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EFEITOS DE UMA PROTEASE MONOCOMPONENTE EM DIETAS DE FRANGOS DE CORTE COM NÍVEIS CRESCENTES DE INIBIDORES DE TRIPSINA

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EFEITO DO TRATAMENTO TERMICO E USO DE PROTEASE MONO-COMPONENTE EM DIETAS DE FRANGOS DE CORTE COM USO DE SOJA INTEGRAL[1]

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RESUMO

A presente tese foi realizada com o objetivo de verificar-se o efeito do tratamento térmico (121 ºC e 1,5 atm por 0, 105, 110 e 135 min) sobre os fatores antinutricionais presentes no grão de soja *in natura* e de sua posterior incorporação em dietas suplementadas ou não com uma protease mono-componente nas respostas de desempenho zootécnico, medidas de rendimento, tamanho de órgão e morfometria intestinal de frangos de corte de 1 a 28 dias de idade. Assim, três experimentos foram conduzidos utilizando dietas que incluíram soja integral termicamente tratada, suplementadas com uma enzima exógena (protease) e linhagens de frango disponíveis no mercado. No primeiro estudo foram determinados os principais fatores antinutricionais presentes na soja *in natura* e na soja termicamente processada. Da mesma maneira foram executados testes empregados rotineiramente na indústria como indicadores indiretos da presença destas substâncias deletérias. Constatou-se que à medida que o tempo de processamento térmico aumenta, diminuem as atividades residuais dos principais inibidores de proteases pancreáticas (tripsina e quimotripsina) presentes no grão. No segundo experimento, a soja termicamente tratada no primeiro estudo foi incorporada as dietas, as quais foram suplementadas ou não com uma protease mono-componente (200 ppm). A suplementação da enzima exógena mostrou-se efetiva na melhora do desempenho zootécnico e medidas de rendimento das aves. Por outro lado, os níveis crescentes de inibidor de tripsina afetaram o desempenho das aves e provocaram o aumento do tamanho relativo de pâncreas e duodeno. No entanto, a hipertrofia desses órgãos não foi observada nas aves suplementadas. No terceiro experimento, a soja foi termicamente tratada (121 ºC e 1,5 atm por 105 min.) e incorporada às dietas, as quais foram suplementadas com níveis crescentes de uma protease mono-componente. Observou-se que ainda depois do tratamento térmico a soja apresentou níveis residuais de inibidor de tripsina e quimotripsina. A suplementação enzimática teve um efeito positivo no ganho de peso e conversão alimentar aos 14 dias de idade. Adicionalmente, a suplementação provocou uma redução na gordura abdominal aos 28 dias, independente do nível adicionado. Finalmente, verificou-se que a protease foi efetiva no aumento de altura de vilo (AV), profundidade de cripta (PC), e relação AV:PC aos 14 dias de idade. Conclui-se que a suplementação de protease melhora o desempenho e rendimento de carcaça, e diminui a gordura abdominal de frangos de corte consumindo dietas com soja integral termicamente tratada. Também, houve melhora dos parâmetros morfométricos os quais são indicativos de boa integridade e funcionalidade intestinal.

[1] Tese de Doutorado em Zootecnia – Nutrição Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (136 p.) Março, 2012.

EFFECT OF THERMAL TREATMENT AND USING OF MONOCOMPONENT PROTEASE IN BROILER DIETS WITH FULLFAT SOYBEAN[2]

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ABSTRACT

This thesis was carried out to understand the effect of thermal treatment (121 ºC and 1.5 atm for 0, 105, 110 and 135 min) on antinutritional factors levels contained in full-fat soybeans, incorporated into broiler diets supplemented or not with a monocomponent protease, on birds' performance, carcass yield, pancreas size and intestinal morphometry from 1 to 28 d. Thus, three experiments were conducted using diets including full-fat soybean heat treated, supplemented or not with a mono-component protease and broiler strain commercially available. In the first study, the main antinutritional factors present in raw and heat treated soybeans were measured, through tests widely used in feed industry and other more specific. It was verified that remaining activities of de antinutritional factors decreased as processing time increased. In the second study, the soybean heat treated previously was incorporated into broiler diets supplemented or not with protease (200 ppm). The addiction of exogenous enzyme improved live performance and carcass yield. Moreover, increasing levels of antinutritional factors negatively affected birds' performance and led to enlargement of pancreas and duodenum. However, that hypertrophy was not observed in supplemented birds. In the third experiment, heat treated soybean (121 ºC and 1.5 atm for 105 min.) was incorporated into broiler diets supplemented with increasing levels of monocomponent protease. It was observed that even after heat processing soybeans presented residual levels of antinutritional factors. Protease addition had a positive effect on body weight gain and feed conversion on day 14. Additionally, protease elicited a reduction of abdominal, regardless of the level used. Similar trend was observed in duodenum relative size. Finally, exogenous protease increased villus heigh (VH), crypt depth (CD) and VH:CD ratio at 14 day of age. It is concluded that protease supplementation improved live performance and carcass yield, and diminished abdominal fat percentage. Furthermore, protease improved the morphometric parameters, which are indicators of good intestinal integrity and functionality.

[2] Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (136 p.) March, 2012.

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RELAÇÃO DE ABREVIATURAS E SÍMBOLOS

Aminoácido(s)	**AA**
Acido clorídrico	**HCL**
Ácido desoxirribonucleico	**DNA**
Ácido fítico	**AF**
Ácidos graxos essenciais	**AGE**
Aminoácidos essenciais	**AAE**
Aminoácidos sulfurados	**AAS**
Atividade ureásica	**AU**
Bicarbonato de sódio	**$NaHCO_3$**
Cianeto de hidrogênio	**HCN**
Colecistoquinina	**CCK**
Fatores antinutricionais	**FANs**
Índice de dispersibilidade de proteína	**IDP**
Inibidores de proteases	**IPs**
Inibidores de tripsina	**ITs**
Inibidores de tripsina tipo Bowman-Birk	**BBI**
Inibidores de tripsina tipo Kunitz	**KTI**
Intestino delgado	**ID**
Lipoxigenases	**LOX**
Lisino-alanina	**LAL**
Oligossacarídeos	**OS**
Pontes dissulfeto	**SS**
Proteína bruta	**PB**
Sistema NADPH-tioredoxina	**NTS**
Sódio	**Na^+**
Tioreoxina redutase	**TrxR**
Trato gastrointestinal	**TGI**

CAPITULO I

1. INTRODUÇÃO

Na indústria da nutrição animal, o farelo e o óleo de soja têm uma importante participação na composição das rações. No entanto, devido às oscilações nos preços destes ingredientes, têm-se procurado outros que dispensem a aplicação de qualquer processamento industrial. Entre estes, a soja *in natura* apresenta-se como um promissor ingrediente, pois esta contém um importante nível protéico e um elevado teor lipídico, o que diminui a necessidade da incorporação de óleo às rações.

Contudo, a soja *in natura* possui baixos teores de aminoácidos, sulfurados, e apresenta diversos compostos fitoquímicos, ou fatores antinutricionais, que atuam como proteção natural da planta, porém capazes de deprimir o crescimento e prejudicar o desempenho dos animais que a consomem. Conseqüentemente, esta deve ser submetida à desativação ou tratamento térmico (tostagem, extrusão, micronização, etc), com o intuito de eliminar as substâncias deletérias, e melhorar o aproveitamento dos nutrientes presentes.

A literatura reporta o efeito positivo deste tipo de processamento sobre as respostas de desempenho zootécnico, cuja efetividade pode ser medida através de testes simples e rápidos utilizados na indústria. Além de refletir o grau de processamento recebido, estes testes são indicadores indiretos da presença de fatores antinutricionais. Contudo, há uma grande variação entre os resultados obtidos, em função da intensidade do processamento aplicado, tipo de equipamento empregado e condições físico-químicas do grão.

Por outro lado, tem-se avaliado o efeito da adição de enzimas, isoladas ou em conjunto, em dietas que incluem soja termicamente processada. No entanto, tem-se registrado grande diferença entre as respostas, devido à sobreposição e/ou mascaramento da ação enzimática sob condições *in vivo*. Estudos *in vitro* mostram que a soja tratada apresenta certa proporção de proteína que permanece indigestível, mesmo após a adição de enzimas. Esta inacessibilidade tem origem na formação de ligações cruzadas (proteína-proteína, proteína-carboidrato) causadas pelo tratamento térmico.

Neste contexto, este trabalho teve por objetivo avaliar o efeito da inclusão de protease mono-componente em dietas para frangos de corte com soja integral termicamente tratada.

2. REVISÃO BIBLIOGRÁFICA

2.1 A origem da soja

A soja (*Glycine soja*) originou-se na região nordeste da China, há cerca de 5.000 anos, durante a dinastia *Zhou*, e acredita-se que sua domesticação teve lugar durante a dinastia *Shang,* nos anos de 1500 a 1100 a.C. Posteriormente, no século I d.C., a soja chega às demais regiões da China, e a outros países da Ásia, através de missões comerciais; por outro lado, a existência de alimentos à base de soja como leite, *tofu* (queijo), *shoyu* (molho) e *missô* (pasta salgada fermentada) é relatada inicialmente por comerciantes europeus, vindos da Ásia, nos séculos XVI e XVII d.C. (Smith, 2003).

No ano de 1765, Samuel Bowen, empregado da *East India Company,* introduziu a soja nas colônias britânicas da América do Norte, a qual foi cultivada e empregada, exclusivamente, na preparação de *shoyu* e *noodle* (massa chinesa) destinados ao mercado inglês, porém estas exportações terminaram abruptamente com a revolução americana de 1776. O cultivo da soja, com fins comerciais, só foi retomado no ano de 1829 e a produção, em grande escala, a partir de 1850. Posteriormente, nos anos de 1858 e 1882, a soja chega à África e a América do sul, sendo cultivada, pela primeira vez, no Egito e no Brasil, mais precisamente na Bahia, respectivamente (Friedman & Brandon, 2001; USSEC, 2006; Shurtleff & Aoyagi, 2009a; Shurtleff & Aoyagi, 2009b).

A partir de 1890, as instituições de pesquisa, ao redor do mundo, determinaram a composição bromatológica da soja, e avaliaram seu potencial na alimentação de diversas espécies de interesse zootécnico, confirmando-se que esta leguminosa era uma valiosa fonte de proteína e óleo comestível; simultaneamente, a soja chegava a Oceânia e a América, central sendo cultivada, pela primeira vez com fins de pesquisa (USSEC, 2006; Shurtleff & Aoyagi, 2009b; Shurtleff & Aoyagi, 2009c; Shurtleff & Aoyagi, 2010).

Na década de 50, registrou-se o auge da utilização da soja, na produção pecuária, devido a seu alto valor nutricional e baixos preços decorrentes do aumento na produção mundial do grão (USSEC, 2006). Depois, na década de 90, a biotecnologia serviu como ferramenta para a obtenção de germoplasmas melhorados, mais resistentes a patógenos e pestes locais; e para o desenvolvimento de variedades adaptadas ao fotoperíodo de regiões de baixa latitude, favorecendo ainda mais a utilização da soja na alimentação humana e animal (Smith, 2003; Vasconcelos *et al.,* 2006). A partir de 2000, a soja apresentou-se como fonte de combustíveis alternativos, polímeros biodegradáveis e compostos com propriedades antioxidantes, e de aplicação industrial, como a lecitina (USSEC, 2006; Daleprane *et al.,* 2009).

2.2 Produção de soja

O território destinado ao plantio da soja é aproximadamente de 47% do total dedicado ao plantio de oleaginosas, similar ao destinado para o plantio de outros cereais como o milho, e maior do que o destinado para o cultivo de outros cultivos como algodão, girassol e outras oleaginosas (USDA, 2012).

Em nível mundial, o farelo de soja representa cerca de 65% de todas as fontes de proteína vegetal usadas na alimentação animal seguidas pelo farelo de canola (16%), farelo de algodão (12%), farelo de girassol (10%) e farelo de amendoim (9%) (USDA, 2012). No entanto, a soja representa aproximadamente 92% do total de farelos de oleaginosas fornecidos a ruminantes e monogástricos. Aproximadamente, 53% deste farelo é usado na avicultura seguido por 27% usado na suinocultura, 14% em ruminantes e 6% em ração para animais de companhia e peixes (Cromwell, 1999).

A soja é a principal fonte de proteína de origem vegetal utilizada na alimentação mundial. Atualmente, estima-se que, aproximadamente, 60% dos alimentos para consumo humano (farinhas e proteínas texturizadas) e animal contenham algum derivado de soja (USSEC, 2006; Daleprane *et al.,* 2009).

O principal subproduto obtido do processo da extração de óleo do grão de soja é o farelo. Seu uso tem sido estimulado pelo banimento da inclusão de fontes protéicas de origem animal, nas rações para não ruminantes; e sua composição nutricional depende do cultivar e das condições de processamento. Comercialmente, são produzidos dois tipos de farelo de soja: o primeiro, sem casca, possui um alto teor protéico, aproximadamente 49% de proteína bruta (PB); já o segundo contém 44% de PB, e inclui a casca que foi retirada na parte inicial do processo; ambos os farelos contem aproximadamente 3% de gordura bruta (Kocher, 2002; Karr-Lilienthal *et al.,* 2005).

2.3 Características da soja

Atualmente mais de 70% da proteína de origem vegetal consumida, em nível mundial, é derivada das leguminosas. As leguminosas, ao contrário dos cereais, acumulam quantidades maiores de proteína**,** devido à fixação de nitrogênio pelas bactérias presentes nas raízes (Friedman & Brandon, 2001; Krishnan, 2005).

Entre as leguminosas, a soja contém uma alta concentração de proteína que varia de 35 a 50%; caracteriza-se pelo maior conteúdo de lisina (aproximadamente 6,5%) em relação a outras oleaginosas como a canola (5,8%), o algodão (4,2%), e o girassol (2,8%); ademais é rica em triptofano, treonina, isoleucina e valina, aminoácidos deficitários no milho, sorgo e outros cereais comumente utilizados na alimentação de aves e suínos. Contudo, sua qualidade nutricional se vê comprometida pela baixa concentração de aminoácidos sulfurados (AAS), como a metionina e cistina (Cromwell, 1999; Krishnan, 2005).

Adicionalmente, a soja é uma importante fonte de óleo comestível. O grão contém de 18 a 25% de óleo o qual contém um bom perfil de ácidos graxos essenciais (AGE), principalmente acido linoléico (ω-6) (8%) e linolênico (ω-3) (Friedman & Brandon, 2001; Karr-Lilienthal *et al.,* 2005). Além disso, a soja também possui oligossacarídeos, fibras (20 a 25%), minerais como ferro, zinco, magnésio, potássio, cálcio, manganês e selênio; e vitaminas como

retinol, tiamina, riboflavina, piridoxina e acido fólico (Karr-Lilienthal *et al.,* 2005).

Sabe-se que a composição da semente (teor de proteína, óleo e perfil de ácidos graxos) é influenciada pelas condições de crescimento e pela localização geográfica das áreas de cultivo, como a latitude, altitude, temperatura, precipitação e fotoperíodo. A literatura reporta que a latitude se correlaciona negativamente com a proteína e positivamente com o conteúdo do óleo, e de ácido linoléico. Entretanto, precipitações intensas, durante o desenvolvimento do grão, correlacionam-se positivamente com conteúdo de óleo, e negativamente com o conteúdo de proteína. Já sojas desenvolvidas sob temperaturas baixas apresentam correlação positiva com o conteúdo de proteína, e negativa com o teor de óleo (Kumar *et al.,* 2006; Vasconcelos *et al.,* 2006; Esteves *et al.,* 2010).

2.4 Fatores antinutricionais presentes na soja

2.4.1 Definição e função

A soja [*Glycine max* (L.) Merril] ocupa um lugar de destaque na alimentação de aves e suínos. No entanto, o valor nutricional do grão, e seus subprodutos, pode ser menor que o esperado, em função da presença de diversos fitoquímicos, ou fatores antinutricionais (FANs), que atuam como antagonistas diretos, ou indiretos, dos nutrientes contidos no grão, limitando a inclusão da soja *in natura* na dieta, especialmente de animais jovens (Olguina *et al.*, 2003; Barać *et al.,* 2005; Feng *et al.,* 2007).

Do ponto de vista bioquímico, os FANs são definidos como metabólitos secundários, isto é, compostos que não são essenciais para o crescimento da planta, porém capazes de transportar e armazenar certos nutrientes, de proteger a planta contra os raios ultravioleta, e de atrair agentes polinizadores e dispersores de sementes. No entanto, sua função mais proeminente é a defesa contra o ataque de insetos herbívoros e patógenos (Bedford, 1996; Kimball & Provenza, 2003; Chen, 2008; Guillamón *et al.,* 2008).

Os FANs, quando ingeridos, exercem efeitos deletérios sobre a saúde e produtividade dos animais. Esses efeitos podem variar de quase imperceptíveis (subclínicos) a manifestamente tóxicos, ou ainda venenosos (Champ, 2002; Putszai *et al.,* 2004), e dependem das características físico-químicas dos FANs e do grau de interação destes com os tecidos e enzimas endógenas do animal (Acamovic & Brooker, 2005).

Segundo o modo de ação, os FANs podem ser divididos em quatro grupos: 1) substâncias que afetam a digestão e utilização dos nutrientes, como os inibidores de proteases, taninos e lectinas; 2) substâncias que afetam a utilização dos minerais, como o fitato e oxalatos; 3) antivitaminas, que inativam ou inibem a função de uma vitamina como cofator enzimático; e 4) substâncias diversas, como glicosídeos cianogênicos, saponinas, alcalóides, e oligossacarídeos, capazes de causar alergias ou hipersensibilidade (Liying *et al.,* 2003; Rubio *et al.,* 2004). Adicionalmente, os FANs podem ser classificados, de acordo com sua aptidão em resistir aos processamentos térmicos, em termolábeis (inibidores de protease, fitatos, lectinas, e antivitaminas) e em termoestáveis (saponinas, oligossacarídeos, e taninos) (Wiryawan & Dingle, 1999; Francis *et al.,* 2001; Friedman & Brandon, 2001).

2.4.1.1 Inibidores de proteases

As proteases presentes nos vegetais participam na mobilização das proteínas de reserva durante a germinação das sementes. Também, agem durante o crescimento de tecidos meristemáticos (folhas, flores e fruto), assim como durante a senescência (Ryan, 1973). Já nos animais, as reações proteolíticas catalisadas pelas serina-proteases são responsáveis pela sinalização, transmissão e término de eventos celulares, como inflamação, apoptose, coagulação sanguínea, ativação de zimogênios e transporte transmembrana (Jongsma & Bolter, 1997; Habib & Fazili, 2007). Estas contêm um íon metálico (Zn^{+2}, Ca^{+2}, Mn^{+2}), ou um único resíduo de aspartato, cisteína ou serina no sítio ativo e são encontradas no fígado, esperma (acrosina), pele e mastócitos (Hartley, 1960; McDonald, 1985; Bond & Butler, 1987; Chen, 2008).

Apesar das proteases serem indispensáveis para as células, e organismos hospedeiros, estas são potencialmente prejudiciais quando sobre-expressas ou presentes em altas concentrações. Conseqüentemente, estas são sintetizadas como pré-enzimas inativas, que possuem uma alta especificidade pelo substrato, ou são suscetíveis de interagir com inibidores que bloqueiam, alteram ou impedem o acesso ao sítio ativo da enzima, através da formação de complexos estáveis (Hartley, 1960; Habib & Fazili, 2007).

Os inibidores de proteases (IPs) são proteínas encontradas nas sementes de cereais, leguminosas e solanáceas, e representam de 5 a 15% da proteína total, concentrando-se, principalmente, nos cotilédones e no endosperma da soja e do milho, respectivamente (Champ, 2002; Otlewski *et al.,* 2005). Estes podem se expressar durante o desenvolvimento da planta, ou serem induzidos em resposta a infecções de origem fúngica e aos ataques de predadores; os ferimentos mecânicos também estimulam a síntese local e transporte dos IPs até o tecido afetado, através do floema. Entretanto, este tipo de defesa sistêmica e mais evidente em plantas jovens, em função da maior suscetibilidade a infestações simples (Ryan, 1973; Masoud *et al.,* 1996; Jongsma & Bolter, 1997; Udedibie & Carlini, 1998). Os IPs inibem as proteases, presentes no trato gastrointestinal (TGI) dos insetos fitófagos, reduzem a disponibilidade dos aminoácidos essenciais (AAE) e, também, afetam o balanço hídrico e síntese de neuropeptídios na larva (Hartley, 1960; Jongsma & Bolter, 1997; Habib & Fazili, 2007).

Os IPs da soja são classificados em duas categorias principais: os inibidores tipo Kunitz (KTI) e os do tipo Bowman-Birk (BBI). O primeiro liga-se com uma molécula de tripsina em uma forma estequiométrica, ou seja, uma molécula do inibidor inativa uma molécula de tripsina, enquanto que o segundo liga simultânea e independentemente as duas moléculas, tripsina e quimotripsina, graças à presença de dois sítios reativos (Lys^{16}-Ser^{17} e Leu^{43}-Ser^{44}, respectivamente). A soja *in natura* contém cerca de 1,67% de KTI e 0,4% de BBI; e dependendo do cultivar, o grão possui três isoformas de KTI, e de 5 a 12 de BBI (Hartley, 1960; Mizubuti & Ida, 1999; Francis *et al.,* 2001; Haq *et al.,* 2004).

O KTI possui um peso molecular relativamente alto (~18 a 22 kDa), é composto por 181 resíduos de aminoácidos (AA) e duas pontes dissulfeto (SS); uma delas essencial para a atividade inibitória; além disso, tem uma ou duas cadeias polipeptídicas, e baixo conteúdo de cistina (quatro resíduos) (Figura 1). Por outro lado, o BBI possui menor peso molecular (~7 a 8 kDa), e

alto conteúdo de AAS, é composto por 70 a 80 resíduos de AA, e é mais termoestável, devido à presença de sete SS (Figura 2) (McNaughton, 1981; Tacon, 1997; Osman *et al.,* 2002; Barać *et al.,* 2005; Zilic *et al.,* 2011).

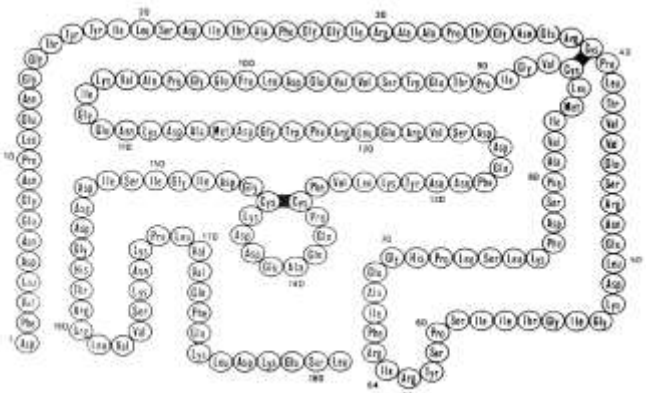

Figura 1. Estrutura primária do inibidor de tripsina da soja tipo Kunitz. Possui duas pontes dissulfeto por mol, em preto, entre os resíduos de cistina. O sítio ativo é Arg^{63}-Ile^{64} (Retirado de Wolf, 1977).

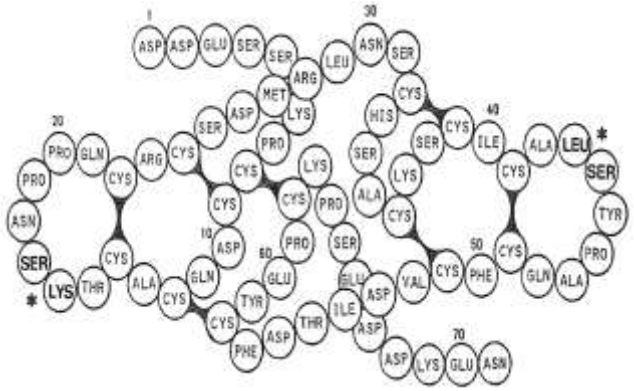

Figura 2. Estrutura primária do inibidor de tripsina da soja tipo Bowman-Birk. Possui sete pontes dissulfeto por mol, em preto, entre os resíduos de cistina. Os sítios ativos estão marcados indicados com asteriscos (Retirado de Wolf, 1977).

A importância dos IPs, na nutrição animal, é decorrente dos efeitos sobre a digestão das proteínas no intestino. A inibição da proteólise provoca o aumento da secreção das serina-proteases pancreáticas endógenas: tripsina (EC 3.4.21.4), quimotripsina (EC 3.4.21.1) e elastase (EC 3.4.21.36), como mecanismo compensatório pela perda das mesmas devido à formação de complexos com seus respectivos inibidores (Brannon, 1990; Mizubuti & Ida, 1999; Champ, 2002; Guillamón *et al.,* 2008). Em aves e ratos, esta hiperestimulação é provocada pelo aumento da liberação do hormônio colecistoquinina (CCK) que, por sua vez, leva à hipertrofia e hiperplasia do pâncreas (Tacon, 1997; Batal & Parsons, 2003).

O pâncreas se adapta rapidamente aos componentes da dieta em função da sua maior proporção de tecido exócrino (~90 a 95%), representado

pelas células acinares, que por sua vez, alteram taxas de síntese e secreção de serina proteases, amilase e lipase (Brannon, 1990). Um número considerável de pesquisas tem sido conduzido para definir a relação entre o consumo de soja crua, a depressão do crescimento e a resposta hipertrófica do pâncreas (Melmed & Bouchier, 1969; Melmed *et al.,* 1973; Struthers *et al.,* 1983; Ge & Morgan, 1993), confirmando-se que os IPs, presentes na soja, são responsáveis por, aproximadamente, 80% da hipertrofia do pâncreas, e por 30 a 50 % da redução no crescimento. Esta redução é provocada pela perda endógena de proteínas pancreáticas ricas em enxofre (tripsina e quimotripsina) e que não pode ser compensada pela ingestão de proteína da soja, a qual é deficitária em AAS (Mizubuti & Ida, 1999; Putszai *et al.,* 2004; Moura *et al.,* 2007; Daleprane *et al.,* 2009).

Alguns dos IPs são moléculas bifuncionais, isto é, capazes de inibir tripsinas assim como α-amilases de mamíferos e insetos (Carlini & Grossi-de-Sá, 2002). Em geral, os inibidores de α-amilase possuem natureza protéica ou glicoprotéica, e formam complexos estáveis com α-amilase inativando-a e, conseqüentemente, reduzindo a digestão do amido no intestino delgado (ID) (Masoud *et al.,* 1996; Mizubuti & Ida, 1999; Champ, 2002; Fasolia *et al.,* 2009).

2.4.1.2 Lipoxigenases

As lipoxigenases (LOX) catalisam a formação de hidroperóxidos a partir de ácidos graxos poliinsaturados, principalmente, o ácido linolênico (C18:3) e o linoléico (C18:2) e estão envolvidas na biossíntese de compostos regulatórios, necessários para o crescimento e a senescência da planta, como a traumatina e o ácido jasmônico. (Hildebrand & Kito, 1984; Silva *et al.,* 2001).

Na soja, as LOX estão representadas por três isoenzimas: LOX-I, LOX-II e LOX-III, que correspondem a 1% da fração protéica total. Sua concentração é influenciada pelas variações genotípicas entre cultivares, as condições edafoclimáticas durante a germinação e desenvolvimento, e pela presença de outros FANs na semente (Zilic *et al.,* 2011).

Nesse sentido, a literatura reporta que variedades de soja cultivadas em regiões com menor precipitação e temperatura exibem maior atividade das três isoenzimas. Por outro lado, a ausência de LOX na semente causa redução da atividade dos inibidores tipo KTI e BBI, em até 72% e 54%, respectivamente (Carvalho *et al.,* 1999; Kumar *et al.,* 2003; Barros *et al.,* 2008).

2.4.1.3 Lectinas

As lectinas ou hemaglutininas foram detectadas, originalmente, no extrato da mamona (*Ricinus communis* L.) e descritas como fatores proteináceos tóxicos capazes de aglutinar os eritrócitos. Posteriormente, estas foram identificadas como glicoproteínas, de origem não imunológica, capazes de se ligar especifica e reversivelmente a oligossacarídeos, ou glicopeptídeos, da superfície celular, através de ligações de hidrogênio e interações de Van der Waals (Brandon & Friedman, 2002; Champ, 2002).

As lectinas nativas da soja são glicoproteínas tetramêricas que se acumulam durante a embriogênese, possuem peso molecular de 120 kDa; e são classificadas em cinco grupos de acordo com o monossacarídeo pelo qual exibem a maior afinidade (Figura 3) (Barać *et al.,* 2005).

A ampla distribuição em diversos tecidos (sementes, raízes, folhas e flores) e a presença ubíqua das lectinas de origem vegetal confirmam a função

protetora contra o ataque de microorganismos fitopatogênicos, insetos fitófagos e herbívoros (Carlini & Grossi-de-Sá, 2002).

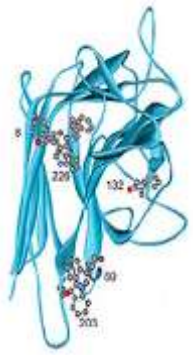

Figura 3. Monômero da lectina presente na soja (Retirado de Sinha & Surolia, 2005).

Em geral, as lectinas aumentam a mortalidade e prejudicam o ganho de peso das larvas; e diminuem a fertilidade de insetos adultos (Mizubuti & Ida, 1999; Francis *et al.,* 2001; Batal & Parsons, 2003).

