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# Novel functional ingredients in pet food – a multifactorial study of alternative diets for dogs

Joana Guilherme-Fernandes

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Tese de Candidatura ao grau de Doutor em Ciência Animal, Especialidade em Nutrição, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

**Orientadora**: Ana Rita Jordão Bentes Cabrita
Categoria: Professora Associada
Afiliação: LAQV, REQUIMTE, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

**Coorientadora**: Margarida Rosa Garcez Maia
Categoria: Professora Auxiliar
Afiliação: LEAF, TERRA, Instituto Superior de Agronomia, Universidade de Lisboa

**Coorientadora**: Sofia Antunes Costa Lima
Categoria: Investigadora
Afiliação: LAQV, REQUIMTE, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

Joana Maria Guilherme Martins Fernandes

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Thesis for applying to a Doctor degree in Animal Science, Specialty in Nutrition, submitted to the School of Medicine and Biomedical Sciences of the University of Porto

**Supervisor**: Ana Rita Jordão Bentes Cabrita
Category: Associate Professor
Affiliation: LAQV, REQUIMTE, School of Medicine and Biomedical Sciences, University of Porto

**Co-supervisor**: Margarida Rosa Garcez Maia
Category: Assistant Professor
Affiliation: LEAF, TERRA, School of Agriculture, University of Lisbon

**Co-supervisor**: Sofia Antunes Costa Lima
Category: Researcher
Affiliation: LAQV, REQUIMTE, School of Medicine and Biomedical Sciences, University of Porto

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# Legal directives and dissemination

In compliance with the provisions of Decree-Law No. 204/2018 of October 23, it is hereby declared that the author of this thesis participated in the conception and execution of the experimental work that led to the presented results, as well as in their interpretation and the writing of the respective manuscripts.

This thesis includes two scientific articles published and one submitted in international peer review journals, presenting and discussing the results of the experimental work, referenced to as:

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“Knowledge alone cannot give rise to value. It is only when knowledge is guided by wisdom that value is created. The font of wisdom is found in the following elements: an overarching sense of purpose, a powerful sense of responsibility and, finally, the compassionate desire to contribute to the welfare of humankind.”

Daisaku Ikeda, 2006
The University of the 21st Century - Cradle of World Citizens

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# List of abbreviations and acronyms

| | |
|---|---|
| AA | Amino acids |
| AAS | Amino acid score |
| ABTS | 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation |
| APPA | The American Pet Products Association |
| ASV | Amplicon sequence variants |
| ATTD | Apparent total tract digestibility |
| AVMA | American Veterinary Medical Association |
| BCS | Body condition score |
| BW | Body weight |
| CC BY 4.0 | Creative Commons Attribution 4.0 International license |
| CC BY NC ND | Creative Commons Attribution-Non Commercial-No Derivatives license |
| CD | Cluster of differentiation |
| ConA | Concanavalin A |
| CP | Crude protein |
| CTF | Compositional Tensor Factorization |
| DHA | Docosahexaenoic acid |
| DM | Dry matter |
| DPPH | Scavenging activity of 2,2-diphenyl-1-picrylhydrazyl radical |
| EE | Ether extract |
| EPA | Eicosapentaenoic acid |
| FAO | Food and Agriculture Organization |
| FBS | Fetal Bovine Serum |
| FC | Folin-Ciocalteu reducing capacity |
| FELASA | Federation of European Laboratory Animal Science Associations |
| FRAP | Ferric reducing antioxidant power |
| FVD | Fixable Viability Dye |
| GE | Gross energy |
| GHG | Greenhouse gases |
| GLP-1 | Glucagon-like peptide-1 |
| IFN-γ | Interferon-gamma |
| Ig | Immunoglobulin |
| IGF-I | Insulin-like growth factor I |
| IL | Interleukin |
| IMTA | Integrated multi-trophic aquaculture |

| | |
|---|---|
| LipL32 | Recombinant antigen from *Leptospira interrogans* |
| LME | Linear mixed-effects models |
| LSD | Least Significant Difference |
| MCP-1 | Monocyte chemoattractant protein-1 |
| ME | Metabolizable energy |
| NDF | Neutral detergent fiber |
| NEPA | The National Environmental Policy Act |
| NGF-β | Nerve growth factor-beta |
| OM | Organic matter |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffer saline |
| PCoA | Principal-coordinate analysis |
| PCR | Polymerase chain reaction |
| PMA | Phorbol myristate acetate |
| PUFA | Polyunsaturated fatty acids |
| Q | Quercetin |
| ROS | Reactive oxygen species |
| SCF | Stem cell factor |
| SD | Standard deviation |
| SDG | Sustainable Development Goal |
| SEM | Standard error of the mean |
| SH5 | Diet with 50 g $kg^{-1}$ inclusion of shrimp hydrolysate |
| SH10 | Diet with 100 g $kg^{-1}$ inclusion of shrimp hydrolysate |
| SH15 | Diet with 150 g $kg^{-1}$ inclusion of shrimp hydrolysate |
| SM5 | Diet with 50 g $kg^{-1}$ inclusion of squid meal |
| SM10 | Diet with 100 g $kg^{-1}$ inclusion of squid meal |
| SM15 | Diet with 150 g $kg^{-1}$ inclusion of squid meal |
| TNF-$\alpha$ | Tumor necrosis factor-alpha |
| VEGF-A | Vascular endothelial growth factor A |
| VFA | Volatile fatty acids |
| VSC | Volatile sulfur compounds |

# Resumo

A população global de animais de estimação tem vindo a crescer e, consequentemente, a produção industrial de alimentos compostos para cães e gatos enfrenta uma pressão significativa para encontrar fontes alimentares sustentáveis e de qualidade elevada. As fontes proteicas convencionais, principalmente as de origem animal, não só sobrecarregam os recursos ambientais, como também competem diretamente com os setores de alimentação humana e de animais de produção. Desta forma, a identificação de fontes proteicas alternativas é crucial para o desenvolvimento sustentável da indústria. Os coprodutos de animais aquáticos, incluindo aqueles provenientes de peixes e invertebrados, como moluscos e crustáceos, oferecem uma solução promissora, mais sustentável e com potencial nutritivo. Estes podem ajudar a reduzir o desperdício e contribuir para a economia circular, promovendo a sustentabilidade dos setores das pescas, da aquacultura e da alimentação de cães e gatos. Em aquacultura e pecuária, os coprodutos de animais aquáticos, incluindo os seus hidrolisados e péptidos bioativos, têm mostrado benefícios nutricionais e funcionais, nomeadamente na saúde intestinal e na resposta imunitária. No entanto, a falta de conhecimento sobre os seus efeitos em cães ainda é significativa. Esta lacuna apresenta uma oportunidade para investigar novas fontes proteicas que possam ser incorporadas em dietas para cães para promover a nutrição e a saúde, ao mesmo tempo que contribuem para a sustentabilidade do setor. O objetivo desta tese foi avaliar, pela primeira vez, o potencial de dois coprodutos aquáticos de invertebrados comercialmente disponíveis, farinha de lulas e hidrolisado de camarão, para serem incluídos em dietas para cães adultos. Especificamente, este trabalho teve como objetivo avaliar critérios relevantes para a inclusão de ingredientes em alimentos para cães, nomeadamente a palatabilidade, o valor nutritivo e parâmetros de saúde dos cães.

O Capítulo 1 destaca a importância dos cães na sociedade moderna e reconhece a crescente procura por soluções sustentáveis para a alimentação animal. Em resposta a esta procura, aborda-se o aumento da produção de aquacultura e como a utilização dos seus coprodutos pode contribuir para a sustentabilidade da indústria de alimentos para cães e gatos. É revisto o conhecimento científico sobre os efeitos dos coprodutos de aquacultura no crescimento, na eficiência alimentar, na resposta imunitária e na saúde intestinal em animais de produção e em animais de estimação, sendo os efeitos dependentes da espécie e dos níveis de inclusão na dieta. Com base na literatura descrita e na disponibilidade de coprodutos de aquacultura, no Capítulo 2, foram avaliadas duas fontes proteicas comercialmente disponíveis, farinha de lulas e

hidrolisado de camarão, nomeadamente quanto à sua composição química, propriedades antioxidantes, palatabilidade e digestibilidade. O hidrolisado de camarão exibiu atividade antioxidante superior à farinha de lulas. Ambas as fontes apresentaram elevado teor em proteína bruta (810 g $kg^{-1}$ de matéria seca (MS) e 658 g $kg^{-1}$ MS, respetivamente para a farinha de lulas e o hidrolisado de camarão), sendo, ainda, de destacar o seu elevado teor de metionina. A palatabilidade foi avaliada através de testes de preferência com 12 cães adultos saudáveis da raça *Beagle*, para comparar a dieta basal (uma dieta comercialmente disponível) sem e com a inclusão de cada uma das fontes proteicas a 15%. Não foram observadas diferenças significativas na primeira aproximação e na primeira prova, mas o rácio de consumo diminuiu com a inclusão de qualquer uma das fontes proteicas. De seguida, foram realizados dois ensaios de digestibilidade *in vivo* de acordo com dois quadrados latinos 3 x 3, com 6 animais (3 machos e 3 fêmeas), 3 períodos experimentais (10 dias cada) e 3 níveis de inclusão (5%, 10% e 15%) de farinha de lulas ou hidrolisado de camarão em substituição da dieta basal. Comparando com a dieta basal, as dietas com inclusão de farinha de lulas e hidrolisado de camarão melhoraram a digestibilidade dos nutrientes e da energia, a inclusão de farinha de lulas reduziu a concentração de butirato fecal, enquanto o hidrolisado de camarão aumentou os ácidos gordos voláteis fecais, exceto o butirato. A farinha de lulas não mostrou ter efeito na microbiota fecal, enquanto o hidrolisado de camarão afetou a abundância de Oscillosperaceae (UCG-005), Firmicutes e *Lactobacillus*, sugerindo ter um papel na modulação da microbiota intestinal. Os resultados indicam que a farinha de lulas e o hidrolisado de camarão poderão ser fontes proteicas inovadoras e promissoras para dietas para cães. Com base nos resultados obtidos, nos Capítulos 3 e 4, o hidrolisado de camarão foi selecionado para ser avaliado num ensaio de longa duração. Foram formuladas duas dietas completas extrudidas utilizando os mesmos ingredientes e quantidades, com exceção da inclusão de 5% de hidrolisado de camarão em substituição de glúten de trigo. A palatabilidade das dietas foi avaliada através de testes de preferência, realizados com 12 cães adultos saudáveis da raça *Beagle*. Seguidamente, foi realizado um ensaio de acordo com um delineamento em blocos completos casualizados com duração de 12 semanas, com os 12 cães distribuídos em 6 blocos, de acordo com o sexo e o peso corporal, e um cão de cada bloco foi aleatoriamente atribuído a uma das duas dietas. As recolhas foram realizadas nas semanas 0, 4, 8 e 12. Os resultados da palatabilidade da dieta, ingestão e digestibilidade, características, metabolitos e microbiota fecal, compostos sulfurosos voláteis orais e qualidade do pêlo estão descritos no Capítulo 3, e os parâmetros hematológicos e bioquímica sérica e função imunitária inata e adquirida no Capítulo 4. A inclusão de hidrolisado de camarão não afetou a palatabilidade, a ingestão e a

digestibilidade da dieta, e os compostos sulfurosos voláteis orais, mas aumentou a produção fecal (MS) e diminuiu a concentração de butirato fecal, enquanto aumentou a abundância de Oscillospiraceae e Clostridia, e diminuiu a abundância de *Sellimonas*, sugerindo potencial prebiótico do hidrolisado de camarão e o efeito na melhoria da digestibilidade de aminoácidos. A inclusão de hidrolisado de camarão reduziu os níveis de eosinófilos e de glicose no sangue, indicando o seu potencial para inclusão em dietas hipoalergénicas e para cães diabéticos. Adicionalmente, a inclusão do hidrolisado de camarão aumentou a concentração de leucócitos, plaquetas, neutrófilos, células T $CD4^+$ e $CD8^+$ que produzem fator de necrose tumoral-alfa, e a proliferação de células T $CD4^+$ em resposta ao antigénio recombinante de *Leptospira interrogans* (LipL32), enquanto reduziu a produção de superóxido em células estimuladas com acetato de miristato de forbol, sugerindo o seu papel imunomodulador. Finalmente, o hidrolisado de camarão promoveu uma boa qualidade do pêlo, destacando-se a melhoria na descamação nas semanas 4 e 12.

O conhecimento produzido no presente trabalho contribui para o desenvolvimento de estratégias alimentares que permitem ganhos de sustentabilidade e, simultaneamente, valor funcional com benefícios para a saúde dos cães, indo ao encontro das preocupações dos tutores. Estudos futuros devem focar-se nos efeitos da farinha de lulas e hidrolisado de camarão em níveis de inclusão mais elevados em dietas extrudidas, explorando o seu potencial como fontes proteicas exclusivas em dietas hipoalergénicas e para promover a sustentabilidade do setor, ao mesmo tempo que avaliam os seus efeitos nutritivos e na saúde dos cães em estudos de longa duração para observar os benefícios e a prolongação dos resultados fisiológicos. Além disso, são necessários mais estudos sobre a palatabilidade destes ingredientes, as propriedades antioxidantes do hidrolisado de camarão, as interações entre nutrientes e o processo de extrusão que podem afetar a digestibilidade, o impacto nos metabolitos e na microbiota fecal, os mecanismos subjacentes aos efeitos na função imunitária e no metabolismo da glicose, e os potenciais benefícios para cães com problemas de saúde, juntamente com o estudo sobre as perceções dos tutores a coprodutos de animais invertebrados e, finalmente, avaliações do ciclo de vida para entender as implicações ambientais destes coprodutos e melhorar a sua integração nos alimentos para animais.

# Abstract

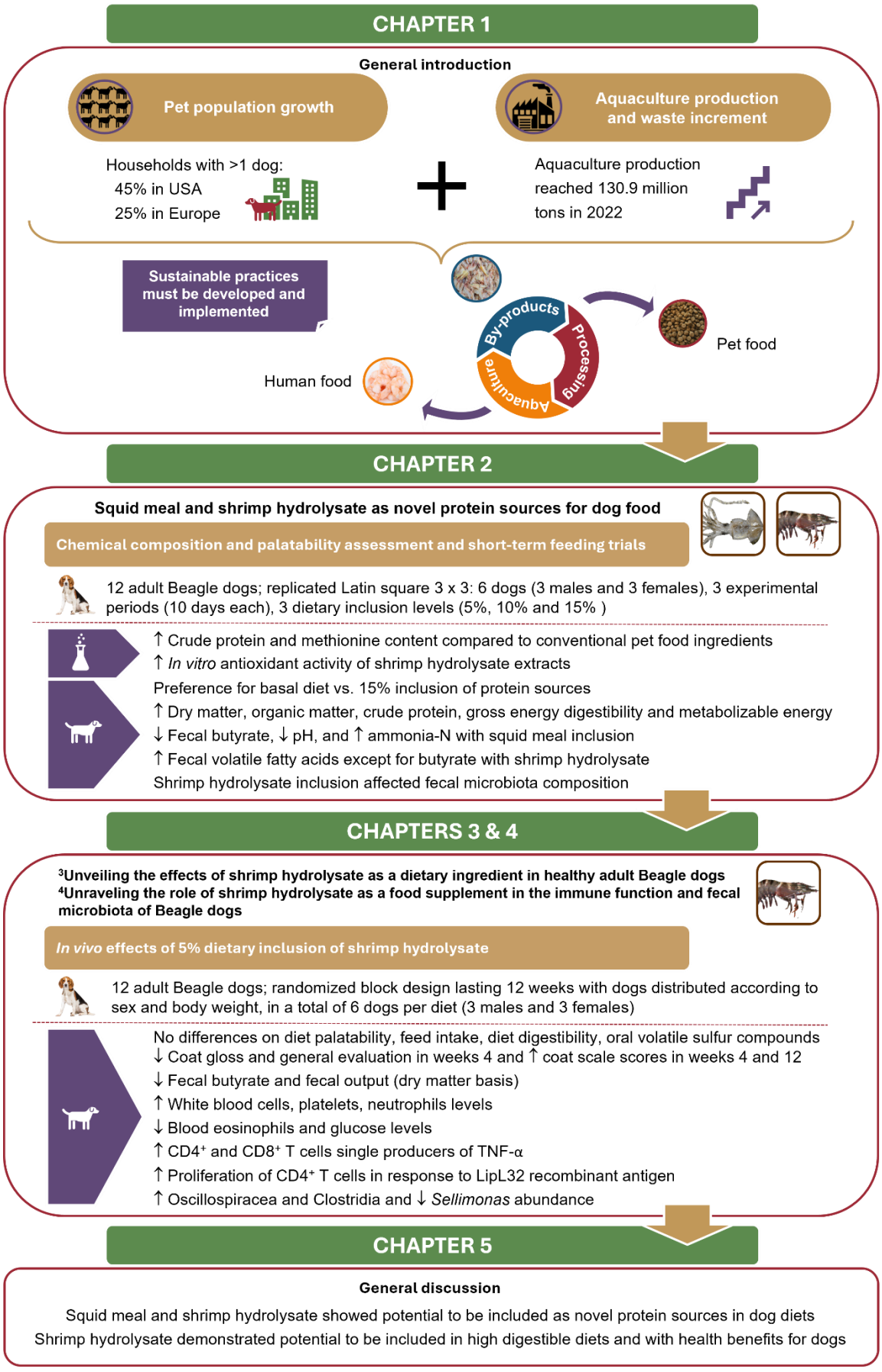
As the global pet population continues to grow, the pet food industry faces significant pressure to find sustainable and high-quality feed sources. Conventional protein sources, primarily animal-based, not only strain environmental resources but also compete directly with human food and animal feed systems. Thus, identifying alternative protein sources is crucial for a sustainable development of the sector. Aquatic animal by-products, including those from fish, and invertebrates such as mollusks and crustaceans, offer a promising sustainable and nutritionally valuable solution. They help to reduce waste, contributing to the circular economy and promoting sustainability within the capture fisheries, aquaculture and pet food sectors. In aquaculture and livestock, aquatic by-products, including their hydrolysates and bioactive peptides, have been shown to possess valuable nutritional and functional benefits, such as improved performance, gut health and immune responses. However, there is still a significant lack of knowledge on their effects in dogs. This gap presents an opportunity to investigate novel protein sources to be incorporated in canine diets to promote health and performance, while contributing to the sustainability of the pet food sector. The objective of this thesis was to evaluate, for the first time, the potential of two commercially available invertebrate aquatic by-products, squid meal and shrimp hydrolysate, to be incorporated into diets for adult dogs. Specifically, this work aimed to assess the relevant criteria for ingredient inclusion in dog food, namely, palatability, nutritive value, and dogs' health parameters.

Chapter 1 highlights the importance of dogs in modern society and acknowledges the growing demand for sustainable pet food solutions. In response to this demand, the rise in aquaculture production and how the use of its by-products can contribute to the sustainability of the pet food industry are addressed. The findings on the effects of aquaculture by-products in growth performance, feed conversion efficiency, immune response, and gut health in livestock, poultry, aquaculture species and pets are revised, being the effects dependent of the species and the dietary inclusion levels. Based on the literature described and on the availability of aquaculture by-products, in Chapter 2, two commercially available protein sources, squid meal and shrimp hydrolysate, were evaluated, namely their chemical composition, antioxidant properties, palatability and digestibility. Shrimp hydrolysate exhibited higher antioxidant activity than squid meal. Both ingredients showed high crude protein content (810 g $kg^{-1}$ dry matter (DM) basis and 658 g $kg^{-1}$ DM, squid meal and shrimp hydrolysate, respectively) and high methionine content. Palatability was assessed through a two-bowl test using 12 healthy adult Beagle dogs, to compare the basal diet (a commercially available diet) and the

inclusion of either protein source at 15%. No significant differences were found in the first approach and the first taste, but the consumption ratio diminished with the inclusion of either protein source. Then, two *in vivo* digestibility trials were conducted according to two Latin square 3 x 3, with 6 animals (3 males and 3 females), 3 experimental periods (10 days each) and 3 dietary inclusion levels (5%, 10% and 15%) of squid meal or shrimp hydrolysate in replacement of the basal diet. Compared to the basal diet, experimental diets with inclusion of squid meal and shrimp hydrolysate improved nutrient and energy digestibility, whereas squid meal inclusion reduced fecal butyrate concentration, and shrimp hydrolysate increased volatile fatty acids in feces, except for butyrate. Squid meal showed no effect on fecal microbiota, whereas shrimp hydrolysate affected Oscillosperaceae (UCG-005), Firmicutes and *Lactobacillus* abundance in feces, suggesting that it plays a role in modulating gut microbiota. The results indicate that squid meal and shrimp hydrolysate represent innovative and promising protein sources for dog diets. From the results obtained, in Chapters 3 and 4, shrimp hydrolysate was selected to be evaluated in a longer-term feeding trial. Two complete extruded diets were formulated using the same ingredients and amounts, except for the inclusion of 5% shrimp hydrolysate in replacement of wheat gluten. Palatability of diets was assessed in a two-bowl test, conducted with 12 healthy adult Beagle dogs, followed by a randomized block design lasting 12 weeks, with the 12 dogs distributed into 6 blocks, according to sex and body weight, and one dog from each block was randomly allocated to each diet. Measurements were performed in weeks 0, 4, 8, and 12. Results regarding diet palatability, intake and digestibility, fecal characteristics, metabolites, and microbiota composition, oral volatile sulfur compounds and coat quality are presented in Chapter 3, and hematological parameters, serum chemistry profile, and innate and adaptive immune function in Chapter 4. The inclusion of shrimp hydrolysate did not affect diet palatability, intake and digestibility, and oral volatile sulfur compounds, but increased fecal output (DM basis), and decreased fecal butyrate concentration, while increased Oscillospiraceae and Clostridia abundance, and decreased *Sellimonas* abundance, suggesting the potential prebiotic effects of shrimp hydrolysate and in enhancing the digestibility of amino acids. Shrimp hydrolysate inclusion resulted in reduced blood eosinophils and glucose levels, indicating its potential for inclusion in hypoallergenic diets and diets for diabetic dogs. It led to the increase in white blood cells, platelets, neutrophils, $CD4^+$ and $CD8^+$ T cells that produce tumor necrosis factor-alpha, and $CD4^+$ T cell proliferation in response to recombinant antigen from *Leptospira interrogans* (LipL32), while lowering superoxide production in stimulated cells with phorbol myristate acetate, suggesting its immunomodulatory role. Finally, shrimp hydrolysate promoted high coat quality, enhancing scale in weeks 4 and 12.

The outcomes of this work contribute to the development of feeding strategies that promote sustainability while providing functional value with benefits for dogs' health, and addressing the concerns of owners. Future research should focus on the effects of incorporating squid meal and shrimp hydrolysate at higher inclusion levels in extruded diets, exploring their potential as sole protein sources in hypoallergenic diets and to promote sustainability in the pet food industry, while also evaluating dog performance and health over longer-term studies to observe sustained physiological effects and benefits. Additional investigations are needed, such as on the palatability of these ingredients, the antioxidant properties of shrimp hydrolysate, the interactions between nutrients and extrusion process that may affect digestibility, the impact on fecal microbiota and metabolites, the mechanisms supporting immune function and glucose metabolism, and the potential benefits for dogs with specific health issues, along with understanding dog owners' perceptions, and the environmental implications through life cycle assessments to enhance the integration of these by-products in pet food.

# Graphical abstract



# CHAPTER 1

General introduction

## 1.1 Dogs in modern society: health and nutrition

For the past 15,000 years, dogs have been companions to humans, sharing a significant journey of domestication (Freedman and Wayne, 2017). In contemporary society, they are primarily kept as companion animals, and often considered integral members of the family (Albert and Bulcroft, 1987; Hirschman, 1994). Bradshaw (2011) stated that for most people "a world without dogs is unthinkable", highlighting the profound impact dogs have on human life. While in the past dogs primarily lived in rural environments, today they are predominantly found in urban areas in close proximity to humans (Puurunen et al., 2020). Research has demonstrated that dog ownership can significantly enhance human physical health, such as encouraging physical activity, reducing the incidence of asthma and obesity, lowering blood pressure, and promoting mental health, by reducing stress and improving emotional support and regulation (Bibbo et al., 2019; Horowitz, 2021). Dogs also serve critical roles as therapy and service dogs, assisting people with disabilities and contributing to public security and welfare (Hart and Yamamoto, 2016). Furthermore, the anthropomorphism of dogs has led to an increased focus on dog welfare and health, with people frequently exhibiting nurturing behaviors towards dogs that are analogous to those directed towards children (Lee Rasmussen and Rajecki, 1995; Mitchell, 2001). This includes providing them with high-quality food, regular health check-ups, grooming, celebrating dogs' birthdays, play with them, create comfortable living spaces for them, among others (Boya et al., 2012). The improvements in nutrition and healthcare have contributed to an increase in lifespan extension. However, increased longevity raises the incidence of age-related diseases in dogs, which, combined with lifestyle alterations, such as exposure to urban stressors (e.g. pollution, encounters with unfamiliar dogs) and reduced physical activity, has led to an increase in health problems among dogs, such as sleep disturbance (Tooley and Heath, 2022), behavioral problems (Amat et al., 2020), obesity (German, 2015), diabetes (O'Kell and Davison, 2023), cancer (Sarver et al., 2022), cognitive dysfunction (Taylor et al., 2023), and cardiac disease (Wess, 2022).

Nutrition plays a crucial role in maintaining the overall health and well-being of dogs, impacting their quality of life and longevity (Vučinić et al., 2023). The growing concern for canine health closely mirrors human dietary considerations, as pet owners increasingly apply their own nutritional standards and preferences to their pets. Therefore, anthropomorphism drives the demand for providing dogs with high-quality and functional food to optimize canine health (Clemens, 2014; de Godoy et al., 2016). This has led to a surge in the popularity of premium diets, and exotic or novel, natural, organic, and sustainable ingredients (Oberbauer and Larsen, 2021). A survey performed

by The American Pet Products Association (APPA) showed that 41% of dog owners buy premium food and 51% of pet owners are willing to pay more for ethically sourced pet products and eco-friendly pet products (APPA, 2021). Therefore, the perception of dog food by owners is crucial, as they are the ones who ultimately decide how to feed their pets.

Hence, when formulating dog food, several key parameters must be thoroughly evaluated to ensure the well-being, health, and safety of the animals. The primary concern in dog food formulation is achieving a balanced diet that meets nutritional needs, including the appropriate levels of proteins, fats, carbohydrates, vitamins, and minerals (FEDIAF, 2021). Moreover, it is essential to evaluate the digestibility of these nutrients to ensure that dogs can efficiently absorb and utilize them (Dust et al., 2005). High digestible diets are especially valuable in promoting better health outcomes, particularly in cases of gastrointestinal dysfunction (Grant Guilford, 1994). It is also important to evaluate ingredients that offer health benefits beyond basic nutrition. Due to their bioactive properties, such as antioxidant and anti-inflammatory effects, functional foods play a significant role in maintaining overall health and reducing the risk of various diseases (Di Cerbo et al., 2017). Nevertheless, the effectiveness of these ingredients should be supported by scientific evidence, demonstrating their impact on overall health and physiological responses, such as gut health, blood biomarkers, and immune function. Additionally, the palatability of foods, such as texture, aroma, and taste, is a critical factor, as even the most nutritious food will be ineffective if a dog refuses to eat it (Taylor, 2014). Therefore, palatability tests are important to ensure that the food is appealing to dogs, which in turn supports consistent intake of the required nutrients (Aldrich and Koppel, 2015; Le Guillas et al., 2024). Finally, given the close relationship between dogs and their owners, food safety is a key aspect to be evaluated (FEDIAF, 2019b). Overall, this holistic approach to dog food formulation is essential for meeting the evolving needs of both dogs and their owners.

## 1.2 Sustainability of pet food industry

The pet food industry has expanded (FEDIAF, 2024) to face the growing dog population. According to the latest statistical reports, by the year of 2020, 45% of USA households owned at least one dog, reaching a total of nearly 88.8 million dogs (AVMA, 2022). In Europe, by 2022, 25% of all households owned at least one dog, and the dog population was approximately 106 million (FEDIAF, 2024). As a result, there has been a heightened awareness of the sustainability of the pet food system (Acuff et al., 2021), and also of the environmental "pawprint", a term that refers to the impact of dogs and cats on the

environment, primarily through food production and consumption (Okin, 2017). Sourcing practices should prioritize sustainability, including the use of by-products and novel protein sources that reduce waste and environmental impact (FEDIAF, 2019a). In fact, as pet owners' awareness of environmental impact grows, increasingly value sustainability in pet food production and pet food products should be evaluated not only for their nutritional value but also for their environmental footprint (European Commission, 2024).

The pet food industry comprises a long chain of activities, from the production through to the consumption, that are responsible for the use of energy and natural resources, the production of waste, and emission of greenhouse gases (GHG), as described in Figure 1.1 (Acuff et al., 2021). The main food sources used in the pet food manufacture are crops, livestock, aquaculture, and also by-products, indicating that pets are close competitors to both livestock and human food systems (Swanson et al., 2013). Therefore, there is an urgent need to take measures that promote a more environmentally, economically, and socially sustainable balance, ensuring that food production and consumption practices protect both canine health and the environment.

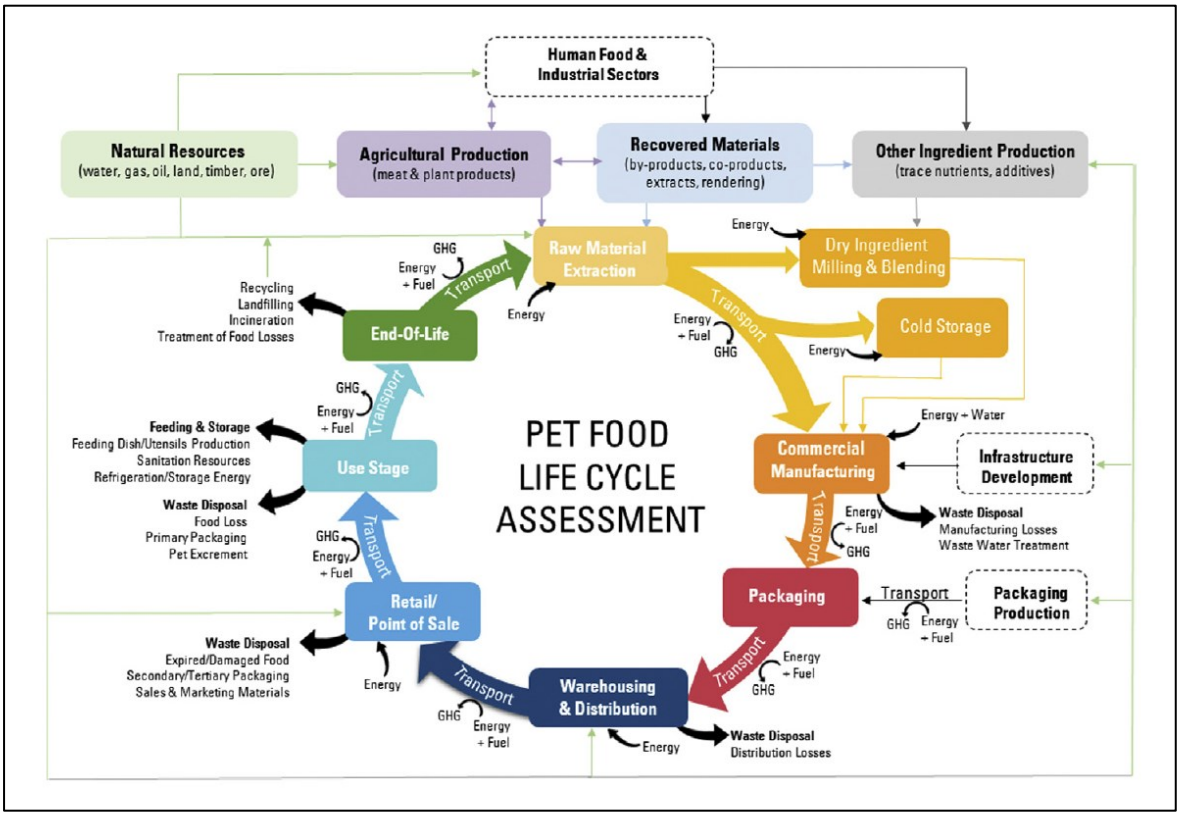



Figure 1.1. Life cycle assessment of the pet food system. Reprinted with permission from Acuff et al. (2021) (CC BY NC ND 4.0).

## 1.3 Protein requirements and sustainability balance

Protein is a macronutrient fundamental to fulfil the nutritional requirements of dogs. It must be fed a minimum of 180 g $kg^{-1}$ of dry matter (DM) based on the maintenance energy requirements of 110 $kcal/kg^{0.75}$ of adult dogs (FEDIAF, 2021). Compared to other nutrients, it is the most expensive nutrient and has the greatest negative impact on the environment (Toujgani et al., 2023). Protein supplies the essential and non-essential amino acids (AA) necessary for structural synthesis in growth, maintenance, and reproduction, serving as an energy source and providing components required for various metabolic functions. Amino acids play important roles in supporting, for instance, muscle development, neurological function, immune system, hormone production, energy metabolism, and enzyme activity (Oberbauer and Larsen, 2021). Although dogs are anatomically carnivores, since they have metabolic characteristics of omnivores and distinctive feeding behaviors, they are broadly considered facultative carnivores (McDonald, 2002; NRC, 2006). For instance, dogs can convert β-carotene into vitamin A, tryptophan into niacin, cysteine into taurine, and linoleic acid into arachidonic acid (Li and Wu, 2023). Dogs fed plant-based diets with low inclusion levels or even the absence of animal-sourced ingredients exhibited AA imbalances that result in health problems, such as dilated cardiomyopathy (Kaplan et al., 2018; Cavanaugh et al., 2021). While most plant-sourced ingredients, namely soy, peas, corn and oats, present low levels of some essential AA, namely lysine, tryptophan, threonine, methionine, and non-essential AA, such as cysteine, glycine and proline, animal-based ingredients typically provide more adequate levels of AA for dogs (Li et al., 2021). The predominant protein sources commonly used by the pet food industry are animal-based ingredients, such as meat and poultry, rendered protein meals (poultry meal, meat meal, fish meal), and by-products and organ meats (Decision Innovation Solutions, 2020). Therefore, achieving the nutritional balance presents challenges, particularly when considering the high environmental impact of the animal-based protein sources commonly used in dog food (Pedrinelli et al., 2022), thus requiring the evaluation of sustainable alternatives to meet both the nutritional needs of dogs and environmental considerations.

Animal by-products, which results mainly from the processing of the remaining portions of animals not used for human consumption (European Union, 2009), have been advocated as sustainable ingredients for animal feed (Meeker and Meisinger, 2015). The application of by-products helps to reduce organic waste, thereby mitigating health hazards for both humans and animals (Meeker and Hamilton, 2006). The National Environmental Policy Act (NEPA) defined sustainability as a tool to “create and maintain the conditions under which animals and nature can exist in productive harmony, that

permits fulfilling the social, economic and other requirements of present and future generations" (NEPA, 1970). Additionally, nutritional sustainability was defined as "the ability of a food system to provide sufficient energy and the amounts of essential nutrients required to maintain good health of the population without compromising the ability of future generations to meet their nutritional needs" (Swanson et al., 2013). Increasing the sustainability of the pet food industry involves evaluating and potentially integrating novel ingredients into pet nutrition (Stevens et al., 2018). These ingredients should meet several criteria, such as providing the nutritional needs of pets, contributing to the improvement of pet health, being cost-effective, and environmentally friendly. Among potential candidates, including insects, bacteria, and yeast, aquatic animals, and particularly their by-products, have emerged as promising sustainable sources of nutrients. Aquatic animal by-products provide valuable protein, essential AA, lipids, and bioactive compounds, making them potentially reliable sources for pet food formulations (Meeker and Meisinger, 2015). The shift towards the use of aquatic animal sources in pet food is closely supported by the growing production and sustainability efforts within the fisheries and aquaculture industries.

## 1.4 Capture fisheries, aquaculture and pet food industries: a sustainable approach

While capture fisheries have remained relatively constant since the 1980s, with annual catches fluctuating between 86 and 93 million tons, global aquaculture production, encompassing aquatic animals and algae, reached a record of 130.9 million tons in 2022 (Figure 1.2) (FAO, 2024). Depending on the aquatic animal and type of processing, large quantities of by-products are generated such as shells, heads, skins, bones, and viscera, constituting up to 80% of the whole animal (Roy et al., 2023). In 2021, the processing of aquatic animals in aquaculture generated 39.1% waste, while capture fisheries produced 35.4% waste (World Economic Forum, 2024). Despite advancements in reducing loss and waste over the past few decades (FAO, 2024), waste management practices, including incineration and improper disposal, continue to negatively affect public health and the environment (Al Khawli et al., 2020). This includes the emission of volatile organic compounds, sulfur dioxide, and hydrogen chloride, as well as the generation of leachate and gas during waste decomposition (Lopes et al., 2015). The negative impact of waste has increased public concerns on sustainability (Martins et al., 2010). Therefore, the success of aquaculture production is closely linked to the adoption of more sustainable practices (Stevens et al., 2018). Circular economy strategies provide a framework for more efficient use of resources and reduce waste (Regueiro et al., 2022).

By repurposing outputs at the end of their lifecycle as resources for different industries (Stahel, 2016), circular economy models enable the transformation of by-products into valuable nutrient-rich feed ingredients for several animal species. This approach enhances resource efficiency and minimizes waste, contributing to the establishment of a more productive and sustainable food system. Integrating these principles in capture fisheries and aquaculture enables the economic growth and environmental management that aligns with the key objectives of the 2030 Agenda for Sustainable Development (United Nations, 2018), such as responsible consumption and production (Sustainable Development Goal, SDG, 12), sustainable use of marine resources (SDG 14), and climate action (SDG 13). This interconnected approach highlights the positive impact of sustainable capture fisheries and aquaculture practices on the pet food industry, ensuring both environmental and economic benefits. Additionally, by leveraging the nutritional and functional value of aquatic by-products, these practices can enhance the quality of ingredients used in pet food.

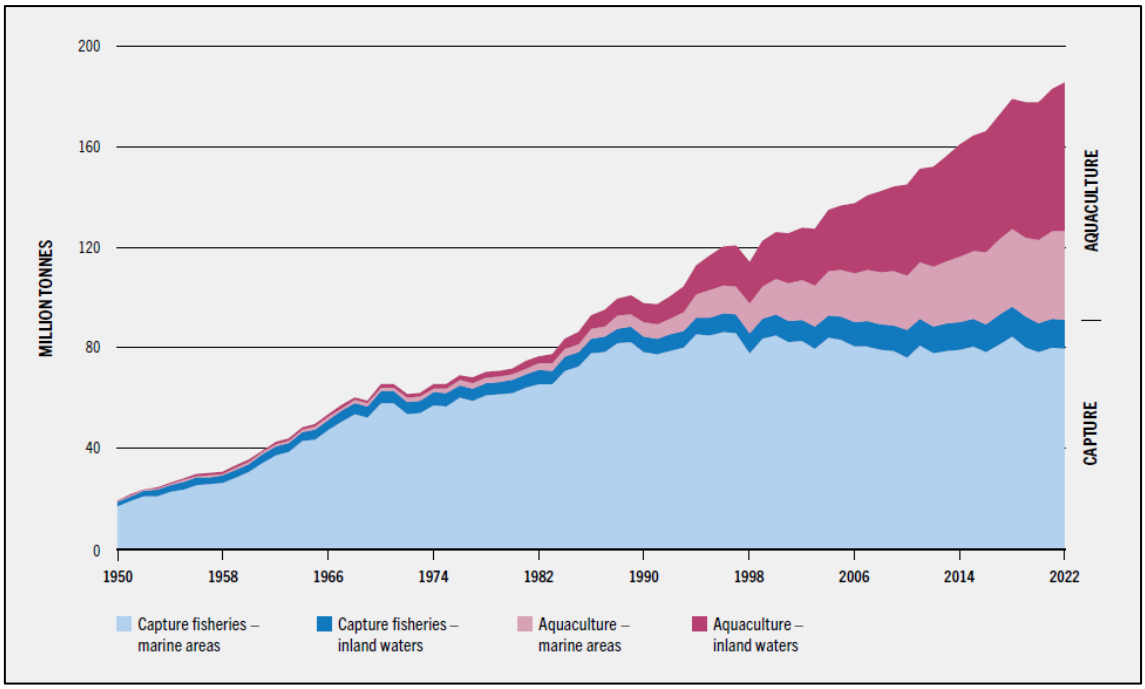



Figure 1.2. Evolution of capture fisheries and aquaculture production of aquatic animals. Reprinted with permission from FAO (2024) (CC BY 4.0).

## 1.5 Valorization of aquatic animal by-products: nutritional and functional values

Aquatic animal by-products were commonly viewed as low-value materials (Olsen et al., 2014). Nowadays, by-products are recognized as valuable resources, and their

utilization is considered a critical measure for reducing aquatic waste (World Economic Forum, 2024). A recently developed stepwise framework for valorization of aquatic by-products is suitable for circular production strategies (Cooney et al., 2023). This framework involves a structured approach to maximize waste materials by converting them into valuable resources, while minimizing the environmental impact associated with traditional waste disposal methods (Figure 1.3). This approach ensures that every stage of by-product lifecycle is optimized for resource efficiency and sustainability, and the pet food industry, among others, can significantly benefit from it. A wide variety of techniques to treat by-products can be employed, ranging from traditional methods such as hot processing, high-pressure treatment, and hydrolysis, to modern technologies such as ultra-high pressure and ultrasonic processing (Bi, 2024). These methods aim to improve quality, palatability, extend shelf life, and preserve the nutritional value of by-products, making them more suitable for use in animal feeds. By integrating by-products into pet food formulations, the industry can not only reduce its reliance on conventional protein sources but also contribute to more sustainable production practices, aligning with the growing demand for environmentally friendly products.

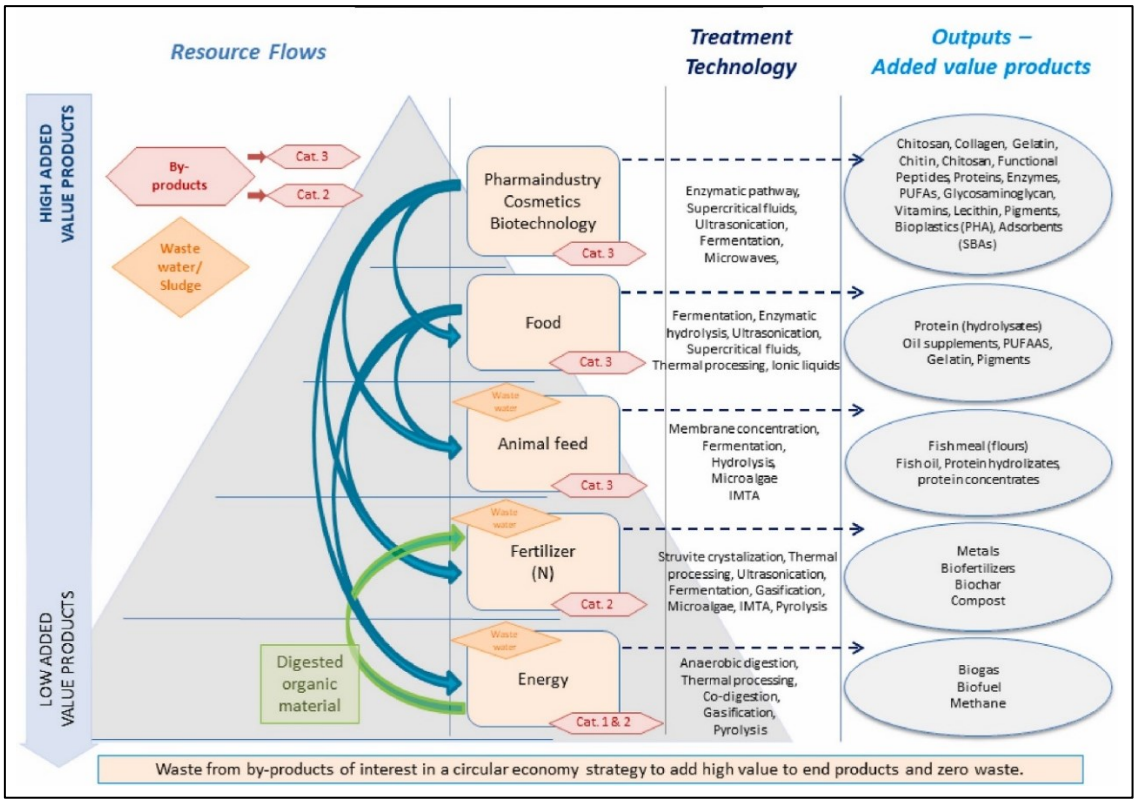



Figure 1.3. Framework for circular economy strategies for aquatic by-products valorization. The triangle represents the added value of products and the required volume, and the blue arrows represent the flows of by-products or low-value discards that are used in industries. Categories (Cat.) of by-products under the European Union Regulation EC No. 1069/2009 are: 1 (materials that present a high risk and must be completely disposed), 2 (materials that have a medium risk and can be used for certain limited purposes after being treated, but not intended for animal consumption) and 3 (materials that pose low risk and can be used in the production of pet food or other animal feed, following appropriate treatment). Abbreviations: IMTA, integrated multi-trophic aquaculture; BSA, sludge-based adsorbents; PHA, polyhydroxyalkanoate; PUFA, polyunsaturated fatty acids. Reprinted with permission from Cooney et al. (2023) (CC BY 4.0).

