UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

CAROLINE FREDRICH DOURADO PINTO

AVALIAÇÃO DA PROTEÍNA HIDROLISADA NA DIETA DE CÃES: POTENCIAL HIPOALERGÊNICO, EFEITO SOBRE DIGESTÃO E FERMENTAÇÃO INTESTINAL, QUALIDADE FECAL, MICROBIOTA INTESTINAL E FORMAÇÃO DE AMINAS BIOGÊNICAS

Porto Alegre
2023

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Tese apresentada como requisito para obtenção do Grau de Doutor em Zootecnia, na Faculdade de Agronomia, da Universidade Federal do Rio Grande do Sul.
Orientador: Luciano Trevizan

Porto Alegre, Rio Grande do Sul, Brasil
Março de 2023

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TESE

Submetida como parte dos requisitos

para obtenção do Grau de

DOUTORA EM ZOOTECNIA

Programa de Pós-Graduação em Zootecnia

Faculdade de Agronomia

Universidade Federal do Rio Grande do Sul

Porto Alegre (RS), Brasil

Aprovada em: 29.03.2023
Pela Banca Examinadora

Documento assinado digitalmente
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CIP - Catalogação na Publicação

Pinto, Caroline Fredrich Dourado
AVALIAÇÃO DA PROTEÍNA HIDROLISADA NA DIETA DE CÃES: POTENCIAL HIPOALERGÊNICO, EFEITO SOBRE DIGESTÃO E FERMENTAÇÃO INTESTINAL, QUALIDADE FECAL, MICROBIOTA INTESTINAL E FORMAÇÃO DE AMINAS BIOGÊNICAS / Caroline Fredrich Dourado Pinto. -- 2023.
155 f.
Orientador: Luciano Trevizan.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Agronomia, Programa de Pós-Graduação em Zootecnia, Porto Alegre, BR-RS, 2023.

1. aminas biogênicas. 2. digestibilidade. 3. produtos de fermentação fecal. 4. proteína hidrolisada. 5. resposta imune. I. Trevizan, Luciano, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

À minha família pelo incentivo e amor incondicional.

Aos colegas e técnicos do Laboratório de Ensino Zootécnico (LEZO) pelo auxílio durante os ensaios experimentais.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos.

À Kemin pelo apoio financeiro, indispensável à execução dos projetos.

Ao meu orientador e professores pelos ensinamentos.

E por último, aos meus queridos colaboradores de pesquisa. Ao longo desta etapa foram muitos, mas citarei todos devido à imensa contribuição: Peppa, Nina, Adele, Tina, Duda, Pandora, Ozzy, Eddie, Remmy, Bruce, Bono, Yuri, Aston, Marla, Matheo, Açaí, Close, Vera, Lennon, Mariane, Joco, Tati, Sauco, Star e Cacau.

AVALIAÇÃO DA PROTEÍNA HIDROLISADA NA DIETA DE CÃES: POTENCIAL HIPOALERGÊNICO, EFEITO SOBRE DIGESTÃO E FERMENTAÇÃO INTESTINAL, QUALIDADE FECAL, MICROBIOTA INTESTINAL E FORMAÇÃO DE AMINAS BIOGÊNICAS[1]

Autor: Caroline Fredrich Dourado Pinto
Orientador: Prof. Dr. Luciano Trevizan

RESUMO

As proteínas hidrolisadas têm despertado interesse devido a disponibilização de peptídeos de baixo peso molecular, compatível com características nutricionais como alta digestibilidade de aminoácidos, reduzida antigenicidade e presença de peptídeos bioativos. Foram conduzidas duas séries experimentais com o objetivo de avaliar os efeitos da inclusão do hidrolisado de fígado de aves (HFA) como fonte proteica majoritária de origem animal em dietas para cães adultos, saudáveis: I) análise da composição química e peso molecular do HFA, seguido pela avaliação de duas dietas: HFA – dieta com 25,6% de HFA e CON – dieta com farinha de vísceras de aves (FVA) e farinha de carne e ossos, sobre a palatabilidade, os coeficientes de digestibilidade aparente (CDA), características fecais e urinárias, parâmetros hematológicos e imunológicos, e microbiota fecal ao longo de 45 dias; e II) avaliação do efeito de duas fontes proteicas (HFA e FVA) e três concentrações de proteína bruta (PB) (24, 32 e 40%) combinados em seis tratamentos: HFA24, HFA32, HFA40, FVA24, FVA32 e FVA40, sobre os CDA, características fecais e urinárias, produtos de fermentação nas fezes, formação de aminas biogênicas, concentrações plasmáticas de MAO (monoamina oxidase) e DAO (diamina oxidase), e CAT (capacidade antioxidante total) após 30 dias de alimentação. Os resultados do experimento I demonstraram que o HFA possui altas concentrações de lisina, ácidos linoleico e araquidônico, e 57% dos peptídeos com peso molecular <10 kDa. A dieta HFA aumentou a umidade e produção fecal diária (P<0,05), mas sem prejuízos a consistência fecal. A concentração plasmática de IL-4 reduziu ao longo do tempo (P<0,001). No grupo CON, houve redução na concentração plasmática de IgA no dia 30 comparado aos dias 0 e 45 (P<0,001). A concentração plasmática de IgE reduziu nos dias 30 e 45 no grupo CON, e nos dias 15 *vs* 30 e 15 *vs* 45 no grupo HFA (P=0,001). Foi verificada maior similaridade da diversidade beta nas fezes dos cães alimentados com HFA. No experimento II, foi verificado maior CDA da PB nas dietas contendo HFA e maiores concentrações de PB (P<0,05). Entretanto, o CDA dos demais nutrientes e energia reduziram (P<0,05). O aumento das concentrações de PB aumentou a produção fecal diária, umidade fecal e escore fecal (P<0,05). As dietas contendo FVA com 32 e 40% de PB aumentaram as concentrações fecais de isovalerato, ácidos graxos de cadeia ramificada e amônia (P<0,05). A inclusão de FVA e maiores concentrações de PB aumentou o consumo de aminas biogênicas putrefativas (P<0,05). As dietas contendo HFA com 32 e 40% de PB aumentaram a excreção fecal de feniletilamina (P=0,045). Por último, as concentrações plasmáticas de MAO foram maiores com as dietas HFA24 e FVA32 (P=0.024).

Palavras-chave: aminas biogênicas; digestibilidade; produtos de fermentação fecal; proteína hidrolisada; resposta imune

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[1]Tese de Doutorado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (155 p.) Março, 2023.

EVALUATION OF HYDROLYZED PROTEIN IN THE DIET OF DOGS: HYPOALLERGENIC POTENTIAL, EFFECT ON DIGESTION AND INTESTINAL FERMENTATION, FECAL QUALITY, INTESTINAL MICROBIOTA, AND BIOGENIC AMINES FORMATION[1]

Author: Caroline Fredrich Dourado Pinto
Advisor: Prof. Dr. Luciano Trevizan

ABSTRACT

Hydrolyzed proteins have aroused interest due to the availability of low molecular weight peptides, compatible with nutritional characteristics such as high digestibility of amino acids, reduced antigenicity and presence of bioactive peptides. Two experimental series were carried out aiming to evaluate the effects of hydrolyzed chicken liver (HCL) inclusion in the diet as the majority animal protein source using healthy adult Beagle dogs: I) analysis of the chemical composition and molecular weight of the HCL, followed by the evaluation of two diets: HCL – diet with 25.6% HCL and CON - with poultry by-product meal (PBPM) and bovine meat and bone meal on palatability, apparent total tract digestibility (ATTD), fecal and urinary characteristics, hematological and immunological parameters, and fecal microbiota over 45 days; and II) evaluation of the effect of two protein sources (HCL and PBPM) and three concentrations of crude protein (CP) (24, 32 and 40%) combined in six treatments: HCL24, HCL32, HCL40, PBPM24, PBPM32 and PBPM40, on ATTD, fecal and urinary characteristics, fermentation products in feces, biogenic amines in feces, plasma concentrations of MAO (monoamine oxidase) and DAO (diamine oxidase), and TAC (total antioxidant capacity) after 30 days of feeding. The results of experiment I demonstrated that HCL has high concentrations of lysine, linoleic and arachidonic acids, and 59% of peptides with molecular weight <10 kDa. The HCL diet increased fecal score and daily fecal production ($P<0.05$), but without impairing fecal consistency. Plasma IL-4 concentration decreased over time ($P<0.001$). In the CON group, there was a reduction in plasma IgA concentration on day 30 compared to days 0 and 45 ($P<0.001$). Plasma IgE concentration decreased on days 30 and 45 in the CON group, and on days 15 *vs*. 30 and 15 *vs.* 45 in the HCL group ($P=0.001$). Greater similarity of beta diversity was verified in the feces of dogs fed with HCL. In experiment II, there was a higher ATTD of CP in the diets containing HCL and higher concentrations of CP ($P<0.05$). However, the ATTD of other nutrients and energy decreased ($P<0.05$). Increasing CP concentration increased daily fecal output, fecal moisture, and fecal score ($P<0.05$). Diets containing PBPM with 32 and 40% CP increased fecal concentrations of isovalerate, branched-chain fatty acids, and ammonia ($P<0.05$). The inclusion of PBPM and higher CP concentrations increased the consumption of putrefactive biogenic amines ($P<0.05$). Lastly, plasma MAO concentrations were higher with the HCL24 and PBPM32 diets ($P=0.024$).

Key words: biogenic amines; digestibility; hydrolyzed protein; immune response; intestinal fermentative end products

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[1]Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (155 p.) March, 2023.

SUMÁRIO

RELAÇÃO DE TABELAS

RELAÇÃO DE FIGURAS

between fat ATTD and TTTD, and nitrogen-free extract (NFE) ATTD and HCLP24 and PBPM24 diets. (B) PCA shows a negative correlation between fecal DM and fecal score. Fecal score was correlated with PBPM40, PBPM32, and HCLP40 diets. Fecal DM was correlated with PBPM24 and HCLP24 diets. Fecal output was correlated with HCLP40. (C) After 30 days of feeding, there was a clear difference in fecal concentrations of valerate, isovalerate, branched-chain fatty acids (BCFA), ammonia, and isobutyrate in PBPM32 and PBPM40 diets compared to the others. Furthermore, fecal concentrations of valerate, isovalerate, BCFA, ammonia, and isobutyrate were negatively correlated with fecal concentrations of lactate, butyrate, acetate, propionate, volatile fatty acids (VFA), and short-chain fatty acids (SCFA).

RELAÇÃO DE ABREVIATURAS E SÍMBOLOS

AGCC	Ácidos graxos de cadeia curta
AGCR	Ácidos graxos de cadeia ramificada
ATTD	Apparent total tract digestibility
DAO	Diamine oxidase
FVA	Farinha de vísceras de aves
HFA	Hidrolisado de fígado de aves
HCLP	Hydrolyzed chicken liver
kDa	Kilodalton
mg	Miligrama
MAO	Monoamine oxidase
NRC	National Research Council
OTU	Operational taxonomic unit
PBPM	Poultry by-product meal
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PB	Proteína bruta
kcal	Quilocaloria
kg	Quilograma
TAC	Total antioxidant capacity
TTTD	True total tract digestibility

CAPÍTULO I

1. INTRODUÇÃO

A produção de alimentos para animais de companhia está voltada ao atendimento das necessidades nutricionais pré-estabelecidas pelo NRC (2006). Os alimentos completos são adequados para animais saudáveis sem qualquer transtorno nutricional em que a dieta possa ter papel coadjuvante. Entretanto, algumas doenças desenvolvidas em cães requerem tratamento nutricional específico para driblar os mecanismos da patogênese. Nestes casos as dietas funcionam como coadjuvantes a terapêutica medicamentosa.

A produção de alimentos coadjuvantes requer ingredientes especiais, que contenham características próprias, sejam seguros e funcionais. Na nutrição de cães a incidência de distúrbios gastrintestinais que comprometem a digestão e absorção de nutrientes, que tenham envolvimento em reações adversas alimentares ou doenças inflamatórias intestinais requerem ingredientes proteicos previamente digeridos. Fontes proteicas de baixo peso molecular, secas e fáceis de ingressarem em formulações estão disponíveis no mercado e são conhecidas como proteínas hidrolisadas.

O uso de proteínas hidrolisadas é preconizado na confecção de dietas para o diagnóstico e manutenção de animais de companhia com distúrbios gastrointestinais, pois tem como principais benefícios a elevada palatabilidade, reduzida imunogenicidade e digestão facilitada (Cave, 2006). O processo de hidrólise promove a ruptura das cadeias peptídicas resultando em aminoácidos livres e pequenos peptídeos, que são mais facilmente absorvidos pela mucosa intestinal comparados a molécula íntegra (Monchi & Rerat, 1993). Além disso, a quebra das ligações peptídicas reduz o peso molecular dos peptídeos, de acordo com o grau de hidrólise aplicado a matéria-prima, possibilitando a redução da antigenicidade e alergenicidade da proteína, uma vez que moléculas de menor calibre resultam em menor reconhecimento como um possível antígeno pelo sistema imune (Verlinden *et al.*, 2006; Hou *et al.*, 2017). Por fim, as proteínas hidrolisadas são amplamente utilizadas como palatabilizantes no recobrimento de alimentos para animais de companhia pois o processo de hidrólise altera as características organoléticas da matéria-prima e promove maior aceitabilidade e consumo (Nagodawithana *et al.*, 2010).

Ademais, a utilização de proteínas hidrolisadas com elevada digestibilidade pode auxiliar na redução do conteúdo de proteína não digerida que alcança o intestino

grosso, em especial o cólon, possibilitando assim a diminuição na síntese de compostos putrefativos, como amônia, sulfeto de hidrogênio, ácidos graxos de cadeia ramificada (AGCR), aminas biogênicas, fenóis e indóis (Blachier *et al.*, 2007). Adicionalmente, o consumo de dietas contendo proteínas hidrolisadas tem sido associado a alterações benéficas na composição e funcionalidade da microbiota intestinal e sinais clínicos de cães com enteropatias crônicas (Mandigers *et al.*, 2010; Wang *et al.*, 2019). Embora estudos recentes avaliando o uso de dietas com proteínas hidrolisadas demonstraram nenhuma ou mínimas alterações na composição microbiana intestinal de cães saudáveis (Pilla *et al.*, 2020; Martínez-Lopez *et al.*, 2021), a investigação do efeito de proteínas hidrolisadas sobre a microbiota intestinal é de extrema relevância em virtude do seu impacto sobre a manutenção da saúde geral de cães (Pilla & Suchodolski, 2020).

O beneficiamento de coprodutos de origem animal através de processamentos altamente tecnificados, como no caso das proteínas hidrolisadas, permite a obtenção de ingredientes com características diferenciadas comparado à sua matéria-prima original. Porém, durante o processo de hidrólise e armazenamento algumas substâncias podem ser produzidas, como aminas biogênicas, devido a elevada concentração de aminoácidos livres, má condição higiênico-sanitário e baixa qualidade microbiológica (Feddern *et al.*, 2019). Aminas biogênicas são compostos orgânicos nitrogenados de baixo peso molecular, derivadas da descarboxilação de aminoácidos por microrganismos. A presença de aminas biogênicas em coprodutos cárneos destinados a alimentação animal tem sido investigada ao longo dos anos, uma vez que estes compostos são termoestáveis e desempenham inúmeras rotas metabólicas e fisiológicas e, potencialmente, tóxicas, podendo comprometer negativamente o metabolismo e desempenho animal (Barnes *et al.*, 2001; den Brinker *et al.*, 2003; Kalač, 2014).

Com vistas em compreender as características químicas da proteína hidrolisada de fígado de frango e os efeitos da sua inclusão como ingrediente proteico sobre o metabolismo de cães adultos saudáveis esta tese foi redigida e dividida em 6 capítulos: O capítulo 1 aborda os conceitos relacionados às proteínas hidrolisadas e seus efeitos sobre a palatabilidade, digestibilidade, produtos de fermentação intestinal e microbiota fecal, bem como o efeito de inclusões crescentes de proteína bruta sobre metabolismo e consumo de aminas biogênicas em cães adultos saudáveis. O capítulo 2 apresenta o artigo publicado caracterizando o hidrolisado de fígado de frango e a

avaliação de duas dietas completas extrusadas secas (hidrolisado de fígado de frango ou farinha de vísceras de aves e farinha de carne e ossos) sobre a palatabilidade e digestibilidade. O capítulo 3 apresenta o artigo publicado avaliando os efeitos das dietas anteriormente citadas sobre a resposta imunológica e microbiota fecal. O capítulo 4 inclui o estudo avaliando o efeito de dietas contendo hidrolisado de fígado de frango substituindo farinha de vísceras de aves e concentrações crescentes de proteína bruta sobre a digestibilidade, características fecais e urinárias e produtos de fermentação intestinal. O capítulo 5 inclui o estudo avaliando o efeito das dietas anteriormente citadas sobre o metabolismo de aminas biogênicas e capacidade antioxidante total. O capítulo 6 apresenta as considerações finais da tese.

2. REVISÃO BIBLIOGRÁFICA

2.1. Proteínas hidrolisadas

As proteínas hidrolisadas têm sido utilizadas na alimentação humana há décadas, com notoriedade em sucedâneos para lactentes com reações alérgicas às proteínas do leite de vaca. Na alimentação animal, o uso de proteínas hidrolisadas está associado a benefícios como digestão facilitada e elevado teor de peptídeos de baixo peso molecular, reduzindo assim o reconhecimento como possíveis antígenos pelo sistema imune (Cave, 2006). Além disso, algumas proteínas hidrolisadas possuem peptídeos bioativos com propriedades funcionais e ação antimicrobiana, antioxidante, anti-hipertensiva e imunomoduladora (Martínez-Alvarez *et al.*, 2015; Hou *et al.*, 2017). Recentemente, foi demonstrado que peptídeos bioativos presentes na farinha de coprodutos de aves enzimaticamente hidrolisada pode reduzir a atividade da enzima conversora de angiotensina no soro de gatos saudáveis, com potencial ação anti-hipertensiva (Miltenburg *et al.*, 2021).

A produção industrial de proteínas hidrolisadas ocorre através da hidrólise ácida, enzimática ou microbiana (Figura 1). A hidrólise viabiliza a ruptura das ligações peptídicas de grandes cadeias de proteínas e as converte em pequenos peptídeos e aminoácidos livres, reduzindo o peso molecular da proteína original, que por consequência reduz a antigenicidade e alergenicidade da proteína (Verlinden *et al.*, 2006). O método de hidrólise enzimática é mais aplicado na produção de proteínas hidrolisadas alimentares pois oferece vantagens aos demais métodos, como condições de processamento moderadas impedindo a perda de aminoácidos, e utilização de proteases de maior especificidade e precisão permitindo maior controle do grau de hidrólise (Hou *et al.*, 2017). Entretanto, a hidrólise de algumas proteínas (p. ex. proteínas da soja e caseína) desencadeia a exposição de cadeias laterais de aminoácidos com propriedades hidrofóbicas que promovem amargor (Nagodawithana *et al.* 2010). Em estudo prévio, Cho *et al.* (2004) observaram que dois hidrolisados de proteína de soja comerciais apresentaram amargor na faixa de peso molecular entre 2 e 4 kDa. Contudo, as proteínas hidrolisadas obtidas a partir de coprodutos de origem animal, principalmente vísceras, são utilizadas como palatabilizantes no recobrimento

de alimentos para animais de companhia devido as suas características organolépticas.