A literatura destaca que, quando consumidas por aves e ratos, as lectinas causam mudanças dramáticas na morfologia celular. Estas mudanças incluem: alteração da proliferação e *turnover* dos enterócitos, rompimento da membrana de borda em escova, e redução a área absortiva superficial (Chen, 2008). Entretanto, a magnitude dos efeitos deletérios exercidos pelas lectinas também depende do status sanitário do animal, da composição da dieta e das características físico-químicas da lectina (Tacon, 1997; Batal & Parsons, 2003; Vasconcelos & Oliveira, 2004).

2.4.1.4 Saponinas

As saponinas são glicosídeos esteroidais presentes nas leguminosas, e em algumas plantas usadas como especiarias. A soja *in natura* contém três tipos de saponinas: αg, βa e βg, em concentrações que variam de 1 a 3 mg/g de soja (Champ, 2002). Estas substâncias formam complexos insolúveis com 3-β-hidroxiesteróides e interferem com a absorção de lipídeos, colesterol, sais biliares e vitaminas lipossolúveis presentes na dieta. Além disso, são capazes de lisar hemácias e reduzir a digestibilidade da proteína através da formação de complexos com proteínas da dieta (Tacon, 1997; Putszai *et al.,* 2004).

Tem sido demonstrado que as saponinas, quando hidrolisadas pela microbiota do ceco de frangos e camundongos, dão origem a sapogeninas e açúcares os quais inibem o transporte ativo de nutrientes e incrementam a permeabilidade da mucosa do ID (Francis *et al.,* 2001), o qual envolve a apertura das junções firmes dos enterócitos, com subsequente penetração de bactérias e macromoléculas como toxinas (MacFie, 2000).

2.4.1.5 Glicosídeos cianogênicos

Os glicosídeos cianogênicos, quando intactos, não são tóxicos, mas os produtos de sua hidrólise, pela ação da enzima tioglucosidase, o são. O

processamento comercial do grão, como a trituração e o dano mecânico gerado pela ação de insetos herbívoros, libera a enzima e conseqüentemente, promove a degradação dos glicosídeos cianogênicos, armazenados em compartimentos celulares diferentes, liberando substâncias tóxicas como cianeto de hidrogênio (HCN), nitrilos e gointrinas (Tacon, 1997; Francis *et al.,* 2001).

Na soja *in natura* têm sido detectadas quantidades variáveis de HCN, assim como em seus subprodutos. Honig *et al.* (1983), utilizando procedimentos colorimétricos, determinaram que a soja crua e a soja tostada apresentaram 0,08 e 0,07 μg de HCN/g de amostra, respectivamente. Por outro lado, o mesmo autor observou que a proteína isolada de soja apresentou maior conteúdo de HCN (0,18 μg/g) em relação ao farelo de soja desengordurado (0,08 μg/g), e que o nível de HCN era 10 vezes maior nas cascas de soja do que no farelo de soja descascado. No farelo de soja desengordurado os níveis de HCN variaram de 0,3 até 61 μg/g

Os glicosídeos cianogênicos e seus subprodutos de degradação inibem o crescimento de insetos, relacionam-se à hemorragia hepática em poedeiras, provocam dano ao tecido hepático e renal em várias espécies e podem induzir insuficiência respiratória e ataque cardíaco quando consumidos em grandes quantidades. Adicionalmente, estes compostos agem como agentes antitireoidianos, isto é, interferem no tamanho, estrutura e função da glândula tireóide, inibindo a síntese e secreção dos hormônios tireoidianos, prejudicando a eficiência alimentar e o ganho de peso (Francis *et al.,* 2001; Champ, 2002; Morandini, 2010). Contudo, estes compostos são suscetíveis à hidrólise microbiana do TGI; e seus efeitos deletérios podem ser reduzidos pela interação com outros FANs presentes, como os taninos (Tacon, 1997; Francis *et al.,* 2001).

2.4.1.6 Proteínas alergênicas

As globulinas da soja, β-conglicinina e glicinina, representam cerca de 80% do total da proteína do endosperma e, portanto, a grande maioria dos AA presentes. Possuem um perfil aceitável de AAE, entretanto são deficitárias em AAS (Katsube *et al.,* 1999; Brandon & Friedman, 2002; Krishnan, 2005; Zilic *et al.,* 2011).

A glicinina (11S) é a proteína de reserva predominante na soja, apresenta-se como hexámero de peso molecular entre 320 a 375 kDa e pode conter até cinco subunidades. Cada subunidade é composta por um polipeptídio ácido (~35 kDa) e um polipeptídio básico (~20 kDa) ligados por uma ponte dissulfeto (Faris *et al.,* 2008). As glicinas podem ser classificadas em dois grupos segundo sua seqüência aminoacídica. O grupo I contém maiores quantidades de metionina (seis a sete resíduos por subunidade) e o grupo II contém de dois a cinco resíduos de metionina. Do ponto de vista nutricional, a glicinina é a fração mais disponível e contém de três a cinco vezes mais AAS em relação à β-conglicinina (Brandon & Friedman, 2002; Krishnan, 2005).

A β-conglicinina (7S), segunda maior proteína de reserva da soja, é uma glicoproteína que contém cerca de 5% de carboidratos, principalmente, manose, ligados covalentemente aos resíduos de asparagina; possui um peso

molecular de aproximadamente 150 kDa, e é composta por três subunidades: α,α' e β. Não contém SS e praticamente carece de metionina (Friedman & Brandon, 2001; Zilic *et al.,* 2011).

Embora a alergenicidade das proteínas de soja em humanos tenha sido descoberta desde a década de 30, seus efeitos em animais só tem sido pesquisados recentemente. Sabe-se que algumas espécies são mais sensíveis à exposição continua às globulinas encontradas na soja e no farelo de soja extraído com solvente ou álcool. Pré-ruminantes (bezerros) são sensíveis a este tipo de proteínas e não são capazes de desenvolver tolerância. Já os leitões desmamados exibem hipersensibilidade local transitória após consumo de β-conglicinina e glicina, contudo conseguem desenvolver tolerância depois de 7 a 10 dias (Francis *et al.,* 2001; Brandon & Friedman, 2002). Durante a reação de hipersensibilidade, evidenciam-se alterações fisiológicas e morfológicas em nível de intestino. Essas alterações incluem: atrofia dos vilos, maior profundidade de cripta, aumento no número de enterócitos imaturos e, conseqüentemente, diminuição da capacidade absortiva e maior suscetibilidade a doenças entéricas (Engle, 1994; Tacon, 1997).

2.4.1.7 Taninos

Os taninos são um grupo ubíquo e complexo de compostos polifenólicos, solúveis em água, que ocorrem naturalmente em leguminosas e com peso molecular alto a intermediário (até 30 kDa) (Sell *et al.,* 2010). Na soja, o conteúdo de taninos pode variar de 0.88 até 2.06 g/kg de amostra dependendo da variedade e da origem da semente (Malenčić *et al.*, 2007).

Estes compostos são classificados em dois grupos: hidrolisáveis e condensados. O acido tânico, ou tanino hidrolisável, é degradado pelo organismo animal, formando compostos menores que podem entrar na corrente sanguínea e exercer efeitos tóxicos sobre vários órgãos (fígado e rim). Já os taninos condensados, ou proantocianidinas, são polímeros de alto peso molecular, e têm o potencial de precipitar até 12 vezes seu peso de proteína (Francis *et al.,* 2001; Champ, 2002).

Em geral, os taninos são capazes de formar complexos insolúveis, com carboidratos, vitaminas, íons metálicos e proteínas (Tacon, 1997; Mizubuti & Ida, 1999). Esta propriedade dos taninos é responsável pela adstringência da alguns alimentos, como vegetais, frutas e chás, especialmente os imaturos, devido à precipitação das proteínas salivares. Ademais, os taninos estão envolvidos nos mecanismos de proteção contra ataques bacterianos, fúngicos ou virais (Schofield *et al.,* 2001; Champ, 2002; Sell *et al.,* 2010).

Os efeitos deletérios dos taninos dependem das propriedades físico-químicas, espécie animal e grau de interação destas substâncias com outros FANs presentes na dieta (Putszai *et al.,* 2004); por exemplo, a interação entre taninos e lectinas neutraliza a ação inibitória dos taninos sobre a amilase (Francis *et al.,* 2001).

2.4.1.8 Ácido fítico ou fitatos

O ácido fítico (AF), ou mio-inositol hexafosfato (IP_6), é uma substância ubíqua contida, principalmente, na fração protéica 7S (β-conglicinina), enquanto que a fração 11S (glicinina) contém cerca de 0,07%. Os fitatos são a principal forma de armazenamento de fósforo nas plantas. Ao

redor de 62 a 73%, e de 46-73% do fósforo total, encontra-se na forma de fitato, em cereais e leguminosas, respectivamente. Por isso, a biodisponibilidade de minerais nas leguminosas é considerada baixa devido à presença de AF e oxalatos (Tacon, 1997; Barać *et al.,* 2005).

Na soja, o AF tem sido detectado durante a embriogênese (18,7 a 33,6 μg/semente/dia), acumulando-se de maneira linear ao longo do desenvolvimento da semente (Raboy & Dickinson, 1987). No grão *in natura* e no farelo de soja desengordurado a concentração de AF é de 0,6 a 5% e de 1,5%, respectivamente (Honig *et al.*, 1984).

A presença de AF na soja é influenciada pelas características do solo e pelos fatores climáticos. Solos ácidos e baixas temperaturas durante a germinação, e presença de óxidos de ferro e alumínio, desfavorável para a mineralização do fósforo, produzem níveis menores de AF no grão. Contrariamente, sojas cultivadas em solos com pH neutro, alto conteúdo de matéria orgânica e sob altas temperaturas exibem maiores teores de AF (Kumar *et al.,* 2006).

Sob condições fisiológicas (pH 6,0-7,0), o AF encontra-se carregado negativamente, o que lhe confere alto potencial como agente quelante, formando complexos insolúveis com íons, di e trivalentes, de carga positiva, como Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+} e Fe^{3+}, tornando-os biologicamente indisponíveis para absorção (Francis *et al.,* 2001; Schlemmer *et al.,* 2009).

Além disso, o AF pode formar complexos com proteínas. Estes são resistentes à ação da pepsina, e provocam hipersecreção de acido clorídrico (HCL), com aumento concomitante do bicarbonato de sódio ($NaHCO_3$), de modo a tamponar o excesso de HCL. Assim, a depleção de sódio (Na^+), induzida pelo AF, compromete o funcionamento dos sistemas de transporte Na^+-dependentes, afetando a absorção intestinal dos AA da dieta e a reabsorção dos AA endógenos (Mizubuti & Ida, 1999; Champ, 2002; Barać *et al.,* 2005; Sell *et al.,* 2010).

2.4.1.9 Oligossacarídeos

Os carboidratos presentes na soja contribuem com ao redor de 35% da matéria seca do grão. Na soja, e na maioria das leguminosas, aproximadamente metade destes carboidratos é representada por polissacarídeos estruturais, incluindo uma grande quantidade de polissacarídeos pécticos; enquanto que a outra metade inclui polissacarídeos não estruturais, como oligossacarídeos (OS) e pequenas quantidades de amido. Sua composição bioquímica e concentração são influenciadas pelo cultivar, tipo de solo, aplicação de fertilizante e condições climáticas próprias da região (Karr-Lilienthal *et al.,* 2005).

Os oligossacarídeos encontrados na soja são, principalmente, α-galactosídeos (rafinose, estaquiose, verbascose), ligam sais biliares e enzimas digestivas, além de diminuir a movimentação da digesta através do TGI pelo aumento da viscosidade (Francis *et al.,* 2001).

Os animais não ruminantes carecem da enzima α-1,6-galactosidase, essencial para sua hidrólise (Wang *et al.,* 2007), porém este tipo de carboidrato pode ser degradado pelas bactérias do ceco. Esta degradação bacteriana resulta na produção de ácidos graxos de cadeia curta e gases, principalmente CO_2 e H_2, os quais causam flatulência e desconforto nos animais (Kocher *et al.,*

2002; Karr-Lilienthal *et al.,* 2005). Nesse sentido, Parsons *et al.* (2000) observaram que a digestibilidade da estaquiose e rafinose foi 30% a 50% maior em galos cecectomizados, em relação aos galos convencionais, indicando que a maior parte da digestão dos OS ocorre através da fermentação no ceco.

2.4.2.0 Alcalóides

Representam um amplo grupo de compostos estruturalmente diversos, são metabólitos secundários, básicos, derivados de diversos precursores de natureza protéica encontrados nas leguminosas (Tacon, 1997).

Os alcalóides, do grupo quinolizidina, são substâncias dotadas de efeitos neurotóxicos e hepatóxicos; e ademais podem alterar as propriedades organolépticas do grão e diminuir o consumo alimentar em algumas espécies; seu mecanismo de ação ainda não é totalmente entendido (Francis *et al.,* 2001).

2.4.2.1 Urease

As ureases (EC 3.5.1.5), ou uréia amidohidrolases, são metaloenzimas, dependentes de Ni^{2+}, que catalisam a hidrólise da uréia em duas moléculas de amônia e dióxido de carbono. Estas enzimas têm sido isoladas de uma ampla variedade de plantas, fungos e bactérias; apresentando-se como trímeros (α3) ou hexámeros (α6) com subunidades de ~90 kDa (Becker-Ritt *et al.,* 2007).

O caso mais bem estudado é o da urease da soja [*Glycine max* (L.) Merril] que possui duas isoformas: uma enzima ubíqua em todos os tecidos em níveis de 0,001-0,01%; e responsável pela reciclagem de uréia derivada do metabolismo; e uma segunda isoenzima, designada embrião-específica, sintetizada somente pelo embrião em desenvolvimento e preservada na semente madura, onde sua atividade é cerca de 1000 vezes maior que a urease ubíqua em todos os tecidos (Olivera-Severo *et al.,* 2006). A urease da soja se acumula, particularmente, nas fases posteriores de desenvolvimento, sugerindo que esta participa no transporte do nitrogênio proveniente da fixação biológica e na utilização das proteínas de reserva da semente durante a germinação concomitantemente com a arginase (Polacco & Winkler, 1984; Stebbins & Polacco,1995).

Contudo, o cultivo *in vitro* de cotilédones de soja, em desenvolvimento, indica que as ureases não cumprem um papel importante na nutrição do embrião, pois a uréia é uma fonte extremadamente pobre em nitrogênio, confirmando que a urease teria um papel importante na defesa química da planta contra insetos predadores, particularmente da ordem coleóptera e hemíptera, devido à alta toxicidade da amônia liberada. Esta entomotoxicidade depende da liberação interna de peptídeos pelas enzimas digestivas do inseto, entretanto o mecanismo exato ainda não foi elucidado (Olivera-Severo *et al.,* 2006; Carlini & Polacco, 2008).

2.5 Eliminação dos fatores antinutricionais

A maioria dos FANs, com exceção do BBI, é termolábil e suscetível a destruição durante processamentos térmicos convencionais; contudo níveis residuais de FANs são encontrados após processamento, e podem ainda interferir com a digestão, absorção e posterior utilização dos nutrientes. Assim,

o grau de desnaturação ou inativação depende das diversas variáveis envolvidas no processo: temperatura, duração, tamanho de partícula e umidade (Barać *et al.,* 2005; Feng *et al.,* 2007).

Atualmente, diversas técnicas de processamento melhoram o valor nutritivo do grão, através da eliminação parcial, ou total, dos diferentes FANs. Entre estas, destacam-se a maceração, aplicação de calor seco ou úmido, filtração, germinação, fermentação e hidrólise enzimática. Todavia, muitas delas são de elevado custo e acabam por inviabilizar o uso da soja *in natura* na alimentação animal; portanto, a escolha da técnica apropriada depende do custo do processamento e da natureza físico-química do FAN que se deseja eliminar (Champ, 2002; Hajos & Osagie, 2004).

2.5.1 Tratamentos físicos

São os métodos mais usados na redução dos efeitos negativos dos FANs e podem ser divididos em tratamentos mecânicos e térmicos.

2.5.1.1 Tratamentos mecânicos

Os mais utilizados são a moagem e a decorticação (remoção da casca). A remoção mecânica da casca produz melhora na qualidade do valor nutritivo da leguminosa devido à redução do conteúdo de polissacarídeos não amiláceos e dos taninos, já que estes residem na testa do grão. As lectinas e IPs, entretanto, estão concentrados nos cotilédones, por isto a decorticação não teria efeito sobre a redução dos mesmos (Champ, 2002; Kocher, 2002; Hajos & Osagie, 2004).

A moagem, por sua vez, remove a camada externa das sementes e reduz o conteúdo de fitato no grão concentrado, principalmente, no endosperma (Mizubuti & Ida, 1999; Wiryawan & Dingle, 1999; Francis *et al.,* 2001). Já a trituração, abrasão e fragmentação mecânica favorecem a ruptura da parede vegetal e aumenta o acesso das enzimas aos nutrientes encapsulados (Karr-Lilienthal *et al.,* 2005).

Outro processo físico importante é a maceração, durante a qual tem lugar várias reações metabólicas que afetam a natureza bioquímica dos FANs, permitindo sua inativação. A resposta das leguminosas à maceração varia em função da espécie, natureza bioquímica do FAN, temperatura e duração do processo (Hajos & Osagie, 2004; Akande & Fabiyi, 2010).

Toledo *&* Canniatti-Brazaca (2008) observaram que grãos submetidos à maceração durante 10 h apresentaram maior digestibilidade *in vitro* da proteína e eliminação significativa dos taninos, além de diminuir o tempo de cocção dos mesmos, em relação aos grãos que não foram macerados. Similarmente, Hajos & Osagie (2004) confirmaram que a maceração de diversos tipos de leguminosas por 18 h a 22 °C provocou a remoção total dos fitatos presentes.

2.5.1.2 Tratamentos térmicos

Os processos térmicos permitem amenizar, reduzir ou inativar os FANs contidos nas leguminosas, incluindo a soja. Dentre esses, pode-se citar a tostagem (em tambor rotativo ou *jetsploder*), a micronização, a extrusão; e processamento de alta pressão hidrostática (APH), como a autoclavagem, os quais modificam a estrutura terciária da proteína e, portanto, a atividade de

muitas enzimas (Tacon, 1997; Francis *et al.,* 2001; Osman *et al.,* 2002). Contudo, estas modificações decorrentes da APH podem levar, também, à desnaturação da proteína através da alteração das interações intra ou intermoleculares (ligações não covalentes, interações hidrofóbicas e eletrostáticas), ou pelo contrário à ativação de algumas enzimas (hidrolases, transferases e oxidorredutases) (Hugas *et al.*, 2002; Hajos & Osagie, 2004). Esta ativação é explicada pelas mudanças diretas na conformação e mecanismos de reação da enzima, na solvatação, isto é, na interação da mesma com o meio circundante, bem como nas propriedades físicas do substrato e/ou solvente (Eisenmenger & Reyes-de-Corcuera, 2009).

Outras técnicas para o tratamento térmico da soja incluem a utilização de vapor combinado com vácuo e a irradiação (Carvalho *et al.,* 2008). Esta última surge como método alternativo para a remoção dos FANs presentes no grão, causando efeitos mínimos sobre a composição do ingrediente (Siddhurajua *et al.,* 2002, Akande & Fabiyi, 2010), visto que os métodos convencionais podem gerar destruição ou perda de alguns nutrientes, e seus efeitos são variáveis. Além disso, em muitas ocasiões, a aplicação de um único tratamento pode não produzir a remoção total dos FANs, fazendo-se necessária a combinação de dois ou mais destes (Abu-Tarboush, 1998).

A melhora na digestibilidade pelo tratamento térmico é atribuída, principalmente, à redução na atividade dos IPs devida às alterações estruturais que estes sofrem, aumentando a susceptibilidade do alimento à hidrólise enzimática no TGI (Araba & Dale, 1990b, Francis *et al.,* 2001; Olguina *et al.,* 2003, Daleprane *et al.,* 2009). Entretanto, a efetividade do tratamento térmico é influenciada pelo tipo de processamento comercial do grão (extração de óleo mecânica ou por solvente), e das condições de processamento (tamanho de partícula, temperatura, pressão, umidade e duração) (Kocher, 2002; Karr-Lilienthal *et al.,* 2005). Sabe-se que a maioria dos farelos de soja, processados comercialmente, retém de 5 a 20% da atividade dos KTI e BBI (Brandon & Friedman, 2002; Champ, 2002).

Por outro lado, a perda da qualidade nutricional, durante a aplicação de calor excessivo ou sobreprocessamento é atribuída à redução da solubilidade protéica, à diminuição da disponibilidade dos AA básicos, à interação com íons metálicos e à formação de compostos tóxicos (Wiryawan & Dingle, 1999; Friedman & Brandon, 2001; Haddad & Allaf, 2007; Mendes *et al.,* 2007).

Durante o sobreprocessamento, as reações de Maillard e Amadori (pardeamento não enzimático) favorecem as interações especificas entre AA básicos e açúcares redutores, ou vitamina C; e a formação de ligações cruzadas entre proteínas adjacentes, o que resulta na formação de compostos que causam alterações na síntese de ácido desoxirribonucleico (DNA) e lesões nas células epiteliais dos túbulos renais (Friedman, 1996). Adicionalmente, ocorre a racemização dos AA, e a geração de AA não naturais como a lisino-alanina (LAL). Do ponto de vista nutricional, estes provocam a diminuição da lisina e cistina e agem como agentes quelantes de íons metálicos (Friedman, 1992; Wiryawan & Dingle, 1999; Friedman & Brandon, 2001).

2.5.2 Tratamentos biológicos

Entre estes se destacam a fermentação e a germinação. A

fermentação, ou bioprocessamento tem sido usada para preparar alimentos tradicionais derivados da soja. Este processo visa à redução dos FANs através da utilização de bactérias lácticas, leveduras e fungos (*Rhizopus oligosporus*) (Wang *et al.,* 2007; Akande & Fabiyi, 2010).

Diversos estudos reportam que a fermentação promove a degradação das proteínas antigênicas (β-conglicinina e glicinina) e dos IPs, e há uma melhora significativa da digestibilidade da proteína. Além disso, a fermentação provoca a redução de mais de 80% da estaquiose e 50% da rafinose; e reduz o teor de fitatos e a atividade da hemaglutinina até em 49% (Francis *et al.,* 2001; Hajos & Osagie, 2004; Feng *et al.,* 2007; Khattab *et al.,* 2009).

A germinação, por sua vez, é um processo geneticamente programado e necessário para a transformação da semente, através da diminuição dos componentes indesejáveis (IPs, alcalóides e fitatos), e do aumento de nutrientes, como a vitamina C (Trugo *et al.,* 2000). Além disso, abrange processos de transporte e controle das rotas metabólicas e caracteriza-se pela formação de gradientes moleculares e celulares (Hajos & Osagie, 2004; Weber *et al.,* 2005).

Este processo inclui três tipos de mudanças bioquímicas na semente: hidrólise dos materiais de reserva, transporte destes materiais, principalmente, do endosperma até o embrião, e síntese de novos materiais a partir dos metabolitos originados na hidrólise (Akande & Fabiyi, 2010).

Vários autores observaram que à medida que a germinação avança há uma diminuição de até 4% no conteúdo de lectinas e uma redução na atividade dos inibidores tipo KTI e BBI, dos níveis de OS e de ácido fítico (Bau *et al.,* 1997; Murthy & Sun, 2000; Trugo *et al.,* 2000). Entretanto, os efeitos da germinação, sobre a composição e constituintes químicos na semente, variam com o cultivar e condições de germinação (temperatura, luz, umidade e duração do período) (Bau *et al.,* 1997).

2.5.3 Químicos

Os FANs, principalmente os inibidores tipo KTI e BBI, podem ser removidos pela extração com solvente (hexano, éter, álcool), ou inativados pela adição de ácidos ou bases (Osman *et al.,* 2002; Kocher, 2002). A redução da atividade dos IPs é favorecida por valores de pH alcalinos em relação a pH ácidos ou neutros, pois valores elevados de pH aceleram a clivagem das pontes dissulfeto (SS) presentes neles, as quais conferem termoestabilidade e maior resistência à desnaturação (Faris *et al.,* 2008; Akande & Fabiyi, 2010). Nesse sentido, Osman *et al.* (2002) observaram que a cocção de soja *in natura* com a adição de 1% de NaOH acelerou a inativação de IPs, porém não houve efeito com a adição de 1% de HCL. Similarmente, Akande & Fabiyi (2010) comprovaram que a maceração de soja em solução de bicarbonato de sódio 0,07% produziu maior diminuição da atividade do IPs do que quando utilizada uma solução de acido cítrico 0,1%, confirmando a maior estabilidade do inibidor em pH acido.

Adicionalmente, o ajuste de pH também permite modificar as propriedades da proteína da soja, seja isoladamente ou em conjunto com a aplicação de calor. Sabe-se que as proteínas globulares da soja (glicina e β-conglicinina) tem mínima solubilidade na faixa de pH 4,0-5,0 e máxima à

medida que os valores de pH se aproximam a 11-12. No entanto, estes valores também provocam a formação de compostos tóxicos como a lisinoalanina, fazendo-se necessária uma elevação de pH moderada (~8,0) com ligeiro aumento na temperatura (50-60°C). Nestas condições a solubilidade dos produtos aumenta de 45 para 56% (Barać *et al.,* 2004).

2.5.4 Enzimáticos

Diversas enzimas comerciais (papaína, pepsina, ficina e oxidases,) são utilizadas para reduzir os níveis de FANs presentes nas leguminosas; estas provocam a alteração da conformação da proteína e reduzem sua atividade biológica, aumentando o valor nutricional do grão (Barać *et al.,* 2004; Hajos & Osagie, 2004).

O pré-tratamento dos grãos com enzimas exógenas visa à ruptura de ligações entre proteínas e componentes voláteis, a oxidação reversível de aldeídos, para posterior remoção, produção de peptídeos com menor peso molecular e o aumento da solubilidade das proteínas nativas (Barać *et al.,* 2004).

Além das enzimas tradicionais, outras têm sido empregadas com o intuito de inativar os FANs, um exemplo destas é o sistema NADPH-tioredoxina (NTS), formado pela enzima tioreoxina redutase (TrxR) e NADPH. Neste sistema, a TrxR reduz o dissulfeto usando NADPH como doador de elétrons, e reduz o número de SS de KTI e BBI, provocando sua inativação e aumentando sua suscetibilidade ao calor e às enzimas exógenas (Faris *et al.,* 2008).

Por tanto, a utilização de enzimas exógenas constitui-se como um método alternativo que visa à remoção dos FANs, na ausência de equipamentos empregados no processamento térmico (Wiryawan & Dingle, 1999).

2.5.5 Genéticos

Os programas de melhoramento genético têm surgido com o intuito de criar cultivares com maior produtividade, maior adaptação a regiões com diversas características geográficas; e com ausência parcial ou total dos FANs que ocorrem naturalmente no grão (Olguina *et al.,* 2003, Putszai *et al.,* 2004; Vasconcelos *et al.,* 2006; Daleprane *et al.,* 2009). Contudo, os resultados obtidos não são consistentes, sugerindo que a transmissão hereditária de alguns FANs não é sistemática; adicionalmente, a obtenção de novos genótipos é um processo ao longo prazo, e pode ser limitada pela dificuldade na identificação e determinação quantitativa dos FANs no ingrediente (Wiryawan & Dingle, 1999; Hajos & Osagie, 2004).

Estudos genéticos comprovam que quatro alelos controlam as isoformas do KTI e que a ausência de todas elas é controlada por um alelo recessivo simples. Porém, o progresso é ainda limitado, pois esta proteína contém níveis relativamente altos de cistina e sua eliminação reduziria o nível total deste AAS na semente. Por outro lado, o desenvolvimento e utilização de linhagens de soja desprovidas de IPs reduziriam os custos decorrentes do processamento térmico, evitando a atividade residual (10-20%) dos IPs no grão e diminuindo as conseqüências indesejáveis do sobreprocessamento (Clarke & Wiseman, 2000; Barros *et al.,* 2008). Além disso, vários autores têm demonstrado que sojas geneticamente modificadas exibiram teores reduzidos

de OS (estaquiose+rafinose) e maior nível de proteína em relação à soja convencional, confirmando que há uma correlação negativa entre o nível de proteína e os açucares solúveis (estaquiose+rafinose) do grão (Parsons *et al.,* 2000; Karr-Lilienthal *et al.,* 2005).

2.6 Uso de enzimas da nutrição

2.6.1 Definição

As enzimas exógenas podem ser definidas como moléculas protéicas que agem como catalisadores biológicos de reações químicas, que atuam sobre substratos específicos, e aumentam a digestibilidade dos nutrientes. A principal característica da ação enzimática é a especificidade. Cada tipo de enzima atua sobre um composto ou substrato associado, cuja estrutura deve encaixar-se à da enzima de modo que os centros ativos coincidam perfeitamente e as enzimas não são alteradas nesse processo (Sifri, 1993; Motta, 2003).

2.6.2 Origem e obtenção

A maioria delas é obtida a partir de animais (amilase pancreática, lipase pancreática, pepsina), vegetais (bromelina, ficina, papaína) e microrganismos (fungos, bactérias e leveduras) (Tabela 1) (Mascarell & Ryan, 1997).

Tabela 1. Principais micro-organismos produtores de enzimas

Aspergillus niger	α-amylase, β-glucanase, cellulase
Aspergillus ficuum, A. candidus, A. sydowi	Fitase
Aspergillus oryzae	α-amylase, neutral protease
Bacillus licheniformis	α-amylase
Bacillus subtilis	α-amylase, neutral protease, β-glucanase
Trichoderma viridae	Xilanase, β-glucanase, cellulase
Aspergillus niger, Rhysopus sp	Lípase
Bacillus subtilis, Endothia parasítica, Mucor pusillus Aspergillus oryzae, A.flavus, A.niger	Proteases

(Adaptado de Mascarell & Ryan, 1997).

