; National Institute of Standards and Technology, Gaithersburg, MD).

Microwave-Assisted Acid Digestion and ICP OES Determination

Samples were digested according to the procedure described previously (Bizzi et al., 2011). Samples (~400 mg) were transferred to quartz vessels, and 6 mL of 3 mol/L HNO_3 was added. After closing and capping the rotor, the vessels were pressurized with 0.75 MPa bar of oxygen using the valve originally designed for pressure release after conventional acid sample digestion. Then, the rotor was placed inside the oven, and the microwave-heating program was started by applying (1) 1,000 W, increasing over 5 min; (2) 1,000 W for 10 min; and (3) 0 W for 20 min (cooling step). Af-

Table 2. Operational parameters for elemental determination by inductively coupled plasma optical emission spectrometry (ICP OES)

Parameter[1]	ICP OES
Radio-frequency power (W)	1,600
Plasma gas flow rate (L/min)	14.0
Auxiliary gas flow rate (L/min)	1.0
Nebulizer gas flow rate (L/min)	0.85
Spray chamber	Double pass, Scott type
Nebulizer	Cross-flow
Observation view	Axial
Analyte emission line (nm)	
Ca (II)	393.366
Cu (I)	324.752
Fe (I)	238.204
K (I)	766.490
Mg (I)	285.213
Mn (II)	257.610
Na (I)	589.592
Zn (I)	213.857

[1]I = atomic emission line; II = ion emission line.

Table 3. Results obtained for the evaluated elements (Ca, Cu, Fe, K, Mg, Mn, Na, and Zn; mg/100 g) in the infant formulas studied (mean ± SD; n = 3)

Sample	Ca	Cu[1]	Fe	K	Mg	Mn[1]	Na	Zn
A1	317 ± 10	316 ± 26	6.50 ± 0.44	456 ± 10	35.0 ± 1.5	51.6 ± 2.8	122 ± 10	4.26 ± 0.26
B1	517 ± 13	307 ± 10	6.68 ± 0.40	678 ± 18	43.2 ± 0.3	34.0 ± 0.5	192 ± 17	4.03 ± 0.14
C1	432 ± 11	345 ± 39	5.32 ± 0.13	396 ± 16	38.1 ± 1.2	63.5 ± 1.7	112 ± 7	2.59 ± 0.14
D1	426 ± 18	274 ± 45	5.37 ± 0.30	456 ± 34	38.5 ± 0.4	57.3 ± 1.1	140 ± 8	1.25 ± 0.12
E1	547 ± 37	322 ± 30	2.69 ± 0.11	660 ± 17	38.2 ± 0.5	37.4 ± 2.0	136 ± 5	4.01 ± 0.13
A2	616 ± 34	476 ± 27	7.22 ± 0.11	679 ± 19	44.4 ± 0.6	34.1 ± 1.3	190 ± 17	5.37 ± 0.34
B2	784 ± 24	514 ± 37	7.63 ± 0.13	977 ± 37	62.9 ± 0.7	33.4 ± 0.9	283 ± 9	6.08 ± 0.32
C2	637 ± 18	165 ± 31	6.69 ± 0.36	778 ± 13	60.0 ± 0.8	50.8 ± 0.7	227 ± 6	2.23 ± 0.10
D2	614 ± 20	183 ± 41	7.44 ± 0.10	606 ± 13	46.1 ± 0.3	60.6 ± 1.8	209 ± 7	3.32 ± 0.11
E2	552 ± 42	494 ± 23	8.53 ± 0.16	723 ± 33	38.3 ± 0.4	52.0 ± 0.9	148 ± 9	4.89 ± 0.11

[1]Results are in micrograms per 100 g.

ter digestion, the pressure in each vessel was carefully released. The resulting solutions were transferred to 30-mL polypropylene vials and diluted up to the mark with water. Digestion vessels were cleaned with 6 mL of concentrated HNO_3 in the microwave oven at 1,000 W for 10 min and 0 W for 20 min for cooling.

RESULTS AND DISCUSSION

Because infant formulas are similar to milk powder, the accuracy of the testing procedure was verified by determining the elements in CRM of skim milk powder and nonfat milk powder (BCR 151, Community Bureau of Reference; SRM NIST 1549, National Institute of Standards and Technology; results not shown). The results obtained for Ca, Cu, Fe, K, Mg, Mn, Na, and Zn for both CRM were in good agreement (better than 93%) with the certified values (Student's t-test, $P > 0.05$). The precision of the CRM measurements was evaluated in 3 independent samples. The relative standard deviations were lower than 10%, with the exception of Cu (up to 14%). This analytical variability is similar to that observed by MacLean et al. (2010; 13%) for Cu determination in some infant formulas.