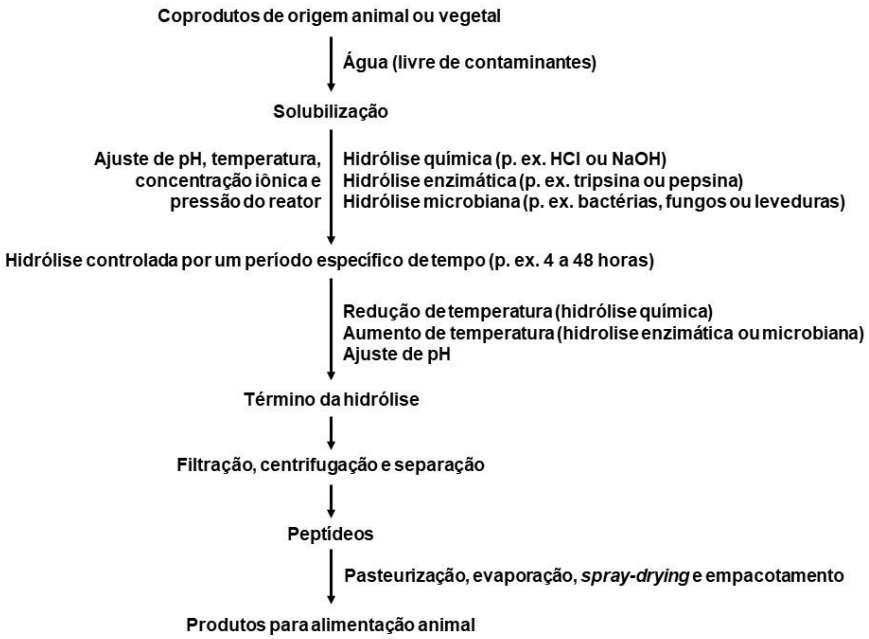


FIGURA 1. Esquema geral do processo de fabricação de proteínas hidrolisadas. (Fonte: Adaptado de Hou *et al.*, 2017)

A hidrólise prévia das matérias-primas pode influenciar o valor nutricional da proteína pois está associada a potencial facilitação dos processos digestivo e absortivo, aspecto importante para animais com trato gastrointestinal comprometido (Cave, 2006). Como resultado do desdobramento e ruptura das ligações peptídicas de macromoléculas proteicas, a presença de grandes quantidades de pequenos peptídeos e aminoácidos livres pode afetar positivamente o desempenho animal, uma vez que pequenos fragmentos proteicos são prontamente absorvidos no intestino delgado sem digestão gastrointestinal prévia (Gilbert *et al.*, 2008). Em ratos, pequenos peptídeos de proteínas parcialmente hidrolisadas do leite são mais eficientemente absorvidos e possuem maior valor nutricional do que aminoácidos livres (Monchi & Rerat, 1993). Por outro lado, maiores proporções de pequenos peptídeos, na forma de di- e tripeptídeos, podem exceder a capacidade absortiva, sem que resulte em diferenças na eficiência de utilização comparado a proteína original íntegra. Ainda, o processo de hidrólise pode eventualmente resultar na formação de produtos da reação de Maillard que não são absorvidos (Rooijen *et al.*, 2013). Em cães adultos saudáveis,

a utilização de dieta hipoalergênica contendo proteína hidrolisada promoveu redução no coeficiente de digestibilidade da matéria seca, mas aumentou a digestibilidade da proteína bruta comparado a dieta contendo proteína intacta (Verlinden *et al.*, 2006). Entretanto, a ampla variabilidade na qualidade da matéria-prima e nas condições de processamento aplicadas durante a manufatura de farinhas de origem animal, em especial a farinha de vísceras de frango, impacta diretamente na qualidade proteica e biodisponibilidade de nutrientes (Murray *et al.*, 1997). Assim, a utilização de proteínas hidrolisadas pode conferir benefícios em virtude da aplicação de processamento mais brando à matéria-prima.

Adicionalmente, o processo de hidrólise pode resultar em aumento da osmolaridade, caracterizado pela concentração de solutos (produtos de fermentação e eletrólitos) podendo ser responsável pela redução na absorção de água no cólon e, consequentemente, na piora do escore fecal e aumento da umidade fecal (Nishinaka *et al.*, 2004). Estudos prévios relataram a incidência de fezes moles e diarreia em cães alimentados com dietas contendo proteínas hidrolisadas (Hekman, 2003; Loeffler *et al.*, 2004). Portanto, a investigação dos efeitos sobre a digestibilidade e características fecais se torna indispensável na avaliação de proteínas hidrolisadas.

2.2. Utilização de proteínas hidrolisadas em alimentos coadjuvantes

As enteropatias crônicas e as reações adversas alimentares são quadros recorrentes na clínica de animais de companhia. As enteropatias crônicas são caracterizadas pela resposta imunológica e inflamatória exacerbada no trato gastrointestinal e de caráter multifatorial. Enquanto as reações adversas alimentares, comumente nomeadas de alergia alimentar, são atribuídas a qualquer resposta clinicamente anormal decorrente da ingestão de um alimento. A prescrição de alimentos com proteínas hidrolisadas tem sido preconizada no manejo nutricional de animais de companhia com doenças gastrointestinais e reações adversas alimentares devido ao sucesso na remissão dos sinais clínicos, como vômito, diarreia, além de sinais dermatológicos no caso das alergias alimentares (Marks *et al.*, 2002; Puigdemont *et al.*, 2006; Mandigers *et al.*, 2010; Olivry *et al.*, 2017).

O mecanismo desencadeador destes distúrbios ocorre pela absorção de macromoléculas íntegras antigênicas, no caso, os alérgenos alimentares que acessam o sistema imune pelo trato gastrintestinal (Cave, 2006). Apesar do processo

digestivo ser eficaz, cerca de 2% das proteínas ingeridas escapam completamente da digestão e são carreadas pela circulação entérica, elicitando uma resposta imunológica (Sampson, 2004). Além disso, fatores como a composição da dieta e alterações na integridade e permeabilidade da barreira da mucosa podem influenciar diretamente na absorção de proteínas, pois alteram a seletividade dos enterócitos permitindo a absorção de macromoléculas com propriedades antigênicas (Verlinden *et al.*, 2006).

Os principais alérgenos alimentares são glicoproteínas hidrossolúveis de elevado peso molecular, estáveis ao tratamento com calor, ácido e proteases (Sampson & Burks, 1996). Os alérgenos alimentares mais comumente identificados em cães estão presentes na carne bovina, derivados lácteos, carne de frango e trigo (Roudebush, 2013; Mueller *et al.*, 2016). Nas reações adversas alimentares, a resposta imunológica é iniciada após a ingestão e absorção da fração proteica alergênica. Posteriormente, ocorre a ligação cruzada do alérgeno aos receptores FcεRI ligados às moléculas de IgE presentes na membrana dos mastócitos, promovendo assim a degranulação e liberação de mediadores pró-inflamatórios, como histamina, prostaglandinas, enzimas e citocinas (Cave, 2006).

Em teoria qualquer alimento ingerido pode induzir ao aumento da resposta imunológica se absorvido integralmente (Verlinden *et al.*, 2006). Dentre os ingredientes utilizados na formulação de alimentos para cães destacam-se os coprodutos de carne bovina e de aves, principais coprodutos de origem animal, e os coprodutos da soja e milho, principais produtos de origem vegetal utilizados em formulações.

O beneficiamento de coprodutos de origem animal através de processos padronizados e altamente tecnificados permite a obtenção de ingredientes com características diferenciadas comparado à sua matéria-prima original. No caso dos distúrbios gastrointestinais e reações adversas alimentares, a introdução de fontes proteicas inéditas à dieta, bem como uso de proteínas hidrolisadas são as principais estratégias nutricionais recomendadas após a conclusão do diagnóstico (Rudinsky, 2018). As proteínas hidrolisadas têm sido utilizadas com frequência na indústria de alimentos para animais de companhia em dietas coadjuvantes devido às características previamente mencionadas, como elevada digestibilidade, palatabilidade, e, principalmente, pelo seu baixo peso molecular que resulta em menor antigenicidade (Cave, 2006).

A redução da antigenicidade nos ingredientes pode ser alcançada mediante tratamento térmico, modificação do pH, hidrólise enzimática e filtração (Hudson, 1995). O tratamento térmico pode não ser totalmente eficaz, uma vez que a maioria dos alimentos destinados a animais de companhia passam previamente por tratamentos térmicos para sua fabricação e grande número de alérgenos são termoestáveis. O tratamento térmico pode ainda aumentar a alergenicidade de algumas proteínas, uma vez que o efeito do processamento térmico afeta apenas a estrutura tridimensional das proteínas (Oobatake, 1993). Adicionalmente, o processamento térmico em elevadas temperaturas permite a ocorrência das reações de Maillard, produzindo compostos chamados melanoidinas através da reação entre alguns aminoácidos e açúcares redutores. As melanoidinas podem ser mais ou menos alergênicas do que as proteínas originais, pois atuam como haptenos ou por reduzirem a absorção de peptídeos, respectivamente (Otani *et al.*, 1985; Sancho *et al.*, 2005). Cave & Marks (2004) observaram que o processo de aquecimento de dietas enlatadas purificadas a base de caseína, amido, sacarose e óleo de milho foi responsável por aumentar a antigenicidade do alimento comparado ao alimento não tratado termicamente.

A modificação do pH exclusivamente pode não ser eficaz pois alguns alérgenos são resistentes ao pH ácido, semelhantes ao pH estomacal (Taylor *et al.*, 1987). O processamento mais confiável para a redução da antigenicidade de um ingrediente é a hidrólise enzimática. Mediante a ação de proteases, sejam elas derivadas do pâncreas de mamíferos, estômago de suínos, proteases bacterianas, fúngicas e de algumas frutas, frações peptídicas e aminoácidos livres são obtidos (Lee, 1992). A eficiência do processo de hidrólise depende do grau de especificidade da enzima proteolítica selecionada, o grau de hidrólise no qual a matéria-prima é submetida e de processamentos posteriores. Além de reduzir a antigenicidade, o processo de hidrólise aumenta a digestibilidade devido a ruptura das cadeias proteicas em fragmentos peptídicos menores. A hidrólise enzimática não altera o valor nutricional da proteína (Pedersen, 1994). Por último, a filtração pode ser realizada para remover as cadeias aminoacídicas de alto peso molecular resistentes ao processamento por hidrólise e, ainda, para remover as enzimas restantes (Cave, 2006).

A avaliação do peso molecular dos peptídeos presentes nas proteínas hidrolisadas pode auxiliar na investigação da sua alergenicidade e antigenicidade. Alérgenos alimentares com peso molecular entre 10 e 70 kDa podem elicitar resposta

alergênica em humanos, assim, quanto menor o tamanho dos fragmentos proteicos no ingrediente processado, menor a possibilidade da permanência de frações alergênicas (Sampson, 1999; Verlinden *et al.*, 2006). Em trabalho prévio, Cave & Guilford (2004) revelaram que a hidrólise reduziu 96,9% da proteína a base de fígado e coração de frango em fragmentos peptídicos com peso molecular <10 kDa. Em humanos, apenas peptídeos com menos de 15 aminoácidos, ou peso molecular entre e 3,5 a 5 kDa podem reduzir a alergenicidade. Embora em cães esse valor permaneça desconhecido, alguns animais mais reativos podem manifestar sintomas a proteínas com peso molecular superior a 4,5 kDa (De Jaham, 2000). No entanto, apenas a avaliação do peso molecular de proteínas hidrolisadas não garante a redução da alergenicidade. A avaliação da resposta imunológica conjuntamente com a observação da ocorrência ou não de sinais clínicos permitem maior acurácia na comprovação da redução da antigenicidade do ingrediente.

2.3. Microbioma intestinal de cães e efeito da proteína hidrolisada sobre a sua composição e funcionalidade

A microbiota intestinal e seus metabólitos influenciam a saúde sistêmica do hospedeiro, através da imunomodulação, proteção contra patógenos, estimulação da função da barreira intestinal, e mais recentemente pela sua ação no eixo intestino-cérebro (Barko *et al.*, 2018; Pilla & Suchodolski, 2020). As bactérias são os principais componentes da microbiota intestinal (Swanson *et al.*, 2011), acompanhadas por fungos, vírus, protozoários e archeas. Além da resiliência e adaptabilidade, a microbiota intestinal de cães saudáveis possui estabilidade na sua composição, sendo formada majoritariamente pelos filos Firmicutes, Bacteroidetes e Fusobacterium (Middelbos *et al.*, 2010; Hand *et al.*, 2013).

O microbioma intestinal é responsivo aos nutrientes, em especial aos acréscimos de fibra e proteína, que promovem a modulação da composição e funcionalidade microbiana (Celi *et al.*, 2017; Pilla & Suchodolski, 2021). Alterações na composição da microbiota intestinal parecem estar mais associadas a grandes alterações nos macronutrientes do que nos ingredientes (Pilla & Suchodolski, 2021).

O monitoramento de bactérias “sentinelas” que são sensíveis a alterações na homeostase intestinal pode auxiliar no monitoramento da funcionalidade intestinal de cães (Félix *et al.*, 2022). Alguns táxons bacterianos, que são as unidades taxonômicas

associadas a classificação científica, são utilizados como biomarcadores da funcionalidade intestinal devido a sua menor abundância em cães com doenças gastrointestinais, dentre os quais destacam-se alguns membros do filo Firmicutes, como *Faecalibacterium* spp, *Blautia* spp, *Turicibacter* spp e *C. hiranonis*, e do filo Fusobacteria, como *Fusobacterium* spp. (Suchodolski *et al.*, 2012; Garcia-Mazcorro *et al.*, 2012; AlShawaqfeh *et al.*, 2017). Um recente estudo de meta-análise demonstrou que além da redução na abundância de táxons relacionados a síntese de metabólitos benéficos à manutenção da funcionalidade intestinal e saúde geral do hospedeiro, cães com doenças gastrointestinais apresentaram menores índices de diversidade alfa, maior índice de disbiose intestinal e aumento na abundância de *Escherichia coli* (Félix *et al.*, 2022). A disbiose intestinal se refere ao desequilíbrio do microbioma intestinal durante a qual ocorrem alterações na sua composição e funcionalidade (Zeng *et al.*, 2017), sendo associada a inúmeras condições em cães, como enteropatias crônicas (Minamoto *et al.*, 2019), obesidade (Bermudez *et al.*, 2020), doenças metabólicas (Jergens *et al.*, 2019) e doenças cardíacas (Li *et al.*, 2021).

Achados prévios evidenciaram os efeitos positivos da utilização de dietas contendo proteínas hidrolisadas sobre a microbiota intestinal, tais como a melhoria na estrutura microbiana e reduções na sintomatologia e disbiose intestinal de cães com enteropatias crônicas (Mandigers *et al.*, 2010; Wang *et al.*, 2019). Deste modo, cães com alterações associadas a doenças podem se beneficiar de planos nutricionais que incluam dietas contendo proteínas hidrolisadas, por exemplo, objetivando melhorias na diversidade da microbiota e consequente síntese de metabólitos (Pilla & Suchodolski, 2021).

2.4. Efeito da fonte e concentração de proteína no metabolismo de cães

Nos últimos anos, houve o aumento na disponibilidade de alimentos completos com alta proteína bruta para animais de companhia seguindo as tendências do mercado e da indústria de alimentos, apesar desta não ser a visão de grande parte dos nutricionistas quanto a sustentabilidade. No entanto, as concentrações de proteína bruta de alguns alimentos completos excedem em muito a recomendação mínima para cães adultos em manutenção. Muito além do nível aumentado de proteína bruta, as formulações devem considerar a qualidade proteica que é afetada pela fonte proteica, concentração e biodisponibilidade de aminoácidos essenciais,

nitrogênio não proteico (ácidos nucleicos, aminas, amidas) e digestibilidade (Gross *et al.*, 2010).

Dentre as fontes proteicas utilizadas em alimentos para animais de companhia, as proteínas animais (derivados da indústria de *rendering*) possuem maior variabilidade na composição química comparado as proteínas vegetais (Parsons *et al.*, 1997; Deng *et al.*, 2016; Donadelli *et al.*, 2019), além do impacto de fatores como a composição das matérias-primas e as condições de processamento dos ingredientes sobre a qualidade proteica e digestibilidade (Murray *et al.*, 1997; Johnson *et al.*, 1998; Cramer *et al.*, 2007; Donadelli *et al.*, 2019).

No organismo, as proteínas não digeridas e absorvidas no intestino delgado, provenientes tanto de proteínas alimentares como endógenas, chegam ao intestino grosso, em especial no cólon, onde são susceptíveis a fermentação pela microbiota intestinal. Diversos fatores influenciam a quantidade de proteína que alcança o cólon, como o teor ingerido de matéria seca e proteína, e a digestibilidade da fonte proteica (Hussein & Sunvold, 2000). A proteína não digerida atua como precursor para a síntese de metabólitos putrefativos, incluindo ácidos graxos de cadeia curta (AGCC), AGCR, amônia, aminas biogênicas, fenóis, indóis, sulfidos, gases (H_2 e CO_2), e produtos intermediários como lactato e succinato (Blachier *et al.*, 2007). Os AGCC, incluindo acetato, propionato e butirato, possuem inúmeros benefícios ao hospedeiro pois são fontes de energia para a mucosa do cólon, inibem microrganismos patogênicos através da diminuição do pH luminal, estimulam a proliferação epitelial e função da barreira, além de possuírem propriedades anti-inflamatórias (Morrison & Preston, 2016). Os AGCR são formados exclusivamente pelo metabolismo de aminoácidos de cadeia ramificada, possibilitando que os AGCR sejam utilizados como marcadores da fermentação proteica no cólon (Blachier *et al.*, 2007). Alguns estudos apontam que os AGCR atuam na supressão de marcadores pró-inflamatórios em células epiteliais intestinais de humanos (Yan *et al.*, 2017) e redução da colite necrosante em ratos neonatais (Ran-Ressler *et al.*, 2011). Entretanto, alguns dos metabólitos da fermentação de proteínas estão associados a efeitos negativos na saúde do hospedeiro, como amônia e fenóis que podem aumentar a permeabilidade intestinal, reduzir a função da barreira epitelial e ter atividade metaplásica (Hughes *et al.*, 2008).

Ephraim *et al.* (2020) observaram o aumento no pH fecal e na concentração fecal de AGCR, diminuição nas concentrações fecais de AGCC e indóis em cães

alimentados com dietas contendo alta concentração de proteína bruta. Os autores revelaram ainda o aumento na abundância de bactérias proteolíticas e níveis aumentados de metabólitos relacionados a inflamação e disfunção renal. Similarmente, Nery *et al.* (2010) observaram o aumento nas concentrações fecais de amônia, AGCR e valerato em cães alimentados com dietas contendo alta proteína bruta. Embora existam estudos demonstrando o aumento da digestibilidade aparente da proteína em resposta a acréscimos de proteína bruta na dieta, os autores previamente mencionados atribuem o aumento da digestibilidade aparente da proteína a um possível efeito da diluição do conteúdo nitrogenado endógeno dado a maior quantidade de proteína *bypass* que alcança o intestino grosso (Nery *et al.*, 2010; Ephraim *et al.*, 2020). Adicionalmente, o consumo de dietas contendo altas concentrações de proteína bruta favorece o aumento na abundância de *Clostridium perfringens*, espécie bacteriana proteolítica, nas fezes de cães (Zentek *et al.*, 2003; Li *et al.*, 2017).

Como previamente evidenciado por Nery *et al.* (2012), a utilização de fontes proteicas altamente digestíveis resulta na diminuição da concentração de metabólitos putrefativos nas fezes de cães devido à redução na entrada de proteínas não digeridas no cólon. Além disso, o uso de proteínas altamente digestíveis poderia permitir a redução na inclusão de proteína bruta na dieta. De acordo com Neis *et al.* (2015), visto que os compostos proteicos não digeridos podem ser utilizados para a síntese de componentes celulares bacterianos ou catabolizados por diferentes vias resultando na produção de metabólitos putrefativos, com efeitos positivos ou negativos no hospedeiro, a modulação do consumo proteico pode fazer parte da estratégia para a modulação das bactérias proteolíticas e suas vias metabólicas, afetando potencialmente o metabolismo do hospedeiro.