Aquatic animal by-products have a significant nutritional value, comprising essential nutrients, such as AA, polyunsaturated fatty acids (PUFA), vitamins, and minerals, as well as bioactive components, which makes them highly valuable for various applications across industries, including food, feed, pharmaceuticals, and biotechnology (Jasrotia et al., 2024). These by-products contain approximately 10-23% (as-is) of high-quality protein (Neves et al., 2016) and can be further processed into high-quality ingredients through hydrolysis, which breaks down proteins into smaller peptides (Malaweera and

Wijesundara, 2014), enhancing their functional and nutritional properties, while also reducing the risk of allergenicity (Pereira et al., 2023). Bioactive peptides have gained significant attention for their potential in developing cost-effective pharmaceuticals, nutraceuticals, and functional food and feed ingredients. Moreover, enzymatic hydrolysis is favored over chemical methods due to their superior physical, chemical, and sensory attributes (Baraiya et al., 2024). Overall, the valorization of aquatic animal by-products, namely fish, mollusks and crustaceans, contributes to waste reduction and to minimize environmental hazards, while also enhancing the economic efficiency of industries and upgrading animals' nutrition and health. Although limited knowledge exists on the impact of valuable aquatic animal by-products on the performance and health of dogs, studies on laboratory animals, livestock and aquaculture species have been conducted.

The following subsections present the effects of several aquatic animal by-products on different species, including fish, livestock, mink, and pets. It should be noted, however, that the studies discussed do not encompass the entirety of the available literature, as a comprehensive review was not conducted.

### 1.5.1 Fish

By-products from fish include heads, viscera, skin, backbone and muscle, and can be used to produce fish meals, protein hydrolysates, among other valuable products (Al Khawli et al., 2020). Fish meals can contain 60-72% (as-is) of crude protein (CP), essential AA, such as arginine, leucine and lysine, and fatty acids, namely PUFA n-3 (7.7-44.2 g/100 g of total fatty acids) and n-6 (1.3-2.9 g/100 g of total fatty acids) (Cho and Kim, 2011). While fish meals have been widely utilized in aquafeeds, concerns have emerged about their sustainability due to growing competition for limited supplies, overexploitation of resources, and rising feed costs (Mitra, 2021). To address this issue, fish meals can be produced from fish processing by-products such as trimmings, offcuts, and offal. Additionally, fish meals made from wild fish may contain elevated levels of heavy metals, dioxins and polychlorinated biphenyls (Mitra, 2021; van Riel et al., 2023). Therefore, evaluation of alternative processing methods is important to promote the valorization and utilization of by-products.

One alternative method is hydrolysis, which not only can reduce toxic elements found in wild fish (de la Fuente et al., 2023), but also yields valuable bioactive peptides with potential health benefits. In fact, fish-derived bioactive peptides exhibit *in vitro* anti-proliferative, antioxidant, anti-hypertensive, and anti-diabetic properties (Baraiya et al., 2024). Only a limited number of *in vivo* studies have been conducted to demonstrate the bioactive effects of fish-derived peptides, showing anti-hypertensive effects in rats (Zou

et al., 2014) and anti-diabetic effects in mice (Harnedy et al., 2018; Daskalaki et al., 2023).

Most studies have assessed the effects of fish hydrolysates dietary inclusion on feed intake, digestibility, growth performance and health status across multiple fish species. Table 1.1 summarizes some examples of those studies showing the beneficial effects of fish hydrolysates, however the responses varied depending on the fish species and the fish hydrolysate source, indicating the need for species-specific evaluation and optimization of inclusion levels in diets.

At levels around 10%-30%, fish hydrolysates generally improve growth, feed efficiency, immune function, and gut health in species such as largemouth bass, barramundi, Atlantic salmon, pike silverside, olive founder, Japanese flounder, Japanese sea bass, turbot, and yellow croaker (Refstie et al., 2004; Hevrøy et al., 2005; Liang et al., 2006; Tang et al., 2008; Kim et al., 2014; Zheng et al., 2014; Ospina-Salazar et al., 2016; Wei et al., 2016; Siddik et al., 2018b; Siddik et al., 2019; Chaklader et al., 2020; Siddik et al., 2020; Chaklader et al., 2021; Fan et al., 2022; Chaklader et al., 2023; Sandbakken et al., 2023; Wei et al., 2023; Sandbakken et al., 2024). Lower inclusion levels (approximately 1%-5%) were also effective for European sea bass, sea bream, olive flounder, Japanese flounder, and striped catfish, enhancing growth, nutrient digestibility, and immune response (Zheng et al., 2012; Zheng et al., 2013; Bui et al., 2014; Khosravi et al., 2015; Leduc et al., 2018b; Gunathilaka et al., 2020; Teoh and Wong, 2021). However, higher levels (above 30% to 100%) negatively impacted growth, survival, and blood parameters in coho salmon, barramundi, pike silverside, olive flounder (Murray et al., 2003; Kim et al., 2014; Ospina-Salazar et al., 2016; Siddik et al., 2018a).

Table 1.1. Effects of fish hydrolysates, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses, and intestinal microbiota of fish.

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Fish hydrolysate (species not reported) | Coho salmon (*Oncorhynchus kisutch*) | 30.3 | ↓ Hematocrit levels | Murray et al. (2003) |
| | Striped catfish (*Pangasius hypophthalmus*) | 2 | ↑ Specific growth rate, weight gain and feed conversion ratio | Teoh and Wong (2021) |
| | Largemouth bass (*Micropterus salmoides*) | 10, 30 and 50 | ↑ Weight gain, specific growth rate, protein efficiency ratio and feed conversion rate at 30%<br>↑ Expressions of superoxide dismutase, glutathione peroxidase, IL-1β, and TNF-β, and ↓ malondialdehyde contents and the expressions of IL-15, Caspase 3, Caspase 9, and Caspase 10 at 30%<br>↓ Actinobacteriota, *Staphylococcus* and *Plesiomonas* abundance | Fan et al. (2022) |
| Kingfish hydrolysate (*Seriola lalandi*)<br>Carp hydrolysate (*Cyprinus carpio*)<br>Tuna hydrolysate (*Thunnus Maccoyii*) | Barramundi (*Lates calcarifer*) | 10 | ↑ Survival<br>↓ Infection rate and total bilirubin levels in a *Vibrio harveyi* infection challenge | Chaklader et al. (2020) |
| | | | ↓ Intestinal TNF-α. IL-10 and a mucin-relevant production gene<br>↑ Intestinal Firmicutes and *Ruminococcus*, *Faecalibacterium*, and *Bacteroides* | Chaklader et al. (2023) |
| Cero hydrolysate (*Scomberomorus regalis*) | Pike silverside (*Chirostoma estor*) | 15, 30 and 45 | ↓ Weight gain and specific growth rates at 30 and 45%<br>↑ Dry matter digestibility at 30%<br>↓ Survival at 45% | Ospina-Salazar et al. (2016) |
| Herring hydrolysate (species not reported) | Atlantic salmon (*Salmo salar*) | 6, 12, 18, 24 and 30 | ↑ Feed conversion ratio and amino acids digestibility<br>↓ Protein efficiency ratio<br>↑ Specific growth rate at 24 and 30% | Hevrøy et al. (2005) |
| Salmon hydrolysate (species not reported) | Atlantic salmon (*Salmo salar*) | 9 and 18 | ↑ Specific growth rate and ash digestibility<br>↑ Crude protein and amino acids digestibility at 18% | Sandbakken et al. (2023) |
| | | | ↑ Spirochaetes abundance and ↓ Cyanobacteria, Proteobacteria and Firmicutes abundance in the hindgut<br>↑ Cyanobacteria and Spirochaetes abundance and<br>↓ Actinobacteria abundance in the midgut | Sandbakken et al. (2024) |
| | Yellow croaker (*Pseudosciaena crocea*) | 5, 10 and 15 | ↑ Weight gain<br>↑ Specific growth rate at 10 and 15%<br>↑ Lysozyme activity, complement activity and total immunoglobulin at 10 and 15% | Tang et al. (2008) |

Table 1.1. Effects of fish hydrolysates, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses, and intestinal microbiota of fish. (continued)

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Tilapia hydrolysate (*Oreochromis niloticus*) | European sea bass (*Dicentrarchus labrax*) | 5 | ↑ Final weight and specific growth rate | Leduc et al. (2018b) |
| | Sea bream (*Pagrus major*) | 4.23 | ↑ Feed conversion ratio and digestibility of crude protein<br>↑ Total immunoglobulin | Bui et al. (2014) |
| Tilapia hydrolysate (species not reported) | Olive flounder (*Paralichthys olivaceus*) | 2 | ↑ Crude protein and dry matter digestibility<br>↑ Lysozyme activity | Khosravi et al. (2015) |
| | | 2.88 | No significant effects found in growth performance, nutrient digestibility, and the immune response | Khosravi et al. (2018) |
| | | 1.5, 3 and 4.5 | ↑ Weight gain<br>↑ Final weight 1.5% and 3% | Gunathilaka et al. (2020) |
| Tuna hydrolysate (species not reported) | Sea bream (*Pagrus major*) | 2 | ↑ Crude protein digestibility<br>↑ Nitro blue tetrazolium activity and lysozyme activity<br>↑ Disease resistance in an *Edwardsiella tarda* challenge | Khosravi et al. (2015) |
| Tuna hydrolysate (*Thunnus maccoyii*) | Barramundi (*Lates calcarifer*) | 10 | ↑ Weight gain and specific growth rate<br>↓ Blood glucose, serum lysozyme activity and survival in a *Vibrio harveyi* infection challenge | Siddik et al. (2019) |
| | | | ↑ Glutathione peroxidase activity<br>↑ IL-1β and TNF-α and ↓ IL-10 mRNA expression levels in the intestine<br>↑ Intestinal Firmicutes and Fusobacteria and *Bacillus*, *Lactococcus* and *Cetobacterium* | Siddik et al. (2020) |
| | | 5 and 10 | ↑ Growth and condition factor<br>↑ Serum total bilirubin and total protein content<br>↓ Infection rate in a *Vibrio harveyi* infection challenge<br>↑ Intestinal abundance of Proteobacteria and Firmicutes | Chaklader et al. (2021) |
| | | 5, 10, 15 and 20 | ↑ Weight gain, specific growth rate at 5 and 10%<br>↓ Blood glucose at 15 and 20% | Siddik et al. (2018b) |
| | | 50 and 75 | ↓ Final weight and specific growth rate at 50 and 75% | Siddik et al. (2018a) |
| Yellowfin tuna hydrolysate (*Thunnus albacares*)<br>Skipjack tuna hydrolysate (*Katsuwonus pelamis*) | Olive flounder (*Paralichthys olivaceus*) | 5, 10, 20, 30, 40, 60, 80 and 100 | ↓ Weight gain and specific growth rates at 40 to 100% | Kim et al. (2014) |

Table 1.1. Effects of fish hydrolysates, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses, and intestinal microbiota of fish. (continued)

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Pollock hydrolysate (*Theragra chalcogramma*) | Atlantic salmon (*Salmo salar*) | 5,10 and 15 | ↑ Specific growth rate, final weight and feed intake at 10 and 15%<br>↑ Crude protein and amino acids digestibility at 5% and 15% | Refstie et al. (2004) |
| | Japanese flounder (*Paralichthys olivaceus*) | 3.7 and 1.2 | ↑ Final weight, specific growth rate, and feed efficiency at 3.7%<br>↓ Protein efficiency ratio<br>↑ Plasma IGF-I levels | Zheng et al. (2012) |
| | | 3.7 and 1.2 | ↑ Final weight, specific growth rate at 1.2%<br>↑ Feed efficiency, protein efficiency ratio at 3.7% | Zheng et al. (2013) |
| | | 6, 11, 16, 21 and 26 | ↑ Final weight, specific growth rate at 11, 16 and 21%<br>↑ Feeding rate and feed efficiency ratio at 26% | Zheng et al. (2014) |
| | | 5, 10 and 20 | ↓ Specific growth rate and feed intake at 20%<br>↓ Feed efficiency ratio and protein efficiency ratio at 10% and 20% | Xu et al. (2016) |
| | Turbot (*Scophthalmus maximus*) | 5, 10, 15 and 20 | ↑ Protein retention at 5% and 10%<br>↓ Weight gain and specific growth rate at 20%<br>↑ Crude protein digestibility | Wei et al. (2016) |
| | | 10 and 30 | ↑ Weight gain at 10%<br>↓ Specific growth rate, feed efficiency ratio, protein efficiency ratio and feed intake at 30%<br>↑ IL-1β and TNF-α mRNA expression levels in middle intestine at 10%<br>↑ *Bacillus* abundance and ↓ *Vibrio* abundance in the intestine | Wei et al. (2023) |
| | Japanese sea bass (*Lateolabrax japonicus*) | 5, 15 and 25 | ↑ Final weight, feed conversion ratio at 15 and 25%<br>↑ Specific growth rate at 15%<br>↑ Lysozyme activity and complement haemolytic activity at 10 and 15% | Liang et al. (2006) |
| | Yellow croaker (*Pseudosciaena crocea*) | 5, 10 and 15 | ↑ Weight gain<br>↑ Specific growth rate at 10 and 15%<br>↑ Lysozyme activity, complement activity and total immunoglobulin at 10 and 15% | Tang et al. (2008) |

Abbreviations: IGF-I, insulin-like growth factor; IL, interleukin; TNF, tumor necrosis factor.

Effects of dietary inclusion of fish hydrolysates have also been evaluated on terrestrial animal, such as pigs, chickens, minks, and cecectomized roosters, with some examples of studies presented in Table 1.2. Overall, data suggests that the benefits of fish hydrolysates are highly dependent on animal species, source of hydrolysate used, and dietary inclusion level. Generally, inclusion levels of approximately 5%-10% enhanced nutrient digestibility, growth, immune response, and gut microbiota in pigs and chickens (Nørgaard et al., 2012; Opheim et al., 2016a; Opheim et al., 2016b; Thuy and Ha, 2017; Zhang et al., 2022; Alizadeh-Ghamsari et al., 2023). In cecectomized roosters and minks fed exclusively salmon hydrolysates, CP and AA digestibility improvement was observed (Folador et al., 2006; Tjernsbekk et al., 2017).

Table 1.2. Effects of fish hydrolysates, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses, and intestinal microbiota of terrestrial animals.

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Fish hydrolysate (species not reported) | Pig | 2, 3 and 5 | ↑ Nutrient digestibility and fecal volatile fatty acid content at 3%<br>↓ Weight gain at 5%<br>↑ Feed intake<br>↑ Total antioxidant capacity, superoxide dismutase and glutathione peroxidase activities, and immunoglobulin (Ig) A at 5% | Zhang et al. (2022) |
| Kilka fish hydrolysate (*Clupeonella* spp.) | Chicken | 2.5, 5 and 7.5 | ↑ Thigh percentage at 5% and 7.5%<br>↑ Relative length of jejunum at 2.5% and 5%<br>↑ Relative weight at 5%<br>↓ Intestinal coliforms, enterobacters, and total gram-negative bacteria | Alizadeh-Ghamsari et al. (2023) |
| Salmon hydrolysate (species not reported) | Pig | 12.3 | ↑ Feed intake | Nørgaard et al. (2012) |
| | Mink | 11.6 and 100 | ↑ Crude protein and amino acids digestibility at 100% | Tjernsbekk et al. (2017) |
| | Chicken | 5 | ↓ Protein efficiency ratio | Folador et al. (2006) |
| Salmon hydrolysate (*Salmo salar*) | Pig | 10 | ↑ Duodenal villi absorption and size (longer) | Opheim et al. (2016a) |
| | Chicken | 5 and 10 | ↑ Weight gain<br>↑ Gain to feed ratio at 10% | Opheim et al. (2016b) |
| Salmon hydrolysate<br>Sole hydrolysate<br>Pink salmon hydrolysate<br>Red salmon hydrolysate<br>Pollock hydrolysate<br>(species not reported) | Cecectomized rooster | 100 | ↑ Total amino acids and total essential and non-essential amino acids digestibility for salmon hydrolysate<br>↓ Total amino acids and total essential amino acids digestibility for pollock hydrolysate | Folador et al. (2006) |
| Tra catfish hydrolysate (*Pangasius hypophthalmus*) | Chicken | 16 | ↑ Total tract retention and crude protein, ether extract, and isoleucine, leucine, lysine, methionine and threonine digestibility | Thuy and Ha (2017) |

In pet food, fish meals are conventional protein sources, with approximately 200,000 tons of fishmeal being used each year by the pet food industry (IFFO, 2024). The limited information available on the effects of fish meals shows no significant differences in diet palatability and digestibility, fecal characteristics, and on the immune responses in dogs fed diets with 10% and 20% of fish meal (Folador et al., 2006; Zinn et al., 2009). Although there are no statistics on the annual usage of fish hydrolysates by the sector, these by-products have been drawn attention as valuable products for pet diets (Cabrita et al., 2024), and already applied in canine diets and supplements.

Moreover, the information presented above (Tables 1.1 and 1.2) is valuable to design studies and formulate diets for dogs. Studies on minks and cecectomized roosters are particularly relevant, as these animals are commonly used as animal models to evaluate complete diets and ingredients, namely *in vivo* nutrients digestibility (Krogdahl et al., 2004; Reilly et al., 2020). Additionally, Table 1.3 provides a summary of some examples of studies performed to evaluate the effects of different fish hydrolysates and commercial diets or supplements containing fish hydrolysates on dogs and cats.

Commercial diets containing partially hydrolyzed salmon or fish hydrolysates showed to improve clinical signs of pruritus and atopic dermatitis of cats and dogs diagnosed with adverse food reactions (Matricoti and Noli, 2018; Noli and Beltrando, 2021; Szczepanik et al., 2022). Capsules containing fish hydrolysates have shown to improve symptomatology of dogs with chronic bilateral otitis externa (Di Cerbo et al., 2016) and keratoconjunctivitis sicca, a dryness of the conjunctiva (Destefanis et al., 2016), while also enhanced stress-related behaviors and levels of stress biomarkers (Landsberg et al., 2015; Sechi et al., 2017; Titeux et al., 2021). Fish hydrolysates containing salmon, sea bream, sea bass, and redfish fed to dogs at 5% increased eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels in red blood cells and decreased neutral detergent fiber (NDF) digestibility, fecal ammonia-N, and valerate concentrations, with no significant effects on coat quality and diet palatability (Cabrita et al., 2024), while salmon hydrolysate at 10% improved diet palatability (Folador et al., 2006). Finally, pink salmon hydrolysate at 20% reduced fecal DM with no effects on nutrient digestibility and immune responses (Zinn et al., 2009)

Overall, fish hydrolysates showed positive effects on the health of both dogs and cats and on the behavior and stress of dogs. However, some of the results presented on fish hydrolysates focused on commercial diets or supplements, often with unknown formulations and fish species sources.

Table 1.3. Effects of fish hydrolysates, according to animal model and dietary inclusion level, on palatability, performance, immunological responses, and health of dogs and cats.

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Partially hydrolyzed salmon included in commercial diet (species not reported) | Dog | Unknown | ↓ Pruritus and atopic dermatitis in cats diagnosed with adverse food reactions | (Szczepanik et al., 2022) |
| | Cat | Unknown | ↓ Pruritus and atopic dermatitis in dogs diagnosed with adverse food reactions | |
| Fish and vegetable hydrolysates capsule (60-80%) (species not reported) | Dog | 6-7 | ↓ Symptom severity in dogs with chronic bilateral otitis externa (occlusion of the ear canal, erythema, discharge quantity, and odor) | Di Cerbo et al. (2016) |
| | Dog | Unknown | ↑ Plasma levels of serotonin, dopamine, and β-endorphins, and ↓ noradrenaline and cortisol levels in dogs with behavioral disorders related to anxiety and chronic stress | Sechi et al. (2017) |
| | Dog | 6-7 | ↓ Corneal pigment density, conjunctival inflammation, corneal keratinization, tear production and mucus discharge in dogs diagnosed with Keratoconjunctivitis sicca | Destefanis et al. (2016) |
| Fish hydrolysate capsule (500 mg) (species not reported) | Dog | 1-2 capsules/day | ↑ Dog-human interactions<br>↓ Stress-related behaviors | Titeux et al. (2021) |
| Fish hydrolysate (cod and mackerel) (species not reported) | Dog | 750 mg/day, and 1500 mg/day | ↓ Fear and anxiety-related behaviors and blood cortisol | Landsberg et al. (2015) |
| Pink salmon hydrolysate (species not reported) | Dog | 20 | ↓ Fecal dry matter, and no differences on digestibility and immune response | Zinn et al. (2009) |
| Salmon hydrolysate (species not reported) | Dog | 10 | ↑ Amount of food consumption and intake ratio | Folador et al. (2006) |
| Fish hydrolysate (salmon, sea bream, sea bass, and redfish) (species not reported) | Dog | 5 | ↑ Eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids concentration in red blood cells<br>↓ Neutral detergent fiber digestibility, fecal ammonia-N and valerate concentrations<br>No differences on coat quality and diet palatability (first approach, first taste and in intake ratio) | Cabrita et al. (2024) |
| Fish hydrolysate included in commercial diet (species not reported) | Dog | Unknown | ↓ Pruritus in dogs diagnosed with adverse food reactions | Matricoti and Noli (2018) |
| | Cat | Unknown | ↓ Pruritus in cats diagnosed with adverse food reactions | Noli and Beltrando (2021) |

In summary, fish hydrolysates present a promising sustainable and valuable alternative to conventional feed sources in both aquaculture and animal feed. Research indicates that depending on species, hydrolysate source and inclusion levels, fish hydrolysates can enhance growth, nutrient digestibility, immune responses and gut microbiota. While studies in pets are limited, fish hydrolysates have demonstrated potential in addressing adverse food reactions, behavioral issues, and chronic pathologies. Further research is needed to fully understand their impact on the health and performance of dogs.

### 1.5.2 Mollusks

Mollusks, including squids, bivalves, snails, abalones, oysters, cuttlefish, and octopuses, hold significant potential as sources for animal feeding due to their high protein (9.45-17.1 g 100 $g^{-1}$, as-is), and PUFA n-3 and n-6 contents, and their antiviral, anti-inflammatory, immunomodulatory, and antimicrobial properties (Khan and Liu, 2019; Zhukova, 2019).

#### *1.5.2.1 Mussels*

Mussels are rich in protein with approximately 16 g protein g 100 $g^{-1}$ (as-is) of edible product (Yaghubi et al., 2021), providing a balanced AA profile, with high contents of taurine (17.8-42.9 g $kg^{-1}$ DM), arginine (3.02-5.72 g $kg^{-1}$ DM), and glycine (3.51-7.63 g $kg^{-1}$ DM; Fuentes et al., 2009). They are also rich in PUFA n-3, such as DHA and EPA (12%-18% and 11%-15% of total fatty acids, respectively; Karakoltsidis et al., 1995; Venugopal and Gopakumar, 2017).

Undersized mussels, which are not suitable for human consumption, and mussels cultivated specifically to reduce nitrogen and phosphorus pollution near urban areas or finfish farms, can be processed into mussel meal for feed formulations (Lindahl, 2013). Therefore, mussels have the potential to become a key source of high-quality protein for animal feeding, while offering numerous environmental benefits, such as low greenhouse gas emissions and a reduction in eutrophication (Gren et al., 2009; Suplicy, 2020; Yaghubi et al., 2021). Additionally, mussels are generally divided into four main components: extracellular fluid, shell, byssus threads, and meat. The latter presents approximately 59% protein (DM), comprising diverse enzymes and carotenoids with food preservative and antioxidant properties, and 7% lipids (DM) with anti-inflammatory properties (Lindqvist et al., 2018; Naik and Hayes, 2019).

Table 1.4 presents studies that evaluated the effects of mussel-derived ingredients (mussel meal, mussel meat, and mussel silage) on growth, feed efficiency, cecal microbiota and immune response of different fish species, chicken and pigs. In general, the effects of mussel meal and meat evaluated from 2% to 75% inclusion levels promoted

growth, feed efficiency, and changes in antioxidant capacity and enzyme activities at levels up to 35.5% in common sole, Japanese flounder, tiger puffer, and ussuri catfish (Kikuchi and Sakaguchi, 1997; Kikuchi et al., 2002; Kikuchi and Furuta, 2009; Mongile et al., 2015; Luo et al., 2019). In chickens, inclusion levels of mussel meal from 3% to 15% often improved feed efficiency, laying percentage and yolk pigmentation (Jönsson and Elwinger, 2009; Jönsson et al., 2011; Afrose et al., 2016), while in pigs, an inclusion of either 28.5% of mussel meal or 70% of mussel silage improved AA and CP digestibility (Nørgaard et al., 2015).

Overall, the studies indicate that mussel-derived ingredients can offer nutritional benefits, such as improved growth, feed efficiency, and digestibility, but the effects are species-specific and inclusion level-dependent. However, it is essential to further explore the optimal inclusion levels across a wider range of species to maximize their nutritional and health benefits, along with their potential environmental advantages. Although there are no specific studies on the inclusion of mussels in the diets of cats and dogs, the promising properties, such as solubility, emulsifying capacity, gel-forming ability, and the high taurine content observed in *Sinanodonta woodiana* suggest the potential benefits for pet nutrition (Konieczny et al., 2022). Moreover, studies on the effects of mussel hydrolysates are scarce, though the hydrolysis of *Mytilus edulis* by-products have been shown to generate bioactive peptides that exert *in vitro* antioxidant, anti-inflammatory, angiotensin-converting enzyme inhibitory, anticancer, anticoagulant and neuroprotective activities (Naik and Hayes, 2019; Naik et al., 2020; Cunha et al., 2021).

Table 1.4. Effects of mussel by-products, according to animal model and dietary inclusion level, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses, and intestinal microbiota of fish and terrestrial animals.

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Mussel meal (*Mytilus chilensis*) | Common sole (*Solea solea*) | 25, 50 and 75 | ↑ Growth rate, feed intake, protein efficiency ratio and gross protein efficiency and ↓ feed conversion rate | Mongile et al. (2015) |
| Blue mussel meal (species not reported) | Turbot (*Psetta maxima*) | 2, 4 and 8 | No significant effects found in growth, feed intake, and the immune response | Nagel (2017) |
| Blue mussel meal (Mytilus galloprovincialis) | Japanese flounder (*Paralichthys olivaceus*) | 8, 17, 34 and 50 | ↓ Feed efficiency at 50% | Kikuchi and Sakaguchi (1997) |
| | | 5, 10 and 20 | ↑ Weight gain, feed efficiency and protein efficiency ratio<br>↑ Plasma protein and ↓ plasma triglyceride | Kikuchi et al. (2002) |
| | Tiger puffer (*Takifugu rubripes*) | 5, 10 and 20 | ↑ Final weight, weight gain and protein efficiency ratio<br>↑ Growth rate and feed utilization at 5% and 10% | Kikuchi and Furuta (2009) |
| Mussel meat (*Cristaria plicata*) | Ussuri catfish (*Pseudobagrus ussuriensis*) | 17.8 and 35.5 | ↑ Protein efficiency ratio and dry matter, crude lipid and gross energy digestibility<br>↓ Final weight, weight gain, specific growth rate and feed intake at 35.5%<br>↓ Hepatic total antioxidant capacity and superoxide dismutase and malondialdehyde level<br>↑ Alpha-amylase and pepsin activities and ↓ hepatic IGF-I gene expression level at 35.5% | Luo et al. (2019) |
| Blue mussel meal (*Mytilus edulis*) | Pig | 28.5 | ↑ Amino acids and crude protein digestibility | Nørgaard et al. (2015) |
| | Chicken | 4, 8 and 12 | ↑ Fat digestibility<br>↑ Nitrogen retention at 12% and metabolizable energy, and amino acid digestibility at 8% and 12%<br>↑ Egg yolk redness and ↓ lightness<br>↑ Egg weight at 8% and 12% | Afrose et al. (2016) |
| Blue mussel silage (*Mytilus edulis*) | Pig | 70 | ↑ Amino acids and crude protein digestibility | Nørgaard et al. (2015) |
| Zebra mussel meal (*Dreissena polymorpha*) | Chicken | 7.5 and 15 | No significant effects found on production performance and egg quality | McLaughlan et al. (2014) |
| Mussel meal (species not reported) | Chicken | 3, 6, 9 and 12 | No significant effects found on growth, feed intake, and in cecal clostridial colony forming units | Waldenstedt and Jönsson (2006) |
| | | 3, 6 and 9 | ↑ Laying percentage at 6%<br>↑ Yolk pigmentation | Jönsson and Elwinger (2009) |
| | | 3.5 and 7 | ↑ Yolk pigmentation at 7%<br>↓ Excreta dry matter at 7% | Jönsson et al. (2011) |

Abbreviations: IGF-I, insulin-like growth factor.

*1.5.2.2 Squids*

Squids contains approximately 60% to 80% edible parts (Packard, 1972), with a CP content of 130-220 g $kg^{-1}$ as-is (Sikorski and Kołodziejska, 1986). Body components, including skin, mantle, ovary, ink, and other visceral parts, can be further processed into squid meal rich in protein and essential AA that can be used in animal feed (Singh et al., 2022). For instance, giant squid meal presents a CP content of 805 g $kg^{-1}$ DM, and high levels of the AA lysine (10% of total protein), and glutamic acid (15% of total protein; Carranco-Jáuregui et al., 2020). Furthermore, several *in vitro* studies have shown that peptides from different squid by-products, such as tunics, gelatin, and hydrolysates possess several bioactivities such as inhibition of angiotensin-converting enzyme, and antioxidant, anticancer, antimutagenic, and antimicrobial activities (Mendis et al., 2005; Alemán et al., 2011; Apostolidis et al., 2016; Mosquera et al., 2016; Suárez-Jiménez et al., 2019).

Table 1.5 summarizes the effects of dietary inclusion of squid by-products on different animal species, including shrimp, fish, and chickens. Squid meal increased weight gain of white leg shrimp when included at 5% and protein digestibility at 5%-23% (Córdova-Murueta and García-Carreño, 2002), while squid hydrolysate at 0.5%-1% improved feed efficiency and reduced inflammation in the gut of tiger shrimp (Pan et al., 2022). In freshwater eel, squid meal at 1%-3% led to improved growth and feed efficiency (Ndobe et al., 2022), while squid hydrolysate decreased growth at 3% in European seabass (Costa et al., 2020) and increased cholesterol levels at 4% in pompano (Novriadi et al., 2017). Fermented squid by-products decreased growth and nutrient retention in Japanese flounder at 24%, but improved antioxidant potential, total serum protein at 18% and 24%, and lysozyme activity at 6% (Abdul Kader et al., 2012). Finally, squid meal improved growth and PUFA n-3 concentration in chicken meat at dietary inclusion levels 1.7%-5% (Hulan et al., 1979; Morales-Barrera et al., 2022).

Currently, there are no studies assessing the effects of squid by-products on dogs, leaving a gap in understanding how these products could benefit canine nutrition, and research on other species has shown variability in results, which may be attributed to species-specific responses, differing processing methods, and variable inclusion levels. Consequently, investigating how dogs specifically respond to squid by-products and identifying optimal inclusion levels will be crucial to uncovering their potential nutritional benefits and understanding their effects in promoting canine health.

Table 1.5. Effects of squid by-products, according to animal model and dietary inclusion level, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses, and intestinal microbiota of shrimp, fish and chicken.

| Source of ingredient | Animal model/species | Dietary inclusion level, % | Effects | Reference |
|---|---|---|---|---|
| Squid meal (*Dosidiscus gigas*) | Pacific white shrimp (*Litopenaeus vannamei*) | 5, 10 and 20 | No significant effects found on growth and survival | Sánchez et al. (2012) |
| | Whiteleg shrimp (*Penaeus vannamei*) | 5, 14 and 23 | ↑ Final weight and ↓ feed conversion ratio at 5%<br>↑ Protein digestibility | Córdova-Murueta and García-Carreño (2002) |
| Squid hydrolysate (species not reported) | Tiger shrimp (*Penaeus monodon*) | 0.5 and 1 | ↑ Feed intake, efficient feed and protein and lipid retention<br>↓ Pro-inflammatory gene RAB-6a and prophenoloxidase gene in the gut at 1% | Pan et al. (2022) |
| Squid meal (*Dosidiscus gigas*) | Pompano (*Trachinotus carolinus*) | 1, 2 and 4 | No significant effects found on growth and survival | Novriadi et al. (2017) |
| Squid meal (*Loligo* sp.) | Freshwater eel (*Anguilla marmorata*) | 1, 3 and 5 | ↑ Weight gain and length, and feed efficiency at 1% and 3% | Ndobe et al. (2022) |
| Squid hydrolysate (*Loligo pealei*) | Pompano (*Trachinotus carolinus*) | 1, 2 and 4 | ↑ Serum cholesterol at 4% | Novriadi et al. (2017) |
| Squid hydrolysate (*Dosidiscus gigas*) | European seabass (*Dicentrarchus labrax*) | 3 | ↓ Final weight and specific growth rate | Costa et al. (2020) |
| Fermented squid by-product (species not reported) | Japanese flounder (*Paralichthys olivaceus*) | 6, 12, 18 and 24 | ↓ Final body mass, weight gain, specific growth rate, and feed efficiency ratio at 24%<br>↓ Protein and leucine retention at 24%<br>↑ Biological antioxidant potential and total serum protein at 18% and 24%<br>↑ Lysozyme activity at 6% | Abdul Kader et al. (2012) |
| Squid meal (*Illex illecebrosus*) | Chicken | 5, 10 and 15 | ↑ Weight gain and food conversion rate | Hulan et al. (1979) |
| Squid meal (*Dosidicus gigas*) | Chicken | 1.7, 3.3 and 5 | ↑ Eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) in chicken meat | Morales-Barrera et al. (2022) |

### 1.5.3 Crustaceans

Crustaceans include shrimp, crabs, prawns, lobster, and krill, and their by-products, such as heads and shells, may account for up to 70% of the total body weight (BW; Cretton et al., 2021). These by-products comprise protein, chitin, carotenoids, phenolic compounds, and polysaccharides (Hamed et al., 2015; Hamed et al., 2016), with bioactive properties, namely antimicrobial, antioxidant, and anticancer (De Aguiar Saldanha Pinheiro et al., 2021).

#### *1.5.3.1 Shrimps*

Shrimp by-products, such as heads and shells, constitutes a substantial portion of their BW, ranging from 48% to 56% (Sachindra et al., 2005), with CP content approximately from 200 to 500 g $kg^{-1}$ DM (Sachindra and Bhaskar, 2008; Yan and Chen, 2015), and rich in valine (4.56%-5.16% of protein content), lysine (3.13-6.65% of protein content), histidine (3.50-5.91% of protein content) and glutamic acid (8.38-10.9% of protein content; Ibrahim et al., 1999). Hence, shrimp by-products have the potential to be used in various applications, including animal feed, as protein-rich meals and hydrolysates, and as flavor enhancers (Abuzar et al., 2023). Moreover, unlike other crustaceans, shrimps have a thin exoskeleton, containing endogenous enzymes that facilitate the degradation of shells. While this degradation facilitates the reprocessing of shell material, it also contributes to environmental contamination by releasing potentially harmful substances into ecosystems during decomposition (Mathew et al., 2020). Therefore, managing shrimp waste disposal effectively is crucial to minimize the environmental risks associated with this process.

The effects of dietary inclusion of shrimp by-products on the growth performance and physiological responses of several aquaculture and livestock species are presented in Table 1.6. Contrasting results have been reported mostly associated with the inclusion level. For instance, shrimp hydrolysate at inclusion levels 1.5%-5% showed to enhance immune function, growth, and feed efficiency in olive flounder, striped catfish, sea bream, and European sea bass (Bui et al., 2014; Khosravi et al., 2018; Leduc et al., 2018a; Gunathilaka et al., 2020; Teoh and Wong, 2021), while at higher inclusion levels (30-40%) decreased growth in pike silverside (Ospina-Salazar et al., 2016).

Shrimp meal fed to lambs at 15% and 25% inclusion levels resulted in decreased growth and nutrient digestibility, particularly at 25%, but increased ruminal chitinolytic bacteria (Cobos et al., 2002). In chickens it revealed inconsistent results on growth and feed efficiency at approximately 20%-30% inclusion levels, while decreased growth at 12%-16%, increased growth at 5%, enhancing intestinal health at 5%-20% through

increased beneficial bacteria and reduced pathogenic bacteria (Rosenfeld et al., 1997; Okoye et al., 2005; Khempaka et al., 2006; Khempaka et al., 2011). Moreover, the use of fermented shrimp by-products in chicken diets enhanced carcass weight at 20% (Abun et al., 2022). Shrimp hydrolysates at 1%-8% inclusion levels showed to benefit growth, feed efficiency and immune responses in poultry (Mahata et al., 2008; Septinova et al., 2011; Parra et al., 2019; Nha and Thuy, 2021).

Overall, while some studies have demonstrated that shrimp hydrolysates and meals can positively influence growth, feed efficiency, and immune responses across a range of species, including fish, chicken, and lambs, particularly at lower inclusion levels, others indicate potential disadvantages, especially at higher inclusion levels, where growth may be negatively impacted. However, there is a lack of studies assessing the effects of shrimp by-products on dogs. Therefore, careful consideration of the type of shrimp by-product processing and source, and the appropriate inclusion level is crucial for optimizing their use in dog nutrition. Ultimately, the effective utilization of shrimp by-products could enhance dogs' health and performance and contribute to more sustainable environmental practices by reducing waste.

Table 1.6. Effects of shrimp by-products, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses of fish, and terrestrial animals.

| Source of ingredient | Animal model/species | Dietary inclusion levels, % | Effects | Reference |
|---|---|---|---|---|
| Shrimp hydrolysate (species not reported) | Olive flounder (*Paralichthys olivaceus*) | 3.34 | ↑ Superoxide dismutase | Khosravi et al. (2018) |
| | Striped catfish (*Pangasius hypophthalmus*) | 2 | ↑ Final weight and ↓ hematocrit levels | Teoh and Wong (2021) |
| Pacific white shrimp hydrolysate (*Litopenaeus vannamei*) | Sea bream (*Pagrus major*) | 4.8 | ↑ Feed conversion ratio, final weight, specific growth rate, protein efficiency ratio and digestibility of crude protein<br>↑ Plasma total immunoglobulin, and serum superoxide dismutase and antiprotease | Bui et al. (2014) |
| | European sea bass (*Dicentrarchus labrax*) | 5 | ↑ Final weight and specific growth rate | Leduc et al. (2018b) |
| | Olive flounder (*Paralichthys olivaceus*) | 1.5, 3 and 4.5 | ↑ Final weight at 3% and 4.5%<br>↑ Weight gain and feed conversion ratio at 4.5% | Gunathilaka et al. (2020) |
| Shrimp hydrolysate (*Pennaeus sp.*) | Pike silverside (*Chirostoma estor*) | 15, 30 and 45 | ↓ Weight gain and specific growth rates at 30 and 45% | Ospina-Salazar et al. (2016) |
| Shrimp meal (species not reported) | Lamb | 15 and 25 | ↓ Weight gain<br>↓ Final weight, total weight gain and dry matter, crude protein and neutral detergent fiber digestibility at 25%<br>↑ Ruminal chitinolytic bacteria concentration | Cobos et al. (2002) |
| | Chicken | 2-3.1, 4.1-6.2, 6.2-9.3, 8.3-12.4, 11.5-18.9, 15.3-25.2, 19.2-31.6, corresponding to 0, 20, 30, 40, 60, 80 and 100 of crude protein, respectively | ↑ Weight gain at 19.2-31.6% | Rosenfeld et al. (1997) |
| | Chicken | 10, 20 and 30 | ↓ Weight gain at 20% and 30% | Okoye et al. (2005) |

Table 1.6. Effects of shrimp by-products, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, immunological responses of fish, and terrestrial animals. (continued)

| Source of ingredient | Animal model/species | Dietary inclusion levels, % | Effects | Reference |
|---|---|---|---|---|
| Back tiger shrimp meal (*Penaeus monodon*) | Chicken | 4, 8, 12 and 16 | ↓ Body gain, final weight, feed efficiency and dry matter digestibility at 12% and 16% | Khempaka et al. (2006) |
| Pacific white shrimp meal (*Litopenaeus vannamei*) | Chicken | 5, 10, 15 and 20 | ↑ Weight gain at 5%<br>↑ Cecal butyric acid and intestinal Lactobacillus and ↓ intestinal *Escherichia coli* and cecal *Salmonella* sp. | Khempaka et al. (2011) |
| Fermented shrimp by-product (species not reported) | Chicken | 5, 10, 15 and 20 | ↑ Carcass weight at 20% | Abun et al. (2022) |
| Shrimp hydrolysate (species not reported) | Duck | 1, 2, 3 and 4 | ↑ Weight gain<br>↑ Feed conversion ratio at 3% and 4%<br>↑ Nitrogen retention at 2%, 3% and 4% | Nha and Thuy (2021) |
| HdrBbanana prawn hydrolysate (*Penaeus merguensis*) | Chicken | 4, 8 and 12 | ↓ Weight gain and nitrogen retention and feed conversion ratio at 12% | Mahata et al. (2008) |
| HydrolysBlack tiger shrimp hydrolysate (*Penaeus monodon*) | Chicken | 5 | ↑ Protein consumption and protein retention | Septinova et al. (2011) |
| Hydrolysa Pacific white shrimp hydrolysate (*Litopenaeus vannamei*) | Chicken | 0.5, 1.5, 2.5 and 5 | ↑ White blood cells at 1.5%, 2.5% and 5% | Parra et al. (2019) |

*1.5.3.2 Krill*

Krill are very small organisms, with Antarctic krill having a maximum weight of approximately 2 g, a high carotenoid content (320-500 g $kg^{-1}$ DM of CP), and a balanced AA profile, being rich in alanine (8.2%-9.6% of total AA), glycine (8.2%-9.3% of total AA), lysine (7.8%-8.3% of total AA), aspartic acid (9.9%-11.5% of total AA), and glutamic acid (12.0%-13.2% of total AA; Saether and Mohr, 1987; Nicol et al., 2000). Moreover, krill have shown to modulate the gut microbiota and the immune response in different fish species (Ringø et al., 2012). Similar to shrimp, krill contain enzymes that leads to rapid spoilage after death (Bykowski and Dutkiewicz, 1986). Therefore, the decomposition of krill can result in significant environmental impacts, including the release of potentially harmful substances into aquatic ecosystems. Among others, implementing processing techniques in waste can help reduce the ecological footprint of krill harvesting and contribute to more sustainable practices in marine resource management (Sun and Mao, 2016).