The results obtained for the evaluated formulas are shown in Table 3. The elements Ca and K had the highest concentrations, ranging from 317 (sample A1) to 784 (sample B2) mg/100 g of formula powder. The formula E1 contained the lowest amount of Fe (2.69 mg/100 g), whereas the largest Fe content was observed in sample E2 (8.53 mg/100 g). The Zn and Na concentrations ranged from 1.25 (sample D1) to 6.08 (sample B2) and 112.1 (sample C1) to 283 (sample C2) mg/100 g, respectively. The Mg concentrations ranged from 35.0 (sample A1) to 62.9 (sample B2) mg/100 g. The lowest contents were observed for the elements Cu and Mn. The Cu concentration varied from 165 (sample C2) to 514 (sample B2) μg/100 g, whereas the Mn content varied from 33.4 (sample B2) to 63.5 (sample C1) μg/100 g.

The experimentally determined mineral contents were compared with the data reported on the labels by the producers (Figure 1). With the exception of sample A1, the Ca content in the formulas was higher than that declared on the label. In general, the difference between the labeled and determined values was less than 11%, but for formulas E1 and E2, the difference reached 37 and 28%, respectively. By contrast, the Fe content was lower than the labeled value, with the exception of samples A1 and B1, which had higher Fe contents than the labeled content. The greatest difference between the labeled and determined value was observed for sample E1 (23%). This difference was less than 13% for the other formulas. For K, Mg, and Na, the maximum difference reached 12 (sample E2), 23 (sample E1), and 26% (sample D1), respectively.

Nevertheless, the most significant difference between the labeled and experimental values was observed for trace elements (Cu, Mn, and Zn). For Cu, differences of 39, 41, and 37% were observed for formulas C2, D2, and E2, respectively. For Zn, differences as high as 68 and 49% were observed for formulas D1 and C2, respectively, whereas a difference of 73% was observed for Mn in formula E2. A similar phenomenon was reported by Lesniewicz et al. (2010), who observed that the experimentally determined values of Mn in Polish infant formulas were 3 times higher than the declared values. The large differences that were observed for Cu, Mn, and Zn could be due to the smaller amounts of these elements in formulas; these small values could be susceptible to large variations in the content of these elements in the raw materials. According to MacLean et al. (2010), Mn can be specifically added during the production of milk-based infant formulas or introduced in the products in small amounts by some of the calcium salts and by ferrous sulfate, which could lead to variations in the final composition. The same authors reported that the majority of the Cu in infant formula is added through the trace element premix during the manufacturing process, but the protein sources (e.g.,