2.5. Aminas biogênicas

A utilização de proteínas hidrolisadas apresenta inúmeros benefícios à nutrição animal, sobretudo devido ao seu elevado conteúdo nutricional, em especial de peptídeos, e à contribuição a características organolépticas, principalmente como palatabilizantes. Entretanto, a matéria-prima da qual o hidrolisado será produzido deve possuir qualidade sanitária, uma vez que o elevado conteúdo de aminoácidos livres presentes nas matérias-primas pode desencadear a proliferação de microrganismos

responsáveis pela síntese de aminas biogênicas, podendo comprometer o metabolismo e saúde animal (Feddern *et al.*, 2019).

As aminas biogênicas são bases orgânicas de baixo peso molecular produzidas a partir da descarboxilação de aminoácidos livres ou aminação e transaminação de aldeídos e cetonas pela aminoácido-transaminase (Liu *et al.*, 2003). A síntese de aminas biogênicas ocorre a partir da descarboxilação de seu aminoácido correspondente pela remoção do grupo α-carboxila.

As aminas são classificadas quanto à origem ou síntese como poliaminas ou biogênicas. As poliaminas são endógenas e formadas naturalmente por animais, plantas e microrganismos. Atuam em inúmeros processos fisiológicos como neurotransmissores, psicoativos, vasoativos, reguladores da expressão gênica, crescimento e diferenciação celular, secreções gástricas, resposta imune, processos inflamatórios. As aminas biogênicas são formadas pela descarboxilação de aminoácidos livres através da ação das enzimas descarboxilases, principalmente de origem microbiana (Ruiz-Capillas & Herrero, 2019). As aminas biogênicas são classificadas quanto a estrutura do aminoácido precursor (alifáticas, aromáticas e heterocíclicas) e quanto ao número de grupos amina (monoaminas, diaminas e poliaminas) (Tabela 1).

Tabela 1. Classificação das aminas biogênicas.

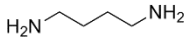
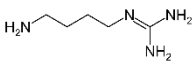
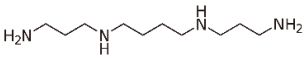
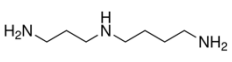
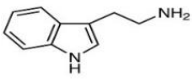
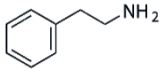
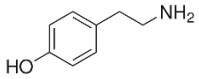
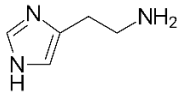
Amina biogênica	Estrutura	Classicação	Precursor
Putrescina		Diamina Alifática	Ornitina
Cadaverina		Diamina Alifática	Lisina
Agmatina		Poliamina Alifática	Arginina
Espermina		Poliamina Alifática	Arginina Ornitina
Espermidina		Poliamina Alifática	Arginina Ornitina
Triptamina		Monoamina Heterocíclica	Triptofano

Tabela 1. Classificação das aminas biogênicas. (continuação)

Feniletilamina		Monoamina Aromática	Fenilalanina
Tiramina		Monoamina Aromática	Tirosina
Histamina		Monoamina Heterocíclica	Histidina

Fonte: Adaptado de Wójcik *et al.*, 2022.

Os principais requerimentos para síntese de aminas biogênicas são as características da matéria-prima (composição, pH, força iônica e disponibilidade de aminoácidos livres), a presença de microrganismos descarboxilase-positivos e as condições que permitem o crescimento bacteriano, como durante o armazenamento e fermentação, a síntese e atividade da descarboxilase (Feddern *et al.*, 2019). Alguns gêneros bacterianos possuem capacidade de descarboxilação de aminoácidos, como *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Shigella*, *Photobacterium* e as bactérias ácido láticas *Lactobacillus*, *Pediococcus* e *Streptococcus* (Figura 2). Assim, a introdução de microrganismos descarboxilase-positivos e, consequentemente, a síntese de aminas biogênicas em alimentos pode resultar na contaminação antes, durante ou após o processamento (Brink *et al.* 1990). Por serem compostos termoestáveis, as aminas biogênicas têm sido utilizadas como indicadores de frescor e deterioração no monitoramento de matérias-primas e produtos ao longo da cadeia produtiva. Além disso são utilizadas no controle de contaminação cruzada, a fim de determinar a qualidade da matéria-prima e condições de higiene durante o processamento (Feddern *et al.*, 2019).

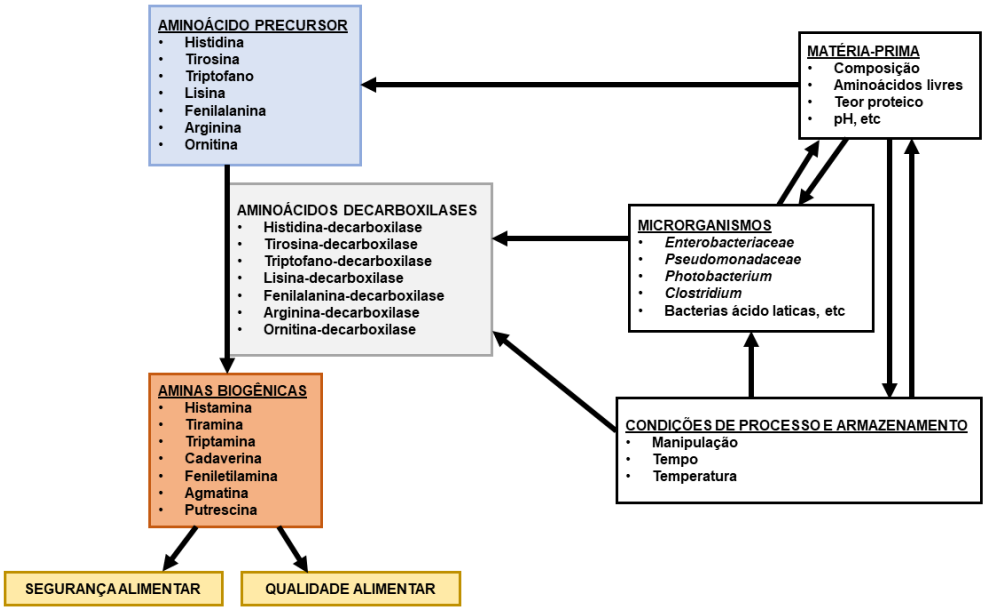


FIGURA 2. Fatores para síntese de aminas biogênicas. (Fonte: Adaptado de Ruiz-Capillas & Herrero, 2019)

De expressiva importância na nutrição animal, os coprodutos de origem animal, em especial as farinhas de origem animal, representam um papel significativo na contaminação de aminas biogênicas por terem passado por algum nível de deterioração (Smith *et al.*, 2000). A contaminação de aminas biogênicas em produtos de origem animal ocorre no período *post mortem*. Neste período, as enzimas proteolíticas intestinais combinadas ao rápido processo autolítico tecidual resultam na liberação de aminoácidos livres que servem de substrato para a ação enzimática de microrganismos e síntese de aminas biogênicas de acordo com o aminoácido correspondente (den Brinker *et al.*, 2003; Cardozo *et al.*, 2013). A maior susceptibilidade à contaminação de aminas biogênicas em coprodutos de origem animal advém da baixa qualidade das matérias-primas. A contaminação microbiológica e condições inadequadas de armazenamento são fatores predisponentes à síntese de aminas biogênicas (EFSA, 2011). Assim, o processamento das matérias-primas de origem animal imediatamente após o abate é preconizado, principalmente em altas temperaturas ambientes, de modo a evitar a produção de altas concentrações de aminas biogênicas e garantir a segurança alimentar (Feddern *et al.*, 2019). Adicionalmente, alguns procedimentos pós-abate, como evisceração e corte, podem resultar em contaminação cruzada devido a presença de microrganismos descarboxilase-positivos. Alguns tecidos como intestino,

pele e brânquias de peixes, que possuem elevada concentração de microrganismos, podem desencadear a disseminação de microrganismos descarboxilase-positivos em superfícies e equipamentos, promovendo o aumento de aminas biogênicas durante processamento e armazenamento posteriores (EFSA, 2011).

Estudos prévios destacam a grande variabilidade nas concentrações de aminas biogênicas em diferentes farinhas de origem animal (Tabela 2), possivelmente como resultado de falhas no controle de qualidade das matérias-primas, processamento e armazenamento. Ao avaliar o efeito da putrefação progressiva em carcaças de frangos em ambiente controlado (30°C e 70% de umidade relativa), Tamim & Doerr (2003) detectaram a presença de aminas biogênicas logo após o abate e verificaram que a maioria das aminas biogênicas permanece relativamente baixa por até 24 horas. Após esse período as concentrações são significativamente aumentadas. Além disso, os autores destacaram que as concentrações de algumas aminas biogênicas excediam os valores associados ao baixo desempenho em frangos de corte comerciais. Desta forma, enfatizaram que a qualidade do coproduto está fortemente associada a boas técnicas de manejo, incluindo coleta e processamento imediato após o abate.

As aminas biogênicas exógenas, ingeridas nos alimentos, são rapidamente detoxificadas no trato intestinal pela ação das enzimas monoamina oxidase (MAO), diamina oxidase (DAO), poliamina oxidase (PAO) e histamina-N-metil transferase (HNMT) (Prester, 2011). As enzimas MAO e DAO estão presentes no epitélio intestinal e detoxificam as mono- e diaminas, enquanto as poliaminas são primeiro acetiladas e posteriormente oxidadas pelas enzimas amina oxidases. As aminas biogênicas desempenham inúmeras ações metabólicas e fisiológicas no organismo, como estabilização de membranas celulares, regulação do crescimento tecidual, transmissão neural e mediação da inflamação (Kalač, 2014). Entretanto, a ingestão de altas quantidades de aminas biogênicas pode resultar em sintomas fisiológicos, como náuseas, dores de cabeça, sudorese, dificuldade respiratória, palpitações cardíacas e hiper ou hipotensão em humanos (Feddern *et al.*, 2019). O risco toxicológico relacionado ao consumo de altas concentrações de aminas biogênicas depende de fatores associados ao próprio alimento e fatores potencializadores de toxicidade, como estado de saúde, suscetibilidade individual pela baixa expressão genética das enzimas de detoxificação e consumo de inibidores da MAO (Ruiz-Capillas & Herrero, 2019).

TABELA 2. Concentração de aminas biogênicas (mg/kg) em diferentes farinhas de origem animal.

Autor	Coproduto	Putrescina	Cadaverina	Histamina	Espermidina	Espermina
Barnes *et al.* (2001)	Farinha de carne e ossos	57 (nd-286)	120 (nd-208)	21 (nd-208)	16 (nd-39)	31 (10-56)
	Farinha de aves	227 (84-390)	451 (140-879)	39 (28-95)	31 (19-53)	74 (55-96)
	Farinha de peixes	99 (12-537)	215 (64-557)	70 (8-1576)	31 (18-97)	27 (120-139)
den Brinker *et al.* (2003)	Farinha de peixes	102 (7-454)	220 (11-1340)	570 (nd-1620)	-	-
	Farinha de aves	82 (7-1340)	121 (nd-1350)	19 (nd-167)	-	-
	Farinha de carne	21 (nd-695)	29 (nd-680)	10 (nd-258)	-	-
	Farinha de penas	31 (5-267)	42 (nd-159)	5 (nd-90)	-	-
	Farinha de sangue	13 (nd-223)	7 (nd-280)	4 (nd-36)	-	-

Os efeitos negativos das aminas biogênicas podem ser dependentes do consumo de ingredientes com altas concentrações, ou ainda da possível ação aditiva ou sinérgica entre diferentes aminas (Barnes *et al.* 2001). Em frangos de corte, o consumo de aminas biogênicas em maiores concentrações pode causar erosão de moela, mortalidade e menor desempenho (Tamim & Doerr, 2003). Entretanto, as informações das concentrações e efeitos das aminas biogênicas sobre o metabolismo de animais de companhia são escassas. Portanto, torna-se essencial investigar os coprodutos de origem animal devido a elevada disponibilidade de aminoácidos livres nestas matérias-primas. Além disso, diante da estabilidade das aminas biogênicas ao processamento térmico, se faz necessária a avaliação do potencial impacto destes compostos sobre a integridade intestinal, através da investigação da atividade plasmática das enzimas detoxificantes MAO e DAO.

3. HIPÓTESES E OBJETIVOS

Hipóteses:

1. O hidrolisado de fígado de frango apresenta perfil de aminoácidos compatível com as formulações de alimentos completos para cães, além de apresentar maior percentual de peptídeos com baixo peso molecular de menor potencial alergênico;
2. O hidrolisado de fígado de frango apresenta elevada palatabilidade e digestibilidade dos nutrientes, e efeito benéfico sobre a microbiota intestinal, podendo ser utilizado em alimentos completos como único ingrediente proteico sem alterações nas características fecais e urinárias de cães adultos;
3. A inclusão de concentrações crescentes de hidrolisado de fígado de frango e proteína bruta promovem aumento na digestibilidade da proteína, reduzindo assim o conteúdo de proteína não digerido no intestino grosso e a produção de compostos da fermentação putrefativa;
4. A presença de aminas biogênicas no hidrolisado de fígado de frango é reduzida comparado a fontes de proteína animal tradicionais;
5. O consumo de alimentos contendo aminas biogênicas altera a atividade plasmática de enzimas detoxificantes, MAO e DAO, demonstrando que as aminas são absorvidas.

Objetivo geral

Este estudo teve como objetivo avaliar o hidrolisado de fígado de frango como ingrediente proteico em dietas para cães adultos saudáveis mediante determinação da palatabilidade, digestibilidade de nutrientes e energia, características fecais e urinárias, microbiota fecal, e efeito sobre a resposta imunológica e metabolismo de aminas biogênicas.

Objetivos específicos

- Avaliar a composição química e peso molecular do hidrolisado de fígado de frango.
- Avaliar a digestibilidade de nutrientes e energia, energia metabolizável, características fecais e urinárias de cães adultos saudáveis alimentados com dieta contendo o hidrolisado de fígado de frango como principal fonte proteica animal.

- Avaliar a resposta imune, mediante quantificação das concentrações plasmáticas de citocinas e imunoglobulinas, e avaliação da microbiota fecal de cães adultos saudáveis alimentados com dieta contendo o hidrolisado de fígado de frango como principal fonte proteica animal.
- Avaliar a digestibilidade de nutrientes e energia, energia metabolizável, características fecais e urinárias, e produtos de fermentação intestinal de cães adultos saudáveis alimentados com dietas contendo o hidrolisado de fígado de frango como principal fonte proteica animal e concentrações crescentes de proteína bruta.
- Avaliar o efeito do consumo de dietas contendo o hidrolisado de fígado de frango como principal fonte proteica animal e concentrações crescentes de proteína bruta sobre o consumo e excreção fecal de aminas biogênicas, e concentrações plasmáticas de MAO e DAO em cães adultos saudáveis.

CAPÍTULO II

Characterisation of spray dried hydrolysed chicken liver powder: effects on palatability and digestibility when included as single source of animal protein in dog diets

Artigo publicado no periódico

ITALIAN JOURNAL OF ANIMAL SCIENCE

Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjas20

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To cite this article: Caroline Fredrich Dourado Pinto, Marcelino Bortolo, Fábio Ritter Marx & Luciano Trevizan (2021) Characterisation of spray dried hydrolysed chicken liver powder: effects on palatability and digestibility when included as single source of animal protein in dog diets, Italian Journal of Animal Science, 20:1, 2086-2094, DOI: 10.1080/1828051X.2021.1993091

To link to this article: https://doi.org/10.1080/1828051X.2021.1993091

ITALIAN JOURNAL OF ANIMAL SCIENCE
2021, VOL. 20, NO. 1, 2086–2094
https://doi.org/10.1080/1828051X.2021.1993091

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Characterisation of spray dried hydrolysed chicken liver powder: effects on palatability and digestibility when included as single source of animal protein in dog diets

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ABSTRACT

This study aimed to evaluate a commercial hydrolysed chicken liver powder (HCLP) as a single source of animal protein in diets for adult dogs. A characterisation of the HCLP was followed by assessment of diets palatability and *in vivo* and *in vitro* digestibility. Two extruded isonitrogenous diets were produced: control (poultry byproduct + bovine meat and bone meal) and HCLP. Twenty-two dogs of different breeds were used to test palatability, and twelve Beagle dogs were used to evaluate digestibility. HCLP had high concentrations of lysine, linoleic and arachidonic acids, and most of peptides with molecular weight <10 kDa. HCLP diet had the highest inclusion of the experimental ingredient based on its chemical composition. Dogs did not show preference among diets ($p > .05$). Ash and fat intake were higher in dogs fed the control diet, ($p < .0001$) and ($p = .0135$), respectively. Crude fibre intake was higher in dogs fed the HCLP diet ($p = .0001$). Dogs fed the HCLP diet had similar faecal score ($p > .05$) compared to control diet, although faecal dry matter was reduced ($p = .0321$) and the daily faecal production was increased ($p = .0361$). The diets in vitro digestibility did not differ ($p > .05$). Based on our results, HCLP included up to 26% in diets for adult dogs presented satisfactory results in palatability, digestibility of nutrients and energy, faecal and urinary characteristics. Although dogs fed the HCLP diet produced slightly moist stools, it had no negative impact on faecal score.

HIGHLIGHTS

- A commercial hydrolysed chicken liver powder (HCLP) was evaluated and presented low molecular weight and high amounts of essential nutrients. HCLP, included as a single source of animal protein, had good digestibility and acceptance for adult dogs.
- Despite findings from previous studies, the inclusion of HCLP at the level of 25.8% did not promote diarrhoea and the final faecal score remained within the ideal range.

ARTICLE HISTORY
Received 10 March 2021
Revised 20 July 2021
Accepted 7 October 2021

KEYWORDS
Hydrolysed protein; hydrolysed chicken liver powder; single protein diet; *in vivo* digestibility; *in vitro* digestibility

Introduction

Adverse food reactions are classified as an abnormal IgE-mediated immune response due to the ingestion of a specific food by sensitive individuals (Cianferoni and Spergel 2009). Recently, hydrolysed protein diets have been highly recommended for the diagnosis and management of adverse food reactions in dogs and cats as an option to homemade diets. Homemade diets were the 'gold standard' in elimination trials since they consist of one protein and one carbohydrate source and both were never consumed previously (Bethlehem et al. 2012). However, it demands full adherence, compliance, and investment of time of owners in order to follow and prepare the prescribed diet with only the selected ingredients. Additionally, if these diets are not nutritionally balanced or complete, they can lead to deficiencies in a long-term feeding period.

Hydrolysis allows the utilisation of ingredients associated with adverse food reactions in dogs, such as beef, chicken, pork, fish, and corn in hypoallergenic diets (Roudebush 2013). In human beings, the main food allergens are water-soluble glycoproteins, with a molecular weight ranging from 10 to 70 kDa, and relatively stable to heat, acid, and protease treatment (Sampson 1999). Hydrolysis breaks large polypeptides chains into smaller peptides and amino acids,

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Table 1. Hydrolysed chicken liver powder ingredient chemical composition.