Krill meals and hydrolysates have been studied extensively for their effects on several aquatic species, particularly fish and shrimp, revealing a range of impacts on growth performance and feed efficiency (Table 1.7). In general, krill meal at 0.5% to 15% inclusion levels promoted growth and feed efficiency in Nile tilapia, gilthead seabream, Atlantic salmon, Atlantic cod, Atlantic halibut, European sea bass, and Oriental river prawn (Gaber, 2005; Olsen et al., 2006; Suontama et al., 2007; Hansen et al., 2011; Hatlen et al., 2017; Saleh et al., 2018; Torrecillas et al., 2021; Yan et al., 2023). At higher inclusion levels (approximately 27%-70%) the results were conflicting with either improved growth in Atlantic salmon, Atlantic cod, Atlantic halibut, and rainbow trout (Moren et al., 2006; Olsen et al., 2006; Suontama et al., 2007; Hansen et al., 2011; Roncarati et al., 2011; Hatlen et al., 2017; Torrecillas et al., 2021; Yan et al., 2023), or decreased growth in Atlantic salmon and rainbow trout (Yoshitomi et al., 2006; Hansen et al., 2010).

Krill hydrolysates at dietary inclusion levels of 5%-25% showed increased growth and feed efficiency in shrimp (Córdova-Murueta and García-Carreño, 2002), and improved growth, feed efficiency, and immune response in Atlantic salmon, olive flounder and sea bream at 1.9% to 4% inclusion levels (Kousoulaki et al., 2013; Bui et al., 2014; Khosravi et al., 2015).

Overall, the inclusion of krill meal and hydrolysate in aquaculture diets promoted growth and feed efficiency in a variety of species, though the effects can vary depending on the inclusion level and the species studied. Future research should explore the effects of krill

meal and hydrolysate on dogs, where studies are currently lacking. Investigating how krill meal and hydrolysates influence canine health and performance could reveal valuable insights and optimize their use in pet food. Moreover, evaluating the sustainable benefits of incorporating krill into pet food is essential. Krill is highly nutritious with a lower environmental impact compared to the conventional sources used, namely fish meals (Dragøy et al., 2024).

Table 1.7. Effects of krill by-products, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, and immunological responses of shrimp and fish.

| Source of ingredient | Animal model/species | Dietary inclusion levels, % | Effects | Reference |
|---|---|---|---|---|
| Krill meal (species not reported) | Nile tilapia (*Oreochromis niloticus*) | 1.5, 3, 4.5 and 6 | ↑ Final weight, weight gain, specific growth rate, feed intake, feed conversion ratio, protein efficiency ratio<br>↑ Protein digestibility at 3%, 4.5% and 6% | Gaber (2005) |
| | Gilthead seabream (*Sparus aurata*) | 3, 6 and 9 | ↑ Final weight at 9% | Saleh et al. (2018) |
| Antarctic krill meal (*Euphausia superba*) | Atlantic salmon (*Salmo salar*) | 13.5, 27, 40, 54 and 68 | ↑ Specific growth rate and feed conversion rate | Olsen et al. (2006) |
| | | 28.1, 30.3 and 34.8 | ↑ Specific growth rate | Moren et al. (2006) |
| | | 28 | ↑ Specific growth rate | Suontama et al. (2007) |
| | | 68.9 | ↓ Growth | Hansen et al. (2010) |
| | | 12.3, 25.7, 38.3 and 59.8 | ↑ Growth and weight gain<br>↓ Starch and lipid digestibility<br>plasma cholesterol | Hansen et al. (2011) |
| | | 7.5, 10 and 15 | ↑ Weight gain, specific growth rate and feed intake<br>↓ Protein and amino acid digestibility | Hatlen et al. (2017) |
| | Atlantic cod (*Gadus morhua*) | 28.1, 30.3 and 34.9 | No significant effects found on growth | Moren et al. (2006) |
| | Atlantic halibut (*Hippoglossus hippoglossus*) | 28 | ↑ Specific growth rate | Suontama et al. (2007) |
| | Rainbow trout (*Oncorhynchus mykiss*) | 60 | ↑ Weight gain and size | Roncarati et al. (2011) |
| | | 7, 15 and 30 | ↓ Weight gain, feed intake and specific growth rate at 30% | Yoshitomi et al. (2006) |
| | European sea bass (*Dicentrarchus labrax*) | 5 and 7.5 | ↑ Final weight, protein and lipid efficiency ratios, specific growth rate and feed conversion ratio | Torrecillas et al. (2021) |
| | Oriental river prawn (*Macrobrachium nipponense*) | 0.25, 0.5, 1 and 2 | ↑ Weight gain and specific growth rate at 0.5% and 1%<br>↑ Hemolymph albumin and ↓ alkaline phosphatase and malondialdehyde<br>↓ Alanine aminotransferase and aspartate aminotransferase at 1% | Yan et al. (2023) |

Table 1.7. Effects of krill by-products, according to animal model and dietary inclusion levels, on performance, feed intake, nutrient digestibility, blood parameters, and immunological responses of shrimp and fish. (continued)

| Source of ingredient | Animal model/species | Dietary inclusion levels, % | Effects | Reference |
|---|---|---|---|---|
| Artic krill meal (*Thysanoessa inermis*) | Atlantic salmon (Salmo salar) | 28.1, 30.3 and 34.8 | ↑ Specific growth rate | Moren et al. (2006) |
| | Atlantic cod (*Gadus morhua*) | 28.1, 30.3 and 34.9 | No significant effects found on growth | Moren et al. (2006) |
| Northern krill meal (*Meganyctiphanes norvegica*) | Atlantic salmon (Salmo salar) | 15, 30 and 46 | ↑ Specific growth rate | Suontama et al. (2007) |
| | Atlantic halibut (*Hippoglossus hippoglossus*) | 15, 30 and 46 | ↑ Specific growth rate | Suontama et al. (2007) |
| Krill hydrolysate (species not reported) | Olive flounder (Paralichthys olivaceus) | 3.12 | No significant effects found on growth and health | Khosravi et al. (2018) |
| Krill hydrolysate (*Euphasia* spp.) | Shrimp (*Penaeus vannamei*) | 5, 15 and 25 | ↑ Final weight and protein digestibility and ↓ feed conversion ratio | Córdova-Murueta and García-Carreño (2002) |
| Antarctic krill hydrolysate (*Euphausia superba*) | Atlantic salmon (Salmo salar) | 1.9 and 3.8 | ↑ Feed intake | Kousoulaki et al. (2013) |
| | Sea bream (*Pagrus major*) | 4 | ↑ Feed conversion ratio, final weight, specific growth rate, protein efficiency ratio and digestibility of crude protein<br>↑ Total immunoglobulin, superoxide dismutase and antiprotease | Bui et al. (2014) |
| | | 2 | ↑ Feed conversion ratio, final weight and digestibility of crude protein<br>↑ Lysozyme activity | Khosravi et al. (2015) |
| | Olive flounder (*Paralichthys olivaceus*) | 2 | ↑ Digestibility of crude protein and dry matter<br>↑ Nitro blue tetrazolium activity and superoxide dismutase | Khosravi et al. (2015) |

### 1.5.4 Conclusion and prospects

In general, the revised literature highlights the potential of fish, mollusks and crustaceans' by-products as sources of valuable nutrients and bioactive compounds for animal feeding. However, the *in vivo* effects of the inclusion of aquatic by-products in diets for aquaculture and livestock are broad and sometimes contrasting. This variability in the results underlines the importance of considering multiple factors, such as the by-product source, inclusion levels, processing methods and study conditions, when evaluating the effects of dietary inclusion of these ingredients. Despite the potential of these aquatic animal by-products, their inclusion in dog food and effects on dogs' health and performance are almost inexistent, turning undeniably the need for research on this topic.

Animal by-products are commonly used in dog food production and can be safely used in the production of foods (AAFCO, 2024). In fact, European Union legislation allows the use of crustaceans and mollusks in pet food (European Commission, 1982) and no constrains are imposed by the Association of American Feed Control Officials (AAFCO, 2023). However, recent research on alternative and more sustainable ingredients has primarily focused on the use of hydrolyzed terrestrial animal by-products and insects (Pinto et al., 2021, Rodríguez-Rodríguez et al., 2022, Valdés et al., 2022, Pinto et al., 2023). Incorporating protein sources such as aquatic animal by-products into pet food offers a promising approach to improving the sustainability of the pet food industry. By repurposing materials that would otherwise be considered waste, the industry can reduce the environmental impact, being cost-effective (Kok et al., 2020), while also benefiting from the nutritional and functional advantages these ingredients offer for canine health. Therefore, the effects of aquatic animal by-products on dogs' health and performance, their palatability, their contribution to the sustainability of the sector, and the processing methods employed are crucial factors to consider in future studies.

## 1.6 Objectives of the thesis

The growing pet population, coupled with the expansion of both pet food and aquaculture sectors, underscores the objectives of this thesis. In the present work, due to the current lack of knowledge on this matter, underexploited and more sustainable aquatic animal by-products were evaluated for inclusion in dog food. These sources hold potential for their high protein content, essential AA, and bioactive compounds that could contribute to improved dog health, while offering a possibility to minimize the negative environmental impact compared to conventional ingredients.

A first study was performed to assess two commercially available protein sources derived from aquatic animal by-products, squid meal and shrimp hydrolysate (Chapter 2). The chemical profile, namely proximate analysis and AA profile, and the *in vitro* antioxidant activity of extracts were assessed. Additionally, the palatability of squid meal and shrimp hydrolysate was assessed at 15% dietary inclusion level, and a short-term trial was conducted to evaluate the *in vivo* effects of protein sources at 5%, 10% and 15% levels on diet digestibility and metabolizable energy content, and fecal characteristics, metabolites, and microbiota composition of adult Beagle dogs.

Based on the results described in Chapter 2, a second study was conducted to evaluate the long-term effects of shrimp hydrolysate (Chapters 3 and 4). After the evaluation of the palatability of an extruded diet with 5% of shrimp hydrolysate, a feeding trial was conducted to assess the effects on diet digestibility and metabolizable energy content, fecal characteristics and metabolites, oral volatile sulfur compounds, coat quality (Chapter 3), hematological parameters, serum chemistry profile, innate and adaptive immune function, and fecal microbiota (Chapter 4).

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# CHAPTER 2

## Squid meal and shrimp hydrolysate as novel protein sources for dog food

**Joana Guilherme-Fernandes[1], Tiago Aires[2], António J. M. Fonseca[1], Timur Yergaliyev[3,4], Amélia Camarinha-Silva[3,4], Sofia A. C. Lima[1], Margarida R. G. Maia[1*], Ana R. J. Cabrita[1]**

[1]REQUIMTE, LAQV, ICBAS, School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal

[2]SORGAL, Sociedade de Óleos e Rações S.A., Lugar da Pardala, 3880-728 S. João Ovar, Portugal

[3]HoLMiR – Hohenheim Center for Livestock Microbiome Research, University of Hohenheim, Stuttgart, Germany

[4]Institute of Animal Science, University of Hohenheim, Stuttgart, Germany

* Present address: LEAF - Linking Landscape, Environment, Agriculture and Food Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

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**Abstract**

The world's growing pet population is raising sustainability and environmental concerns for the pet food industry. Protein-rich marine by-products might contribute to mitigating negative environmental effects, decreasing waste, and improving economic efficiency. The present study evaluated two marine by-products, squid meal and shrimp hydrolysate, as novel protein sources for dog feeding. Along with the analysis of chemical composition and antioxidant activity, palatability was evaluated by comparing a commercial diet (basal diet) and diets with the inclusion of 150 g $kg^{-1}$ of squid meal or shrimp hydrolysate using 12 Beagle dogs (2.2 ± 0.03 years). Two *in vivo* digestibility trials were conducted with six dogs, three experimental periods (10 days each) and three dietary inclusion levels (50, 100 and 150 g $kg^{-1}$) of squid meal or shrimp hydrolysate in place of the basal diet to evaluate effects of inclusion level on apparent total tract digestibility (ATTD), metabolizable energy content, fecal characteristics, metabolites, and microbiota. Both protein sources presented higher protein and methionine contents than ingredients traditionally used in dog food formulation. Shrimp hydrolysate showed higher antioxidant activity than squid meal. First approach and taste were not affected by the inclusion of protein sources, but animals showed a preference for the basal diet. Effects on nutrient intake reflected the chemical composition of diets, and fecal output and characteristics were not affected by the increasing inclusion levels of both protein sources. The higher ATTD of dry matter, most nutrients and energy of diets with the inclusion of both by-products when compared to the basal diet, suggests their potential to be included in highly digestible diets for dogs. Although not affected by the inclusion level of protein sources, when compared to the basal diet, the inclusion of squid meal decreased butyrate concentration and shrimp hydrolysate increased all volatile fatty acids, except butyrate. Fecal microbiota was not affected by squid meal inclusion, whereas inclusion levels of shrimp hydrolysate significantly affected abundances of Oscillosperaceae (UCG-005), Firmicutes and *Lactobacillus*. Overall, results suggest that squid meal and shrimp hydrolysate constitute novel and promising protein sources for dog food, but further research is needed to fully evaluate their functional value.

## 2.1 Introduction

The pet population is increasing worldwide, and, according to recent statistics, 45% of USA households own at least one dog (AVMA, 2022), while in Europe the number of households owning dogs ranges from 5% in Turkey to 49% in Poland (FEDIAF, 2023). As a result, the pet food industry is predicted to continue steadily developing (FEDIAF, 2023), raising concerns about the sustainability and environmental impact of ingredient

sources used in pet food production (Cucurachi et al., 2019). Protein sources are the most expensive both in environmental and economic terms, thus being the macronutrient requiring greater attention with regard to sustainability (Swanson et al., 2013). Moreover, as dogs are frequently seen as family members, pet owners are increasingly favoring palatable pet foods with high nutritional and functional values that ensure the welfare and health of their pets (Schleicher et al., 2019). Therefore, there is an undeniable need for alternative protein sources with lower environmental impact, while at the same time offering increased food palatability and nutritional and functional values, thus contributing to dogs' nutritional and health status and to the sustainability of the pet food sector.

Pet owner's mindset on food ingredients choice is also changing. According to a recent survey (GlobalPETS, 2023), only 19% of respondents would continue to buy pet foods with conventional protein sources, while 54% of respondents were interested in foods containing by-products, although 66% of them were not familiar with the term "by-products". Increasing interest has emerged in alternative protein sources from terrestrial (Mevliyaoğulları et al., 2023; Shields et al., 2023; Sieja et al., 2023; Smith and Aldrich, 2023) and aquatic (Folador et al., 2006; Cabrita et al., 2023) origin to replace conventional terrestrial ones due to their high nutritive value and lower environmental impact (Hilborn et al., 2018; Newton et al., 2023). Under a circular economy perspective, the utilization of by-products from aquatic sources offers additional advantages as it contributes to the reduction of waste and food-feed competition, and to a greater economic and environmental efficiency (Olsen et al., 2014; Stevens et al., 2018). This approach is particularly important as the volume of waste from aquatic production was reported to range from 1% to 20% for fish, from 40% to 85% for crustaceans, and from 60% to 80% for mollusks (Roy et al., 2023). The use of by-products from aquatic resources, namely crustaceans and mollusks, with high protein content and bioactive compounds (Olsen et al., 2014; Al Khawli et al., 2020), such as carotenoids, glycosaminoglycans, bioactive peptides, and chitin/chitosan (Al Khawli et al., 2020), which may have antioxidant, anticoagulant, antibacterial, anticancer, anti-inflammatory, and antimicrobial effects (Al Khawli et al., 2020; Ghosh et al., 2022), could be appealing to dog owners who are looking for functional pet foods (de Godoy et al., 2016). Furthermore, as novel protein sources, squid meal and particularly shrimp hydrolysate may play a role in the diagnosis of adverse food reactions and in the prevention of allergic reactions due to food hypersensitivity (Verlinden et al., 2006).

Although squid meal and shrimp hydrolysate have been studied as alternative feeds in livestock and aquaculture species, to the best of authors' knowledge, there are no studies

on the use of these by-products in dog feeding. Therefore, the aim of the current study was to evaluate the chemical composition and antioxidant activity of squid meal and shrimp hydrolysate and the effects of increasing levels of their dietary inclusion on palatability, digestibility, fecal characteristics, metabolites, and microbiota of healthy adult Beagle dogs, contributing to the recent trend in the pet food industry to provide more sustainable high protein diets with novel protein sources.

## 2.2 Materials and Methods

Trials were approved by the Animal Ethics Committee of School of Medicine and Biomedical Sciences, University of Porto (Permit Nº 344). Procedures and animal care were carried out by scientists trained by FELASA, category C, and in line with the recommendations on the ethical use of animals for scientific purposes (European Union Directive 2010/63/EU). Animals were clinically examined to ensure their suitability to participate in the studies. All dogs received regular vaccinations and were treated for endoparasites.

### 2.2.1 Animals and housing

Twelve Beagle dogs, six males and six females (2.2 ± 0.03 years-old; 12.6 ± 1.55 kg initial body weight, BW) were used in the palatability and digestibility assays. Sample size followed the minimum number of animals recommended for digestibility trials (FEDIAF, 2021). Animals were housed in pairs in environmentally enriched communicating boxes with sliding doors to allow their individual feeding, and with inside and outside areas of 1.8 and 3.5 $m^2$, respectively. Animals were allowed to daily exercise and socialize between meals in an outdoor park and had at least 30 min leash walks. During the feces collection period of the digestibility assays, animals were housed individually, had daily access to the outdoor park between meals under supervision and leash walked for at least 30 min. Kennel temperature and relative humidity were monitored daily.

### 2.2.2 Protein sources and experimental diets

Squid meal was provided by Inproquisa (Madrid, Spain) and comprises a by-product from the canning industry of *Dosidicus gigas* obtained through steaming and pressing for oil extraction. Shrimp hydrolysate, provided by Symrise Aqua Feed (Elven, France), resulted from the enzymatic hydrolysis of heads and cephalothoraxes of *Litopenaeus vannamei*. Both marine by-products were provided as a dry powder and kept at room temperature until use.

A commercial extruded complete diet formulated for medium size adult dogs containing (label information), animal meals, vegetable by-products, oils and fats, and beet pulp without the inclusion of squid meal and shrimp hydrolysate was used as the basal diet (SilverDog, Sorgal Pet Food, Ovar, Portugal). The experimental diets included 50, 100 or 150 g $kg^{-1}$ of squid meal in experiment 1 (SM5, SM10, and SM15) and shrimp hydrolysate in experiment 2 (SH5, SH10, and SH15) in place of the basal diet. The studied protein sources were thoroughly mixed with the basal diet shortly before being offered to each dog. During the digestibility trials all dogs consumed the total daily food offered.

### 2.2.3 Palatability assays

Two-bowl tests (Aldrich and Koppel, 2015) were conducted to evaluate the palatability by the pairwise comparison of the basal diet with either the experimental diet SM15 or SH15. In two consecutive days and after an overnight fast, animals (n=12) were offered the choice between the two diets in two bowls placed in opposite positions (left and right, 45 cm apart) each containing half amount of the daily food allowance calculated to supply the metabolizable energy (ME) requirements of dogs (FEDIAF, 2021). The bowls were placed in alternated positions between days to control side bias. The first bowl approached and the first food tasted in each trial were recorded. Trials ended after 30 min or when the animals had consumed all the food available in a bowl. The food offered and the food refusals were weighed to calculate the ratio of consumption of the two diets.

### 2.2.4 Digestibility assays

The method of total fecal collection was used to assess the apparent total tract digestibility (ATTD) of the basal and experimental diets. The *in vivo* ATTD of the basal diet was determined previously to experiments 1 and 2, using 12 animals for 10 days (5 days for adaptation and 5 days for feces collection), as recommended by FEDIAF and earlier described (Cabrita et al., 2023). The two digestibility trials performed to evaluate the effects of inclusion levels of squid meal and shrimp hydrolysate were designed according to a replicated Latin square 3 x 3, with six animals (three males and three females, from the 12 animals used for the determination of the *in vivo* digestibility of the basal diet), three experimental periods of 10 days (5 days for adaptation to the diet and 5 days for total feces collection) and three dietary inclusion levels (50, 100 or 150 g $kg^{-1}$).

At the beginning of each adaptation period and prior to the morning feeding, the animals were weighed and the body condition was assessed according to a 9 point-scale, with 5 considered the ideal body condition score (BCS, Laflamme, 1997). Daily food allowance

was defined according to the ME requirements considering the ideal BW of individuals, ME (kcal/day) = 110 × $BW^{0.75}$ (FEDIAF, 2021), and adjusted to body condition score. Animals were individually fed their daily ration in two equal meals (8.30 a.m. and 5.00 p.m.). Fresh water was provided *ad libitum*. During the feces collection periods, the number of defecations were recorded and individual fresh feces were weighed and scored with a 5-point scale to evaluate the consistency of stools, with score (1) reflecting watery diarrhea, (3.5) firm, shaped, and dry stools, and (5) powdery hard mass pellets (Félix et al., 2013). Diarrhea was scored from 1 to 2, according to the scale. Fecal samples were mixed, subsampled, and stored in plastic bags at -20 °C until analysis of chemical composition, pH, ammonia-N and volatile fatty acids (VFA) concentrations and fecal microbiota. Analyses were carried out in fecal samples pooled per dog and period.

### 2.2.5 Analytical procedures

#### *2.2.5.1 Proximate analysis*

Protein sources, basal diet and fecal samples were dried until constant weight in an air-forced oven at 65 °C, 1-mm milled, and analyzed in duplicate, according to official methods (AOAC, 2005), as previously described (Pereira et al., 2021a). Samples were analyzed for dry matter (DM; ID 934.01), ash (ID 942.05), ether extract (EE; ID 920.39), and Kjeldahl N (ID 990.03; in fresh feces samples). Crude protein (CP) was calculated as Kjeldahl N × 6.25. Gross energy (GE) analysis was performed with an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). The basal diet was also analyzed for neutral detergent fiber (with α-amylase, without sodium sulfite, and expressed exclusive of residual ash, NDF; Van Soest et al., 1991), and for starch (in 0.5-mm milled samples; Salomonsson et al., 1984) contents.

Amino acids (AA) were determined as described by Aragão et al. (2020). Briefly, samples were hydrolyzed with 6 M HCl solution at 116 °C for 48 h. Precolumn derivatization was performed according to the AccQ Tag method (Waters, Milford, MA, USA) using the Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and the analyses carried out by ultra-high-performance liquid chromatography on a Waters reversed-phase AA analysis system with norvaline as the internal standard. Peaks were then analyzed with EMPOWER software (Waters). The analyses were carried out in duplicate.

#### *2.2.5.2 Antioxidant activity assays*

Squid meal and shrimp hydrolysate extracts were prepared, in quadruplicate, as reported by Zaharah and Rabeta (2017). A volume of 20 mL of Milli-Q water was added to 2 g of squid meal and to 0.8 g of shrimp hydrolysate. Samples were then incubated in an orbital

shaker overnight, in the dark, at 160 rpm and 27 °C, and centrifuged for 30 min at 2500 rpm at 20 °C. The supernatant was collected and diluted to obtain a final concentration based on the initial dry weight and the final supernatant volume yielding 2.5 mg $mL^{-1}$ for squid meal extracts and 1 mg $mL^{-1}$ for shrimp hydrolysate. The antioxidant activity was determined through the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization test (ABTS assay; Barbosa et al., 2019), the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH assay; Esposito et al., 2020), the ferric reducing antioxidant power (FRAP assay), and the Folin-Ciocalteu reducing capacity (FC assay; Agregán et al., 2018). For the ABTS assay, ABTS solution was prepared with equal volumes of 7 mM ABTS (Sigma-Aldrich, A1888, Saint Louis, MO, USA) and 2.45 mM potassium persulfate solution and left overnight in dark at room temperature. ABTS solution was diluted in water to achieve an absorbance of 0.90 ± 0.02 at 734 nm (Conde et al., 2021). In a 96 well plate, 50 µL of ABTS was added to 50 µL of each sample and solvent (blank), and absorbance was assessed after an incubation period of 30 min at 25 °C in the dark. For the DPPH assay, a 0.2 mM DPPH solution (Sigma-Aldrich, D9132) was freshly prepared with methanol. In a 96 well plate, 100 µL of DPPH solution was added to 100 µL of each sample and solvent (blank). Absorbance was read at 517 nm after an incubation period of 30 min in the dark at 22 °C. For the FRAP assay, a fresh FRAP solution was prepared by mixing 10 mM TPTZ stock solution, acetate buffer (300 mM at pH of 3.6), and 20 mM $FeCl_3$ solution in a proportion of 10:1:1 (v/v/v). Samples were incubated for 30 min at 37 °C in the dark in a 96 well plate, preceding the addition of 300 µL of FRAP solution to 10 µL of samples diluted in 30 µL of Milli-Q water. The absorbance was read at 593 nm. Finally, for the FC assay, in a 96 well plate, 12 µL of Folin-Ciocalteu phenol reagent (Sigma-Aldrich, F9252) was added to 15 µL of samples diluted in 170 µL of Milli-Q water, followed by the addition of 30 µL of $Na_2CO_3$ solution (10% w/v). After a period of 1 h of incubation in the dark at room temperature, 73 µL of Milli-Q water was added to each well and absorbance was read at 765 nm. Absorbance of samples, solvent (blank), and standard solution (quercetin from Sigma-Aldrich, Q4951) were measured using a Synergy™ HT Multimode plate reader (BioTek® Instruments Inc., Winooski, VT, USA). Analyses were performed in triplicate. A calibration curve with the standard solution was performed in all assays and the results were expressed in milligram of quercetin equivalents per gram of DM (mg Q $g^{-1}$ DM).

### 2.2.6 Fecal end-fermentation products

The fecal pH was measured with a potentiometer (pH and Ion-Meter GLP 22, Crison, Barcelona, Spain) after dilution of thawed feces to 1:10 (w/v) in water and incubation for

10 min in a sonication bath at room temperature. The concentration of fecal ammonia-N was determined according to the methodology of Chaney and Marbach (1962) adapted to dog feces. Briefly, fecal samples were thawed, diluted to 1:10 (w/v) in 2 M KCl and centrifuged at 5200 × g at 4 °C for 60 min. The supernatant was filtered with a 0.45 µm pore size polyethersulfone syringe filter (FILTER-LAB, Barcelona, Spain). Forty µL of water were added to 40 µL of sample, followed by the addition of 2.5 mL of phenol solution and 2 mL of 0.37% alkaline hypochlorite solution. Samples were firstly incubated for 10 min at 37 °C, followed by 40 min at 22 °C in the dark. An ammonia solution (32 mg $dL^{-1}$) was used as standard. The absorbance was read at 550 nm in a Synergy™ HT Multimode plate reader (BioTek® Instruments Inc.). Analysis was done in duplicate.

For VFA analysis, fecal samples were diluted to 1:10 (w/v) in 25% ortho-phosphoric acid solution with an internal standard (4 mM 3-methyl valerate, Sigma-Aldrich), and centrifuged for 60 min at 2360 × g at 4 °C. The supernatant was filtered with a 0.45 µm pore size polyethersulfone syringe filter (FILTER-LAB) and analyzed by gas chromatography as described by Pereira et al. (2021b).

### 2.2.7 Fecal microbiota

DNA from fecal samples was extracted by Fast DNA™ Spin Kit for soil and quantified using a NanoDrop 2000 spectophotometer (Thermo Scientific, Waltham, MA, USA). For bacterial amplicons library preparation, V1–V2 hypervariable regions of the 16S rRNA gene were amplified (Kaewtapee et al., 2017). Unique barcodes (6-nt) were linked to forward primers, while index adapters were attached to reverse. 16S library was created by two rounds of polymerase chain reaction (PCR). In short, 1 µL of extracted DNA was used for the first round of PCR, in a total of 20 µL reaction mix volume, with 0.2 µL of PrimeSTAR HS DNA polymerase and 0.5 µL of each forward and reverse primers (in the concentration of 0.2 µM each). The second round of PCR was performed with 1 µL of the first PCR product with a total volume of 50 µL. An initial denaturation was performed at 95 °C for 3 min and was followed by 15 cycles for the first round of PCR and 20 cycles for the second, with denaturation at 98 °C (10 sec) and subsequent annealing at 55 °C (10 sec), elongation at 72 °C (45 sec) and a final extension at 72 °C (2 min). Library normalization was carried out by the SequalPrepTM Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) and sequencing was performed with the 250 bp paired-end Illumina NovaSeq 6000 platform.

Raw sequences were demultiplexed with Sabre (https://github.com/najoshi/sabre). Downstream analyses were implemented using Qiime2 (Bolyen et al., 2019).

Primers/adapters trimming was performed by the q2-cutadapt plugin (Martin, 2011). Denoising, quality filtering, merging of paired reads, and chimeras removal were completed by the q2-dada2 (Callahan et al., 2016). Taxonomy assignation of amplicon sequence variants (ASVs) was performed with VSEARCH-based consensus (Rognes et al., 2016) and pre-fitted sklearn-based classifiers (Pedregosa et al., 2012) against the Silva database (v138.1, 16S 99%; Quast et al., 2013). The reference reads were preprocessed by RESCRIPt (Robeson et al., 2021). A phylogenetic tree was built by the q2-phylogeny, utilizing MAFFT (v7.3; Katoh and Standley, 2013) and FastTree (v2.1; Price et al., 2010). Alpha diversity was assessed by Shannon's entropy (Shannon, 1948) and Faith's phylogenetic diversity (Faith, 1992) indices, and beta diversity by Jaccard (Jaccard, 1912) and Bray-Curtis (Bray and Curtis, 1957) distances. Beta diversity ordination was performed by principal-coordinate analysis (PCoA; Halko et al., 2011). Alpha diversity metrics were compared by the Wilcoxon test (Wilcoxon, 1945), and beta diversity distances by the Adonis test (999 permutations; Anderson, 2001). Differentially abundant genera (only for counts of genera with relative abundance ≥ 1% and prevalence ≥ 10%) were detected by ALDEx2 (Fernandes et al., 2013). All *P*-values obtained from multiple comparisons were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Raw sequences are available at the European Nucleotide Archive (ENA) under accession number PRJEB71521.

### 2.2.8 Calculations and statistical analysis

A chi-square test was used to analyze the first approach and first taste results from the two-bowl tests, being the ratio of consumption analyzed through a paired *t*-test, both at 5% probability level (n=12). Fecal production was calculated as:

$$\text{Fecal production (\%)} = \frac{\text{dried fecal output } (\frac{g}{d})}{\text{dry matter intake } (\frac{g}{d})} \; x\; 100$$

The ATTD of the basal and experimental diets was determined using the following equation:

$$\text{ATTD (\%)} = \frac{\text{nutrient intake } \left(\frac{g}{d}\right) \text{ - fecal nutrient } (\frac{g}{d})}{\text{nutrient intake } (\frac{g}{d})} \; x\; 100$$

Metabolizable energy content of diets was calculated according to FEDIAF (2021), as follows:

$$\text{ME (MJ/kg DM)} = \frac{(\text{GE intake } \left(\frac{\text{MJ}}{\text{d}}\right) - \text{fecal GE } \left(\frac{\text{MJ}}{\text{d}}\right)) - (\text{CP intake } (\frac{\text{g}}{\text{d}}) - \text{fecal CP } \left(\frac{\text{g}}{\text{d}}\right)) \times 5.23}{\text{DM intake } (\frac{\text{g}}{\text{d}})}$$

For each digestibility trial, data on BW, BCS, diet and nutrient intake, ATTD, ME content, fecal output and characteristics, and fecal metabolites were analyzed according to a replicated 3 x 3 Latin square considering the fixed effects of square, dog within the square, period, inclusion level of protein source and the residual error (SAS, 2022, release 3.81., SAS Institute Inc., Cary, NC, USA). Means were compared by the least significant difference test when significant differences ($P < 0.05$) among experimental diets were found. A paired *t*-test was performed to compare the basal diet with experimental diets with inclusion of squid meal or shrimp hydrolysate (SAS, 2022, release 3.81.) to mimic the at-home scenario of dog owners changing the diet of their animals, thus understand the perceived effects. For that, data from the six dogs collected during the digestibility trial with each studied protein source were used for comparison with the values obtained for the same animals during the digestibility trial on the basal diet.

## 2.3 Results

### 2.3.1 Chemical composition

The proximate composition of protein sources, basal diet, and experimental diets with increasing levels of squid meal and shrimp hydrolysate is shown in Table 2.1. Compared to shrimp hydrolysate, squid meal presented a higher content of CP (658 g $kg^{-1}$ DM and 810 g $kg^{-1}$ DM, respectively), and lower ash (151 g $kg^{-1}$ DM and 103 g $kg^{-1}$ DM) and EE (93.8 g $kg^{-1}$ DM and 31.2 g $kg^{-1}$ DM) contents. The basal diet presented 252 g $kg^{-1}$ CP (DM basis) and 91.4 g $kg^{-1}$ (DM basis) of EE. The chemical composition of the experimental diets of both experiments reflected the chemical composition of the basal diet and the studied protein sources.

Squid meal and shrimp hydrolysate presented 652 g $kg^{-1}$ DM and 445 g $kg^{-1}$ DM total AA content, being the main essential and non-essential AA found in both studied protein sources respectively arginine, lysine and leucine, and glutamic acid plus glutamine and tyrosine (Table 2.2).

Table 2.1. Proximate composition (g $kg^{-1}$ dry matter, DM) and gross energy (MJ $kg^{-1}$ DM) of protein sources, basal diet, and experimental diets with increasing levels of inclusion of squid meal (Experiment 1) and shrimp hydrolysate (Experiment 2) in substitution of the basal diet.

| | Protein sources | | Basal diet | Experiment 1[1] | | | Experiment 2[2] | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | Squid meal | Shrimp hydrolysate | | SM5 | SM10 | SM15 | SH5 | SH10 | SH15 |
| DM, g $kg^{-1}$ | 937 | 962 | 924 | 925 | 925 | 926 | 926 | 928 | 930 |
| Ash | 103 | 151 | 125 | 124 | 123 | 122 | 126 | 128 | 129 |
| Crude protein | 810 | 658 | 252 | 280 | 308 | 336 | 272 | 293 | 313 |
| Ether extract | 31.2 | 93.8 | 91.4 | 88.4 | 85.4 | 82.4 | 91.5 | 91.6 | 91.8 |
| Neutral detergent fiber | ND | ND | 228 | 217 | 205 | 194 | 217 | 205 | 194 |
| Starch | ND | ND | 311 | 295 | 280 | 264 | 295 | 280 | 264 |
| Gross energy | 21.0 | 21.3 | 183 | 175 | 167 | 159 | 175 | 167 | 159 |

[1]SM5, diet with 50 g $kg^{-1}$ inclusion of squid meal; SM10, diet with 100 g $kg^{-1}$ inclusion of squid meal; SM15, diet with 150 g $kg^{-1}$ inclusion of squid meal.
[2]SH5, diet with 50 g $kg^{-1}$ inclusion of shrimp hydrolysate; SH10, diet with 100 g $kg^{-1}$ inclusion of shrimp hydrolysate; SH15, diet with 150 g $kg^{-1}$ inclusion of shrimp hydrolysate.
ND, reaction not detected.

Table 2.2. Total, essential and non-essential amino acids (g $kg^{-1}$ dry matter, DM, basis) of protein sources, basal diet, and experimental diets with increasing levels of inclusion of squid meal (Experiment 1) and shrimp hydrolysate (Experiment 2) in substitution of the basal diet.

| Item | Protein sources | | Basal diet | Experiment 1[1] | | | Experiment 2[2] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Squid meal | Shrimp hydrolysate | | SM5 | SM10 | SM15 | SH5 | SH10 | SH15 |
| Essential amino acids | | | | | | | | | |
| Arginine | 57.9 | 31.6 | 20.50 | 22.4 | 24.2 | 26.1 | 21.1 | 21.6 | 22.2 |
| Histidine | 14.0 | 8.8 | 6.43 | 6.81 | 7.19 | 7.56 | 6.55 | 6.67 | 6.79 |
| Lysine | 54.4 | 38.5 | 16.00 | 17.9 | 19.8 | 21.8 | 17.1 | 18.3 | 19.4 |
| Threonine | 27.9 | 17.5 | 10.50 | 11.4 | 12.2 | 13.1 | 10.9 | 11.2 | 11.6 |
| Isoleucine | 26.0 | 17.6 | 9.63 | 10.4 | 11.3 | 12.1 | 10.0 | 10.4 | 10.8 |
| Leucine | 45.7 | 30.3 | 21.20 | 22.4 | 23.7 | 24.9 | 21.7 | 22.1 | 22.6 |
| Valine | 33.1 | 30.0 | 16.20 | 17.0 | 17.9 | 18.7 | 16.9 | 17.6 | 18.3 |
| Methionine | 29.7 | 16.3 | 3.68 | 4.98 | 6.28 | 7.58 | 4.31 | 4.95 | 5.58 |
| Methionine+cystine | 34.1 | 18.9 | 8.24 | 9.53 | 10.8 | 12.1 | 8.77 | 9.30 | 9.84 |
| Phenylalanine | 27.1 | 24.0 | 12.2 | 12.9 | 13.7 | 14.4 | 12.8 | 13.4 | 14.0 |
| Phenylalanine+tyrosine | 84.1 | 53.9 | 19.0 | 22.3 | 25.5 | 28.8 | 20.8 | 22.5 | 24.2 |
| Total | 316 | 215 | 116 | 126 | 136 | 146 | 121 | 126 | 131 |
| Non-essential amino acids | | | | | | | | | |
| Cystine | 4.41 | 2.54 | 4.56 | 4.55 | 4.55 | 4.54 | 4.46 | 4.36 | 4.26 |
| Tyrosine | 57.0 | 29.9 | 6.82 | 9.33 | 11.8 | 14.4 | 7.97 | 9.13 | 10.3 |
| Aspartic acid + Asparagine | 42.4 | 29.3 | 21.8 | 22.8 | 23.9 | 24.9 | 22.2 | 22.6 | 22.9 |
| Glutamic acid + Glutamine | 75.8 | 53.4 | 39.9 | 41.7 | 43.5 | 45.3 | 40.6 | 41.2 | 41.9 |
| Alanine | 34.9 | 34.2 | 19.4 | 20.2 | 21.0 | 21.7 | 20.1 | 20.9 | 21.6 |
| Glycine | 41.2 | 30.5 | 26.7 | 27.4 | 28.2 | 28.9 | 26.9 | 27.1 | 27.3 |
| Proline | 51.6 | 34.3 | 24.8 | 26.1 | 27.5 | 28.8 | 25.3 | 25.7 | 26.2 |
| Serine | 28.6 | 16.7 | 15.5 | 16.2 | 16.8 | 17.5 | 15.6 | 15.6 | 15.7 |
| Total | 336 | 231 | 159 | 168 | 177 | 186 | 163 | 167 | 170 |

[1]SM5, diet with 50 g $kg^{-1}$ inclusion of squid meal; SM10, diet with 100 g $kg^{-1}$ inclusion of squid meal; SM15, diet with 150 g $kg^{-1}$ inclusion of squid meal.
[2]SH5, diet with 50 g $kg^{-1}$ inclusion of shrimp hydrolysate; SH10, diet with 100 g $kg^{-1}$ inclusion of shrimp hydrolysate; SH15, diet with 150 g $kg^{-1}$ inclusion of shrimp hydrolysate.

### 2.3.2 Antioxidant activity

The antioxidant activity of shrimp hydrolysate was higher than that of squid meal in all the assays performed (19.8 vs. 4.35 mg Q $g^{-1}$ DM for ABTS, 10.4 vs. 1.58 mg Q $g^{-1}$ DM for FC, and 2.27 vs. 0.36 mg Q $g^{-1}$ DM for FRAP; Table 2.3). No reaction in the DPPH assay was observed with squid meal extract.

Table 2.3. Antioxidant activity of protein sources extracts expressed in milligram of quercetin, Q, per gram of dry matter.

| Protein sources extracts | Quercetin equivalent | | | |
|---|---|---|---|---|
| | ABTS assay | DPPH assay | FC assay | FRAP assay |
| Squid meal | 4.35±0.23 | ND | 1.58±0.09 | 0.36±0.03 |
| Shrimp hydrolysate | 19.8±0.03 | 1.90±0.120 | 10.4±0.23 | 2.27±0.084 |

Data expressed as mean ± SD; ND, reaction not detected.

### 2.3.3 Palatability assays

The results of the two-bowl tests are shown in Figure 2.1. No differences were found on first diet approached and tasted in both tests. The consumption of either SM15 (22.9%) or SH15 (24.5%) was significantly lower ($P < 0.05$) in comparison with the consumption of the basal diet (77.4% and 75.5%, respectively for squid meal and shrimp hydrolysate tests).

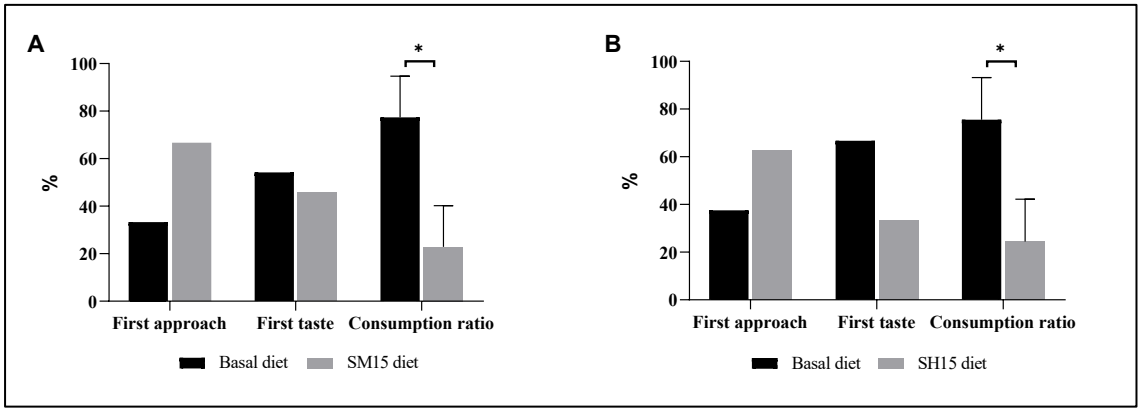


Figure 2.1. Frequency of first approach and first taste (mean, n = 12), and consumption ratio (mean ± SEM, n = 12) of basal diet in comparison with either SM15 diet (A) or SH15 diet (B) in the two-bowl tests. *$P < 0.05$.

### 2.3.4 Digestibility assays

Dogs remained healthy throughout the studies with no episodes of emesis, diarrhea, and food refusal.

#### *2.3.4.1 Experiment 1. Squid meal*

Increasing levels of inclusion of squid meal kept unaffected diet intake, and significantly increased ($P < 0.05$) intake of organic matter (OM), CP and GE while decreasing EE intake (Table 2.4). No effects of squid meal inclusion levels were observed on fecal output and characteristics, and ATTD of DM, nutrients and energy and ME content, except for ATTD of CP that was significantly higher ($P = 0.024$) in SM15 diet (79.1%). Fecal metabolites were not affected by the level of squid meal inclusion, with the only exception of fecal pH, that was the highest with SM15 and the lowest with SM10 ($P = 0.024$).

Compared to the basal diet, the dietary inclusion of squid meal significantly increased ($P < 0.05$) DM intake and ATTD of DM, OM, CP, and GE as well as ME content, but decreased EE intake and fecal output. Fecal DM and GE content, consistency score, pH and butyrate content were significantly lower ($P < 0.05$) whereas ammonia-N concentration and acetate:propionate ratio were higher in diets with squid meal inclusion compared to the basal diet (Table 2.4).

#### *2.3.4.2 Experiment 2. Shrimp hydrolysate*

Increasing levels of inclusion of shrimp hydrolysate significantly increased ($P < 0.05$) the intake of DM, OM, CP, EE, and GE, but decreased fecal GE, with no differences being observed on ATTD of DM, nutrients, and energy, ME content, and fecal output, characteristics, and metabolites (Table 2.5). Compared to the basal diet, the inclusion of shrimp hydrolysate significantly increased ($P < 0.05$) intake of DM, CP, EE, and GE, ATTD of DM, OM, CP, and GE, ME content, total VFA production and the concentration of the individual VFA (except butyrate) and decreased fecal output, consistency score and the number of defecations (Table 2.5).