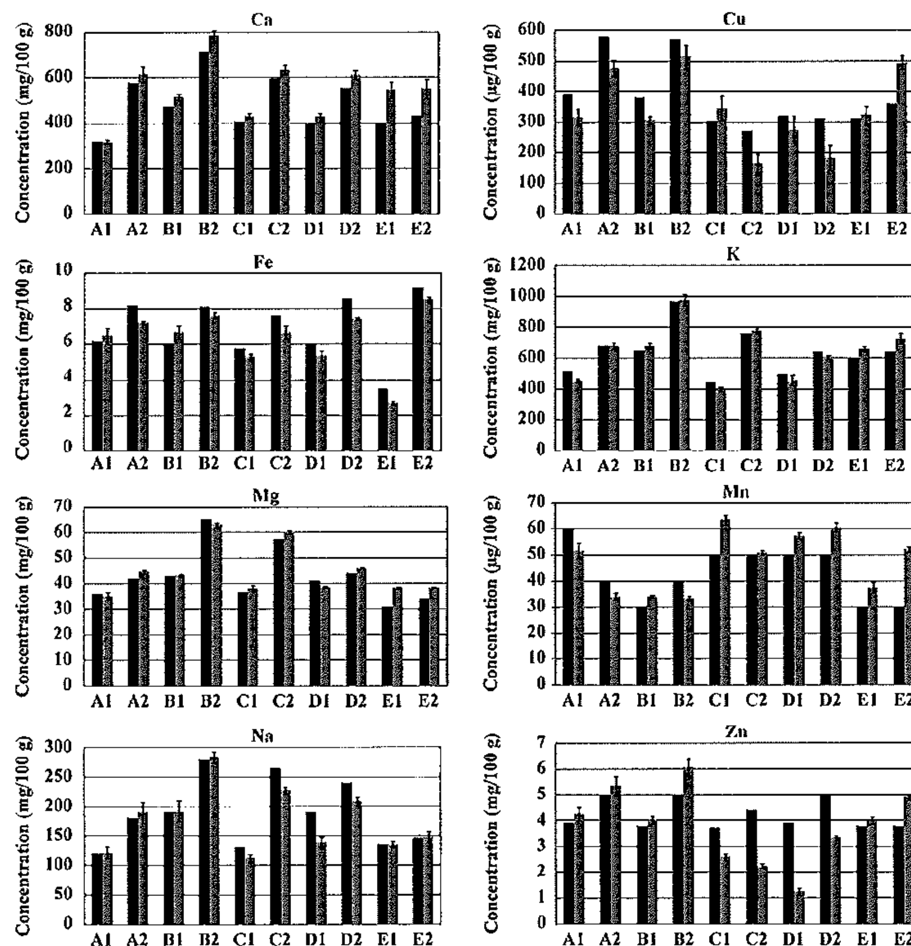

Figure 1. Comparison of the obtained results for elements (gray bars) with the values declared by the infant formula producers (black bars). The standard deviation is indicated by the error bars.

nonfat milk protein, whey protein concentrates, and soy protein isolates) variably contribute to the Cu level of infant formulas and could explain the behavior of this element in the formulas studied in their work. However, this variation is of concern due to the important role of these elements in infant metabolism (Kobla and Volpe, 2000).

A Student's *t*-test was also used for comparison between declared and experimental data for each element (5% significance level). A significant difference was observed for some formulas for Ca (B1, B2, C1, C2, D2, E1, and E2), Cu (A1, A2, B1, C2, D2, and E2), Fe (A2, B2, C1, C2, D1, D2, E1, and E2), K (A1, C1, D2, E1, and E2), Mg (A2, B2, C2, D1, D2, E1, and E2), Na (C1, C2, D1, and D2), and Zn (B2, C1, C2, D1, D2, E1, and E2). For Mn, all formulas presented labeled values different from experimental data obtained. The starting formulas showed better results than follow-up formulas regarding the comparison of obtained results with labeled values as well as the producers A and B in relation to the other ones. The formula D2 showed all values in disagreement with the label.

To evaluate if the obtained values were in agreement with those recommended for formulas, the obtained results were compared with international and Brazilian standards (Tables 4 and 5). For proper comparison, the elemental levels in all of the evaluated powder formulas were converted to micrograms or milligrams per 100 kcal using the specified feeding tables supplied by the infant formula manufacturers. Experimental results

Table 4. Content of Ca, Cu, Fe, K, Mg, Mn, Na and Zn (mg/100 kcal) in starting formulas (mean ± SD) and recommended values

	Formula					Codex[1]			Brazil		
Element	A1	B1	C1	D1	E1	Minimum	Maximum	GUL[2]	Minimum	Maximum	GUL[2]
Ca	61.1 ± 1.9	103 ± 3	89.3 ± 2.3	83.9 ± 3.5	107 ± 7.2	50	NSP[3]	140	50	NSP	140
Cu[4]	60.9 ± 5.0	61.3 ± 2.0	71.3 ± 10.1	53.9 ± 8.9	62.8 ± 5.8	35	NSP	120	35	NSP	120
Fe	1.25 ± 0.08	1.33 ± 0.08	1.10 ± 0.03	1.06 ± 0.06	0.524 ± 0.021	0.45	NSP	NSP	0.45	1.3	NSP
K	87.9 ± 1.9	135 ± 4	81.8 ± 3.3	89.8 ± 6.7	129 ± 3	60	180	NSP	60	180	NSP
Mg	6.74 ± 0.29	8.62 ± 0.06	7.87 ± 0.25	7.58 ± 0.08	7.45 ± 0.10	5	NSP	15	5	NSP	15
Mn[4]	9.94 ± 0.54	6.79 ± 0.10	13.1 ± 0.3	11.3 ± 0.2	7.29 ± 0.39	1	NSP	100	1	NSP	100
Na	23.5 ± 1.9	38.3 ± 3.3	23.1 ± 1.4	27.6 ± 1.6	26.5 ± 1.0	20	60	NSP	20	60	NSP
Zn	0.821 ± 0.050	0.804 ± 0.027	0.535 ± 0.029	0.246 ± 0.024	0.782 ± 0.025	0.5	NSP	1.5	0.5	NSP	1.5

[1]Codex = Codex Alimentarius (Codex Alimentarius Commission, 2007).
[2]GUL = guidance upper levels.
[3]NSP = not specified.
[4]Results are in micrograms per 100 kcal.