Nutrient profile [g/kg, as fed basis]	
Water	61.0
Protein	619
Fat	224
Ash	59.0
Amino acids [g/kg]	
Alanine	38.4
Arginine	37.5
Aspartic acid	56.0
Cystine	4.6
Glutamic acid	78.6
Glycine	31.7
Histidine	14.0
Isoleucine	29.2
Leucine	54.6
Lysine	58.1
Methionine	14.8
Phenylalanine	28.7
Proline	26.8
Serine	27.4
Threonine	28.6
Tryptophan	8.9
Tyrosine	23.1
Valine	37.2
Fatty acids [g/kg]	
C08:0 Octanoic (caprylic)	<0.20
C10:0 Decanoic (capric)	<0.20
C11:0 Undecanoic (hendecanoic)	<0.20
C12:0 Dodecanoic (lauric)	<0.20
C14:0 Tetradecenoic (myristic)	0.60
C14:1 Tetradecenoic (myristoleic)	<0.20
C15:0 Pentadecanoic	<0.20
C15:1 Pentadecenoic	<0.20
C16:0 Hexadecanoic (Palmitic)	41.60
C16:1 Hexadecenoic (Palmitoleic)	5.90
C16:2 Hexadecadienoic	<0.20
C16:3 Hexadecatrienoic	<0.20
C16:4 Hexadecatetraenoic	<0.20
C17:0 Heptadecanoic (margaric)	0.20
C17:1 Heptadecenoic (margaroleic)	<0.20
C18:0 Octadecanoic (stearic)	26.60
C18:1 Octadecenoic (oleic + isomers)	55.50
C18:2 Octadecadienoic (linoleic + isomers)	38.40
C18:2 Octadecadienoic omega 6 (linoleic)	37.70
C18:3 Octadecatrienoic (linolenic + isomers)	2.20
C18:3 Octadecatrienoic Omega 3 (alpha linolenic)	1.70
C18:3 Octadecatrienoic Omega 6 (gamma linolenic)	0.50
C18:4 Octadecatetraenoic Omega 3 (stearidonic)	<0.20
C20:0 Eicosanoic (arachidic)	0.30
C20:1 Eicosanoic (Gondoic + isomers)	0.80
C20:2 Eicosadienoic Omega 6	0.50
C20:3 Eicosatrienoic	1.60
C20:3 Eicosatrienoic Omega 3	<0.20
C20:3 Eicosatrienoic Omega 6	1.60
C20:4 Eicosatetraenoic (arachidonic + isomers)	11.60
C20:4 Eicosatetraenoic Omega 3	<0.20
C20:4 Eicosatetraenoic Omega 6 (arachidonic)	11.50
C20:5 Eicosapentanoic Omega 3	0.30
C21:5 Heneicosapentaenoic Omega 3	<0.20
C22:0 Docosanoic (behenic)	0.40
C22:1 Docosenoic (erucic + isomers)	<0.20
C22:2 Docosadienoic Omega 6	<0.20
C22:3 Docosatrienoic Omega 3	<0.20
C22:4 Docosatetraenoic Omega 6	1.40
C22:5 Docosapentaenoic	1.60
C22:5 Docosapentaenoic Omega 3	0.60
C22:5 Docosapentaenoic Omega 6	1.00
C22:6 Docosahexaenoic Omega 3	1.20
C24:0 Tetracosanoic (lignoceric)	0.20
C24:1 Tetracosenoic (nervonic)	0.20
Minerals [mg/kg]	
Calcium	240
Phosphorus	8830
Magnesium	620
Sodium	2710
Potassium	8360
Chloride	6100
Zinc	68.00
Copper	6.80
Iron	329
Manganese	7.30
Selenium	1.84

Amino acids and fatty acids values reported as a % of the total product.

reducing molecular weight and therefore decreasing the antigenicity of the original protein, preserving its nutritional properties. Properly hydrolysed proteins contain peptides with reduced molecular weight that do not allow the IgE cross-linking, therefore, preventing degranulation and release of mediators (Corlde 1994 ; Verlinden et al. 2006).

In this way, testing novel hydrolysed protein sources have become an important way to validate their availability for inclusion in commercial diets specific for dogs with adverse food reaction and gastrointestinal disorders, as well as in premium commercial diets for healthy dogs. Thereby, the present study aimed to describe the nutritional composition of a commercial hydrolysed chicken liver powder ingredient, followed by the evaluation of palatability, and digestibility in vivo and in vitro when hydrolysed protein replaces the most traditional sources of animal protein in diets for dogs: poultry byproduct + bovine meat and bone meals.

Materials and methods

All animal care and handling procedures were approved by The Institutional Animal Care and Use Committee at the Universidade Federal do Rio Grande do Sul (UFRGS), protocol number 36,138.

Nutritional characterization of the hydrolyzed chicken liver powder

Chemical analysis

Hydrolysed chicken liver powder (HCLP) was obtained from a commercial industry (PROSURANCE® CHX Liver HD, Kemin® Industries). The product is reviewers a spray-dried protein hydrolysate produced by enzymatic hydrolysis under controlled temperature and pressure conditions. The final product is a fine caramel brown powder with low molecular weight protein and low ash content (Table 1). Using Size Exclusion

Table 2. Ingredients and chemical composition of experimental diets.

	Treatments	
Items	Control	HCLP*
Ingredient [g/kg, as fed basis]		
Brewers rice	527	527
Full-fat rice bran	80.00	80.00
Poultry byproducts meal	186	–
Bovine meat and bone meal	50.00	–
HCLP†	–	258
Cellulose	33.40	42.20
Poultry fat#	87.50	43.00
Soybean oil#	–	18.00
Canola oil#	13.70	4.60
L-lysine	6.30	–
DL-methionine	3.30	2.70
Potassium chloride	2.30	0.70
L-tryptophan	0.30	–
Limestone	–	20.10
Premix mineral/vitamin$	5.00	5.00
Salt	5.00	5.00
Analysed composition [g/kg, DM-basis]		
Dry matter	941	945
Organic matter	943	954
Ash	56.70	46.20
Crude protein	248	248
Acid-hydrolysed fat	151	140
Crude fibre	37.50	46.70
Gross energy [kJ/g]	20.70	21.20
Peroxide index [meq/kg] ‡	2.23	3.83

*HCLP: hydrolysed chicken liver powder diet; †HCLP: hydrolysed chicken liver powder ingredient; #Added on top; $Premix mineral/vitamin (supplied per kilogram of diet): vitamin A (10,800U), vitamin D3 (980 U), vitamin E (60 mg), vitamin K3 (4.8 mg), vitamin B1 (8.1 mg), vitamin B2 (6.0 mg), vitamin B6 (6.0 mg), 12 vitamin (30 mcg), pantothenic acid (12 mg), niacin (60 mg), folic acid (0.8 mg), biotin (0.084 mg), manganese (7.5 mg), zinc (100 mg), iron (35 mg), copper (7.0 mg), cobalt (10 mg), iodine (1.5 mg), selenium (0.36 mg), choline (2.400 mg), taurine (100 mg), and, antioxidant BHT (150 mg); ‡milliequivalents of active oxygen/kg of sample.

Chromatography (Kemin Nutrisurance Proprietary Method), the HCLP was analysed for protein molecular weight. Also, the HCLP was analysed for dry matter (DM—AOAC 934.01), crude protein (CP—AOAC 954.01; model TE 036/2, Tecnal, Piracicaba, Brazil), acid-hydrolysed fat (AHF—AOAC 954.02; model 170/3, Fanem, São Paulo, Brazil), ash (AOAC 1995), amino acids (AOAC 994.12 [Alt. III]; AOAC 994.12 [Alt. I]; AOAC 988.15 [mod]), fatty acids (AOAC 985.01 [mod]), and minerals (AOAC 985.01 [mod]). All analyses were performed in duplicate, assuming a coefficient of variation of <5% for all analyses. The amino acid score (AAS) was calculated based on the equation described by Kerr et al. (2013), using minimal requirements for the growth of puppies and kittens provided by NRC–National Research Council (2006) as reference values:

$$AAS = \frac{mg\ of\ limiting\ AA\ in\ 1g\ of\ test\ protein}{mg\ of\ limiting\ AA\ in\ 1g\ of\ reference\ protein} \times 100$$

For the *AAS* calculation, the amino acid content of poultry byproduct and bovine meat and bone meals were obtained from Rostagno et al. (2017).

Diets

Two experimental diets were formulated and extruded (model 2000, TNL Tecnal, Ourinhos, Brazil) to be isonitrogenous, differing only in the animal protein added: control (poultry byproduct + bovine meat and bone meals) and HCLP (hydrolysed chicken liver powder) (Table 2). For the palatability assay, diets were coated with a mix of soybean and canola oil at 2% instead of poultry fat. For the digestibility assay, diets were coated with poultry fat only. No commercial palatability enhancer was added, as these contain amino acids and peptides that alter the molecular weight and mono-protein concept of the HCLP diet.

Palatability assay

Animals

Twenty-two healthy adult dogs of different breeds (2 Rottweilers, 4 Labradors, 4 Siberian Huskies, 2 Basenjis, 4 Beagles, 2 Shih Tzus, and 4 Spitzes) were used to evaluate the palatability of the experimental diets. The dogs were allocated into individual kennels, and two diets were offered at the same time. During the rest of the period, the dogs were maintained outdoors. Water was provided *ad libitum*.

Assessment of palatability

Palatability was determined using the '2-pan' choice method (Griffin 2003). One comparison was made to evaluate dietary preference: Control vs. HCLP in a completely randomised design, with two comparisons and four meals for a total of 88 observations. During the testing phase, at 8:00 h, each diet used in the comparison was offered side by side, simultaneously, in identical feeders for 30 min. After this period, the dogs were released outdoors. At 18:00 h, the dogs were placed back into their metabolic cages, where they stayed until the following morning and were fed the experimental diets. The leftovers were collected, weighed, and discarded. The feeders were alternated for every meal to eliminate any bias effects. Food intake and first choice were observed during the trial. Food intake was calculated based on the total consumption of each diet. The first choice, observed when food was first offered, is the number of times that a given diet was chosen first.

Statistical analyses

The results of the palatability assessment were tested for homogeneity of variances and normality of errors and then subjected to Student's *t*-test ($p < .05$) to determine whether the food intake differed for dietary comparison. The first choice was analysed using a

Chi-square test ($p < .05$) based on the frequency of each meal choice using Statistix 10 (Analytical Software, Tallahassee, FL, USA). Based on the number of dogs used in this test, values of first choice and feed intake of >.80 were considered to be significant at a p value of <.05, according to the method described by Griffin (2003).

In vivo digestibility, fecal, and urinary characterization

Animals

Twelve healthy, intact adult Beagles (six males and six females) from the Animal Science Department, UFRGS, Porto Alegre, Brazil, were used in this study. All individuals were 5 years of age, weighing 11.8 ± 1.45 kg, with a body condition score (BCS) ranging from 5 to 6 out of 9 points (Laflamme 1997), and were free of endo- and ectoparasites. All dogs were regularly immunised and submitted to clinical and laboratory tests to measure complete blood count (CBC) and to perform biochemical and coproparasitological analyses before the start of the study. The dogs were housed and kept in conditions similar to those used in the palatability study. Dogs were fed experimental diets twice a day (at 08:30 h and 17:00 h) to meet their daily maintenance energy requirements (110 kcal of metabolisable energy × body weight $(kg)^{0.75}$/day), as recommended by the NRC– National Research Council (2006). Water was provided *ad libitum* throughout the experiment.

Experimental procedures

Digestibility was measured using the total faecal collection method. The assay was conducted as a randomised block design with two treatments and six dogs per treatment for a total of six replicates per treatment according to the American Association of Feed Control Officials protocol (AAFCO – Association of American Feed Control Officials 2020). Sex (female and male) was used as a criterion for blocking, and body weight was used to randomise the treatments. The experimental period lasted 10 days, with 5 days for adaptation to the cage and experimental diet, followed by 5 days of total faeces and urine collection and measurement of urinary pH.

Sample procedure

To establish the beginning and the end of each period of faeces and urine collection, gelatine capsules containing 1 g of iron oxide (III) Fe_2O_3 were orally administered to the dogs. Faeces were collected for five consecutive days, every 3 h except at night (0 AM/midnight), and scored as follows: 1 = very hard and dry stool, 2 = hard, dry, firm stool, 3 = soft, moist stool, well formed, 4 = soft and shapeless stool, and 5 = liquid stool and diarrhoea. Faecal score analysis was conducted by a single trained person using the WALTHAM Faeces Scoring System (Moxham 2001). After daily collection, faeces were weighed and stored in a freezer at −20 °C until the end of the trial for analysis. Total urine collection was performed daily in the morning and then stored in plastic bottles containing 0.1 g of thimol/100 mL of urine (EXÔDO CIENTÍFICA®, Hortolândia, Brazil), an aliquot of urine was used to measure the pH using a bench pH metre (AKSO® pH Plus, São Leopoldo, Brazil) and urinary density by a portable refractometer (BEL ENGINEERING® model RPI).

Chemical analysis

Stools from each dog were thawed, homogenised, and dried in a forced-air oven at 55 °C for 72 h, according to the recommendations of the AOAC (1995). Faeces and diets were ground through a 1 mm screen in a Wiley hammer mill (DeLeo Equipamentos Laboratoriais, Porto Alegre, Brazil), and analysed for DM (AOAC 934.01), AHF (AOAC 954.02; model 170/3, Fanem, São Paulo, Brazil), CP (AOAC 954.01; model TE 036/2, Tecnal, Piracicaba, Brazil), crude fibre (CF—AOAC 962.10; model MA 450/8, Marconi, Piracicaba, Brazil), and ash (AOAC 985.01 [mod]). The total urine produced was thawed, homogenised, and 150 mL aliquots were lyophilised (Micromodulyi-Fis; Thermo Fisher Scientific Inc., Lanham, MD, USA) for analysis of DM. Another 50 mL aliquot was collected for the analysis of CP. Dietary and faecal GE were determined using isoperibolic bomb calorimetry (calorimeter model C2000 basic, Ika-werke, Staufen, Germany). All analyses were performed in duplicate, assuming a coefficient of variation <0.01 for energy and <0.05 for the other analyses.

Statistical analyses

Data were tested for homogeneity of variances and normality of errors, and then analysed using ANOVA in SAS 9.4 (SAS Inst. Inc., Cary, NC, USA). Means were compared using Tukey's test ($p < .05$).

In vitro digestibility assay

Chemical analysis

The *in vitro* digestibility of dry matter (IVDDM) and organic matter (IVDOM) was determined based on the method proposed by Hervera et al. (2007) using 1 g of

Table 3. Nutrient intake, coefficient of total tract apparent digestibility, metabolisable energy, faecal and urinary characteristics of dogs fed experimental diets.

	Treatments			
Item	Control	HCLP*	*p*-Value	SEM†
Nutrient intake [g/d]				
Dry matter (DM)	171	167	.4749	9.29
Organic matter	161	159	.6978	8.78
Ash	9.71[a]	7.72[b]	<.0001	0.52
Crude protein	42.5	41.50	.4835	2.29
Acid-hydrolysed fat	25.8[a]	23.30[b]	.0135	1.39
Nitrogen-free extract	93.2	94.60	.6472	5.12
Crude fibre	6.42[b]	7.81[a]	.0001	0.38
Gross energy [kJ/d]	3546	3536	.9329	193
Apparent total tract digestibility [%]				
Dry matter	87.2	86.10	.3037	1.67
Organic matter	90.0	89.10	.2637	1.33
Crude protein	88.0	89.30	.2335	1.74
Acid-hydrolysed fat	92.4	90.80	.0928	1.47
Nitrogen-free extract	90.3	88.60	.0964	1.57
Gross energy	89.8	89.00	.3636	1.36
Digestible energy [kJ/g]	18.6	18.80	.2454	0.30
Metabolisable energy [kJ/g]	17.4	17.60	.2273	0.27
Faecal characteristics				
Faecal score#	1.98	2.17	.1071	0.18
Faecal DM [%]	51.7[a]	43.30[b]	.0321	5.76
Faecal output [g/d]	42.9[b]	53.70[a]	.0361	7.63
Faecal output [g/d, DM]	29.6	27.70	.5719	5.61
Urinary characteristics				
Volume [mL/d]	219	159	.4431	130
Dry matter [%]	6.87	7.85	.6113	3.22
Nitrogen [g/d]	3.48	2.93	.4296	1.15
Urine pH	7.85	8.33	.1981	0.60
Urine density	1032	1034	.7196	10.90

*HCLP: hydrolysed chicken liver powder diet; †SEM: standard error of the mean; #Scored as follows: 1 = very hard and dry stool, 2 = hard, dry, firm stool, 3 = soft, moist stool, well formed, 4 = soft and shapeless stool, 5 = liquid stool, diarrhoea.
[a,b]Means in the same row with different lowercase letters are significantly different ($p < .05$).

each experimental diet. The analysis was conducted in duplicate. This method simulates the stomach and small intestine compartments with the action of pepsin followed by pancreatin.

Results

The HCLP had high concentrations of protein, fat, and amino acids lysine, aspartic acid, and leucine, and low ash content (Table 1). Formulation adjustments were necessary in order to formulate the diet to contain an exclusive source of animal protein as HCLP or the most common sources based in beef and poultry byproducts meal (Table 2).

The experimental diets had similar chemical compositions, with small differences on ash and fibre content (Table 2), specially on HCLP diet which increased differences in nutrient intake for the diets (Table 3). The intake of ash ($p<.0001$) and fat ($p = .0135$) was higher in dogs fed the control diet. Dogs fed the HCLP diet consumed more crude fibre ($p = .0001$), probably due to the higher addition of cellulose in this diet.

The AAS of the HCLP showed high amounts of all amino acids based on the minimal requirement for puppies and kittens (Table 4), except for combination of Met + Cys and Phe + Tyr for kittens that had AAS values under 100. The HCLP contained considerable amount of essential fatty acids, mainly the polyunsaturated fatty acids (Table 1). The AAS of the bovine meat and bone meal showed values below 100 of Leu, Met, Met + Cys, Phe + Tyr, Thr, and Try for dogs, and Met, Met + Cys, Phe + Tyr, and Try for cats. The poultry byproducts meal had AAS below 100 of Try for dogs and Met, Met + Cys, and Phe + Tyr for cats.

The molecular weight profile differed between samples (Table 5), especially in content (%) <10 kDa, in which HCLP had 57%, poultry byproducts meal had 41%, bovine meat and bone meal had 35%, control diet had 39% and HCLP diet had 59%.

The dogs consumed the experimental diets, without refusals and leftovers. There were not significant differences in feed intake and first choice between both experimental diets (Table 6). The inclusion of HCLP at a level of 258 g/kg did not affect the palatability of the diet, and no vomit, diarrhoea, or other gastrointestinal clinical signs were observed during the study.

Consumption of the HCLP diet promoted an increase in the faecal water content ($p = .0321$) and increased daily faecal production ($p = .0361$) (Table 3). In spite of these changes, the mean faecal score did not differ between diets (average score 2.2), resulting in well formed, but slightly moist stools. No changes were observed in the urine volume, dry matter, nitrogen, pH, and density ($p > .05$).

The comparison between *in vivo* and *in vitro* digestibility of dry matter and organic matter was slightly different for each diet tested (Table 7). The *in vitro* method was effective for estimating the digestibility of the ingredient. The *in vitro* method underestimated the digestibility of DM and OM of control diet (84.5 and 84.0%, respectively) and HCLP diet (84.4 and 84.3%, respectively).

Discussion

The growing demand for therapeutic diets for dogs and cats diagnosed with adverse food reactions and food sensitivities highlights the importance of investigating new protein sources. The presence of thermal, chemical, and enzymatic resistant glycoproteins associated with the high molecular weight of these compounds, are the main factors which limit the inclusion of regular sources of protein in diets for sensitive

Table 4. Amino acid score (AAS) of the protein ingredients.

	AAS								
	Puppies 4–14w*			Puppies > 14w†			Kittens#		
Amino acid	HCLP§	PBM‡	BMBM§	HCLP§	PBM‡	BMBM§	HCLP§	PBM‡	BMBM§
Arginine	173	203	211	160	187	195	142	166	173
Histidine	131	111	100	158	133	121	157	132	119
Isoleucine	163	139	101	165	140	102	197	168	122
Leucine	154	124	99	190	153	122	156	125	100
Lysine	241	148	136	235	144	132	248	153	140
Methionine	154	123	87	159	127	90	123	98	70
Methionine + cystine	101	109	75	104	113	78	81	87	60
Phenylalanine	160	143	102	162	145	104	209	186	133
Phenylalanine + Tyrosine	145	120	86	146	121	87	98	81	59
Threonine	128	113	92	129	115	93	160	142	114
Tryptophan	144	94	54	144	94	54	199	130	75
Valine	200	169	148	187	158	138	212	179	157

*Calculated based on the minimal requirement for growing puppies 4–14 weeks old as reference values (NRC– National Research Council 2006); †Calculated based on the minimal requirement for growing puppies 14 weeks and older as reference values (NRC– National Research Council 2006); #Calculated based on the minimal requirement for growing kittens as reference values (NRC– National Research Council 2006); §HCLP: chicken liver powder ingredient; ‡PBM: poultry byproducts meal; §BMBM: bovine meat and bone meal.