Para a obtenção das enzimas a partir das fontes citadas são empregados diferentes processos, os quais são selecionados de acordo com o grau de pureza desejado para o preparado enzimático a ser comercializado. Em termos genéricos, as etapas para a obtenção de enzimas, a partir das diferentes fontes, são: → Extração → Filtração/Centrifugação → Precipitação → Purificação → Secagem → Estabilização → Padronização → Embalagem. Na obtenção de enzimas deve-se levar em conta o fato da enzima de interesse ser extracelular ou intracelular, pois conforme o caso a seqüência das operações de separação/purificação será diferente (Vitolo, 2001).

2.6.3 Aspectos tecnológicos da utilização de enzimas

Durante sua elaboração, as rações são submetidas a diferentes processos que incluem moagem, mistura, tratamentos térmicos (extrusão/expansão), condicionamento e peletização. Cada um deles afeta negativa ou positivamente a qualidade do alimento, o desempenho do animal (Amerah *et al.*, 2011), assim como a atividade especifica da enzima adicionada

à ração. Sabe-se que as enzimas exógenas são suscetíveis às altas temperaturas e pressões geradas durante o processamento das rações. Por tanto, entre os vários aspectos que devem ser levados em conta quando se pretende suplementar uma enzima exógena na ração, a estabilidade e origem são, sem dúvida, os mais importantes.

Assim, enzimas que são mais termoestáveis, através de modificação genética ou por revestimento com ceras ou gorduras, rapidamente dissolvidas no intestino, têm vantagens sobre outras (Mascarell & Ryan, 1997; Silva & Smithard*,* 2002). Em geral, a temperatura ótima, para enzimas termoestáveis, é 85 a 90 °C, contudo esta temperatura é maior que a fisiológica do animal (~40 °C). Por isso, enzimas que exibem alto grau de termoestabilidade, podem ter sua atividade comprometida sob as temperaturas menores encontradas no intestino de aves e suínos (Silva & Smithard*,* 2002; Cowieson *et al.,* 2006b).

A estabilidade da enzima pode também ser modificada durante o trânsito através dos diferentes segmentos do TGI, devido às mudanças súbitas, e significativas de pH, e à ação das enzimas proteolíticas de origem endógena ou microbiana. Por conseguinte, seu uso dependerá do pH e da temperatura do local onde a enzima desenvolverá sua atividade máxima (Marquardt *et al.,* 1996; Mascarell & Ryan, 1997).

Quanto à origem, sabe-se que a maioria das enzimas possui origem microbiana (bacteriana ou fúngica) que determina a estrutura molecular e o padrão de atividade da enzima. Deste modo, a atividade das enzimas fúngicas é favorecida por valores de pH baixos (>5,0), enquanto que o pH ótimo para enzimas de origem bacteriana está próximo à neutralidade; estas, por sua vez, são mais resistentes ao calor que aquelas derivadas de fungos (Mascarell & Ryan, 1997; Ao *et al.,* 2008; Bharathidhasan *et al.,* 2009).

2.6.4 Função

O aumento significativo da utilização das enzimas exógenas, nas últimas décadas, ocorreu em função do aumento do custo das matérias-primas convencionais, da utilização de ingredientes alternativos e da tentativa de redução da excreção de nutrientes não digestíveis no meio ambiente (Araujo *et al.,* 2007).

Durante o trânsito da digesta através do TGI, principalmente intestino delgado, há um declínio na concentração de enzimas endógenas e de ácidos biliares à medida que eles são catabolizados e/ou absorvidos, tornando-se um ambiente altamente propício à colonização bacteriana (Bedford, 2000). Assim as enzimas exógenas deslocam o sítio de digestão e absorção de nutrientes para um local anterior, principalmente duodeno, no qual a ave possui uma maior vantagem competitiva sobre sua microbiota residente, devido às altas concentrações de enzimas pancreáticas e enterócitos altamente ativos (Bedford, 1996).

Em geral a adição de enzimas exógenas visa: a) o incremento do valor nutritivo das matérias primas convencionais e alternativas, através da remoção ou hidrólise de fatores antinutricionais; b) a suplementação das enzimas endógenas, produzidas em níveis insuficientes, em animais jovens, ou inativadas na presença de FANs e c) o aumento da digestibilidade dos nutrientes (Mascarell & Ryan, 1997; Choct, 2006; Cowieson *et al.,* 2006b; Plumstead & Cowieson, 2007).

Atualmente, as enzimas majoritariamente empregadas são as carboidrases e as fitases seguidas das α-amilases e proteases, em menor proporção. As carboidrases liberam o amido e a proteína encapsulados na matriz da parede celular e reduzem a quantidade de substrato não digerido disponível para fermentação ileal ou cecal (Mateos *et al.,* 2002; Plumstead & Cowieson, 2007). Já as fitases disponibilizam o fósforo complexado e diminuem sua presença nas fezes, reduzindo seu potencial poluente do meio ambiente. Além disso, reduzem os custos de formulação, pelo aporte de P adicional, que quando contabilizado, substitui as fontes de fosfato inorgânico (Silva & Smithard*,* 2002; Araujo *et al.,* 2007; Costa *et al.,* 2007).

Contudo, o fato das enzimas serem específicas em suas reações determina que os produtos que tenham só uma enzima sejam insuficientes para produzir o máximo benefício. Isto sugere que misturas de enzimas sejam mais efetivas no aproveitamento dos nutrientes das dietas. Em função disso, tem sido proposta a adição de enzimas exógenas, principalmente na forma de *blends* ou complexos multienzimáticos de modo a melhorar a qualidade nutricional de ingredientes alternativos e reduzir a variação no valor nutricional entre ingredientes convencionais (Yadav & Sah, 2005).

Os *blends* são combinações de enzimas ou de produtos de fermentação, obtidos a partir de cepas selvagens de microrganismos, que expressam uma ampla gama de actividades enzimáticas. Estes produtos têm diferentes concentrações de xilanases, β-glucanases, e celulases, mas também têm outras actividades enzimáticas, tais como amilases, proteases e lipases. Em contraste, as enzimas mono-componentes são oriundas de um microorganismo único e produzidas pela selecção do DNA que codifica a enzima, o qual é clonado num hospedeiro microbiano. Durante a fermentação, o hospedeiro que contém o DNA clonado libera a enzima desejada a um meio de cultivo, o qual é purificado· posteriormente (Freitas *et al.*, 2011).

O efeito da adição de *blends* às dietas para frangos de corte à base de milho e farelo de soja, ou de cereais de inverno (trigo, cevada, centeio) os quais aumentam a viscosidade da digesta, interferindo com a atividade das enzimas endógenas no TGI (Gracia *et al.,* 2003), é amplamente reportado na literatura (Silva & Smithard*,* 2002; Cowieson & Adeola, 2005; Olukosi *et al.,* 2007; Cowieson & Ravindran, 2008). Em geral, estes agem sobre uma ampla gama de substratos e são capazes de melhorar o desempenho zootécnico dos animais devido à redução de perdas endógenas e ao aumento na digestibilidade dos nutrientes, devido ao maior acesso aos nutrientes encapsulados associado à hidrólise da fibra e dos FANs proteináceos (Cowieson, 2005; Cowieson *et al.,* 2006c).

O efeito da adição de enzimas mono-componentes como a protease é avaliado majoritariamente sob condições *in vitro*. A literatura reporta que o pré-tratamento de farelo de soja com protease melhora a solubilidade da proteína e diminui a atividade residual dos ITs, após a inativação parcial destes durante a extração com solvente e tostagem, demonstrando que a enzima provoca a hidrólise das proteínas da soja, especificamente das proteínas de reserva, (conglicinina e β-conglicinina) e dos FANs de natureza protéica (Caine *et al.,* 1998; Rooke *et al.,* 1998; Sorbara *et al.,* 2009). Entretanto, a melhora da qualidade nutricional do farelo de soja depende das condições de incubação (temperatura e pH) e da origem da protease (bacteriana ou fúngica). Por outro

lado, estudos *in vivo* apresentaram a eficácia da suplementação de protease em dietas vegetais para frangos de corte, demonstrando que a enzima mono componente aumenta a digestibilidade da proteína bruta da dieta e melhora a conversão alimentar, porém sem exibir efeito sobre o rendimento e composição da carcaça (Garcia, 1997; Ghazi *et al.,* 2002, Yadav & Sah, 2005).

Em geral, a adição de protease suplementa a produção de serina proteases endógenas, reduzindo o requerimento por AA e energia (Mascarell & Ryan, 1997; Cowieson, 2005). Esta informação sugere que esta pode melhorar o valor nutricional da soja pela hidrólise dos FANs proteináceos, das proteínas antigênicas e daquelas que têm sido danificadas durante o processamento (Cowieson *et al.,* 2006b), entretanto as respostas são específicas de cada espécie e dependem da idade, tamanho do TGI, e da capacidade de consumo (Cowieson *et al.,* 2006a).

2.7 Avaliação da efetividade do tratamento

O conteúdo de FANs e/ou a composição química da soja não são influenciados só pelo genótipo, região geográfica de crescimento, maturidade, mas também pelas condições de processamento (Qin *et al.,* 1998). Portanto, diversos métodos físico-químicos têm sido desenvolvidos com o intuito de avaliar a efetividade dos processamentos térmicos empregados na inativação dos FANs e na melhora da qualidade nutricional do grão. Usualmente, a indústria de alimentos para animais mostra resistência ao uso de estudos de alimentação conduzidos em animais devido ao fato de que eles são trabalhosos, mais longos e mais caros do que os métodos físico-químicos caracterizados pela relativa facilidade de execução, baixo custo e rapidez (Newkirk, 2010).

No entanto, a qualidade do tratamento térmico também pode ser testada através da determinação de FANs específicos como as lectinas e inibidores de tripsina (ITs). As lectinas são quantificadas pelo ensaio de hemaglutinação. O extrato de soja é colocado em placas contendo eritrócitos. Diluições variáveis de extrato de soja são usadas e a menor concentração requerida para aglutinar os eritrócitos e é determinada e usada para calcular a concentração de lectinas viáveis na soja (Kennedy *et al.,* 1995).

A quantificação dos ITs, FANs termolábeis mais importantes da soja, é acompanhada pela medida da atividade residual da enzima tripsina após que um extrato de soja que contém inibidor ter sido adicionado a uma fonte purificada da enzima. O grau ao qual o extrato inibe a atividade é calculado e expresso como atividade inibitória da tripsina (Kakade *et al.,* 1974). Contudo, estes ensaios são muito técnicos e não fazem parte dos sistemas rotineiros de controle de qualidade das plantas de processamento de soja.

Adicionalmente, os níveis de ITs estão estreitamente relacionados ao conteúdo de urease. Esta enzima não é um antinutriente *per se*, mas desnatura-se quando o calor é aplicado de maneira similar aos ITs, podendo ser usada como indicador da destruição destes (Lilburn, 1996). A atividade ureásica (AU) é amplamente usado na indústria como indicador do processamento térmico adequado (AOCS, 1980a). Contudo, seu uso é limitado, pois somente identifica o subprocessamento do farelo de soja. Em geral, AU igual ou menor a 0,3 ∆pH indica que a amostra foi processada apropriadamente, já valores acima de 0,5 ∆pH indicam que foi subprocessada

e valores próximos de 2,5 ΔpH são encontrados em sojas cruas. Entretanto, a utilização do AU como critério para avaliar a qualidade do processamento térmico sempre foi fortuita e questionada (Vohra & Kratzer, 1991; Kimball & Provenza, 2003; Willis, 2003; Palić *et al.,* 2008). Adicionalmente, ainda com a total inativação da urease há uma atividade residual dos ITs (até 45%), pois esta é mais sensível ao calor do que os ITs, desnaturando-se mais rápido (Wang & Johnson, 2001).

O sobreprocessamento favorece a formação de ligações cruzadas ou de reações de Maillard, as quais afetam a digestibilidade da proteína; e pode ser avaliado através do ensaio de solubilidade protéica em KOH 0,2%. Neste ensaio, a soja é misturada com 0,2% de solução de KOH e o nível de proteína solúvel é medida e reportada como uma fração da proteína total. Já a aplicação excessiva ou prolongada de calor produz valores baixos de solubilidade protéica, implicando numa maior hidrólise dos ITs e menor proporção de AA disponíveis para o animal, enquanto que a aplicação insuficiente de calor produz valores altos de solubilidade protéica, indicando uma hidrólise menor dos ITs e, portanto baixa digestão das proteínas pelas enzimas intestinais (Araba & Dale, 1990a, Vohra & Kratzer, 1991; Lilburn, 1996; Shin, 2002).

O índice de dispersibilidade de proteína (IDP) é determinado segundo o método da AOCS (1980b), destacando-se pela técnica de rápida agitação e a medição da proteína presente no sobrenadante (Batal *et al.,* 2000), cujo objetivo é verificar o sobreprocessamento do farelo de soja. De maneira geral os valores de IDP diminuem linearmente à medida que o período de processamento térmico aumenta (Căpriţă & Căpriţă, 2009). Em geral, o farelo de soja processado adequadamente apresenta valores de IDP entre 45 e 50%; valores de solubilidade protéica entre 78 a 84% e valores de AU entre 0,05 até 0,2. No entanto, estas são apenas diretrizes, pois ainda há controvesias sobre os padrões exatos que devem ser usados devido aos problemas associados com a aplicação desses métodos nos diferentes laboratórios (Willis, 2003; Newkirk, 2010).

3. HIPÓTESES E OBJETIVOS

3.1 Hipóteses

O tratamento térmico da soja inativará ou reduzirá a atividade dos fatores antinutricionais presentes no grão *in natura*.

Frangos de corte machos consumindo dietas que com soja integral termicamente tratada obterão respostas positivas no desempenho zootécnico, no rendimento de carcaça e na integridade do pâncreas e no intestino delgado pela suplementação de protease mono-componente.

Haverá resposta linear positiva do desempenho zootécnico, do rendimento de carcaça e da integridade da mucosa intestinal à suplementação de níveis crescentes de protease mono-componente em dietas com soja integral termicamente tratada.

3.2 Objetivos

Entender o efeito do processamento térmico sobre a atividade dos fatores antinutricionais presentes na soja integral termicamente tratada.

Determinar as respostas de desempenho zootécnico, rendimento de carcaça e peso relativo de pâncreas e de intestino delgado de frangos de corte consumindo dietas com soja integral termicamente tratada e suplementadas com protease mono-componente.

Avaliar as respostas de desempenho zootécnico, rendimento de carcaça e morfometria intestinal de frangos de corte consumindo dietas com soja integral termicamente tratada e suplementadas com protease mono-componente.

CAPÍTULO II[3]

[3] Artigo escrito conforme as normas do Food Chemistry

Quantitative analysis of antinutritional factors in commercial raw soybean grain after thermal processing

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Abstract

Raw soybean contains antinutritional factors (ANFs) which are inactivated by heat processing. Processing efficacy was evaluated through *in vitro* estimation and the ANFs in autoclaved soybean (0, 105, 110, 135 min) and soybean meal (SBM) was measured. Urease index, determined indirectly, was not linear with increasing autoclaving time and it felt rapidly as autoclaving time augmented. Protein solubility slightly decreased after 135 min and approached 100% in raw soybeans. Soybeans autoclaved for 135 min and SBM showed the highest residual trypsin activity, and raw soybean the lowest. Quimotrypsin residual activities showed a similar trend, however reduction magnitude was lower than those seen in trypsin activity. Urease activity, determined directly, was higher in raw soybean *vs.* SBM, and decreased as autoclaving time increased. Only raw soybean contained lectins, which was fully destroyed after 105 min. SDS-PAGE from raw and processed soybeans showed quantitative differences which became less evident as autoclaving time increased.

Keywords: Antinutritional factor, Broiler chicken, Heat treatment, Soybean, Urease

1. Introduction

Soybean [*Glycine max* (L) Merril] is important as a protein source, being part of diets for non-ruminant livestock. Soybeans provide an excellent source of proteins, with an acceptable amino acid profile, and essential lipids (Haddad & Allaf, 2007).

However, raw soybean contains high concentrations of secondary plant metabolites, also referred as antinutritional factors (ANFs) (Becker-Ritt et al., 2004). These bioactive substances regulate protein turnover, enzyme secretion, and play an important role in plant protection through biological control (Oliva & Sampaio, 2009). They also may impair growth rate, feed efficiency, nutrient digestibility and health status due to a variety of underlying mechanisms (Acamovic & Brooker, 2005; Jezierny, Mosenthin & Bauer, 2010). The most commonly ANFs found in soybeans are condensed tannins, lectins, glycosides, phytate, alkaloids, saponins and, mainly, protease inhibitors (PIs) (Becker-Ritt et al., 2004).

Nevertheless, most of the ANFs are inactivated by proper thermal treatments (Haddad & Allaf, 2007). They range from methods of treating the whole bean, resulting in a full-fat feed ingredient, to processes that combine oil extraction and heat treatment to produce an oil-free product devoid of ANFs. The method used is determined by the requirements of the intended use, the scale of production, and the cost of the process (both capital and operating) (Newkirk, 2010).

Heat processing induces conformational changes in storage proteins, which may render them more accessible to digestive enzymes, and thus may increase protein digestibility (Jezierny et al., 2010), whereas, over-processing leads to Maillard reactions that reduce protein digestibility (Zarkadas & Wiseman, 2005; Valencia, Serrano, Centeno, Lázaro, & Mateos, 2008). Other ANFs, such as the antigenic proteins and the oligosaccharides, are not destroyed by heat but can be deactivated by alcohol extraction or fermentation (Valencia et al., 2008).

Thus, it is necessary to evaluate the adequacy of soybean processing to ensure they have been processed sufficiently to eliminate the ANFs present (Newkirk, 2010). Soybean quality can be assessed using *in vivo* feeding studies; however they are time-consuming, costly, and

complex. However, the quality of processing treatment can also be evaluated by either measuring specific anti-nutrients, such as trypsin inhibitors (TIs) and lectins, or using *in vitro* assays, such as protein solubility (PS) or urease index (UI), which indirectly determines the ANFs destruction by heat processing. (Van Eys, Offner, & Bach, 2004). Therefore, this study aimed to compare specific *in vitro* estimators of quality product with actual content of ANFs in processed soybeans used in broiler chickens diets.

2. Materials and methods

2.1. Collection of the seed sample

Soybean seeds [*Glycine max* (L.) Merr] were purchased as *BMX Impacto RR* cultivar from a local commercial source (Viamão, RS, Brazil). The immature and damaged seeds were removed.

2.2. Processing of soybeans

To obtain decreasing levels of UI, as indicator of presence of ANFs, a batch of whole seeds of soybeans was randomly split into four fractions; they were not crushed before the thermal processing. Every fraction was autoclaved at 121 °C and 1.5 atm for 0, 105, 110, 135 min, respectively; at the Laboratório de Pesquisas Micológicas (LAPEMI), Universidade Federal de Santa Maria, RS, Brazil. Immediately after steam processing, the soybeans were put into trays and spread in one layer for cooling down for 40 min afterwards, processed soybeans were stored until analysis in a cooling chamber.

2.3. Analytical methods

2.3.1. Proximate composition

Moisture and protein contents were determined in raw, autoclaved soybeans and soybean meal (SBM) according to standard methods (AOAC, 2000).

2.3.2. Urease index

UI was determined by the pH change method (AOCS, 1980). After incubation, the pH of the solutions is determined rapidly and the difference between pH of test and pH of blank is calculated as UI.

2.3.3. Urease activity

A 20 μl aliquot of urea solution (100mM) was mixed with 20 μl of seed crude extracts and

buffered with 0.02M sodium phosphate pH 7.5 to give a final volume of 500 μl. The reaction mixtures were incubated for 10min at 37 ºC and the ammonia released was measured colorimetrically (Weatherburn, 1967). One unit of urease activity was defined as the amount of enzyme required to release 1 μmol NH_3 min^{-1} at 37 °C and pH 7.5 under the conditions described.

2.3.4. Protein solubility (PS)

PS was determined according to the procedure of Araba & Dale (1990). The procedure involves incubation of 1.5 g sample with 75 mL 0.2% KOH (w/v; 0.036 N*)* solution for 20 min at room temperature using a magnetic stirrer. Following this incubation, the sample is centrifuged for 5 min at 6,000 rpm and the supernatant is analyzed for the protein concentration by the biuret method. The solubility of the protein, expressed as a percentage, was calculated by dividing the protein content of the KOH-extracted solution by the protein content of the original sample.

2.3.5. Antinutritional compounds

Autoclaved soybean seeds were finely ground and defatted by petroleum ether extraction (1:5 w/v, 24 h). For preparation of the crude extracts the meals were suspended in 10mM sodium phosphate buffer pH7.5 containing 10mM *β*-mercaptoethanol at 1:5 w/v ratio and stirred at 4 °C for 4 h. The extracts were kept at 4 °C in the presence of 0.02% sodium azide as preservative (Becker-Ritt et al 2004).

2.3.5.1. Trypsin inhibitory activity

The presence of compounds with trypsin inhibitory activity was analyzed using bovine trypsin (Sigma Chem Co) and two different substrates, according to Kakade, Simons & Liener (1969) with modifications, as follows. (1) Aliquots (10 μl) of crude extracts (1:5 w/v) were mixed with bovine trypsin (50 μg) and azocasein (Sigma Chem Co) at 0.8% w/v final

concentration, pH 8.0, in 500 μl final volume. After incubation for 30 min at 37 ◦C the reaction was stopped by adding trichloroacetic acid (TCA, 5% v/v), the mixture was centrifuged and the TCA-soluble material in the supernatant was quantified by absorbance at 420 nm. The inhibitory activity of soybean extracts was expressed as residual activity (%) of bovine trypsin assayed alone under the same conditions. (2) Aliquots (10 μl) of 20-fold diluted crude extracts were mixed with bovine trypsin (10 μg) and *N*-*α*-benzoyl-DLarginine- *ρ*-nitroanilide (BAPNA) at 1.5mM final concentration, pH 8.2, in 100 μl final volume (Becker-Ritt et al 2004). The hydrolysis of the substrate was followed at room temperature by taking readings at 405nm every 15 s using a SpectraMax (Molecular Devices Corporation, Sunnyvale, CA, USA) apparatus programmed with kinetic analysis software. The inhibitory activity of soybean extracts was expressed as residual activity (% of *V*max) of bovine trypsin assayed alone under the same conditions. Controls were run in parallel for each substrate in the absence of added trypsin in order to correct the experimental points for endogenous proteolytic activity of the soybean cultivars.

2.3.5.2. Quimotrypsin inhibitory activity

The presence of compounds with chymotrypsin inhibitory activity was analysed using bovine chymotrypsin (Sigma Chem Co) and its inhibition was assayed using the chromogenic substrate N-benzoyl-L-tyrosine-*ρ*-nitroanilide (BTPNA) in 0.08 M Tris–HCl buffer pH 7.8 containing 20% DMSO band 20 mM $CaCl_2$, according to Kumar, Sreeram, & Gowda (2002).

2.3.5.3. Total tannins

The content of tannins in the seeds was analyzed by a radial diffusion method in albumin-containing gels. For that purpose the defatted seed meals were extracted with 50% v/v methanol and centrifuged at 10 000 rpm for 15 min. The supernatants were collected, freeze-dried and suspended in 1/20 of the original volume in 50% methanol. Aliquots (5 and 10 μl) of the extracts were applied to wells in a 1% agarose gel containing 0.1% w/v bovine albumin, 50mM

acetic acid and 60 μM ascorbic acid. The diameter of rings representing tannin–albumin complexes was determined after 48 h at room temperature. A standard curve of tannic acid (50–600 μg, Sigma Chem Co) was run in parallel (Hagerman, 1987).

2.3.5.4. Lectin content

The haemagglutination activity of dialyzed crude extracts was assayed upon human type A^+ fresh erythrocytes in round-bottomed wells of microtiter plates; the reaction mixture was incubated for 2 hours, at room temperature (Vasconcelos, Cavada, Moreira, & Oliveira, 1991). The minimum concentration required for haemagglutination was visually determined. The haemagglutination titer, defined as the reciprocal of the highest dilution exhibiting haemagglutination, was reckoned as 1 haemagglutination unit. Specific activity is the number of haemagglutination units or per mg protein.

2.3.5.5. SDS-PAGE

Polyacrylamide gels (12%) containing 0.1% SDS (both Sigma Chem Co reagents) were prepared as described previously (Laemmli, 1970). Samples of 500 μg of defatted seed meal were extracted directly by vortexing with 500 μl of sample buffer containing 0.1% SDS. Samples were run for 2–3 h at room temperature at 30mA and protein bands were visualized by staining with Coomassie blue. Molecular weight marker from Fermentas (Thermo Fisher Scientific Inc.) was used.

2.4. Statistical Analysis

Data from all assays were subjected to ANOVA for completely randomized designs and the significance of difference among means determined by Tukey's test. The statistically significant difference was defined as $P < 0.05$.

3. Results and discussion

3.1. Proximate composition

The determined values of moisture in raw and autoclaved soybeans [*Glycine max* (L) Merril] and SBM (Table 1) were close to expected values (Agunbiade, 2000; Van Eys et al., 2004) and they are in agreement to those indicated by ANEC (National Grain Exporters Association Standards) for Brazilian raw whole soybean (maximum 14%) and SBM (12.5 to 13%). As a rule, 13% is the acceptable moisture level for soybean marketing, storage or processing. Lower moisture content is necessary to prevent grain spoilage, caused by molds and insect infestation. However, safe moisture levels depend on grain variety, length of storage and geographical location.

Furthermore, this value is within the range of values reported by Van Eys et al. (2004) (42 to 50%) and by Edwards, Douglas, Parsons, & Baker (2000) (47.5 to 62.7%) for SBM solvent extracted. In addition, the composition of SBM can be influenced by climatic conditions, geographical location, ANFs content in the seed and processing (Edwards et al., 2000).

Raw soybean (38.38%) and heat treated soybeans (38.01 to 39.01%) had considerably lower CP values since their high oil content. However, they were similar to those reported by Agunbiade (2000) (38.63%) and by Senkoylu, Samli, Akyurek, Agma, & Yasar, (2005) (38.00%) for raw soybean; and to those reported by Mendes, Oliveira, Costa, Pires, & Hoffmam (2009) (38.02 to 48%). Generally, autoclaving had little effect on moisture or CP contents of raw and heat treated soybeans.

3.2. Urease index (UI), urease activity (UA) and protein solubility (PS)

It is accepted that the presence of active TIs and other ANFs can be indirectly determined by measuring the residual urease activity present in soybean since both enzymes, TIs and urease, are deactivated during heating (Căpriţă & Căpriţă, 2009).

Determination of UI is based on measurement of pH change (ΔpH) and, due to its

simplicity, is probably the most widely used method for evaluating the quality of soybean processing in laboratories. Generally, raw soybeans will produce a pH change of approximately 2.5 ΔpH, but a properly heat-processed SBM will have a pH change of less than 0.2 units. Moreover, the recommended minimum level of urease, for SBM, is controversial, with acceptable values varying from 0.05 to 0.2 ΔpH (White, Campbell, & McDowell, 2000; Palacios et al., 2004).

In this study, UI was determined in raw and autoclaved soybeans and also in SBM. It was confirmed that the UI is not linear with increasing heating time and it falls rapidly from approximately 2.0 ΔpH to near 0.5 ΔpH as soybeans were incrementally heat processed (Table 1). Values above of 0.35 ΔpH are suggested to be safe for poultry and swine (Waldroup, Ransey, Hellwig, & Smith, 1985).

Literature reports that this enzyme is progressively destroyed by heat (Prachayawarakorn, Prachayawasin & Soponronnarit, 2004; Căpriţă & Căpriţă, 2009). However, UI values obtained here remained nearly constant up to 105 min and solely dropped to 0.49 when autoclaved for 135 min. and are higher than those reported in literature after a few minutes, even seconds, of heat-processing (i.e. extrusion, roasting) (Waldroup et al., 1985). These higher UI values, even after a larger time of autoclaving, are probably due to the lower grain temperature obtained in whole grains. Maybe soybean hulls impeded the release of latent heat from material surface and thereafter a rapid increase in internal grain temperature (Wiriyaumpaiwong, Soponronnarit, & Prachayawarakorn, 2002). Additionally, the lower UA values reported by several authors were obtained from commercially produced SBM or raw dehulled, solvent-extracted soybean flour placed on trays or in Erlenmeyer flasks, in layers not exceeding 2.5 cm in depth (Araba & Dale, 1990; Machado, Queiróz, Oliveira, Piovesan, & Moreira, 2008).

UA, determined directly by the phenol-nitroprussiate method (Weatherburn, 1967), was also performed. The relevance of direct urease determination in raw and heat treated soybeans is justified by the association of bacterial urease with ulceration of the gastric mucosa of vertebrates, and by the fact that the embryo-specific urease (from soybean) might mimic the

effects observed for microbial urease due to the homology observed among the plant seed and bacterial enzymes (Vasconcelos, Maia, Siebra, Oliveira, Carvalho, Melo, Carlini & Castelar, 2001).

UA was found in all soybean samples. Raw soybean had the highest value, whilst autoclaved soybeans UA values ranged from 3.46 to 1.92 urease units g^{-1} soluble protein, suggesting that urease was significantly reduced upon heat treatment (Table 3). Besides, raw soybean presented urease content six-fold higher than those found for SBM. These results confirm that residual urease still remains in autoclaved full-fat soybeans and in SBM after oil extraction, desolventization, and toasting processes, suggesting the insufficiencies of some processing techniques, which have led to the application of complementary methods, such as such enzyme addition, for the elimination of residual ANFs in processed soybeans.