Table 5. Content of Ca, Cu, Fe, K, Mg, Mn, Na and Zn (mg/100 kcal) in the analyzed follow-up infant formulas (mean value ± SD) and recommended values

	Formula					Codex[1]			Brazil		
Element	A2	B2	C2	D2	E2	Minimum	Maximum	GUL[2]	Minimum	Maximum	GUL[2]
Ca	128 ± 7	162 ± 5	139 ± 4	127 ± 4	108 ± 8	90	NSP[3]	NSP	50	NSP	140
Cu[4]	98.8 ± 5.6	106 ± 8	36.1 ± 6.8	37.9 ± 8.5	96.3 ± 4.5	NSP	NSP	NSP	35	NSP	120
Fe	1.50 ± 0.02	1.58 ± 0.03	1.46 ± 0.08	1.54 ± 0.02	1.66 ± 0.03	1	2	NSP	0.9	2.0	NSP
K	141 ± 4	202 ± 8	170 ± 3	125 ± 3	141 ± 6	80	NSP	NSP	60	180	NSP
Mg	9.21 ± 0.12	13.0 ± 0.1	13.1 ± 0.2	9.54 ± 0.06	7.47 ± 0.08	6	NSP	NSP	5	NSP	15
Mn[4]	7.07 ± 0.27	6.92 ± 0.19	11.1 ± 0.2	12.6 ± 0.4	10.1 ± 0.2	NSP	NSP	NSP	1	NSP	100
Na	39.4 ± 3.5	58.6 ± 1.9	49.7 ± 1.3	43.3 ± 1.4	28.8 ± 1.8	20	85	NSP	20	60	NSP
Zn	1.11 ± 0.07	1.26 ± 0.07	0.488 ± 0.022	0.687 ± 0.023	0.953 ± 0.021	0.5	NSP	NSP	0.5	NSP	1.5

[1]Codex = Codex Alimentarius (Codex Alimentarius Commission, 1987).
[2]GUL = guidance upper levels.
[3]NSP = not specified.
[4]Results are in micrograms per 100 kcal.

regarding the starting formulas are shown in Table 4. The amount of Fe in formula B1 (1.33 ± 0.08) was close to the limit specified in the Brazilian legislation (1.3 mg/100 kcal). The mineral content was in accordance with levels established by the Codex (Codex Alimentarius Commission, 2007) and Brazilian standards, and only 1 brand had a level of zinc (sample D1; 0.246 ± 0.024 mg/100 kcal) that was not in accordance with both standards (0.5 mg/100 kcal; Ministry of Health, National Health Surveillance Agency, 2011a). Similar behavior was observed by De Castro et al. (2010) for infant formulas in Brazil in which Zn levels were less than the minimum recommended by both standards; if only the Codex minimum values were considered for Ca, the same formula contained levels lower than the recommended value. By contrast, Winiarska-Mieczan and Tupaj (2009) determined Ca values in Polish infant formulas that were higher than the limits of the Codex.

As shown in Table 5, for follow-up infant formulas, the Codex and Brazilian standards differ in the limits established for each element. If only the Codex is considered, 1 formulation (D1) had unsatisfactory levels of Zn (2.6% below the minimum amount recommended), and all others would be considered suitable for consumption. However, if the Brazilian standard is used, 3 formulations would be considered unsuitable. For Ca, the levels observed in formula B2 were 15% greater than the Brazilian standard guidance upper levels, and formula C2 was very close to this limit. For K, the level in formula B2 was also greater than the Brazilian recommended maximum value (12%), and for Zn, the formulation (C2) was below the minimum limit. It is important to mention that the C2 formula is supplemented with prebiotics (fructooligosaccharide and galactooligosaccharide) that stimulate the absorption of some elements, such as Ca and Zn (Scholz-Ahrens et al., 2007). Thus, the use of prebiotics could improve zinc bioavailability, and the small amount of this element may not represent a problem. However, if the same behavior is expected for Ca, the amount of this element could exceed the maximum value recommended when supplemented with prebiotics.