Table 5. Molecular weight profile of the protein ingredients.

	Percentage of total sample [%]				
	Ingredients			Diets	
Molecular weight [kDa]	HCLP*	PBM†	BMBM#	Control	HCLP§
<1	31.0	14.0	17.0	15.0 (14.6)‡	31.0 (31.0)‡
1–10	26.0	27.0	18.0	24.0 (25.1)‡	28.0 (26.0)‡
10–20	16.0	27.0	23.0	24.0 (26.2)‡	17.0 (16.0)‡
>20	27.0	32.0	42.0	37.0 (34.3)‡	24.0 (27.0)‡

*HCLP: chicken liver powder ingredient; †PBM: poultry byproducts meal; #BMBM: bovine meat and bone meal; §HCLP: hydrolysed chicken liver powder diet, ‡Values within parentheses were estimated based on food formulation.

Table 6. Preference of experimental diets in adult dogs from different breeds.

	Treatments		
Item	Control	HCLP*	*p*-Value
Feed intake [g]	195	202	.2405
First choice [%]	45.5	54.6	.5465

*HCLP: hydrolysed chicken liver powder diet.

Table 7. *In vitro* coefficient of digestibility of experimental diets.

	Treatments	
Item	Control	HCLP*
Dry matter [%]	84.5	84.4
Organic matter [%]	84.0	84.3

*HCLP: hydrolysed chicken liver powder diet.

patients. Thus, the present study aimed to describe the chemical composition of a commercial HCLP and its acceptability and availability of nutrients and energy when it is replacing the most common sources of protein in diet for dogs: poultry byproduct meal and bovine meat and bone meal. And, finally, to compare *in vivo* and *in vitro* digestibility of the diet based on HCLP.

Adverse food reaction may occur due to an exacerbated immune response to food antigens (Mueller and Unterer 2018). The gastrointestinal tract has some mechanisms to avoid the entrance of foreign bodies into the bloodstream. The gut associated lymphoid tissue (GALT) is one of them, providing an active barrier to harmful substances such as food antigens. However, due to the higher permeability of the intestinal mucosa some food antigens may pass through inducing an immune response (Verlinden et al. 2006). The absorbed antigen cross-links between two high-affinity IgE receptor (FCεRI) present on the surface of mast cells and basophils, eliciting the release of mediators, such as histamine, prostaglandins, enzymes, and cytokines (Cave 2006).

The most common ingredients associated with adverse food reactions in dogs are beef, dairy products, chicken, and wheat. Less frequently are chicken egg, soy, lamb, pork, fish, and corn (Roudebush 2013). Through chemical analysis investigation, the HCLP provided adequate nutritional composition to be used as protein source in diets for dogs with no prior adverse reactions.

Molecular weight is one of the main tools for selecting protein sources, since small peptides can retain allergenicity and induce adverse food reactions (Cave 2006). In humans, peptides with molecular weight between 10 and 70 kDa are absorbed entirely by the enteric mucosa inducing an allergic reaction by IgE binding (Sampson 1999). In dogs, the molecular weight associated with allergenicity remains unknown, but the selection of new and/or low molecular weight ingredients is recommended for adverse food reaction in dogs. The ingredients used in the experimental diets had different proportions of molecular weights. The control diet had 39% proteins with molecular weight <10 kDa, and the HCLP diet had 59%, based on the calculation according to the molecular weight

present in the ingredients. Thus, seems that the extrusion process did not alter the fraction of molecular weight <10 kDa in both diets, as predicted in the estimated calculation (39.7% proteins with molecular weight <10 kDa on control diet and 57% on HCLP diet). Combined with the hydrolysed chicken liver powder, HCLP diet included brewers rice and full-fat rice bran, which contributed to increase the fraction of proteins with molecular weight >10 kDa. However, adverse food reactions related to rice are rare in dogs and cats (Roudebush 2013).

Cave and Guilford (2004) evaluated the molecular weight profile of a hydrolysate derived from chicken heart and liver, that presented 96.9% of its molecular weight <10 kDa. Compared with the intact protein, the chicken hydrolysate showed a residual antigenic mass of 1.5% analysed by the inhibition ELISA using IgG. According to De Jaham (2000), peptides with a molecular weight >45 kDa are still capable of eliciting an immune response in dogs. Differences in molecular weight profile in protein hydrolysates varies with the type of protein material used and the degree of hydrolysis applied to the protein material. Olson et al. (2000) recommends that at least 50% of the protein material should be hydrolysed to prevent allergic reaction in dogs.

The hydrolysis process of the chicken liver powder was conducted enzymatically and, following this was spray dried. The spray drying technique consists of producing a dry powder from a liquid by drying with a hot gas. This process allows the preservation of functional characteristics of the original raw material compared to the conventional drying process applied to meat by-products that may impact negatively on the nutritional content (Murray et al. 1997). The experimental diet based on HCLP was formulated to meet the complete nutritional requirements for adult dogs, and based on its amino acids content, such as lysine (58.1 g/kg), we were able to include the HCLP at a level of 258 g/kg. Analysis of subsequent batches of HCLP indicate that there was a reduction in the lysine concentration in the ingredient, with an average level of 48.0 g/kg of lysine. In addition, chemical analyzes of new batches of HCLP showed high concentration of taurine (5.00 g/kg) and choline (5120 mg/kg). Thus, HCLP becomes a viable option in diets for cats due to their high requirement for these nutrients. Finally, in order to attain the complete protein requirement, we selected rice, added as brewers rice and full-fat rice bran, due to its low association with dogs with adverse food reactions in dogs, as mentioned above.

The HCLP ingredient had all amino acid scores (AAS) above 100, based on the minimum requirement for growth of puppies and kittens, except for the combined requirement for Met + Cys and Phe + Tyr for kittens. In comparison, the poultry byproduct meal and the bovine meat and bone meal had more than one amino acid scored below 100 for dogs and cats, which indicates fewer limiting amino acids present on the HCLP ingredient evaluated in this study. Kerr et al. (2013) evaluated raw meat diets based on beef, bison, elk and horse and verified that in all diets the first limiting amino acid was the combined requirement of Met + Cys, scored below 100 (AAS ranged from 81 to 95) based on the minimum requirement for growth of kittens. Based on these findings, the HCLP ingredient presents high levels of most amino acids, which indicates high protein quality of the ingredient.

The palatability of the HCLP diet was not affected by the high inclusion of hydrolysed chicken liver powder (258 g/kg) compared to the control diet based on poultry byproducts and bovine meat and bone meals. Hydrolysis reduces the size of protein chains in small peptides and free amino acids, with the goal to reduce the molecular weight to avoid the protein recognition as an antigen by the immune system (Cave 2006). However, hydrolysis can expose side chain peptides, especially the hydrophobic side chain, which elicits the bitterness of some hydrolysed proteins. Cho et al. (2004) evaluated two commercial soy protein hydrolysates and noted that bitterness increased as the molecular weight of the peptide ranged between 4 to 2 kDa, and peptides with molecular weight <1 kDa showed the lowest bitterness. At the same time, hydrolysed protein sensorial characteristics are associated with mixture of peptides and the original protein source that highly affects palatability (Adler-Nissen 1986). Despite reports of bitterness associated to hydrolysed proteins, previous studies showed an adequate consumption of commercial dog diets containing chicken and soy isolate hydrolyzates (Biourge et al. 2004; Loeffler et al. 2004), indicating good palatability. Indeed, hydrolysed proteins have been long used as palatability enhancers in commercial diets for dogs and cats. Additionally, the peroxide index of both experimental diets remained in acceptable range.

Small peptides from partially hydrolysed proteins are more efficiently absorbed from the intestine and have a higher nutritional value than free amino acids (Monchi and Rerat 1993). Thus, the digestibility of protein hydrolysates was expected to be superior to the intact protein (Cave 2006). However, the digestibility of both experimental diets, control and HCLP, did not differ.

According to Rouanet et al. (1990), hydrolysate diets containing di- and tripeptides are efficiently digested, but not better utilised than diets composed of the original protein in healthy growing rats. Hekman (2003) found an apparent ileal protein digestibility of 82.4% for a commercial diet based on hydrolysed chicken. Compared to our results, both diets showed superior results (88.0% for the control diet and 89.3% for the HCLP diet). In order to attain some amino acids requirements and to obtain isonitrogenous diets, synthetic amino acids were added to the control diet, which may have improved its digestibility due to the bioavailability of these components. However, differences in digestibility occurred to a small extent. Though, we use the apparent total tract digestibility that does not account for part of the metabolism of nutrients in the large intestine, mainly due to the microbiota metabolism and epithelial desquamation. Thus, this method may increase the digestibility coefficient of protein due to the microbiota degradation (Zebrowska 1975). Although the diets were formulated to be isonutritive, some differences were observed, such as a lower fat and a higher crude fibre content of the HCLP diet. In addition, the HCLP diet had a lower ash content compared to the control diet (10.5 g/kg less), which is a favourable aspect to the selection of the HCLP ingredient for addition in high digestibility diets.

The increase on the faecal water content and daily faecal production in dogs fed HCLP diet may be due to the hydrolysed chicken liver powder osmolarity. High osmolarity solutions attract water to the intestinal luminal promoting severe diarrhoea and it is increased with hydrolysis. Therefore, extensively hydrolysed proteins could promote diarrhoea in some dogs. In a study conducted by Loeffler et al. (2004) with 46 dogs fed a commercial diet with chicken hydrolysate, only 4 developed soft faeces. In addition, 21 of the 46 dogs had gastrointestinal symptoms prior to the study and all showed improvement on these signs. In our research, one of our first concern was the high inclusion of the HLCP (258 g/kg) that could promote severe diarrhoea in dogs as reported by Hekman (2003), in which dogs fed a commercial diet based on hydrolysed chicken showed diarrhoea. However, despite changes in faecal DM and faecal volume, the faecal output in dry matter did not differ between the dietary treatments, and the final faecal score remained within the recommended by Moxham (2001).

Ribeiro et al. (2019) observed that poultry by-product meals from two integrated rendering plants showed different *in vitro* digestibility of organic matter (IVDOM) based on their oxidative stability. From 100 samples analysed, 18 had 84.8% of IVDOM, classified by the authors as high IVDOM. The control diet showed 84.0% of IVDOM and the HCLP, 84.3% of IVDOM. We did not analyse the ingredients separately, only the complete diets. Biagi et al. (2016) analysed the *in vitro* digestibility of dry matter (IVDDM) of commercial diets for dogs in two different durations of gastric digestive phase and found that IVDDM for 2 h of incubation was 86.4% and for 4 h of incubation was 84.2%. According to these authors, *in vitro* digestibility method is a quick procedure to predict the digestibility of commercial diets, thus reducing the need for *in vivo* assays. However, more assays and replicates are necessary to guarantee the accuracy of this method.

Conclusions

Based on the current high demand for protein hydrolysates of high nutritional value and acceptable for dogs as a viable option in therapeutic diets, the hydrolysed chicken liver powder evaluated in this study has nutritional characteristics compatible with those required for adult dogs, especially due to the high content of some essential amino acids and fatty acids. The inclusion level of 258 g/kg of hydrolysed chicken liver powder promoted good acceptance and digestibility, and did not promote diarrhoea in dogs fed the HCLP diet. Further studies are needed to evaluate its effects in dogs diagnosed with adverse food reactions and gastrointestinal disorders.

Disclosure statement

No potential conflict of interest was reported by the author(s). Marcelino Bortolo and Fábio Ritter Marx are employees of Kemin Industries.

Funding

This study was supported by the Brazilian governmental research support institution Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (grant no. 88887.354521/2019-00) and Kemin Nutrisurance, the pet food and rendering technologies division of Kemin Industries.

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Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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CAPÍTULO III

Hydrolyzed chicken liver used as single source of animal protein in diet and its effects on cytokines, immunoglobulins, and fecal microbiota profile of adult dogs

Artigo publicado no periódico

PLOS ONE

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RESEARCH ARTICLE

Hydrolyzed chicken liver used as single source of animal protein in diet and its effect on cytokines, immunoglobulins, and fecal microbiota profile of adult dogs

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OPEN ACCESS

Citation: Pinto CFD, de Oliveira BB, Bortolo M, Guldenpfennig R, Marx FR, Trevizan L (2022) Hydrolyzed chicken liver used as single source of animal protein in diet and its effect on cytokines, immunoglobulins, and fecal microbiota profile of adult dogs. PLoS ONE 17(7): e0271932. https://doi.org/10.1371/journal.pone.0271932

Editor: Ewa Tomaszewska, University of Life Sciences in Lublin, POLAND

Received: May 18, 2022

Accepted: July 10, 2022

Published: July 22, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (grant number 88887.354521/2019-00) as a PhD scholarship to CFDP, and by Kemin Nutrisurance, the pet food and rendering technologies division of Kemin Industries, Inc., that provided the hydrolyzed

Abstract

Dogs with food allergies and enteropathies may require hydrolyzed diets to prevent or reduce clinical signs, therefore the protein sources used in these diets must be previously characterized and evaluated in healthy dogs. This study aimed to investigate the effects of a hydrolyzed chicken liver powder-based diet (HCLP) versus a poultry by-product meal and bovine meat and bone meal-based diet (Control), on complete blood count (CBC), cytokine, immunoglobulins responses (assessed on days 0, 15, 30 and 45), and fecal microbiota (assessed on day 45) in healthy adult dogs. The CBC did not differ between diets ($P>0.05$), remaining within reference range. Total plasma IL-4 concentrations were decreased over time independent of the dietary treatment ($P<0.001$). Total plasma IgA decreased on day 30 compared to days 0 and 45 in dogs fed the control diet ($P<0.001$). Total plasma IgE concentrations were reduced on days 30 and 45 in dogs fed the control diet, and on days 15 vs 30 and 15 vs 45 in dogs fed HCLP diet ($P = 0.001$). The 16S rRNA gene sequencing showed similar species richness and abundances of phyla and genera between diets ($P>0.05$). β-diversity principal coordinate analysis plots demonstrated that HCLP group had a higher similarity than control. Based on our results, healthy adult dogs fed a HCLP based diet maintained normal values for hematological and immunological characteristics, and fecal microbiota after 45 days of feeding.

Introduction

Hydrolyzed proteins are commonly used in therapeutic diets for dogs with adverse food reactions (AFR) and gastrointestinal diseases. These therapeutic diets possess a high bioavailability of nutrients and peptides with reduced molecular weight that decrease the risk of antigenic stimulation in the intestine [1]. Food antigens elicit an immune response at the Gut Associated

chicken liver powder and experimental financial support. CAPES was not involved in the study design, analysis, interpretation, and writing of this article. Kemin Industries, Inc. was involved in the review and decision to publish the manuscript.

Competing interests: CFDP is a PhD student supported by CAPES, BBO is an Undergraduate student supported from Universidade Federal do Rio Grande do Sul, and LT is a Full Professor at the Universidade Federal do Rio Grande do Sul and holds the grant provided by Kemin Industries for this study. MB, RG, and FRM are employees of Kemin Industries. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Lymphoid Tissue (GALT) after passing through the intestinal mucosa, which can lead to immediate (IgE-mediated, Type I reaction) or delayed (immune complex or cell mediated, also known as Types III and IV, respectively) hypersensitivities. Whether through IgE induced mast cell degranulation or by humoral and cell-mediated reactions, the constant stimulus induces symptoms such as pruritus, vomiting, and diarrhea in dogs [2]. Several researchers reported increased TNF-α and IL-4, and decreased IL-10 in humans and mice with food allergies [3–5]. In dogs, previous studies highlighted the benefits of hydrolyzed proteins in preventing allergenic reactions. Puigdemont et al. [6] observed that experimentally soy-sensitized dogs did not develop cutaneous and gastrointestinal reactions after oral administration of hydrolyzed soy protein. In a different approach, Olivry et al. [7] showed that only an extensively hydrolyzed poultry feather extract was able to prevent recognition by serum IgE from poultry-sensitized dogs.

Since hydrolysis allows the cleavage of the protein chains into small peptides and free amino acids [1], we presumed that the hydrolyzed protein diet could modulate the gut microbiota composition and functionality due to the high bioavailability of peptides present in the chicken liver hydrolyzed protein resulting in the formation of less protein fermentation end products. Bacterial metabolic products synthesized in the colon from "bypass" undigested nutrients [8] generated from proteins can be harmful to the host gastrointestinal health, such as ammonia, sulfides, phenols, and biogenic amines [9, 10].

While hydrolyzed proteins may stimulate less cytokine production than intact proteins, in order to guarantee the safety of novel ingredients it is highly recommended to investigate their effects on healthy dogs. Thus, this study aimed to evaluate the effects of a hydrolyzed chicken liver diet on hematologic, pro-inflammatory (IFN-γ, TNF-α, IL-2, IL-6, IL-7) and anti-inflammatory (IL-4, IL-10) cytokines, immunoglobulins (IgA and IgE), and fecal microbiome response of healthy adult dogs.

Materials and methods

Animal care and handling procedures were approved by The Institutional Animal Care and Use Committee at the Universidade Federal do Rio Grande do Sul (protocol number 36138).

Animals and installations

Twelve healthy, intact adult Beagle dogs (6 males and 6 females), 5 years old, weighing 11.8 ± 1.45 kg, body condition score ranging from 5 to 6 out of 9 points [11], and free of ectoparasites were supplied by the Animal Science Department, Universidade Federal do Rio Grande do Sul–UFRGS, Porto Alegre, Brazil. The dogs were regularly immunized, dewormed, and submitted to clinical and laboratory tests to measure complete blood count and biochemistry analyses before the trial beginning. The dogs were allocated into individual stainless steel metabolic cages (1.0 × 1.0 × 1.5 m) equipped with a feces and urine collector, feeders, and drinkers, in a controlled room at 24˚C, with a light: dark cycle of 14:10 h. During the study, they were fed twice daily inside the metabolic cages and stayed there all through the night. During the day dogs remained all together in an outdoor area for socialization. After the study, the dogs remained in the lab for further nutritional studies.

Experimental design

The study was conducted in a randomized design with two dietary treatments, with six dogs per diet. The experimental period lasted 45 days in which was measured: hematological indicators, cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-7, IL-10) and immunoglobulins (IgA and IgE) on days 0, 15, 30 and 45; and fecal microbiota on day 45.

Dietary treatments

Before the start of the study, all dogs were fed the same commercial diet for 30 days, during a wash-in period to stabilize the gastrointestinal tracts. Experimental diets ingredients and chemical composition were previously reported by Pinto et al. [12] and are presented in Table 1. The dietary treatments were a Control diet (based in poultry by-product + bovine meat and bone meals) and HCLP diet (based in hydrolyzed chicken liver powder—PROSURANCE® CHX Liver HD, Kemin® Industries). The dogs received the dietary treatments twice a day (at 08:30 h and 17:00 h) to attend their daily maintenance energy requirements (110 kcal of metabolizable energy x body weight $(kg)^{0.75}$/day), as recommended by NRC [13]. The dogs were weighed weekly to adjust the amount of food and maintain a body condition score ranging from 5 to 6 out of 9 points. Water was provided *ad libitum* during the trial.

Table 1. Ingredients and chemical composition of experimental diets.

Ingredient, % as fed basis	Treatments	
	Control	HCLP
Brewers rice	52.7	52.7
Full-fat rice bran	8.00	8.00
Poultry by-product meal	18.6	-
Bovine meat and bone meal	5.00	-
HCLP[1]	-	25.8
Cellulose	3.34	4.22
Poultry fat[2]	8.75	4.30
Soybean oil[2]	-	1.80
Canola oil[2]	1.37	0.46
L-lysine	0.63	-
DL-methionine	0.33	0.27
Potassium chloride	0.23	0.07
L-tryptophan	0.03	-
Limestone	-	2.01
Premix mineral/vitamin[3]	0.50	0.50
Salt	0.50	0.50
Analyzed composition, % DM basis		
Dry matter	94.1	94.5
Organic matter	94.3	95.4
Ash	5.67	4.62
Crude protein	24.8	24.8
Acid-hydrolysed fat	15.1	14.0
Crude fiber	3.75	4.67
Gross energy, kcal/kg	4950	5055

Control, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet.