Table 2.4. Experiment 1. Body weight of animals, diet, nutrient and energy intake, apparent total tract digestibility (ATTD), metabolizable energy content, fecal output and characteristics, and fecal metabolites of dogs fed experimental diets with squid meal inclusion and basal diet (n = 6).

| Item | Experimental diets[1] | | | SEM | *P*-value | Basal diet | Basal diet vs. Experimental diets[2] |
|---|---|---|---|---|---|---|---|
| | SM5 | SM10 | SM15 | | | | *P*-value |
| Body weight | 13.1 | 12.7 | 12.6 | 0.27 | 0.479 | 12.5±1.73 | 0.167 |
| Body condition score | 5.50 | 5.33 | 5.33 | 0.192 | 0.785 | 5.33±0.471 | 0.717 |
| Diet intake | | | | | | | |
| g diet $d^{-1}$, as-is | 316.6 | 316.6 | 316.5 | 0.13 | 0.730 | 316.7±42.13 | 0.071 |
| g diet $d^{-1}$, dry matter (DM) | 292.7 | 292.9 | 293.0 | 0.13 | 0.199 | 292.6±38.92 | 0.020 |
| Nutrient intake, g $d^{-1}$ | | | | | | | |
| Organic matter | 256.3$^{a}$ | 256.9$^{b}$ | 257.3$^{c}$ | 0.12 | 0.002 | 255.9±34.04 | <0.001 |
| Crude protein | 82.0$^{a}$ | 90.4$^{b}$ | 98.7$^{c}$ | 0.50 | <0.001 | 73.7±9.81 | <0.001 |
| Ether extract | 25.9$^{c}$ | 25.0$^{b}$ | 24.1$^{a}$ | 0.05 | <0.001 | 26.7±3.56 | <0.001 |
| Gross energy, MJ $d^{-1}$ | 5.40$^{a}$ | 5.45$^{b}$ | 5.49$^{c}$ | 0.004 | <0.001 | 5.36±0.007 | <0.001 |
| Fecal output and characteristics | | | | | | | |
| g feces $d^{-1}$, as-is | 285 | 279 | 270 | 6.0 | 0.268 | 300±56.4 | 0.006 |
| g feces $d^{-1}$, DM | 85.1 | 86.0 | 81.8 | 2.12 | 0.378 | 93.0±13.67 | 0.001 |
| Gross energy, MJ $d^{-1}$ | 1.18 | 1.24 | 1.14 | 0.046 | 0.346 | 1.36±0.214 | <0.001 |
| Production, % | 28.9 | 29.4 | 27.9 | 0.73 | 0.388 | 31.8±2.33 | 0.001 |
| DM, % | 30.4 | 30.9 | 30.7 | 0.26 | 0.401 | 31.8±1.72 | 0.002 |
| Consistency score | 3.29 | 3.31 | 3.09 | 0.061 | 0.065 | 3.35±0.297 | 0.021 |
| Defecations no. $d^{-1}$ | 2.60 | 2.43 | 2.50 | 0.098 | 0.508 | 2.63±0.243 | 0.207 |
| ATTD, % | | | | | | | |
| DM | 71.1 | 71.0 | 73.3 | 0.72 | 0.099 | 68.2±2.33 | <0.001 |
| Organic matter | 76.9 | 76.6 | 78.4 | 0.64 | 0.173 | 74.4±2.13 | <0.001 |
| Crude protein | 74.8$^{a}$ | 75.7$^{a}$ | 79.1$^{b}$ | 0.91 | 0.024 | 69.2±3.90 | <0.001 |
| Ether extract | 90.6 | 90.2 | 90.9 | 0.39 | 0.463 | 91.1±0.99 | 0.067 |
| Gross energy | 78.3 | 77.2 | 79.3 | 0.80 | 0.245 | 74.6±2.38 | <0.001 |
| Metabolizable energy, MJ $kg^{-1}$ | 13.3 | 13.1 | 13.5 | 0.14 | 0.317 | 12.8±0.39 | <0.001 |
| Fecal metabolites, mg $kg^{-1}$, DM | | | | | | | |
| pH | 6.87$^{a,b}$ | 6.82$^{a}$ | 6.94$^{b}$ | 0.025 | 0.024 | 6.96±0.122 | 0.006 |
| Ammonia-N | 175 | 183 | 170 | 10.2 | 0.690 | 138±13.5 | <0.001 |
| VFA | | | | | | | |
| Total | 785 | 729 | 703 | 0.1 | 0.443 | 766±81.0 | 0.659 |
| Acetate | 471 | 437 | 427 | 0.1 | 0.486 | 447±54.9 | 0.965 |
| Propionate | 211 | 202 | 188 | 0.1 | 0.532 | 209±23.5 | 0.629 |
| Butyrate | 68.5 | 59.9 | 57.3 | 0.01 | 0.157 | 78.3±16.11 | 0.011 |
| *Iso*-butyrate | 11.7 | 10.3 | 9.21 | 0.001 | 0.218 | 10.6±2.11 | 0.867 |
| *Iso*-valerate | 14.8 | 13.3 | 12.4 | 0.01 | 0.405 | 11.3±1.93 | 0.111 |
| Valerate | 2.42 | 2.76 | 2.86 | 0.002 | 0.539 | 3.38±1.513 | 0.121 |
| *Iso*-caproate | 5.01 | 3.27 | 5.32 | 0.001 | 0.375 | 5.46±2.220 | 0.222 |
| Caproate | 1.34 | 1.09 | 1.21 | 0.000 | 0.891 | 1.28±0.352 | 0.736 |
| Acetate:propionate | 2.25 | 2.21 | 2.32 | 0.060 | 0.495 | 2.14±0.103 | 0.016 |

[1]SM5, diet with 5% inclusion of squid meal; SM10, diet with 10% inclusion of squid meal; SM15, diet with 15% inclusion of squid meal.
[2]Data from the same dogs used in the squid meal digestibility trial.
[a,b,c]Values with different superscript letters in the same row are significantly different ($P < 0.05$).

Table 2.5. Experiment 2. Body weight of animals, diet, nutrient and energy intake, apparent total tract digestibility (ATTD), metabolizable energy content, fecal output and characteristics, and fecal metabolites of dogs fed experimental diets with shrimp hydrolysate inclusion and basal diet (n = 6).

| Item | Experimental diets[1] | | | SEM | *P*-value | Basal diet | Basal diet vs. Experimental diets[2] |
|---|---|---|---|---|---|---|---|
| | SH5 | SH10 | SH15 | | | | *P*-value |
| Body weight | 12.9 | 13.1 | 13.0 | 0.21 | 0.768 | 12.6±1.19 | 0.075 |
| Body condition score | 5.67 | 5.33 | 5.33 | 0.173 | 0.342 | 5.17±0.373 | 0.056 |
| Diet intake | | | | | | | |
| g diet $d^{-1}$, as-is | 312.2 | 312.1 | 312.6 | 0.13 | 0.051 | 312.6±33.41 | 0.148 |
| g diet $d^{-1}$, dry matter (DM) | 289.0$^{a}$ | 289.5$^{b}$ | 290.6$^{c}$ | 0.12 | <0.001 | 288.7±30.87 | <0.001 |
| Nutrient intake, g $d^{-1}$ | | | | | | | |
| Organic matter | 252.4$^{a}$ | 252.5$^{a}$ | 253.0$^{b}$ | 0.10 | 0.004 | 252.5±27.00 | 0.549 |
| Crude protein | 78.9$^{a}$ | 85.1$^{b}$ | 91.5$^{c}$ | 0.25 | <0.001 | 72.8±7.78 | <0.001 |
| Ether extract | 26.4$^{a}$ | 26.5$^{b}$ | 26.7$^{c}$ | 0.01 | <0.001 | 26.4±2.82 | <0.001 |
| Gross energy, MJ $d^{-1}$ | 5.34$^{a}$ | 5.40$^{b}$ | 5.46$^{c}$ | 0.003 | <0.001 | 5.29±0.006 | <0.001 |
| Fecal output and characteristics | | | | | | | |
| g feces $d^{-1}$, as-is | 266 | 262 | 253 | 3.8 | 0.117 | 282±25.3 | <0.001 |
| g feces $d^{-1}$, DM | 85.9 | 82.7 | 82.9 | 1.74 | 0.398 | 88.8±8.17 | 0.002 |
| Gross energy, MJ $d^{-1}$ | 1.22$^{b}$ | 1.18$^{a}$ | 1.15$^{a}$ | 0.011 | 0.007 | 1.27±0.116 | <0.001 |
| Production, % | 29.7 | 28.7 | 28.6 | 0.55 | 0.356 | 28.5±0.86 | 0.248 |
| DM, % | 32.8 | 32.0 | 33.0 | 0.67 | 0.561 | 32.2±1.42 | 0.472 |
| Consistency score | 3.44 | 3.20 | 3.25 | 0.061 | 0.052 | 3.54±0.286 | 0.006 |
| Defecations no. $d^{-1}$ | 2.53 | 2.53 | 2.37 | 0.081 | 0.295 | 2.70±0.396 | 0.009 |
| ATTD, % | | | | | | | |
| DM | 69.9 | 71.3 | 71.4 | 0.55 | 0.156 | 69.2±0.93 | 0.001 |
| Organic matter | 76.5 | 77.4 | 77.5 | 0.38 | 0.182 | 75.3±0.67 | <0.001 |
| Crude protein | 75.7 | 76.9 | 77.3 | 0.76 | 0.357 | 71.9±1.36 | <0.001 |
| Ether extract | 92.5 | 92.3 | 91.8 | 0.26 | 0.178 | 92.1±0.86 | 0.799 |
| Gross energy | 77.1 | 78.0 | 78.4 | 0.39 | 0.124 | 76.0±0.65 | <0.001 |
| Metabolizable energy, MJ $kg^{-1}$ | 13.2 | 13.3 | 13.5 | 0.07 | 0.064 | 13.0±0.11 | <0.001 |
| Fecal metabolites, mg $kg^{-1}$, DM | | | | | | | |
| pH | 6.98 | 6.97 | 6.99 | 0.052 | 0.969 | 7.08±0.197 | 0.062 |
| Ammonia-N | 151 | 147 | 142 | 7.7 | 0.745 | 159±45.1 | 0.223 |
| VFA | | | | | | | |
| Total | 943 | 937 | 1030 | 0.1 | 0.363 | 720±111.5 | <0.001 |
| Acetate | 547 | 549 | 602 | 0.1 | 0.368 | 414±80.6 | <0.001 |
| Propionate | 248 | 255 | 267 | 0.1 | 0.659 | 207±30.1 | <0.001 |
| Butyrate | 69.6 | 61.0 | 64.8 | 0.01 | 0.569 | 71.4±15.18 | 0.115 |
| *Iso*-butyrate | 20.8 | 18.6 | 22.5 | 0.01 | 0.397 | 9.71±1.474 | <0.001 |
| *Iso*-valerate | 27.4 | 24.3 | 34.3 | 0.01 | 0.380 | 11.3±0.97 | <0.001 |
| Valerate | 11.6 | 8.79 | 12.7 | 0.002 | 0.529 | 3.30±1.725 | <0.001 |
| *Iso*-caproate | 12.8 | 13.6 | 14.6 | 0.01 | 0.802 | 3.32±0.696 | <0.001 |
| Caproate | 5.78 | 6.82 | 11.9 | 0.002 | 0.073 | 0.835±0.2792 | <0.001 |
| Acetate:propionate | 2.22 | 2.16 | 2.26 | 0.079 | 0.677 | 1.99±0.173 | <0.001 |

[1]SH5, diet with 5% inclusion of shrimp hydrolysate; SH10, diet with 10% inclusion of shrimp hydrolysate; SH15, diet with 15% inclusion of shrimp hydrolysate.
[2]Data from the same dogs used in the shrimp hydrolysate digestibility trial.
[a,b,c]Values with different superscript letters in the same row are significantly different ($P < 0.05$).

### *2.3.5* Fecal microbiota

To assess samples distribution based on bacterial communities, PCoA plots were created using Jaccard and Bray-Curtis distances (Figure 2.2). The Adonis test revealed no significant effect of including squid meal or shrimp hydrolysate in diets for both Jaccard and Bray-Curtis metrics. Regarding alpha diversity, no effect of the inclusion of protein sources in diets on Shannon entropy and Faith's phylogenetic diversity was detected by the Wilcoxon test (Figure 2.3). In the feces of dogs fed the basal and experimental diets, *Turicibacter* was the most abundant genus, followed by unclassified genus of Peptostreptococcaceae and *Blautia* (Figure 2.4). When tested with ALDEx2, the experimental diet SH15 resulted in increased abundances of Oscillospiracea (UCG-005 in Silva database) in comparison to the basal diet, while SH5 and the SH10 experimental diets respectively decreased abundances of Firmicutes and *Lactobacillus* (Figure 2.5). According to the same test, no differentially abundant genera were discovered between dog feces fed the basal diet and experimental diets with squid meal inclusion.

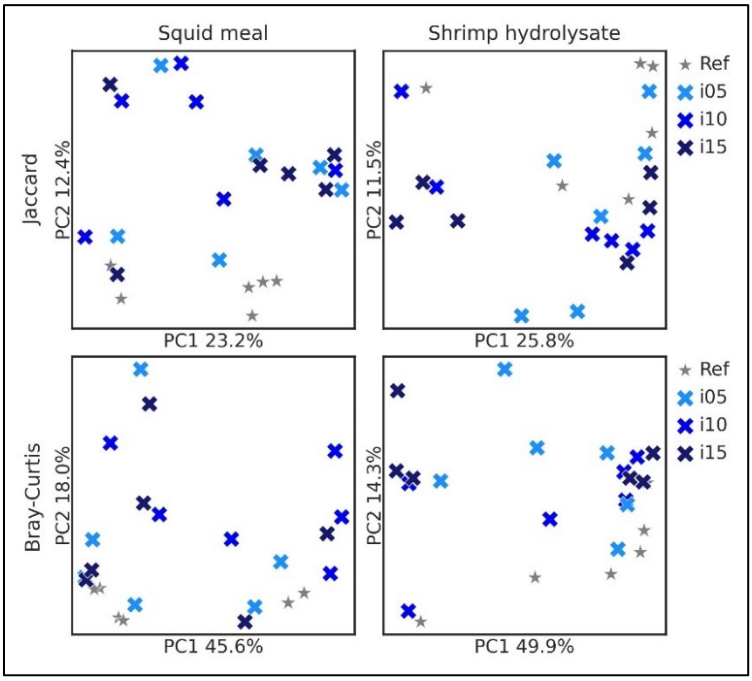


Figure 2.2. Beta diversity metrics. Principal-coordinate analysis (PCoA) based on Jaccard and Bray-Curtis distances of fecal bacteria of dogs fed the basal diet (ref), and the experimental diets with increasing levels of inclusion of squid meal or shrimp hydrolysate (i05, i10, i15) in place of the basal diet. Each cross indicates one sample. Basal diet and experimental diets are differentiated by shapes and color and inclusion levels by the color gradient.

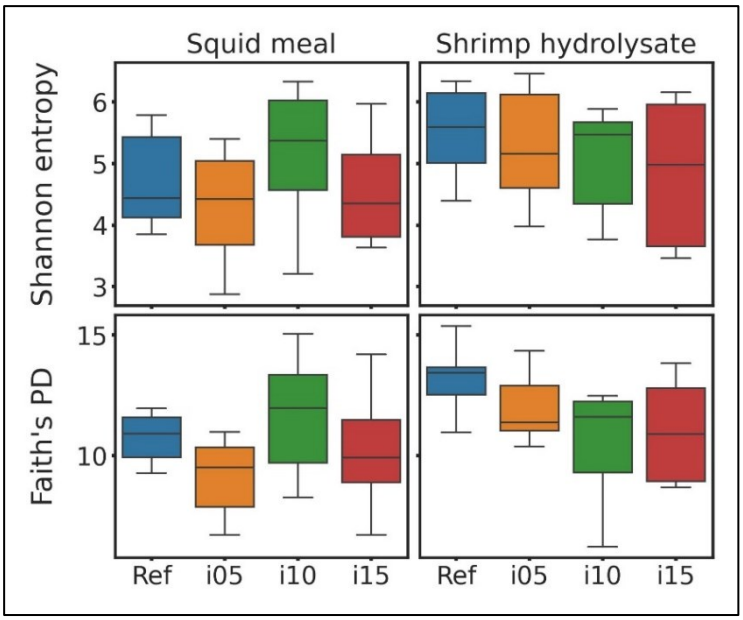


Figure 2.3. Alpha diversity metrics. Bloxpots of Sannon entropy and Faith's PD indices of fecal bacteria of dogs fed the basal diet (ref), and the experimental diets with increasing levels of inclusion of squid meal or shrimp hydrolysate (i05, i10, i15) in place of the basal diet.

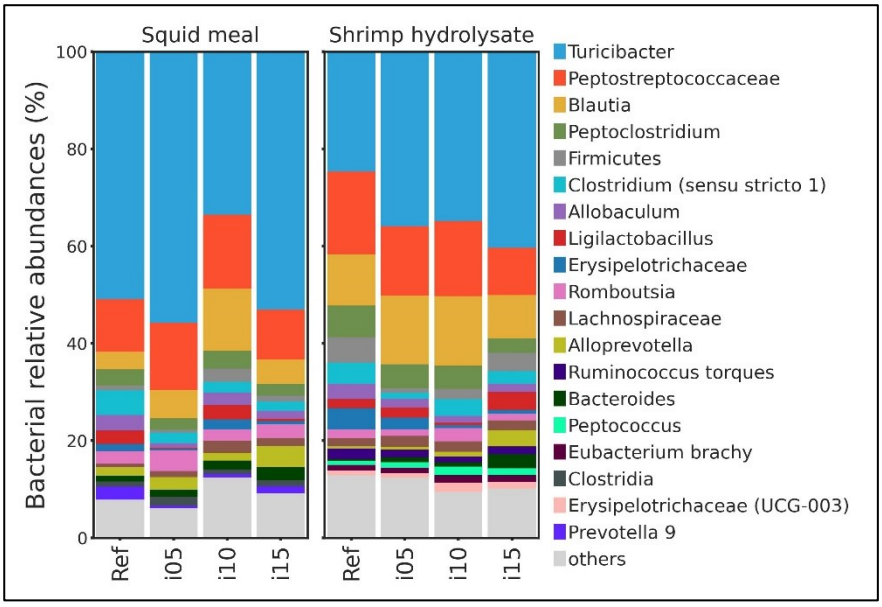


Figure 2.4. Bacterial relative abundance (%). Taxonomy barplots at the genus level of dogs fed the basal diet (ref), and the experimental diets with increasing levels of inclusion of squid meal or shrimp hydrolysate (i05, i10, i15) in place of the basal diet. If genus level was not assigned, the last available taxonomy rank was used for the label.

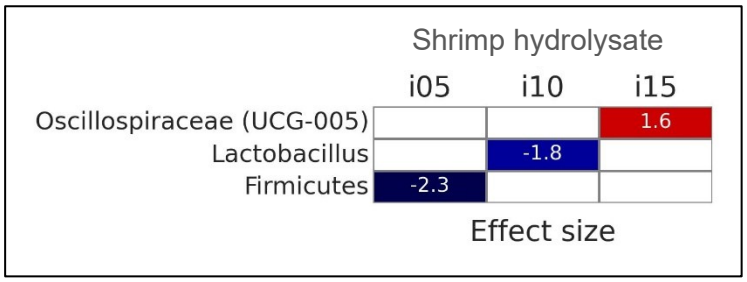


Figure 2.5. Differentially abundant genera ($P$ < 0.05), according to ALDEx2, of fecal bacteria of dogs fed the experimental diets with increasing levels of inclusion of shrimp hydrolysate (i05, i10, i15) in place of the basal diet in comparison with the basal diet. The value in red indicates increased abundance and the values in blue indicate decreased abundance.

## 2.4 Discussion

The European Union legislation allows the use of crustaceans and mollusks in pet food (European Commission, 1982) and no constrains were found to be imposed by the Association of American Feed Control Officials (AAFCO, 2023). However, the use of protein-rich by-products obtained from these aquatic organisms in pet food has not been studied yet, so the present study aimed to evaluate the chemical composition, including the amino acid profile, and antioxidant activity of squid meal and shrimp hydrolysate, and the effect of increasing levels of their dietary inclusion on the palatability, digestibility, fecal characteristics and metabolites, and microbiota in adult healthy dogs.

### 2.4.1 Chemical composition

The chemical composition of the experimental diets in both experiments reflected the chemical composition of the protein studied sources, namely by enhancing the CP and AA content compared to the basal diet. All diets met the nutritional requirements for adult dogs (FEDIAF, 2021).

Protein was the main chemical constituent of the studied by-products, with squid meal presenting the highest amount. The CP content of squid meal agrees with the wide range of values reported in the literature (680 g $kg^{-1}$ DM (Córdova-Murueta and García-Carreño, 2002) to 805 g $kg^{-1}$ DM (Carranco-Jáuregui et al., 2020)), as well as for shrimp hydrolysate (436 g $kg^{-1}$ DM (da Silva et al., 2017) to 888 g $kg^{-1}$ DM (Cao et al., 2009)). Both by-products, particularly the squid meal, presented higher CP content than animal and vegetal protein sources commonly used in dog food formulation such as poultry by-product meal (590 g $kg^{-1}$ DM), meat and bone meal (509 g $kg^{-1}$ DM), corn gluten meal (563 g $kg^{-1}$ DM), and soybean meal (445 g $kg^{-1}$ DM; NRC, 2006).

The CP content of both protein sources was higher than the total AA content, as previously reported in related sources (Hulan et al., 1979; Dey and Dora, 2014), questioning the use of 6.25 as the conversion factor to calculate CP. As no recommendations exist regarding the conversion factor to be applied to these sources, AA analysis is considered more suitable than CP to evaluate the protein content of foods, as suggested by FAO (2003). The total content of AA of squid meal observed in the current study corresponds to the range of values previously reported (457 g $kg^{-1}$ DM (Hulan et al., 1979) to 628 g $kg^{-1}$ DM (Querol et al., 2015)), but the AA profile differs from comparable sources. While in the present study, the essential AA found at the highest concentrations were arginine and lysine, and at the lowest concentrations were histidine and isoleucine, other studies (Hulan et al., 1979; Querol et al., 2015) reported leucine, valine, and arginine at the highest concentrations, and histidine, phenylalanine, and methionine at the lowest concentrations. Similarly, the AA content of shrimp hydrolysate is within the wide range of values previously reported (345 g $kg^{-1}$ DM (Bueno-Solano et al., 2009) to 862 g $kg^{-1}$ DM (Cao et al., 2009)), but its AA profile varies from comparable sources earlier reported. Indeed, whereas, in the current study, the essential AA found at the highest concentrations were arginine and lysine, and at the lowest concentrations were histidine and methionine, in other studies (Bueno-Solano et al., 2009; Cao et al., 2009) arginine, isoleucine, leucine and lysine were present at the highest concentrations and at the lowest concentrations were histidine, phenylalanine, and methionine. The variations observed between studies on CP and AA contents might be explained by the well-known effects of species, growth stage, feeding conditions, part of the animal used (e.g., head, cephalothorax, in shrimp, or head, tentacles, viscera, in squid), conditions of processing and hydrolysis, including enzymes applied, duration, temperature, and pH, and storage, among others (Hulan et al., 1979; Dey and Dora, 2014; da Silva et al., 2017).

According to the National Research Council (NRC, 2006), methionine is frequently the most limiting AA in protein sources routinely used in pet food, such as poultry by-product meal, meat and bone meal, and soybean meal prompting manufacturers to employ synthetic methionine supplements (D'Onofrio et al., 2023). The high methionine content of shrimp hydrolysate and, especially, of squid meal suggests the potential of these by-products to overcome the dietary deficiency in this essential AA.

### 2.4.2 Antioxidant activity

The evaluation of the antioxidant activity of novel protein sources contributes to the understanding of their functional potential for animal health. As the response of antioxidants to different radicals or oxidants may vary, leading to a lack of consensus on

the optimal method for demonstrating antioxidant activity (Prior et al., 2005; Karadag et al., 2009), four distinct antioxidant assays were used in the present study: ABTS, DPPH, FC and FRAP. ABTS assay relies on a hydrogen atom transfer (HAT) or a single-electron transfer (SET) mechanism and it quantifies the amount of ABTS•+ radical cation quenched and the residual ABTS•+ concentration (Re et al., 1999), and can be performed in a wide pH range and in hydrophilic and lipophilic samples (Magalhães et al., 2008). DPPH assay relies on a SET mechanism, whereas the DPPH• is scavenged, which is affected by the solvent and the pH of the reaction (Magalhães et al., 2007; Staško et al., 2007). Folin–Ciocalteu assay is also based on a SET mechanism, measuring the reducing capacity of compounds in an alkaline medium, but only applicable to hydrophilic solvents (Huang et al., 2005; Magalhães et al., 2008). Lastly, FRAP assay evaluates the capacity of compounds to reduce the ferric 2,4,6-tripyridyl-s-triazine complex through a SET mechanism in an acidic medium (Benzie and Strain, 1996). Shrimp hydrolysate extracts showed higher antioxidant activity in all methods tested in comparison to squid meal extracts. The reaction of squid meal extracts in the DPPH assay was below the threshold of detection, requiring higher concentrations for the reaction to occur (data not shown), as previously reported (Magalhães et al., 2008). Although bioactive compounds are known to exert antioxidant properties, scavenging free radicals, thus preventing their harmful effects on health (Losada-Barreiro and Bravo-Díaz, 2017; Karasahin et al., 2021), additional research is needed to identify the specific compounds responsible for the observed antioxidant activity in these extracts.

The antioxidant activity of squid meal extracts was assessed for the first time, thus precluding the comparison of the results herein obtained with the literature. Earlier research has demonstrated the antioxidant properties of components from *D. gigas* with higher antioxidant activity of arms collagen hydrolysates in contrast to fins collagen hydrolysates (Suarez-Jimenez et al., 2015), and higher antioxidant activity of skin gelatine in contrast to gelatine from arms and fins (Chan-Higuera et al., 2016). The antioxidant activity of *L. vannamei* shrimp hydrolysates has been demonstrated before (Latorres et al., 2018; Nikoo et al., 2021), but the variety of the process of hydrolysis, along with the diversity of methodology, such as the extraction and the method of antioxidant activity used, makes it difficult to compare findings among studies. Hydrolyzed peptide size may contribute to the antioxidant properties of shrimp hydrolysate (Ambigaipalan and Shahidi, 2017; Djellouli et al., 2020), with smaller fractions exhibiting the highest activity (Zhao et al., 2011; Djellouli et al., 2020). Moreover, other compounds present in shrimp, such as phenolic compounds, might contribute to the antioxidant activity (Seymour et al., 1996; Cho et al., 2019). Additionally, as food

processing influences the antioxidant properties (Toydemir et al., 2022), more research is needed to evaluate the impact of dog food extrusion on the antioxidant potential of novel protein sources.

### 2.4.3 Palatability

The palatability of novel ingredients is of crucial importance as it can affect food consumption. Thus, the addition of palatability enhancers is a common practice in the pet food industry, being protein hydrolysates extensively employed for this purpose (Nagodawithana et al., 2008). Palatability refers to taste, odor, and mouth feel (texture, shape, and size; Thombre, 2004), and it is influenced by the composition of diets, such as the content of DM, fiber, carbohydrates, fat and protein (Alegría-Morán et al., 2019). In the present study, although no food refusal was observed, the palatability of diets was negatively affected by the inclusion of 150 g $kg^{-1}$ of shrimp hydrolysate or squid meal in place of the basal diet. This result was unexpected as both squid meal and shrimp hydrolysate presented high levels of glutamic and aspartic acids, two AA found in various foods, including seafood, known to induce umami, a taste highly attractive to dogs (Kurihara and Kashiwayanagi, 1998; Tanase et al., 2022). Indeed, earlier studies found no negative or even positive effects on palatability with higher inclusion levels of marine by-products than the level used in the current study (Folador et al., 2006; 2023). The lower palatability of diets with 15% inclusion of squid meal or shrimp hydrolysate might be due to protein sources being mixed with the basal diet immediately before being offered to dogs, instead of included in the kibble, as previously shown with microalgae supplementation (Cabrita et al., 2023). Future research should be performed to evaluate the palatability of diets containing squid meal and shrimp hydrolysate included in the extruded complete diet.

### 2.4.4 Body weight, food intake, fecal output and characteristics, *in vivo* digestibility, and metabolizable energy

To the best of authors knowledge this is the first study that assessed the in vivo effects of inclusion of squid meal and shrimp hydrolysate in diets for dogs, so the results obtained here cannot be compared with the literature. Based on studies performed with other monogastric species (Leal et al., 2010; Fuente-Martínez et al., 2023), and taken into consideration the results obtained in the palatability trials, the levels of dietary inclusion in the digestibility trials were set at 50, 100, and 150 g $kg^{-1}$. As daily food allowance was defined according to the ME requirements and adjusted to the ideal BW, inclusion levels of squid meal and shrimp hydrolysate did not affect BW. Diet intake was not affected by the level of squid meal, but increasing levels of shrimp hydrolysate linearly

increased diet intake, while comparing to the basal diet, both studied protein sources increased food intake. However, these effects lack biological meaning. Differences in intake of nutrients and energy reflect the different chemical composition of the basal and experimental diets in both experiments.

The number of defecations, fecal DM content and consistency score, parameters highly valuable for dog owners, were not affected by increasing levels of squid meal and shrimp hydrolysate inclusion. Compared to the basal diet, although differences reached significance on consistency score, feces were all classified between soft, shaped, and moist stools leaving spots on the floor (3.0) and approximately firm, shaped, and dry stools (3.5), which is considered the optimum (Hernot et al., 2005). The higher fecal output observed with the basal diet relative to the diets with the novel protein sources, reflects the modest digestibility of this diet.

Along with fecal output and quality, diet digestibility assumes relevant importance to pet owners. In experiment 1, the ATTD of CP was the highest with 150 g $kg^{-1}$ inclusion of squid meal. Despite being known that indigestible protein might reduce fecal quality due to the high osmotic pressure promoted by the fermentation of protein by proteolytic bacteria (Hall et al., 2013), the effect on CP ATTD was not reflected in fecal DM and consistency score. Although, the decreased molecular weight resulting from hydrolysis of protein sources is expected to improve CP ATTD, the inclusion of increasing levels of shrimp hydrolysate had no effect on this parameter. Compared to the basal diet, the inclusion of the studied protein sources increased ATTD of DM, nutrients, and energy, with the major difference being observed for CP. This demonstrates the high potential of these ingredients to be included in highly digestible diets for dogs. Moreover, despite not being herein studied the allergic responses to squid meal and shrimp hydrolysate, these novel protein sources can also have the potential to be included in therapeutic diets to prevent adverse food reactions (Verlinden et al., 2006).

### 2.4.5 Fecal end-fermentation products

Despite the significant differences observed among squid meal inclusion levels and between basal diet and squid meal supplementation, fecal pH was nearly neutral in both experiments. These high fecal pH values are known to favor the fermentation of undigested protein in the colon (Windey et al., 2012), generating *iso*-butyrate, *iso*-valerate and ammonia-N (Blachier et al., 2007) that might have negative effects on gut health and fecal odor (Windey et al., 2012). In the current study, despite the observed differences in CP ATTD with increasing levels of squid meal, no effects were observed on these parameters. Compared to the basal diet, the inclusion of squid meal increased

ammonia-N and slightly reduced fecal quality, suggesting a higher amount of protein reaching the large intestine. Indeed, the higher CP intake observed with squid meal inclusion in relation to the basal diet could have resulted in higher protein fermentation in the colon, culminating in higher ammonia-N levels (Kroupina et al., 2022). Moreover, the inclusion of squid meal decreased fecal butyrate concentration when compared to the basal diet. As this VFA constitutes the main source of energy for colonocytes, thus contributing for the maintenance of cell growth and differentiation in the gut, its reduction might suggest a lower preventing role in inflammation and colon cancer (Binder, 2010). Conversely, a positive correlation between ammonia-N and butyrate concentrations have been previously observed in humans and rats (Bajka et al., 2008; McOrist et al., 2011). The majority of ammonia-N is produced by bacteria through the deamination of amino acids (Blachier et al., 2007), and can be absorbed by colonocytes or utilized by bacteria for protein synthesis and metabolism (Windey et al., 2012).

Despite the absence of effects of increasing levels of shrimp hydrolysate on VFA production, when compared to the basal diet, the dietary inclusion of this protein source significantly increased total VFA and individual concentrations except for butyrate, tended to decrease fecal pH, and did not influence ammonia-N concentration. An earlier *in vitro* study has also reported increased production of VFA with hydrolyzed soy protein, such as acetate, iso-butyrate and iso-valerate, compared to non-hydrolyzed soy protein (Ashaolu et al., 2019). The high concentrations of acetate and propionate observed with shrimp hydrolysate inclusion diets might benefit dogs health, namely by regulating host metabolic, immune and neuro-immunoendocrine responses (Tremaroli and Bäckhed, 2012; Sridharan et al., 2014; Silva et al., 2020), and lowering cholesterol (Nybroe et al., 2023), being also observed decreased acetate and propionate concentrations in dogs diagnosed with chronic enteropathy in comparison to healthy dogs (Minamoto et al., 2019). On the other hand, the increased iso-butyrate and iso-valerate concentrations, known to be originated from the bacterial fermentation of leucine, isoleucine and valine (Rios-Covian et al., 2020), results in increased concentration of fermentation products, such as ammonia-N, that might be detrimental to host health (Windey et al., 2012).

### 2.4.6 Fecal microbiota

The fecal microbiota is a complex ecosystem influencing host health by modulating the immune system (Suchodolski, 2016; Ziese and Suchodolski, 2021), and also by regulating nutrient utilization of substances entering the colon, where the production of fecal metabolites occurs (Sieja et al., 2023). While there are some variations of microbiota along the dog intestinal tract (Suchodolski et al., 2008; Honneffer et al., 2017), fecal microbiota is mostly studied due to its ease of sampling and non-invasiveness

(Pereira and Clemente, 2021). Changes in diet composition, such as protein content and source, are normally accompanied by variations in the microbiome profile of the gut (Pilla and Suchodolski, 2020), within a short period of time, namely 2 d for metabolites, such as VFA and ammonia-N, and 6 d for microbiota (Lin et al., 2022). In the current study, the inclusion of squid meal or shrimp hydrolysate in the diets did not significantly affect the beta and alpha diversity and relative abundance of bacteria. All diets presented higher abundances of *Turicibacter,* Peptostreptococcaceae, and *Blautia*, all pertaining to the phylum Firmicutes the most common phylum in dog gut (Pilla and Suchodolski, 2020). Higher levels of *Turicibacter* and *Blautia* are indicators of a healthy gut microbiota, while lower levels of these genera have been observed in dogs diagnosed with chronic inflammatory enteropathy (Félix et al., 2022).

The inclusion of 150 g $kg^{-1}$ of shrimp hydrolysate increased the abundance of a genus pertaining to Oscillospiraceae. A positive correlation of *Oscillospira*, a genus from Oscillospiraceae, with the production of acetate, butyrate, propionate (Yang et al., 2021), and valerate (Ecklu-Mensah et al., 2023) has been shown, thus suggesting the potential of shrimp hydrolysate as a prebiotic (Yang et al., 2021). The inclusion of 100 g $kg^{-1}$ of shrimp hydrolysate, decreased the abundance of *Lactobacillus* that is known to have the ability to cross-feed other commensals to produce butyrate (Zhang et al., 2021) and was earlier reported to decrease when dogs are fed diets high in protein and fat obtained from natural sources (Sandri et al., 2017). However, in the current study, the effect of shrimp hydrolysate inclusion on butyrate concentration did not reach significance and diet SH10 presented a lower CP content than diet SH15, thus being not clear the mechanism for a decreased *Lactobacillus* genus with this diet. The inclusion of 50 g $kg^{-1}$ of shrimp hydrolysate decreased the abundance of a genus pertaining to Firmicutes, in agreement with a previous study showing a decrease in Firmicutes abundance when dogs were fed a protein hydrolysate from leather waste (Li et al., 2021). Conversely, the increased concentration of acetate, butyrate and propionate have been associated with the increased relative abundance of Firmicutes (Atasoy et al., 2019). Further research is needed to fully understand the effects of shrimp hydrolysate on microbiota profile.

## 2.5 Conclusions

The present study demonstrated, for the first time, the potential of squid meal and shrimp hydrolysate as novel protein sources for dog nutrition. Both by-products presented higher protein and methionine contents than commonly used protein sources, and their dietary inclusion increased diet digestibility, suggesting their potential to be included in high protein digestible diets. Despite the general absence of effects of inclusion levels on fecal

metabolites, and conversely to squid meal, feeding dogs with shrimp hydrolysate diets affected microbiota composition. In summary, the findings support the potential of these protein rich by-products for dog feeding. However, additional research is needed to fully evaluate their functional properties.

## Conflict of Interest

TA is employed by SORGAL, Sociedade de Óleos e Rações S.A. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

ARJC and MRGM designed the study. ARJC and AJMF obtained funding. TA supplied the diets. JG-F performed the experimental protocols. JG-F and ARJC analyzed the data. TY and AC-S performed the microbiota analysis. JG-F drafted the original manuscript and elaborated the final version of the manuscript. SL, MRGM, ARJC, AJMF, TA, AC-S and TY critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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**Data Availability Statement**

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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# CHAPTER 3

## Unveiling the effects of shrimp hydrolysate as a dietary ingredient in healthy adult Beagle dogs

**Joana Guilherme-Fernandes[1], António J. M. Fonseca[1], Tiago Aires[2], Sofia A. C. Lima[1], Margarida R. G. Maia[1*], Ana R. J. Cabrita[1]**

[1]REQUIMTE, LAQV, ICBAS, School of Medicine and Biomedical Sciences, University of Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

[2]SORGAL, Sociedade de Óleos e Rações S.A., Lugar da Pardala, 3880-728 S. João Ovar, Portugal

[*]Present address: LEAF - Linking Landscape, Environment, Agriculture and Food Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal

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**Abstract**

To be more sustainable, the pet food industry could increase inclusion of animal by-products from the human food chain and fish hydrolysates have been reported to benefit dogs' health. However, there is limited research on the impact of alternative marine hydrolysates in dog food. The current study evaluated the effects of including shrimp hydrolysate as replacement for wheat gluten (experimental diet) in an extruded complete diet (control diet) on diet palatability, intake, digestibility, fecal characteristics and metabolites, oral volatile sulfur compounds (VSC) and coat quality in dogs. Palatability of diets was assessed in a two-bowl test, conducted with twelve healthy adult Beagle dogs. No differences were observed in first approach, first taste or intake ratio. A randomized block design lasting 12 weeks was performed with 12 dogs distributed into six blocks, according to sex and body weight; one dog from each block was randomly allocated to each diet. Fecal characteristics and metabolites were measured in weeks 0, 4, 8, and 12, VSC and coat quality in weeks 4, 8 and 12, and apparent total tract digestibility (ATTD) of nutrients and energy in week 12. The inclusion of shrimp hydrolysate did not affect intake, but increased fecal output (dry matter, DM, basis, $P < 0.05$). Fecal butyrate concentration was lower ($P < 0.05$) in dogs fed the experimental diet. The inclusion of shrimp hydrolysate did not affect ATTD of nutrients and energy, and VSC. Both diets promoted high coat quality. The experimental diet decreased gloss and general evaluation scores in week 4 ($P < 0.05$), but improved scale score in weeks 4 and 12 ($P < 0.05$). Overall, the findings indicate the potential of including shrimp hydrolysate in diets for dogs, fostering a more sustainable industry.

## 3.1 Introduction

Trends in the pet food industry are strongly driven by the humanization of pets (Gray, 2021), which favors the inclusion of sustainable and functional ingredients (Vasconcellos et al., 2023). From a circular economy perspective, the use of by-products from the human food chain can contribute to a more sustainable sector. Animal by-products are regularly used in pet food with a recent report stating that this strategy is accepted by two thirds of pet owners (GlobalPETS, 2023). Moreover, it has been demonstrated that protein hydrolysates obtained through hydrolysis of by-products, thus containing small peptides and amino acids (AA), have remarkable functional properties, such as antioxidant and antihypertensive activity (Karami and Akbari-Adergani, 2019; Shahidi and Ambigaipalan, 2019). Among marine sources, fish hydrolysates, such as from salmon and white fish, are the most commonly used in pet food. Salmon protein hydrolysates have been found to present high digestibility of AA in cecectomized roosters

(Folador et al., 2006) and in mink (Tjernsbekk et al., 2017), and to reduce cutaneous adverse food reaction in dogs (Szczepanik et al., 2022). Additionally, fish protein hydrolysates have been shown to help improve pruritus (Matricoti and Noli, 2018), to alleviate symptoms in dogs with chronic otitis externa (Di Cerbo et al., 2016), and to improve the levels of neuroendocrine and stress biomarkers in dogs (Sechi et al., 2017). Similarly, a supplement comprising cod and mackerel protein hydrolysates positively affected fear and anxiety-related behaviors in dogs (Landsberg et al., 2015). However, the effects of alternative marine protein hydrolysates in dog food, such as shellfish, remain largely unknown.

According to the Food and Agriculture Organization (FAO, 2023), world shrimp production, primarily for human consumption, reached 9.4 million tons in 2022 with *Litopenaeus vannamei* accounting for over half of the total global shrimp production. During the processing of shrimp for human consumption, wasted body parts, including heads, shells and tails, account for nearly half of the animal's weight (Roy et al., 2023). Although a small amount of shrimp waste is used in animal feeding (Okoye et al., 2005; Goncalves et al., 2022), large quantities are still being wasted. This waste results in the loss of valuable components, such as chitin/chitosan, protein, carotenoids, polyunsaturated fatty acids, α-tocopherol, and minerals (Nirmal et al., 2020). Additionally, it contributes to the increase of environmental pollution namely through the emission of volatile organic compounds, sulfur dioxide, and hydrogen chloride during waste decomposition (Lopes et al., 2015). Although methods currently used to reuse waste are based on corrosive or hazardous reagents, more environmentally friendly alternatives are increasing, such as the use of enzymatic hydrolysis (Mao et al., 2017). *In vitro* studies have shown that protein hydrolysates originated from shrimp by-products, namely from *L. vannamei*, have several functional properties, such as angiotensin-converting enzyme inhibition (Feng et al., 2016; Ambigaipalan and Shahidi, 2017), and antioxidant (Ambigaipalan and Shahidi, 2017; Djellouli et al., 2020), metal chelating (Ambigaipalan and Shahidi, 2017), anticancer (Kannan et al., 2011), antimicrobial (Djellouli et al., 2020), calcium binding (Huang et al., 2011a), and iron binding (Huang et al., 2011b) activities. Furthermore, diets containing shrimp hydrolysate have been found to improve the health and performance, namely growth, feed efficiency, nutrient digestibility, and immune response in fish (Gisbert et al., 2018; Gunathilaka et al., 2020), avian (Mahata et al., 2008; Nha and Thuy, 2021), and pigs (Sun et al., 2009). However, research into the effects of shrimp hydrolysate on health and performance of dogs is scarce. Recently, in a short-term study, Guilherme-Fernandes et al. (2024) demonstrated the potential of shrimp hydrolysate for canine nutrition, namely due to its valuable methionine content,

antioxidant activity, and high digestibility. Given these previous results, the present study aimed to assess the long-term effects of shrimp hydrolysate inclusion in an extruded canine kibble diet compared to a control diet with similar ingredients and chemical composition without the inclusion of shrimp hydrolysate. It was hypothesized that inclusion of shrimp hydrolysate at 5% would enhance diet palatability and digestibility, and benefit fecal characteristics and metabolites, oral malodor, and coat quality in dogs.

## 3.2 Materials and methods

Study design and procedures were approved by the Animal Ethics Committee of the School of Medicine and Biomedical Sciences, University of Porto (Permit Nº 408/2021). Animal handling and procedures were conducted in accordance with good animal welfare practices (European Union Directive 2010/63/EU), by trained scientists in Laboratory Animal Science (FELASA, category C). Before the start of the study, dogs were routinely vaccinated and clinically evaluated to determine suitability for the study. Dogs were continuously monitored throughout the entire study.

### 3.2.1 Animals and housing

Twelve adult Beagle dogs, six females and six males, 4.5 ± 0.65 years-old, with an initial body weight (BW) of 12.4 ± 2.53 kg and median (interquartile range) body condition score (BCS) of 5.0 (1) out of 9 (Laflamme, 1997) participated in the study. Dogs from different blocks but undergoing the same dietary treatment were housed in pairs in environmentally enriched interconnected boxes with inside and outside areas (1.8 and 3.5 $m^2$, respectively). Between daily meals, dogs were allowed to exercise and socialize in an outdoor park and taken on 30 min leash walks. During feces collection, dogs were housed separately, allowed to exercise and socialize under supervision, and walked daily on a leash.