The protein solubility in a solution of 0.2% potassium hydroxide (PS) is the most commonly used assay to detect over-processed SBM, although very high values are indicative of under-processed soybean. The reduction in protein solubility is caused by the destruction of helical regions of the proteins and subsequent disulfide bond breakage. Consequently, the proton of hydrogen in water molecules inside proteins will react with the sulfur of disulfide to form a sulfhydryl (Prachayawarakorn et al., 2004). PS is inversely related to degree of heat treatment. Therefore, the PS of raw soybean flour approaches 100%. In general, values above of 85% are indicative of under-processed SBM, while values below 70% may suggest a meal received excess heating (Wiriyaumpaiwong et al., 2002; Căpriţă, Căpriţă & Creţescu, 2010). In this study, KOH-PS changed very little up to 105 min of autoclaving and then slightly decreased to a solubility of 81.76% in soybeans autoclaved for 135 min (Table 1); however, those values are higher than those reported in literature (Wiriyaumpaiwong et al., 2002; Căpriţă & Căpriţă, 2009). The differences may be due to the variety of soybean or the method of autoclaving used. A large autoclave was used in this study to facilitate processing of larger quantities of soybeans, whereas a small laboratory autoclave was used in those studies cited in literature.

Usually, higher PS values are obtained when soybeans are heat processed prior to being

ground, than when they are ground first and then heat processed. In this study, soybeans were autoclaved intact thus decreasing the surface area exposed to the heat of the autoclave.

Thus, PS test is not sensitive enough to estimate the level of heat processing that a soybean product has undergone, but it is effective in differentiating overheated products from correctly processed ones. Solubility values between 78 and 84 % are recommended for SBM considered adequately heat processed (Newkirk, 2010).

3.3. Antinutritional compounds

Use of soybean in animal feeds is limited due to the presence of a number of ANFs. These mainly consist of heat labile (trypsin inhibitors, lectins, goitrogens) and heat stable (oligosaccharides) factors, which can be minimized or even inactivated through thermal processing and plant breeding (Clarke & Wiseman, 2000). However, excessive heating (over-heating) reduces the availability of lysine (via the Maillard reaction) and possibly, to a lesser extent, of other amino acids, whilst insufficient heating (under-processing) fails to completely destroy the antinutritional factors. Hence, laboratory tests are used to distinguish adequately processed SBM from under or over-processed meal (Căpriţă, Căpriţă, Gheorghe, Cretescu, & Simulesku, 2010).

Among the tests commonly used, UI is the easiest to perform, and is especially useful in detecting under-processed SBM. PS test is the most commonly used assay to detect over-processed soybean meal, although very high values are indicative of under-processed meal (Căpriţă et al., 2010). Nevertheless, these values indicated that, besides urease index, complementary measures of specific ANFs, such as TIs and lectins, should be performed to predict the effectiveness of the heat processing and the integrity of proteins Van Eys et al. (2004).

3.3.1. Trypsin and Quimotrypsin inhibitory activity

PIs are a unique class of proteinaceous ANFs found in raw soybeans that inhibit serine

proteases (trypsin, chymotrypsin and elastase) in the gastrointestinal tract (GIT). Proteases catalyze many physiological processes such as food digestion, tissue remodeling, host defense and blood coagulation (Losso, 2008). There are two main classes of TIs found in raw soybeans: Kunitz (KTI) and Bowman-Birk (BBI). They reduce trypsin activity and, to a lesser extent, chymotrypsin, and, therefore, impair protein digestion in poultry, swine and young ruminants (Newkirk, 2010).

Since their proteic nature, PIs are inactivated under the conditions of heat treatment leading to irreversible protein denaturation by ordinary cooking and moist-heat treatment, although some residual activity can be found even after thermal treatments. It is known that soy products retain 5-20% of the trypsin inhibitory activity originally present in raw soybeans (Lajolo & Genovese, 2002).

Therefore, PIs activities were assayed with azocasein (for general protease activity) or BAPNA (for trypsin-like protease activity). The procedure described to determine TIs activity is based on the ability of the inhibitors to form a complex with the enzyme and thus to reduce the enzyme activity. Uninhibited trypsin catalyzes the hydrolysis of a synthetic substrate BAPNA, forming a yellow-colored product and thus producing a change in absorbance Van Eys et al. (2004). Differences between soybeans submitted to different times of thermal processing were observed (Fig 1). The crude extracts from raw and autoclaved soybean were able to inhibit trypsin when two different substrates were used. Nevertheless, differences among the heat treated soybean evaluated appeared as the time of autoclaved increased, confirming that the rupture of disulfide bonds in the KTI molecules by heat treatment leads to reduction of TIs activity (Liener, 1994).

Generally, moist heat is essential for obtaining the marked reduction of TIs and that steaming under pressure produces the effect at a faster rate than steaming under atmospheric conditions. Values ranged from 12.72 to 98.10% and from 11.62 to 64.21% for BAPNA and azocasein, respectively (Fig 1). Soybeans autoclaved for 135 min and commercial SBM exhibited the highest residual trypsin activity, without significant differences between them.

Furthermore, solvent extraction *per se* has no effect on TIs inactivation, since extraction solvents used are hydrophobic, they are capable of dissolving soybean oil, but has a little effect or no effect on soybeans proteins. Thereby, TIs inactivation appears to be affected by the degree of heat treatment applied in the desolventizing step. Moreover, extraction is essentially done under anhydrous conditions, and it has been established that dry heat has no effect on protein quality (Del Valle, 1981).

Also, raw soybean presented the lowest residual trypsin activity value as expected. However it was no significant differences between raw and autoclaved soybean for 105 and 110 min, indicating that there was not complete inactivation of TIs (Fig 1). It was expected that the maximum autoclaving time (135 min) would allow a complete degradation of TIs; however, this did not occur, possibly due to soybean particle size (Fig 1). It was reported that steaming whole soybeans or cotyledons only partially inactivates TIs, apparently because of the large particle size, which retards heat penetration (Del Valle, 1981). Thus, reduced particle size prior to heat treatment could improve the effectiveness of temperature during thermal processing.

As a whole, the most common grain legume processing methods, such as cooking at atmospheric pressure and cooking under pressure, are efficient in inactivating most or even all of the protease inhibitory activity in raw and SBM samples. In this regard, Giami (2002) compared two processing methods (autoclaving or boiling) on TIs activity reduction. They noted that autoclaving was observed to be less effective in lowering TIs activity compared with boiling, suggesting that further processing, such as soaking prior to heat processing, may be necessary in order to completely destroy them. Thereby, this inactivation is time and temperature dependent process and can vary in the grain matrix compared to the more stable purified forms (Lajolo & Genovese, 2002).

In regard with BBI activity, raw soybean presented the lowest Residual Quimotrypsin Activity (Table 2). Significant differences among raw and autoclaved soybeans autoclaved for 105, 110 and 135 min were detected. Quimotrypsin residual activities increased as autoclaving times increased. In addition, SBM presented the highest value. Reduction magnitude in BBI

activity was lower than those seen in KTI activity, possibly for two reasons. Firstly, the method used for measuring Trypsin inhibitory activity reflects the concentration and effects of two distinctively different types of inhibitors namely the KTI and the BBI, hence measure chymotrypsin inhibitory activity only express the BBI activity through the utilization of BATPNA substrate. Secondly, BBIs are highly structured proteins. The rigid structure of BBI proteins consisting of a well conserved skeleton of cysteine residues, which form seven disulfide bridges, is thought to prevent structural changes induced by heat treatment, acidic conditions or the action of proteolytic enzymes of the upper GIT *in vitro* (Clemente, Jimenez, Marin-Manzano & Rubio, 2008).

As a whole, the extent to which TI activity is destroyed by heating is a function of temperature, duration of heating, particle size, and moisture conditions. In this regard, literature reports that reduction of TIs content varied between samples, which is attributable to many factors including source of soybean and processing conditions (Haddad & Allaf, 2007; Clarke & Wiseman, 2007). However, not all of the processing variables are given in the description of the method of these studies which makes comparisons difficult.

3.3.2. Total tannins

Literature reports variable tannin content in heat treated soybean samples (i.e. extruded, raw, soaked, toasted) (Gehan & Amin, 2010; Odumodu, 2010; Stech, Carneiro, & Carvalho, 2010). These values were obtained from different (i.e. the Folin-Denis, Prussian blue, and vanillin-HC1 reaction) assays which detect all phenolic groups. Although they may give little quantitative information about tannin content, and some may seriously overestimate tannin content. Also, hydrolysable tannins are not detected by these assays, moreover reliable chemical assays specific for hydrolysable tannins have not been developed (Mehansho, Butler & Carlson, 1987). Thus, in this study, tannins were determined in all samples of raw and autoclaved soybeans according to the radial diffusion assay. In this assay, tannin diffuses through a protein-containing gel, and a visible disk-shaped precipitate develops as the tannin interacts with the

protein. The area of the ring is proportional to the amount of tannin in the extract. Besides, the rings expanded slowly, and reached their equilibrium sizes only after long incubation period. The amount of time required for the rings to reach equilibrium increased with increasing tannin concentration. An incubation time of 96 h after sample application was performed in this study, nonetheless any ring was observed, suggesting that this cultivar does not possess neither condensed nor hydrolysable tannins (Hagerman, 1987).

Furthermore, it is possible that soybeans extracts analyzed in this study contain low levels of tannins. Hence, polyphenolic metabolites present in soybeans may bind strongly to protein without causing precipitation (Bennick, 2002). Possibly, soybean cultivar used in this study may be an improved genotype, originated from breeding programs with reduced the content of AFNs to a safe extent, including tannins. These free-ANFs cultivars allow a wide utilization in both human consumption and for animal feeding. It seems that the most of the single-cross hybrids shows lower levels of tannins which recommend them as good source for livestock feed (Malencic, Maksimovic, Popovic, & Miladinovic, 2008).

In addition, it is known that tannin levels of soybean cultivars vary greatly with stage of development, seasonal changes and magnitude of fertilization. Also, condensed tannins are higher in plants growing under nutrient stress (Kraus, Zasoski1, & Dahlgren, 2004). Thus, the absence of tannins in those extracts may be due favorable soil conditions and suitable nutrient supply of soybeans assessed.

3.3.3. Lectin content

Soybean agglutinins (SBAs), also known as lectins, are tetrameric glycoproteins that specifically binds to terminal N-acetyl-D-galactosamine with greatest affinity and to a lesser extent with D-galactose (Fasina, Classen, Garlich, Swaisgood, & Clare, 2003; Michiels, Van Damme, & Smagghe, 2010).

Furthermore, SBAs are stable over a wide pH range and are resistant to insect and animal the TGI enzymes (Murdock & Shade, 2002). Moreover, SBA retains at least 50% of its

biological activity during passage through the small intestine (Zang, Li, Piao, & Tang, 2006).

SBAs activity was measured against human type A erythrocytes, since SBA binds extensively to N-acetyl-D-galactosamine (Maenz et al., 1999), which is the sugar determinant conferring A blood group specificity (Sharon & Lis, 2004). In this study, it was seen that only raw soybean contained SBA, which was completely destroyed after 105 min of autoclaving (moist-heat), confirming that although the compact globular structure, molecular aggregation and glycosylation of SBA confer high structural stability to SBA; thermal processing is a powerful denaturing agent leading to protein unfolding through breaking of hydrogen bonds, which results in a disrupted protein structure and therefore a loss of their biological properties (Paiva, Gomes, Napoleão, Sá, Correia, & Coelho, 2010). Hence, the degree and duration of heat exposure and the moisture levels employed here is a cost-saving option effective in inactivating SBA contained in whole raw soybean.

Although SBAs are usually reported as being heat-labile, their stability varies between plant species. It is known that they are more resistant to inactivation by dry heat and require the presence of moisture for more complete destruction. This may explain why low residual, but significant levels of SBA activity were detected in conventionally processed SBM (Mikić, Perić, Đorđević, Srebrić, & Mihailović, 2009). Nevertheless, SBM used in this study did not show any SBA activity, confirming that industrial feed processing destroyed the quaternary structure required for multiple binding sites and agglutination activity of SBA (Maenz et al., 1999).

3.3.4. SDS-PAGE

The seed flours were extracted directly into the denaturing sample buffer for electrophoresis and the protein patterns were visualized on SDS-containing polyacrylamide gels. The electrophoretic patterns of distribution or relative abundance of proteins from raw and processed soybean reveal quantitative differences in protein bands among the soybean samples. Protein bands became less evident as autoclaving time increased (Fig 2).

It was seen that processing whole soybeans without removing the oil involves the elimination of ANFs through the application of heat, in measured amounts and lengths, which denatures TIs and possibly storage proteins present in raw soybeans (Fasina et al., 2003), producing changes in the intensity of protein bands of each soybean sample (Fig 2).

As a rule, soybean storage proteins (7S and 11S) and the Kunitz and Bowman-Birk TIs present in raw grains are collected into protein bodies. Opportunities for interactions and reactions among these proteins should be enhanced as a result of their close proximity in protein bodies (Anderson, 1992). It is believed that ANFs, such as TIs present in raw soybeans were destroyed for the most part by a moist heat treatment. Although steam would be expected to heat protein bodies from the outside surface toward the center, formation of an insoluble, and denatured layer of protein on the surface would likely retard penetration of moisture into the interior of the protein body (Anderson, 1992). Hence, in an incomplete denaturation of ANFs is possible. The close proximity of proteins contained within the protein bodies would be expected to lend a measure of stability to the remaining undenatured protein. At the same time, these proteins present could represent SBAs. They remain in small but significant amount of active forms, even after dry or moist heating at 70 °C for several hours (Li, Qiao, Zhu & Huang, 2003).

4. Conclusion

It was identified variations in amount of proteinaceous ANFs among whole raw and autoclaved soybeans during different periods. This variation may be due to the effect of moist-heat on protein denaturation and, therefore, on ANFs activities. This research provides useful data for nutritionists considering variables involved in thermal process and its impact on ANFs inactivation. Moreover, it was demonstrated that UA can reflect protein quality and ANFs levels contained in processed soybeans, which is important since a more detailed quantification of ANFs in extract of soybeans performed here is too technically challenging for day-to-day use on-site by soybean processors.

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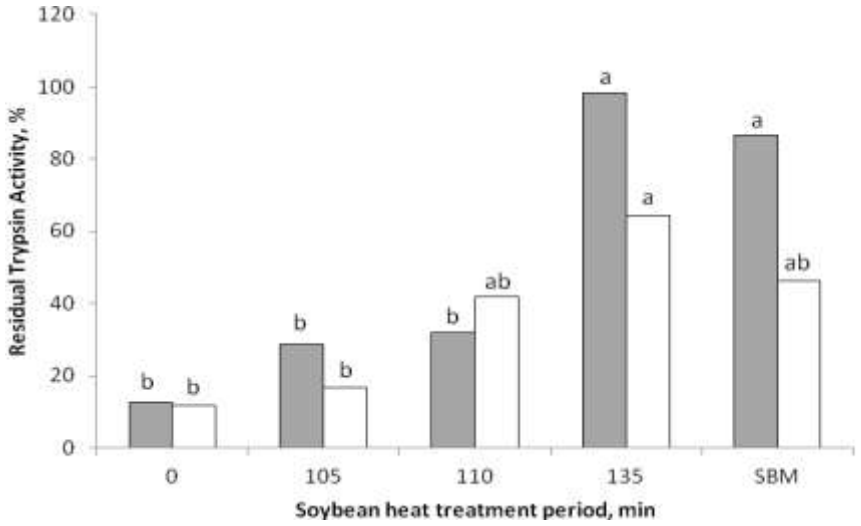

Fig. 1. Trypsin inhibitory activity of crude extracts of heat treated soybean. The dialyzed crude seed extracts were tested for inhibition of bovine trypsin using BAPNA (shaded bars) or azocasein (white bars) as substrate. Values are the mean ± SD of three determinations with triplicated points and represent the residual activity of a given amount of bovine trypsin in the presence of the seed extracts. Means with different letters differ significantly *(α* = 0.05).

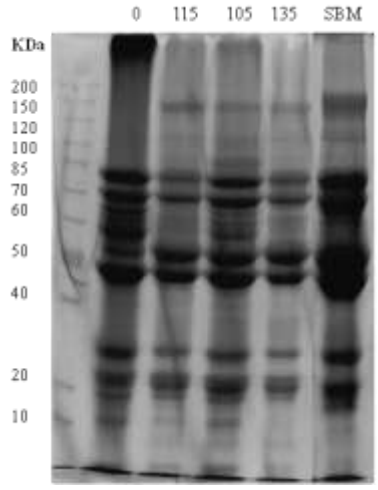

Fig.2. 12% polyacrylamide/0.1% SDS gels of raw, autoclaved soybean and commercial SBM extracts. Samples of 500 μg of defatted meal were suspended in 500 μl of denaturing sample buffer. After centrifugation, 15 μl of the supernatant was applied to the gel. Protein bands were stained with coomassie blue. Lane 1 molecular mass markers; lane 2 whole raw soybean; lanes 3 to 5 whole raw soybean autoclaved at 120°C and 1.5 atm. for 110, 105 and 135 min; lane 6 comercial SBM solvent-extracted.

Table 1

Proximate composition, Urease Index (UI) and Protein Solubility (PS) values of whole raw soybeans autoclaved during different times.

Thermal treatment (min.)[a]	Moisture (%)	Crude Protein (%)	UI (Δ pH)[b]	PS (%)
0	12.21	38.48	2.02	87.30
105	11.55	38.01	1.86	86.91
110	14.08	39.01	1.03	81.53
135	11.35	38.36	0.49	81.76
SBM	12.69	46.48	0.04	81.23

[a]Whole soybeans were autoclaved for 105, 110, 135 minutes (120°C and 1.5 atm.)
[b]The measurement method followed the AOCS procedure (1980) and it was performed in duplicate.

Table 2

Residual chymotrypsin activity of crude extracts of whole raw soybeans autoclaved during different times using BATPNA as substrate.

UI (Δ pH)	Residual Chymotrypsin activity, %
0	5.19 ± 0.18 d
105	9.43 ± 1.41 c
110	20.19 ± 0.83 b
135	23.45 ± 1.94 b
SBM	33.03 ± 1.08 a

Means with different superscripts are significantly different by Tukey test at 5%.

Table 3

Urease activity (UA) in crude extracts of whole raw soybeans autoclaved during different times.

UA (Δ pH)	Urease units g^{-1} soluble protein
0	4.36 ± 0.03 a
105	3.45 ± 0.14 b
110	3.46 ± 0.30 b
135	1.92 ± 0.11 c
SBM	0.78 ± 0.01 d

Means with different superscripts are significantly different by Tukey test at 5%.
The crude extracts were assayed for urease activity by the phenol–nitroprussiate method (Weatherburn, 1967). One unit of urease activity was defined as the amount of enzyme required to release 1 μmol NH_3 min^{-1} at 37 °C, and pH 7.5. Values are the mean ± SD of three determinations (in triplicate).

CAPITULO III[4]

[4] Artigo escrito conforme as normas do Food Chemistry

Effect of a monocomponent protease on broiler performance fed diets containing heat processed raw soybean

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Abstract

Raw soybean contains antinutrients, which depresses growth and enlarges chickens' pancreas. Those compounds can be reduced, but not completely inactivated by heat treatment. The effect of protease supplementation in diets containing soybean autoclaved 0 (RSB), 105 (ASB105), 110 (ASB110), and 135 min (ASB135) on performance, carcass yield and organ size was evaluated. Urease index (UI), determined indirectly, was similar for diets with or without protease. RSB and RSB+P presented the lowest trypsin residual activity, and RSB exhibited the lower quimotrypsin residual activity. Body weight gain and feed conversion were influenced positively by enzyme supplementation. ASB135 diet elicit better carcass yield, and protease supplementation enhanced carcass yield and diminished abdominal fat deposition.

Keywords: Antinutritional factor, Broiler chicken, Heat treatment, Soybean, Organ size, Protease

1. Introduction

Soybean meal is the primary source of vegetal protein in animal feeds, which is produced by extracting oil from soybeans (Clarke & Wiseman, 2007). Nonetheless, there is significant interest in the use of whole soybeans in poultry and swine diets in order to eliminate the cost of oil extraction and to allow its fat content ensures that high energy diets can be formulated. However, whole raw soybeans contain antinutritional factors (ANFs), such as protease inhibitors, lectins and saponins. These secondary metabolites are not essential for normal plant growth and development, and serve as chemical defenses against pathogenic microorganisms in the soil, during germination, as well as airborne pests prior to seed maturity (Chen, 2008).

Furthermore, these ANFs may interfere with nutrient digestion, and cause damaging hypersensitivity reaction (antigenic proteins), pancreatic enlargement and growth depression in monogastric animals (Vasconcelos, Brasil, Oliveira, Campello, Farias, & Carvalho, 2009). Nevertheless, most of these ANFs could be reduced or inactivated by heat treatment with moist treatment involving atmospheric pressure (autoclaving) preferred to dry heat treatment (Ilheukwuere, Ndubisi, Mazi, & Etusim, 2008). Additionally, heat treatment improves nutritional value of raw soybeans by unfolding the protein structure, thus making them more susceptible to attack by digestive enzymes.

However, differences in the soybean heat treatment conditions may have a marked influence on inactivation of ANFs and nutrient quality (Clarke & Wiseman, 2007). Thereby, many in vitro analytical methods, such as urease index (UI) and protein solubility (PS) (Yu, Wu, Liu, Gauthier & Chiou, 2007), are widely used as indicators of adequacy of processing and its consequences on nutritional value.

In fact, ANFs content in raw soybean is not always entirely neutralized by heat treatment. Thus, leading to addition of exogenous enzymes to supplement endogenous enzyme production and to alleviate deleterious effects of ANFs found in feed raw materials (Yu et al., 2007). Nonetheless, information on influence of supplementation of mono component enzymes, such as protease, in diets containing raw or heat processed soybean on animal performance is

scarce. On the whole, protease supplementation could help to neutralize the negative effects of the proteinaceous ANFs in addition to breaking down the large storage protein molecules into smaller, absorbable fractions increasing the solubility of soybean meal protein (Caine, Sauer, Tamminga, Verstegen, & Schulze, 1997).

Therefore, the present study was carried out to establish the effect of thermal treatment on ANF levels of soybeans and the effect of mono component protease supplementation on growth performance, carcass yield and organ size of broiler chickens.

2. Materials and methods

2.1. Collection of the seed sample

Seeds of Soybeans [*Glycine max* (L.) Merr] were purchased as BMX Impacto RR cultivar from a local commercial source (Viamão, RS, Brazil). The immature and damaged seeds were removed.

2.2. Processing method and preparation of the experimental diets

One batch of whole seeds of soybeans was randomly split into four fractions; they were not crushed before the thermal processing. Every fraction was autoclaved at 121 ºC and 1.5 atm for 0 (RSB), 105 (ASB105), 110 (ASB110), and 135 min (ASB135), respectively; at the Laboratório de Pesquisas Micológicas (LAPEMI), Universidade Federal de Santa Maria, RS, Brazil. Autoclaved soybean was cooled down for 40 min afterwards and stored in a cooling chamber until the elaboration of diets.

Every processed fraction was ground and used to compose the broiler diets (RSB, ASB105, ASB110 and ASB135) at a concentration of 20%, with (+P) or without 200 ppm of monocomponent serine protease (Ronozyme ProAct, 75,000 PROT units/g of enzyme, DSM Nutritional Products). All diets were formulated to be isonitrogenous and isocaloric (22% crude protein, 3,100 Kcal/kg ME energy) according to nutrients standards for the southern region of Brazil (Table 1).

Moisture and protein contents were determined in raw and processed soybeans, and in experimental diets were determined according to standard methods (AOAC, 2000). Additionally, urease index (UI) was measured in raw and autoclaved soybean prior to feed manufacture to verify increasing levels of urease, as indicator of the presence of ANFs, and also in all final diets by the pH change method (AOCS, 1980).

2.3. Animals

Four hundred, day-old, male broiler chicks were housed in a temperature-controlled

metabolism room, under continuous lighting. They were randomly allocated to 8 dietary treatments with 5 replicates each. Birds were allowed *ad libitum* access to feed in mash form and water throughout the 28 d of the trial. Dead birds were weighed daily and the used to correct feed conversion ratio (FCR).

2.4. Growth performance traits, carcass yield and organ weights

Body Weight (BW) and Feed Intake (FI) of birds were recorded at 7, 14, 21, and 28 d of age, for each treatment, and FCR and Body Weight Gain (BWG) were calculated subsequently. At 28 days of age, eight birds per pen were selected and identified. They were fasted, individually weighed and slaughtered to evaluate carcass yield and abdominal fat content according to the standard slaughter procedures (stunning, bleeding, scalding, plucking, chilling and dripping). Eviscerated carcass (without head, neck and feet) percentage was calculated as the ratio between the post-chilled eviscerated carcass and live Body Weight. The weight percentage of abdominal fat was calculated as a percentage of post-chilled eviscerated carcass weight. Simultaneously, the pancreas and duodenum were excised and weighed. Relative organ weights were expressed as percentages of whole live bird weight.

Additionally, on day 7, 14, 21 and 28 two birds per treatment were killed by cervical dislocation, their weight recorded and the pancreas also removed, freed from connective tissue and fat; and kept frozen for subsequent protein and DNA analysis. The protein content of the pancreatic homogenate was measured according to the method of Bradford (1976). Genomic DNA was extracted by using a PureLink™ Genomic DNA mini kit from Invitrogen (Carlsbad, CA) according to the manufacturer's recommendations; and quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) in order to calculate total pancreatic protein DNA and RNA.

2.5. Preparation of to the extracts measure antinutritional compounds

Autoclaved soybean seeds and the experimental diets were finely ground and defatted by

petroleum ether extraction (1:5 w/v, 24 h). Crude extracts were prepared as described by (Becker-Ritt, Mulinari, Vasconcelos, & Carlini, 2004).

2.5.1. Trypsin inhibitory activity

The presence of compounds with trypsin inhibitory activity was analyzed using bovine trypsin (Sigma Chem Co) and two different substrates, according to a modified method of Kakade, Simons & Liener (1969) (Becker-Ritt et al., 2004).

2.5.2. Quimotrypsin inhibitory activity

The presence of compounds with chymotrypsin inhibitory activity was analyzed using bovine chymotrypsin (Sigma Chem Co) and its inhibition was assayed using the chromogenic substrate N-benzoyl-L-tyrosine-ρ -nitroanilide (BTPNA) in 0.08 M Tris–HCl buffer pH 7.8 containing 20% DMSO band 20 mM $CaCl_2$, according to Kumar, Sreeram & Gowda (2002).

2.5.3. Urease activity (UA)

A 20 μl aliquot of urea solution (100mM) was mixed with 20 μl of autoclaved soybean seeds and experimental diets crude extracts and buffered with 0.02M sodium phosphate pH 7.5 to give a final volume of 500 μl. The reaction mixtures were incubated for 10min at 37 ºC and the ammonia released was measured colorimetrically (Weatherburn, 1967).

2.5.4. Lectin content

The haemagglutination activity of dialyzed autoclaved soybean seeds and experimental diets crude extracts was assayed upon human type A^+ fresh erythrocytes in round-bottomed wells of microtiter plates; the reaction mixture was incubated for 2 hours, at room temperature (Vasconcelos, Cavada, Moreira, & Oliveira, 1991).

2.5.5. SDS-PAGE

Polyacrylamide gels (12%) containing 0.1% SDS (both Sigma Chem Co reagents) were prepared as described previously (Laemmli, 1970). Samples of 500 μg of defatted meal from autoclaved soybean seeds and experimental diets were extracted directly by vortexing with 500 μl of sample buffer containing 0.1% SDS. Samples were run for 2–3 h at room temperature at 30mA and protein bands were visualized by staining with Coomassie blue. Molecular weight marker from Fermentas (Thermo Fisher Scientific Inc.).

2.6. Statistical Analysis

The experiment was completely randomized with 8 treatments arranged factorially with 4 periods of autoclaving (0, 105, 110, 135 min) and 2 levels of monocomponent serine protease supplementation (0 or 200 ppm). The results were subjected to a one-way analysis of variance and the significance of difference among means determined by Tukey's test. The statistically significant difference was defined as $P < 0.05$.

3. Results and discussion

3.1. Proximate composition and urease index

Formulated crude protein (CP) in the diets was 22.0% and the resulting concentrations were similar (Table 2).

The presence of ANFs in soybeans, particularly proteins, is often associated with impaired growth and nutrient utilization (Vasconcelos et al., 2009). However, many of these ANFs, such as trypsin inhibitors, which inhibit the proteolytic action of the pancreatic enzymes, are easily inactivated or minimized by proper heat processing. Generally, raw soybeans are submitted to extrusion or dry toasting prior to incorporation into feeds, or then used as soybean meal (SBM) produced by the solvent (usually hexane) extraction process.

Nevertheless, careful control of heating conditions is required to prevent under- or over-processing of SBM. Thereafter, feed manufacturers (or commercial soybean processing plants)

apply analytical methods to assess processing adequacy.

Today, UI measurement, based on change in pH (ΔpH), is probably the most widely analysis used to correlate processing conditions with improvement in protein quality and reduction in ANFs levels. This is due to the simplicity of the assay compared to the cumbersome analytical procedures required for measuring each antinutrient (Fasina, Classen, Garlich, Swaisgood, & Clare, 2003). However, this assay is used only for detecting undercooking, because the UI rapidly falls to zero as the SBM is heated. Generally, the optimum pH increase is considered to be between 0.05 and 0.20. Moreover, it is recommended that, when using soybean products for swine or poultry the increase in pH is not greater than 0.35 (Waldroup et al., 1985).

Nevertheless, analytical procedures are solely applied to determine whether SBM have received adequate heat treatment following oil extraction, which even after heat processing retain up to 20% of the residual TI activity (Stanojević et al., 2004). Furthermore, no data is available on the quality assessment of poultry feed (i.e. the presence of specific ANFs). Thus, in this study it was determined the presence of ANFs through direct and indirect test, such as UI, in diets fed to male broilers from 1 to 28 days of age.