[1]HCLP: hydrolysed chicken liver powder ingredient.

[2]Added on top.

[3]Premix mineral/vitamin (supplied per kilogram of diet): vitamin A (10,800U), vitamin D3 (980 U), vitamin E (60 mg), vitamin K3 (4.8 mg), vitamin B1 (8.1 mg), vitamin B2(6.0 mg), vitamin B6 (6.0 mg), 12 vitamin (30 mcg), pantothenic acid(12 mg), niacin (60 mg), folic acid (0.8 mg), biotin (0.084 mg), manganese(7.5 mg), zinc (100 mg), iron (35 mg), copper (7.0 mg), cobalt (10 mg), iodine (1.5 mg), selenium (0.36 mg), choline (2.400 mg), taurine (100 mg),and, antioxidant BHT (150 mg).

https://doi.org/10.1371/journal.pone.0271932.t001

Immunological analyses

To assess the effect of a hydrolyzed chicken liver diet on systemic humoral responses, circulating total IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-7, IL-10, IgA, and IgE were measured in the plasma.

Sample collection. Blood samples were collected via jugular venipuncture before morning feeding sessions on days 0, 15, 30, and 45 of the experimental periods. Three mL blood samples were placed into blood collection tubes with EDTA per dog on each time of the evaluation. One tube was used for complete blood count (CBC). The other two tubes were centrifuged at 1,000 *g* for 15 min within 30 minutes after blood collection. Then the plasma was immediately divided into aliquots, transferred to 2 mL Eppendorf tubes, and stored frozen at -20˚C until cytokine and immunoglobulin analyses were performed.

Cytokines and immunoglobulins analyses. IFN-γ, TNF-α, IL-2, IL-6, IL-7, and IL-10 were quantified using a Luminex xMAP kit canine specific, according to the manufacturer's instructions (CCYTOMAG-90K - MILLIPLEX MAP Canine Cytokine Panel). The presence of IL-4 was quantified using an ELISA kit specific to dogs, according to the manufacturer's instructions (Canine IL-4 ELISA, SEA077CA). The plasmatic levels of immunoglobulins, IgA (IgA Dog ELISA ab157699, Abcam®) and IgE (IgE Dog ELISA ab157700, Abcam®) were performed using ELISA kits specific to dogs, used according to the manufacturer's instructions.

Fecal microbiota analyses

Fecal samples from each dog were collected the morning of day 45. Samples were divided into aliquots, stored in a 2 mL Eppendorf tube, and stored in a freezer at -20˚C until analyzed. Total DNA was extracted using ZR Fecal DNA MiniPrep (Zymo Research Corporation, Irvine, CA), followed by quantification of extracted DNA by spectrophotometry at 260nm. Quality of extracted DNA was assessed by electrophoresis using a 1% agarose gel. A segment of approximately 460 bases from the hypervariable region V3V4 of the ribosomal 16S rRNA gene was amplified, under the following PCR conditions: 95˚C for 3 min; 25 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, followed by step at 72˚C for 5 min. The metagenomic library was built from these amplifiers using the commercial kit Nextera DNA Library Preparation Kit (Illumina Inc., San Diego, CA). Amplicons were pooled and sequenced using the Illumina MiSeq sequencer (Illumina Inc., San Diego, CA) [14]. Sequence data was analyzed using the QIIME (Quantitative Insights Into Microbial Ecology) platform [15, 16], followed by a low quality sequence removal workflow, filtration, chimera removal and taxonomic classification. Sequences were classified into bacterial genera through the recognition of operational taxonomic units (OTU), in this case, the homology between the sequences when compared to a database. Sequences were compared using the update 2017 version (SILVA 128) from the database of ribosomal RNA genes sequences SILVA [17]. In total, 63000 readings were used per sample to generate the classification of bacterial communities through the identification of OTU, aiming to normalize the data and not compare samples with different numbers of readings, avoiding taxonomic bias. Alpha diversity was estimated using OTU, Simpson Index, and Shannon Index. Beta diversity was calculated using Bray-Curtis and D_0.5 UniFrac distance measures and presented with Principal Coordinate Analysis (PCoA) plots.

Statistical analysis

Complete blood count, cytokines and immunoglobulins were analyzed as delta values, calculated by the difference between each evaluated day and day 0. A positive delta represents an increase in the measure while a negative delta represents a reduction in time. All data were

analyzed using the GEE procedure (Generalized Estimating Equation) with the Bonferroni post-hoc test for days. Data without normal distribution were previously transformed and analyzed with gamma distribution for transformed variables. Analyses were performed using the software IBM SPPS v. 18 (Statistical Package for Social Sciences) and the level of 5% was considered significant. Additionally, hematological and immunological markers were submitted to a Multivariate Analysis of Variance (MANOVA) and Canonical Discriminant Analysis to verify the differences and associations between the dietary treatments throughout the experimental period, also considering the level of 5% as significant. Alpha diversity was estimated by Kruskal-Wallis H-test. Differences between the relative abundances of the taxonomic groups were estimated by T-test using the software Minitab 18 (Minitab Inc. State College, PA) and the level of 5% was considered significant.

Results

All hematological indicators were within the reference values. There was an increase over time independent of the dietary treatment for erythrocytes ($P = 0.0110$) and hemoglobin on day 15 ($P = 0.0010$), hematocrit on days 15 and 45 vs day 0 ($P<0.001$) and mean corpuscular volume on days 15 and 45 vs day 0 ($P<0.001$) (Table 2). A reduction over time regardless of dietary treatment was detected in mean corpuscular hemoglobin ($P = 0.0360$), leucocytes on days 15 and 30 vs day 0 ($P<0.001$), total segmented neutrophils on day 30 vs day 0 ($P<0.001$), total monocytes on days 15 vs 0 and 15 vs 30 ($P<0.001$). Changes over time were also observed for total lymphocytes on days 30 vs 45 ($P = 0.0140$), monocytes on days 15 vs 0 and days 15 vs 30 ($P<0.001$), and lymphocytes on days 30 and 45 vs 0 ($P = 0.0010$). Interaction effect was observed for total eosinophils, in which the HCLP group had a difference on day 45 ($P = 0.040$). Eosinophils had a difference in the control group on days 15 vs 30, and HCLP group on days 45 vs 0, and 45 vs 30 ($P = 0.0280$). Total protein differed on the control group on days 15 vs 30, and on HCLP group on days 0 vs 15 and 45, 15 vs 30, 30 vs 45 ($P = 0.008$).

Although MANOVA did not identify differences between dietary treatments and blood collection days ($P>0.05$), the canonical correlation identified differences between dietary treatments regarding hematological markers (Fig 1A), in which leukocytes, segmented neutrophils (/μL), eosinophils, MCV, total protein and platelet counts were associated with the control diet, while erythrocytes, hemoglobin, hematocrit, segmented neutrophils (%), monocytes and MCHC were associated with the hydrolyzed diet. Considering the day of blood collection (Fig 1B), the medians of all markers on day 0 were smaller than on other days, but with greater variability. Multivariate analysis did not identify a difference between the days of blood collection, but averages appeared to be greater on day 45, especially when compared to day 0. Through the two-dimensional correlation, it is possible to assess that leukocytes and segmented neutrophils are associated with day 0, while platelet count and total protein are with day 15. Monocytes point to day 30, while hemoglobin, hematocrit, and erythrocytes appear to be associated with day 45 (Fig 1C).

No changes were observed in the total plasma concentrations of IFN-γ, TNF-α, IL-2, and IL-10 during the experimental period ($P>0.05$) (Table 3). Total plasma IL-4 decreased over time regardless of dietary treatment ($P<0.001$), with an increase in delta values on days 30 ($P = 0.006$) and 45 ($P<0.001$) compared to day 0. In addition, there was a significant difference between the delta values of days 15 and 30 ($P = 0.002$), and delta values of days 15 and 45 ($P<0.001$). Although there was a difference between the delta values over time in the total plasma concentrations of IL-6 ($P = 0.015$) and IL-7 ($P = 0.036$), it was not possible to verify differences with the Bonferroni post-hoc test. There were differences over time for both dietary treatments in IgA and IgE concentrations ($P<0.05$). Total plasma IgA delta values differed on

Table 2. Hematology of dogs fed the experimental diets.

Items	RV	Treatments[1]				Δ-values[2]			P-values		
		D0	D15	D30	D45	D 15–0	D 30–0	D 45–0	Diet	Day	Diet x Day
Erythrocytes, 106/μL[3]											
Control	5.5–8.5	6.64±0.19	6.74±0.09	6.79±0.09	6.94±0.06	0.10	0.15	0.30	0.251	0.011	0.145
HCLP		6.63±0.27	7.11±0.11	6.97±0.09	7.21±0.04	0.48	0.34	0.59			
Hemoglobin, g/dL[3]											
Control	12–18	15.4±0.43	15.8±0.18	15.8±0.16	16.1±0.10	0.40	0.43	0.75	0.441	0.001	0.083
HCLP		15.4±0.55	16.6±0.20	16.0±0.17	16.7±0.07	1.15	0.58	1.25			
Hematocrit, %[3]											
Control	37–55	44.7±1.22	46.0±0.49	46.0±0.32	47.5±0.24	1.33	1.33	2.83	0.405	<0.001	0.263
HCLP		44.5±1.56	48.0±0.40	46.7±0.43	48.8±0.20	3.50	2.17	4.3			
MCV, fL[3]											
Control	60–77	67.3±0.71	68.5±0.32	67.9±0.41	68.5±0.22	1.15	0.58	1.22	0.391	<0.001	0.638
HCLP		67.3±0.40	67.7±0.31	67.1±0.34	67.8±0.32	0.35	-0.22	0.43			
MCHC, %[3]											
Control	32–36	34.5±0.18	34.3±0.08	34.4±0.13	34.0±0.05	-0.15	-0.07	-0.50	0.847	0.036	0.8177
HCLP		34.6±0.15	34.5±0.09	34.3±0.06	34.2±0.06	-0.13	-0.33	-0.48			
Leucocytes, /μL[4]											
Control	6000–17000	10167±367	9417±240	9133±189	8733±215	-400	-800	-1150	0.743	<0.001	0.415
HCLP		9717±508	8367±272	8017±192	8917±422	-1850	-2050	-1350			
Total segmented neutrophils, /μL[4]											
Control	3000–11500	6489±263	5787±250	5280±174	5395±188	-937	-1196	-995	0.910	<0.001	0.724
HCLP		6480±363	5384±168	4922±87.3	6249±388	-1276	-1652	-619			
Total eosinophils, /μL[4]											
Control	100–1250	828±128	678±39.6	477±29.2	516±27.4	-66.5^{Aa}	-291^{Aa}	-200^{Aa}	0.893	<0.001	0.040
HCLP		706±88.3	526±38.9	541±42.2	413±14.6	-217^{Ab}	-183^{Ab}	-382^{Aa}			
Total monocytes, /μL[4]											
Control	150–1350	583.5±62.5	381±24.5	589±43.5	455±23.9	-253	-80.5	-212	0.844	<0.001	0.582
HCLP		636±76.1	398±36.9	599±28.7	660±52.2	-336	-6.00	168			
Total lymphocytes, /μL[3]											
Control	1000–4800	2266±213	2619±96.8	2787±98.2	2368±98.5	353	521	102	0.322	0.014	0.586
HCLP		1895±259	2113±141	1955±103	1706±76.2	218	59.3	-189			
Segmented neutrophils, %[3]											
Control	60–77	64.2±2.54	61.0±1.22	57.7±1.33	61.5±1.57	-3.17	-6.50	-2.67	0.567	0.073	0.480
HCLP		66.8±2.93	65.0±1.76	62.2±1.57	68.8±1.63	-1.83	-4.67	2.00			
Eosinophils, %[3]											
Control	2–10	8.00±0.95	7.17±0.33	5.17±0.33	6.00±0.36	-0.83^{Ab}	-2.83^{Aa}	-2.00^{Aab}	0.915	0.002	0.028
HCLP		7.17±0.64	6.17±0.34	6.50±0.44	5.00±0.44	-1.00^{Aab}	-0.67^{Ab}	-3.50^{Aa}			
Monocytes, %[3]											
Control	3–10	5.67±0.45	4.20±0.30	6.50±0.45	5.17±0.25	-2.00	0.83	-0.50	0.838	<0.001	0.866
HCLP		6.67±0.91	4.80±0.45	7.50±0.31	7.33±0.44	-2.50	0.83	0.67			
Lymphocytes, %[3]											
Control	12–30	22.2±1.68	28.2±1.03	30.7±1.04	27.3±1.20	6.00	8.50	5.17	0.303	0.001	0.169
HCLP		19.3±2.18	24.7±1.20	23.8±0.98	20.2±1.10	5.33	4.50	0.83			
Total protein, g/L[4]											
Control	60–80	54.0±7.35	65.7±0.80	62.7±0.54	55.3±3.43	0.00^{Aab}	-2.00^{Ab}	1.00^{Aa}	0.026	<0.001	0.008
HCLP		62.3±1.54	66.7±0.74	61.0±0.43	68.7±0.74	4.00^{Ba}	-1.00^{Ab}	5.00^{Aa}			
Platelet count, /μL[4]											

(*Continued*)

Table 2. (Continued)

Items	RV	Treatments[1]				Δ-values[2]			P-values		
		D0	D15	D30	D45	D 15–0	D 30–0	D 45–0	Diet	Day	Diet x Day
Control	200000–500000	366667±14062	3733333±5274	340000±7912	354000±3871	0.00	-15000	-37000	0.103	0.069	0.357
HCLP		309500±30074	389167±18410	331333±8277	315000±5696	48000	-12000	-20000			

RV, reference values; Control, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.

[1]Values expressed as mean ± standard error of the mean.

[2]Values expressed as delta values.

[3]GEE (Generalized Estimating Equations) analysis with normal distribution.

[4]GEE (Generalized Estimating Equations) analysis with gamma distribution for transformed variables.

[a,b]Comparisons of days in each treatment in the same row with different lowercase letters are significantly different by Bonferroni post-hoc test (P<0.05).

[A,B]Comparisons of treatments on each day in the same row with different capital letters are significantly different by Bonferroni post-hoc test (P<0.05).

https://doi.org/10.1371/journal.pone.0271932.t002

day 30 compared to days 0 and 45 for the control group (P<0.001). However, the delta values of the HCLP group showed no differences. The delta values of total plasma IgE decreased for both treatments over time, in the control group the delta values on days 30 and 45 differed from the others, while for the HCLP group the delta values differed on days 15 vs 30 and 15 vs 45 (P = 0.001).

MANOVA indicated a difference between dietary treatments (P<0.05), but not for the days of blood collection (P = 0.45). The canonical correlation showed the difference between the control and hydrolyzed treatment (Fig 2A), in which markers such as IFN-γ, IL-2, IL-6, IL-7, IL-10, and TNF-α were associated with the control diet, while IL-4, IgE and IgA were associated with the hydrolyzed diet. Regarding the association between immunological markers and days of blood collection (Fig 2B), it seems that the medians were progressively reduced throughout the experimental period, but no statistical difference was observed (P>0.05). The two-dimensional canonical correlation evidenced the difference between day 0 and days 30 and 45 since the circles did not overlap between these days (Fig 2C). In addition, from the attribution of colors, day 30 and day 45 were considered equal.

In this study, 11 fecal samples were processed and a total of 63000 sequence reads per dog were used. One fecal sample from the control group was removed due to the low number of read sequences. Bacterial community was composed of 27 phyla, in which 6 phyla (Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Proteobacteria, Tenericutes) represented more than 99% of sequence reads, considering mean relative abundance higher than 0.5%. There was no difference for the relative abundance of phyla between dietary treatments (P>0.05). Both treatments had the majority of bacterial community composed by Firmicutes, Bacteroidetes and Fusobacteria, counting for more than 85% of the OTU (Fig 3A). At the genera level, 152 were identified with 19 of those with a mean relative abundance higher than 0.5%. No changes were observed in the genus abundance between the dietary treatments (P>0.05). The most abundant genera in both treatments were *Fusobacterium*, [*Prevotella*], *Allobaculum*, *Bacteroides*, *Sutterella*, *Blautia*, *Collinsella*, [*Ruminococcus*], *Dorea*, *Clostridium* and *Faecalibacterium*, which counted for more than 50% of the OTU (Fig 3B). Each dog was considered an experimental unit, totaling 6 replicates for the HCLP group and 5 replicates for the control group. Clear differences on abundances of phyla (Fig 3C), but mainly at the genera level (Fig 3D), were detected among dogs within the same dietary treatment on day 45.

Diversity indices estimators OTU (mean ± SD; HCLP = 1042 ± 96.8; Control = 1066 ± 34.6) (Fig 4A), Simpson Index (HCLP = 0.96 ± 0.007; Control = 0.94 ± 0.039) (Fig 4B) and Shannon

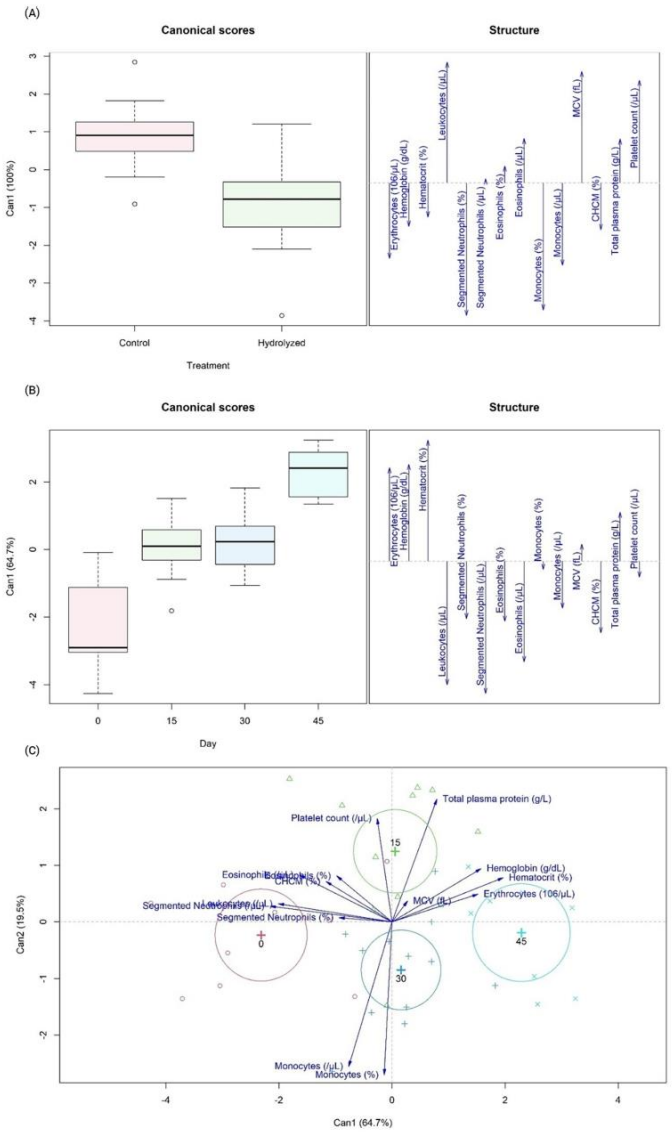

Fig 1. Canonical correlations of dietary treatments (A), days of blood collection (B) and two-dimensional canonical correlation of days of blood collection (C) for hematological markers from healthy Beagle dogs fed the experimental diets.

https://doi.org/10.1371/journal.pone.0271932.g001

Table 3. Plasma cytokines and immunoglobulins concentrations of dogs fed the experimental diets.