### 3.2.2 Diets and feeding

Two extruded diets, a control diet (commercial complete diet) and an experimental diet were used. Diets were produced with the same base ration except for the addition of 5% (w/w) shrimp hydrolysate in the experimental diet at the expense of wheat gluten to obtain isoproteic diets (Table 3.1). Diets were produced following common methodology for industrial production of dry food for dogs with ingredient grinding, mixing, extrusion (120-130 ºC plus 25% moisture), drying, coating with liquid ingredients, cooling, and packaging. The shrimp hydrolysate (Symrise Aqua Feed, Elven, France) was obtained by enzymatic hydrolysis of heads and cephalothoraxes of *L. vannamei*. The solid waste (shell) was separated by centrifugation at the end of the hydrolysis process and the liquid

fraction was spray-dried to obtain the protein hydrolysate (Leduc et al., 2018). Daily food allowance was calculated to meet the metabolizable energy (ME) requirements according to individual BW of dogs (ME (kcal/d) = 110 × $BW^{0.75}$; FEDIAF, 2021) and adjusted for BCS. Body weight and BCS were assessed every four weeks throughout the duration of the study. The ideal BCS was considered between 4 and 6 (Laflamme, 1997). Dogs were individually fed their daily ration in two equal meals (8.30 a.m. and 5.00 p.m.) and provided with *ad libitum* fresh water.

### 3.2.3 Experimental design

The study was performed according to a complete randomized block design with dog as the experimental unit. The 12 dogs were distributed into six blocks of two dogs each, according to sex and BW, and one animal from each block was randomly assigned to one of the two diets, for a total of six dogs per diet, the minimum recommended by FEDIAF (2021). The study lasted 12 wks, with measurements taken at weeks 0, 4, 8, and 12. A two-bowl test was performed in week 0. Assessment of body weight and body condition and feces collection were performed in weeks 0, 4, 8, and 12. Oral volatile sulfur compounds (VSC) and coat quality were evaluated in weeks 4, 8, and 12. Apparent total tract digestibility (ATTD) of diets was determined in week 12.

### 3.2.4 Palatability

On two consecutive days of week 0 and after an overnight fast, a two-bowl test (Aldrich and Koppel, 2015) was performed on 12 dogs to assess palatability by pairwise comparison of the control and experimental diets. The sample size and duration of the trial were defined according to an earlier study (Guilherme-Fernandes et al., 2024) that found significant differences in intake ratio among diets without or with inclusion of shrimp hydrolysate. Each bowl contained the calculated amount of food to meet the morning meal ME requirements of each dog, with dogs thus being offered twice their ME requirements (FEDIAF, 2021). Bowls were placed 45 cm apart in opposite positions (left and right) for two days to control side bias. Each session was limited to 30 min or when a dog had consumed all food from a bowl. The bowl approached first and diet tasted first by each dog were recorded. At the end of each session, the remaining food was weighed to determine the ratio of food consumption between the control and experimental diets.

### 3.2.5 Chemical analyses of diets

Diet samples were dried in an air-forced oven at 65 °C until constant weight, ground (1-mm), and analyzed according to official methods (AOAC, 2005). Samples were analyzed for dry matter (DM; ID 934.01), ash (ID 942.05), ether extract (EE; ID 920.39), insoluble dietary fiber (ID 991.42), soluble dietary fiber (ID 993.19), and nitrogen (ID

990.03). Crude protein (CP) was calculated as nitrogen × 6.25. Neutral detergent fiber (NDF, with α-amylase and expressed exclusive of residual ash) was determined as described by Van Soest et al. (1991), and starch (in 0.5-mm milled samples) determined according to Salomonsson et al. (1984). Gross energy (GE) was analyzed using an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). Amino acid profile was assessed as described by Aragão et al. (2020). Briefly, samples were hydrolyzed with 6 M HCl solution at 116 °C for 48 h, and then derivatized in a precolumn Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate), according to the AccQ Tag method (Waters, Milford, MA, USA). Analyses were performed by ultra-high-performance liquid chromatography on a Waters reversed-phase AA analysis system using norvaline as the internal standard. Peaks were analyzed with EMPOWER software (Waters). All analyses were performed in duplicate.

### 3.2.6 Fecal output, characteristics, and metabolites analysis

During two consecutive days of weeks 0, 4, 8, and 12, the number of defecations by each dog was recorded. Individual feces were weighed and scored for consistency according to a 5-point scale, ranging from watery diarrhea (1) to powdery hard mass pellets (5; Félix et al., 2013). Fresh fecal samples were composited per dog per week and stored at -20 °C until further analysis. pH was measured in thawed and homogenized samples with a potentiometer (sensION+ PH3, Hach-Langue, S.L.U., Barcelona, Spain). For ammonia-nitrogen concentration (Chaney and Marbach, 1962), 1 g of feces was diluted in 10 mL of 2 M KCl, centrifuged at 5200 × *g* at 4 °C for 60 min. Supernatant was obtained using a polyethersulfone syringe filter with 0.45 µm pore size (FILTER-LAB, Barcelona, Spain). Forty µL of the supernatant was mixed with 40 µL of water, 2.5 mL of phenol solution and 2 mL of alkaline hypochlorite solution. The resulting solution was incubated for 10 min at 37 °C, and 1 h at 22 °C in the dark. The absorbance was read at 550 nm in a SynergyTM HT Multimode plate reader (BioTek Instruments Inc., Winooski, VT, USA). An ammonia solution (32 mg $dL^{-1}$) was used as the standard. To determine volatile fatty acids (VFA) concentrations, 1 g of feces was diluted in 10 mL of 25% ortho-phosphoric acid solution with an internal standard (4 mM 3-methyl valerate, Sigma-Aldrich, Saint Louis, MO, USA). The solution was centrifuged for 16 min at 18000 × *g* at 4 °C, and the supernatant stored at -20 °C until analysis by gas chromatography as previously described (Pereira et al., 2021). All analyses were performed in duplicate.

### 3.2.7 Apparent total tract digestibility

During week 12, total feces eliminated by each dog was collected for five consecutive days to assess the ATTD of the diets. Samples were weighed and stored at -20 °C. Feces

were later thawed and homogenized. Representative samples were immediately analyzed for nitrogen (CP calculated as nitrogen × 6.25). Ash, EE, NDF, starch (0.5-mm milled), and GE were analyzed from dried (65 ºC) and milled (1-mm) feces pooled per dog as previously described for diet samples.

### 3.2.8 Oral volatile sulfur compounds

Measurements of VSC concentrations were conducted using a Halimeter following the instructions provided by the manufacturer (Interscan Corporation, Simi Valley, CA, USA). A plastic cup with an attached straw was used to cover each dog's snout, which was gently immobilized during measurements. The measurements were performed in one day of weeks 4, 8 and 12, before the morning meal and 1 h and 5 h later (Rawlings and Culham, 1998).

### 3.2.9 Coat evaluation

On one day at weeks 4, 8 and 12, coat quality was scored by a naïve 11-person panel comprising 2 veterinarians, 1 animal science technician, 3 dog owners and 5 animal science and veterinary students. The panel was blinded to the diets. A five-point scale was used to score gloss, greasiness, softness, scale and general evaluation of coat from the least desirable (1) to the most desirable (5; Hester, 2004). Briefly, the parameters were evaluated as following: a) gloss, from very dull (1) to very shiny (5); b) greasiness, from very greasy (1) to not greasy (5); c) softness, from very brittle (1) to very soft (5); d) scale, from very scaly (1) to no scale present (5); and e) general evaluation of coat, from poor (1) to excellent (5). The panel was instructed to use both whole numbers (e.g., 3, 4) and half-point increments (e.g., 2.5, 4.5) when scoring coats. Dogs were bathed the previous day and groomed immediately before the scoring. The evaluation was performed in indoor rooms with natural daylight. The panel could freely interact with and handle the dogs.

### 3.2.10 Calculations and statistical analyses

Amino acid scores (AAS) of diets were calculated as described by Kerr et al. (2013), following the equation:

$$AAS = \frac{AA\ (mg) \Big/ diet\ CP\left(\frac{g}{kg}\right)}{minimum\ recommended\ level\ of\ AA\ (mg) \Big/ CP\left(\frac{g}{kg}\right)}\ x\ 100$$

The minimum levels of AA and CP recommended by FEDIAF (2021) for adult dogs (maintenance energy requirements of 110 kcal/kg$^{0.75}$) was used as reference.

Geometric mean of AAS was calculated to determine the indexes of essential AA of diets (Oser, 1959).

Metabolizable energy content of diets was determined according to FEDIAF (2021), using the equation:

$$ME\ (MJ/kg\ DM) = \frac{(GE\ intake\ (\frac{MJ}{d}) - fecal\ GE\ (\frac{MJ}{d})) - (CP\ intake\ (\frac{g}{d}) - fecal\ CP\ (\frac{g}{d})\ x\ 5.23)}{DM\ intake\ (\frac{g}{d})}$$

Fecal production was determined as:

$$Fecal\ production\ (\%) = \frac{dried\ fecal\ output\ (\frac{g}{d})}{dry\ matter\ intake\ (\frac{g}{d})}\ x\ 100$$

Apparent total tract digestibility was calculated as:

$$ATTD\ (\%) = \frac{nutrient\ intake\ (\frac{g}{d}) - fecal\ nutrient\ (\frac{g}{d})}{nutrient\ intake\ (\frac{g}{d})}\ x\ 100$$

All data were analyzed using SAS software (2022, release 3.81., SAS Institute Inc., Cary, NC, USA). The results of bowl approached first and diet tasted first from the two-bowl test were analyzed with the chi-square test, and the ratio of consumption with the Student's *t*-test. Median and interquartile range were determined for BCS. Data on fecal consistency and coat quality were analyzed with the Wilcoxon rank sum test to compare scores in each week between dogs fed control and experimental diets (Scheff, 2016). The 12-wk study followed a randomized design with dog as the experimental unit. Data was analyzed according to a mixed model with repeated measurements, including diet, week, and the interaction between diet and week as fixed effects, block as a random effect, and week as a repeated measure. The results from week 0 were considered as a covariate in the model. Means were compared by the LSD post hoc test. Apparent total tract digestibility of nutrients and energy and diet ME content were analyzed using a mixed model, with diet as fixed effect and block as a random effect. The statistical level of significance was considered for $P < 0.05$.

## 3.3 Results

### 3.3.1 Chemical composition of diets

Shrimp hydrolysate and wheat gluten respectively presented (DM basis) 151 g $kg^{-1}$ and 9.32 g $kg^{-1}$ of ash, 658 g $kg^{-1}$ and 815 g $kg^{-1}$ of CP, 93.8 g $kg^{-1}$ and 2.26 g $kg^{-1}$ of EE, 21.3 g $kg^{-1}$ and 12.0 g $kg^{-1}$ of insoluble fiber and 21.2 g $kg^{-1}$ and 18.9 g $kg^{-1}$ of soluble fiber (Table 3.1). The essential AA found in greater concentrations in shrimp hydrolysate were arginine and lysine (7.10 g/100 g total AA and 8.65 g/100 g total AA, respectively). The diets studied had similar nutrient and GE contents, being isoproteic (293 g $kg^{-1}$ CP) and isoenergetic (19.2 MJ $kg^{-1}$ GE). Among the essential AA, leucine and arginine were present in the greatest concentrations, averaging 7.97 g/100 g AA and 6.28 g/100 g AA, respectively. Compared to the control diet, the experimental diet presented greater concentrations of arginine, histidine, lysine, and methionine, and lower concentration of the other essential AA. Both diets had an AAS greater than 100 for all AA and the same index of essential AA (Table 3.2).

Table 3.1. Chemical composition (dry matter basis) and amino acid (AA) profile (g/100g of total AA) of wheat gluten, shrimp hydrolysate, control diet and experimental diet.

| Item | Wheat gluten | Shrimp hydrolysate[1] | Diet[2] | |
|---|---|---|---|---|
| | | | Control | Experimental |
| Dry matter, g $kg^{-1}$ | 897 | 962 | 942 | 957 |
| Ash, g $kg^{-1}$ | 9.32 | 151 | 77.7 | 79.9 |
| Crude protein, g $kg^{-1}$ | 815 | 658 | 293 | 293 |
| Ether extract, g $kg^{-1}$ | 2.26 | 93.8 | 136 | 131 |
| Neutral detergent fiber, g $kg^{-1}$ | ND | ND | 146 | 143 |
| Insoluble dietary fiber, g $kg^{-1}$ | 12.0 | 21.3 | 77.0 | 76.0 |
| Soluble dietary fiber, g $kg^{-1}$ | 18.9 | 21.2 | 31.0 | 37.2 |
| Starch, g $kg^{-1}$ | ND | ND | 330 | 319 |
| Gross energy, MJ $kg^{-1}$ | 22.2 | 21.3 | 19.2 | 19.2 |
| Essential AA, g/100 g total AA | | | | |
| Arginine | 3.70 | 7.10 | 6.17 | 6.39 |
| Histidine | 2.16 | 1.98 | 2.22 | 2.27 |
| Lysine | 1.70 | 8.65 | 5.34 | 5.72 |
| Threonine | 2.80 | 3.93 | 4.04 | 4.13 |
| Isoleucine | 3.46 | 3.94 | 4.85 | 4.83 |
| Leucine | 6.73 | 6.79 | 8.24 | 7.69 |
| Valine | 3.68 | 6.73 | 5.22 | 5.14 |
| Methionine | 1.48 | 3.67 | 3.09 | 3.32 |
| Methionine+cystine | 4.14 | 4.24 | 6.27 | 6.44 |
| Phenylalanine | 5.29 | 5.38 | 5.52 | 5.35 |
| Phenylalanine+tyrosine | 9.4 | 12.1 | 11.0 | 10.7 |
| Non-essential AA, g/100 g AA | | | | |
| Cystine | 2.66 | 0.57 | 3.18 | 3.11 |
| Tyrosine | 4.13 | 6.72 | 5.44 | 5.37 |
| Aspartic acid + Asparagine | 3.19 | 6.59 | 8.00 | 8.37 |
| Glutamic acid + Glutamine | 35.7 | 12.0 | 14.6 | 14.2 |
| Alanine | 2.47 | 7.68 | 5.97 | 5.86 |
| Glycine | 3.46 | 6.84 | 6.70 | 7.02 |
| Proline | 11.9 | 7.70 | 6.62 | 6.45 |
| Serine | 5.57 | 3.75 | 4.80 | 4.75 |

[1]Data reported in Guilherme-Fernandes et al. (2024).
[2]Control diet: commercial diet including, in descending order, poultry by-product meal, corn, wheat, broken rice, pea starch, wheat gluten, poultry and mammal fat, pea protein concentrate, autolyzed brewers' yeast, beet pulp, lucerne, fish oil; Experimental diet: control diet with the inclusion of 5% of shrimp hydrolysate in replacement of wheat gluten, including, in descending order, poultry by-product meal, corn, wheat, broken rice, pea starch, wheat gluten, shrimp hydrolysate, poultry and mammal fat, pea protein concentrate, autolyzed brewers' yeast, beet pulp, lucerne, fish oil.
Abbreviation: ND, not determined.

Table 3.2. Amino acid scores and index of essential amino acids of control and experimental diets.

| Item | Diet[1] | |
|---|---|---|
| | Control | Experimental |
| AAS[2] | | |
| Arginine | 232 | 237 |
| Histidine | 188 | 191 |
| Lysine | 247 | 263 |
| Threonine | 151 | 154 |
| Isoleucine | 206 | 203 |
| Leucine | 196 | 181 |
| Valine | 173 | 169 |
| Methionine | 150 | 161 |
| Methionine+cystine | 161 | 164 |
| Phenylalanine | 199 | 191 |
| Phenylalanine+tyrosine | 240 | 233 |
| Index of essential AA[3] | 192 | 192 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[2]Amino acid score (AAS) determined according to Kerr et al. (2013) using the minimum recommended levels of amino acids by FEDIAF (2021) for adult dogs (maintenance energy requirement of 110 kcal/kg$^{0.75}$).
[3]Index of essential amino acids (AA) calculated according to Oser (1959).

### 3.3.2 Palatability

No significant differences were found in the diet approached first (50% for each diet), and diet tasted first (50% for each diet; Figure 3.1). The ratio of consumption between the control and experimental diets was similar (40.1% vs 59.9%, respectively).

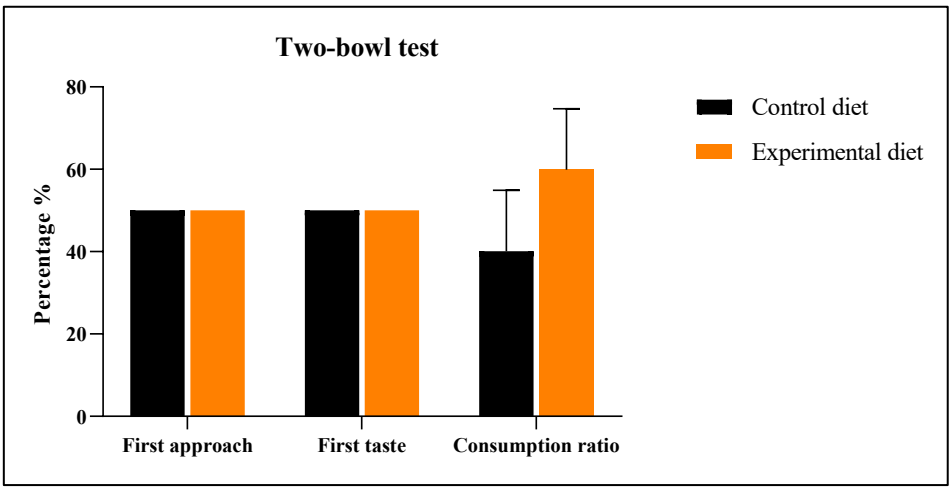


Figure 3.1. Frequency (%) of diet approached first and diet tasted first (mean, n = 12), and consumption ratio (mean ± SEM, n = 12) of control and experimental diets.

### 3.3.3 Body weight, body condition score, and diet and nutrient intake

All dogs remained healthy throughout the study and no emesis or food refusal were observed. The average BW of dogs fed the experimental diet was greater than that of dogs fed the control diet (12.1 kg vs. 11.5 kg, $P < 0.05$; Supplementary Table S1), but week (Supplementary Table S1) and interaction between diet and week kept BW unaffected (Table 3.3). The median BCS of all dogs during the entirety of the study was 5.0 (0) out of 9 in dogs fed both diets. Diet, nutrient and energy intake were not significantly affected by diet, week, and their interaction.

### 3.3.4 Fecal output, characteristics, and metabolites

Replacing wheat gluten with shrimp hydrolysate increased DM fecal output of dogs (26.1 g $d^{-1}$ vs. 23.9 g $d^{-1}$, $P < 0.05$; Supplementary Table S2). Butyrate concentration decreased in feces of dogs fed the experimental diet (75.3 mg $kg^{-1}$, DM, vs. 102 mg $kg^{-1}$, DM, $P < 0.05$). No differences in fecal consistency scores were observed between diets in each week (Figure 3.2 and Supplementary Table S3). Diet had no effect on other fecal parameters measured. Number of defecations ($P < 0.05$) were greatest in week 12, while valerate concentration was greatest in week 8 ($P < 0.05$). Fecal ammonia-nitrogen tended to be greater in week 4 ($P < 0.10$). The interaction between diet and week did not affect any of the parameters evaluated (Table 3.4).

Table 3.3. Body weight, body condition score and diet, nutrient, and energy intake in weeks 4, 8 and 12 of dogs fed control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| Body weight | 11.6 | 11.6 | 11.4 | 11.9 | 12.1 | 12.3 | 0.20 | 0.023 | 0.656 | 0.155 |
| Diet intake | | | | | | | | | | |
| g diet $d^{-1}$, as-is | 194 | 191 | 191 | 193 | 198 | 198 | 11.4 | 0.648 | 0.893 | 0.172 |
| g diet $d^{-1}$, dry matter, DM | 183 | 180 | 180 | 185 | 189 | 189 | 10.8 | 0.424 | 0.888 | 0.173 |
| Nutrient intake, g $d^{-1}$, DM | | | | | | | | | | |
| Organic matter | 168 | 166 | 166 | 170 | 174 | 174 | 10.0 | 0.454 | 0.888 | 0.173 |
| Crude protein | 56.2 | 55.3 | 55.3 | 58.8 | 60.3 | 60.3 | 3.38 | 0.137 | 0.875 | 0.175 |
| Ether extract | 24.9 | 24.5 | 24.5 | 24.1 | 24.8 | 24.8 | 1.45 | 0.953 | 0.902 | 0.171 |
| Neutral detergent fiber | 26.6 | 26.2 | 26.2 | 26.4 | 27.0 | 27.0 | 1.56 | 0.691 | 0.895 | 0.172 |
| Gross energy intake, MJ $d^{-1}$, DM | 3.51 | 3.45 | 3.45 | 3.54 | 3.64 | 3.64 | 0.210 | 0.437 | 0.888 | 0.173 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[a,b,c]Means with different lowercase superscript letters in same row are significantly different ($P < 0.05$).

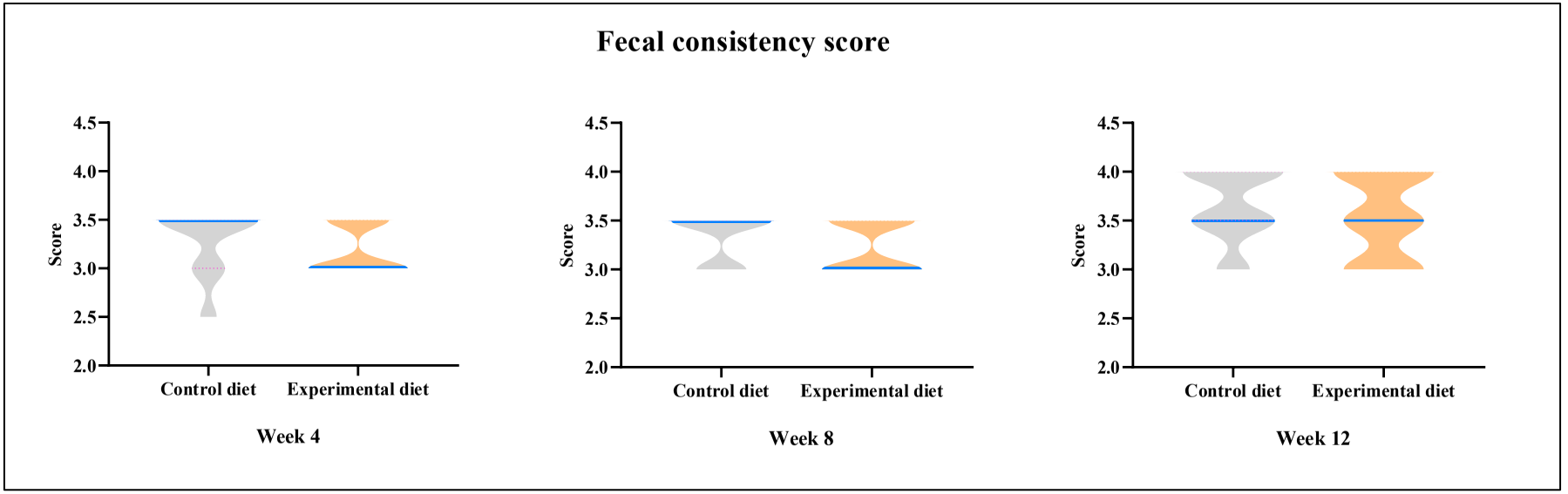


Figure 3.2. Violin plot of fecal consistency scores in weeks 4, 8 and 12 of dogs fed control and experimental diets. The violin plot illustrates the distribution of the scores. Wider sections of the plot indicate a higher concentration of data points, and narrower sections indicate fewer data points. The median scores are represented by the blue line within the plot.

Table 3.4. Fecal output, characteristics, and metabolites in weeks 4, 8 and 12 of dogs fed control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| Fecal output and characteristics | | | | | | | | | | |
| g feces $d^{-1}$, as-is | 67.5 | 89.4 | 81.0 | 92.9 | 96.5 | 71.5 | 10.58 | 0.110 | 0.327 | 0.326 |
| g feces $d^{-1}$, dry matter, DM, basis | 20.4 | 26.6 | 24.8 | 28.7 | 28.2 | 21.3 | 3.03 | 0.014 | 0.436 | 0.241 |
| Fecal production, % | 11.8 | 15.1 | 14.0 | 15.5 | 15.0 | 11.0 | 1.82 | 0.645 | 0.416 | 0.231 |
| Feces DM, % | 29.8 | 29.7 | 30.9 | 31.5 | 29.7 | 30.5 | 0.92 | 0.342 | 0.156 | 0.129 |
| Number of defecations per day | 2.05 | 2.21 | 3.21 | 2.12 | 2.12 | 3.45 | 0.344 | 0.848 | <0.001 | 0.840 |
| Fecal metabolites | | | | | | | | | | |
| Fecal pH | 6.25 | 6.34 | 6.17 | 6.51 | 6.32 | 6.36 | 0.097 | 0.225 | 0.247 | 0.109 |
| Fecal ammonia-nitrogen, mg $kg^{-1}$ DM | 187 | 157 | 160 | 166 | 150 | 157 | 12.6 | 0.469 | 0.065 | 0.644 |
| Volatile fatty acids, mg $kg^{-1}$ DM | | | | | | | | | | |
| Total | 666 | 621 | 618 | 652 | 644 | 604 | 42.1 | 0.966 | 0.521 | 0.877 |
| Acetate | 355 | 323 | 332 | 364 | 338 | 331 | 25.4 | 0.710 | 0.419 | 0.954 |
| Propionate | 163 | 137 | 164 | 177 | 177 | 152 | 12.3 | 0.173 | 0.523 | 0.128 |
| Butyrate | 106 | 116 | 85.0 | 65.4 | 80.8 | 79.9 | 10.41 | 0.004 | 0.223 | 0.161 |
| *Iso*-butyrate | 10.3 | 9.35 | 8.72 | 10.5 | 9.93 | 8.92 | 1.445 | 0.772 | 0.541 | 0.987 |
| *Iso*-valerate | 13.2 | 12.3 | 12.8 | 13.1 | 12.2 | 11.7 | 1.53 | 0.719 | 0.777 | 0.925 |
| Valerate | 9.60 | 13.2 | 6.60 | 5.77 | 11.5 | 7.98 | 1.411 | 0.292 | 0.002 | 0.179 |
| *Iso*-caproate | 6.74 | 8.07 | 7.97 | 9.63 | 8.73 | 7.78 | 1.728 | 0.512 | 0.939 | 0.631 |
| Caproate | 3.16 | 3.45 | 3.06 | 6.60 | 5.25 | 3.47 | 1.419 | 0.118 | 0.518 | 0.573 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.

### 3.3.5 Apparent total tract digestibility

No differences were observed in ATTD and ME content between control and experimental diets (Table 3.5). Dry matter ATTD varied from 76.7% to 79.6%, CP ATTD from 76.6% to 78.1%, and ME from 14.7% to 15.1% in dogs fed the control and the experimental diets, respectively.

Table 3.5. Apparent total tract digestibility of nutrients and metabolizable energy of dogs fed control and experimental diets.

| Item | Diet[1] | | SEM | *P*-value |
|---|---|---|---|---|
| | Control | Experimental | | |
| Dry matter | 76.7 | 79.6 | 1.03 | 0.098 |
| Organic matter | 82.3 | 84.7 | 0.75 | 0.072 |
| Crude protein | 76.6 | 78.1 | 1.96 | 0.711 |
| Ether extract | 94.1 | 94.7 | 0.34 | 0.275 |
| Neutral detergent fiber | 59.0 | 64.6 | 2.08 | 0.051 |
| Starch | 99.4 | 99.5 | 0.08 | 0.896 |
| Gross energy | 83.1 | 85.2 | 0.70 | 0.081 |
| Metabolizable energy, MJ/kg | 14.7 | 15.1 | 0.12 | 0.115 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.

### 3.3.6 Oral volatile sulfur compounds

No differences in oral volatile sulfur compounds of dogs were observed between control and experimental diets before, 1 h and 5 h after the morning meal (Supplementary Table S4). Week had a significant effect ($P < 0.05$) on the concentration of VSC with the greatest values observed in week 4 regardless of the diet offered, particularly 5 h after the morning meal (Supplementary Table S4). The interaction between diet and week did not affect VSC concentrations (Table 3.6).

### 3.3.7 Coat quality

Coat gloss and general evaluation rank sums were greater in dogs fed the control diet than the experimental diet in week 4 (5091 vs. 3688, $z = -3.48$, and 4831 vs. 3947, $z = -2.32$, respectively, $P < 0.05$; Figure 3.3 and Supplementary Table S5). The experimental diet increased rank sums of the coat scale score in weeks 4 and 12 (4932 vs. 3846, $z = 2.70$, and 4780 vs. 3998, $z = 1.98$, respectively, $P < 0.05$).

Table 3.6. Oral volatile sulfur compounds measured in parts per billion (ppb) in weeks 4, 8, and 12 of dogs fed control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| Before meal | 29.2 | 16.5 | 16.7 | 23.7 | 14.0 | 23.0 | 3.47 | 0.841 | 0.010 | 0.207 |
| 1h after meal | 40.8 | 20.8 | 14.3 | 14.7 | 21.7 | 7.00 | 6.60 | 0.054 | 0.049 | 0.132 |
| 5h after meal | 32.8 | 21.4 | 20.4 | 28.0 | 14.2 | 21.0 | 3.02 | 0.105 | 0.001 | 0.365 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.

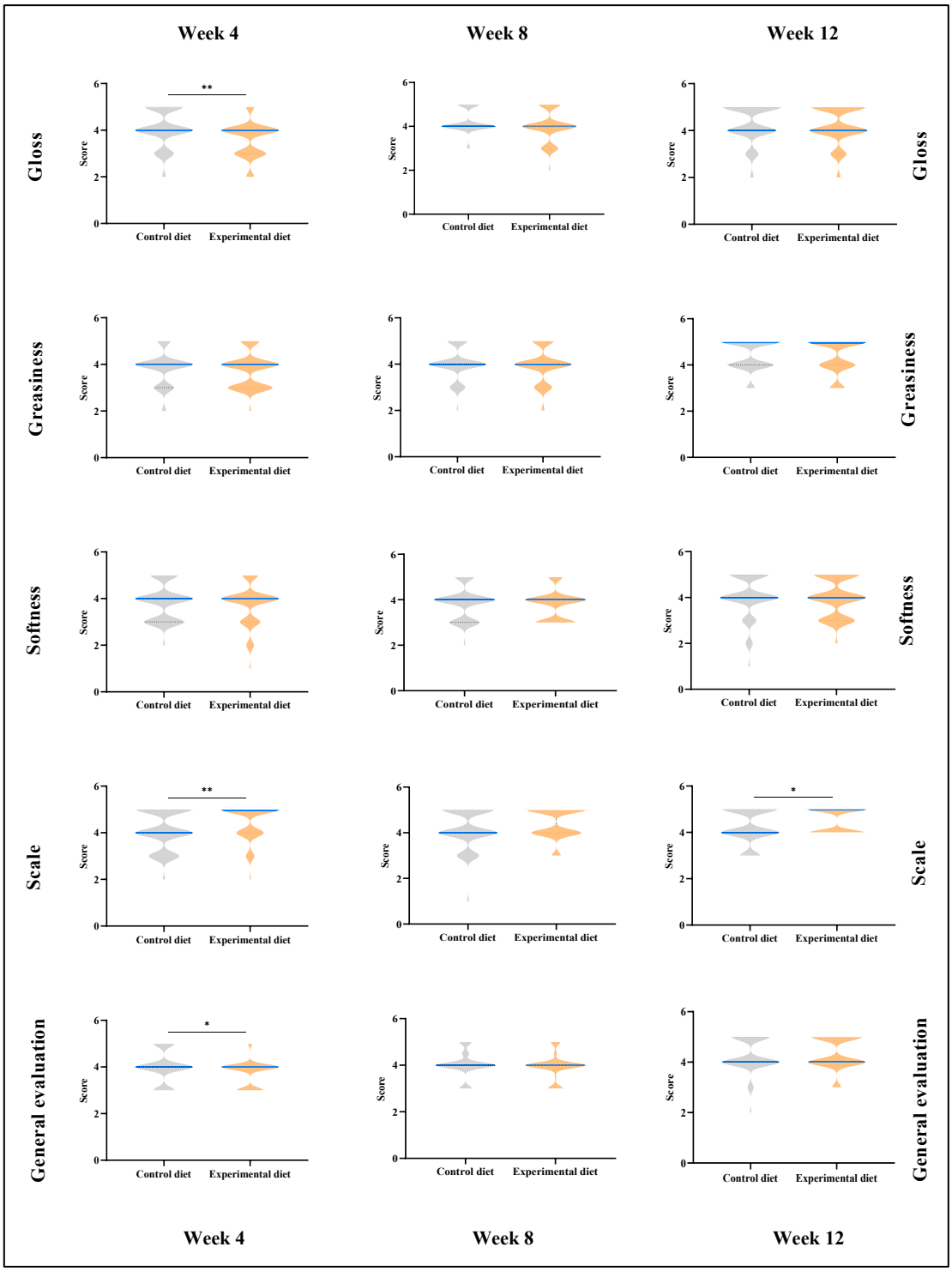


Figure 3.3. Violin plot of gloss, greasiness, softness, scale, and general evaluation scores in weeks 4, 8 and 12 of dogs fed control and experimental diets. The violin plot illustrates the distribution of the scores for each parameter. Wider sections of the plot indicate a higher concentration of data points, and narrower sections indicate fewer data points. The median scores are represented by the blue line within the plot. *$P < 0.05$ and **$P < 0.001$.

## 3.4 Discussion

While shrimp hydrolysate is mainly used in diets for fish, its impact on dog nutrition remains largely unexplored. An earlier short-term study, with 4 periods of 10 days each, revealed the potential of shrimp hydrolysate as a dietary ingredient for dogs when included up to 15% (Guilherme-Fernandes et al., 2024). In this earlier study, performance of shrimp hydrolysate was evaluated blended as-is with a commercial diet, offering valuable insights for potential future applications as an ingredient or for incorporation into diets post-extrusion. Conversely, in the present study, shrimp hydrolysate was included before the extrusion process to facilitate the industrial manufacturing of pet food. Due to high temperature and pressure, the extrusion process can affect nutrient digestibility, starch gelatinization, protein denaturation (Tran et al., 2008), and bioactive properties of protein hydrolysates (Chávez-Ontiveros et al., 2022).

### 3.4.1 Chemical composition of diets

Compared to wheat gluten, shrimp hydrolysate presented a lower CP content and a greater EE content. The low inclusion level of shrimp hydrolysate (5%) did not greatly alter the chemical composition of diets. The AAS indicated that the minimum requirements for AA were ensured in both diets. Lysine and methionine concentrations were greater in the experimental diet, being consistent with the previous study (Guilherme-Fernandes et al., 2024) that showed the potential of shrimp hydrolysate to enhance the levels of these AA in diets, the most common limiting essential AA particularly in vegetable protein sources (Li and Wu, 2023).

### 3.4.2 Palatability

In addition to ensuring that dogs are provided with complete and balanced diets, palatability is a critical factor to consider in dog food as it might affect food intake, the provision of nutrients, and strongly determines food repurchase among pet owners. Protein hydrolysates, such as salmon protein hydrolysate and poultry-liver hydrolysate, have been used by the pet food industry to improve the palatability of diets (Folador et al., 2006; Friesen and Yamka, 2008; Nagodawithana et al., 2008). During the production of these ingredients, hydrolysis breaks proteins into peptides and AA that can positively influence the flavor of foods (Belitz et al., 2009). Salts of glutamic acid and aspartic acid produce the umami taste that play a role in enhancing food flavor (Zhang et al., 2013). The palatability of a commercial diet with shrimp hydrolysate inclusion at 15% was reduced to a consumption of 24.5% compared to 75.5% for the non-supplemented diet (Guilherme-Fernandes et al., 2024). This result might be attributed to the higher inclusion level as well as the mixture of the shrimp hydrolysate to the commercial diet right before

feeding, rather than extruded into the kibble. Moreover, it must be emphasized that despite the sample size (12 dogs) used in the current study defined according to the previous study (Guilherme-Fernandes et al., 2024), the number of dogs was lower than the generally recommended (20 dogs; Aldrich and Koppel, 2015), suggesting some caution when interpreting the results obtained. Even though the sufficient amount for the feeding period was offered in both bowls to allow dogs to choose based on preference rather than caloric need, two-bowl tests often lack sensitivity to the long-term satiating effects of food, even when nutritional and caloric values are considered (Samant et al., 2021), and results are solely dependent on the control diet, not elucidating any particular preference for a specific element in a complex food (Aldrich and Koppel, 2015). Nevertheless, since no food refusal was observed and food was immediately consumed throughout the entirety of the study, it suggests both diets were well accepted by dogs (Le Guillas et al., 2024).

### 3.4.3 Body weight, body condition score, nutrient intake, and fecal output and characteristics

Daily food allowance was adjusted every four weeks according to ME requirements and the ideal BW and BCS of dogs. Although diet significantly affected BW, the results had no biological significance as BW only differed by 0.6 kg between diets. Dogs had ideal BCS, representative of palpable ribs covered with fat, a waist behind the ribs (viewed from above) and an abdomen tucked up (viewed from the side). As feed intake was similar among diets that presented similar chemical composition, diet had no effect on nutrient and energy intake.

Many factors can influence fecal characteristics, such as diet composition, intake and digestibility, microbiota and fecal metabolites, and water intake (Wakshlag et al., 2011; Algya et al., 2018; El-Wahab et al., 2021). In this study, DM fecal output was greater in dogs fed the experimental diet than dogs fed the control diet, whereas fecal production, DM content and consistency score, and number of defecations were unaffected by diet. Although DM fecal output was significantly greater with the experimental diet, the slight difference among diets (average 2.2 g per dog per day) might be insufficient to be detected by pet owners as a negative outcome. However, as this effect might be driven by differences in ATTD, future studies should include evaluation of ATTD of diets over time. Week had no effect on fecal output, production and DM content, but number of defecations increased with time. The current study commenced in winter and ended in spring, encompassing fluctuations in temperature and humidity, which could have influenced the activity levels of dogs in the outdoor parks, as physical exercise has been

shown to increase fecal consistency (Templeman et al., 2020) and colonic motor activity (Dapoigny and Sarna, 1991).

### 3.4.4 Apparent total tract digestibility of diets

The experimental diet did not influence ATTD of nutrients and energy. Previous research indicated no differences in the ATTD of DM, and either no differences or a decrease in the ATTD of organic matter and GE in dogs fed diets including animal hydrolyzed protein sources compared to non-hydrolyzed protein sources (hydrolyzed feather meal vs. diet with bovine meat and bone meal and poultry viscera meal, Machado et al., 2021; hydrolyzed chicken meal vs poultry by-product meal, Pinto et al., 2023). The presence of smaller peptides and AA, being more easily digestible than non-hydrolyzed proteins (Singh et al., 2021), was expected to benefit ATTD of CP. However, no differences were found among diets in the present study. The lack of effect on CP ATTD when wheat gluten was replaced by shrimp hydrolysate might be explained by the high digestibility of the wheat gluten (Kendall and Holme, 1982; Nery et al., 2010). Indeed, the ATTD of CP of dogs fed a wheat gluten meal diet was greater than diets including deboned chicken, chicken by-product meal, and corn gluten meal (Sieja et al., 2023). Furthermore, diets with equal amounts of poultry liver hydrolyzed protein, wheat gluten and poultry meal resulted in a decrease of ATTD of CP compared to diets containing wheat gluten alone or a combination of wheat gluten and poultry meal (Urrego et al., 2017). Additionally, protein digestibility of hydrolysates could potentially be compromised by molecular interactions between ingredients, such as the formation of structures between proteins and other nutrients or the rearrangement of protein aggregates (Schmid et al., 2022). Further factors such as the extrusion process, which can induce protein denaturation, and storage conditions, which may decrease protein solubility and thermal stability, could also influence protein digestibility (Schmid et al., 2022; Gu et al., 2023; Orlien et al., 2023). These aspects should be further exploited in future studies.

### 3.4.5 Fecal metabolites

Fecal pH and metabolites, except butyrate concentration, were unaffected by diet, week and their interaction. Similarly, earlier studies comparing diets without or with protein hydrolysates have reported absence of effects on fecal pH of healthy dogs, with a range of pH values observed (6.49 to 6.84) identical to the present study (Machado et al., 2021; Pinto et al., 2023). Fecal pH is correlated with VFA production in the hindgut, with greater VFA production promoting a decrease in fecal pH, thus contributing to prevent the growth of pathogenic species (Swanson et al., 2002). Fecal ammonia-nitrogen and branched-chain fatty acids arise from the fermentation of protein, and thus could be affected by

dietary protein content and digestibility (Pinto et al., 2023). Diets in the current study presented similar CP and branched-chain AA contents, as well as CP ATTD, agreeing with the absence of effects on fecal ammonia-nitrogen and branched-chain fatty acids.

Butyrate is one of the major VFA present in the gut microbiome, with great intestinal health benefits, constituting a source of energy for colonocytes (Rivera-Chávez et al., 2016), modulating the immune and inflammatory responses and maintaining the integrity of the intestinal barrier (Tan et al., 2014). The decrease of fecal butyrate concentration could be due to greater absorption in the intestinal tract, an increased utilization by the enterocytes, or most likely a decrease in production by gut bacteria (Pilla and Suchodolski, 2020). The amount of soluble and insoluble fiber is known to affect the intestinal microbiota or its metabolic activity (Sunvold et al., 1995; Zentek, 1996), with soluble fiber being more fermentable by gastrointestinal microorganisms, thus increasing the production of VFA. Moreover, the eventual presence of chitin, indigestible by dogs (Okamoto et al., 2001), in the shrimp hydrolysate could also contribute to a decrease VFA production. However, in the present study, the soluble and insoluble fiber contents were similar among diets, and the chitin content is suggested to be residual due to the process of obtention of shrimp hydrolysate. Therefore, the decreased fecal butyrate concentration with the experimental diet might be explained by effects on fecal microbiota composition. Indeed, in an earlier study, the inclusion of shrimp hydrolysate at 5%, 10% and 15% did not affect fecal butyrate concentration. However, when compared to a basal diet (without the inclusion of shrimp hydrolysate), 5% inclusion decreased the abundance of Firmicutes (Guilherme-Fernandes et al., 2024). It is known that most Firmicutes, such as *Clostridium*, *Faecalibacterium and Eubacterium*, produce butyrate through the fermentation of carbohydrates (Garrigues et al., 2022), and in minor extent through glutamate and lysine pathways (Singh et al., 2022). The long-term impact of dietary inclusion of shrimp hydrolysate on the microbiota of dogs should be studied.

### 3.4.6 Oral volatile sulfur compounds

Oral malodor is typically caused by bacteria in the mouth, such as the anaerobic Gram-negative bacteria *Porphyromonas* spp. and *Treponema* spp., the major pathogens causing periodontal disease in dogs (Nordhoff et al., 2008; Ito et al., 2023). These bacteria produce VSC as a result of the metabolism of proteinaceous substrates with sulfur-containing AA. Volatile sulfur compounds mainly comprise hydrogen sulfide, methyl mercaptan and dimethyl sulfide, and can be toxic to tissues even at low concentrations (Culham and Rawlings, 1998; Ratcliff and Johnson, 1999; Milella, 2015). Diet can influence the oral microbiota (Baker and Edlund, 2018), with healthy bacterial populations associated with reduced oral malodor (Oba et al., 2022). In the present

study, no differences on VSC were observed. Bell et al. (2020) found no differences in the oral microbiota of dogs fed diets differing in the main protein source, namely chicken, lamb and salmon. Conversely, Di Cerbo et al. (2015) has shown a significant reduction in halitosis-related sulfur compounds in dogs fed a diet comprising fish protein hydrolysate, *Ribes nigrum* L., *Salvia officinalis*, *Thymus vulgaris*, lysozyme, propolis, bioflavonoids, and vitamin C. The potential role of shrimp hydrolysate, a source of AA and small peptides with bioactive properties, such as antioxidant or antimicrobial activity (Djellouli et al., 2020), on the oral microbiota and VSC concentration in dogs should be studied.