It was observed that UI in processed soybeans incorporated into broiler diets was not linear with increasing autoclaving time and it ranged from 2.0, for raw soybeans, to near 0.5 when raw soybeans were autoclaved, at 120°C and 1.5 atm., for 135 min (results not tabulated). Additionally, increasing urease levels were obtained through the incorporation of autoclaved soybeans during different lengths of time. UI values for final diets varied from 0.13 to 1.52 ΔpH (Table 2). In this experiment, UI was relatively similar for diets with or without enzyme supplementation, however, values for ASB105, RSB, ASB105+P, and RSB+P are above of 0.35 ΔpH value suggested to be safe for poultry and swine (Waldroup, Ransey, Hellwig, & Smith, 1985). As a rule, adequately processed soybeans possess UI values in the range of 0.02 to 0.35 ΔpH, which corresponds to residual urease activity (%) between 10 and 20% (Prachayawarakorn et al., 2004). Thus, final diets may contain residual urease activity above

20%.

3.2. Antinutritional compounds

3.2.1. Trypsin and chymotrypsin inhibitory activity of experimental diets crude extracts

Protease inhibitors activities were assayed with azocasein (for general pancreatic protease activity) or BAPNA (for trypsin-like protease activity) in autoclaved soybeans, from 0 to 135 min, incorporated into experimental broiler diets. With either BAPNA or Azocasein as a substrate processed soybeans extracts exhibited an enhanced residual activity as autoclaving time increased, showing that heat processing (moist-heat) causes, at least in part, denaturation and inactivation of TIs. Autoclaving for 135 min caused a significant (P<0.05) improvement of residual pancreatic protease inhibitor activity (%) and residual Trypsin activity (%). Differences between soybeans submitted to different times of processing were observed.

In general, most commercially available soybean edible products have received sufficient heat treatment to cause inactivation of at least 80% of the trypsin inhibitor activity present in raw soybeans. However, this level of TI destruction is well above the threshold level of 50 to 60% inactivation found to be necessary for eliminating significant growth inhibition and pancreatic hypertrophy in rats (Liener, 1994). Hence, it was observed that crude extracts from all final diets contain TIs, which are able to inhibit trypsin when two different substrates were used.

It is known that soybean seeds contain large amounts of stored material, which are enzymatically degraded, to support early plant growth, mostly proteinaceous compounds such as TIs. They protect seed against invading microorganisms and insects and can have a major impact on nutritional value as they inhibit pancreatic serine proteases, thus impairing protein digestion (Guillamón et al., 2008). Moreover, cereal grains, such corn, must rely on other mechanisms to protect themselves from pathogen attack and infection. These mechanisms include synthesis of inhibitory compounds, such as phenols or tannins, as well as accumulation of proteins that can directly inhibit fungal growth. These include protease inhibitors and lectins (Chen, Brown, Russin, Lax, & Cleveland, 1999). In fact, TIs of both trypsin and α-amylase have

been isolated from Corn (*Zea mays*) kernels (Cowieson, 2005). Thus, residual activity level fond in broiler diets may also due to the presence of inhibitors in the main diet ingredients. Additionally, it was confirmed that Trypsin residual activities of broiler diets varied according to the type of soybean included. As expected, diets including raw soybean (RSB and RSB+P) presented the lowest trypsin residual activity (Table 2).

Residual quimotrypsin activity showed that raw soybean presented the lowest value. There was a significant difference among soybeans autoclaved for 105, 110 and 135 min. Quimotrypsin residual activities augmented as autoclaving times increased (results not shown). Final broiler diets including raw soybean exhibited the lower quimotrypsin residual activity values. There were not significant differences among soybeans autoclaved for 105, 110, and 135 min (Table 3) and they still show reduced quimotrypsin residual activity since the compact structure of the BBI and its stability with heat in its isolated form. This may be the result of a disulfide interchange of the disulfide bridges of the BBI with other cysteine-rich protein components comprising the soybean matrix, and these are reactions that may be accelerated by heat (Liener, 1994). Hence, increases, to various extents, in Trypsin and Quimotrypsin inhibitor residual activity depend upon the heat-processing times to which the raw soybeans, incorporated in broiler diets, were exposed. Besides, TIs inactivation is different in the grain matrix compared to the more stable purified forms (Lajolo & Genovese, 2002).

3.2.2. Urease activity

Processed soybeans incorporated at 20% level in broiler diets produced variable levels of urease activities in final diets. The resulting UA of these diets varied from 0.86 to 7.62. Experimental diets containing soybeans autoclaved for 135 min showed UA approximately nine-fold and 7-fold lower than those containing raw soybeans for unsupplemented and supplemented diets, respectively.

These results confirm that residual urease still remains in broiler diets even after commercial processing or traditional heat treatments, such as autoclaving. The UA of broiler

was also evaluated since the urease content varied between autoclaved soybeans incorporated. The concern with urease in this study is due to the association of bacterial urease with ulceration of the gastric mucosa on vertebrates (Cussac, Ferrero & Labigne, 1992).

3.2.3. Lectin content

The nutritional value of soybean meal is much lower than expected, in spite of its protein content and amino acid (AA) profile of the proteins. This is largely attributed to the presence of antinutritional factors, such as protease inhibitors and lectins, which constitute ~6% and ~0.5% of the protein of soybeans, respectively (Brandon & Friedman, 2002). Soybean agglutinins (SBA), or lectins, are glycoproteins that have erythrocyte-agglutinating activity. Polyvalent SBAs may induce toxic effects in animals by damaging the intestinal epithelium upon binding to the enterocytes, interfering with nutrient absorption (Fasina et al., 2003).

The inactivation of the SBA by moist heat treatment closely matches the destruction of TIs in soybeans, and, as in the case of the TI, lectins activity can be used to monitor the improvement in the nutritive value of the protein affected by heat treatment. In general, the higher the SBAs level in commercially processed meals the greater the potential toxicity of the poultry or animal diet in which they are included. In this study, SBA activity was tested by the haemagglutination assay, which is based on the ability of SBA to agglutinate erythrocytes. It was observed that broiler diets containing soybean autoclaved for 110 min (ASB110) and raw soybean (RSB) supplemented or not with protease did not show any SBA activity (Table 3). In general, common processing methods, such as autoclaving, are efficient in inactivating SBA. However, low residual levels of SBA activity were detected in broiler diets containing soybean autoclaved for 135 (ASB135) and 105 min (ASB105) supplemented or not with protease (Table 3). Those results were expected since measurable amounts of SBA were detected in a number of soybean products intended for human consumption and also in soybean oil (Liener, 1994).

Furthermore, final diets also contain other ingredients which may contain lectins. It is known that corn contains different ANFs (primarily phytate, amylase inhibitors and resistant

starches). Corn also contains variable concentrations of other ANFs such as TIs and lectins (Cowieson, 2005). Moreover, it was demonstrated that corn endosperm contain not one but several lectins distributed in protein fractions, namely globulins, zein, and glutelins. Also, corn kernel contained a Lectin with weak haemagglutinating activity as well (Jankovic, Cuperlovic, & Hajdukovic, 1990).

3.2.4. SDS-PAGE

The seed and experimental diets flours were extracted directly into the denaturing sample buffer for electrophoresis and the protein patterns were visualized on SDS-containing Polyacrylamide gels. Results show that no significant differences could be observed in the distribution or relative abundance of the major protein bands among diets, supporting that all diets possess similar protein content.

Evaluation of the native gel (not shown) shows some degree of variation in broiler diets proteins. This variation may be partially explained by the presence of autoclaved soybeans during different periods into broiler diets, which contain denatured protein, and by the contribution of other animal and vegetal origin ingredients present in all broiler diets. The stained bands represent soybean storage globulins mainly β-conglycinin (7S fraction) and glycinin (11S fraction), products of denaturation of enzyme inhibitors of serine proteases and α-amylase found in soybean and corn, and of others ANFs, such lectins and saponins, phytates, tannins which may remains active in feed even after heat treatment. Typically, protein denaturation begins with unfolding or dissociation of the protein quaternary structure into constituent subunits (the tertiary structures), which in turn associates through intermolecular interaction to form aggregates of irreversibly denatured molecules (Fasina et al., 2003).

3.3. Growth performance traits, carcass yield and organ weights

There were not any differences between FI of birds fed with diets including raw or autoclaved soybean. Overall, birds fed heat treated soybean had higher values of weight gain

compared with data for chicks fed diets with raw soybean. Heat-processing of soybeans increased the feed efficiency relative to raw soybeans, and the feed efficiency for birds fed diets with autoclaved soybeans for 135 min was significantly higher than that for 0, 105 and 115 min. Birds performance (body weight, body weight gain and feed conversion ratio) on d 28 of broiler chickens were influenced positively by enzyme supplementation besides the dilution of the substrate and possible interference by other diet components.

In general, literature reports that protease alone or in combination with other enzymes improves nutritive value of poultry diets, however results have been inconsistent. Positive responses to protease supplementation in broiler chickens were observed by Angel, Saylor, Vieira, & Ward (2011) supplementing increasing levels of the enzyme in diets with reductions in CP, lysine, total sulfur amino acids, threonine and metionine, by Yu et al. (2007) using either a mixture of protease and carbohydrase or a single protease, in a corn-SBM broiler diet, and by Cowieson, Singh, & Adeola (2006b) supplementing exogenous xylanase, amylase, and protease in a low nutrient density diet. Conversely, no responses were observed by Olukosi, Cowieson, & Adeola (2007) who used a blend of xylanase, amylase, and protease. Moreover, pre-feed treatment of SBM with proteases has produced inconclusive responses as well. Ghazi, Rooke, Galbraith, & Bedford (2002) obtained positive responses elicited by protease (isolated from an *Aspergillus* species) applied under optimum conditions of pH and temperature for the enzyme prior to inclusion of SBM in the broiler diets, whilst Caine et al. (1997) observed no effect on nutrient digestibility when SBM was treated with protease, either as a topical spray or by incubation before inclusion in pigs' diet. Moreover, Ao, Cantor, Pescatore, Pierce, & Dawson (2010), confirmed that although increasing levels of protease resulted in a linear increase in the release of α-amino N from raw soybean, it did not reduce TI activity.

In this study, it was observed that there was not interaction between heat processing and protease supplementation. It was also noticed that birds supplemented with monocomponent protease (200 mg/kg) were 4% heavier and gained 3.3 % more than those fed the unsupplemented diet, confirming that this enzyme is able to inactivate proteinaceous ANFs

improving the nutritional value of poultry feeds. Additionally, improvement in FCR was observed in birds fed supplemented diets (Table 4). There was no effect of enzyme supplementation on FI suggesting that the observed performance responses were likely due to changes in the digestibility of nutrients rather than improved digestible nutrient intake. Moreover, performance impairment increased as level of UA augmented (Table 4), thus UA is an indicator of the presence of TIs in broiler diets evaluated in this study, confirming that loss of the sulfur-rich endogenous proteins as a consequence of hypersecretion of pancreatic enzymes (trypsin and chymotrypsin) caused by TIs would result in growth depression as soy proteins are also deficient in these amino acids (Lajolo & Genovese, 2002).

It was confirmed that exogenous enzymes complement enzymes that birds cannot produce in sufficient quantity by itself, or even reduce the requirement for the endogenous enzyme production, thus making more nutrients and energy available for growth of the chick at early stages of growth (Olukosi et al., 2007). On the other hand, monocomponent protease could hydrolyze proteinaceous ANFs, antigenic proteins and proteins damaged during heat processing. Those ANFs stimulates endogenous losses (mucins, endogenous enzymes etc.), which represent a nutritional cost to the birds, especially in instances where hyper-production occurs (i.e. trypsin inhibitors presence) (Bedford, 2000). Hence, enzyme supplementation could lead to the reduction in the secretion of these endogenous compounds and more digestible nutrients could be available for birds, specially the young ones, which may also explain the effects noted on performance.

Carcass yield improvements occurred when heat-processing time increased. Birds fed diets with soybeans autoclaved for 135 min presented better carcass yield. On the other hand, protease supplementation enhanced carcass yield and diminished abdominal fat deposition (Table 5).

It was observed that consumption of diets containing ANFs led to organ weight alterations. Raw soybean consumption induced an enlargement of the pancreas and duodenum. Pancreatic secretion is controlled by negative feedback mechanism whereby enzyme secretion is

inversely related to the level of trypsin present in the small intestine. Thus, when dietary trypsin/chymotrypsin inhibitors reach the duodenum they neutralize the proteases present, this reduction in duodenal protease level is the signal for the release of cholecystokinin (CCK) from the duodenal epithelial endocrine cells that, in turn, after reaching the exocrine pancreas, stimulate the synthesis of large amounts of more serine proteases, and cause pancreatic enlargement (Pusztai, Bardocz, & Martín-Cabrejas, 2004).

The improvement in the nutritional quality of diets trough enzyme supplementation was verified by an attenuation of pancreas and duodenum weight alteration (Table 5). Hence, part of positive effects seen on birds' performance due exogenous enzyme was mediated through mechanisms such as a reduced mass of gastrointestinal tract segments and/or support organs (i.e. pancreas). Moreover, reduction in specific endogenous secretions allows more energy to be used in protein accretion (Cowieson & Ravindran, 2008).

3.4. Weight, protein and DNA contents of tissue

It was observed that there was not interaction between heat processing and protease supplementation. On day 7, relative weight of the pancreas was not affected by protease supplementation. Whilst, UI of the diets ($P<0.05$) increased organ size. However there were not significant differences between birds fed RSB and ASB135. Neither enzyme supplementation nor UA of the diets had significant impact on protein, DNA content and Protein/ DNA ratio. On day 14, relative weight of the pancreas remains unaffected protease supplementation; whereas, organ size was affected by UI of the diets. Birds fed RSB and ASB105 exhibited the higher weights. On the other hand, Protein/ DNA ratio was affected by protease supplementation. Unsupplemented birds exhibited higher Protein/ DNA ratio (cell size) with no change in DNA content or in pancreas weight, which indicates a hypertrophic response (Table 6).

On day 28, relative weight and Protein/ DNA ratio increased in unsupplemented birds; whilst, a decrease in DNA content was observed. Increasing in organ weight with concomitant increasing of Protein/DNA ratio and decreasing in DNA content indicates hypertrophy. Furthermore, UA of the diets elicited the higher and the lower pancreas weight in birds fed RSB

and RSB135 (P=0.0566), respectively; whereas, DNA content decreased in the pancreas of birds fed ASB135 and ASB110. Hence, DNA content did not increase with increasing relative weights, which indicates hypertrophy, rather than hyperplasia (cell number increase) of pancreatic cells (Struthers, MacDonald, Prescher & Hopkins, 1983.). Any effect on relative weight, protein, DNA-mass and Protein/DNA ratio by enzyme supplementation or UA of the diets were detected on day 21 (Table 6).

Although, pancreatic enlargement, which accompanies the consumption of raw soybean has been generally ascribed to hypertrophy, hyperplasia, or a combination of both effects (Liener, Nitsan, Srisangnam, Rackis & Gumbmann, 1985), in this study, it was verified that increasing in pancreas size of birds was due to hypertrophy. Moreover, information on protease effects on relative weight and pancreatic growth variables (total-pancreatic protein and DNA) in broiler chickens is not available.

4. Conclusion

It can be concluded that ANFs present in poultry diets were influenced by the type of processed soybean previously incorporated. Diets containing raw seeds showed the highest levels of ANFs, which decreased as soybean autoclaving period into poultry diets increased. Hence, after conducting a growth trial and extensive evaluation of ANFs content in processed soybeans, autoclaving treatment during 110 min appears to be effective in reducing ANFs levels without causing impaired performance, and it could be recommended as a processing time appropriate in the poultry feed, without causing impaired growth performance.

The monocomponent protease supplementation has proven to be effective in improving performance and carcass yield measures of bird. Increasing levels of urease affected bird's performance and caused an increase in the size of the pancreas and duodenum. However monocomponent enzyme, at concentrations of 200 mg/kg, was able to overcome the organ enlargement, which is due to hypertrophy according with the results obtained in this study.

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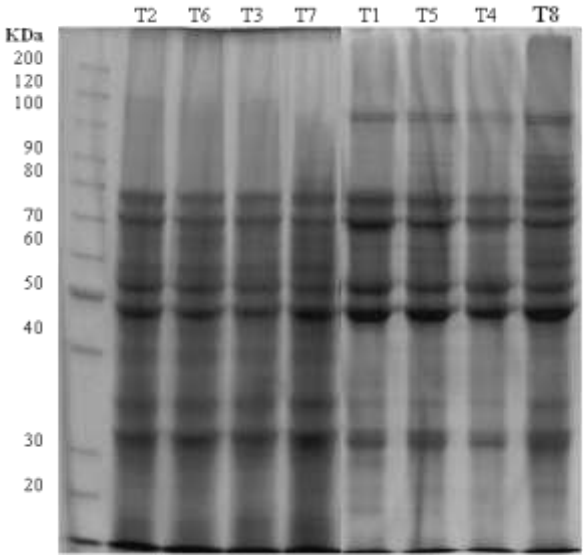

Fig. 1. 12% polyacrylamide/0.1% SDS gels of experimental diets[1] extracts. Samples of 500 μg of defatted meal were suspended in 500 μl of denaturing sample buffer. After centrifugation, 15 μl of the supernatant were applied to the gel. Protein bands were stained with Coomassie blue. Lanes: 1, molecular mass markers; 2= ASB110 (T2); 3= ASB110+P (T6); 4= ASB105 (T3); 5= ASB105+P (T7); 6= ASB135 (T1); 7= ASB135+P (T5); 8= RSB (T4); 9= RSB+P (T8). [1]Whole soybeans were autoclaved for 0 (RSB), 105 (ASB105), 110 (ASB110), 135 (ASB135) minutes (120°C and 1.5 atm.) and then incorporated at 20% inclusion level in broiler diets supplemented or not with monocomponent protease (P)= Ronozyme ProAct® added at 200 mg/kg.

Table 1

Feed composition and calculated nutrient value of experimental diets containing processed soybeans without or with or 200 ppm of mono component serine protease fed to high-performance broiler chickens from 1 to 28 d of age (%).

Ingredients	%
Corn	52.51
Processed soybean[a]	20.00
Soybean meal	19.89
Meat and bone meal	3.65
Celite[b]	1.00
Limestone	0.98
Soybean oil	0.87
Salt	0.43
DL-Methionine	0.28
L-Lysine	0.11
Vitamin premix[c]	0.10
Maxiban	0.05
Mineral premix[c]	0.05
Choline	0.02
Linco-Spectin 440	0.02
Kaolin[d]	0.02
Ronozyme NP CT[e]	0.02
L-Threonine 98%	0.01
Calculated analysis	
ME (kcal/kg)	3,100
CP (%)	22.00
Ca (%)	0.86
Av. P (%)	0.38
Lys dig. (%)	1.15
Met dig. (%)	0.58

[a] Autoclaved (moist-heat) soybeans at 121 ºC and 1.5 atm for 0, 105, 110 and 135 min

[b] Celite is an acid-insoluble ash marker.

[c] All diets were formulated to provide the following vitamins/minerals per kilogram of diet: vitamin A, 18,000 IU; vitamin D_3, 6,000 IU; vitamin E, 100 IU; vitamin K_3, 7 mg; vitamin B_1, 6 mg; vitamin B_2, 16 mg; vitamin B_6, 12 mg; vitamin B_{12}, 80 mg; Niacin, 100 mg; Pantothenic acid, 30 mg; Biotin, 0,4mg ; Folic acid, 3.0 mg; Zn, 100 mg; Mn, 160 mg; Cu, 20 mg; Fe, 100 mg; I, 2 mg; Co, 2mg; Se, 0,8mg.

[d] Treatments with enzyme addition were supplemented with Ronozyme ProAct® (200 g/ton) in substitution of inert vehicle (kaolin).

[e] Ronozyme NP was used to add 0.1% AvP and 0.1% Ca in all diets.

Table 2

Moisture, crude protein, urease index (UI) and residual trypsin activity of poultry diets containing processed soybeans without or with or 200 ppm of mono component serine protease fed to high-performance broiler chickens from 1 to 28 d of age (%).

Treatment[a]	Moisture (%)	Crude protein (%)	UI [b] (Δ pH)	Residual trypsin activity[c] (%)	
				BAPNA	Azocasein
ASB135	12.10	22.35	0.13	85.56 ± 2.37 a	40.57 ± 1.26 ab
ASB110	11.96	22.50	0.23	83.47 ± 5.09 a	38.18 ± 0.26 ab
ASB105	12.36	22.39	0.72	71.24 ± 1.03 ab	32.57 ± 1.79 ab
RSB	12.37	22.55	1.52	60.07 ± 9.90 b	22.58 ± 3.20 b
ASB135+P	12.11	22.65	0.13	88.54 ± 20.62 a	60.62 ± 29.85 a
ASB110+P	12.36	22.64	0.21	80.60 ± 23.74 ab	42.89 ± 0.57 ab
ASB105+P	12.43	21.60	0.73	71.49 ± 5.02 ab	35.07 ± 1.06 ab
RSB+P	12.30	22.94	1.49	57.82 ± 5.51 b	27.69 ± 1.29 b

Means within a column followed by different letters differ by Tukey Test at 5%.

[a]Whole soybeans were autoclaved for 0 (RSB), 105 (ASB105), 110 (ASB110), 135 (ASB135) min (120°C and 1.5 atm.) and then incorporated at 20% inclusion level in broiler diets supplemented or not with monocomponent protease (P)= 200 mg/kg.

[b]The measurement method followed the AOCS official Method Ba 9-58 (1980) and it was performed in duplicate.

[c] The crude seed extracts were tested for inhibition of bovine trypsin using BAPNA or azocasein as substrate. Values are the mean ± SD of three determinations and they represent the residual activity of a given amount of bovine trypsin in the presence of the seed extracts.

Table 3

Residual Chymotrypsin activity and lectin content of poultry diets containing processed soybeans without or with or 200 ppm of mono component serine protease fed to high-performance broiler chickens from 1 to 28 d of age (%).

Treatment[a]	Residual Chymotrypsin activity[b] (%)	HUμg−1 soluble protein[c]
ASB135	41.28 ± 0.52 abc	8
ASB110	49.83± 18.36 a	-
ASB105	37.20 ± 2.69 abc	16
RSB	20.93 ± 0.88 c	-
ASB135+P	44.34 ± 11.31 ab	3.2
ASB110+P	39.46 ± 1.61 abc	-
ASB105+P	38.79 ± 0.50 abc	4
RSB+P	27.69 ± 3.53 bc	-

Means within a column followed by different letters differ by Tukey Test at 5%.

[a] Whole soybeans were autoclaved for 0 (RSB), 105 (ASB105), 110 (ASB110), 135 (ASB135) min (120°C and 1.5 atm.) and then incorporated at 20% inclusion level in broiler diets supplemented or not with monocomponent protease (P)= Ronozyme ProAct® added at 200 mg/kg.

[b] The crude seed extracts were tested for inhibition of bovine trypsin using BATPNA as substrate. Values are the mean ± SD of three determinations and they represent the residual activity of a given amount of bovine trypsin in the presence of the seed extracts.

[c] The dialyzed crude seed extracts (20% w/v) were serially diluted (twofold) and tested for haemagglutination activity upon human type A^+ fresh erythrocytes. One haemagglutination unit (HU) was defined as the amount of protein (μg; spectrophotometry method) required to agglutinate 10^6 erythrocytes under the conditions used. The Lectin content of each sample is also expressed as HUμg−1 seed protein. Values are the mean ± SD of three determinations.

(-) Not detected.

Table 4

Growth performance of broiler chickens fed diets containing processed soybeans without or with or 200 ppm of mono component serine protease fed to high-performance broiler chickens from 1 to 28 d of age (%).

	Body weight (g)	Feed intake (g)	Body weight gain (g)	Feed Conversion Ratio
Enzyme [a] (E)				
0	1,436 b	2,168	1,393b	1.54a
200	1,491a	2,174	1,438a	1.47b
Urease index[b] (UI)				
ASB135	1,540a	2,186	1,494a	1.46a
ASB110	1,507a	2,231	1,463a	1.49a
ASB105	1,433 b	2,109	1,388b	1.49a
RSB	1,370b	2,157	1,317b	1.60b
Pooled SEM	22.29	2.82	22.14	0.03
			P-value	
E	0.0464	0.1555	0.0401	0.0317
UI	<.0001	0.8840	<.0001	0.0115
E x UI	0.6889	0.1913	0.8402	0.8410

Means within a column followed by different letters differ by Tukey Test at 5%.
[a] Experimental diets were supplemented or not with monocomponent protease (P) = Ronozyme ProAct® added at 200 mg/kg.
[b] Whole soybeans were autoclaved for 0 (RSB), 105 (ASB105), 110 (ASB110), 135 (ASB135) min (120°C and 1.5 atm.) with UI values of 1.52, 0.72, 0.23 and 0.13 ΔpH, respectively, and then incorporated at 20% inclusion level in broiler diets.

Table 5

Carcass and abdominal fat yields and organ relative weights of broiler chickens fed diets containing processed soybeans without or with or 200 ppm of monocomponent serine protease fed to high-performance broiler chickens from 1 to 28 d of age (%).

	Carcass	Abdominal fat	Pancreas	Duodenum
	%		g/100 g LW	
Enzyme[a] (E)				
0	69.76b	1.17b	0.295a	0.870a
200	71.22a	1.04a	0.281b	0.832b
Urease index[b] (UI)				
ASB135	71.25a	1.06	0.232d	0.835b
ASB110	70.48ab	1.10	0.265c	0.814b
ASB105	70.29b	1.10	0.290b	0.833b
RSB	69.85b	1.20	0.365a	0.937a
Pooled SEM	0.32	0.06	0.005	0.01
	P-value			
E	<.0001	0.0159	0.0088	0.0004
UI	0.0276	0.3656	<.0001	<.0001
E x UI	0.6491	0.1242	0.8135	0.2219

Means within a column followed by different letters differ by Tukey Test at 5%.
[a] Experimental diets were supplemented or not with monocomponent protease (P) = Ronozyme ProAct® added at 200 mg/kg.
[b] Whole soybeans were autoclaved for 0 (RSB), 105 (ASB105), 110 (ASB110), 135 (ASB135) min (120°C and 1.5 atm.) with UI values of 1.52, 0.72, 0.23 and 0.13 ΔpH, respectively, and then incorporated at 20% inclusion level in broiler diets.
LW=live weight

Table 6. Relative weight and pancreatic growth variables (total-pancreatic protein and DNA) at 7, 14, 21 and 28 d of age.

	7d				14d				21d				28d			
	Pancreas, g/100 g LW	Prot., µg/g	DNA, µg/g	Prot./ DNA	Pancreas, g/100 g LW	Prot., µg/g	DNA, µg/g	Prot./ DNA	Pancreas, g/100 g LW	Prot., µg/g	DNA, µg/g	Prot./ DNA	Pancreas, g/100 g LW	Prot., µg/g	DNA, µg/g	Prot./ DNA
Enzyme[a] (E)																
0	0.613	1467	1287	1.40	0.547	2334	1353	2.27a	0.365	2829	800	4.74	0.335a	2382	584b	4.34a
200	0.618	2021	1243	1.92	0.502	1917	1915	1.08b	0.325	2662	1500	2.67	0.266b	2252	1012a	2.58b
UI[b]																
ASB135	0.507b	1327	1612	1.01	0.370c	1873	1750	1.12	0.294	3178	950	5.16	0.249b	2936	618b	5.18
ASB110	0.571b	1921	893	2.15	0.387c	1817	1700	1.76	0.341	2876	1031	5.09	0.285b	1971	606b	3.40
ASB105	0.802a	2352	1137	2.47	0.609b	2643	1181	2.60	0.337	2577	1118	2.33	0.296b	2042	1087a	2.22
RSB	0.582b	1378	1418	0.99	0.733a	2169	1906	1.23	0.407	2350	1500	2.25	0.372a	2318	881ab	3.04
Pooled SEM	0.04	197.5	207.35	0.30	0.03	216.2	199.2	0.36	0.03	348.38	324.26	0.98	0.02	198.1	75.36	0.46
								P-value								
E	0.9178	0.0827	0.8851	0.2721	0.3095	0.2097	0.1194	0.0497	0.3974	0.7430	0.1654	0.1774	0.0302	0.6552	0.0039	0.0262
UI	0.0226	0.0934	0.3841	0.0909	0.0006	0.2802	0.4608	0.2387	0.4086	0.6719	0.8375	0.3185	0.0566	0.1378	0.0359	0.0613
E x UI	0.5880	0.6267	0.2015	0.4407	0.5670	0.9039	0.5579	0.4554	0.6453	0.4833	0.8611	0.5871	0.6899	0.2990	0.4895	0.8301

Means within a column followed by different letters differ by Tukey Test at 5%.

[a] Experimental diets were supplemented or not with monocomponent protease (P) = Ronozyme ProAct® added at 200 mg/kg.

[b] Whole soybeans were autoclaved for 0 (RSB), 105 (ASB105), 110 (ASB110), 135 (ASB135) min (120°C and 1.5 atm.) with UI values of 1.52, 0.72, 0.23 and 0.13 ΔpH, respectively, and then incorporated at 20% inclusion level in broiler diets.

LW=live weight.

