



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

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The inclusion of protein hydrolysates from animal by-products in petfood may enhance the 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 ($P = 0.017$) and glucose levels ($P = 0.023$), while increasing levels of white blood cells ($P = 0.002$), platelets ($P = 0.038$), neutrophils ($P = 0.036$), and CD4⁺ ($P < 0.001$) and CD8⁺ T cells ($P < 0.001$) single producers of tumor necrosis factor-alpha. It also led to a more extensive proliferation of CD4⁺ T cells in response to LipL32 antigen ($P = 0.020$) and to a decrease in superoxide production in stimulated cells ($P = 0.002$). Additionally, supplemented diets increased *Oscillospiraceae* ($P\text{-adj} = 0.005$) and *Clostridia* ($P\text{-adj} = 0.017$) abundance, and decreased such of *Sellimonas* ($P\text{-adj} = 0.025$). 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.

Hydrolyzed protein from animal by-products, such as those derived from the human food chain, could benefit dog health while contributing to the petfood industry's economic and environmentally sustainable growth¹. 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 immunomodulatory² being also reported to modulate gut microbiota composition³.

Protein hydrolysates are commonly used in petfood, especially to prevent allergic reactions in sensitive dogs⁴. 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 levels⁵. 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 hydrolysates⁶. 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 14th day), lactic acid bacteria (at 42nd day) and clostridia (at 42nd day), and an increase in the serum aspartate aminotransferase at 28 days⁷. Including up to 15% shrimp hydrolysate from *Litopenaeus*

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vannamei in diets of Beagle dogs over a 10-day feeding trial affected the abundances of *Oscillospiraceae*, *Bacillota* (formerly Firmicutes), and *Lactobacillus* in the fecal microbiota⁸. 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 trial⁹. 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 days¹⁰. 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 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 IgM¹¹ and to decrease gut pathogenic bacteria abundance¹². 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 distribution¹³. 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 levels¹⁴ decrease glucose levels, and improve innate immunity by enhancing the lysozyme activity and survival rates of fish infected with *Edwardsiella tarda*¹⁵. 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 activities¹⁶. Moreover, the hydrolysis of *Penaeus monodon* with alkaline protease has reduced in vitro IgE reactivity to tropomyosin¹⁷.

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 dogs¹⁸ this study focused on assessing its impact on the hematological parameters, serum chemistry profile, innate and adaptive immune function, and fecal microbiota composition.

Results

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 vs. 92.8 mg/dL, $P=0.023$), and increased the concentration of white blood cells ($7.67 \times 10^3/\mu\text{L}$ vs. $6.71 \times 10^3/\mu\text{L}$, $P=0.002$), platelets ($300 \times 10^3/\mu\text{L}$ vs. $274 \times 10^3/\mu\text{L}$, $P=0.038$), and the percentage of neutrophils (53.2% vs. 56.8%, $P=0.036$; Table 1 and Table S1). Regarding the time effect, 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 eight. Platelet concentration ($P=0.040$) and mean platelet volume ($P=0.004$) were greater in week four, whereas albumin concentration was lower ($P<0.001$).

Item	Week 4		Week 8		Week 12		SEM ²	P - value		
	Control ¹	Experimental ¹	Control ¹	Experimental ¹	Control ¹	Experimental ¹		Diet	Week	Diet*Week
White blood cells, $\times 10^3/\mu\text{L}$	6.86	7.09	6.83	7.62	6.45	8.29	0.433	0.002	0.601	0.139
Neutrophils, %	54.7	54.7	50.8	56.2	54.2	59.3	1.93	0.036	0.129	0.168
Lymphocytes, %	35.0	35.8	37.2	32.7	34.9	30.7	2.05	0.183	0.265	0.219
Monocytes, %	5.54	4.91	5.42	5.21	5.79	5.81	0.467	0.437	0.347	0.734
Eosinophils, %	4.80	4.25	6.63	5.50	5.11	3.75	0.500	0.017	0.003	0.671
Red blood cells, $\times 10^6/\mu\text{L}$	7.10 ^a	7.30 ^{a, b, c}	7.43 ^{b, c, d}	7.38 ^{a, b, c}	7.49 ^{c, d}	7.18 ^{a, b}	0.153	0.636	0.068	0.044
Hemoglobin, g/dL	16.6 ^a	17.1 ^{a, b, c}	17.6 ^{b, c}	17.4 ^{b, c}	17.7 ^c	16.9 ^{a, b}	0.37	0.596	0.013	0.037
Platelets, $\times 10^3/\mu\text{L}$	301	318	252	294	270	289	17.0	0.038	0.040	0.632
Mean platelet volume, fL	10.1	10.1	9.87	9.88	9.64	9.76	0.192	0.914	0.004	0.650
Total protein, g/dL	5.51	5.61	5.85	6.07	5.78	5.89	0.098	0.106	0.001	0.740
Albumin, g/dL	3.59	3.64	3.81	3.89	3.83	3.82	0.061	0.565	<0.001	0.657
Globulin, g/dL	1.93	1.97	2.04	2.18	1.95	2.06	0.062	0.067	0.054	0.720
Glucose, mg/dL	87.2	82.6	101	95.3	90.3	81.4	2.60	0.023	<0.001	0.608
Creatinine, mg/dL	0.764	0.836	0.880	0.793	0.762	0.808	0.0635	0.894	0.456	0.143
Urea, mg/dL	26.5	28.7	38.0	28.0	34.5	28.9	3.55	0.098	0.229	0.172
Alanine aminotransferase, U/L	27.7	36.6	27.2	29.4	30.4	29.3	3.34	0.218	0.471	0.273
Alkaline phosphatase, U/L	43.5	45.7	43.3	45.8	42.3	44.0	2.16	0.396	0.618	0.966
C-reactive protein, $\mu\text{g/mL}$	7.37	8.23	8.41	9.09	5.19	9.01	1.054	1.22	0.234	0.318
Immunoglobulin E, $\mu\text{g/mL}$	216	195	241	177	55.9	71.3	26.1	0.438	<0.001	0.135

Table 1. Hematology, serum chemistry, C-reactive protein, plasma Immunoglobulin E in weeks 4, 8 and 12 in dogs fed control and experimental diets. ¹Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten. ²SEM: Standard error of the mean. ^{a, b, c, d}Means with different superscript letters in the same row are significantly different ($P<0.05$).