CONCLUSIONS

The values obtained for the evaluated infant formulas (starting and follow-up) consumed in Brazil revealed that some are not in accordance with the Codex and Brazilian standards with respect to Ca, K, and Zn levels. The values declared by the producers were significantly different than the observed values, particularly for trace elements (Cu, Mn, and Zn). Despite the limited number of sample evaluated in this work (only 1 batch of each product was used), these results demonstrate that further investigations about the mineral composition of infant formulas are needed. If the levels of some elements remain out of specification, special attention by the Brazilian government on the content of minerals in infant formulas will be needed. Finally, it is important to mention that no guidance upper levels have been established in the Codex Alimentarius for follow-up formulas, and the maximum value has been established for only 2 of the analyzed minerals. This lack of a standard could be a problem; as shown in the present study, if the Brazilian standard is used, several formulas could be considered out of specification.

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5 DISCUSSÃO

Esta tese foi desenvolvida com o objetivo de avaliar as propriedades das proteínas do soro do leite bovino (concentrado, hidrolisado e isolado) com relação a sua composição, valor nutricional e propriedades funcionais. Os resultados obtidos foram comparados com a caseína com o intuito de identificar diferenças que apontem vantagens e desvantagens da utilização desses produtos como fonte proteica na dieta.

Os resultados apresentados demonstraram que as proteínas do leite bovino podem ser consideradas não apenas uma fonte de aminoácidos essenciais e não-essenciais, mas também de minerais - macro e microelementos (ARTIGO 1). A concentração de macroelementos (Ca, K, Mg, Na and P) foi maior para WPC35, WPC80 e WPH enquanto que maior quantidade de microelementos (Cu, Fe, Mn and Zn) foi encontrada na caseína, perfazendo de 40 a 93% da Ingestão Diária Recomendada (DRIs) desses elementos se 100 g dos produtos avaliados forem consumidos. Já para iodo a concentração é cerca de uma ordem de magnitude superior para as proteínas do soro do leite em comparação com a caseína, mostrando que esse elemento pode ser concentrado durante a produção, revelendo a necessidade de monitoramento já que até 70% do limite máximo tolerado de ingestão diária pode ser atingido pelo consumo dessas proteínas (ARTIGO 2). Outro resultado importante é que sódio foi encontrado em grande quantidade nas proteínas do soro do leite, podendo atingir até 59,3% da DRIs.

Após a determinação de diversos componentes das proteínas do soro do leite bovino (ARTIGOS 1 e 2), foram realizados ensaios biológicos para avaliação do valor nutricional e das propriedades funcionais desses produtos. WPC80, WPH e o WPI foram consideradas fontes proteicas de alto valor nutricional com teores de aminoácidos essenciais adequados quando comparados ao padrão estabelecido pela FAO/WHO (1997) (ARTIGO 3). Além disso, de acordo com o escore químico de aminoácidos essenciais (EAE) e o escore químico de aminoácidos corrigido pela digestibilidade proteica (PDCAAS) a caseína, o WPC80, WPH e o WPI foram consideradas fontes proteicas de alto valor nutricional, com efeitos biológicos semelhantes identificados pelos parâmetros quociente de eficácia alimentar (QEA), quociente de eficiência proteica (PER) e quociente de eficiência líquida da proteína (NPR). Já os parâmetros bioquímicos dos animais alimentados com essas proteínas, tais como colesterol total, HDL colesterol, LDL colesterol, triglicerídeos, glicose, albumina séria e proteínas totais indicaram efeitos mais favoráveis das fontes proteicas WPC80, WPH e WPI. Os resultados da

histomorfometria do intestino delgado dos animais indicaram significativa diferença entre o grupo que recebeu dieta aproteica com os demais grupos avaliados. No tratamento com o WPC35 verificou-se uma maior preservação morfológica particularmente nos parâmetros área e largura do vilo e espessura do epitélio o que evidência as diferenças na absorção das proteínas do leite bovino.