### 3.4.7 Coat quality

A healthy coat is highly valued by pet owners and often plays a significant role in their decision-making when purchasing pet food (Vinassa et al., 2020). Diet strongly influences skin and coat appearance (Watson, 1998), but studies evaluating effects of hydrolyzed protein sources on coat quality are almost nonexistent. A recent study found no variations in the coat quality of dogs fed diets containing either non-hydrolyzed or hydrolyzed chicken protein (Hsu et al., 2023). In the present study, the inclusion of shrimp hydrolysate reduced coat scale, but worsened gloss. Scale is a biomarker of unhealthy skin and is characterized by excessive renewal of the epidermis, resulting in an over-exaggeration of the shedding of dead skin cells. Gloss is influenced by several factors, including the condition of hair cuticles and sebum production (Marsh et al., 2000). Despite the differences among diets, median scores varied between 4 and 5, being representative of great coat health. Coat quality observed in the current study should be interpreted with caution as an earlier study suggested that improvements on coat quality might require a period of study longer than 12 weeks (Geary et al., 2022).

## 3.5 Conclusion

In summary, shrimp hydrolysate supplementation at 5% did not affect diet palatability and ATTD. The absence of effects on ATTD of CP demonstrates the similar digestibility of wheat gluten and shrimp hydrolysate in dogs. Fecal characteristics and metabolites were generally not affected by diet and week, except for butyrate concentration which was reduced in dogs fed the shrimp hydrolysate diet. Differences in the coat quality were not biologically relevant, as both diets promoted a healthy coat. Overall, the results suggest the potential of shrimp hydrolysate inclusion in pet food, contributing to a more sustainable sector. However, further research is needed to fully understand the health benefits of incorporating shrimp hydrolysate into dog food.

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**Conflict of interest statement**

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## Supplementary material

Table S1. Body weight and diet, nutrient, and energy intake of dogs fed control and experimental diets over time.

| Item | Diet[1] | | SEM | Week | | | SEM | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Experimental | | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| Body weight | 11.5 | 12.1 | 0.17 | 11.7 | 11.9 | 11.9 | 0.14 | 0.023 | 0.656 | 0.155 |
| Diet intake | | | | | | | | | | |
| g diet $d^{-1}$, as-is | 192 | 196 | 11.2 | 193 | 194 | 194 | 10.3 | 0.648 | 0.893 | 0.172 |
| g diet $d^{-1}$, dry matter, DM | 181 | 188 | 10.7 | 184 | 185 | 185 | 9.8 | 0.424 | 0.888 | 0.173 |
| Nutrient intake, g $d^{-1}$, DM | | | | | | | | | | |
| Organic matter | 167 | 173 | 9.8 | 169 | 170 | 170 | 9.0 | 0.454 | 0.888 | 0.173 |
| Crude protein | 55.6 | 59.8 | 3.33 | 57.5 | 57.8 | 57.8 | 3.06 | 0.137 | 0.875 | 0.175 |
| Ether extract | 24.6 | 24.5 | 1.43 | 24.5 | 24.6 | 24.6 | 1.32 | 0.953 | 0.902 | 0.171 |
| Neutral detergent fiber | 26.3 | 26.8 | 1.54 | 26.5 | 26.6 | 26.6 | 1.42 | 0.691 | 0.895 | 0.172 |
| Gross energy intake, MJ $d^{-1}$, DM | 3.47 | 3.61 | 0.205 | 3.53 | 3.55 | 3.55 | 0.188 | 0.437 | 0.888 | 0.173 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.

Table S2. Fecal output, characteristics, and metabolites of dogs fed control and experimental diets over time.

| Item | Diet[1] | | SEM | Week | | | SEM | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Experimental | | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| Fecal output and characteristics | | | | | | | | | | |
| g feces $d^{-1}$, as-is | 79.3 | 87.0 | 5.06 | 80.2 | 92.9 | 76.3 | 7.96 | 0.110 | 0.327 | 0.326 |
| g feces $d^{-1}$, dry matter, DM, basis | 23.9 | 26.1 | 1.23 | 24.5 | 27.4 | 23.1 | 2.27 | 0.014 | 0.436 | 0.241 |
| Fecal production, % | 13.6 | 13.8 | 0.92 | 13.6 | 15.0 | 12.5 | 1.41 | 0.645 | 0.416 | 0.231 |
| Feces DM, % | 30.1 | 30.6 | 0.81 | 30.6 | 29.7 | 30.7 | 0.83 | 0.342 | 0.156 | 0.129 |
| Number of defecations | 2.49 | 2.56 | 0.256 | 2.08[a] | 2.17[a] | 3.33[b] | 0.241 | 0.848 | <0.001 | 0.840 |
| Fecal metabolites | | | | | | | | | | |
| Fecal pH | 6.25 | 6.40 | 0.080 | 6.38 | 6.33 | 6.26 | 0.067 | 0.225 | 0.247 | 0.109 |
| Fecal ammonia-nitrogen, mg $kg^{-1}$ DM | 168 | 158 | 9.6 | 177 | 153 | 158 | 8.8 | 0.469 | 0.065 | 0.644 |
| Volatile fatty acids, mg $kg^{-1}$ DM | | | | | | | | | | |
| Total | 635 | 634 | 25.2 | 659 | 633 | 611 | 30.4 | 0.966 | 0.521 | 0.877 |
| Acetate | 337 | 344 | 15.4 | 360 | 330 | 331 | 18.3 | 0.710 | 0.412 | 0.954 |
| Propionate | 154 | 169 | 7.1 | 170 | 157 | 158 | 8.7 | 0.173 | 0.523 | 0.128 |
| Butyrate | 102 | 75.3 | 6.81 | 85.6 | 98.6 | 82.4 | 7.70 | 0.004 | 0.227 | 0.161 |
| *Iso*-butyrate | 9.44 | 9.77 | 0.916 | 10.4 | 9.64 | 8.82 | 1.074 | 0.772 | 0.541 | 0.987 |
| *Iso*-valerate | 12.8 | 12.3 | 0.99 | 13.2 | 12.3 | 12.2 | 1.15 | 0.719 | 0.777 | 0.925 |
| Valerate | 9.79 | 8.43 | 0.864 | 7.68[a] | 12.4[b] | 7.28[a] | 0.987 | 0.292 | 0.002 | 0.179 |
| Iso-caproate | 7.60 | 8.71 | 1.185 | 8.19 | 8.40 | 7.88 | 1.220 | 0.512 | 0.939 | 0.631 |
| Caproate | 3.22 | 5.11 | 0.820 | 4.88 | 4.35 | 3.27 | 1.003 | 0.118 | 0.518 | 0.573 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[a,b,c]Means with different lowercase superscript letters in same row are significantly different ($P < 0.05$).

Table S3. Fecal consistency results of Wilcoxon rank sum tests between dogs fed control and experimental diets in weeks 4, 8 and 12.

| Item | Week 4 | | Week 8 | | Week 12 | |
|---|---|---|---|---|---|---|
| | *z* score | *P* - value | *z* score | *P* - value | *z* score | *P* - value |
| Fecal consistency | 0.906 | 0.365 | 0.187 | 0.374 | -0.659 | 0.516 |

Table S4. Oral volatile sulfur compounds measured in parts per billion (ppb) of dogs fed control and experimental diets over time.

| Item | Diet[1] | | SEM | Week | | | SEM | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Experimental | | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| Before meal | 20.8 | 20.2 | 2.14 | 26.4[a] | 15.3[b] | 19.8[a,b] | 2.54 | 0.841 | 0.010 | 0.207 |
| 1h after meal | 25.3 | 14.4 | 3.81 | 27.8[a] | 21.3[a,b] | 10.7[b] | 4.66 | 0.054 | 0.049 | 0.132 |
| 5h after meal | 24.9 | 21.1 | 2.02 | 30.4[a] | 17.8[b] | 20.7[b] | 2.31 | 0.105 | 0.001 | 0.365 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[a,b,c]Means with different lowercase superscript letters in same row are significantly different ($P < 0.05$).

# CHAPTER 4

## Unraveling the role of shrimp hydrolysate as a food supplement in the immune function and fecal microbiota of Beagle dogs

**Joana Guilherme-Fernandes[1], Carolina Barroso[1], Alexandra Correia[2,3], Tiago Aires[4], Timur Yergaliyev[5,6], Amélia Camarinha-Silva[5,6], Manuel Vilanova[2,3], António J. M. Fonseca[1], Sofia A. C. Lima[1], Margarida R. G. Maia[1*], Ana R. J. Cabrita[1]**

[1]REQUIMTE, LAQV, ICBAS, School of Medicine and Biomedical Sciences, University of Porto, Portugal

[2]i3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

[3]ICBAS, School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal

[4]SORGAL, Sociedade de Óleos e Rações S.A., S. João Ovar, Portugal

[5]HoLMiR, Hohenheim Center for Livestock Microbiome Research, University of Hohenheim, Stuttgart, Germany

[6]Institute of Animal Science, University of Hohenheim, Stuttgart, Germany

[*]Present address: LEAF - Linking Landscape, Environment, Agriculture and Food Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

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**Abstract**

The inclusion of protein hydrolysates from animal by-products in dog food may enhance the pet food industry's sustainability, while contributing to dogs' health due to their nutritional and functional properties. However, inconsistent results of protein hydrolysates on gut microbiota, immune responses, among other health parameters, have been observed in dogs. This study aimed to evaluate the effects of diets supplemented with 5% shrimp hydrolysate, compared to non-supplemented diets, on hematological parameters, serum chemistry profile, innate and adaptive immune function, and fecal microbiota composition in adult Beagle dogs during a 12-week feeding trial. Dietary inclusion of shrimp hydrolysate decreased blood eosinophils and glucose levels, while increasing levels of white blood cells, platelets, neutrophils, and $CD4^+$ and $CD8^+$ T cells single producers of tumor necrosis factor-alpha. It also led to a more extensive proliferation of $CD4^+$ T cells in response to LipL32 antigen and to a decrease in superoxide production in stimulated cells. Additionally, supplemented diets increased Oscillospiracea and Clostridia abundance, and decreased *Sellimonas* abundance. Overall, the results indicate that diet supplementation with shrimp hydrolysate at 5% modulates the immune response and fecal microbiota, highlighting its potential to be included in hypoallergenic and gastrointestinal diets, and in diets for diabetic dogs.

## 4.1 Introduction

Hydrolyzed protein from animal by-products, such as those derived from the human food chain, could benefit dog health while contributing to the pet food industry's economic and environmentally sustainable growth (Hou et al., 2017). Protein hydrolysates comprise low molecular weight peptides and free amino acids with several reported *in vitro* functional properties, such as antioxidant, anti-microbial, anti-inflammatory, and immunomodulatory (Sánchez and Vázquez, 2017), being also reported to modulate gut microbiota composition (Wu et al., 2021).

Protein hydrolysates are commonly used in dog food, especially to prevent adverse food reactions in sensitive dogs (Cave, 2006). However, studies evaluating diets with protein hydrolysates have shown inconsistent effects on gut microbiota, immune response and hematological and biochemical parameters in dogs. For instance, Beagle dogs fed diets containing up to 15% of a commercial mix of black soldier fly larvae hydrolysate and microalgae-like *Schizochytrium* sp. during 28 days had decreased plasma concentrations of the pro-inflammatory cytokine interleukin (IL)-8, triglycerides and total cholesterol, and increased immunoglobulins (Ig) A and G, and albumin levels (Wei et al., 2024). Greater levels of fecal IgA were observed with Beagle dogs fed diets including

chicken hydrolysate at 25% for 28 days, but not with 25% of chicken liver and heart hydrolysates (Hsu et al., 2024). German Shepherd dogs supplemented with 0.3% of hydrolyzed yeast *Saccharomyces cerevisiae* for 42 days exhibited an increase in the abundance of fecal bifidobacteria (at 14$^{th}$ day), lactic acid bacteria (at 42$^{nd}$ day) and clostridia (at 42$^{nd}$ day), and an increase in the serum aspartate aminotransferase at 28 days (Strompfová et al., 2021). Including up to 15% shrimp hydrolysate from *Litopenaeus vannamei* in diets of Beagle dogs over a 10-day feeding trial affected the abundances of Oscillosperaceae, Firmicutes, and *Lactobacillus* in the fecal microbiota (Guilherme-Fernandes et al., 2024a). Conversely, 20% dietary inclusion of pink salmon hydrolysate have failed to demonstrate significant alterations in the immune response of Pointer dogs in a 26-day feeding trial (Zinn et al., 2009). No variations in fecal microbiota, immune response and hematology were observed in Beagle dogs fed with 25.8% (as fed basis) of hydrolyzed chicken liver for 45 days (Pinto et al., 2022). The source of protein hydrolysate, the duration of the study, and the specific breed of dogs used might contribute to these conflicting results.

Despite the effects of shrimp hydrolysate that have been investigated in mice and fish from aquaculture, to the best of the authors' knowledge, there is no information available on the immune response in dogs, and limited data on its effects on microbiota. In mice, a shrimp hydrolysate from *Penaeus chinensis* has been shown to enhance macrophage activation, phagocytosis, the levels of the cytokines interferon-gamma (IFN-γ) and IL-2, and the levels of the antibodies IgA and IgM (Khan et al., 2022b) and to decrease gut pathogenic bacteria abundance (Khan et al., 2022a). In mice exposed to chronic stress, shrimp hydrolysate derived from the heads of unidentified species has been shown to restore fecal short-chain fatty acid levels and improve gut microbiota by modulating alpha diversity and maintaining microbiota distribution (Hu et al., 2024). Shrimp hydrolysate from *L. vannamei* has been shown to benefit different fish species. In red seabream, it has been shown to increase hemoglobin and hematocrit levels (Gunathilaka et al., 2021), decrease glucose levels, and improve innate immunity by enhancing the lysozyme activity and survival rates of fish infected with *Edwardsiella tarda* (Khosravi et al., 2015). In seabass, it positively influenced the survival of fish affected by an epizootic outbreak, with additional benefits in the non-specific immune responses, such as in the lysozyme, alternative complement and bacteriolytic activities (Gisbert et al., 2018). Moreover, the hydrolysis of *Penaeus monodon* with alkaline protease has reduced *in vitro* IgE reactivity to tropomyosin (Lasekan, 2017).

Building on previous research that evaluated the effects of dietary inclusion of 5% shrimp hydrolysate on diet palatability and digestibility, fecal characteristics, coat quality and oral

volatile sulfur compounds of healthy adult Beagle dogs (Guilherme-Fernandes et al., 2024b), this study focused on assessing its impact on the hematological parameters, serum chemistry profile, innate and adaptive immune function, and fecal microbiota composition.

## 4.2 Methods

The trial was approved by the Animal Ethics Committee of the School of Medicine and Biomedical Sciences, University of Porto, licensed by the Portuguese General Directorate of Food and Veterinary Medicine (Permit Nº 0421/000/000/2021). Animal handling and procedures were performed by trained scientists in laboratory animal science (FELASA, Category C) in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. This study was carried out in agreement with the ARRIVE guidelines.

### 4.2.1 Animals, diet and experimental design

Details on animals, diets and experimental design of the trial were earlier reported (Guilherme-Fernandes et al., 2024b). Briefly, twelve adult Beagle dogs, six females and six males, 4.5 ± 0.65 years-old, 12.4 ± 2.53 kg of body weight (BW), and body condition score with a median (interquartile range) of 5.0 (1) out of 9 (Laflamme, 1997), were selected for this study. Dogs were kept in pairs within environmentally enriched boxes in the university kennel. Each dog received its daily food ration individually, divided into two meals at 8:30 a.m. and 5:00 p.m. Daily food allowance was calculated according to requirements of metabolizable energy (ME) and ideal BW (FEDIAF, 2021), following the equation ME (kcal/d) = 110 × $BW^{0.75}$. Fresh water was provided *ad libitum*.

Two extruded isoproteic diets were formulated to meet the nutritional requirements of adult medium dogs (FEDIAF, 2021), using the same ingredients, except for the inclusion of 5% (w/w) shrimp hydrolysate (experimental diet) in replacement of wheat gluten (control diet; Table S1). The shrimp hydrolysate (Symrise Aqua Feed, Elven, France) was obtained by enzymatic hydrolysis of heads and cephalothoraxes of *L. vannamei* (Leduc et al., 2018). A detailed characterization of the diets was earlier reported (Guilherme-Fernandes et al., 2024b). The feeding trial was performed according to a complete randomized block design with 12 dogs distributed into six blocks of two dogs each, according to sex and BW. Within each block, one dog was randomly assigned to one of the two diets, totaling six dogs per diet. The study lasted 12 weeks, comprising four time-points of blood and feces collection at weeks 0, 4, 8 and 12.

### 4.2.2 Blood collection and analyses

Blood samples were collected before the morning meal via jugular vein puncture into VACUETTE ETDA (Greiner Bio-one, Kremsmunster, Austria), VACUETTE Lithium Heparin (Greiner Bio-one) and VACUETTE Serum Blood Collection (Greiner Bio-one) tubes. The blood samples were centrifuged at 500 × g at 22 °C, for 10 min, to allow the isolation of white blood cells. The plasma was recovered and stored at -80 °C for later IgE quantification. For peripheral blood mononuclear cells (PBMC) isolation, the buffy coat was diluted 1:2 in phosphate buffer saline 1× (PBS, Sigma Aldrich, St. Louis, MO, USA). The PBMC were separated by gradient density centrifugation using Histopaque 1.077 (Sigma Aldrich) and washed using PBS. The PBMC were stained with a Tuerk's solution (Sigma Aldrich) and counted using a Neubauer counting chamber. The PBMC were re-suspended in PBS for reactive oxygen species (ROS) assay, and in fetal bovine serum (FBS, heat inactivated South America origin, S181H, BioWest, Nuaillé, France) with dimethyl sulfoxide (25-950-CQC, Corning, Glendale, AZ, USA) at 10% (v/v) to be stored at -80 °C until usage in lymphocyte proliferation and intracellular staining assays. Serum samples were centrifuged at 3000 rpm at 20 °C, for 10 min (Thermo Scientific Heraeus Megafuge 16R, Thermo Fisher Scientific, Carlsbad, MA, USA), for evaluation of serum biochemistry and C-reactive protein, and stored at -80 °C for later cytokine quantification assays.

### 4.2.3 Hemogram, serum chemistry, C-reactive protein and plasma IgE

The hemogram was performed using a hematology analyzer (Sysmex XN-V, Norderstedt, Germany) and the serum chemistry using a Roche Cobas c501 analyzer (Roche Diagnostics, Basel, Switzerland). C-reactive protein was assessed in serum by immunoturbidimetry using a Roche Cobas c501 analyzer (Roche Diagnostics) with the Gentian Canine CRP Immunoassay Kit (Gentian Diagnostics, Stockholm, Sweden). IgE levels in plasma were determined using a commercial canine IgE ELISA kit (MyBioSource, San Diego, CA, USA), following manufacturer's instructions.

### 4.2.4 Cytokine, chemokine, and growth factor quantification in serum

Serum samples were thawed and the concentrations of IFN-γ, IL-10, IL-12/IL-23p40, IL-2, IL-6, IL-8 (CXCL8), monocyte chemoattractant protein-1 (MCP-1/CCL2), nerve growth factor-beta (NGF-β), stem cell factor (SCF), tumor necrosis factor-alpha (TNF-α) and vascular endothelial growth factor A (VEGF-A) were determined using the ProcartaPlex Canine Cytokine/Chemokine/Growth Factor Panel 1, 11plex (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. The analysis was performed in the i3S Scientific Platform Bioimaging with the Bio-Plex 200 system with high-throughput fluidics

(Bio-Rad, Hercules, CA, USA). All washing steps were carried out with the washing buffer in an automated Bio-Plex Pro Wash Station (Bio-Rad). Data acquisition and analysis were performed on the Bio-Plex 200 system, using the Bio-Plex Manager Software version 6.2 (Bio-Rad).

#### 4.2.5 Reactive oxygen species production

For the evaluation of ROS production, PBMC ($1\times10^6$ cells/well) were stimulated with 100 nM phorbol myristate acetate (PMA) for 30 and 60 min at 37 °C and 5% $CO_2$. Non-stimulated cells were used to set basal ROS production. For the quantification of ROS, cells were stained using the ROS-ID Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Lausen, Switzerland), following manufacturer's instructions. Data were acquired by flow cytometry using a FACSCanto II system (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the FlowJo v10 software (BD Biosciences).

#### 4.2.6 Lymphocyte proliferation and cytokine measurement

The PBMC were thawed using complete RPMI 1640 Medium (Sigma Aldrich) at 37 °C and left resting overnight at 37 °C and 5% $CO_2$. Cell populations were stained with trypan blue (Sigma Aldrich) and counted using a Neubauer counting chamber. For the assessment of lymphocyte proliferation, cells were stained with CellTrace Violet Cell Proliferation Kit (Life Technologies Corporation, Eugene, OR, USA). Cells were plated at $2.5\times10^4$ cells/well in 96-well plates and incubated for 4 days at 37 ºC and 5% $CO_2$, with 1 μg $mL^{-1}$ of concanavalin A (ConA) from *Canavalia ensiformis* (C0412, Sigma Aldrich) or 10 μg $mL^{-1}$ of recombinant antigen from *Leptospira interrogans* (LipL32, Rekom Biotech, Granada, Spain). Non-stimulated cells were used as negative controls of cell proliferation. Plates were centrifuged at 300 × g for 5 min, and supernatants were collected and stored at -80 ºC for later cytokine measurements. Cells were stained with the antibodies anti-dog CD3 FITC-conjugate (clone CD3-12, MCA1174F, Bio-Rad), anti-dog CD4 PE-Cy7-conjugate (clone YKIX302.9, 25-5040-42, eBioscience, San Diego, CA, USA) and anti-dog CD8 AlexaFluor700-conjugate (clone YCATE55.9, MCA1039A700, Bio-Rad), and incubated protected from light during 20 min at 4 ºC. Propidium iodide was added to tubes prior to acquisition to assess cell viability. UltraComp eBeads (Invitrogen) were used for compensation. Samples were analyzed by flow cytometry using a LSR Fortessa analyzer (BD Biosciences). Culture supernatants were later thawed to determine the concentration of TNF-α, IFN-γ, IL-10, and IL-17A, using commercial canine ELISA kits (Canine DuoSet ELISA, R&D Systems, Oxford, UK), according to the manufacturer's instruction. The colorimetric detection was assessed

with a Multiskan EX microplate reader (Thermo Fisher Scientific), equipped with Ascent software (Thermo Fisher Scientific).

### 4.2.7 Intracellular staining

Peripheral blood mononuclear cells were thawed, washed in complete RPMI at 37 °C, and left resting overnight at 37 °C and 5% $CO_2$. Cells were plated at $1\times10^6$ cells/well and incubated at 37 °C and 5% $CO_2$ for 4 h in the presence of 1× eBioscience Cell Stimulation Cocktail (Invitrogen) and 3 μg $mL^{-1}$ of eBioscience brefeldin A (Invitrogen). Cell viability was assessed using eBioscience Fixable Viability Dye (FVD) eFluor 506 (Invitrogen). Samples were first stained with FVD at 1:1000 in PBS, protected from light, for 20 min at 4 ºC. After washing with PBS, cells were stained with the antibodies anti-dog CD3 FITC-conjugate (Bio-Rad), anti-dog CD4 PE-Cy7-conjugate (eBioscience), anti-dog CD8 AlexaFluor700-conjugate (Bio-Rad) and anti-dog CD5 PE-conjugate (clone YKIX322.3, 12-5050-42, eBioscience), at pre-titrated dilutions in FACS buffer (PBS, 10 mM of $NaN_3$, 2% FBS) and incubated, protected from light, for 25 min at 4 ºC. Cells were then washed with PBS and fixed with 2% formaldehyde. For Fcγ receptor nonspecific binding, cells were pre-treated with Canine Fc Receptor Binding Inhibitor Polyclonal Antibody (14-9162-42, eBioscience), for 10 min at room temperature, protected from light. After cell fixation with formaldehyde 2%, cells were permeabilized with permeabilization buffer [0.5% saponin (Sigma Aldrich) in FACS buffer] for intracellular staining with the antibodies anti-bovine IFN-γ Alexa Fluor 647-conjugated (clone CC302, MCA1783A647, Bio-Rad) and anti-human TNF-α eF450-conjugated (clone MAb11, 48-7349-42, eBioscience) that cross-react with canine IFN-γ and TNF-α, respectively (Moreira et al., 2015). The PBMC were incubated, protected from light, for 30 min at room temperature, washed twice in permeabilization buffer, and transferred into cytometry tubes. Data acquisition was performed by flow cytometry with a LSR Fortessa analyzer (BD Biosciences).

For intracellular staining of the transcription factor Foxp3, PBMC ($1\times10^6$ cells/well) were stained with FVD eFluor 780 (Invitrogen) at 1:1000 in PBS and incubated, protected from light, for 20 min, at 4 ºC. The PBMC were washed with PBS and stained with anti-dog CD3 FITC-conjugate (Bio-Rad), anti-dog CD4 PE-Cy7-conjugate (eBioscience), and anti-dog CD25 Super Bright 436-conjugate (clone P4A10, 62-0250-42, eBioscience), protected from light, for 25 min at 4 ºC. The PBMC were washed with FACS buffer, fixed with Foxp3 Fixation/Permeabilization solution (eBioscience) for 45 min, and permeabilized using Foxp3 Permeabilization Buffer (eBioscience). The PBMC were pre-treated with Canine Fc Receptor Binding Inhibitor Polyclonal Antibody (eBioscience) and incubated with anti-mouse/rat Foxp3 eF506-conjugate (clone FJK-16s, 69-5773-82,

eBioscience), protected from light, for 30 min at room temperature. The PBMC were transferred into cytometry tubes for data acquisition by flow cytometry with a LSR Fortessa (BD Biosciences). UltraComp eBeads were used for antibody-fluorescence compensation.

#### 4.2.8 Fecal collection and analyses

Individual fresh feces were collected during two consecutive days at weeks 0, 4, 8, and 12. Fecal samples were pooled per dog per week and stored at -20 ºC until further analysis.

#### 4.2.9 Fecal IgA extraction and determination

Fecal IgA extraction was performed based on Peters et al. (2004). Briefly, 1 g of thawed and homogenized feces was diluted in 10 mL of extraction buffer (PBS with 0.5% Tween 20, Sigma-Aldrich) and centrifuged at 1500 × g at 5 ºC for 20 min. Eighty µL of a 25 × concentrated solution of cOmplete EDTA-free Protease Inhibitor Cocktail (04693132001, Roche Diagnostics) were added to 2 mL of supernatant, and centrifuged at 15000 × g at 5 ºC for 15 min. Supernatant was stored at -20 ºC until further analysis. Fecal IgA concentration was assessed using a commercial canine IgA ELISA kit (Dog IgA ELISA Quantitation Set, E44-104, Bethyl Laboratories Inc., Montgomery, TX, USA), following manufacturer's instructions. After optimal dilution determination, samples were diluted in 1:300 or 1:400 in dilution buffer. Absorbance was read in a Multiskan EX microplate reader (Thermo Fisher Scientific), equipped with Ascent software (Thermo Fisher Scientific). The analyses were performed in duplicate.

#### 4.2.10 Fecal microbiota analyses

Fecal DNA was extracted with E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc., Georgia, GA, USA), following manufacturer's instructions. Primers targeting the V4 region of the 16S rRNA gene (forward: GTGYCAGCMGCCGCGGTAA, reverse: GGACTACNVGGGTWTCTAAT) with attached adapters and barcodes were then used for amplification. The produced sequences were purified, quantified, and homogenized. Qualified libraries were sequenced on an Illumina Novaseq 6000 sequencer. Bioinformatic analyses of microbial data were performed using the Qiime2 pipeline (Bolyen et al., 2019). Primers and adapters were removed from the sequences by the cutadapt (Martin, 2011). After trimming, reads were denoised and merged by the dada2 (Callahan et al., 2016). Resulted amplicon sequence variants were classified by VSEARCH-based consensus (Rognes et al., 2016) and pre-fitted sklearn-based classifiers (Pedregosa et al., 2012) against the Silva database (v138.1, 16S 99%) (Quast

et al., 2013). The reference reads were preprocessed by RESCRIPt (Robeson et al., 2021).

### 4.2.11 Calculations and statistical analysis

The $CD4^+/CD8^+$ ratio was calculated from the percentage of $CD4^+$ and $CD8^+$ T cells. Data on blood parameters and fecal IgA were analyzed according to a mixed model with repeated measurements, including diet, week, and the interaction between diet and week as fixed effects, block as a random effect, and week in the subject dog as a repeated measure, using the SAS software (2022, release 3.81., SAS Institute Inc., Cary, NC, USA). Means were compared by the Least Significant Difference post hoc test. The statistical level of significance was considered for $P \leq 0.05$. For alpha diversity estimation of fecal microbiota, Shannon's entropy (Shannon, 1948) and Faith's phylogenetic diversity (Faith, 1992) indices were calculated, and for beta diversity, Compositional Tensor Factorization (CTF) (Martino et al., 2021) distances were used. Alpha diversity metrics were compared by the Wilcoxon test for dependent and the Kruskal-Wallis test for independent samples. Shannon differences were calculated as longitudinal differences of Shannon entropy at weeks 4, 8 and 12 to week 0, which served as baseline. For beta diversity, log ratios of features that contributed to the separation of subjects based on the CTF Principal coordinate analysis plot were extracted by Qurro (Fedarko et al., 2020). Shannon differences and log ratios were then analyzed with linear mixed-effects models (LME) (Seabold and Perktold, 2010). Differentially abundant genera (only for counts of genera with relative abundance ≥ 0.1% and prevalence ≥ 10%) were detected by Ancom-BC (Lin and Peddada, 2020).

## 4.3 Results

### 4.3.1 Hematological and biochemical blood profile

The inclusion of shrimp hydrolysate led to a decrease in the percentage of eosinophils (4.50% vs. 5.51%, $P = 0.017$) and in the levels of glucose (86.4 mg $dL^{-1}$ vs. 92.8 mg $dL^{-1}$, $P = 0.023$), and increased the concentration of white blood cells ($7.67 \times 10^3$ $\mu L^{-1}$ vs. $6.71 \times 10^3$ $\mu L^{-1}$, $P = 0.002$), platelets ($300 \times 10^3$ $\mu L^{-1}$ vs. $274 \times 10^3$ $\mu L^{-1}$, $P = 0.038$), and the percentage of neutrophils (56.8% vs. 56.8%, $P = 0.036$; Table 4.1 and Table S2). Greater percentage of eosinophils in the blood ($P = 0.003$), and greater concentrations of total protein ($P = 0.001$), glucose ($P < 0.001$), and hemoglobin ($P = 0.013$) were observed in week 8. Platelet concentration ($P = 0.040$) and mean platelet volume ($P = 0.004$) were greater in week 4, whereas albumin concentration was lower ($P < 0.001$). Concentration of IgE in plasma was lower in week 12 ($P < 0.001$). The interaction

between week and diet affected the concentrations of the red blood cells, ranging from $7.10 \times 10^6$ $\mu L^{-1}$ in week 4, to $7.49 \times 10^6$ $\mu L^{-1}$ in week 12 in dogs fed the control diet ($P$ = 0.044), and the hemoglobulin levels that varied from 16.6 g $dL^{-1}$ in week 4 to 17.7 g $dL^{-1}$ in week 12 in dogs fed the control diet ($P$ = 0.037).

### 4.3.2 Serum cytokine, chemokine, and growth factor concentrations

Concentrations of IFN-γ, IL-10, IL-6, NGF-β, and TNF-α were below the detection limits (8.42 pg $mL^{-1}$, 6.47 pg $mL^{-1}$, 16 pg $mL^{-1}$, 4.98 pg $mL^{-1}$ and 5.22 pg $mL^{-1}$, respectively). Diet and week did not affect the production of the different cytokines, chemokine, and growth factors in serum (Table 4.2 and Table S3). The interaction between diet and week affected the production of IL-8 ($P$ = 0.038), ranging from 539 pg $mL^{-1}$ in week 8 to 964 pg $mL^{-1}$ in week 12 in dogs fed the control diet. The interaction between diet and week also affected the production of SCF ($P$ = 0.027), from 29.6 pg $mL^{-1}$ in week 4 to 50.6 pg $mL^{-1}$ in week 12 in dogs fed the control diet.

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Table 4.1. Hematology, serum chemistry, C-reactive protein, plasma immunoglobulin E in weeks 4, 8 and 12 in dogs fed control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM[2] | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| White blood cells, × $10^3$ µL$^{-1}$ | 6.86 | 6.83 | 6.45 | 7.09 | 7.62 | 8.29 | 0.433 | 0.002 | 0.601 | 0.139 |
| Neutrophils, % | 54.7 | 50.8 | 54.2 | 54.7 | 56.2 | 59.3 | 1.93 | 0.036 | 0.129 | 0.168 |
| Lymphocytes, % | 35.0 | 37.2 | 34.9 | 35.8 | 32.7 | 30.7 | 2.05 | 0.183 | 0.265 | 0.219 |
| Monocytes, % | 5.54 | 5.42 | 5.79 | 4.91 | 5.21 | 5.81 | 0.467 | 0.437 | 0.347 | 0.734 |
| Eosinophils, % | 4.80 | 6.63 | 5.11 | 4.25 | 5.50 | 3.75 | 0.500 | 0.017 | 0.003 | 0.671 |
| Red blood cells, × $10^6$ µL$^{-1}$ | $7.10^{a}$ | $7.43^{b,c,d}$ | $7.49^{c,d}$ | $7.30^{a,b,c}$ | $7.38^{a,b,c}$ | $7.18^{a,b}$ | 0.153 | 0.636 | 0.068 | 0.044 |
| Hemoglobin, g dL$^{-1}$ | $16.6^{a}$ | $17.6^{b,c}$ | $17.7^{c}$ | $17.1^{a,b,c}$ | $17.4^{b,c}$ | $16.9^{a,b}$ | 0.37 | 0.596 | 0.013 | 0.037 |
| Platelets, × $10^3$ µL$^{-1}$ | 301 | 252 | 270 | 318 | 294 | 289 | 17.0 | 0.038 | 0.040 | 0.632 |
| Mean platelet volume, fL | 10.1 | 9.87 | 9.64 | 10.1 | 9.88 | 9.76 | 0.192 | 0.914 | 0.004 | 0.650 |
| Total protein, g dL$^{-1}$ | 5.51 | 5.85 | 5.78 | 5.61 | 6.07 | 5.89 | 0.098 | 0.106 | 0.001 | 0.740 |
| Albumin, g dL$^{-1}$ | 3.59 | 3.81 | 3.83 | 3.64 | 3.89 | 3.82 | 0.061 | 0.565 | <0.001 | 0.657 |
| Globulin, g dL$^{-1}$ | 1.93 | 2.04 | 1.95 | 1.97 | 2.18 | 2.06 | 0.062 | 0.067 | 0.054 | 0.720 |
| Glucose, mg dL$^{-1}$ | 87.2 | 101 | 90.3 | 82.6 | 95.3 | 81.4 | 2.60 | 0.023 | <0.001 | 0.608 |
| Creatinine, mg dL$^{-1}$ | 0.764 | 0.880 | 0.762 | 0.836 | 0.793 | 0.808 | 0.0635 | 0.894 | 0.456 | 0.143 |
| Urea, mg dL$^{-1}$ | 26.5 | 38.0 | 34.5 | 28.7 | 28.0 | 28.9 | 3.55 | 0.098 | 0.229 | 0.172 |
| Alanine aminotransferase, U L$^{-1}$ | 27.7 | 27.2 | 30.4 | 36.6 | 29.4 | 29.3 | 3.34 | 0.218 | 0.471 | 0.273 |
| Alkaline phosphatase, U L$^{-1}$ | 43.5 | 43.3 | 42.3 | 45.7 | 45.8 | 44.0 | 2.16 | 0.396 | 0.618 | 0.966 |
| C-reactive protein, µg mL$^{-1}$ | 7.37 | 8.41 | 5.19 | 8.23 | 9.09 | 9.01 | 1.054 | 0.234 | 0.318 | 0.277 |
| Immunoglobulin E, µg mL$^{-1}$ | 216 | 241 | 55.9 | 195 | 177 | 71.3 | 26.1 | 0.438 | <0.001 | 0.135 |

[1]Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[2]SEM: Standard error of the mean.
[a,b,c,d]Means with different superscript letters in the same row are significantly different ($P < 0.05$).

Table 4.2. Concentration of cytokines, chemokine, and growth factor in serum in weeks 4, 8 and 12 of dogs fed the control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM[2] | *P* – value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| Il-12/IL-23p40, pg mL$^{-1}$ | 666 | 749 | 963 | 1209 | 1223 | 1182 | 328.8 | 0.371 | 0.240 | 0.115 |
| IL-8, pg mL$^{-1}$ | 774$^{a,b}$ | 539$^{a}$ | 964$^{b}$ | 783$^{a,b}$ | 877$^{a,b}$ | 709$^{a,b}$ | 144.3 | 0.862 | 0.502 | 0.038 |
| IL-2, pg mL$^{-1}$ | 28.2 | 39.3 | 62.0 | 95.9 | 104 | 97.4 | 49.90 | 0.161 | 0.901 | 0.899 |
| SCF, pg mL$^{-1}$ | 29.6$^{a}$ | 33.8$^{a}$ | 50.6$^{b}$ | 43.9$^{a,b}$ | 47.1$^{a,b}$ | 41.7$^{a,b}$ | 15.64 | 0.750 | 0.214 | 0.027 |
| MCP-1, pg mL$^{-1}$ | 106 | 113 | 90.3 | 103 | 99.0 | 98.4 | 10.17 | 0.713 | 0.366 | 0.464 |
| VEGF-A, pg mL$^{-1}$ | 6.00 | 5.47 | 5.85 | 5.62 | 5.08 | 8.36 | 1.016 | 0.622 | 0.059 | 0.103 |

[1]Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[2]SEM: Standard error of the mean.
[a,b]Means with different superscript letters in the same row are significantly different ($P < 0.05$).
Abbreviations: IL, interleukin; SCF, stem cell factor; MCP-1, monocyte chemoattractant protein-1; VEGF-A, vascular endothelial growth factor A.

### 4.3.3 Reactive oxygen species production

Cells of both control and experimental diets groups produced greater amounts of total ROS after 30 min of PMA stimulation than after 60 min (Figure 4.1A and Table S4). Conversely, superoxide production was lower after 30 min than after 60 min, with the control diet showing greater levels of production of superoxide after 60 min of PMA stimulation compared to the experimental diet ($P$ = 0.002; Figure 4.1B). The interaction between diet and week affected the production of total ROS after 60 min of PMA stimulation with higher fold increases in week 12 in the control and experimental diets ($P$ = 0.030), and the production of superoxide after 60 min of PMA stimulation with greater fold increase in week 12 with the control diet ($P$ = 0.030).

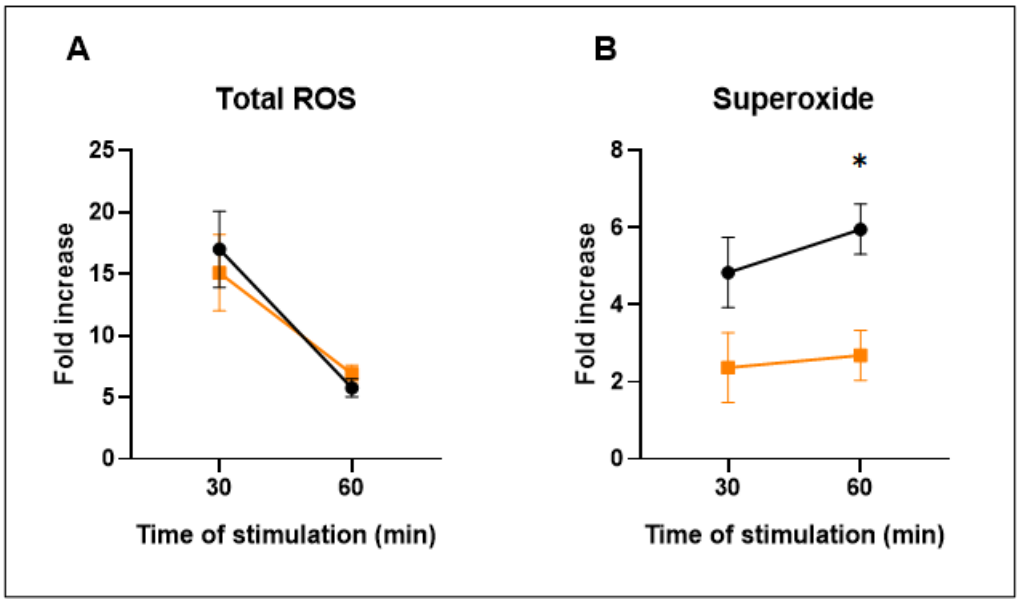


Figure 4.1. Reactive oxygen species (ROS) production evaluated by flow cytometry. A) Fold increase in the production of total ROS; B) Fold increase in the production of superoxide in cells stimulated with phorbol myristate acetate for 30 and 60 min over the basal production (non-stimulated) in control diet (round black dots) and experimental diet (square orange dots). Bars correspond to mean plus standard error of the mean. *$P$ < 0.05.

### 4.3.4 Lymphocyte proliferation and cytokine production

The percentages of proliferation of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells stimulated with ConA were not affected by the diet. The experimental diet induced more extensive proliferation of $CD3^+CD4^+$ cells in response to LipL32, when compared to the control diet (10.8% vs. 2.07%, respectively, $P$ = 0.020), and no diet effects were observed in the proliferation of $CD3^+CD8^+$ cells stimulated with this antigen (Figure 4.2).

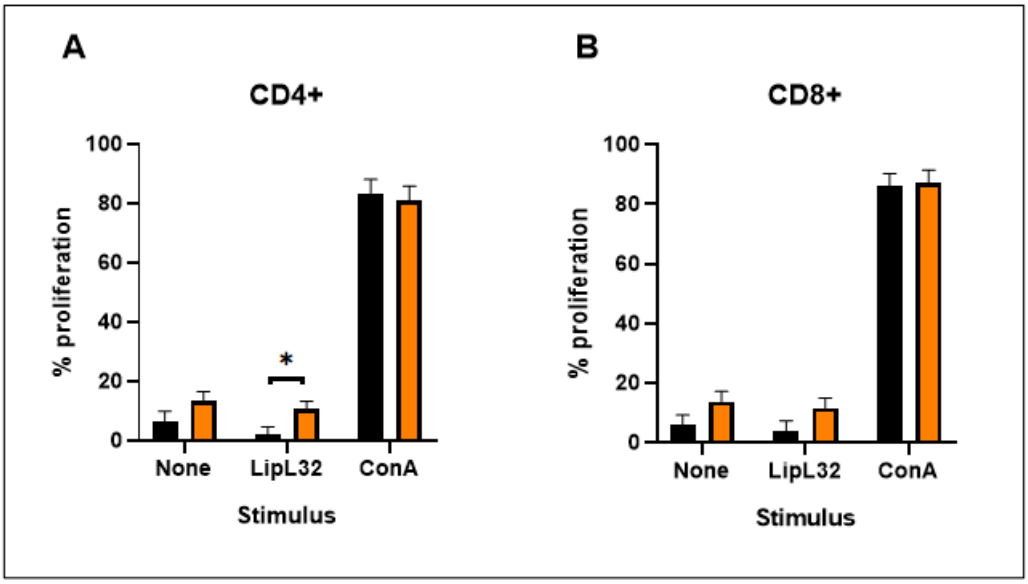


Figure 4.2. T lymphocyte proliferation evaluated by flow cytometry. A) Percentage of $CD3^+CD4^+$ cells; B) Percentage of $CD3^+CD8^+$ cells, from dogs fed control diet (black columns) and experimental diet (orange columns), that proliferated at least once when non-stimulated (None) or in response to recombinant antigen from *Leptospira interrogans* (LipL32) and concanavalin A (ConA). Bars correspond to mean plus standard error of the mean. * $P < 0.05$.

The levels of IL-17, IFN-γ, TNF-α and IL-10 in culture supernatants of non-stimulated PBMC or stimulated with LipL32 were below the detection limits (62.5 pg $mL^{-1}$, 31.3 pg $mL^{-1}$, 31.3 pg $mL^{-1}$ and 15.6 pg $mL^{-1}$, respectively). In week 12, the PBMC stimulated with ConA produced increased levels ($P < 0.05$) of IL-17, TNF-α, and IL-10, and decreased levels of IFN-γ ($P = 0.011$; Table S5). No effect of diet and of interaction between diet and week were found in the production of IL-17, IFN-γ, TNF-α and IL-10 in cells stimulated with ConA (Table 4.3).