CAPITULO IV[5]

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EFFECTS OF A MONOCOMPONENT PROTEASE ON PERFORMANCE AND GUT MORPHOMETRY IN 1- TO 28-DAY-OLD BROILER CHICKENS

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SUMMARY

A study was conducted to determine the effect exogenous protease addition in broiler diets containing autoclaved soybean. 400 male Cobb broiler chicks were placed in 40 pens, with 10 birds per pen. There were 5 treatments, with 8 replicates each, fed a grower diet from d 1 to 28. Processed soybean was incorporated at 20% inclusion level in all diets, formulated with 3,100 kcal of ME/ kg and 22% CP. Diet 1 (ASB 0) was the unsupplemented control diet. Diets 2 through 5 were supplemented with monocomponent protease (75,000 PROT units/g of enzyme) at 200, 400, 800 and 1600 ppm (ASB 200, ASB 400, ASB 800 and ASB 1,600, respectively). On d 14, BWG was improved when protease was added at inclusion levels of 800 mg/kg. There was no enzyme effect on BWG on d 28. The ASB800 and ASB1,600 diets at 14 d of age, and the ASB200 and ASB 1,600 diets at 28 d of age elicited an improved FCR *vs.* unsupplemented diet. Regression analysis of graded protease supplementation on Feed intake showed no significant linear or quadratic effects in any age. Carcass traits were affected by protease supplementation. A quadratic improvement in carcass yield was observed in birds fed ASB 800, while, protease supplementation reduced abdominal fat, regardless of protease. Unsupplemented birds showed the greater pancreas and duodenum size. Protease-supplemented diets elicited smaller organ sizes vs. unsupplemented diet. The protease tested in this study improved microvilli height, villus height and width, total mucosal thickness, crypt depth and VCR. Additionally, surface area was increased on d 28.

DESCRIPTION OF PROBLEM

Soybean is a valuable protein-rich feed ingredient used extensively in poultry and swine diet. However, the availability of nutrients in raw grain is often limited by the presence of anti-nutritional factors (ANFs), which limit their use in diets for monogastric and immature ruminants. These substances, which are not susceptible to digestion by the endogenous enzymes, interfere with normal digestion and absorption of nutrients, causing poor performance and also leading to damage and destruction to intestinal absorptive surface area. Moreover, ANFs content not only depend on the genotype and geographical region of their growing, but also on processing conditions [1, 2, 3, 4].

Thus, many of these ANFs, such as lectins and trypsin inhibitors (TI), can be reduced or even eliminated by proper thermal processing [5, 6], such as steaming or autoclaving, dry heating, toasting and extrusion [7]. These processes should be carefully controlled since under-heating can result in inadequate inactivation of ANFs, [8, 9], whilst over-heating leads to denaturation of amino acids, oxidation of Cysteine and Methionine, and the Maillard reaction between the lysine and aldehyde groups of carbohydrates that reduce amino acid availability [7, 9].

Furthermore, the efficacy of heat processing could be determined indirectly using laboratory analytical methods, in order to replace its *in vivo* determination, which is laborious, longer, and more expensive [9]. Both protein solubility in 0.2% KOH solution and the urease activity index (indirect method) have been widely used as indicators of soybean meal quality. Hence, it is generally accepted that the KOH protein solubility measures excessive heat-treatment, while the urease activity is used to detect insufficient heat-treatment [10]. As a rule, lack of urease activity indicates adequate TIs destruction, however some residual activity could still remain even after thermal processing [11, 12].

Thus, maximum inactivation of ANFs can be also achieved through the addition of exogenous enzymes [13], which improve availability of proteins and release more nutrients in the small intestine [14]. Moreover, the addition of a protease could help to neutralize the

negative effects of the residual proteinaceous ANFs in addition to breaking down the large storage protein molecules (glycinin and β-conglycinin) into smaller, absorbable fractions [2, 5]. Nonetheless, information on the influence of monocomponent enzyme supplementation and heat processing of raw soybean on size and morphology of the digestive organs is scarce.

Therefore, the purpose of this study was to assess the influence of increasing levels of monocomponent serine protease on live performance, carcass yield and fat deposition of male broiler fed diets with autoclaved raw soybean, and to determine whether enzyme supplementation produce changes in morphology and size of digestive organs.

MATERIALS AND METHODS

Animals and Diets

Four hundred day-old, Cobb 500, male broiler chicks were individually weighed and distributed to 40 pens (0.80 x 0.90m^2), five pens per treatment and ten chicks per pen, placed in a temperature and humidity-controlled metabolism room until 28 days of age at the Universidade Federal do Rio Grande do Sul, RS, Brazil. The temperature of the room with continuous lighting was maintained at 32 ºC initially, and then reduced until it reached 22 °C, at which temperature the room was maintained for the rest of the feeding period. Birds had free access to water and feed in stainless steel troughs.

Soybean [*Glycine max* (L.) Merr.] was purchased from a local commercial source (Viamão, RS, Brazil). A batch of whole soybeans was autoclaved at 121 ºC and 1.5 atm for 105 min at the Laboratório de Pesquisas Micológicas (LAPEMI), Universidade Federal de Santa Maria, RS, Brazil. Immediately after steam processing, the soybeans were put into trays and spread in one layer for cooling down for 40 min afterwards, processed soybeans were stored in a cooling chamber until the elaboration of diets.

Five diets were formulated according to nutrients standards for the southern region of Brazil and fed in mash form 1 to 28 d of age (Table 1). The processed soybean was ground and then incorporated at 20% inclusion level in all experimental diets**.** Diet 1 (ASB 0) was the

unsupplemented control diet. Diets 2 through 5 were supplemented with monocomponent protease (*Bacillus licheniformis*) Ronozyme ProAct® (minimum protease activity of 75,000 PROT/g) at increasing levels: 200, 400, 800 and 1600 ppm (ASB 200, ASB 400, ASB 800 and ASB 1,600, respectively). Each dietary treatment had 8 replicates of 10 chicks each.

Proximate composition and urease activity (indirect method)

Moisture and protein contents were determined in Autoclaved raw soybean and experimental diets were determined according to standard methods [15]. Urease activity (UA) in autoclaved soybean and the experimental diets samples was determined by the pH change method. The urease assay is based on the pH increase from ammonia released from urea by residual urease enzyme in soybean meal (SBM) [16].

Additionally, Protein Solubility in a solution of 0.2% potassium hydroxide (KOH-PS) was determined in autoclaved soybean and in SBM according to the procedure of Araba & Dale [17].

Antinutritional compounds

Samples of autoclaved raw soybean and experimental diets were finely ground and defatted by petroleum ether extraction (1:5 w/v, 24 h). For preparation of the crude extracts the meals were suspended in 10mM sodium phosphate buffer Ph 7.5 containing 10mM β-mercaptoethanol at 1:5 w/v ratio and stirred at 4 °C for 4 h. The extracts were kept at 4 ºC in the presence of 0.02% sodium azide as preservative.

Urease activity (direct method)

A 20 µl aliquot of urea solution (100mM) was mixed with 20 µl of seed crude extracts and buffered with 0.02M sodium phosphate pH 7.5 to give a final volume of 500 µl. The reaction mixtures were incubated for 10min at 37 ºC and the ammonia released was measured colorimetrically [18]. One unit of urease activity was defined as the amount of enzyme required

to release 1 μmol NH3 min^{-1} at 37 ºC and pH 7.5 under the conditions described.

Trypsin inhibitory activity

The presence of compounds with trypsin inhibitory activity was analyzed using bovine trypsin (Sigma Chem Co) and two different substrates, according to Kakade et al. [19] with modifications, as follows. (1) Aliquots (10 μl) of crude extracts (1:5 w/v) were mixed with bovine trypsin (50 μg) at 0.8% w/v final concentration, pH 8.0, in 500 μl final volume. After incubation for 30 min at 37 ◦C the reaction was stopped by adding trichloroacetic acid (TCA, 5% v/v), the mixture was centrifuged and the TCA-soluble material in the supernatant was quantified by absorbance at 420 nm. The inhibitory activity of soybean extracts was expressed as residual activity (%) of bovine trypsin assayed alone under the same conditions. (2) Aliquots (10 μl) of 20-fold diluted crude extracts were mixed with bovine trypsin (10 μg) and *N*-α-benzoyl-DLarginine-ρ-nitroanilide (BAPNA) at 1.5mM final concentration, pH 8.2, in 100 μl final volume. The hydrolysis of the substrate was followed at room temperature by taking readings at 405 nm every 15 s using a SpectraMax (Molecular Devices Corporation, Sunnyvale, CA, USA) apparatus programmed with kinetic analysis software. The inhibitory activity of soybean extracts was expressed as residual activity (% of V_{max}) of bovine trypsin assayed alone under the same conditions. Controls were run in parallel for each substrate in the absence of added trypsin in order to correct the experimental points for endogenous proteolytic activity of the soybean cultivars.

Quimotrypsin inhibitory activity

The presence of compounds with chymotrypsin inhibitory activity was analysed using bovine chymotrypsin (Sigma Chem Co) and its inhibition was assayed using the chromogenic substrate N-benzoyl-L-tyrosine-ρ -nitroanilide (BTPNA) in 0.08 M Tris–HCl buffer pH 7.8 containing 20% DMSO band 20 mM $CaCl_2$, according to Kumar et al. [20].

Performance, Carcass yield, organ size and sampling for histomorphometry

Feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR), adjusted for mortality (FC) were measured on 7, 14, 21 and 28 d of age. Mortality and culled birds were also weighed and recorded daily. At 28 days of age, eight birds per each replicate were randomly selected and were slaughtered to evaluate carcass yield and abdominal fat content according to the standard slaughter procedures (stunning, bleeding, scalding, plucking, chilling and dripping). Eviscerated carcass (without head, neck and feet) percentage was calculated as the ratio between the post-chilled eviscerated carcass and live body weight. The weight percentage of abdominal fat was calculated as a percentage of post-chilled eviscerated carcass weight. Simultaneously, pancreas of all birds were also excised, freed from adhering fatty tissue and weighed individually. Relative organ weights were calculated as organ weights as percentages of live body weight.

Additionally, three birds selected randomly per treatment were at day 14 and 28 and then killed by cervical dislocation. Then small intestine was removed immediately from coelomic cavity without any press on its tissue. Tissue sections (2 cm long each) were then taken from the duodenum, flushed with cold saline, and fixed in 10% neutral buffered formalin for histological morphometric analysis. The tissue segments were then trimmed and embedded in paraffin according to standard procedures. Embedded tissue samples were cut, stained with hematoxylin and eosin, and photomicrographs were obtained using an Olympus® CX40 trinocular microscope coupled to a C-7070 Olympus camera, and analyzed using Motic Images Plus v.2.0 software (Motic China Group, 1999-2004). Measurements taken included: villus height (from the tip of the villus to the crypt-villus junction), crypt depth (defined as the difference between the total mucosal thickness and the villus height), villus height:crypt depth ratio (VCR) and total mucosal thickness (from the top of the villus to the border over the *muscularis mucosa*) [21].

Statistical Analysis

One-way ANOVA was used to analyze the data and regression analysis was performed

on protease level. When the effects were found to be significant, treatment means were separated using Tukey's test. The statistically significant difference was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Formulated and analyzed moisture and CP concentrations were similar (Table 2). UA values of the diets ranged from 0.04 to 0.06 (Δ pH). UA is mainly based on the measurement of the amounts of products released during the reaction, and quantifies urease activity [22]. This test is routinely done to assess the adequacy of heat treatment during soybean processing, but not for broiler diets. Whereas, UA and KOH-PS values of autoclaved soybean, incorporated diets, were 0.30 Δ pH and 81.22%, respectively (results not tabulated).

Autoclaved soybean, soybean meal (SBM) and experimental diets were also analyzed to confirm the presence of antinutritional factors (urease, trypsin and chymotrypsin inhibitors) (Table 3 and 4). Protease inhibitors activities were assayed with azocasein (for general protease activity), BAPNA (for trypsin-like protease activity) or BATPNA (for chymotrypsin-like protease activity). In addition, the urease activity of the diets remained constant (mean=0.99). Results obtained in this study indicated that autoclaved soybean presented a higher UA, measured directly, compared to SBM (2.53 vs. 0.78). Thus, even after denaturation caused by thermal processing grains may contain remaining ANFs activities. In this study, a significant portion of trypsin and chymotrypsin inhibitors (~42% and ~80%, respectively) remained active in autoclaved soybean; whilst, approximately 14 % and 67% of trypsin and chymotrypsin inhibitors, respectively, remained active in SBM, obtained from a standard extraction plant (Table 3). These results confirm that the rigid structure of the quimotrypsin inhibitor molecule, provided by the seven disulfide bridges, confers thermal stability [23].

Furthermore, UA remain on average constant in all diets. They presented trypsin inhibitors activities ranging from ~8 to 27% (Table 4). It is known that commercial SBM retain up to 20% of the residual trypsin inhibitor activity [24], however there is not published information about mean values in poultry diets. According to Ao [25] trypsin inhibitors can be

reduced by 80 - 95% of the activity originally present by heat processing. Nonetheless, results obtained here indicate that heat treatment was not able to reach full inactivation of the AFNs contained in soybeans. Autoclaving involves cooking the soybeans in hot, pressurized steam, which is more effective than dry heat. Nevertheless, the effectiveness of ANFs reduction depends on the samples preparation that varies between laboratories and on the treatment conditions (i.e. milling size, moisture, time and pressure) [24]. Moreover, ANFs contained in the diets may be derive from other ingredients as cereals, such as corn (*Zea mays*), which contains trypsin inhibitors with antifungal activities, capable of inhibit serine proteases as well as amylases and lipases [26, 27].

Effects of combination of exogenous enzymes, such as phytase, xylanase and β-glucanase have been widely used in poultry diets [28, 29]. However, exogenous proteases have very limited application in poultry diets, and their effects on live performance are not likely to be consistent. Differences in the type of proteases used (isolated from *Bacillus* or *Aspergillus* strains) and the use of the enzyme during prefeed treatment of SBM rather than added directly in feed, can partially explain the variable and inconclusive results reported [30, 31].

At 14d of age, birds supplemented with 400, 800 and 1,600 ppm of protease exhibited the higher BWG ($P<0.005$) *vs.* unsupplemented birds. There were not any improvements of BWG by protease on 28 d of age. As the bird ages it seems to be able to cope better with ANFs due to a fully developed GIT. Hence, exogenous enzymes might be most beneficial in young birds (Table 5). Improvement ($P<0.005$) in FCR was observed in supplemented birds regardless the level of protease on day 14. However, on day 28 only ASB 200 and ASB 1,600 showed a slight improvement in FCR compared with those fed the ASB diet (Table 5). In contrast, there were not differences between the FCR of birds fed any level of protease. Regression analysis of graded protease supplementation on FI showed no significant linear or quadratic effects in any age.

The lack of major effects on performance may be explained by the method of applying enzyme. In this study, the monocomponent protease was added to an experimental diet, so the

target substrates (protease inhibitors mainly) were considerably diluted, since those ANFs, which only constitute a small proportion of the total diet. In addition, there was a possible interference by other ingredients present in diets. Furthermore, it is known that protease has very narrow pH range for maximal activity. Ao et al. [32] measured the activities of different feed-grade enzymes under which simulate pH levels prevailing in birds' gastrointestinal tract (GIT). They observed that protease had the highest activity at pH 3.0, and decreased dramatically when the pH was higher than 5.5. Those pH values are lower than those commonly found in avian duodenum 6.4 (ranged from 5.2 to 7.6) and that may be a limiting factor for maximum activity of protease.

Carcass traits were positively affected by protease supplementation (Table 6). A quadratic improvement in carcass yield was observed in birds fed ASB 800 ($y = 67.718 + 9.08 \times 10^{-3} - 4.22 \times 10^{-6}\ X^2$; $P < 0.0057$, $R^2 = 0.2624$). While, protease supplementation reduced abdominal fat percentage, regardless of protease concentration, when compared with unsupplemented birds ($y = 1.355 - 6.807 \times 10^{-4} + 3.651 \times 10^{-7}\ X^2$; $P < 0.0260$, $R^2 = 0.2655$) (Table 6). In this study, protease supplementation, regardless of concentration used, decrease abdominal fat percentage. Reduction in abdominal fat with the addition of protease occurred through increased AA availability, which requires a larger amount of energy to metabolize them. Thus, less energy can be directed to fat deposition. Freitas et al. [33], using the same protease, did not observe any change in carcass yield or abdominal fat percentage. In contrast, Dozier et al. [34] reported that protease supplementation elicited increases in carcass and total breast meat weights. More published works reporting the effect of monocomponent proteases on carcass yields were not found.

Unsupplemented birds showed the greater pancreas size (Table 7). Graded inclusion of protease led to a quadratic response in pancreas size. ($y = 0.296 - 9.157 \times 10^{-5} + 3.656 \times 10^{-8}\ X^2$; $P < 0.0026$, $R^2 = 0.5801$). Similarly, protease-supplemented diets (ASB 200 and ASB 1,600) elicited smaller duodenum relative size when compared with unsupplemented birds (Table 7), leading to a cubic response in organ size ($y = 0.823 + 4.490 \times 10^{-4} - 6.073 \times 10^{-7}\ X^2 + 2.265 \times$

10^{-10} X^3; P <0.0162, R^2 = 0.4495) (Table 7). Although heating is the most commonly used treatment for the elimination of ANFs, such as proteases inhibitors, some residual activity may remains. The data presented herein indicate that pancreas and duodenum sizes increased by the action of protease inhibitors, confirming that the inactivation or removal of proteolytic activity, from the proximal small intestine, by the protease inhibitors produce a large increase in pancreatic exocrine secretion and, consequently, organ enlargement [35, 36]. On the other hand, organs size was reduced by protease supplementation, regardless of the concentration used, suggesting that exogenous protease can partially hydrolyze ANFs. Hence, part of the beneficial effects of protease supplementation on performance were mediated through a reduction in specific endogenous secretions rich in AAS, such as pancreatic enzymes, and through a reduction in GTI mass and support organs, thus allowing more energy to be used in protein accretion [37].

In the domestic fowl the capacity to absorb nutrients depends mainly on the mucosal surface area of the small and large intestine, as well as on the functional properties of the specific nutrient transporters present in the brush border membranes [38]. Hence, the study of intestinal cell dynamics is crucial to understanding both digestive physiology and the efficiency of animal production [39]. Literature reports that multi-enzyme complex containing NSP-enzymes result in an increase in the villus height and crypt depth of intestinal mucosa of broilers [40, 41]. Nevertheless, information about protease effect on duodenum morphometry is not available.

n this study, only the lower level of monocomponent protease (200 ppm) produced the highest microvilli height, total mucosa thickness and VCR at 14 d *vs.* unsupplemented birds (Table 8). Moreover, protease addition increased crypt depth when supplemented at 800 and 1,600 ppm. Furthermore, deeper crypts indicate fast tissue turnover, thus allowing villus renewal [42]. Also, increasing the villus height provides more surface area for nutrient absorption [43]. Regression analysis of protease supplementation on duodenum histomorphometry showed significant cubic effects for villus crypt depth (y = 222.937 – 0.207 +

$7.076 \times 10^{-4}\ X^2 - 3.353 \times 10^{-7}\ X^3$; P <0.0001, $R^2 = 0.4968$) and VCR ($y = 6.481 + 8.580 \times 10^{-3} - 2.229 \times 10^{-5}\ X^2 + 9.987 \times 10^{-9}\ X^3$; P <0.0001, $R^2 = 0.3912$).

On day 28, protease supplementation (400 ppm) improved the villus height and total mucosal thickness. ASB 400 and ASB 800 showed a higher VCR *vs.* unsupplemented birds. However, crypt depth remained unaffected by protease supplementation (Table 8).

These results evidence the major duodenal development occurred during this phase. The largest area of absorption observed could be attributed to lower cell loss by sloughing and to the greater development of microvilli, positively affected by protease supplementation. Also, enzyme supplementation increased the thickness of intestinal tissue layers. These results are in agreement with those reported by Yuan et al. (2008) [44]. They observed that xylanase, ß-glucanase and protease increased the thickness of intestinal mucosa. This effect of enzyme was found to be more pronounced for the duodenum than the caecum and colon.

CONCLUSIONS AND APPLICATIONS

1. A significant amount of serine proteases inhibitors remained active even after thermal processing of soybeans.
2. The monocomponent protease supplementation had a slight beneficial effect on BWG and FCR on day 14.
3. The monocomponent protease reduced abdominal fat.
4. The monocomponent protease increased villus height, total mucosa thickness and VCR at 14 d. Thus, functionality of the intestinal epithelium was improved by exogenous enzyme.

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Table 1. Composition of basal diet containing increasing levels of monocomponent serine protease (as-fed)

	Treatment[1]
Ingredients, %	
Corn	52.36
Heat-treated raw soybean[2]	20.00
Soybean meal	19.89
Meat and bone meal (41%)	3.65
Celite[3]	1.00
Limestone	0.98
Soybean oil	0.87
Salt	0.43
DL-Methionine 99%	0.28
L-Lysine 78%	0.11
Vitamin premix[4]	0.10
Maxiban	0.05
Mineral premix[4]	0.05
Choline 60%	0.02
Linco-Spectin 440	0.02
Ronozyme NP CT[5]	0.02
L-Threonine 98%	0.01
Ronozyme Proact[1]	0.00
Caolin	0.16
Calculated nutrient value	
ME, kcal/kg	3,100
CP, %	22.00
Ca, %	0.86
Av. P, %	0.38
K, %	0.91
Na, %	0.21
Cl, %	0.34
Lys, %	1.15
Met + Cys, %	0.86
Met dig, %	0.58
Thr dig, %	0.72
Choline, ppm	1650

[1]Treatments: 1= basal diet (ASB 0); 2= basal diet+ 200 ppm protease (ASB 200); 3= basal diet+ 400 ppm protease (ASB 400); 4= basal diet+ 800 ppm protease (ASB 800); 5= basal diet+ 1,600 ppm protease (ASB 1600).
Treatments were supplemented with monocomponent serine protease (Ronozyme ProAct, 75,000 PROT units/g of enzyme, DSM Nutritional Products) in substitution of inert vehicle (caolin).
[2]Whole raw soybeans were autoclaved at 121 ºC and 1.5 atm for 105 min.
[3]Celite is an acid-insoluble ash marker.
[4]All diets were formulated to provide the following vitamins/minerals per kilogram of diet: vitamin A, 18,000 IU; vitamin D3, 6,000 IU; vitamin E, 100 IU; vitamin K_3, 7 mg; vitamin B_1, 6 mg; vitamin B_2, 16 mg; vitamin B_6, 12 mg; vitamin B_{12}, 80 mg; Niacin, 100 mg; Pantothenic acid, 30 mg; Biotin, 0,4mg ; Folic acid, 3.0 mg; Zn, 100 mg; Mn, 160 mg; Cu, 20 mg; Fe, 100 mg; I, 2 mg; Co, 2mg; Se, 0,8mg.
[5]Ronozyme NP was used to add 0,1% AvP and 0,1% Ca in all diets.

Table 2. Proximate composition and Urease Activity (UA) values of broiler diets fed to broiler chickens from 1 to 28 d of age (%)

Item	Moisture, %	Crude Protein, %
Treatment[2]		
ASB 0	12.35	22.15
ASB 200	12.17	22.44
ASB 400	12.31	21.52
ASB 800	12.27	21.83
ASB 1,600	12.40	21.75

[1]The measurement method followed the AOCS official Method Ba 9-58 (1980) and it was performed in duplicate.
[2]Whole soybeans were autoclaved 120°C and 1.5 atm. during 105 min and then incorporated at 20% inclusion level in all diets supplemented with increasing levels of monocomponent protease= 0 (ASB 0), 200 (ASB 200), 400 (ASB 400), 800 (ASB 800) and 1,600 mg/kg (ASB 1600).

Table 3. Urease activity[1], residual trypsin[2] and chymotrypsin[3] activity in crude extracts of autoclaved soybean and SBM incorporated into experimental diets

Item	Urease units g^{-1} soluble protein	Residual trypsin activity, %	Residual chymotrypsin activity, %
ASB[4]	2.53 ± 0.20^{a}	58.34 ± 17.11	17.99 ± 1.85^{b}
SBM	0.78 ± 0.01^{b}	86.42 ± 18.53	33.03 ± 1.08^{a}

Means within a column with different superscripts are significantly different by Tukey test at 5%.
[1]The crude extracts were assayed for urease activity by the phenol–nitroprussiate method [18]. One unit of urease activity was defined as the amount of enzyme required to release 1 μmol NH_3 min^{-1} at 37 °C, and pH 7.5.
[2]The crude seed extracts were tested for inhibition of bovine trypsin using BAPNA as substrate. Values represent the residual activity of a given amount of bovine trypsin in the presence of the seed extracts.
[3]The crude seed extracts were tested for inhibition of bovine trypsin using BATPNA as substrate.
Values are the mean ± SD of three determinations and they represent the residual activity of a given amount of bovine trypsin in the presence of the seed extracts.
[4]ASB=whole soybeans autoclaved at 120°C and 1.5 atm. during 105 min

Table 4. Residual trypsin[2] and chymotrypsin[3] activity in crude extracts of poultry diets[4]

Item	Residual Trypsin activity, %	Residual Chymotrypsin activity, %
Treatment[5]		
ASB 0	87.10 ± 8.63^{abc}	24.03 ± 1.99
ASB 200	$91.08 \pm ab$	23.67 ± 1.22
ASB 400	92.89 ± 0.19^{a}	22.59 ± 0.70
ASB 800	77.22 ± 4.02^{bc}	23.40 ± 0.29
ASB 1,600	73.48 ± 6.64^{c}	22.54 ± 0.49

Means within a column with different superscripts are significantly different by Tukey test at 5%.
[2]The crude seed extracts were tested for inhibition of bovine trypsin using BAPNA as substrate.
[3]The crude seed extracts were tested for inhibition of bovine trypsin using BATPNA as substrate.
Values are the mean ± SD of three determinations and they represent the residual activity of a given amount of bovine trypsin in the presence of the seed extracts.
[4]Raw whole soybeans were autoclaved at 120°C and 1.5 atm. for 105 min and then incorporated to all treatments.
[5]Treatments: ASB 0 = 0 mg of protease/kg; ASB 200 = 200 mg of protease/kg; ASB 400 = 400 mg of protease/kg; ASB 800= 800 mg of protease/kg; ASB 1,600=1,600 mg of protease/kg.

Table 5. Live performance of 28-d-old broilers fed diets supplemented with graded levels of protease[1]

	BWG, g		Feed intake, g		FCR, g/g	
Item	1-14d	1-28d	1-14d	1-28d	1-14d	1-28d
Treatment[2]						
ASB	378[b]	1,372	537	2,079	1.41[a]	1.51[a]
ASB 200	394[ab]	1,406	534	2,050	1.35[b]	1.45[b]
ASB 400	402[a]	1,404	533	2,086	1.33[b]	1.48[ab]
ASB 800	409[a]	1,427	550	2,181	1.34[b]	1.50[a]
ASB 1,600	406[a]	1,429	541	2,080	1.33[b]	1.45[b]
SEM	8.21	31.68	8.91	44.33	0.02	0.02
P-*value*	0.0303	0.5102	0.4825	0.1452	0.0377	0.0139

Means with different superscripts are significantly different by Tukey test at 5%.
[1]Monocomponent protease enzyme Ronozyme ProAct® (minimum protease activity of 75,000 PROT/g).
[2]Treatments: ASB 0 = 0 mg of protease/kg; ASB 200 = 200 mg of protease/kg; ASB 400 = 400 mg of protease/kg; ASB 800= 800 mg of protease/kg; ASB 1,600=1,600 mg of protease/kg.

Table 6. Carcass yield and abdominal fat of 28-d-old broilers fed diets supplemented with graded levels of protease[1]

Item	Carcass yield, %	Abdominal fat, %
Treatment[2]		
ASB	69.87[b]	1.36[b]
ASB 200	69.47[ab]	1.20[a]
ASB 400	70.10[ab]	1.17[a]
ASB 800	72.78[c]	1.03[a]
ASB 1,600	71.38[abc]	1.20[a]
SEM	1.22	0.08
P-*value*	0.0294	0.0424

Means with different superscripts are significantly different by Tukey test at 5%.
[1]Monocomponent protease enzyme Ronozyme ProAct® (minimum protease activity of 75,000 PROT/g).
[2]Treatments: ASB 0 = 0 mg of protease/kg; ASB 200 = 200 mg of protease/kg; ASB 400 = 400 mg of protease/kg; ASB 800= 800 mg of protease/kg; ASB 1,600=1,600 mg of protease/kg.

Table 7. Relative pancreas and duodenum weights[1] of broilers fed diets supplemented with graded levels of protease[2]

Item	Pancreas, g/100g PV	Duodenum, g/100g PV
Treatment[3]		
ASB	0.302[a]	0.823[a]
ASB 200	0.270[b]	0.893[b]
ASB 400	0.266[b]	0.919[b]
ASB 800	0.250[c]	0.911[b]
ASB 1,600	0.242[c]	0.915[b]
SEM	0.007	0.019
P-*value*	<.0001	0.0003

Means with different superscripts are significantly different by Tukey test at 5%.
[1]Weight expressed in grams per 100 g of body weight.
[2]Monocomponent protease enzyme Ronozyme ProAct® (minimum protease activity of 75,000 PROT/g).
[3]Treatments: ASB 0 = 0 mg of protease/kg; ASB 200 = 200 mg of protease/kg; ASB 400 = 400 mg of protease/kg; ASB 800= 800 mg of protease/kg; ASB 1,600=1,600 mg of protease/kg.