Items	Treatments[1]				Δ-values[2]			P-values		
	D0	D15	D30	D45	D 15–0	D 30–0	D 45–0	Diet	Day	Diet x Day
IFN-γ, pg/mL[3]										
Control	1.37±0.43	1.27±0.41	1.33±0.44	1.24±0.37	-0.11	-0.04	-0.13	0.900	0.388	0.354
HCLP	0.88±0.13	0.83±0.11	0.79±0.07	0.79±0.12	-0.05	-0.09	-0.09			
TNF-α, pg/mL[4]										
Control	21.0±7.65	14.4±3.70	10.0±2.18	10.3±2.48	-0.05	-0.73	0.04	0.249	0.225	0.118
HCLP	4.07±0.51	3.31±0.32	3.46±0.38	3.16±0.29	-0.75	-0.68	-0.92			
IL-2, pg/mL[4]										
Control	44.1±15.8	31.2±9.70	27.8±8.27	28.1±9.03	-0.68	-4.27	-0.07	0.163	0.342	0.381
HCLP	7.24±1.25	6.44±1.08	6.99±1.51	5.94±0.85	-0.54	-0.50	-1.25			
IL-4, pg/mL[4]										
Control	174±20.1	163±9.82	125±14.4	115±14.9	-5.22	-42.6	-53.1	0.166	<0.001	0.107
HCLP	178±20.1	174±11.3	157±7.22	142±12.0	-12.8	-29.4	-41.2			
IL-6, pg/mL[4]										
Control	19.9±6.42	15.6±4.70	13.2±3.75	14.7±3.64	-0.64	-3.73	-0.06	0.502	0.015	0.074
HCLP	13.4±5.07	12.3±4.07	11.2±3.19	10.9±3.38	-0.66	-0.11	-1.15			
IL-7, pg/mL[4]										
Control	90.3±34.8	61.3±17.8	42.9±11.1	45.7±12.6	-3.37	-10.6	-2.95	0.160	0.036	0.304
HCLP	21.2±6.14	20.0±5.34	17.3±4.17	11.6±2.09	-0.73	-1.40	-5.43			
IL-10, pg/mL[4]										
Control	6.30±1.42	6.13±1.00	6.25±1.04	7.91±1.57	-0.09	0.00	0.43	0.293	0.168	0.367
HCLP	5.57±0.43	5.26±0.54	6.05±0.69	4.90±0.30	-0.13	0.33	-0.67			
IgA, ng/mL[4]										
Control	3535419±152407	3568147±78565	3474936±100862	3524653±97926	-13702^{Aab}	-68848^{Ab}	-5322^{Aa}	0.047	0.032	<0.001
HCLP	3512716±147054	3674435±34772	3688204±26921	3560697±61867	14990^{Aa}	43240^{Aa}	-12102^{Aa}			
IgE, ng/mL[4]										
Control	249910±26681	235248±16910	192412±14109	203169±15047	-4825Ac	-57157Aa	-47380Ab	0.619	<0.001	0.001
HCLP	276237±45083	261632±26731	243326±25138	232666±25006	-2200Ab	-18840Aa	-26866Aa			

Control, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet.

[1]Values expressed as mean ± standard of the error of the mean.

[2]Values expressed as delta values.

[3]GEE (Generalized Estimating Equations) analysis with normal distribution.

[4]GEE (Generalized Estimating Equations) analysis with gamma distribution for transformed variables.

[a,b,c]Comparisons of days in each treatment in the same row with different lowercase letters are significantly different by Bonferroni post-hoc test (P<0.05).

[A,B]Comparisons of treatments on each day in the same row with different capital letters are significantly different by Bonferroni post-hoc test (P<0.05).

https://doi.org/10.1371/journal.pone.0271932.t003

Index (HCLP = 5.56 ± 0.27; Control = 5.38 ± 0.59) (Fig 4C) were not affected by the dietary treatments (P = 0.70). Principal Coordinate Analysis (PCoA) based on the Bray-Curtis and D_0 UniFrac distance metrics, the HCLP dogs presented a clustering effect demonstrating a higher similarity degree (Fig 5A and 5B, respectively) differently from those belonging to the control group.

Discussion

In this study, we replaced the traditional sources of animal protein used in pet food, poultry by-product and bovine meat and bone meals, with a source of hydrolyzed chicken liver powder and observed the effects on CBC, immunological, and fecal microbiota of healthy dogs.

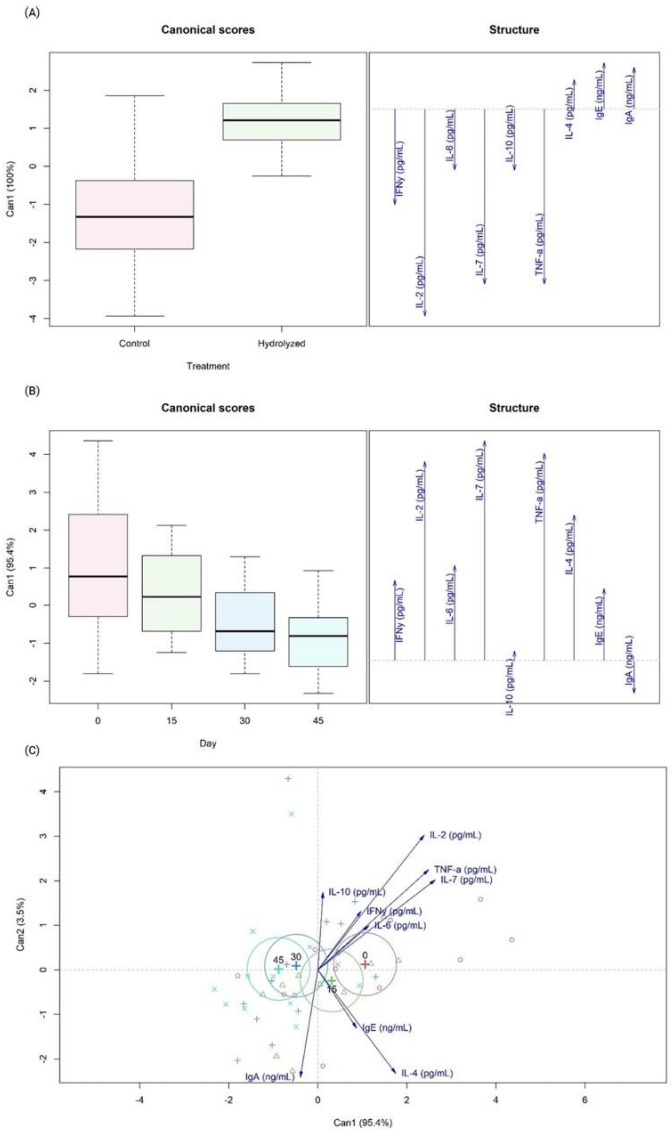

Fig 2. Canonical correlation of dietary treatments (A), days of blood collection (B) and two-dimensional canonical correlation of days of blood collection (C) for plasmatic cytokines and immunoglobulins from healthy Beagle dogs fed the experimental diets.

https://doi.org/10.1371/journal.pone.0271932.g002

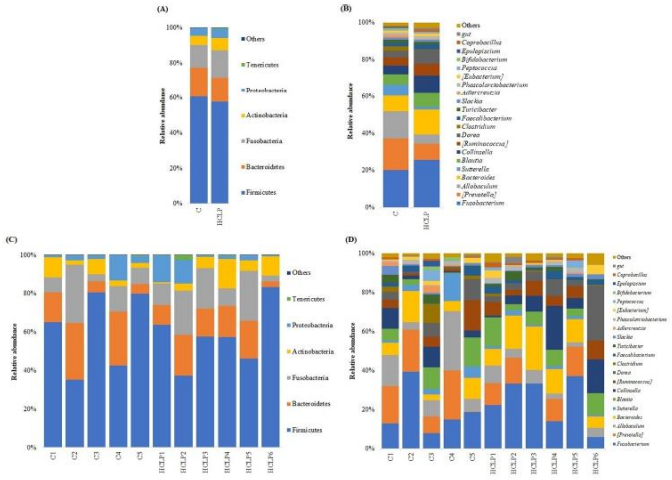

Fig 3. Relative abundances of phylum (A for treatments and C for individuals) and genera (B for treatments and D for individuals) based on 16S sequencing analysis of fecal samples from healthy Beagle dogs after 45 days consuming the experimental diets. Results are displayed for 11 dogs, as 1 dog on the control diet was removed due to the low number of reads. Others: Bacteria taxa with < 1% abundance. C, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet.

https://doi.org/10.1371/journal.pone.0271932.g003

Since dietary management of dogs diagnosed with AFR and chronic enteropathies generally includes hypoallergenic diets based on low-molecular weight hydrolyzed proteins to reduce intestinal antigenic stimulation [1, 18], we hypothesized that healthy, immunotolerant dogs fed a hydrolyzed chicken liver diet would not develop strong disturbances in the markers assessed during a period of 45 days.

Most hematological biomarkers were modified throughout the study, but all remained within the reference ranges for healthy adult dogs [19] and the colony norms. According to Sasaki et al. [20], the production of erythropoietin increases as the amount of protein in the diet increases. Based on this, we assume that both diets provided adequate amounts of protein, allowing an increase in erythropoiesis and, consequently, in the erythrocyte, hemoglobin and

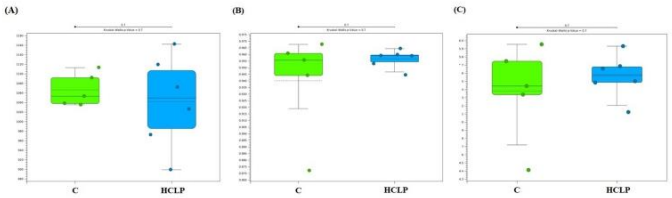

Fig 4. Bacterial alpha diversity indices of canine fecal samples assessed by the observed operational taxonomic units (OTU) (A), Simpson Index (B) and Shannon Index (C). C, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet.

https://doi.org/10.1371/journal.pone.0271932.g004

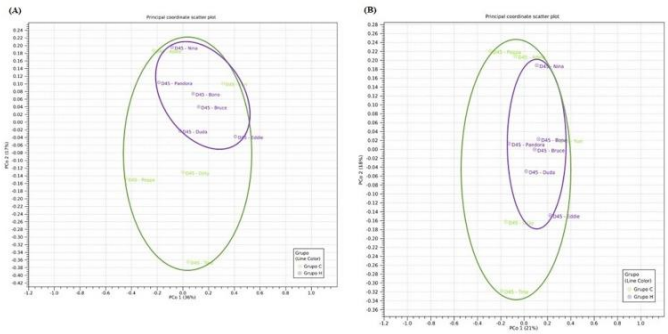

Fig 5. Principal Coordinate Analysis (PCoA) plots of Bray-Curtis (A) and D_0.5 UniFrac (B) distances of fecal samples. Treatments are represented as: Control in green, and HCLP in purple. Control, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet.

https://doi.org/10.1371/journal.pone.0271932.g005

hematocrit count, verified in both treatments over time. Although there were no significant differences in these markers between dietary treatments, it was possible to observe a greater increase from day 15 onwards in dogs fed the HCLP diet.

Eosinophil counts were affected by both dietary treatments and time, with a progressive reduction throughout the study. However, the HCLP diet promoted a greater and sustained reduction after 45 days of consumption. The increased concentration of eosinophils reflects an immune response to parasites and more recently with some specific cases of diet-related gastrointestinal disorders in dogs [21, 22]. Animals with chronic enteropathies may respond to a single dietary management based on the use of single novel protein or hydrolyzed protein diets, or to a combination of dietary intervention with antibiotics or immunosuppressive agents [23]. The reduction in eosinophil count in dogs fed HCLP diet at day 45 suggests that a beneficial impact on CBC is occurring. As eosinophils are related to the allergic process, the reduction in their counts may be related to the positive impact of the diet. Furthermore, other biomarkers related to immune responses such as leukocytes, neutrophils, and monocytes, were decreased over time in both dietary treatments, which may corroborate the positive impact of HCLP diet consumption in the long term.

Regarding the immunological response, all dogs had been naturally sensitized to poultry by-product meal and bovine meat and bone meal prior to this study, as both ingredients are commonly present in commercial diets for dogs. However, none of them consumed HCLP previously, so if a dog developed a response to HCLP it would be related mainly to this ingredient.

Anti-inflammatory Th2 cytokine, IL-4, was reduced from day 15 to the end of the experimental period in both treatments. IL-4 is one of the signature cytokines involved in inflammatory responses triggered by allergens or parasites [24]. The presence of food allergens in the intestinal mucosa activates antigen-presenting cells (APC) that signal Th2 lymphocytes to secrete cytokines, including IL-4, which stimulate the production of IgE. In addition, APC secrete pro-inflammatory mediators that induce epithelial cells to produce chemokines that attract eosinophils [25]. Thus, the reduction in IL-4 over 45 days in this study may be correlated with the decrease in eosinophil and IgE concentrations. Interestingly, previous studies reported an increase in IL-4 concentrations in humans and mice affected by food-induced

gastrointestinal disorders [4, 5]. Additionally, recent reports did not find detectable amounts of IL-4 using ex-vivo whole blood stimulation with commercial hydrolyzed protein diets in dogs with chronic enteropathy and healthy immunotolerant cats [26, 27].

Although plasma IgA was reduced on day 30 in dogs fed the control diet, at the end of the experimental period there was no difference between groups. Similarly, Verlinden et al. [2] also did not observe differences on total serum IgA between dogs fed diets containing hydrolyzed or intact protein. Indeed, some researchers demonstrated that serum IgA concentrations poorly correlates with secretory IgA, the most predominant secretory antibody at intestinal mucosa that protect against microorganisms, allergens, and other substances to adhere and pass-through enterocytes [28, 29]. In this way, future studies should measure secretory IgA to provide a better understanding between dietary treatments and their interference in the immune status of the intestinal mucosa.

IgE concentrations were measured as IgE-mediated reactions are the most recognized category of food allergies. Total plasma IgE concentrations were decreased from day 30 onwards in the control group, while the reduction in the HCLP group was observed from day 15 onwards. Consumption of hydrolyzed diets, containing smaller peptides, rich in free amino acids and low-molecular weight proteins, may reduce the cross binding between two molecules on the surface of the mast cell, thus preventing mast cell degranulation and cytokines release [30]. Verlinden et al. [2] did not observe differences on total serum IgE concentrations of healthy adult dogs fed hydrolyzed protein or intact protein diets. The authors evaluated the total serum IgE only after 24 days of feeding and did not analyze levels at the beginning of the trial, which may have indicated possible changes in this immunoglobulin during the study. Apart from healthy individuals, previous studies on sensitized dogs challenged with hydrolyzed protein diets have shown positive results. Soy-sensitized dogs did not present cutaneous or gastrointestinal reactions after being fed with hydrolyzed soy protein diet [6]. On a different approach, Olivry et al. [7] evaluating the recognition of several hydrolyzed poultry extracts on sera from dogs and cats with elevated chicken-specific serum IgE, noted that only extensively hydrolyzed poultry feather prevented the IgE recognition by poultry-specific IgE. Though the most known food allergens come from animal sources of protein [1], some grains may also have potential protein allergens, triggering an IgE-mediated response in dogs and cats [31]. Reports by Olivry and Bexley [32] show that serum from corn-sensitized dogs and cats did not have measurable IgE against proteins isolated from cornstarch, while kernel and flour extracts promoted IgE recognition. Therefore, the combination of hydrolyzed proteins and carbohydrate sources with limited protein content and proteome are highly recommended for hypoallergenic diets.

The 16S rRNA gene sequencing did not show differences in fecal communities between dogs fed hydrolyzed or common protein source diet. Although no differences were observed for fecal bacterial phyla between groups, the relative abundance verified for the HCLP group is in accordance with a previous study by Martínez-López et al. [33], in which healthy adult dogs also fed a hydrolyzed chicken liver diet for 6 weeks showed Firmicutes (median of 55%), Fusobacteria (median of 17%) and Bacteroidetes (median of 16%) as the most abundant bacterial phyla. Similarly, Bresciani et al. [34] did not find changes on fecal microbiota of healthy dogs after 60 days of an animal protein-free diet consumption. Interestingly, the fecal microbiota of healthy dogs is co-dominated by these three phyla: Firmicutes, Fusobacteria and Bacteroidetes [35, 36].

Fusobacterium, the most predominant genera in both groups, is associated with health in dogs [37] and can be used as a therapeutic marker for selected ingredients since it is reported to be reduced in dogs with gastrointestinal diseases [38]. Also, some *Fusobacterium* species are associated with SCFA synthesis from protein sources [39].

Although we did not evaluate end-fermentation products, a higher abundance of genera related to SCFA production were verified. Followed by *Fusobacterium*, dogs fed both dietary treatments showed a high abundance of *Bacteroides*, [*Prevotella*] and *Blautia*, genera associated with SCFA production. Among SCFA, butyrate is widely recognized by its preferred role as energy source for colonocytes, thus improving gut health [40].

Alpha diversity was not affected by dietary treatments, but PCoA plots based on Bray-Curtis and D_0.5 UniFrac distance metrics showed a clear separation between control group and HCLP group, which was also verified previously in dogs fed hydrolyzed and anallergenic diets [33, 41].

Additionally, the 16S rRNA sequencing evidenced the pronounced variability among dogs within the same dietary treatment in both phyla and genera relative composition, as previously described by Garcia-Mazcorro et al. [42]. The authors also found that the microbiome is relatively stable after a period of 2 weeks, so our feeding program was long enough to stabilize the gut microbiome.

In contrast to different types of fibers widely recognized for their prebiotic impact on microbiota composition and fermentative end-products related to gut health [43–45], some authors suggest that hydrolyzed diets affect the canine microbiome on a functional level instead of a taxonomic [33]. Perhaps a metabolomic approach could be more specific for identifying possible modifications in the HCLP group.

In general, the microbiome composition of dogs tends to be more affected by macronutrient modifications than by the ingredient itself. A recent study verified that dogs fed diets with similar macronutrient contents, one based on vegetable proteins and the other with mixed animal and vegetable proteins, did not showed differences in the microbiome composition [34]. This study could explain the absence of significant differences in our study, as both control and HCLP diet were formulated to be isonutritive and the main difference was the animal protein ingredient.

Overall, the present study showed that a diet based on HCLP was able to maintain all hematological markers within the reference intervals for adult dogs. Furthermore, 45 days of feeding with both diets decreased the concentrations of IL-4, IL-6, IL-7, IgA and IgE, cytokines and immunoglobulins related to the allergic response. Future studies are needed to evaluate different levels of the hydrolyzed protein and, more specifically, the end fermentation products that could be improved by this ingredient, since a higher abundance of specific genera related to SCFA production was verified in HCLP dogs.

Supporting information

S1 File. Full data set. Control, poultry by-product + bovine meat and bone meals-based diet; HCLP, hydrolyzed chicken liver powder-based diet.
(XLSX)

Acknowledgments

The authors are thankful to Nutribarrasul, Brasil, for the diets extrusion.

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Supervision: Luciano Trevizan.

Validation: Caroline Fredrich Dourado Pinto.

Visualization: Caroline Fredrich Dourado Pinto.

Writing – original draft: Caroline Fredrich Dourado Pinto.

Writing – review & editing: Caroline Fredrich Dourado Pinto, Marcelino Bortolo, Ryan Guldenpfennig, Fábio Ritter Marx, Luciano Trevizan.

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CAPÍTULO VI

CONSIDERAÇÕES FINAIS

As proteínas hidrolisadas têm sido demandadas pela indústria de alimentos para animais de companhia para a formulação de alimentos coadjuvantes. O diagnóstico das doenças gastrointestinais e reações adversas alimentares requerem dietas especiais, com ingredientes diferenciados, que passem pelo trato gastrointestinal sem acionar os mecanismos inflamatórios presentes nesta via. Associados a estas patologias, diversos distúrbios gastrointestinais requerem alimentos com alta digestibilidade, que sejam palatáveis como formas coadjuvantes no tratamento clínico. Estas características são potenciais dos ingredientes hidrolisados proteicos, que conferem às dietas elevado conteúdo de peptídeos de baixo peso molecular, relacionados com a reduzida antigenicidade e consequente alergenicidade.