Table 4.3. Concentration of cytokines after lymphocyte stimulation with concanavalin A in weeks 4, 8 and 12 of dogs fed the control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM[2] | *P* – value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| IL-17, pg $mL^{-1}$ | 470 | 1284 | 2177 | 334 | 1202 | 1422 | 509.3 | 0.405 | 0.027 | 0.752 |
| IFN-γ, pg $mL^{-1}$ | 2762 | 1586 | 1308 | 1626 | 1883 | 875 | 471.1 | 0.452 | 0.011 | 0.122 |
| TNF-α, pg $mL^{-1}$ | 25.2 | 18.6 | 47.8 | 29.4 | 19.7 | 35.3 | 5.90 | 0.594 | 0.004 | 0.317 |
| IL-10, pg $mL^{-1}$ | 135 | 156 | 289 | 72.7 | 148 | 211 | 44.27 | 0.124 | 0.002 | 0.729 |

[1]Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of 5% of wheat gluten.
[2]SEM: Standard error of the mean.
Abbreviations: IFN-γ: interferon-gamma; IL, interleukin; TNF-α: tumor necrosis factor alpha.

### 4.3.5 Production of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells and Foxp3 in $CD4^+$

The inclusion of shrimp hydrolysate did not influence the percentage of $CD4^+$ and $CD8^+$ T cells single IFN-γ producers (Figure 4.3A), IFN-γ and TNF-α double producers (Figure 4.3B), and the $CD4^+/CD8^+$ T cell ratio (Figure 4.3C). However, it positively influenced the $CD4^+$ TNF-α T cells single producers (from 13.1%, in dogs fed the control diet, to 20.2%, in those fed the experimental diet, $P < 0.001$), and the $CD8^+$ TNF-α T cells single producers (3.78% and 7.09% for dogs fed the control and the experimental diet, respectively, $P < 0.001$; Figure 4.3A; 4.3B). The percentage of $CD4^+$ T cells was greatest in week 4 (63.9%, $P = 0.050$; Table S6). The $CD4^+$ T cells double producers of IFN-γ and TNF-α and single producers of TNF-α presented the greatest values in week 12 (15.2%, $P = 0.002$, and 20.3%, $P < 0.001$, respectively), while $CD4^+$ T cells single producers of IFN-γ presented the greatest value in week 8 (8.62%, $P < 0.001$). $CD8^+$ T cells double producers of IFN-γ and TNF-α presented the greatest value in week 12 (32.7%, $P < 0.001$). No differences were found in the percentage of $CD4^+CD25^+Foxp3^+$ T cells between diets (Figure 4.3D), over time and in the interaction of diet and week.

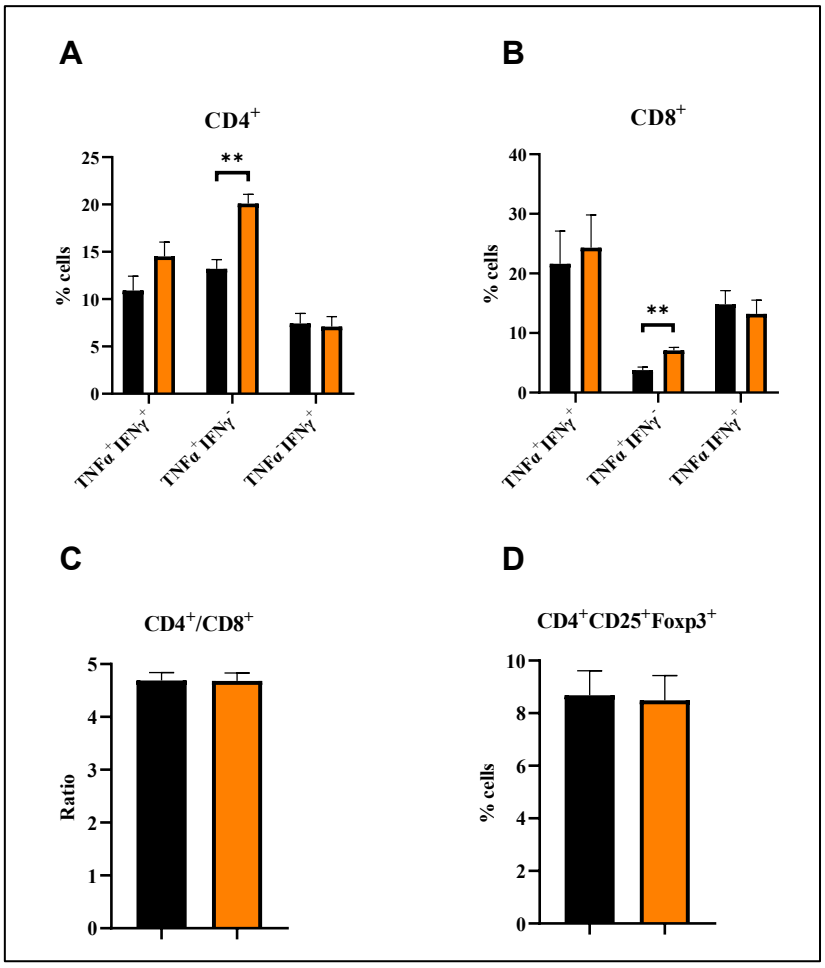


Figure 4.3. Intracellular cytokine measurement by flow cytometry. A) Percentage of $CD3^+CD4^+$ cells expressing interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and both cytokines; B) Percentage of $CD3^+CD8^+$ cells expressing IFN-γ, TNF-α and both cytokines; C) Ratio of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells calculated from the percentage of $CD4^+$ and $CD8^+$ T cells; D) Percentage of $CD3^+CD4^+CD25^+$ cells expressing Foxp3 from dogs fed control diet (black columns) and experimental diet (orange columns). Bars correspond to mean plus standard error of the mean. ** $P < 0.01$.

### 4.3.6 Fecal IgA and microbiota

Fecal IgA concentration was not affected by diet, week, and their interaction (Figure 4.4). Regardless of the diet, *Fusobacterium* was the most abundant genus, followed by genus pertaining to Muribaculaceae and genus *Bacteroides* (Figure 4.5A). Beta diversity metrics indicate a clear separation of bacterial communities between diets, by using CTF distance (Figure 4.5B). Linear mixed-effects analysis performed on log-ratios of feature loadings of abundances that contributed to diets separation confirmed a separation between control and experimental diets (Figure 4.5C). Regarding alpha diversity, no differences were observed between diets on Shannon entropy and Faith's phylogenetic

diversity through Kruskal-Wallis and Wilcoxon tests between diets within weeks (Figure 4.5D). The experimental diet led to an increased abundance of genera pertaining to Oscillospiracea and Clostridia, while abundance of *Sellimonas* decreased ($P < 0.05$; Figure 4.5E).

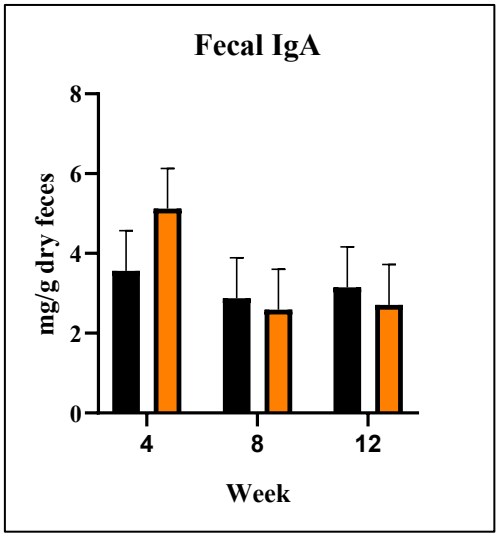


Figure 4.4. Fecal immunoglobulin A (IgA) concentrations in weeks 4, 8 and 12 in dogs fed control diet (black columns) and experimental diet (orange columns). Bars correspond to mean plus standard error of the mean.

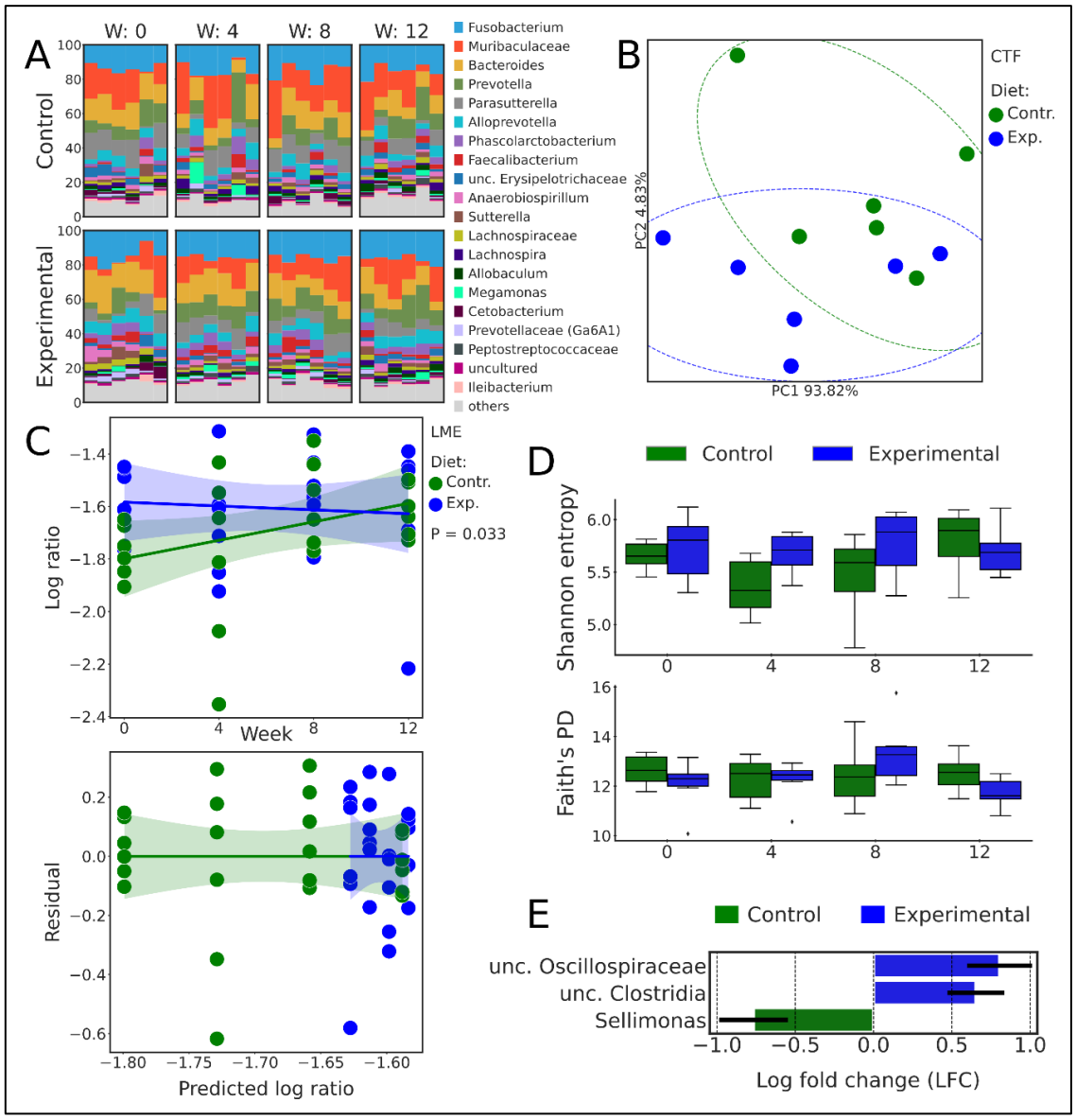


Figure 4.5. Bacterial relative abundances, composition and diversity. A) Taxonomy barplots at the genus level of dogs fed control and experimental diets in weeks 0, 4, 8, and 12. If genus level was not assigned, the last available taxonomy rank was used for the label; B) Beta diversity metrics. Compositional Tensor Factorization (CTF) distance of fecal bacteria of dogs fed control and experimental diets. Each dot represents one dog fed on either diet; C) Linear mixed-effects analysis based on log-ratios of fecal bacteria of dogs fed control and experimental diets; D) Alpha diversity metrics. Bloxplots of Shannon entropy and Faith's PD indices of fecal bacteria of dogs fed control and experimental diets; E) Differentially abundant genera (*P*-adj < 0.05), according to ANCOM-BC. The fecal bacteria of dogs fed the experimental diet were compared to those of the control diet.

## 4.4 Discussion

The incorporation of animal by-products in pet nutrition has been a subject of growing interest due to its potential impact on animal health and industry sustainability (Acuff et al., 2021). From a nutritional standpoint, hydrolyzed protein from animal by-products, which comprise smaller peptides and amino acids, may offer essential nutrients and functional properties that can benefit dogs' health (Vasconcellos et al., 2024). This study aimed to evaluate the effects of the dietary inclusion of 5% shrimp hydrolysate on hematological, serum chemistry, immunological parameters, and fecal microbiota of healthy adult Beagle dogs.

Overall, the blood profiles of dogs showed values within the established reference ranges for healthy adult dogs (Kahn, 2005; Kaneko et al., 2008). The dietary inclusion of shrimp hydrolysate led to an increase in neutrophils, platelets and white blood cells, suggesting that shrimp hydrolysate might play a role in modulating the immune function and supporting a healthy bone marrow function (Nothdurft and Kreja, 1998). Furthermore, the inclusion of shrimp hydrolysate led to a decrease in eosinophils concentration, but no variations in IgE were observed among diets. Eosinophils are known to play a role in allergic reactions and combating certain infections. Their differentiation and activation can be mediated by cytokines, such as IL-5 and IL-3, chemokines, prostaglandin D2, and indirectly be influenced by the IgE pathway (Matucci et al., 2018). The levels of eosinophils observed in the present study were within the normal range values for dogs (0-9%; Kahn, 2005), likewise the levels of IgE in blood (25-410 μg $mL^{-1}$; Wilkie et al., 1990). Nevertheless, a reduction in the concentration of eosinophils suggests that shrimp hydrolysate may modulate the immune system, potentially decreasing inflammation or allergic responses. This is consistent with the finding that shrimp hydrolysate did not affect IgE-mediated allergic responses in the dogs. Previous research has shown that the dietary inclusion of chicken liver hydrolysate induced a decrease in the levels of eosinophils and IgE in the plasma of dogs over a 45-day feeding trial (Pinto et al., 2022). The IgE levels decreased in week 12 in dogs fed either diet, whereas the eosinophils levels were lower in weeks 4 and 12.

The inclusion of shrimp hydrolysate led to a significant decrease in glucose levels in the blood compared to the control diet, despite all values remaining within the normal range for healthy dogs (76-119 mg $dL^{-1}$; Kahn, 2005). Fish hydrolysates have been demonstrated to lower glucose levels and regulate hyperglycemia *in vitro*, in murine models, and in human subjects (Sharkey et al., 2020). Furthermore, reduced glucose levels were observed in red seabream fed diets containing shrimp hydrolysate from *L.*

*vannamei* (Khosravi et al., 2015). Little is known on the effect of hydrolyzed proteins in blood glucose levels in dogs. In a study using an *in vitro* gastrointestinal digestion model for dogs, it was observed that, compared to raw tilapia, its hydrolysate enhanced the secretion of active glucagon-like peptide-1 (GLP-1), a hormone involved in blood glucose regulation, and improved the inhibition of dipeptidyl peptidase-IV enzymatic activity in Caco-2 cells, crucial in degrading GLP-1 (Theysgeur et al., 2020). Moreover, diets containing hydrolyzed yeast *S. cerevisiae* have been shown to increase blood glutathione concentration, an antioxidant which may protect cells from oxidative damage caused by ROS (Kim et al., 2012). The excessive production of superoxide radicals is considered the main mechanism responsible for tissue damage in diabetes mellitus (Brownlee, 2005), and superoxide production may stimulate insulin release through the metabolism of branched-chain keto-acids in mitochondria (Plecitá-Hlavatá et al., 2020). Additionally, high blood glucose levels increase the generation of ROS in both mitochondria and cytosol, which contributes to the development of various diabetes-related pathologies (González et al., 2023). Therefore, the decreased levels of blood glucose herein observed might be associated with the decreased production of superoxide compared to dogs fed the control diet, suggesting that lowering superoxide production could have led to upregulation of glucose metabolism. These results agree with the reported *in vitro* antioxidant activity of shrimp hydrolysate (Guilherme-Fernandes et al., 2024a), which possibly helped reduce superoxide production by neutralizing free radicals. However, no differences were found in total ROS production between the control and experimental groups.

The interaction between diet and week affected serum concentrations of IL-8 and SCF, with the lowest and greatest values observed in weeks 8 and 12 in dogs fed the control diet. In dogs fed the experimental diet, the concentrations remained unaltered over time. IL-8 is a chemokine produced by various immune cells and it plays important roles in a wide variety of functions, such as recruiting neutrophils, basophils and T cells during immune responses to infection, inflammation, and white blood cells activation (Brennan and Zheng, 2007). Stem cell factor is a cytokine that binds to the c-kit, a tyrosine kinase receptor, and interacts with other cytokines, protecting the viability of hematopoietic cells, while also inducing their proliferation and differentiation (Hassan and Zander, 1996). The increase of both IL-8 and SCF could be linked, as previously demonstrated (Gooya et al., 1996), potentially resulting from the production of IL-8 by hematopoietic progenitors cells (Laterveer et al., 1995).

No differences were found between control and experimental groups in the production of cytokines by lymphocytes stimulated with ConA. Similarly, gene expression of IL-10

and IFN-γ in dogs were not affected by the dietary inclusion of pink salmon hydrolysate (Zinn et al., 2009). Additionally, dog diets including black soldier fly larvae hydrolysate and *Schizochytrium* sp. did not affect the production of TNF-α in non-stimulated cells, while the levels of IL-8 in plasma decreased (Wei et al., 2024). Although the levels of IL-8 in serum were similar between diets, the inclusion of shrimp hydrolysate tended to result in decreased IL-8 levels, suggesting a possible anti-inflammatory effect. Lymphocytes stimulated with ConA produced increased levels of IL-17, TNF-α and IL-10 over time, whereas the production of IFN-γ decreased. The levels of IL-17 and IFN-γ can exhibit inverse or corresponding patterns in immune responses due to complex regulatory networks (Belpaire et al., 2022; Shao et al., 2022), and diet might influence their regulation with important effects in autoimmune diseases (Zhang et al., 2024). Moreover, TNF-α can promote a reduction of Th1 cells producing IFN-γ and an increase of Th17 cells secreting IL-17 (Pesce et al., 2022), and an increment of the anti-inflammatory cytokine IL-10 (Mitoma et al., 2005). IL-10 can inhibit pro-inflammatory responses of innate and adaptive immune cells by suppressing the production of various cytokines, such as IFN-γ and TNF-α (de Waal Malefyt et al., 1991). It has also been shown to play an important role in the homeostasis of intestinal mucosal (Shouval et al., 2014).

Diet did not influence lymphocyte proliferation, except for the greater proliferation of $CD4^+$ T cells stimulated with LipL32 from dogs fed shrimp hydrolysate compared to the control diet. LipL32 is a protein present in outer membrane of leptospires that may induce a proliferative response by memory $CD4^+$ T cells (Teixeira et al., 2022). Dogs that participated in the study were annually vaccinated for leptospires. Recently, it has been shown that the percentage of central memory $CD4^+$ T cells and proliferation of $CD4^+$ T cells in response to different *Leptospira* serovars increased in dogs after vaccination (Novak et al., 2023). Diet plays an important role in the regulation of memory T cells, opening the possibility of developing and implementing diet-based therapies that can be used to attain more effective vaccination strategies (Collins, 2020). Therefore, the results might indicate a positive effect of shrimp hydrolysate in the generation of memory $CD4^+$ T cells, which could lead to a more effective immune response against leptospiral infections in dogs. Furthermore, lymphocyte proliferation has been observed in PBMC isolated from blood of dogs with suspected food hypersensitivity when cultured with extracts of commercially hydrolyzed canine diets (Masuda et al., 2020). Despite no research on lymphocyte proliferation of healthy dogs fed protein hydrolysates has been conducted, hydrolysates from Alaska pollock frame, oyster and *Paphia undulata* were

shown to enhance lymphocyte proliferation of cells isolated from mouse spleens (Wang et al., 2010; Hou et al., 2012; Cai et al., 2013; He et al., 2015).

No differences between control and experimental groups were found in the percentage of $CD4^+$ and $CD8^+$ T cells, and in their ratio. Similar results were observed in dogs fed diets with hydrolyzed yeast inclusion (Strompfová et al., 2021). Dietary inclusion of shrimp hydrolysate promoted the increment in $CD4^+$ and $CD8^+$ T cells single producers of TNF-α. While TNF-α plays an important role in the defense against pathogens (Zannoni et al., 2020), high circulating levels in blood have been associated with the development of insulin resistance, diabetes and cardiovascular disease (Vykoukal and Davies, 2011). However, experiments conducted in ob/ob mice and TNFR1/R2 double knockout mice have demonstrated that increasing TNF-α levels can enhance glucose homeostasis (Wu et al., 2018). The authors suggest that TNF-α plays a more complex role in glucose regulation than previously assumed, through an alternative receptor-independent mechanism that positively influences glucose homeostasis. Therefore, further research is needed to understand whether the increase of $CD4^+$ and $CD8^+$ T cells single producers of TNF-α in dogs fed shrimp hydrolysate may be beneficial to dogs' health. The levels of $CD4^+CD25^+Foxp3^+$ regulatory T cells remained similar among diets, which indicates no alterations in the general immune homeostasis of both groups of dogs, probably influenced by a healthy gut environment with either diet (Arroyo Hornero et al., 2020).

Immunoglobulin A is a secretory immunoglobulin present, among others, in the intestinal mucosa, protecting it from pathogens that can be used as an inflammatory biomarker (Alhalwani et al., 2023). The levels of IgA might be influenced by diet (Maria et al., 2017; Hiney et al., 2024). Supplementing the diet with shrimp hydrolysate did not affect fecal IgA levels. There is a lack of research studying the effects of dietary inclusion of protein hydrolysates in IgA levels in dog feces. Studies on the effects of marine protein hydrolysates on intestinal IgA levels in other animal species have generated conflicting results. The IgA levels in the middle and distal intestine of turbot were not influenced by the inclusion of fish protein hydrolysate in diets (Wei et al., 2023), whereas feeding mice with fish protein hydrolysate led to an increase in IgA concentrations in the small intestine (Duarte et al., 2006). Additionally, IgA helps to regulate the intestinal microbiota, such as their colonization, invasion, growth, and motility (Takeuchi and Ohno, 2022). In turn, the microbiota stimulates the production of IgA, and, unlike classical immunological memory responses, the immune system produces IgA antibodies that are specific for gut microbial species (Hapfelmeier et al., 2010). Furthermore, similar to human patients, also higher

concentrations of $IgA^+$ bacteria, which specifically bound to IgA, were observed in dogs with inflammatory bowel disease compared to healthy dogs (Soontararak et al., 2019).

The greatest relative abundance of genus found in both diets pertained to phyla Bacteroidota, previously called Bacteroidetes, and Fusobacteria. These phyla abundance is in accordance with data reported in dog feces (Hand et al., 2013; Alessandri et al., 2019). The four most predominant genera found (*Fusobacterium*, Muribaculaceae, *Bacteroides*, *Prevotella*) were previously observed in Beagle dogs fed diets with comparable macronutrients composition (Pereira et al., 2021), suggesting that microbiota composition is mainly a consequence of the amount of macronutrients that comprise diets than of the ingredients (Pinto et al., 2022). *Fusobacterium* was the most predominant genera found in both diets, agreeing with a previous study in which dogs fed diets without and with chicken liver hydrolysate (Pinto et al., 2022). According to our previous findings (Guilherme-Fernandes et al., 2024a), the inclusion of shrimp hydrolysate led to an increment in the abundance of genus belonging to Oscillospiraceae. *Oscillospira*, a genus from Oscillospiraceae, has been suggested to be a great probiotic candidate in future treatments, such as in obesity and chronic inflammation (Yang et al., 2021). Increased production of volatile fatty acids beneficial to animal health (Tremaroli and Bäckhed, 2012; Sridharan et al., 2014; Silva et al., 2020), such as acetate, butyrate, propionate and valerate have been associated with the presence of *Oscillospira* in the gut (Yang et al., 2021; Ecklu-Mensah et al., 2023). However, a decrease in fecal butyrate levels was observed in dogs fed shrimp hydrolysate, compared to a diet without hydrolysate inclusion (Guilherme-Fernandes et al., 2024b). Clostridia, a class of Firmicutes, was also increased in feces of dogs fed diets supplemented with shrimp hydrolysate. Different species of this class have been associated with various disorders of the gastrointestinal tract of dogs (Mentula et al., 2005). Previous research has suggested an association between improved digestibility of protein and increased abundance of Clostridiaceae in dogs (Bermingham et al., 2017), being bacteria of the *Clostridium* genus crucial in the fermentation of amino acids, such as lysine and proline (Lin et al., 2017). Although digestibility of amino acids was not analyzed in the current trial, no alterations in the digestibility of protein were observed among control and experimental diets (Guilherme-Fernandes et al., 2024b). Nevertheless, because the hydrolysis process breaks down proteins into small peptides, bacteria from Clostridia might be able to metabolize these peptides in the intestinal tract, prompting their growth. *Sellimonas* decreased in dogs fed the experimental diet. Higher abundances of *Sellimonas* and lower abundances of Oscillospiraceae have been observed in human patients with inflammatory bowel disease, compared to healthy

humans (Vestergaard et al., 2024). However, in dogs diagnosed with inflammatory bowel disease, a decrease in the relative abundance of *Sellimonas* and Oscillospiraceae *UCG–005* has been observed (Díaz-Regañón et al., 2023). Further studies are needed to understand the role of *Sellimonas* in dog gut.

Overall, the current study has shown the potential of shrimp hydrolysate to be included in dog diets, by maintaining the general health of dogs, namely their immune function and fecal microbiota, while promoting the sustainability of the pet food industry. The findings suggest that it has an immunomodulatory role, evidenced by increased neutrophils, white blood cells, and platelets, alongside decreased eosinophil levels, indicating its potential for inclusion in hypoallergenic diets. The observed reduction in blood glucose levels and superoxide production in PBMCs suggests its potential as a supplement in diets for diabetic dogs. Furthermore, the increased proliferation of $CD4^+$ T cells in response to LipL32 indicates a potential benefit in vaccination. Additionally, shrimp hydrolysate positively impacted the relative abundance of genera pertaining to Oscillospiraceae and Clostridia, suggesting it could function as a prebiotic and enhance amino acid digestibility, suggesting its potential in gastrointestinal diets. Future studies are needed to explore the underlying mechanisms responsible for these outcomes. Understanding these mechanisms will provide further insights into the role of shrimp hydrolysate in promoting health and its potential applications in hypoallergenic and gastrointestinal diets, diets for diabetic dogs, and immunomodulatory therapies.

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**Author contributions**

JG-F: Formal analysis, Investigation, Writing – original draft; CB: Investigation, Writing–review & editing; AC: Investigation, Writing – review & editing; TA: Resources; TY: Investigation, Writing – review & editing; AC-S: Writing – review & editing; MV: Writing – review & editing; AF: Conceptualization, Funding acquisition, Resources, Writing – review & editing. SL: Supervision, Writing – review & editing. MM: Supervision, Writing – review & editing. AC: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

**Data availability statement**

The data generated and analyzed during the current study are available from the corresponding author on reasonable request. Fecal DNA raw sequences obtained in this study are available at the European Nucleotide Archive (ENA) under accession number PRJEB75174.

**Competing Interests Statement**

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## Supplementary material

Table S1. Chemical composition of the control and experimental diets.

| Item | Diet[1] | |
|---|---|---|
| | Control | Experimental |
| Dry matter, DM, g $kg^{-1}$ | 942 | 957 |
| Ash, g kg $DM^{-1}$ | 77.7 | 79.9 |
| Crude protein, g kg $DM^{-1}$ | 293 | 293 |
| Ether extract, g kg $DM^{-1}$ | 136 | 131 |
| Neutral detergent fiber, g kg $DM^{-1}$ | 146 | 143 |
| Starch, g kg $DM^{-1}$ | 330 | 319 |
| Gross energy, MJ kg $DM^{-1}$ | 19.2 | 19.2 |

Data reported in Guilherme-Fernandes et al. (2024b).
[1]Complete diets included, in descending order, poultry by-product meal, corn, wheat, broken rice, pea starch, wheat gluten, poultry and mammal fat, pea protein concentrate, autolyzed brewers' yeast, beet pulp, lucerne, fish oil; Control diet: complete diet without the inclusion of shrimp hydrolysate; Experimental diet: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.

Table S2. Hematology, serum chemistry, C-reactive protein, and plasma immunoglobulin E in dogs fed the control and experimental diets over time.

| Item | Diet[1] | | SEM[2] | Week | | | SEM[2] | *P* – value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Experimental | | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| White blood cells, × $10^3$ µL$^{-1}$ | 6.71 | 7.67 | 0.291 | 6.97 | 7.23 | 7.37 | 0.344 | 0.002 | 0.601 | 0.139 |
| Neutrophils, % | 53.2 | 56.8 | 1.46 | 54.7 | 53.5 | 56.8 | 1.52 | 0.036 | 0.129 | 0.168 |
| Lymphocytes, % | 35.7 | 33.1 | 1.54 | 35.4 | 34.9 | 32.8 | 1.53 | 0.183 | 0.265 | 0.219 |
| Monocytes, % | 5.58 | 5.31 | 0.321 | 5.23 | 5.32 | 5.80 | 0.362 | 0.437 | 0.347 | 0.734 |
| Eosinophils, % | 5.51 | 4.50 | 0.323 | 4.53$^{a}$ | 6.07$^{b}$ | 4.43$^{a}$ | 0.372 | 0.017 | 0.003 | 0.671 |
| Red blood cells, × $10^6$ µL$^{-1}$ | 7.34 | 7.29 | 0.135 | 7.20 | 7.40 | 7.33 | 0.133 | 0.636 | 0.068 | 0.044 |
| Haemoglobin, g dL$^{-1}$ | 17.3 | 17.1 | 0.332 | 16.9$^{a}$ | 17.5$^{b}$ | 17.3$^{a,b}$ | 0.323 | 0.596 | 0.013 | 0.037 |
| Platelets, × $10^3$ µL$^{-1}$ | 274 | 300 | 12.4 | 309$^{b}$ | 273$^{a}$ | 280$^{a}$ | 13.7 | 0.038 | 0.040 | 0.632 |
| Mean platelet volume, fL | 9.88 | 9.90 | 0.177 | 10.1$^{c}$ | 9.88$^{b}$ | 9.70$^{a}$ | 0.162 | 0.914 | 0.004 | 0.650 |
| Total protein, g/dL$^{-1}$ | 5.71 | 5.86 | 0.066 | 5.56$^{a}$ | 5.96$^{b}$ | 5.84$^{a,b}$ | 0.072 | 0.106 | 0.001 | 0.740 |
| Albumin, g dL$^{-1}$ | 3.74 | 3.78 | 0.046 | 3.61$^{a}$ | 3.85$^{b}$ | 3.83$^{b}$ | 0.042 | 0.565 | <0.001 | 0.657 |
| Globulin, g dL$^{-1}$ | 1.98 | 2.07 | 0.034 | 1.95 | 2.11 | 2.01 | 0.043 | 0.067 | 0.054 | 0.720 |
| Glucose, mg dL$^{-1}$ | 92.8 | 86.4 | 1.84 | 84.9$^{a}$ | 98.1$^{b}$ | 85.8$^{a}$ | 1.83 | 0.023 | <0.001 | 0.608 |
| Creatinine, mg dL$^{-1}$ | 0.802 | 0.812 | 0.0536 | 0.800 | 0.837 | 0.785 | 0.0446 | 0.894 | 0.456 | 0.143 |
| Urea, mg/dL | 33.0 | 28.5 | 2.42 | 27.6 | 33.0 | 31.7 | 2.74 | 0.098 | 0.229 | 0.172 |
| Alanine aminotransferase, U L$^{-1}$ | 28.4 | 31.8 | 2.20 | 32.1 | 28.3 | 29.9 | 2.50 | 0.218 | 0.471 | 0.273 |
| Alkaline phosphatase, U L$^{-1}$ | 43.1 | 45.2 | 1.71 | 44.6 | 44.6 | 43.1 | 1.53 | 0.396 | 0.618 | 0.966 |
| C-reactive protein, µg mL$^{-1}$ | 6.99 | 8.78 | 0.853 | 7.80 | 8.75 | 7.10 | 0.758 | 0.234 | 0.318 | 0.277 |
| Immunoglobulin E, µg mL$^{-1}$ | 171 | 148 | 21.0 | 206$^{b}$ | 209$^{b}$ | 63.6$^{a}$ | 18.5 | 0.438 | <0.001 | 0.135 |

[1]Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[2]SEM: Standard error of the mean.
[a,b]Means with different lowercase superscript letters in the same row are significantly different ($P < 0.05$).

Table S3. Concentration of cytokines, chemokine, and growth factors in serum of dogs fed the control and experimental diets over time.

| Item | Diet[1] | | SEM[2] | Week | | | SEM[2] | *P* – value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Experimental | | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| Il-12/IL-23p40, pg mL$^{-1}$ | 580 | 1418 | 352.6 | 938 | 986 | 1072 | 253.4 | 0.105 | 0.239 | 0.114 |
| IL-8, pg mL$^{-1}$ | 1112 | 437 | 239.6 | 778 | 708 | 837 | 180.6 | 0.057 | 0.501 | 0.038 |
| IL-2, pg mL$^{-1}$ | 43.2 | 99.1 | 38.79 | 62.1 | 71.6 | 79.7 | 40.31 | 0.161 | 0.901 | 0.899 |
| SCF, pg mL$^{-1}$ | 27.3 | 54.9 | 14.44 | 36.7 | 40.5 | 46.1 | 10.52 | 0.188 | 0.219 | 0.026 |
| MCP-1, pg mL$^{-1}$ | 103 | 100 | 12.3 | 104 | 106 | 94.3 | 9.74 | 0.846 | 0.281 | 0.379 |
| VEGF-A, pg mL$^{-1}$ | 5.97 | 6.16 | 0.903 | 5.81 | 5.27 | 7.10 | 0.770 | 0.884 | 0.058 | 0.102 |

[1]Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[2]SEM: Standard error of the mean.
Abbreviations: IL, interleukin; MCP-1, monocyte chemoattractant protein-1; SCF, stem cell factor; VEGF-A, vascular endothelial growth factor A.

Table S4. Fold increase of total reactive oxygen species (ROS) and superoxide production in cells stimulated with phorbol myristate acetate (PMA) for 30 and 60 min over the basal (non-stimulated) in weeks 4, 8 and 12 in dogs fed the control and experimental diets.

| Item | Control[1] | | | Experimental[1] | | | SEM[2] | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Week 4 | Week 8 | Week 12 | Week 4 | Week 8 | Week 12 | | Diet | Week | Diet*Week |
| Total ROS production | | | | | | | | | | |
| 30 min PMA stimulation | 6.20 | 1.02 | 43.7 | 1.85 | 0.785 | 42.7 | 5.638 | 0.642 | <0.001 | 0.930 |
| 60 min PMA stimulation | 2.58[a] | 1.51[a] | 13.2[b] | 1.92[a] | 0.800[a] | 18.0[b] | 1.176 | 0.174 | <0.001 | 0.030 |
| Superoxide production | | | | | | | | | | |
| 30 min PMA stimulation | 1.82 | 2.26 | 10.4 | 1.28 | 1.90 | 3.94 | 1.523 | 0.066 | 0.002 | 0.088 |
| 60 min PMA stimulation | 2.86[a] | 4.32[a] | 10.7[b] | 1.23[a] | 3.04[a] | 3.79[a] | 1.144 | 0.002 | <0.001 | 0.038 |

[1]Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten.
[2]SEM: Standard error of the mean.
[a,b]Means with different lowercase superscript letters in the same row are significantly different ($P < 0.05$).

Table S5. Concentration of cytokines in the supernatants of PBMC stimulated with concanavalin A in dogs fed the control and experimental diets over time.

| Item | Diet[1] | | SEM[2] | Week | | | SEM[2] | *P* - value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Experimental | | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| IL-17, pg mL$^{-1}$ | 1310 | 986 | 306.8 | 402$^{a}$ | 1243$^{a,b}$ | 1800$^{b}$ | 374.4 | 0.405 | 0.027 | 0.752 |
| IFN-γ, pg mL$^{-1}$ | 1885 | 1461 | 384.1 | 2194$^{b}$ | 1735$^{a,b}$ | 1092$^{a}$ | 328.6 | 0.452 | 0.011 | 0.122 |
| TNF-α, pg mL$^{-1}$ | 30.6 | 28.2 | 3.31 | 27.3$^{a}$ | 19.2$^{a}$ | 41.6$^{b}$ | 4.23 | 0.594 | 0.004 | 0.317 |
| IL-10, pg mL$^{-1}$ | 193 | 144 | 28.1 | 104$^{a}$ | 152$^{a}$ | 250$^{b}$ | 33.7 | 0.124 | 0.002 | 0.729 |

[1]Control: complete diet without inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of 5% of wheat gluten.
[2]SEM: Standard error of the mean.
[a,b]Means with different lowercase superscript letters in the same row are significantly different ($P < 0.05$).
Abbreviations: IFN-γ: interferon-gamma; IL, interleukin; TNF-α: tumor necrosis factor alpha.

Table S6. Percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells expressing interferon-gamma, tumor necrosis factor alpha and both cytokines, and the $CD4^+/CD8^+$ ratio in weeks 4, 8, and 12.

| Item | Week | | | SEM[1] | *P* - value | | |
|---|---|---|---|---|---|---|---|
| | 4 | 8 | 12 | | Diet | Week | Diet*Week |
| $CD3^+CD4^+$, % | 63.9[b] | 59.6[a] | 59.0[a] | 1.46 | 0.190 | 0.050 | 0.585 |
| $CD4^+TNF\alpha^+IFN\gamma^+$, % | 8.76[a] | 14.1[b] | 15.2[b] | 1.413 | 0.113 | 0.002 | 0.290 |
| $CD4^+TNF\alpha^+IFN\gamma^-$, % | 13.4[a] | 16.2[a] | 20.3[b] | 1.07 | <0.001 | <0.001 | 0.607 |
| $CD4^+TNF\alpha^-IFN\gamma^+$, % | 5.92[a] | 8.62[c] | 7.26[b] | 1.041 | 0.748 | 0.023 | 0.927 |
| $CD3^+CD8^+$, % | 13.6 | 13.8 | 14.6 | 0.59 | 0.180 | 0.491 | 0.187 |
| $CD8^+TNF\alpha^+IFN\gamma^+$, % | 8.69[a] | 27.6[b] | 32.7[b] | 4.534 | 0.251 | <0.001 | 0.832 |
| $CD8^+TNF\alpha^+IFN\gamma^-$, % | 4.41 | 6.32 | 5.58 | 0.586 | <0.001 | 0.054 | 0.890 |
| $CD8^+TNF\alpha^-IFN\gamma^+$, % | 7.73[a] | 17.4[b] | 16.9[b] | 2.669 | 0.216 | <0.001 | 0.526 |
| $CD4^+/CD8^+$ | 5.23 | 4.56 | 4.26 | 0.385 | 0.951 | 0.317 | 0.354 |

[1]SEM: Standard error of the mean.
[a,b,c]Means with different lowercase superscript letters in the same row are significantly different ($P < 0.05$).
Abbreviations: IFN-γ: interferon-gamma; TNF-α: tumor necrosis factor alpha.

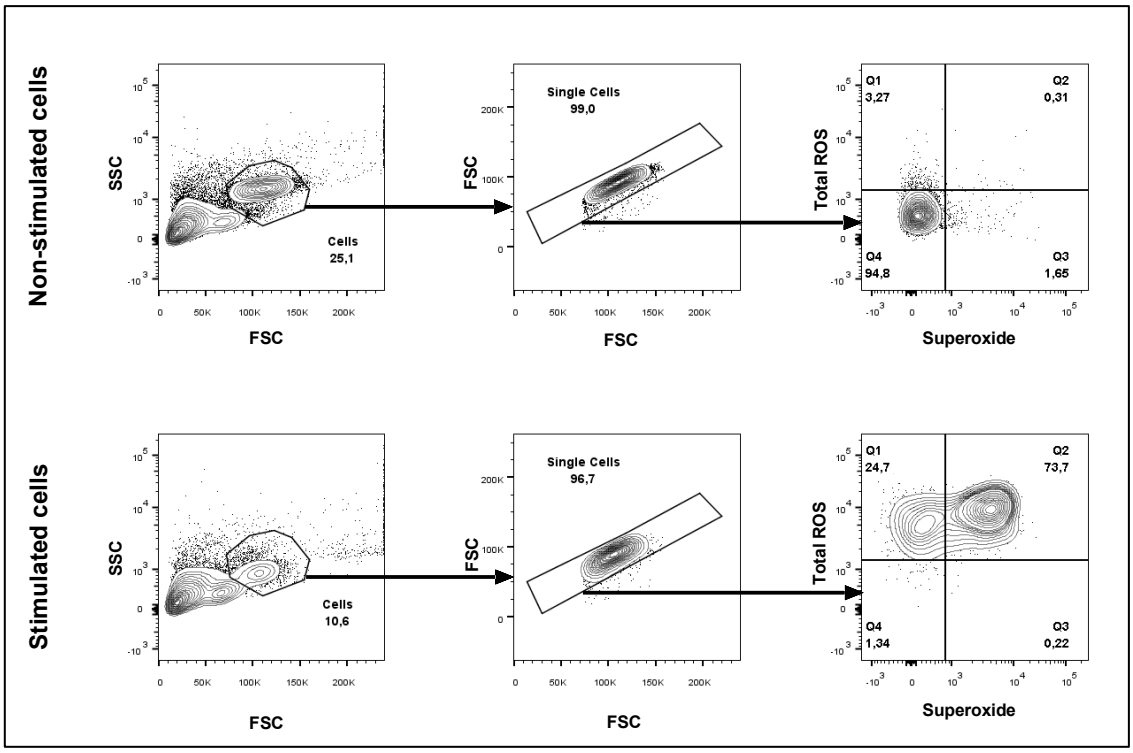


Figure S1. Representative example of flow cytometry gating strategy used to define the production of total reactive oxygen species (ROS) and superoxide in non-stimulated cells (basal ROS production) and in cells stimulated with phorbol myristate acetate for 30 and 60 min. Cells were gated based on side scatter (SSC) and forward scatter (FSC) parameters and doublets were excluded (Single cells) in FSC versus FSC plots. Mean fluorescence intensity was used to quantify total ROS and superoxide production.