Table 8. Histomorphometry of duodenum of broilers fed diets supplemented with graded levels of protease[1] at 14 and 18 days of age

	Treatments[2]					Level of significance	
Variable, μm	ASB	ASB 200	ASB 400	ASB 800	ASB 1,600	SEM	P-*value*
d 14							
Villus height	1,342^{b}	1,582^{a}	1,382^{b}	1,385^{b}	1,245^{b}	119.29	<.0001
Total mucosal thickness	1,565^{b}	1,792^{a}	1,610^{ab}	1,724^{ab}	1,575^{ab}	116.71	0.0003
Crypt depth	222.18^{b}	209.58^{b}	228.55^{b}	338.89^{a}	329.26^{a}	25.89	<.0001
VCR[3]	6.33^{b}	7.90^{a}	6.31^{b}	4.38^{c}	4.04^{c}	0.78	<.0001
d 28							
Villus height	1,516^{c}	1,695^{bc}	2,051^{a}	1,651^{bc}	1,858^{ab}	119.60	<.0001
Total mucosal thickness	1,804^{c}	1,964^{bc}	2,311^{a}	1,865^{c}	2,147^{ab}	117.05	<.0001
Crypt depth	288.35^{a}	268.36^{a}	259.98^{a}	214.35^{b}	289.34^{a}	25.06	<.0001
VCR[3]	5.42^{b}	6.95^{ab}	8.27^{a}	8.14^{a}	6.52^{ab}	0.96	<.0001

Within the same row, means with different superscripts are significantly different by Tukey test at 5%.

[1]Monocomponent protease enzyme Ronozyme ProAct® (minimum protease activity of 75,000 PROT/g).

[2]Treatments: ASB 0 = 0 mg of protease/kg; ASB 200 = 200 mg of protease/kg; ASB 400 = 400 mg of protease/kg; ASB 800= 800 mg of protease/kg; ASB 1,600=1,600 mg of protease/kg.

[3]VCR= Villus height:crypt depth ratio

CAPITULO V

CONSIDERAÇÕES FINAIS

O presente trabalho permitiu uma visão integrada e multidisciplinar a respeito da utilização de soja *in natura* termicamente processada. A utilização da biotecnologia como ferramenta que permite, com maior precisão, a quantificação dos FANs mais relevantes para a nutrição de aves permitiu constatar o efeito da aplicação de calor sobre a estrutura protéica desses fatores e, conseqüentemente, sobre a atividade residual destes fitoquímicos contidos no grão e incorporados posteriormente na ração para frangos de corte. Contudo, os testes *in vitro,* mais simples e rápidos, empregados rotineiramente pela indústria mostraram-se bons indicadores de um adequado processamento térmico.

Usualmente, as respostas obtidas com o uso de enzimas multicomponentes são mais previsíveis comparadas com aquelas decorrentes da utilização de enzimas monocomponentes, principalmente das proteases. Apesar disso, o estudo permitiu comprovar o efeito desta enzima quando suplementada individualmente. Portanto, a suplementação de uma protease monocomponente permitiu a obtenção de respostas positivas no desempenho zootécnico das aves, ainda daquelas que consumiram soja crua na ração. Além disso, a adição desta protease exógena conseguiu contrarrestar a hipertrofia no pâncreas, causada pelos fatores antinutricionais presentes na soja integral.

Considerando o grande potencial que as proteases exógenas tem de melhorar a utilização da proteína em animais monogástricos e de complementar a ação das proteases endógenas (pepsina, tripsina, quimotripsina, elastase e carboxipeptidase) cuja atividade vai diminuindo ao longo do intestino delgado, recomenda-se a execução de novos estudos que avaliem o comportamento *in vitro* de uma protease mono-componente, bem como o efeito na digestibilidade da proteína e o impacto deste tipo de enzima exógena no meio ambiente.

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APÊNDICES

Apêndice 1. Dados referentes à analise especifica do farelo de soja comercial (SBM) e da soja integral termicamente tratada. Capítulo II.

Trat.	Proteína (mg/ml)	Urease g^{-1} proteína solúvel	Atividade residual da tripsina, %		Atividade residual quimotripsina %
			BAPNA	Azocaseína	
0min	60,25	4,34	13,21	9,23	5,40
0min	62,10	4,34	12,41	10,87	5,17
0min	74,90	4,41	12,55	14,79	5,03
105min	47,15	3,31	28,42	29,00	8,57
105min	65,35	3,59	29,05	11,11	11,07
105min	52,85	3,47	28,45	9,97	8,67
110min	47,65	3,41	34,55	44,28	19,66
110min	47,65	3,19	31,54	39,22	21,15
110min	46,85	3,79	29,31	41,91	19,77
135min	61,75	1,86	98,54	47,79	21,68
135min	56,15	2,05	87,56	98,69	23,15
135min	55,70	1,86	108,22	46,16	25,53
SBM	36,35	0,78	86,40	46,65	34,13
SBM	36,15	0,77	73,31	45,18	33,03
SBM	36,50	0,80	99,53	46,90	31,96

Apêndice 2. Dados referentes à analise especifica das rações para frango de corte com soja integral termicamente tratada. Capítulo III.

Trat.	Proteína (mg/ml)	Urease g^{-1} proteína solúvel	Atividade residual da tripsina, %		Atividade residual quimotripsina %
			BAPNA	Azocaseína	
T1	28,60	0,97	86,94	39,30	41,54
T1	28,40	0,90	82,82	40,60	41,63
T1	25,75	0,71	86,94	41,83	40,69
T2	28,20	1,67	89,33	38,48	40,35
T2	23,80	3,56	80,11	38,07	38,26
T2	27,25	1,55	80,97	37,99	70,90
T3	41,05	5,81	71,55	33,17	34,19
T3	43,95	5,59	70,09	33,99	39,41
T3	40,90	6,00	72,10	30,56	38,01
T4	33,25	7,21	57,05	22,14	19,96
T4	29,55	7,30	71,15	19,61	21,66
T4	27,75	7,59	52,04	25,98	21,19
T5	51,50	1,63	80,08	95,10	56,47
T5	36,25	0,84	73,51	43,06	34,09
T5	36,75	0,78	112,06	43,71	42,45
T6	28,80	1,29	53,20	42,65	41,18
T6	29,00	1,75	94,94	42,48	37,97
T6	25,35	1,40	93,67	43,55	39,24
T7	52,55	6,02	66,00	34,07	38,32
T7	38,45	6,19	75,87	36,19	38,74

T7	38,55	5,93	72,60	34,97	39,32
T8	27,35	7,69	63,77	29,17	26,01
T8	29,35	7,63	56,85	27,21	27,25
T8	28,35	7,56	52,87	26,72	20,61

Apêndice 3. Dados referentes à analise especifica do farelo de soja comercial (SBM) e de soja autoclavada durante 105 min. Capítulo IV.

Trat.	Proteína (mg/ml)	Urease g^{-1} proteína solúvel	Atividade residual da tripsina, %		Atividade residual quimotripsina %
			BAPNA	Azocaseína	
105min	49,80	2,30	51,13	78,46	15,90
105min	45,30	2,65	46,03	74,54	19,42
105min	48,15	2,66	77,89	88,25	18,66
SBM	36,35	0,78	86,39	46,65	34,13
SBM	36,15	0,77	73,31	45,18	33,03
SBM	36,50	0,80	99,53	46,90	31,96

Apêndice 4. Dados referentes à analise especifica das rações para frango de corte que contém soja autoclavada durante 105 min. Capítulo IV.

Trat.	Proteína (mg/ml)	Atividade residual da tripsina (BAPNA), %	Atividade residual quimotripsina %
T1	40,40	97,07	26,23
T1	43,15	82,38	22,33
T1	53,05	81,87	23,53
T2	58,45	89,05	24,95
T2	36,10	92,15	22,51
T2	45,55	91,08	23,57
T3	62,60	93,03	22,57
T3	44,10	91,26	23,31
T3	44,45	92,76	21,91
T4	50,15	74,70	23,71
T4	49,30	81,86	23,36
T4	51,70	75,09	23,14
T5	51,20	71,18	22,99
T5	49,80	80,98	22,01
T5	46,65	68,30	22,64

Apêndice 5. Consumo de ração (Cons.), ganho de peso (GP) e conversão alimentar (CA) de 1 a 28 dias de idade. Capítulo III.

Trat.	Cons. 1-7	GP 1-7	CA 1-7	Cons. 1-14	GP 1-14	CA 1-14	Cons. 1-21	GP 1-21	CA 1-21	Cons. 1-28	GP 1-28	CA 1-28
T1	0,139	0,118	1,175	0,465	0,351	1,324	1,240	0,848	1,463	2,166	1,522	1,423
T1	0,134	0,112	1,194	0,461	0,379	1,215	1,176	0,878	1,340	2,169	1,543	1,406
T1	0,133	0,107	1,248	0,450	0,350	1,286	1,170	0,850	1,377	2,433	1,510	1,612
T1	0,130	0,103	1,262	0,441	0,346	1,274	1,151	0,797	1,444	2,129	1,435	1,484
T1	0,142	0,120	1,182	0,418	0,283	1,478	1,045	0,727	1,437	2,221	1,344	1,653
T2	0,122	0,095	1,290	0,444	0,321	1,383	1,072	0,783	1,370	2,166	1,344	1,612

T2	0,125	0,082	1,527	0,453	0,307	1,476	1,076	0,722	1,490	1,998	1,325	1,509
T2	0,120	0,091	1,320	0,428	0,308	1,389	1,077	0,755	1,427	2,046	1,382	1,481
T2	0,114	0,081	1,407	0,422	0,300	1,407	1,105	0,728	1,517	2,082	1,373	1,516
T2	0,116	0,097	1,198	0,449	0,335	1,341	1,051	0,731	1,438	2,207	1,335	1,653
T3	0,131	0,112	1,177	0,467	0,374	1,251	1,192	0,841	1,417	2,221	1,536	1,446
T3	0,145	0,118	1,228	0,487	0,367	1,327	1,215	0,846	1,435	2,244	1,531	1,465
T3	0,124	0,077	1,614	0,425	0,292	1,454	1,064	0,750	1,418	1,946	1,299	1,498
T3	0,124	0,097	1,281	0,469	0,357	1,315	1,089	0,757	1,439	2,302	1,385	1,663
T3	0,129	0,107	1,197	0,471	0,351	1,341	1,158	0,831	1,393	2,425	1,458	1,663
T4	0,126	0,077	1,636	0,439	0,281	1,562	1,058	0,675	1,566	2,321	1,308	1,774
T4	0,122	0,085	1,436	0,463	0,311	1,491	1,151	0,743	1,549	2,161	1,373	1,574
T4	0,126	0,090	1,388	0,431	0,298	1,448	1,106	0,730	1,516	2,082	1,338	1,556
T4	0,116	0,088	1,318	0,451	0,323	1,394	1,072	0,697	1,538	2,306	1,260	1,830
T4	0,111	0,073	1,528	0,461	0,285	1,616	1,212	0,696	1,743	2,250	1,330	1,692
T5	0,134	0,110	1,224	0,484	0,372	1,301	1,231	0,883	1,394	2,187	1,525	1,434
T5	0,146	0,128	1,142	0,503	0,409	1,230	1,286	0,905	1,421	2,317	1,617	1,434
T5	0,138	0,114	1,208	0,468	0,360	1,299	1,098	0,828	1,326	2,158	1,450	1,488
T5	0,138	0,111	1,240	0,498	0,369	1,348	1,149	0,831	1,383	2,103	1,488	1,414
T5	0,141	0,124	1,132	0,484	0,400	1,210	1,074	0,877	1,224	2,240	1,508	1,485
T6	0,121	0,094	1,294	0,442	0,320	1,381	1,160	0,794	1,462	2,139	1,459	1,466
T6	0,118	0,089	1,327	0,430	0,311	1,381	1,005	0,705	1,427	1,894	1,289	1,469
T6	0,114	0,091	1,257	0,479	0,344	1,392	1,229	0,810	1,517	2,233	1,473	1,516
T6	0,117	0,091	1,292	0,429	0,324	1,326	1,071	0,715	1,499	2,246	1,372	1,637
T6	0,121	0,096	1,264	0,447	0,345	1,294	0,977	0,792	1,234	2,081	1,392	1,496
T7	0,140	0,118	1,180	0,497	0,388	1,282	1,219	0,882	1,381	2,209	1,542	1,432
T7	0,141	0,110	1,285	0,494	0,380	1,300	1,198	0,849	1,411	2,468	1,539	1,604
T7	0,131	0,097	1,356	0,446	0,319	1,401	1,157	0,799	1,448	2,148	1,469	1,462
T7	0,139	0,114	1,211	0,473	0,366	1,293	1,164	0,830	1,403	2,139	1,457	1,468
T7	0,131	0,101	1,293	0,492	0,369	1,336	1,202	0,802	1,499	2,462	1,418	1,736
T8	0,118	0,082	1,439	0,450	0,297	1,513	1,102	0,723	1,525	2,003	1,295	1,546
T8	0,124	0,087	1,424	0,461	0,320	1,441	1,172	0,724	1,617	2,140	1,316	1,627
T8	0,115	0,079	1,457	0,431	0,300	1,436	1,097	0,746	1,471	2,072	1,391	1,490
T8	0,121	0,090	1,349	0,461	0,332	1,389	1,058	0,750	1,411	2,151	1,292	1,666
T8	0,123	0,093	1,319	0,469	0,322	1,458	1,092	0,709	1,541	2,322	1,300	1,786

Apêndice 6. Rendimento de carcaça, gordura abdominal, peso relativo de pâncreas e duodeno de frangos de corte aos 28 dias de idade. Capítulo III.

Trat.	Carcaça, %	Gordura Abdominal, %	Peso relativo Pâncreas, %	Peso relativo Duodeno, %
T1	71,350	1,247	0,225	0,742
T1	70,135	1,056	0,235	0,875
T1	70,578	0,959	0,240	0,829
T1	70,287	1,196	0,262	0,875
T1	69,820	1,005	0,235	0,907
T2	70,337	1,402	0,296	0,790
T2	69,766	1,191	0,284	0,860
T2	70,354	0,981	0,287	0,789
T2	69,400	1,226	0,333	0,839
T2	69,234	1,101	0,288	0,832
T3	72,429	1,119	0,266	0,850
T3	70,151	1,076	0,246	0,792
T3	68,565	1,177	0,280	0,846

T3	69,935	1,017	0,294	0,899
T3	70,387	1,036	0,265	0,826
T4	70,034	1,068	0,396	0,959
T4	69,640	1,277	0,367	0,874
T4	68,976	1,504	0,353	0,986
T4	68,930	1,248	0,386	0,916
T4	68,368	1,042	0,341	0,961
T5	72,901	1,764	0,247	0,775
T5	72,326	1,036	0,212	0,829
T5	71,674	0,969	0,220	0,753
T5	73,801	1,141	0,206	0,784
T5	70,800	1,039	0,232	0,860
T6	71,224	1,007	0,281	0,783
T6	71,881	1,275	0,304	0,833
T6	70,208	0,670	0,281	0,828
T6	69,723	0,920	0,307	0,933
T6	72,721	0,987	0,288	0,844
T7	72,457	0,988	0,271	0,826
T7	70,138	1,089	0,262	0,822
T7	70,343	1,299	0,260	0,853
T7	70,198	0,909	0,273	0,799
T7	71,036	1,292	0,268	0,871
T8	71,872	1,086	0,367	0,912
T8	70,455	1,362	0,383	0,916
T8	69,440	1,317	0,389	0,928
T8	68,962	0,760	0,370	0,956
T8	70,068	1,113	0,370	0,921

Apêndice 7. Consumo de ração (Cons.), ganho de peso (GP) e conversão alimentar (CA) de 1 a 14 e de 14 a 28 dias de idade . Capitulo IV.

Trat.	**GP 1-14**	**CA 1-14**	**Cons. 1-14**	**GP 1-28**	**CA 1-28**	**Cons. 1-28**
T1	0,414	1,359	0,563	1,473	1,521	2,240
T1	0,386	1,354	0,523	1,352	1,504	2,034
T1	0,412	1,350	0,557	1,421	1,490	2,118
T1	0,386	1,338	0,517	1,405	1,467	2,062
T1	0,399	1,311	0,523	1,369	1,506	2,062
T1	0,405	1,295	0,524	1,415	1,482	2,097
T1	0,418	1,303	0,545	1,436	1,494	2,145
T1	0,378	1,372	0,519	1,363	1,494	2,036
T1	0,414	1,359	0,563	1,473	1,521	2,240
T1	0,386	1,354	0,523	1,352	1,504	2,034
T1	0,412	1,350	0,557	1,421	1,490	2,118
T1	0,386	1,338	0,517	1,405	1,467	2,062
T2	0,398	1,310	0,522	1,306	1,515	1,979
T2	0,402	1,324	0,532	1,428	1,475	2,105
T2	0,394	1,336	0,526	1,417	1,426	2,020
T2	0,406	1,371	0,557	1,503	1,563	2,349
T2	0,398	1,394	0,555	1,312	1,584	2,078
T2	0,383	1,403	0,537	1,398	1,477	2,065
T2	0,371	1,423	0,528	1,422	1,485	2,112
T2	0,408	1,275	0,520	1,464	1,482	2,169
T3	0,413	1,303	0,539	1,426	1,478	2,108
T3	0,370	1,429	0,530	1,312	1,570	2,061

T3	0,409	1,351	0,553	1,361	1,557	2,119
T3	0,391	1,392	0,545	1,396	1,517	2,118
T3	0,384	1,344	0,516	1,300	1,566	2,035
T3	0,359	1,415	0,508	1,406	1,499	2,108
T3	0,375	1,544	0,580	1,384	1,594	2,206
T3	0,389	1,372	0,534	1,395	1,509	2,105
T4	0,391	1,345	0,526	1,345	1,512	2,033
T4	0,404	1,363	0,550	1,470	1,502	2,209
T4	0,410	1,283	0,526	1,348	1,537	2,072
T4	0,434	1,289	0,559	1,430	1,481	2,117
T4	0,443	1,275	0,565	1,267	1,700	2,155
T4	0,377	1,454	0,548	1,467	1,538	2,256
T4	0,430	1,369	0,588	1,603	2,105	3,374
T4	0,384	1,399	0,537	1,488	1,712	2,547
T5	0,441	1,340	0,591	1,592	1,431	2,278
T5	0,420	1,275	0,535	1,465	1,430	2,095
T5	0,385	1,376	0,529	1,369	1,540	2,107
T5	0,430	1,284	0,552	1,368	1,514	2,071
T5	0,416	1,308	0,544	1,360	1,515	2,060
T5	0,396	1,327	0,525	1,410	1,516	2,137
T5	0,384	1,368	0,525	1,427	1,486	2,122
T5	0,343	1,381	0,474	1,445	1,444	2,088

Apêndice 8. Rendimento de carcaça, gordura abdominal, peso relativo de pâncreas e duodeno de frangos de corte aos 28 dias de idade. Capítulo IV.

Trat.	Carcaça, %	Gordura Abdominal, %	Peso relativo pâncreas, %	Peso relativo duodeno, %
T1	71,350	1,247	0,324	0,858
T1	70,135	1,056	0,312	0,822
T1	70,578	0,959	0,287	0,860
T1	70,287	1,196	0,296	0,710
T1	69,820	1,005	0,290	0,828
T2	70,337	1,402	0,302	0,894
T2	69,766	1,191	0,301	0,817
T2	70,354	0,981	0,307	0,797
T2	69,400	1,226	0,278	0,901
T2	69,234	1,101	0,295	0,906
T3	72,429	1,119	0,281	0,881
T3	70,151	1,076	0,266	0,850
T3	68,565	1,177	0,272	0,909
T3	69,935	1,017	0,255	0,908
T3	70,387	1,036	0,258	0,933
T4	70,034	1,068	0,260	0,860
T4	69,640	1,277	0,281	0,887
T4	68,976	1,504	0,268	0,951
T4	68,930	1,248	0,280	0,879
T4	68,368	1,042	0,254	0,910
T5	72,901	1,764	0,278	0,923
T5	72,326	1,036	0,271	0,910
T5	71,674	0,969	0,250	0,914
T5	73,801	1,141	0,250	0,978
T5	70,800	1,039	0,263	0,907
T6	71,224	1,007	0,268	0,935
T6	71,881	1,275	0,238	0,947

T6	70,208	0,670	0,247	0,853
T6	69,723	0,920	0,248	0,939
T6	72,721	0,987	0,244	0,982
T7	72,457	0,988	0,248	0,814
T7	70,138	1,089	0,245	0,910
T7	70,343	1,299	0,266	0,933
T7	70,198	0,909	0,216	0,885
T7	71,036	1,292	0,247	0,917
T8	71,872	1,086	0,257	0,920
T8	70,455	1,362	0,267	0,937
T8	69,440	1,317	0,251	0,865
T8	68,962	0,760	0,257	0,898
T8	70,068	1,113	0,183	0,969

Apêndice 9. Medidas morfométricas de duodeno de frangos de corte aos 14 dias de idade (objetiva 4X). Capítulo IV.

Trat.	Altura vilo, μm	Espessura total da mucosa, μm	Profundidade de cripta, μm	Relação vilo:cripta
T1	1491,00	1692,28	201,28	7,41
T1	1546,48	1800,31	253,82	6,09
T1	1749,01	2003,48	254,47	6,87
T1	1773,30	1996,38	223,08	7,95
T1	1600,34	1799,03	198,69	8,05
T1	1433,52	1597,80	164,28	8,73
T1	1584,96	1774,07	189,11	8,38
T1	1406,56	1560,83	154,27	9,12
T1	1327,10	1540,39	213,29	6,22
T1	1267,13	1396,48	129,35	9,80
T1	1449,62	1704,22	254,60	5,69
T1	1594,80	1832,88	238,09	6,70
T1	1190,67	1368,77	178,10	6,69
T1	1527,81	1774,41	246,60	6,20
T1	1574,90	1811,71	236,81	6,65
T1	1461,58	1654,69	193,11	7,57
T1	1422,08	1629,00	206,92	6,87
T1	1447,23	1589,13	141,89	10,20
T1	1374,71	1574,39	199,68	6,88
T1	1284,11	1487,29	203,19	6,32
T1	1169,44	1367,42	197,99	5,91
T1	1118,08	1328,89	210,81	5,30
T1	1146,90	1360,84	213,95	5,36
T1	1154,92	1340,17	185,25	6,23
T1	1214,11	1478,87	264,76	4,59
T1	1190,45	1441,93	251,48	4,73
T1	1293,88	1458,50	164,63	7,86
T1	1148,25	1376,18	227,93	5,04

T1	1260,45	1467,24	206,80	6,10
T1	1085,30	1318,12	232,82	4,66
T1	1490,57	1693,43	202,87	7,35
T1	1403,87	1647,65	243,78	5,76
T1	1080,82	1363,16	282,33	3,83
T1	991,69	1278,24	286,55	3,46
T1	1178,89	1385,97	207,07	5,69
T1	1203,57	1547,78	344,21	3,50
T1	1095,10	1400,90	305,81	3,58
T1	1302,37	1529,29	226,92	5,74
T1	1195,67	1514,11	318,44	3,75
T1	1441,06	1709,15	268,09	5,38
T1	1433,45	1625,71	192,26	7,46
T1	1297,46	1513,81	216,35	6,00
T2	1806,67	2033,59	226,92	7,96
T2	1792,71	1939,55	146,84	12,21
T2	1948,77	2144,42	195,65	9,96
T2	1797,28	1952,18	154,91	11,60
T2	1727,26	1946,78	219,52	7,87
T2	1088,08	1278,10	190,02	5,73
T2	1333,09	1602,61	269,53	4,95
T2	1598,03	1854,08	256,05	6,24
T2	1646,03	1889,00	242,97	6,77
T2	1274,16	1500,97	226,81	5,62
T2	1315,35	1549,68	234,33	5,61
T2	963,87	1237,69	273,82	3,52
T2	1808,34	2022,29	213,95	8,45
T2	1816,78	2039,90	223,12	8,14
T2	1495,55	1783,48	287,92	5,19
T2	1532,32	1736,36	204,04	7,51
T2	1580,04	1795,21	215,17	7,34
T2	1345,01	1560,03	215,02	6,26
T2	2015,84	2364,51	348,67	5,78
T2	1908,65	2207,80	299,15	6,38
T2	1871,29	2116,22	244,93	7,64
T2	1911,62	2154,90	243,28	7,86
T2	1984,03	2169,32	185,29	10,71
T2	1981,34	2197,16	215,82	9,18
T2	1924,76	2118,04	193,28	9,96
T2	1659,17	1805,11	145,95	11,37
T2	1634,08	1804,35	170,27	9,60
T2	1600,43	1762,59	162,16	9,87
T2	1661,42	1778,99	117,57	14,13
T2	1486,29	1654,91	168,62	8,81

T2	1279,38	1433,49	154,11	8,30
T2	1188,17	1343,92	155,75	7,63
T2	1220,28	1405,93	185,65	6,57
T2	1038,78	1239,07	200,30	5,19
T2	1586,14	1796,95	210,81	7,52
T2	1641,91	1844,65	202,74	8,10
T2	1591,02	1822,25	231,22	6,88
T2	1467,41	1646,98	179,57	8,17
T2	1205,53	1367,54	162,01	7,44
T3	1248,20	1426,58	178,38	7,00
T3	1210,76	1394,32	183,55	6,60
T3	1086,62	1237,50	150,87	7,20
T3	1131,33	1308,32	176,99	6,39
T3	1142,27	1338,75	196,49	5,81
T3	1078,88	1292,83	213,95	5,04
T3	1080,12	1262,23	182,12	5,93
T3	1037,86	1207,17	169,30	6,13
T3	911,84	1113,61	201,77	4,52
T3	767,79	1080,58	312,79	2,45
T3	1590,09	1817,31	227,21	7,00
T3	1841,10	2013,80	172,70	10,66
T3	1634,76	1807,46	172,70	9,47
T3	1757,08	1949,27	192,19	9,14
T3	1720,96	1912,13	191,17	9,00
T3	1614,00	1890,78	276,77	5,83
T3	1501,16	1769,47	268,32	5,59
T3	1678,66	1907,47	228,82	7,34
T3	1296,76	1559,20	262,44	4,94
T3	1176,63	1448,87	272,24	4,32
T3	1364,64	1662,90	298,26	4,58
T3	1728,93	2020,11	291,18	5,94
T3	1680,70	1911,69	230,99	7,28
T3	1532,47	1873,94	341,48	4,49
T3	1573,03	1874,11	301,07	5,22
T3	1555,42	1803,88	248,46	6,26
T4	1686,97	1977,97	291,01	5,80
T4	1702,81	2017,04	314,23	5,42
T4	1542,16	1820,74	278,57	5,54
T4	1463,91	1773,07	309,16	4,74
T4	1576,87	1971,67	394,80	3,99
T4	1141,68	1553,69	412,01	2,77
T4	1121,80	1452,25	330,45	3,39
T4	928,96	1274,33	345,38	2,69
T4	1271,03	1640,29	369,26	3,44

T4	1510,74	1850,44	339,70	4,45
T4	1282,16	1656,35	374,20	3,43
T4	1675,38	1994,25	318,87	5,25
T4	1765,76	2035,72	269,96	6,54
T4	1707,03	2007,33	300,30	5,68
T4	1725,36	1999,66	274,30	6,29
T4	1348,31	1648,54	300,23	4,49
T4	1814,73	2034,78	220,05	8,25
T4	1964,10	2229,76	265,66	7,39
T4	1580,02	1882,98	302,96	5,22
T4	1629,62	1939,71	310,09	5,26
T4	1784,80	2067,15	282,36	6,32
T4	1447,62	1759,54	311,92	4,64
T4	1514,46	1822,03	307,56	4,92
T4	1212,10	1464,29	252,19	4,81
T4	1583,64	1948,86	365,23	4,34
T4	1515,69	1892,86	377,17	4,02
T4	1044,80	1383,48	338,69	3,08
T4	1311,20	1727,51	416,30	3,15
T4	1148,60	1511,41	362,81	3,17
T4	705,49	1068,20	362,71	1,95
T4	867,80	1307,92	440,12	1,97
T4	1199,37	1646,41	447,04	2,68
T4	1343,84	1670,76	326,92	4,11
T4	813,59	1170,25	356,66	2,28
T4	1219,75	1621,60	401,85	3,04
T4	1124,40	1515,18	390,79	2,88
T4	1000,96	1478,26	477,30	2,10
T5	1385,89	1669,33	283,45	4,89
T5	1456,96	1702,03	245,07	5,95
T5	1326,25	1678,44	352,20	3,77
T5	1220,36	1577,24	356,87	3,42
T5	1014,29	1399,13	384,84	2,64
T5	1084,66	1454,30	369,64	2,93
T5	1280,02	1677,02	397,00	3,22
T5	1396,51	1910,35	513,85	2,72
T5	1109,65	1572,51	462,86	2,40
T5	998,36	1327,47	329,11	3,03
T5	1337,67	1853,31	515,63	2,59
T5	1507,70	1804,80	297,10	5,07
T5	1388,32	1667,50	279,18	4,97
T5	1426,83	1683,45	256,62	5,56
T5	1058,83	1361,67	302,84	3,50
T5	976,56	1333,02	356,46	2,74

T5	1085,54	1343,80	258,25	4,20
T5	878,92	1161,33	282,40	3,11
T5	856,24	1156,26	300,02	2,85
T5	941,96	1408,67	466,71	2,02
T5	1016,80	1504,37	487,57	2,09
T5	955,66	1328,35	372,69	2,56
T5	1131,20	1376,73	245,53	4,61
T5	1159,79	1501,47	341,68	3,39
T5	1178,01	1392,76	214,75	5,49
T5	1610,88	1875,98	265,10	6,08
T5	1567,29	1866,51	299,22	5,24
T5	1231,66	1532,01	300,35	4,10
T5	1608,69	1917,17	308,48	5,21
T5	1001,18	1306,40	305,22	3,28
T5	1285,23	1545,65	260,42	4,94
T5	1868,19	2124,80	256,62	7,28
T5	1412,18	1692,58	280,41	5,04
T5	1604,21	1850,85	246,64	6,50

Apêndice 10. Medidas morfométricas de duodeno de frangos de corte aos 28 dias de idade (objetiva 4X). Capítulo IV.