Considerando que os resultados biológicos obtidos para as diferentes proteínas do soro do leite bovino (WPC 80, WPH e WPI) foram de maneira geral semelhantes, foi escolhido o WPH para dar sequência ao trabalho visando a elaboração de alimentos para fins especiais, tais como fórmulas infantis. O uso do WPH em tais produtos facilitaria a digestão e absorção das proteínas, além de reduzir a incidência de reações alérgicas. Porém, devido ao marcante sabor amargo, sua utilização tem sido limitada como ingrediente alimentar. Assim, o WPH foi microencapsulado por *spray-drying* e o efeito desse processo nas propriedades sensoriais e biológicas foram investigados (ARTIGO 4). A avaliação sensorial mostrou uma melhoria significativa na aceitação do atributo sabor para o WPH microencapsulado, enquanto que a avaliação biológica em animais revelou que não houve diferenças significativas entre as fontes proteicas WPH e WPH microencapsulado. Dessa forma, o processo de microencapsulação do WPH mostrou-se promissor minimizando o sabor desagradável do produto sem perda da sua atividade biológica.

Contudo, antes da utilização do WPH microencapsulado para elaboração de fórmulas infantis, foi feita uma investigação acerca das propriedades desses produtos disponíveis comercialmente (ARTIGO 5). Assim, várias fórmulas de partida (0 a 6 meses) e segmento (6 a 12 meses) foram avaliadas com relação ao teor de macro e microelementos. A quantidade de Ca, K e Zn encontradas foram consideradas fora dos padrões do *Codex Alimentarius* e da legislação brasileira, enquanto que os teores de Cu, Mn e Zn determinados foram muito diferentes daqueles descritos no rótulo. Contudo, devido ao tempo limitado para desenvolvimento desta tese de doutoramento, os experimentos foram encerrados nesta etapa.

6 CONCLUSÃO

Os resultados apresentados nesta tese permitem concluir, de maneira geral, que as proteínas do soro do leite bovino podem ser consideradas não somente como uma fonte de aminoácidos essenciais e não-essenciais, mas também de elementos minerais importantes. Contudo, elevada concentração de sódio foi encontrada e pode ser considerada como um fator importante e limitante para o uso desses produtos como ingredientes alimentares. Os experimentos com animais revelaram que todas as fontes proteicas são de alto valor nutricional (com exceção do WPC35) e que, maiores efeitos biológicos benéficos foram observados para WPC80, WPH e WPI. Para WPH, percebeu-se uma melhoria significativa na aceitação do atributo sabor na avaliação sensorial pela microencapsulação do produto, o que foi considerado importante pela possibilidade de aplicação em alimentos para fins especiais, tais como fórmulas infantis. Por fim, algumas formulações de fórmulas infantis foram caracterizadas com relação à concentração de elementos minerais e resultados em desacordo com os rótulos e com a legislação brasileira foram observados.

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APÊNDICES

APÊNDICE A – Composição (g/kg) das dietas experimentais fornecidas aos animais (ARTIGO 3).

Tabela 1. Composição (g/kg) das dietas experimentais fornecidas aos animais (ARTIGO 3).

Ingredientes	Fontes proteicas					
	Aproteica	Caseína	WPC35	WPC80	WPH	WPI
Amido de milho	729,49	532,89	266,89	511,10	517,69	537,09
Proteína	0	200,00	479,30	221,90	223,00	194,70
Sacarose	100,00	100,00	100,00	100,00	100,00	100,00
Óleo de soja	70,00	66,60	53,30	69,00	58,80	67,70
Fibra (celulose)	50,00	50,00	50,00	50,00	50,00	50,00
Mix de minerais*	35,00	35,00	35,00	35,00	35,00	35,00
Mix de vitaminas**	10,00	10,00	10,00	10,00	10,00	10,00
L-cistina	3,00	3,00	3,00	3,00	3,00	3,00
Bitartarato de colina	2,50	2,50	2,50	2,50	2,50	2,50
Terc-butil-hidroquinona (TBHQ)	0,0014	0,0014	0,0014	0,0014	0,0014	0,0014

Ingredientes	Fontes proteicas					
	Aproteica	Caseína	WPC35	WPC80	WPH	WPI
Carboidrato (%)	84,04	66,63	65,49	67,55	66,56	66,89
Proteína (%)	0	17,00	17,57	16,53	17,03	16,86
Lipídio (%)	15,96	16,39	16,94	15,94	16,41	16,25
Valor energético total (kcal)	394,80	384,37	372,00	395,30	383,84	387,72

*Mix mineral (por kg de mix): Ca 142,94 g; P 44,61 g; K 102,81 g; Na 29,11 g; Cl 44,89 g; S 8,57 g; Mg 14,48 g; Fe 1,00 g; Zn 0,86 g; Si 0,14 g; Mn 0,30 g; Cu 0,17 g; Cr 0,03 g; B 14,26 mg; F 28,73 mg; Ni 14,31 mg; Li 2,85 mg; Se 4,28 mg; I 5,93 mg; Mo 4,32 mg; V 2,87 mg.