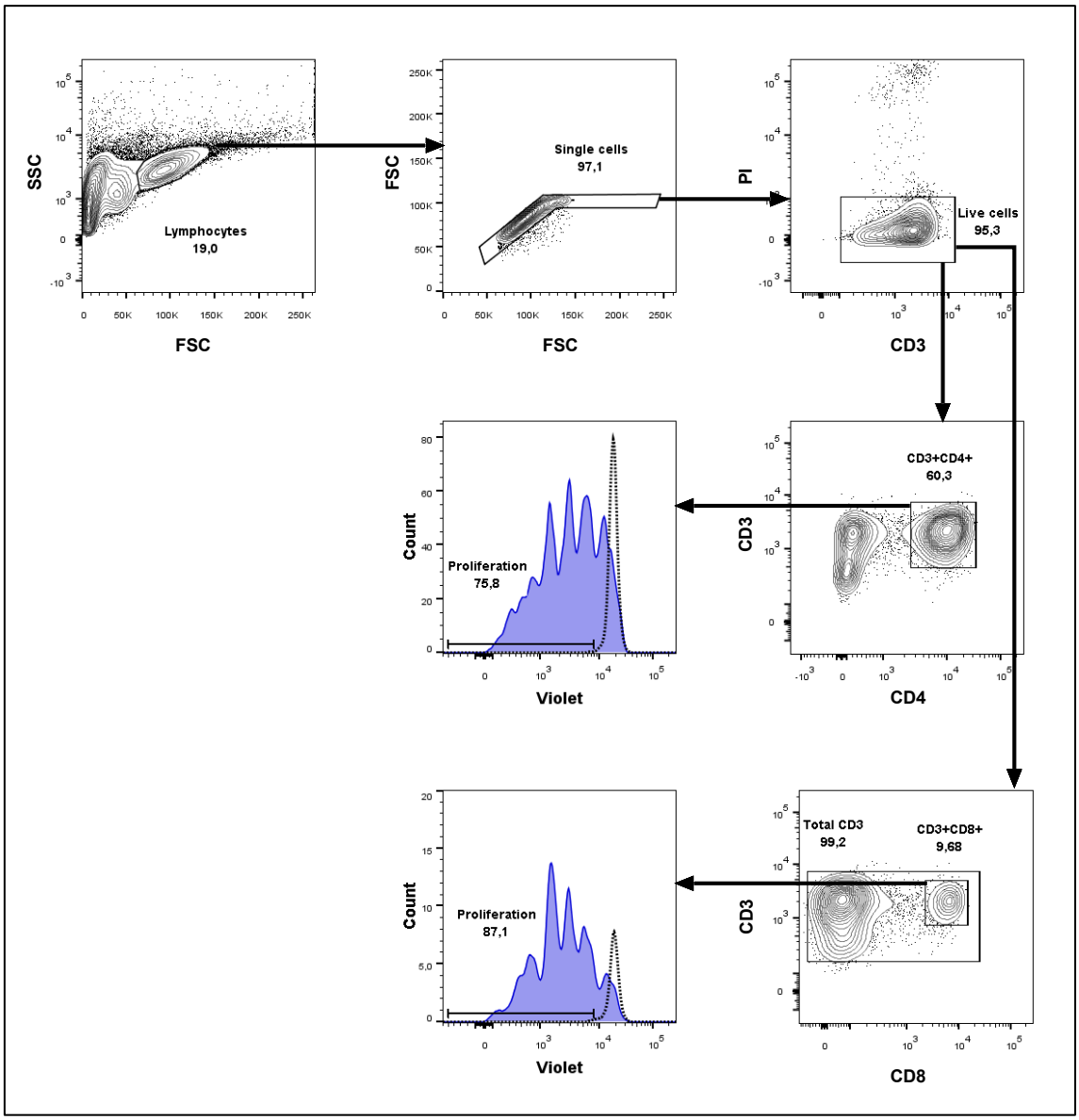


Figure S2. Representative example of flow cytometry gating strategy used to define lymphocyte proliferation of non-stimulated $CD3^+CD4^+$ cells or in response to recombinant antigen from *Leptospira interrogans* and concanavalin A. Lymphocytes were gated based on side scatter (SSC) and forward scatter (FSC) parameters and doublets were excluded (Single cells) in FSC versus FSC plots. Viable cells (Live cells) were defined as propidium iodide (PI) negative cells. $CD3^+CD8^+$ cells and $CD3^+CD4^+$ cells were set in contour plots, based on fluorescence minus one (FMO) staining.

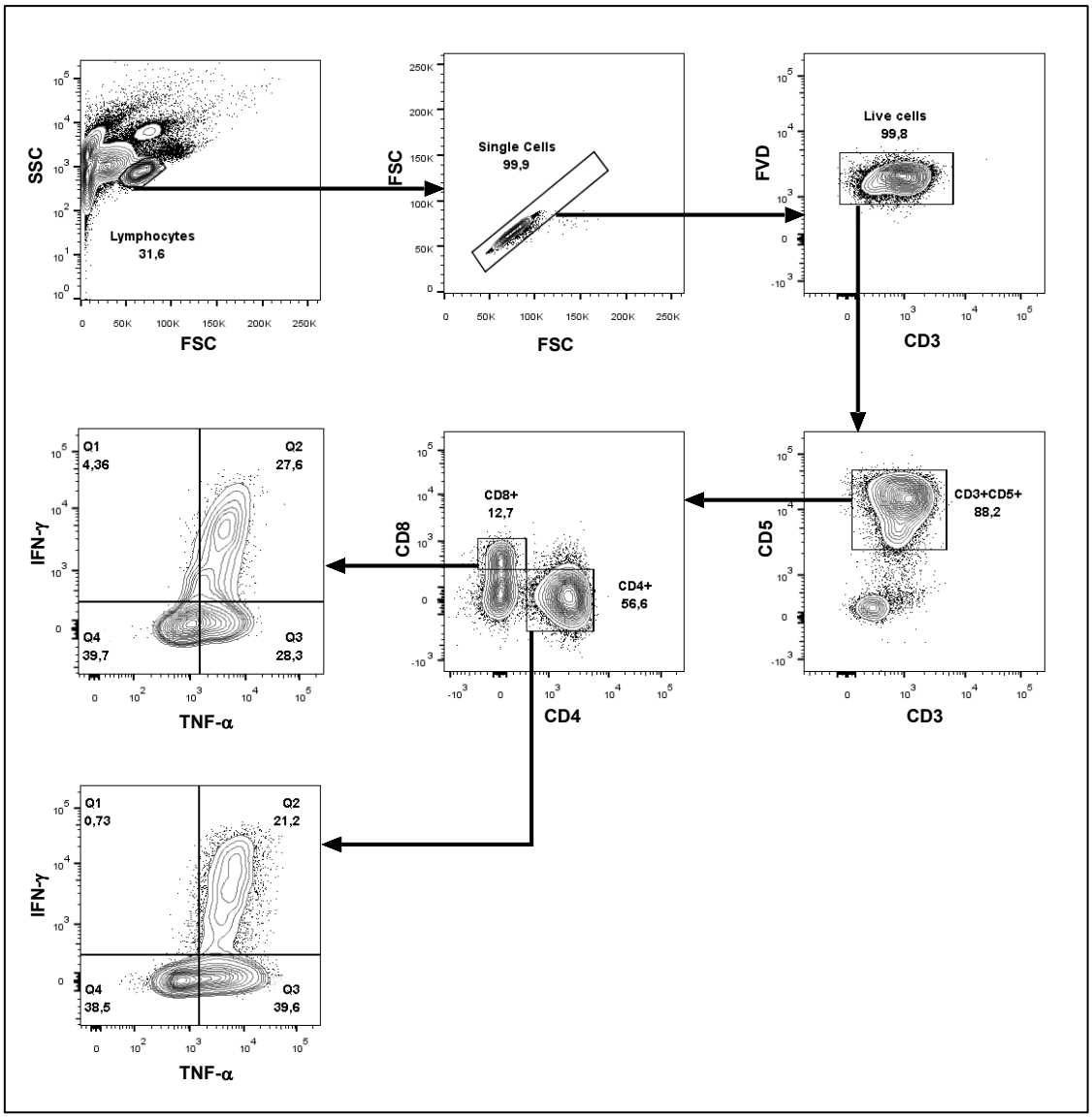


Figure S3. Representative example of flow cytometry gating strategy used to define lymphocyte intracellular cytokines tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). Lymphocytes were gated based on side scatter (SSC) and forward scatter (FSC) parameters and doublets were excluded (Single cells) in FSC versus FSC plots. Viable cells (Live cells) were defined as Fixable Viability Dye (FVD) negative cells. $CD3^+CD8^+$ cells and $CD3^+CD4^+$ cells expressing TNF-α and/or IFN-γ were set in contour plots, based on fluorescence minus one (FMO) staining.

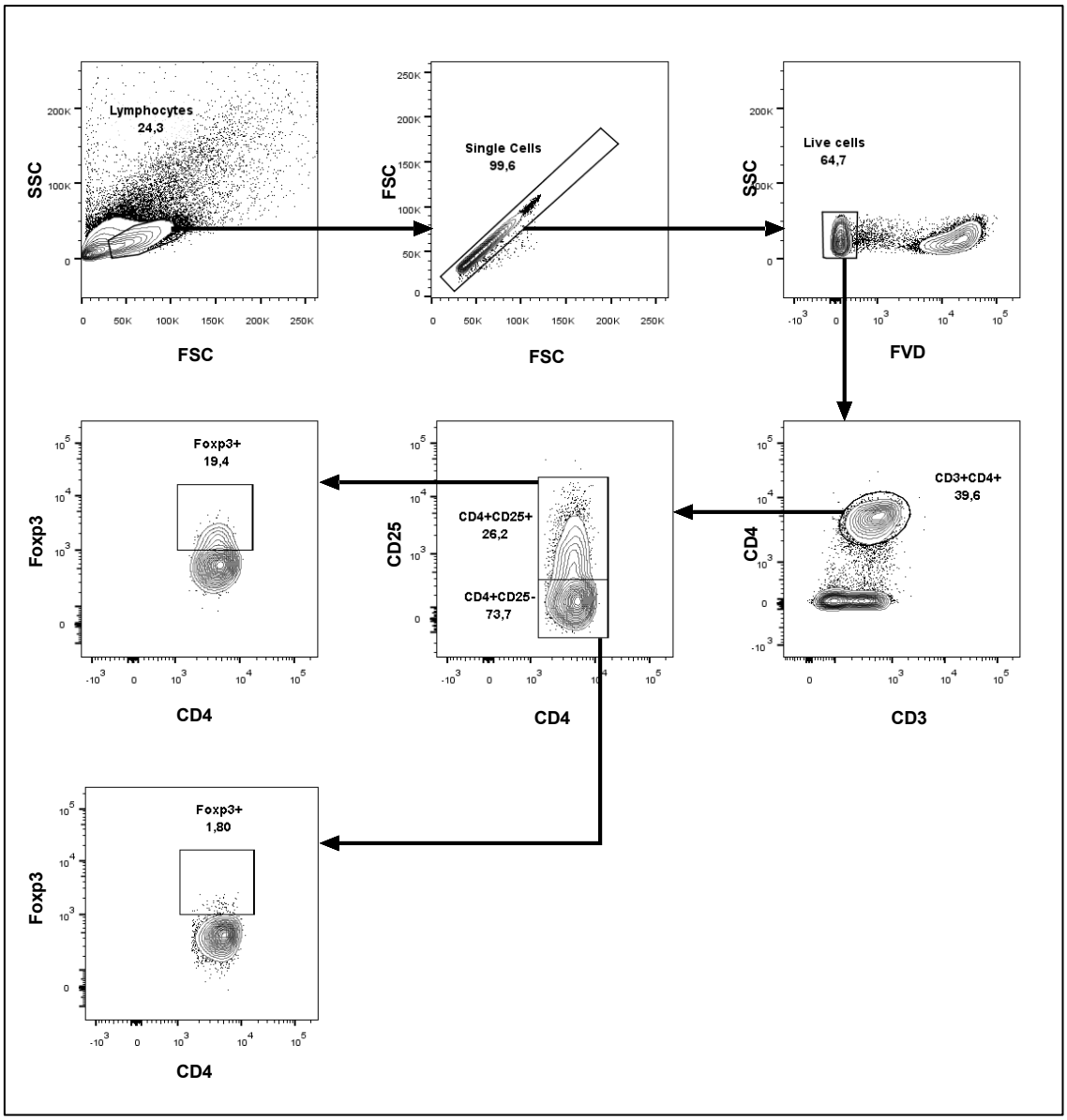


Figure S4. Representative example of flow cytometry gating strategy used to define lymphocyte intracellular cytokine Foxp3. Lymphocytes were gated based on side scatter (SSC) and forward scatter (FSC) parameters and doublets were excluded (Single cells) in FSC versus FSC plots. Viable cells (Live cells) were defined as Fixable Viability Dye (FVD) negative cells. $CD4^{+}CD25^{+}$ expressing Foxp3 were set in contour plots, based on fluorescence minus one (FMO) staining.

# CHAPTER 5

General discussion, conclusions and future directions

## 5.1 General discussion

Dogs have been human companions for thousands of years, and today they live as integral members of the family, especially in urban areas. As a result, dog owners are increasingly motivated to provide high-quality and nutritionally balanced diets that support the health, longevity, and quality of life of their dogs. This has driven demand for pet food that aims to meet specific nutritional needs and to promote health benefits, such as improved digestion, gut health, and immune support. As the global dog population grows, so does the pet food industry, raising concerns about its sustainability and environmental impact. Therefore, there is a need to find nutritious and functional ingredients, through more sustainable practices, to promote canine health and protect the environment. Regarding protein sources, fundamental to supply amino acids (AA) crucial for maintenance, growth, and reproduction of dogs, there is a undeniable need to find more sustainable ingredients than those conventionally used in pet food. With the growth of aquaculture in recent decades, integrating circular economy strategies into the sector can enhance resource efficiency and reduce waste, while contributing to the sustainability of pet food by supplying the industry with valuable by-products.

The present thesis aimed to evaluate the potential of underexploited aquatic animal by-products for inclusion in canine diets. In Chapter 1, *in vivo* effects of dietary inclusion of aquatic animal by-products, such as fish, mollusks, and crustaceans on various species, including livestock, poultry and aquaculture species, were reviewed, showing general improvements in growth, feed efficiency, immune function and gut health, depending on the species and dietary inclusion levels. Fish hydrolysates have been shown to benefit dogs in a variety of pathologies and diseases, stress and behavior. However, there is a lack of knowledge on the effects of dietary inclusion of mollusks and crustaceans' by-products in dogs. In this context, two commercially available aquatic animal by-products, squid meal and shrimp hydrolysate, were chosen for a detailed chemical composition and *in vivo* assessment. This work is the first to explore aquatic invertebrate by-products within the context of canine nutrition, contributing to both improved dog health and the sustainability of pet food production.

### 5.1.1 Squid meal and shrimp hydrolysate chemical characterization

In Chapter 2, the chemical characterization of squid meal and shrimp hydrolysate highlighted their potential as protein sources. Squid meal showed greater levels of crude protein (CP, 810 g $kg^{-1}$ dry matter, DM, basis) compared to most conventional protein sources used in dog food (Table 5.1), but similar to wheat gluten (815 g $kg^{-1}$ DM). Shrimp hydrolysate contained a CP content of 658 g $kg^{-1}$ DM, which is comparable to fish meal

(580-740 g $kg^{-1}$ DM) poultry meal (473-781 g $kg^{-1}$ DM), and corn gluten meal (545-779 g $kg^{-1}$ DM), but greater than meat and bone meal (509 g $kg^{-1}$ DM).

Moreover, squid meal and shrimp hydrolysate showed to be good sources of essential AA, particularly of methionine, arginine, lysine, and leucine. Squid meal had the greatest content of methionine (29.7 g $kg^{-1}$ DM), while shrimp hydrolysate (16.3 g $kg^{-1}$ DM) presented a methionine content comparable to corn gluten meal (10.6-23.1 g $kg^{-1}$ DM), and fish meal (8.30-28.7 g $kg^{-1}$ DM), but greater than wheat gluten (12.1 g $kg^{-1}$ DM), soybean meal (5.20-7.70 g $kg^{-1}$ DM), poultry meal (3.40-15.0 g $kg^{-1}$ DM), and meat and bone meal (7.30 g $kg^{-1}$ DM). Squid meal (57.9 g $kg^{-1}$ DM) contained arginine content comparable to fish meal (19.5-72.0 g $kg^{-1}$ DM), while shrimp hydrolysate (31.6 g $kg^{-1}$ DM) showed arginine content comparable to poultry meal (28.4-55.1 g $kg^{-1}$ DM), lower than meat and bone meal (35.3 g $kg^{-1}$ DM) and soybean meal (32.2-42.4 g $kg^{-1}$ DM), but higher than wheat gluten (30.2 g $kg^{-1}$ DM), and corn gluten meal (14.1-27.3 g $kg^{-1}$ DM). Squid meal contained the greatest lysine content (54.4 g $kg^{-1}$ DM), while shrimp hydrolysate (38.5 g $kg^{-1}$ DM) had comparable levels to fish meal (26.8-69.0 g $kg^{-1}$ DM) and poultry meal (18.7-44.1 g $kg^{-1}$ DM), but surpassing wheat gluten (13.9 g $kg^{-1}$ DM), corn gluten meal (7.80-15.0 g $kg^{-1}$ DM), soybean meal (26.9-35.9 g $kg^{-1}$ DM) and meat and bone meal (29.3 g $kg^{-1}$ DM). Both squid meal (45.7 g $kg^{-1}$ DM) and shrimp hydrolysate (30.3 g $kg^{-1}$ DM) had leucine content lower than wheat gluten (54.9 g $kg^{-1}$ DM) and corn gluten meal (85.9-135 g $kg^{-1}$ DM), but squid meal showed similar levels to fish meal (32.0-63.6 g $kg^{-1}$ DM) and poultry meal (29.1-50.7 g $kg^{-1}$ DM), and above soybean meal (33.7-43.4 g $kg^{-1}$ DM), followed by shrimp hydrolysate and meat and bone (32.9 g $kg^{-1}$ DM). While most dog breeds can synthetize taurine when fed adequate amounts of methionine and cysteine, insufficient methionine intake may lead to the development of pathologies, such as dilated cardiomyopathy (Li and Wu, 2023). Deficient intake of arginine may result in decreased feed intake, hyperammonemia, emesis, frothing at the mouth, and muscle tremors (NRC, 2006). Lysine is the first limiting AA in most plant-based sources, and it is crucial in muscle development, tissue repair, and the production enzymes, while its deficiency can limit body protein synthesis (Ball et al., 2007). Also, leucine is involved in protein synthesis pathways, particularly important in maintaining muscle tissue (Li and Wu, 2023). As a result, squid meal and shrimp hydrolysate may offer great nutritional value in terms of protein and essential AA, making them attractive options for high-quality dog food formulations.

Shrimp hydrolysate extracts demonstrated higher antioxidant activity compared to squid meal extracts, and while antioxidant activity of squid meal extracts was assessed for the first time, the *in vitro* antioxidant properties of shrimp hydrolysate extracts are

acknowledged (Latorres et al., 2018; Nikoo et al., 2021). Differences in the antioxidant properties of body components, processing methods such as hydrolysis, and the assays used to measure antioxidant potential, among other factors, make it challenging to compare the present data with published results. Nevertheless, the greater antioxidant activity of shrimp hydrolysate compared to squid meal observed in the present work might be due to the size of peptides that is consequent of the hydrolysis process, as smaller peptides have been shown to have greater antioxidant activity (Zhao et al., 2011; Djellouli et al., 2020). Additionally, the AA sequence in peptides, particularly those containing valine, leucine, proline, histidine, tyrosine, tryptophan, methionine, and cysteine, may be associated with high antioxidant activity (Li et al., 2022). However, further research is needed to identify the compounds that were extracted and responsible for the levels of antioxidant activity found. Moreover, also the evaluation of how dog food processing affects the antioxidant potential of these novel protein sources should be performed.

Table 5.1. Dry matter (DM), ash, crude protein and essential amino acids of studied novel ingredients, squid meal and shrimp hydrolysate, and pet food conventional ingredients, wheat gluten, corn gluten meal, soybean meal, fish meal, poultry meal, and meat and bone meal.

| Item | Studied novel ingredients | | Pet food conventional ingredients | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Squid meal | Shrimp hydrolysate | Wheat gluten[1] | Corn gluten meal[2] | Soybean meal[2] | Fish meal[2] | Poultry meal[2] | Meat and bone meal[3] |
| Dry matter, g $kg^{-1}$ | 937 | 962 | 897 | 842-975 | 801-963 | 782-978 | 803-987 | 940 |
| Ash, g $kg^{-1}$ DM | 103 | 151 | 9.32 | 9.00-70.0 | 57.0 -112 | 58.0-279 | 24.0 -277 | 192 |
| Crude protein, g $kg^{-1}$ DM | 810 | 658 | 815 | 545-779 | 438-584 | 580-740 | 473-781 | 509 |
| Essential amino acids g $kg^{-1}$ DM | | | | | | | | |
| Arginine | 57.9 | 31.6 | 30.2 | 14.1-27.3 | 32.2-42.4 | 19.5-72.0 | 28.4-55.1 | 35.3 |
| Histidine | 14.0 | 8.80 | 17.6 | 9.20-18.6 | 11.9-15.5 | 5.90-31.8 | 5.50-24.3 | 10.4 |
| Lysine | 54.4 | 38.5 | 13.9 | 7.80-15.0 | 26.9-35.9 | 26.8-69.0 | 18.7-44.1 | 29.3 |
| Threonine | 27.9 | 17.5 | 22.8 | 16.5-28.4 | 16.9-22.9 | 17.1-39.0 | 16.1-31.8 | 16.9 |
| Isoleucine | 26.0 | 17.6 | 28.3 | 19.1-33.4 | 18.3-27.2 | 10.8-39.2 | 15.5-29.0 | 15.6 |
| Leucine | 45.7 | 30.3 | 54.9 | 85.9-135 | 33.7-43.4 | 32.0-63.6 | 29.1-50.7 | 32.9 |
| Valine | 33.1 | 30.0 | 30.1 | 21.4-35.9 | 22.0-29.5 | 21.0-48.5 | 19.3-39.8 | 23.1 |
| Methionine | 29.7 | 16.3 | 12.1 | 10.6-23.1 | 5.20-7.70 | 8.30-28.7 | 3.40-15.0 | 7.30 |
| Methionine+cystine | 34.1 | 18.9 | 33.8 | 20.5-34.6 | 12.2-18.5 | 18.3-35.2 | 15.0-33.6 | 11.6 |
| Phenylalanine | 27.1 | 24.0 | 43.2 | 31.4-51.5 | 23.1-28.9 | 17.1-34.8 | 15.9-29.7 | 18.2 |
| Phenylalanine+tyrosine | 84.1 | 53.9 | 76.9 | 55.4-91.3 | 40.1-49.5 | 32.4-62.7 | 26.9-49.7 | 31.2 |

[1]Data analyzed in the present work.
[2]Data reported in INRAE-CIRAD-AFZ (2024).
[3]Data reported in NRC (2006), Sulabo and Stein (2013)

### 5.1.2 Dogs' preference of diets

Dietary inclusion of shrimp hydrolysate or squid meal at 15% negatively impacted the consumption ratio compared to the basal diet, but no differences were observed in the first approach and first taste (Chapter 2). Both ingredients presented high levels of glutamic and aspartic acids, which are AA associated with the umami taste and typically appealing to dogs (Kurihara and Kashiwayanagi, 1998; Tanase et al., 2022). Previous studies reported either no adverse effects or improved palatability with fish meals and fish hydrolysates at 10% inclusion level (Folador et al., 2006; Poppi et al., 2023). Among the factors that could influence dogs' choice are taste, texture and odor (Thombre, 2004; Pétel et al., 2018), highlighting the importance of understanding how these sensory attributes are impacted during the development of attractive dog food. The lack of differences in the first approach and first taste may indicate that odor of squid meal and shrimp meal was attractive to dogs. Therefore, in this case, the reduced palatability might be attributed to the texture, as the protein sources were mixed with the basal diet just before feeding, as shown in studies with microalgae supplementation at a level of 5% (Cabrita et al., 2023), rather than being incorporated into the extruded kibble. When 5% of shrimp hydrolysate was included in the extruded diet (Chapter 3), neither enhanced nor reduced effects of the ingredient on palatability were observed. This may also be due to the texture, which is equal between control and experimental diets, though the impact of the inclusion level should not be dismissed as a contributing factor.

Nevertheless, no food refusal was observed throughout the feeding trials, and food was consumed immediately. This suggests that diets containing squid meal and shrimp hydrolysate are well accepted by dogs. Further research is needed to assess the palatability of extruded diets with different dietary inclusion levels of squid meal and shrimp hydrolysate. Additionally, palatability assays should be performed with larger sample sizes (> 20 dogs) as recommended by Aldrich and Koppel (2015). Smaller sample sizes, such as the 12 dogs used in two-bowl tests, may limit the ability to detect subtle differences in preferences and could lead to results that are more prone to variability. Larger sample sizes provide more robust and reliable data on palatability, reducing the likelihood of sampling bias and improving the generalizability of the findings.

### 5.1.3 Impact of squid meal and shrimp hydrolysate on diet digestibility and fecal characteristics

The findings of the present thesis highlight the complexities involved in understanding the nutritional value of protein sources. In Chapter 2, squid meal and shrimp hydrolysate diets (5%, 10%, and 15% inclusion levels), compared to the basal diet, increased

digestibility of nutrients and energy, except for ether extract (EE), and the digestibility of CP increased with the increased levels of inclusion of squid meal, whereas the increment of inclusion levels of shrimp hydrolysate failed to improve CP digestibility. In Chapter 3, shrimp hydrolysate had no effect on digestibility of nutrients and energy. This lack of effect could be attributed to the fact that the ingredient it replaced in the experimental diet, wheat gluten, is highly digestible. Additionally, the CP digestibility of diets containing shrimp hydrolysate could have been compromised by molecular interactions between ingredients or by processing factors such as extrusion and storage conditions, which can alter protein structure and stability, potentially affecting their bioavailability (Schmid et al., 2022; Gu et al., 2023; Orlien et al., 2023).

The inclusion of squid meal and shrimp hydrolysate in dog diets had minimal impact on body weight (BW), nutrient intake, and fecal characteristics, with only slight adjustments made to food amounts as needed to maintain optimal BW and body condition score (BCS). This suggests that these novel protein sources can be incorporated into dog food without adverse effects on these parameters. Dogs consistently presented ideal BCS, mostly representative of palpable ribs covered with fat, a waist behind the ribs (viewed from above) and an abdomen tucked up (viewed from the side). Additionally, the fecal consistency scores observed mainly ranged from soft, shaped, and moist stools leaving spots on the floor (3.0) and approximately firm, shaped, and dry stools (3.5), which is considered the optimum (Hernot et al., 2005).

In Chapter 3, although diet digestibility was measured in week 12, the variations in the number of defecations among weeks and the differences of DM fecal output between diets support the importance of assessing digestibility over time, particularly considering seasonal changes, such as the environmental conditions and dogs' physical activity.

### 5.1.4 The role of squid meal and shrimp hydrolysate in modulating fecal microbiota composition and metabolites

Dietary changes, particularly in protein content and source, can rapidly alter the gut microbiota and its metabolites (Pilla and Suchodolski, 2020; Lin et al., 2022). In Chapter 2, the fecal microbiota and metabolites were analyzed at the end of the 10-day periods for each inclusion level of squid meal and shrimp hydrolysate, whereas in Chapters 3 and 4, the effects of 5% shrimp hydrolysate dietary inclusion on fecal microbiota, and metabolites were evaluated every 4 weeks over a period of 12 weeks.

Squid meal and shrimp hydrolysate did not affect the alpha and beta diversity or the relative abundance of bacteria, but different profiles were observed between studies regarding shrimp hydrolysate diets. Both squid meal and shrimp hydrolysate dietary

inclusion were associated with greatest relative abundances of *Turicibacter*, Peptostreptococcaceae, and *Blautia*, members of the phylum Firmicutes, while the greatest bacteria relative abundance observed with the shrimp inclusion in the extruded diet were *Fusobacterium*, Muribaculaceae, *Bacteroides*, and *Prevotella*, pertained to phyla Bacteroidota and Fusobacteria. Overall, the genera found in the greatest abundance in the diets described in Chapters 2 and 4 were more prevalent in the feces of healthy dogs compared to non-healthy dogs (Hand et al., 2013; Alessandri et al., 2019; Félix et al., 2022). The differential prominence of bacteria described in Chapters 2 and 4 suggests that fecal microbiota profile may be influenced by the ingredients used and the overall diet macronutrient content.

Nevertheless, the variations observed in genera in dogs fed the shrimp hydrolysate diets compared to the basal and control diets in both Chapters 2 and 4, respectively, suggest that this ingredient plays a role in modulating the gut microbiota. The inclusion of shrimp hydrolysate at 10% in Chapter 2 led to a decrease in *Lactobacillus*, but, in Chapter 4 (5% inclusion), no variations in this genus, known for its probiotic properties and its role in producing beneficial metabolites, were observed. This difference could be due to the inclusion level, the effects of the extrusion process, or the overall ingredients of basal formulation. On the other hand, the inclusion of shrimp hydrolysate at 5% in extruded diet resulted in an increase in genera from the *Clostridia* and a decrease in *Sellimonas*. While *Sellimonas* role is poorly studied in dogs, some species pertaining to Clostridia have been associated with canine disorders of the gastrointestinal tract (Mentula et al., 2005), but also with improved AA digestibility (Lin et al., 2017). Therefore, it remains unclear whether these variations are beneficial or detrimental to gut health. Nevertheless, throughout the entirety of the feeding trials, no clinical signs of an unhealthy gut were observed.

Moreover, shrimp hydrolysate inclusion at 5% (Chapter 4) and at 15% (Chapter 2) consistently led to an increment in the abundance of bacteria from the Oscillospiraceae family. *Oscillospira*, a genus from this family, has been associated with increased production of volatile fatty acids (VFA) that are beneficial to gut health, such as acetate, butyrate, propionate, and valerate, suggesting the potential prebiotic effects of shrimp hydrolysate (Yang et al., 2021; Ecklu-Mensah et al., 2023). Despite this promising association, while the beneficial VFA increased with the dietary inclusion of shrimp hydrolysate (5%-15%) in Chapter 2, it did not result in an expected increase in butyrate levels and, in fact, it decreased butyrate at 5% inclusion level in Chapter 3. Additionally, even though decreased levels of butyrate were observed in feces of dogs fed diets with squid meal inclusion, no variations were observed in differentially abundant genera.

These results underscore the complexity of gut microbiota and its metabolites, highlighting the multifaceted interactions within the gut microbiota, such as other microbiota dynamics or metabolic pathways (Liu et al., 2022). While increases in genera associated with butyrate production do not always correlate with higher fecal butyrate levels, a decrease in butyrate may not be solely attributed to the microbiota profile. Other factors such as butyrate absorption or utilization, substrate availability impacting microbial production, and alterations in hydrogen-consuming bacteria or the presence of inorganic electron acceptors, can also play a role in influencing butyrate levels (Vital et al., 2014; Louis and Flint, 2017; Singh et al., 2022).

Therefore, the effects of dietary inclusion of squid meal and shrimp hydrolysate on the fecal microbiota and gut health of dogs warrants further investigation. Understanding these effects is crucial for optimizing dietary interventions aimed at improving gut health.

### 5.1.5 Shrimp hydrolysate enhanced immune function

The dietary inclusion of 5% shrimp hydrolysate in extruded diets demonstrated promising findings regarding immune function. The fact that blood profiles remained within reference ranges for healthy dogs indicates that shrimp hydrolysate is a safe and beneficial ingredient for dogs. Moreover, the increased concentrations of neutrophils, platelets, and white blood cells observed with shrimp hydrolysate inclusion suggest an enhancement in the dogs' immune system. Neutrophils are the first line of defense against infections of bacteria and fungi, and they play a predominant role in shaping the host response to infection and immune system homeostasis (Malech et al., 2014). An elevation in neutrophil levels could indicate an improved capacity to respond to pathogens, potentially enhancing the overall condition of the immune system. Platelets are crucial for promoting tissue health, regeneration, and repair, being natural sources of growth factors and substances, such as fibronectin, vitronectin, and sphingosine 1-phosphate that help to maintain homeostasis (Locatelli et al., 2021). Their elevated levels may indicate a more responsive and prepared immune system. The rise in white blood cells also supports that shrimp hydrolysate may boost immune health, as these cells are vital for identifying and combating infections (Tizard, 2018). Additionally, the lack of differences in the IgE levels, couple with the reduced blood eosinophil levels in dogs fed 5% shrimp hydrolysate diet, may indicate a reduction in inflammation or allergic responses (Lombardi et al., 2022). Since hydrolyzed proteins are commonly used in hypoallergenic dog diets to prevent allergic reactions and manage food sensitivities, these results further support the beneficial role of shrimp hydrolysate in maintaining the immune system and reducing potential allergic responses.

Furthermore, lowered blood glucose levels with 5% shrimp hydrolysate inclusion, while keeping levels within the normal range for healthy dogs, highlights the potential of this ingredient to support metabolic health. This observation is consistent with existing research demonstrating that hydrolyzed proteins can positively impact glucose regulation (Khosravi et al., 2015). One of the mechanisms by which shrimp hydrolysate may exert this effect is by reducing superoxide production. By mitigating the production of superoxide, shrimp hydrolysate may help alleviate oxidative stress, which is often linked to various metabolic disorders, including insulin resistance and diabetes (González et al., 2023). Although the antioxidant potential of diets studied in Chapters 3 and 4 was not evaluated, in Chapter 2, extracts of shrimp hydrolysate showed its antioxidant properties, which may play a critical role in shrimp hydrolysate ability to enhance glucose metabolism. Additionally, the observed increased levels of $CD4^+$ and $CD8^+$ T cells producing TNF-α observed in dogs fed shrimp hydrolysate could potentially be linked to the lowered glucose levels in these animals. Although elevated TNF-α levels are commonly associated with insulin resistance and metabolic disorders, such as diabetes and cardiovascular disease (Vykoukal and Davies, 2011), recent findings suggest that TNF-α plays a more complex role in glucose regulation possibly due to a receptor-independent mechanism that positively influences glucose homeostasis (Wu et al., 2018). Therefore, future research should further explore the specific mechanisms by which shrimp hydrolysate affects glucose metabolism and investigate its broader implications for canine health and dietary strategies, especially in managing conditions associated with glucose dysregulation, such as diabetes.

Moreover, a significant increase in $CD4^+$ T cells proliferation was observed in response to LipL32 in dogs fed shrimp hydrolysate. Given that dogs used in the study were vaccinated annually for leptospirosis, LipL32, a protein found in the outer membrane of leptospiral bacteria, may have stimulated memory $CD4^+$ T cells, potentially enhancing immune responses. This suggests that shrimp hydrolysate might improve memory T cell generation and support more effective vaccination against leptospiral infections, with potential for the development of diets that could be strategically used to improve the effectiveness of vaccinations, particularly for diseases like leptospirosis.

Overall, these findings indicate that incorporating shrimp hydrolysate into canine diets could enhance immune function, improve metabolic health, and increase the efficacy of vaccinations, ultimately promoting canine well-being.

### 5.1.6 Coat quality variations with shrimp hydrolysate diets

The inclusion of 5% shrimp hydrolysate in extruded diets decreased coat gloss and general evaluation only in week 4, with no significant changes observed thereafter. This early variation could suggest that initial responses to shrimp hydrolysate dietary inclusion may manifest quickly but stabilize over time. However, the scores observed indicate an optimal coat quality with both control and 5% shrimp hydrolysate diets. Longer studies should be conducted, as coat quality may require a period longer than 12 weeks to fully stabilize and reveal any long-term effects of dietary interventions (Geary et al., 2022).

Moreover, the shrimp hydrolysate inclusion reduced coat scale (in weeks 4 and 12), an indicator of unhealthy skin, characterized by excessive renewal of the epidermis and increased shedding of dead skin cells. This improvement in skin condition may be linked to the observed decrease in eosinophil levels (Chapter 4), which possibly contributed to a reduction in the inflammatory response in the skin. In fact, eosinophils contain granules loaded with toxic proteins, cytokines and chemokines that can be released, amplifying the inflammatory response (Tizard, 2018). Furthermore, eosinophils infiltration in the dermis is a prevalent histopathological characteristic observed across diverse dermatological disorders in humans (Radonjic-Hoesli et al., 2021) and pets (Bloom, 2006), and lower eosinophil levels might benefit the reduction in inflammation and the improvement of skin barrier function (Naidoo et al., 2018). Therefore, the present work suggests that the reduction in eosinophil levels may have not only diminished inflammation but also supported improved skin barrier function, further contributing to a healthier coat. Future research should focus on understanding how shrimp hydrolysate affects eosinophil activity and skin health, exploring its potential as a dietary intervention for skin disorders such as atopic dermatitis.

## 5.2 Conclusions

This thesis highlights the potential of underexploited aquatic animal by-products, squid meal and shrimp hydrolysate, as sustainable and functional ingredients in canine diets. The integration of circular economy strategies in aquaculture and pet food industries offers a promising approach to enhance resource efficiency and reduce the environmental impact, while promoting health and performance of dogs.

A) **Squid meal and shrimp hydrolysate as novel protein sources for pet food**: This thesis demonstrated that both aquatic invertebrate by-products are rich in protein and essential AA, such as methionine, arginine, lysine, and leucine, offering diverse nutritional benefits. Shrimp hydrolysate extracts exhibited greater antioxidant activity

than squid meal extracts. Dogs preferred the basal diet over squid meal and shrimp hydrolysate added on top at moderate inclusion levels (15%), but shrimp hydrolysate proved to be equally palatable at lower levels (5%) in extruded diet compared to the control diet.

B) **Shrimp hydrolysate modulates fecal microbiota**: Shrimp hydrolysate altered the microbiota profile, and consistently increased the abundance of bacteria from the Oscillospiraceae family, which is associated with prebiotic effects and the production of beneficial VFA like acetate and propionate.

C) **Shrimp hydrolysate supports immune function**: Shrimp hydrolysate at 5% dietary inclusion helped to maintain healthy blood levels and boosted immune cells, suggesting it plays a role in supporting the immune system. It also lowered blood glucose levels, which could help with managing insulin resistance. Additionally, shrimp hydrolysate reduced coat scale, probably by decreasing inflammation. However, the mechanisms that lead to these outcomes are unknown.

The present work was the first to approach the value of aquatic invertebrate by-products and highlighted the potential of squid meal and shrimp hydrolysate to be included in canine diets. Shrimp hydrolysate further suggested potential to be included in hypoallergenic and gastrointestinal diets, diets for diabetic dogs, and immunomodulatory therapies. Overall, the results demonstrated the complexity of interactions between diets, their ingredients and nutrients, and how these factors impact a wide variety of physiological responses in dogs. Ongoing research is therefore crucial to optimize the use of these novel protein sources in dog diets. By continuing to explore their functional benefits and understanding the mechanisms behind their effects, it will be possible to develop more sustainable and health-promoting nutritious diets for dogs, contributing to pet welfare and sustainability of the pet food industry.

Moreover, this thesis makes a significant contribution to the One Health concept by exploring dietary interventions that promote both canine health and broader environmental and human health benefits (Figure 5.1). Incorporating ingredients into canine diets that can significantly enhance dogs' health by improving immune function, gut microbiota, coat health, and overall welfare, contribute to reduce the need for veterinary interventions and medications. Healthier pets can lead to healthier human-pet interactions, reducing the risk of zoonotic diseases and improving the quality of life for pet owners. Additionally, utilizing by-products supports sustainable practices by reducing waste and promoting the efficient use of natural resources. This approach not only contributes to environmental sustainability but is also consistent with global efforts to

ensure the health of ecosystems, animals, and humans, supporting the interconnectedness emphasized by the One Health concept.

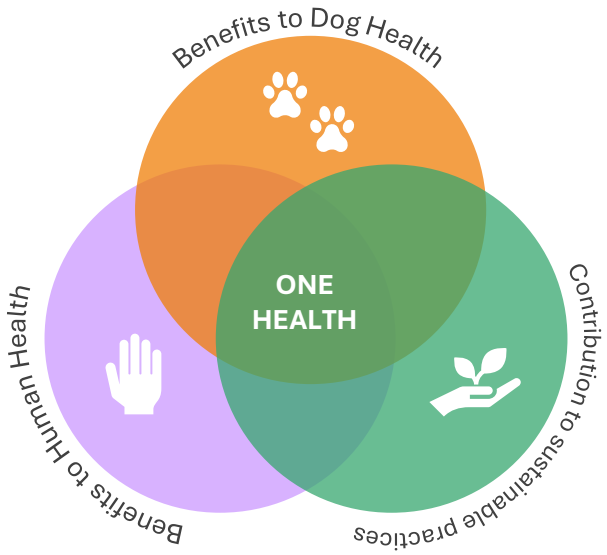


Figure 5.1 Integration of squid meal and shrimp hydrolysate into dog food in the One Health approach.

## 5.3 Future directions

For future research, it would be interesting to assess the effects of incorporating squid meal and shrimp hydrolysate in extruded diets at higher inclusion levels. There is potential for including these by-products in canine diets at significant levels or as the sole protein source, such as in hypoallergenic diets, which could promote the sustainability in both aquaculture and pet food industries. As a result, a comprehensive and holistic evaluation of dog performance and health across a broad range of physiological responses will be necessary. Longer-term studies (> 12 weeks) are particularly crucial, as they allow the observation of sustained physiological effects and potential long-term health benefits, which may not be apparent in shorter trials. Additionally, other crucial criteria for the success of pet food, such as palatability of these ingredients at higher inclusion levels when incorporated into extruded diets needs further investigation, since they must be generally well-accepted and appealing to dogs.

Key findings of the present work include the antioxidant properties of squid meal and shrimp hydrolysate, with the latter showing particularly high activity. However, further research is needed to identify the compounds responsible for these effects and to determine how food processing might influence their activity. Additionally, interactions

between nutrients or ingredients can affect nutrient bioavailability and the expected high digestibility of shrimp hydrolysate, as well as the extrusion process, by exposing ingredients to high heat, pressure, and moisture. Exploring the bioavailability of AA, bioactive peptides, and antioxidant compounds from squid meal and shrimp hydrolysate in dogs, could provide valuable insights into how effectively these components are absorbed and utilized, offering important considerations into their practical benefits and implementation.

The impact of squid meal and shrimp hydrolysate on fecal microbiota and metabolites also requires further exploration. Although squid meal did not alter the microbiota profile, shrimp hydrolysate influenced the abundance of certain beneficial bacteria, though results varied between studies. Additionally, the effect of squid meal on fecal butyrate levels was negative, while the impact of shrimp hydrolysate was either neutral or negative. These findings suggest that the overall effects of these protein sources on fecal microbiota and their metabolites are complex. Therefore, more research is needed to fully understand how nutrients from squid meal and shrimp hydrolysate are absorbed in the gut, how they interact with the microbiota, and in what manner these interactions lead to variations in bacterial composition and in metabolites production.

Similarly, while shrimp hydrolysate was found to enhance immune function - evidenced by increased levels of neutrophils, platelets, and white blood cells, along with decreased eosinophils - it also showed potential improvements in glucose metabolism. However, the precise mechanisms underlying these beneficial effects are not yet identified. Further research is needed to clarify how shrimp hydrolysate influences immune cell activity and glucose regulation at a molecular level. Investigating these mechanisms could provide deeper insights into the specific pathways through which shrimp hydrolysate exerts its effects, ultimately leading to a better understanding of its role in supporting immune health and metabolic function. Additionally, understanding the correlation between these immune effects and improvements in coat quality, such as by assessing skin inflammation and eosinophil levels through biopsies or additional diagnostic methods, is important. This could lead to the development of targeted dietary treatments for dogs with dermatological conditions, improving both their health and quality of life.

Moreover, the findings indicating that $CD4^+$ T cell proliferation was stimulated by Lilp32 suggest that shrimp hydrolysate could potentially enhance the effectiveness of vaccinations. Further research is needed to confirm these effects and to understand the underlying mechanisms. Specifically, studies should evaluate how shrimp hydrolysate influences T cell activity, particularly memory $CD4^+$ T cells, and vaccine-induced immune

responses. Such insights could help in the development of dietary strategies to boost immune function and vaccination outcomes, offering a valuable tool for improving health and disease prevention in dogs.

The beneficial effects of incorporating squid meal and shrimp hydrolysate into diets for dogs with specific health issues, such as overweight or diabetic, and those suffering from adverse food reactions or gastrointestinal disorders could be investigated. The exploration of these dietary interventions could provide valuable insights into how these by-products can effectively support the health and well-being of these vulnerable dog populations.

Other key areas requiring investigation include dog owners' acceptance, concerns, and motivations regarding the purchase of products containing aquatic invertebrate by-products. While some owners may be driven to purchase these by-products for their nutritional and health benefits, and sustainability, others may have reservations. Concerns regarding potential allergies, sensitivities, and safety might impact acceptance. This complex interplay of acceptance, concerns, and motivations highlights the need for targeted communication to address dog owners' apprehensions and promote informed decision-making.

A life cycle assessment could provide valuable insights into the environmental impacts of these by-products throughout their life cycle, from aquaculture production to processing and use in pet food. Developing strategies to enhance the circular economy within the involved industries and to improve the feasibility of incorporating these by-products into pet food need to be developed. Research should focus on understanding market demand, assessing cost-effectiveness, and identifying potential barriers to adoption, ultimately helping to design effective strategies for integrating sustainable and functional ingredients into the pet food industry.

Finally, evaluating the potential of the inclusion of other aquatic invertebrate by-products in pet food could provide valuable opportunities to improve the sustainability and nutritional value of dog diets, thereby enhancing the industry's capacity to meet the needs of both pets and their owners.

## 5.4 References

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