Trat.	**Altura vilo, μm**	**Espessura total da mucosa, μm**	**Profundidade de cripta, μm**	**Relação vilo:cripta**
T1	1582,60	1790,63	208,03	7,61
T1	1304,90	1509,61	204,71	6,37
T1	1368,41	1580,95	212,53	6,44
T1	1311,19	1536,15	224,96	5,83
T1	1403,26	1677,45	274,19	5,12
T1	1186,60	1459,92	273,32	4,34
T1	1644,06	1916,28	272,22	6,04
T1	1355,38	1641,93	286,56	4,73
T1	1816,30	2056,02	239,72	7,58
T1	1840,70	2221,38	380,68	4,84
T1	1745,07	2089,94	344,87	5,06
T1	1442,93	1778,98	336,05	4,29
T1	2144,79	2447,43	302,64	7,09
T1	1750,01	2053,87	303,86	5,76
T1	1227,28	1573,49	346,21	3,54
T1	1148,92	1500,43	351,51	3,27
T1	1393,39	1702,58	309,19	4,51
T1	1463,75	1827,85	364,10	4,02
T1	1155,51	1504,86	349,35	3,31
T1	1372,72	1712,96	340,24	4,03
T1	1278,06	1533,45	255,40	5,00
T1	1226,26	1481,39	255,13	4,81

T1	1440,59	1715,79	275,20	5,23
T1	1499,51	1731,09	231,58	6,48
T1	1666,56	2038,14	371,58	4,49
T1	1666,31	1965,70	299,39	5,57
T1	1382,43	1682,30	299,88	4,61
T1	1592,98	1888,59	295,61	5,39
T1	1723,18	1997,37	274,19	6,28
T1	1748,39	1992,03	243,64	7,18
T1	1942,29	2164,50	222,21	8,74
T1	1703,55	1982,23	278,68	6,11
T2	1737,60	2092,18	354,58	4,90
T2	1631,72	1963,97	332,24	4,91
T2	1175,91	1443,07	267,16	4,40
T2	1498,09	1904,20	406,11	3,69
T2	1557,42	1822,85	265,43	5,87
T2	1592,33	1798,13	205,79	7,74
T2	1641,69	1889,74	248,06	6,62
T2	1619,26	1846,95	227,69	7,11
T2	1583,57	1811,76	228,19	6,94
T2	1454,01	1725,87	271,85	5,35
T2	1415,91	1652,23	236,32	5,99
T2	1864,83	2092,52	227,69	8,19
T2	1873,63	2099,56	225,92	8,29
T2	1926,79	2123,67	196,88	9,79
T2	1648,91	1836,74	187,83	8,78
T2	1788,10	1979,83	191,73	9,33
T2	1881,01	2137,31	256,30	7,34
T2	1578,10	1801,59	223,49	7,06
T2	1838,15	2064,92	226,77	8,11
T2	1989,44	2107,26	117,82	16,89
T2	1852,46	1997,74	145,28	12,75
T2	1533,92	1713,28	179,36	8,55
T2	1613,32	1796,58	183,26	8,80
T2	1304,52	1677,71	373,19	3,50
T2	1808,42	2142,05	333,63	5,42
T2	2004,42	2386,78	382,37	5,24
T2	1601,53	1971,01	369,48	4,33
T2	1667,79	2052,95	385,16	4,33
T2	1918,07	2326,75	408,68	4,69
T2	1794,94	2119,58	324,64	5,53
T2	1922,73	2235,61	312,88	6,15
T2	1875,56	2142,84	267,28	7,02
T2	1768,47	2061,49	293,02	6,04
T3	2056,19	2312,40	256,21	8,03

T3	2334,27	2534,60	200,33	11,65
T3	2400,52	2577,19	176,67	13,59
T3	2437,98	2688,35	250,38	9,74
T3	2415,34	2631,64	216,31	11,17
T3	2514,75	2719,64	204,90	12,27
T3	2045,41	2241,58	196,18	10,43
T3	2460,67	2737,69	277,02	8,88
T3	2439,08	2690,10	251,02	9,72
T3	2507,60	2762,49	254,89	9,84
T3	1919,73	2281,61	361,87	5,30
T3	1903,43	2194,84	291,41	6,53
T3	2000,98	2271,84	270,86	7,39
T3	1584,38	1871,55	287,17	5,52
T3	1439,66	1704,51	264,85	5,44
T3	1593,97	1846,27	252,31	6,32
T3	1753,93	2050,42	296,49	5,92
T3	1585,16	1935,90	350,74	4,52
T3	1680,33	2003,05	322,72	5,21
T3	1611,05	1943,03	331,99	4,85
T3	1483,01	1786,69	303,68	4,88
T3	1446,56	1788,40	341,84	4,23
T3	2457,51	2657,73	200,22	12,27
T3	2471,35	2691,19	219,84	11,24
T3	2326,84	2547,20	220,36	10,56
T3	2422,12	2728,68	306,56	7,90
T3	2204,01	2427,29	223,28	9,87
T3	2114,34	2347,11	232,77	9,08
T3	2064,59	2344,62	280,03	7,37
T3	2079,89	2325,26	245,37	8,48
T3	2110,94	2365,83	254,89	8,28
T3	2076,10	2327,61	251,51	8,25
T3	2047,26	2280,46	233,20	8,78
T3	1947,50	2180,80	233,30	8,35
T3	1874,01	2112,15	238,14	7,87
T4	1962,93	2140,90	177,98	11,03
T4	1965,65	2152,91	187,26	10,50
T4	1460,60	1653,29	192,69	7,58
T4	1866,77	2041,64	174,87	10,67
T4	2011,41	2167,82	156,41	12,86
T4	2017,22	2186,04	168,82	11,95
T4	1795,14	2027,25	232,11	7,73
T4	1741,89	1962,01	220,12	7,91
T4	1563,98	1766,61	202,63	7,72
T4	1854,64	2057,26	202,63	9,15

T4	1605,37	1900,69	295,32	5,44
T4	1275,61	1552,30	276,69	4,61
T4	1452,33	1691,52	239,19	6,07
T4	1434,49	1720,13	285,63	5,02
T4	1770,68	1963,80	193,11	9,17
T4	1736,74	1997,74	261,01	6,65
T4	1360,14	1585,57	225,43	6,03
T4	1627,18	1869,14	241,96	6,73
T4	1081,10	1339,36	258,25	4,19
T4	1498,82	1647,89	149,07	10,05
T4	1590,75	1751,12	160,38	9,92
T5	2341,13	2603,74	262,61	8,91
T5	1915,33	2212,27	296,94	6,45
T5	1607,91	1876,37	268,46	5,99
T5	1659,87	1963,93	304,05	5,46
T5	1562,53	1824,01	261,48	5,98
T5	2142,90	2572,42	429,52	4,99
T5	1472,08	1738,57	266,49	5,52
T5	1904,98	2157,93	252,95	7,53
T5	1857,34	2131,73	274,39	6,77
T5	1778,25	2059,50	281,25	6,32
T5	1973,56	2290,84	317,28	6,22
T5	2112,56	2492,60	380,05	5,56
T5	2063,33	2355,25	291,92	7,07
T5	2134,93	2375,04	240,12	8,89
T5	1971,07	2298,04	326,97	6,03
T5	1987,97	2229,38	241,41	8,23
T5	1764,17	2003,67	239,50	7,37
T5	1924,43	2176,59	252,17	7,63
T5	1452,74	1711,35	258,60	5,62
T5	2072,02	2403,78	331,76	6,25
T5	1850,15	2163,99	313,84	5,90
T5	1338,75	1612,55	273,79	4,89

Apêndice 11. Quantificação de DNA do pâncreas de frango aos 7, 14, 21 e 28 dias de idade. Capítulo III.

Trat.	**7d**	**14d**	**21d**	**28d**
T1	119180,63	53886,93	48418,97	15384,62
T1	42513,86	67748,09	14563,11	16666,67
T2	42628,77	29203,54	9666,08	14545,45
T2	44750,43	86538,46	23465,70	20833,33
T3	26737,97	57788,94	39305,30	28046,42
T3	17825,31	111111,11	32882,01	26411,66
T4	32967,03	64150,94	36630,04	21100,92
T4	51587,30	107495,07	36190,48	29783,39

T5	36328,87	87837,84	63432,84	37072,24
T5	41516,25	52102,38	17889,09	22140,22
T6	19298,25	31818,18	27722,77	27938,34
T6	57692,31	27629,23	94517,96	31000,00
T7	44194,11	66729,32	40747,03	43137,25
T7	37366,55	16977,93	48128,34	71713,15
T8	61657,03	56350,63	21186,44	34722,22
T8	6768,95	62022,90	117697,59	42857,14

Apêndice 12. Relação proteína total:DNA do pâncreas de frango aos 7, 14, 21 e 28 dias de idade. Capítulo III.

Trat.	**7d**	**14d**	**21d**	**28d**
T1	0,4799	1,3514	1,2324	5,0537
T1	0,7589	0,8636	10,5661	7,1771
T2	1,5161	1,7729	8,6543	4,3744
T2	1,8229	1,4192	6,9779	3,6343
T3	1,9583	0,6559	2,7726	2,4008
T3	2,3825	0,8125	2,1896	3,3361
T4	1,3199	1,2253	2,8131	6,1358
T4	0,9988	0,5880	2,7291	2,6781
T5	1,8078	0,7756	2,3460	3,0232
T5	1,0281	1,5002	6,5068	5,4890
T6	4,8877	3,3023	4,0202	2,6535
T6	1,6879	3,9096	0,7108	2,9755
T7	2,4082	0,8432	2,8241	2,3570
T7	1,8625	4,7313	1,5390	0,7879
T8	0,4419	1,4237	2,8082	2,0137
T8	1,2383	1,6935	0,6547	1,3546

Apêndice 13. Peso relativo de pâncreas de frangos de corte aos 7, 14, 21 e 28 dias de idade. Capítulo III.

Trat.	**7d**	**14d**	**21d**	**28d**
T1	0,5660	0,4137	0,2930	0,3308
T1	0,4981	0,3604	0,3968	0,2411
T2	0,7019	0,7332	0,3052	0,3709
T2	0,7708	0,4397	0,3320	0,3183
T3	0,4677	0,2729	0,4785	0,3284
T3	0,6710	0,3668	0,2863	0,2914
T4	0,6867	0,7174	0,4687	0,4179
T4	0,5419	0,7190	0,3622	0,3878
T5	0,5118	0,3288	0,2063	0,1670
T5	0,4545	0,3781	0,2823	0,2580
T6	0,9746	0,5991	0,3997	0,2889
T6	0,7642	0,6667	0,3277	0,1638
T7	0,5540	0,4343	0,2500	0,3055

T7	0,5914	0,4750	0,3371	0,2595
T8	0,6804	0,7605	0,2882	0,3048
T8	0,4195	0,7356	0,5114	0,3812

Apêndice 14. Proteína total de pâncreas de frangos de corte aos 7, 14, 21 e 28 dias de idade. Capítulo III.

Trat.	7d	14d	21d	28d
T1	57196,91	72823,30	59673,77	77749,57
T1	32263,31	58504,07	153875,27	119618,92
T2	64630,20	51773,69	83652,76	63628,26
T2	81574,14	122818,19	163740,42	75714,83
T3	52360,86	37901,32	108976,49	67334,77
T3	42468,25	90275,38	71997,25	88111,19
T4	43513,63	78606,34	103043,85	129471,29
T4	51523,09	63204,83	98769,03	79763,66
T5	65675,57	68129,58	148816,37	112077,44
T5	42682,90	78161,78	116401,00	121528,16
T6	94324,23	105074,18	111450,16	74135,34
T6	97376,44	108019,26	67184,94	92241,05
T7	106428,24	56268,84	115075,27	101674,00
T7	69596,55	80327,73	74071,90	56504,77
T8	27244,53	80226,26	59495,87	69920,13
T8	8382,17	105035,99	77055,80	58054,55

Apêndice 15. Análises de variância referentes ao Capítulo II.

Análise de variância de atividade residual da quimotripsina da soja comercial (SBM) e das soja integral termicamente tratada.

Fontes de Variação	GL	SQ	QM	F	p
Tratamento	4	1492,808836	373,202209	242,80	<,0001
Erro	10	15,370781	1,537078		
Total	14	1508,179617			

Análise de variância de atividade de urease da soja comercial (SBM) e das soja integral termicamente tratada.

Fontes de Variação	GL	SQ	QM	F	p
Tratamento	4	24,47943077	6,11985769	242,64	<,0001
Erro	10	0,25221448	0,02522145		
Total	14	24,73164525			

Apêndice 16. Análises de variância referentes ao Capítulo III.

Análise de variância de atividade residual da tripsina (azocaseína) das rações para frango de corte com soja integral termicamente tratada.

Fontes de Variação	GL	SQ	QM	F	p
Tratamento	7	2767,683787	395,383398	3,48	0,0185
Erro	16	1819,756800	113,734800		
Total	23	4587,440586			

Análise de variância de atividade residual da tripsina (BAPNA) das rações para frango de corte com soja integral termicamente tratada.

Fontes de Variação	GL	SQ	QM	F	p

Tratamento	7	2826,375843	403,767978	2,75	0,0446
Erro	16	2351,227386	146,951712		
Total	23	5177,603229			

Análise de variância de atividade residual da quimotripsina das rações para frango de corte com soja integral termicamente tratada.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	7	1972,211014	281,744431	4,64	0,0052
Erro	16	970,739277	60,671205		
Total	23	2942,950291			

Análise de variância do consumo de ração de frangos de 1 a 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,07437305	0,02479102	1,88	0,1555
Enzima	1	0,00028604	0,00028604	0,02	0,8840
IU x Enzima	3	0,06688486	0,02229495	1,69	0,1913
Erro	29	0,38290039	0,01320346		
Total	36	0,53086168			

Análise de variância do ganho de peso de frangos de 1 a 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,16735096	0,05578365	14,18	<,0001
Enzima	1	0,01818041	0,01818041	4,62	0,0401
IU x Enzima	3	0,00329019	0,00109673	0,28	0,8402
Erro	29	0,11409012	0,00393414		
Total	36	0,30634143			

Análise de variância da conversão alimentar de frangos de 1 a 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,07491349	0,02497116	4,91	0,0115
Enzima	1	0,02758270	0,02758270	5,43	0,0317
IU x Enzima	3	0,00422857	0,00140952	0,28	0,8410
Erro	18	0,09150203	0,00508345		
Total	25	0,20714391			

Análise de variância do peso corporal de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,15249989	0,05083330	12,73	<,0001
Enzima	1	0,01729268	0,01729268	4,33	0,0464
IU x Enzima	3	0,00592369	0,00197456	0,49	0,6889
Erro	29	0,11578847	0,00399271		
Total	36	0,29982938			

Análise de variância do peso relativo do pâncreas de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,09550052	0,03183351	135,44	<,0001
Enzima	1	0,00182943	0,00182943	7,78	0,0088
IU x Enzima	3	0,00022298	0,00007433	0,32	0,8135
Erro	32	0,00752138	0,00023504		
Total	39	0,10507432			

Análise de variância do peso relativo do duodeno de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,07791124	0,02597041	31,15	<,0001

Enzima	1	0,01319359	0,01319359	15,83	0,0004
IU x Enzima	3	0,00389382	0,00129794	1,56	0,2219
Erro	28	0,02334105	0,00083361		
Total	35	0,11877100			

Análise de variância da porcentagem de gordura abdominal de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,07964728	0,02654909	1,10	0,3656
Enzima	1	0,15969633	0,15969633	6,63	0,0159
IU x Enzima	3	0,15154603	0,05051534	2,10	0,1242
Erro	27	0,65079893	0,02410366		
Total	34	0,99471555			

Análise de variância do rendimento de carcaça dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	8,76872344	2,92290781	3,53	0,0276
Enzima	1	18,17135480	18,17135480	21,93	<,0001
IU x Enzima	3	1,37969183	0,45989728	0,55	0,6491
Erro	28	23,20331988	0,82869000		
Total	35	53,24327955			

Análise de variância peso relativo de pâncreas de frangos aos 7 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,19934910	0,06644970	5,64	0,0226
Enzima	1	0,00013369	0,00013369	0,01	0,9178
IU x Enzima	3	0,02408936	0,00802979	0,68	0,5880
Erro	8	0,09433643	0,01179205		
Total	15	0,31790858			

Análise de variância do peso relativo de pâncreas de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,37362492	0,12454164	18,61	0,0006
Enzima	1	0,00787790	0,00787790	1,18	0,3095
IU x Enzima	3	0,01447428	0,00482476	0,72	0,5670
Erro	8	0,05353502	0,00669188		
Total	15	0,44951212			

Análise de variância do peso relativo de pâncreas de frangos aos 21 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,02610776	0,00870259	1,09	0,4086
Enzima	1	0,00640109	0,00640109	0,80	0,3974
IU x Enzima	3	0,01390166	0,00463389	0,58	0,6453
Erro	8	0,06407201	0,00800900		
Total	15	0,11048252			

Análise de variância do peso relativo de pâncreas de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	0,03245512	0,01081837	3,85	0,0566
Enzima	1	0,01944899	0,01944899	6,92	0,0302
IU x Enzima	3	0,00425293	0,00141764	0,50	0,6899
Erro	8	0,02248538	0,00281067		
Total	15	0,07864242			

Análise de variância da proteína total de pâncreas de frangos aos 7 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	2834937,950	944979,317	3,03	0,0934

Enzima	1	1227079,405	1227079,405	3,93	0,0827
IU x Enzima	3	572247,404	190749,135	0,61	0,6264
Erro	8	2496536,600	312067,075		
Total	15	7130801,359			

Análise de variância da proteína total de pâncreas de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	1715855,251	571951,750	1,53	0,2802
Enzima	1	696073,348	696073,348	1,86	0,2097
IU x Enzima	3	207253,396	69084,465	0,18	0,9039
Erro	8	2993367,388	374170,924		
Total	15	5612549,384			

Análise de variância da proteína total de pâncreas de frangos aos 21 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	1555123,642	518374,547	0,53	0,6719
Enzima	1	111891,060	111891,060	0,12	0,7430
IU x Enzima	3	2615224,198	871741,399	0,90	0,4833
Erro	8	7767918,84	970989,85		
Total	15	12050157,74			

Análise de variância da proteína total de pâncreas de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	2311453,459	770484,486	2,46	0,1378
Enzima	1	67470,364	67470,364	0,21	0,6552
IU x Enzima	3	1365596,024	455198,675	1,45	0,2990
Erro	8	2510711,885	313838,986		
Total	15	6255231,732			

Análise de variância da quantificação de DNA do pâncreas de frango aos 7 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	1193906,250	397968,750	1,16	0,3841
Enzima	1	7656,250	7656,250	0,02	0,8851
IU x Enzima	3	2003906,250	667968,750	1,94	0,2015
Erro	8	2751875,000	343984,375		
Total	15	5957343,750			

Análise de variância da quantificação de DNA do pâncreas de frango aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	1187656,250	395885,417	0,95	0,4608
Enzima	1	1265625,000	1265625,000	3,04	0,1194
IU x Enzima	3	922812,500	307604,167	0,74	0,5579
Erro	8	3330000,000	416250,000		
Total	15	6706093,750			

Análise de variância da quantificação de DNA do pâncreas de frango aos 21 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	710312,500	236770,833	0,28	0,8375
Enzima	1	1960000,000	1960000,000	2,33	0,1654
IU x Enzima	3	624062,500	208020,833	0,25	0,8611
Erro	8	6729375,00	841171,88		
Total	15	10023750,00			

Análise de variância da quantificação de DNA do pâncreas de frango aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	638554,6875	212851,5625	4,69	0,0359

Enzima	1	733164,0625	733164,0625	16,14	0,0039
IU x Enzima	3	120429,6875	40143,2292	0,88	0,4895
Erro	8	363437,500	45429,688		
Total	15	1855585,938			

Análise de variância da relação proteína total:DNA do pâncreas de frangos aos 7 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	7,04135039	2,34711680	3,07	0,0909
Enzima	1	1,06357125	1,06357125	1,39	0,2721
IU x Enzima	3	2,29611695	0,76537232	1,00	0,4407
Erro	8	6,11703234	0,76462904		
Total	15	16,51807092			

Análise de variância da relação proteína total:DNA do pâncreas de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	5,46201112	1,82067037	1,73	0,2387
Enzima	1	5,62966048	5,62966048	5,34	0,0497
IU x Enzima	3	3,05131409	1,01710470	0,96	0,4554
Erro	8	8,43867673	1,05483459		
Total	15	22,58166243			

Análise de variância da relação proteína total:DNA do pâncreas de frangos aos 21 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	32,18360626	10,72786875	1,38	0,3185
Enzima	1	17,06744250	17,06744250	2,19	0,1774
IU x Enzima	3	15,98111503	5,32703834	0,68	0,5871
Erro	8	62,4136223	7,8017028		
Total	15	127,6457861			

Análise de variância da relação proteína total:DNA do pâncreas de frangos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Índice de urease (IU)	3	18,75587841	6,25195947	3,71	0,0613
Enzima	1	12,48905593	12,48905593	7,41	0,0262
IU x Enzima	3	1,47705271	0,49235090	0,29	0,8301
Erro	8	13,48358828	1,68544853		
Total	15	46,20557533			

Apêndice 17. Análises de variância referentes ao Capítulo IV.

Análise de variância de atividade de urease da soja comercial (SBM) e de soja autoclavada durante 105 min.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	1	4,62500099	4,62500099	218,62	0,0001
Erro	4	0,08462256	0,02115564		
Total	5	4,70962355			

Análise de variância de atividade residual da tripsina (BAPNA) da soja comercial (SBM) e de soja autoclavada durante 105 min.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	1	945,666459	945,666459	3,05	0,1789
Erro	3	929,401957	309,800652		
Total	4	1875,068416			

Análise de variância de atividade residual da quimotripsina de soja comercial (SBM) e de soja autoclavada durante 105 min.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**

Tratamento	1	339,4390837	339,4390837	146,85	0,0003
Erro	4	9,2458693	2,3114673		
Total	5	348,6849531			

Análise de variância da atividade residual da tripsina (BAPNA) das rações para frango de corte que contém soja autoclavada durante 105 min.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	1209,289851	302,322463	0,40	0,8017
Erro	8	6000,268788	750,033599		
Total	12	7209,558639			

Análise de variância da atividade residual da quimotripsina das rações para frango de corte que contém soja autoclavada durante 105 min.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	5,22408384	1,30602096	1,03	0,4361
Erro	10	12,62312444	1,26231244		
Total	14	17,84720828			

Análise de variância de ganho de peso dos frangos de 1 a 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,00414177	0,00103544	3,07	0,0303
Erro	32	0,01080824	0,00033776		
Total	36	0,01495001			

Análise de variância de conversão alimentar dos frangos de 1 a 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,03124292	0,00781073	2,89	0,0377
Erro	32	0,08646417	0,00270201		
Total	36	0,11770709			

Análise de variância de consumo de ração dos frangos de 1 a 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,00140878	0,00035219	0,89	0,4825
Erro	35	0,01391401	0,00039754		
Total	39	0,01532279			

Análise de variância de ganho de peso dos frangos de 14 a 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,01683232	0,00420808	0,84	0,5102
Erro	35	0,17566163	0,00501890		
Total	39	0,19249396			

Análise de variância de consumo de ração dos frangos de 14 a 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,41273634	0,10318409	2,39	0,0696
Erro	35	1,51167771	0,04319079		
Total	39	1,92441406			

Análise de variância de conversão alimentar dos frangos de 14 a 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,02059486	0,00514872	3,71	0,0139

Erro	31	0,04298408	0,00138658		
Total	35	0,06357894			

Análise de variância de rendimento de carcaça de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	92,9916960	23,2479240	3,09	0,0294
Erro	32	240,7965043	7,5248908		
Total	36	333,7882003			

Análise de variância da porcentagem de gordura abdominal de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,38591099	0,09647775	2,82	0,0424
Erro	30	1,02562560	0,03418752		
Total	34	1,41153659			

Análise de variância do peso relativo do pâncreas de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,01657143	0,00414286	13,80	<,0001
Erro	34	0,01020407	0,00030012		
Total	38	0,02677550			

Análise de variância do peso relativo do duodeno de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	0,05061334	0,01265334	6,96	0,0003
Erro	34	0,06183144	0,00181857		
Total	38	0,11244478			

Análise de variância da altura de vilo de duodeno de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	2248964,41	562241,10	7,90	<,0001
Erro	173	12310271,50	71157,64		
Total	177	14559235,92			

Análise de variância da espessura total da mucosa duodenal de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	1521256,76	380314,19	5,58	0,0003
Erro	173	11782790,00	68108,61		
Total	177	13304046,76			

Análise de variância de profundidade de cripta do duodeno de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	573700,277	143425,069	42,77	<,0001
Erro	173	580075,957	3353,040		
Total	177	1153776,233			

Análise de variância da relação vilo:cripta do duodeno de frangos aos 14 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	377,4394257	94,3598564	30,96	<,0001
Erro	173	527,2535504	3,0477084		
Total	177	904,6929761			

Análise de variância da altura de vilo de duodeno de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	5474326,42	1368581,60	19,13	<,0001

Erro	138	9870663,04	71526,54		
Total	142	15344989,46			

Análise de variância da espessura total da mucosa duodenal de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	5415748,61	1353937,15	19,76	<,0001
Erro	138	9453506,27	68503,67		
Total	142	14869254,88			

Análise de variância da profundidade de cripta do duodeno de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	85437,1115	21359,2779	6,80	<,0001
Erro	138	433336,0203	3140,1161		
Total	142	518773,1318			

Análise de variância da relação vilo:cripta do duodeno de frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	4	168,4488600	42,1122150	8,96	<,0001
Erro	138	648,6564108	4,7004088		
Total	142	817,1052708			

Análise de regressão para rendimento de carcaça dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	2	88,62751	44,31375	6,15	0,0053
Erro	34	245,16069	7,21061		
Total	36	333,78820			
	GL	**Parâmetro estimado**	**Erro padrão**	**t**	**p**
Intercepto	1	67,71856	0,85665	79,05	<,0001
Linear	1	0,00908	0,00313	2,90	0,0065
Quadrática	1	-0,00000422	0,00000181	-2,33	0,0260

R^2= 0,2655

Análise de regressão para porcentagem de gordura abdominal dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	2	0,37039	0,18519	5,69	0,0077
Erro	32	1,04115	0,03254		
Total	34	1,41154			
	GL	**Parâmetro estimado**	**Erro padrão**	**t**	**p**
Intercepto	1	1,35522	0,05486	24,70	<,0001
Linear	1	-0,00068073	0,00020695	-3,29	0,0024
Quadratica	1	3,651944E-7	1,230892E-7	2,97	0,0057

R^2= 0,2624

Análise de regressão para tamanho relativo de pâncreas dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	2	0,01553	0,00777	24,87	<,0001
Erro	36	0,01124	0,00031232		
Total	38	0,02678			
	GL	**Parâmetro estimado**	**Erro padrão**	**t**	**p**
Intercepto	1	0,29666	0,00534	55,56	<,0001
Linear	1	-0,00009157	0,00001938	-4,72	<,0001
Quadratica	1	3,656938E-8	1,131762E-8	3,23	0,0026

R^2= 0,5801

Análise de regressão para tamanho relativo de duodeno dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	3	0,05054	0,01685	9,53	<,0001

Erro	35	0,06190	0,00177		
Total	38	0,11244			
	GL	**Parâmetro estimado**	**Erro padrão**	**t**	**p**
Intercepto	1	0,82396	0,01445	57,03	<,0001
Linear	1	0,00044904	0,00011292	3,98	0,0003
Quadrática	1	-6,07362E-7	2,063124E-7	-2,94	0,0057
Cúbica	1	2,2655E-10	8,96289E-11	2,53	0,0162

R^2= 0,4495

Análise de regressão para tamanho relativo de duodeno dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	3	573152	191051	57,25	<,0001
Erro	174	580625	3336,92315		
Total	177	1153776			
	GL	**Parâmetro estimado**	**Erro padrão**	**t**	**p**
Intercepto	1	222,93753	8,71755	25,57	<,0001
Linear	1	-0,20725	0,07357	-2,82	0,0054
Quadrática	1	0,00070760	0,00013340	5,30	<,0001
Cúbica	1	-3,35326E-7	5,742938E-8	-5,84	<,0001

R^2= 0,4968

Análise de regressão para tamanho relativo de duodeno dos frangos aos 28 dias de idade.

Fontes de Variação	**GL**	**SQ**	**QM**	**F**	**p**
Tratamento	3	353,87357	117,95786	37,26	<,0001
Erro	174	550,81940	3,16563		
Total	177	904,69298			
	GL	**Parâmetro estimado**	**Erro padrão**	**t**	**p**
Intercepto	1	6,48134	0,26850	24,14	<,0001
Linear	1	0,00858	0,00227	3,79	0,0002
Quadrática	1	-0,00002229	0,00000411	-5,43	<,0001
Cúbica	1	9,987762E-9	1,76885E-9	5,65	<,0001

R^2= 0,3912

VITA

María Esperanza Mayorga Cortés, filha de Oscar Mayorga e Maria Cortés, nasceu em 25 de novembro de 1977, na cidade de Bogotá, DC, Colômbia.

Cursou o ensino fundamental e médio no colégio San José em Bogotá, DC, Em 1996 ingressou no curso de Zootecnia da Universidade Nacional de Colômbia, em Bogotá, DC, obtendo o grau de Zootecnista em junho de 2002.

Iniciou seus estudos de mestrado em marco de 2006, na Área de Produção Animal no programa de Pós-graduação em Zootecnia da Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, No ano de 2008 terminou seus estudos de mestrado e iniciou seu doutorado na mesma instituição, permanecendo nesta até o presente momento.