**Mix vitamínico (por kg de mix): ácido nicotínico 3,00 g; pantotenato de cálcio 1,60 g; piridoxina-HCl 0,70 g; tiamina-HCl 0,60 g; riboflavina 0,60 g; ácido fólico 0,20 g; biotina 0,02 g; vitamina B_{12} 2,50 mg; vitamina E 7.500 UI; vitamina A 400.000 UI; vitamina D3 100.000 UI; vitamina K1 0,075 g.

APÊNDICE B – Composição (g/kg) das dietas experimentais fornecidas aos animais (ARTIGO 4).

Tabela 1. Composição (g/kg) das dietas experimentais fornecidas aos animais (ARTIGO 4).

Ingredientes	Fontes proteicas			
	Aproteica	Caseína	WPH	WPH microencapsulado
Amido de milho	729,49	532,89	517,69	445,37
Proteína	0	200,00	223,00	291,90
Sacarose	100,00	100,00	100,00	100,00
Óleo de soja	70,00	66,60	58,80	64,73
Fibra (celulose)	50,00	50,00	50,00	50,00
Mix de minerais*	35,00	35,00	35,00	35,00
Mix de vitaminas**	10,00	10,00	10,00	10,00
L-cistina	3,00	3,00	3,00	3,00
Bitartarato de colina	2,50	2,50	2,50	2,50
Terc-butil-hidroquinona (TBHQ)	0,0014	0,0014	0,0014	0,0014

Ingredientes	Fontes proteicas			
	Aproteica	Caseína	WPH	WPH microencapsulado
Carboidrato (%)	84,04	66,63	66,56	67,35
Proteína (%)	0	17,00	17,03	16,62
Lipídio (%)	15,96	16,39	16,41	16,02
Valor energético total (kcal)	394,80	384,37	383,84	393,16

*Mix mineral (por kg de mix): Ca 142,94 g; P 44,61 g; K 102,81 g; Na 29,11 g; Cl 44,89 g; S 8,57 g; Mg 14,48 g; Fe 1,00 g; Zn 0,86 g; Si 0,14 g; Mn 0,30 g; Cu 0,17 g; Cr 0,03 g; B 14,26 mg; F 28,73 mg; Ni 14,31 mg; Li 2,85 mg; Se 4,28 mg; I 5,93 mg; Mo 4,32 mg; V 2,87 mg.

**Mix vitamínico (por kg de mix): ácido nicotínico 3,00 g; pantotenato de cálcio 1,60 g; piridoxina-HCl 0,70 g; tiamina-HCl 0,60 g; riboflavina 0,60 g; ácido fólico 0,20 g; biotina 0,02 g; vitamina B_{12} 2,50 mg; vitamina E 7.500 UI; vitamina A 400.000 UI; vitamina D3 100.000 UI; vitamina K1 0,075 g.

APÊNDICE C – Modelo de ficha utilizada no Teste Triangular (ARTIGO 4)

Figura 1. Modelo de ficha utilizada no Teste Triangular (ARTIGO 4)

Teste Triangular

Julgador: ____ Data: _____/_____/_____

Você está recebendo três amostras codificadas.

Duas amostras são iguais e uma é diferente.

Por favor, avalie as amostras da esquerda para a direita.

Circule a amostra DIFERENTE.

587 246 894

Comentários: ____

____

APÊNDICE D – Carta de Aprovação

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais - UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação das propriedades nutritivas, funcionais e tecnológicas das proteínas do soro de leite bovino".

Numero do Parecer: 127/2011

Pesquisador Responsável: Neila Pereira dos Santos Richards

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 16 de janeiro de 2012.

Marta Lizandra do Rêgo Leal
Coordenadora da Comissão de Ética no Uso de Animais-UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362