A primeira série experimental possibilitou a investigação aprofundada da composição química de uma proteína hidrolisada comercial, o hidrolisado de fígado de aves. O ingrediente avaliado apresentou altas concentrações de proteína e gordura, além do aminoácido lisina e ácidos graxos linoleico e araquidônico. Tais características evidenciam o alto valor nutricional do ingrediente, potencialmente possibilitando a redução na utilização de fontes purificadas dos nutrientes acima mencionados em formulações contendo o hidrolisado de fígado de aves. O hidrolisado de fígado de aves apresentou ainda 57% dos peptídeos com peso molecular <10 kDa, comparado a duas fontes proteicas tradicionais a farinha de vísceras de aves e farinha de carne e ossos (41% e 35% dos peptídeos com peso molecular <10 kDa, respectivamente). O elevado conteúdo de peptídeos de baixo peso molecular é um dos fatores primordiais na seleção de proteínas hidrolisadas para inclusão em dietas para animais com distúrbios gastrointestinais e reações adversas alimentares. Assim, o principal fator limitante do presente estudo pode ser atribuído a avaliação do hidrolisado de fígado de aves como fonte proteica animal apenas em animais saudáveis, sem sintomas de alergias alimentares e doenças gastrointestinais. Embora os achados nas concentrações plasmáticas de citocinas e imunoglobulinas sejam de extrema importância, os resultados não são necessariamente aplicáveis a população de cães com os distúrbios previamente mencionados. Diante dessa limitação, estudos posteriores poderiam investigar a antigenicidade e alergenicidade do hidrolisado de

fígado de aves em cães sensibilizados, para atestar a eficácia e inocuidade do ingrediente no suporte nutricional.

Quanto ao impacto do hidrolisado de fígado de aves sobre as características fecais, os resultados obtidos nas duas séries experimentais demonstraram que apesar do acréscimo na umidade fecal o ingrediente não promoveu diarreia nos cães quando incluído até 31% na formulação. Tal achado foi relevante devido a resultados prévios obtidos em cães alimentados com dietas comerciais contendo proteínas hidrolisadas. Ainda a nível intestinal, durante a primeira série experimental não foram observadas alterações significativas na composição da microbiota intestinal dos cães alimentados com a dieta contendo o hidrolisado de fígado de aves. Entretanto, é importante considerar que o ingrediente possa promover alterações a nível metabólico, que poderia ser acessado através da análise metabolômica. Adicionalmente, e ainda de encontro com a principal limitação do estudo, referente a falta da avaliação do hidrolisado de fígado de aves em cães com distúrbios gastrointestinais e reações adversas alimentares, é possível que o uso do ingrediente possa resultar em modificações benéficas na estrutura e funcionalidade da microbiota intestinal de cães sensibilizados. Esta pressuposição parte da digestão facilitada atribuída às proteínas hidrolisadas, ponto a ser considerado em cães com distúrbios digestivos e absortivos. Desta forma, a inclusão do hidrolisado de fígado de aves poderia auxiliar na redução do conteúdo não digerido no intestino delgado, o qual é encaminhado e fermentado no intestino grosso. Por fim, há de se considerar o elevado custo das proteínas hidrolisadas comparado às principais fontes proteicas tradicionais. Assim, a sua inclusão pode ser justificável apenas para algumas categorias, como cães com comprometimentos gastrointestinais.

As elevadas concentrações de aminoácidos livres nas proteínas hidrolisadas podem representar um risco à qualidade das matérias-primas devido a formação de aminas biogênicas. A síntese destes compostos depende da disponibilidade de aminoácidos livres e presença de microrganismos com atividade da enzima descarboxilase. Em humanos, o consumo elevado de algumas aminas biogênicas promove vasodilatação, hipertensão, crescimento e proliferação celular, além de sintomas associados a reações alérgicas. Apesar da relevância para a segurança alimentar, não existem informações a respeito das concentrações máximas de consumo de aminas biogênicas associadas a efeitos tóxicos em cães. Com base nos

resultados obtidos na segunda série experimental, os cães alimentados com as dietas contendo farinha de vísceras de aves consumiram maiores concentrações de aminas biogênicas putrefativas, como putrescina, cadaverina e histamina. Entretanto, não houve diferença na excreção fecal e no balanço de aminas biogênicas, calculado pela diferença da quantidade consumida e excretada, entre a farinha de vísceras de aves e o hidrolisado de fígado de aves. Este achado reforça a elevada eficiência das enzimas detoxificantes MAO e DAO presentes na mucosa do epitélio intestinal, podendo estar relacionado ao hábito alimentar ancestral de cães que consistia, eventualmente, de carcaças em diferentes estágios de putrefação. Diante da presença de aminas biogênicas nas matérias-primas, particularmente nos coprodutos cárneos, por se tratar de compostos termoestáveis, e da falta de informações a respeito dos limites tóxicos de consumo e efeitos metabólicos a longo prazo, torna-se indispensável a realização de novas pesquisas direcionadas ao estudo das aminas biogênicas em cães.

Além dos benefícios nutricionais, o processo de hidrólise de proteínas pode gerar peptídeos bioativos com potencial atividade antimicrobiana, antioxidante, anti-hipertensiva e imunomoduladora, garantindo resultados positivos em parâmetros de desempenho, como crescimento e eficiência alimentar, em animais de produção. Entretanto, os estudos voltados a avaliação dos efeitos de peptídeos bioativos nos animais de companhia são escassos, mas prometem otimização da nutrição, saúde e bem-estar.

Por último, foi constatado que as dietas experimentais contendo o hidrolisado de fígado de aves em altas concentrações (acima de 25%), resultou em retenção da massa no canhão da extrusora, com a parada do processo para ajuste nas configurações do equipamento. Assim, estudos futuros poderiam avaliar diferentes concentrações de inclusão do ingrediente sobre a extrusão mediante coleta de dados de parâmetros do processo.

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APÊNDICES

Apêndice A – Carta de aprovação do Comitê de ética no uso de animais

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 36138
Título: COMPORTAMENTO INGESTIVO E DE SACIEDADE EM SUÍNOS E CÃES

Vigência: 01/01/2019 à 01/01/2022

Pesquisadores:

Equipe UFRGS:

LUCIANO TREVIZAN - coordenador desde 01/01/2019
PEDRO HENRIQUE SESSEGOLO FERZOLA - Outra Função desde 01/01/2019
Bruna Cristina Kuhn Gomes - Aluno de Doutorado desde 01/01/2019
Caroline Fredrich Dourado Pinto - Aluno de Mestrado desde 01/01/2019
Aline Kummer de Souza - Aluno de Mestrado desde 01/01/2019

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 14/01/2019 - Sala 330 do anexo I do prédio da Reitoria, Campus Centro. , em seus aspectos éticos e metodológicos, para a utilização de provenientes de 12 cães da raça Beagle, seis machos e seis fêmeas com 10 a 14 Kg e idade entre 4 e 5 anos, provenientes do canil experimental da Faculdade de Agronomia da UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 8 de Fevereiro de 2019

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Vice Coordenador da comissão de ética

1

Apêndice B – Carta de aprovação do Comitê de ética no uso de animais

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 40920
Título: Nível de proteína na dieta e seu impacto sobre a fermentação no cólon e formação de aminas biogênicas em cães

Vigência: 01/08/2021 à 31/08/2023

Pesquisadores:

Equipe UFRGS:

LUCIANO TREVIZAN - coordenador desde 01/08/2021
Camila Figueiredo Carneiro Monteiro - desde 01/08/2021
JÉSSICA FERREIRA BARCELLOS - desde 01/08/2021
GIOVANE KREBS - desde 01/08/2021
Caroline Fredrich Dourado Pinto - desde 01/08/2021
BIANCA BRUM DE OLIVEIRA - zzz Outra Função zzz desde 01/08/2021
MATHEUS NUNES PERES - zzz Outra Função zzz desde 01/08/2021
ARIANE MIRANDA DA SILVA - zzz Outra Função zzz desde 01/08/2021

Comissão De Ética No Uso De Animais aprovou o mesmo, em reunião realizada em 26/07/2021 - Reunião remota via portal Mconf, em seus aspectos éticos e metodológicos, para a utilização de 12 cães (machos e fêmeas, com peso entre 6 -12 kg) provenientes do canil Champions Line Beagle (CNPJ:304.795.330-91), de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 5 de Agosto de 2021

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Coordenador da comissão de ética

1

Apêndice C – Normas para redigir os capítulos II e V – Publicação no periódico Italian Journal of Animal Science

Taylor & Francis Word Template for journal articles

Author Name[a]* and A. N. Author[b]

[a]*Department, University, City, Country;* [b]*Department, University, City, Country*

Provide full correspondence details here including e-mail for the *corresponding author

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Repeat the title of your article here

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Keywords: word; another word; lower case except names

Subject classification codes: include these here if the journal requires them

Heading 1: use this style for level one headings

Paragraph: use this for the first paragraph in a section, or to continue after an extract.

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Display quotations of over 40 words, or as needed.

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Apêndice C – Normas para redigir o capítulo III – Publicação no periódico PLOS One

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Symbol Legend		
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John Doe[1¶], Antonie Data[1¶], Johannes van Stats[1,#a], Marie Testperson[2*], David Ribosome Jr.[3,4], Gregory H.T. McBio[5,#b], Angela Reviewerson[1,2&], Marina Measure[1&], on behalf of The Bunny Genome Sequencing Consortium[^]

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[1] Department, Institution, City, State, Country

[2] Department of Dermatology, Division of Rabbit Health, Section of Veterinary Medicine, St. Hare Hospital, San Francisco, California, United States of America

[3] Department of Libraries and Archives, National Contemporary Bunny Museum, Lagomorph, Connecticut, United States of America

[4] Department of Restoration, National Contemporary Bunny Museum, Lagomorph, Connecticut, United States of America

[5] Department of Archaeology, Bunny University, Lagomorph, Connecticut, United States of America

[#a]Current Address: Department of Carrot Science, Bunny University, Lagomorph, Connecticut, United States of America

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[¶]These authors contributed equally to this work.

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Abstract

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Level 1 Heading

- Use Level 1 heading for all major sections (Abstract, Introduction, Materials and methods, Results, Discussion, etc.).
- Bold type, 18pt font.
- Only use italics and text formatting where needed (e.g. genus and species names, genes, etc.).
- Headings should be written in sentence case (capitalize only the first word of the heading, the first word of the subheading, and any proper nouns and genus names).

NOTE: Do not cite figures, tables, supporting information, or references in the Abstract.

Introduction

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Level 1 heading

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Figure Citations

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- Cite multiple figures as "Figs 1 and 2", "Figs 1-3", etc.

Fig 1. This is the Fig 1 Title. This is the Fig 1 legend.

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- Use bold type for the figure titles.

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- Figure files should be saved as "Fig1.tif", "Fig2.eps", etc.
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- Figures should be uploaded separately as individual files.

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$$p^2 + 2pq + q^2 = 1 \quad (1)$$

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Level 2 heading

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Level 1 heading

Lorem ipsum dolor sit amet, consectetur adipiscing elit. Vestibulum adipiscing urna ut lectus gravida, et bland Table 1 Donec tincidunt porta sem nec hendrerit. Vestibulum nec pharetra quam, vitae convalli. Fido nemo.

Table 1. This is the Table 1 Title.

	Chemical W	Chemical X	Chemical Y	Chemical Z
Chemical 1	Reaction 1W	Reaction 1X	Reaction 1Y	Reaction 1Z
Chemical 2	Reaction 2W	Reaction 2X	Reaction 2Y	Reaction 2Z
Chemical 3	Reaction 3W[a]	Reaction 3X	Reaction 3Y[b]	Reaction 3Z
Chemical 4	Reaction 4W	Reaction 4X	Reaction 4Y	Reaction 4Z
Chemical 5	Reaction 5W	Reaction 5X	Reaction 5Y	Reaction 5Z

This is the Table 1 legend.
[a]Table footnotes belong here.
[b]Footnotes should have corresponding symbols in the table.

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- Cite multiple tables as "Tables 1 and 2", "Tables 1-3", etc.
- Tables should be included directly after the paragraph in which they are first cited.
- Tables must be cell-based in Microsoft Word or embedded with Microsoft Excel.
- Do not use empty rows to create spacing.
- Do not include graphic objects, images, or colored text.

Conclusion

Lorem ipsum dolor sit amet, consectetur adipiscing [1-5]. Vestibulum adipiscing urna ut lectus gravida, vitae blandit tortor interdum. Donec tincidunt porta sem nec hendrerit. Vestibulum nec pharetra quam, vitae convallis nunc. Mauris in mattis sapien. Fusce sodales vulputate auctor S1 Fig. Dolor sit amet S1 and S2 Tables.

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Acknowledgments

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Acknowledgments

- Do not include funding or competing interests information in Acknowledgments.

References

1. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr, McBio GHT, et al. This is the article title. PLoS ONE. 2017;12(12):e00000. doi: 10.1371/journal.pone.0000000
2. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr, McBio GHT, et al. Bunny dynamics in cartoon landscapes. PLoS ONE. Forthcoming 2017.

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S1 Table. This is the S1 Table Title. This is the S1 Table legend.

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Apêndice D – Normas para redigir o capítulo IV – Publicação no periódico Journal of Animal Science

Appeals

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JAS Ethics Policy
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biological sex.

- The hierarchy for brackets and parentheses is [( )]. For example, [(2 + 3) × (12 ÷ 2)] × 2 = 60.
- Meat shear force should be expressed in kilograms (kg), although newtons (N) may also be acceptable.
- Report time using the 24-h system (e.g., 1410 h rather than 2:10 p.m.).
- Use italics to designate genus and species.
- Names of muscles are not italicized.
- Specify the basis (i.e., as-fed or dry matter) for dietary ingredient and chemical composition data listed in text or in tables. Similarly, specify the basis for tissue composition data (e.g., wet or dry basis).
- Calculations of efficiency should be expressed as output divided by input (i.e., gain:feed, not feed:gain).
- A diet is a feedstuff or a mixture of feedstuffs; a ration is the daily allotment of the diet.
- The word "Table" is capitalized and never abbreviated.
- Except to begin a sentence, the word "Figure" should be abbreviated to "Fig."
- Except to begin a sentence, experiment and equation should be abbreviated to Exp. And Eq., respectively, when preceding a numeral (e.g., Exp. 1).
- Avoid jargon unfamiliar to scientists from other disciplines. Do not use the term "head" to refer to an animal or group of animals. Instead, use animal, sow, ewe, steer, heifer, cattle, etc.
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Tables and figures should be placed at the end of the manuscript, following the references. Tables and figures should be numbered consecutively, in the order in which they are cited in the manuscript. Tables and figures must be prepared so they can be understood without referring to information in the body of the manuscript.

Tables

1. Tables can be created in in Word using the Table function.
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3. Each table should be placed on a separate page. Tables should fit on a single 8.5 X 11-inch page in either landscape or portrait view.
4. Every column should include a heading.
5. Align column values to the decimal point whenever possible. Columns containing a mix of values, symbols and words may be aligned to the center of the heading. Columns using ± should be aligned to the symbol.
6. Units (e.g., kg) are separated from descriptor by a comma.
7. Numerals are used to reference footnotes. Each footnote should begin on a new line immediately below the table.
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9. The order of footnotes below the table is numbers first followed by letters and special symbols.
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Additional Usage Notes

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A clear justification for conducting the research with a stated hypothesis and objective(s) is required. The rationale for the experiments should place the work into the context of existing literature. There is NO word limit on the section but brevity is encouraged.

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The American Society of Animal Science (ASAS) supports rigor, reproducibility and transparency in science and seeks to ensure that publications of the society reflect these values while also minimizing the burden on authors in preparation of scientific results for publication. There are many available resources describing principles and practices to enhance rigor, reproducibility, and transparency in science. Authors considering the Journal of Animal Science are encouraged to consult these resources when during preparation of their submissions.

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Results

Experimental results are presented in tables and figures. The results should contain sufficient detail to allow the reader to interpret the data. Quantitative measures of significance (*P*-values) should be presented. Authors may use either absolute *P*-values or a defined significance level as long as usage is consistent.

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The section contains the interpretation of the results. It should be clear and concise, address the biological mechanisms and their significance, and integrate the results into existing literature. The Discussion may offer an interpretation that is consistent with the data. Do NOT include any reference to tables and figures or include *P*-values in the Discussion. Authors have the option to create a single RESULTS AND DISCUSSION section.

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Journal articles

Perez, V. G., A. M. Waguespark, T. D. Bidner, L. L. Southern, T. M. Fakler, T. L. Ward, M. Steidinger, and J. E. Pettigrew. 2011. Additivity of effects from dietary copper and zinc on growth performance and fecal microbiotia of pigs after weaning. J. Anim. Sci. 89:414–425. doi:10.2527/jas.2010-2839.

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Robinson, P. H., E. K. Okine, and J. J. Kennelly. 1992. Measurement of protein digestion in ruminants. In: S. Nissen, editor, Modern methods in protein nutrition and metabolism. Academic Press, San Diego, CA. p. 121–127.

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Bailey, E. A., J. R. Jaeger, J. W. Waggoner, G. W. Preedy, L. A. Pacheco, and K. C. Olson. 2012. Effect of weaning method on welfare and performance of beef calves during receiving. Proc. West. Sec. Amer. Soc. Anim. Sci. 63:25–29.

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VITA

Caroline Fredrich Dourado Pinto, filha de Mirla Luiza Fredrich Dourado e Paulo César Flores Pinto, nasceu em 21 de março de 1993, Porto Alegre, Rio Grande do Sul.

Ingressou no curso de Zootecnia da Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rio de Janeiro, no segundo semestre de 2011.

Ao longo da graduação foi bolsista de Apoio Técnico na Comissão de Bem-Estar Animal. Foi monitora das disciplinas Reconhecimento de Plantas Forrageiras no período 07/2013 a 03/2014 sob orientação do professor Mauro Portela Piña Rodrigues, e Fisiologia Animal no período 05/2014 a 07/2014 sob orientação da professora Dra. Magda Alves de Medeiros. Foi bolsista de Iniciação Científica PIBIC/CNPq – UFRRJ no período de 08/2014 a 07/2015, sob orientação da professora Dra. Magda Alves de Medeiros no projeto “Efeito do 17β-estradiol no modelo de lesão medular por compressão em ratos” na área de Fisiologia Animal. Foi monitora da disciplina Nutrição Animal no período 08/2015 a 02/2017 sob orientação do professor Dr. Vinicius Pimentel Silva, onde realizou atividades no Laboratório de Bromatologia Animal do Instituto de Zootecnia da UFRRJ.

Em abril de 2017 deu início ao curso de Mestrado do Programa de Pós-Graduação em Zootecnia da Faculdade de Agronomia da Universidade Federal do Rio Grande do Sul (UFRGS), na área de Produção Animal, com ênfase em Nutrição de Cães e Gatos, sob orientação do professor Dr. Luciano Trevizan. No ano de 2019 defendeu a dissertação intitulada “Avaliação de ossos autoclavados sobre a remoção de cálculo dentário, impacto sobre o periodonto e esmalte dentário de cães adultos”, da qual foram publicados dois artigos científicos: “Evaluation of teeth injuries in Beagle dogs caused by autoclaved beef bones used as a chewing item to remove dental calculus” (PLoS One, 2020) e “Short-term changes in the oral microbiota of dogs after chewing different types of autoclaved bones” (Veterinarski Arhiv, 2021). No mesmo ano deu início ao curso de Doutorado pelo mesmo Programa de Pós-Graduação e sob a orientação do professor Dr. Luciano Trevizan.