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

Table 2. Concentration of cytokines, chemokine, and growth factor in serum in weeks 4, 8 and 12 of dogs fed the control and experimental diets. ¹Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten. ²SEM: Standard error of the mean. ^{a, b, c, d}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.

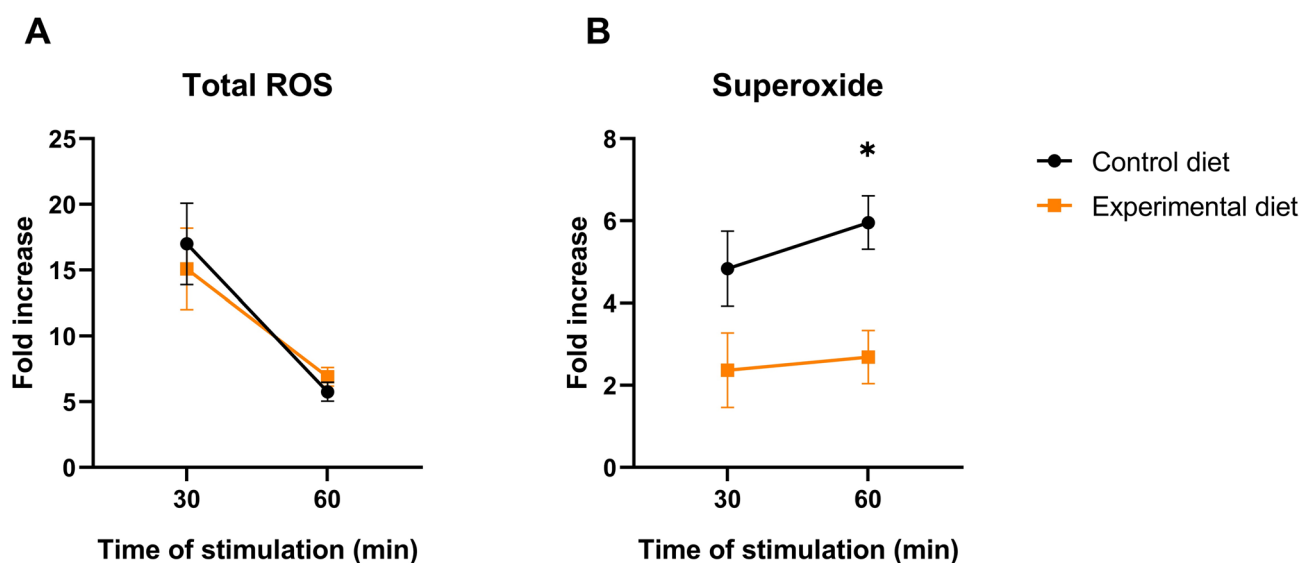


Fig. 1. Reactive oxygen species (ROS) production evaluated by flow cytometry. (A) Fold increase in the production of total ROS; (B) Fold increase in the production of superoxide in cells stimulated with phorbol myristate acetate for 30 and 60 min over the basal production (non-stimulated) in control diet and experimental diet. Bars correspond to mean plus standard error of the mean. * $P < 0.05$.

Concentration of IgE in plasma was lower in week 12 ($P < 0.001$; Table S1). The interaction between week and diet affected the concentrations of the red blood cells ($P = 0.044$) and hemoglobin levels ($P = 0.037$), with dogs fed the control diet showing higher levels than those fed the experimental diet at week 12, no differences being observed among diets for weeks four and eight (Table 1).

Serum cytokine, chemokine, and growth factor concentrations

Concentrations of IFN- γ , IL-10, IL-6, nerve growth factor-beta (NGF- β), and tumor necrosis factor-alpha (TNF- α) were below the detection limits (8.42 pg/mL, 6.47 pg/mL, 16 pg/mL, 4.98 pg/mL and 5.22 pg/mL, respectively). Diet and week did not affect the production of IL-12/IL-23p40, IL-8, IL-2, stem cell factor (SCF), monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor A (VEGF-A; Table 2 and Table S2). The interaction between diet and week affected the production of IL-8 ($P = 0.038$) that was significantly lower at week eight (539 pg/mL) and higher at week 12 (964 pg/mL) in dogs fed the control diet (Table 2). The interaction between diet and week also affected the production of SCF ($P = 0.027$), with lower values being observed at weeks four (29.6 pg/mL) and eight (33.8 pg/mL) and a higher value at week 12 (50.6 pg/mL) in dogs fed the control diet (Table 2). No differences were observed for IL-8 and SCF among weeks in dogs fed the experimental diet.

Reactive oxygen species production

Cells of both control and experimental diets groups produced greater amounts of total reactive oxygen species (ROS) after 30 min of phorbol myristate acetate (PMA) stimulation than after 60 min (Fig. 1A and Table S3).

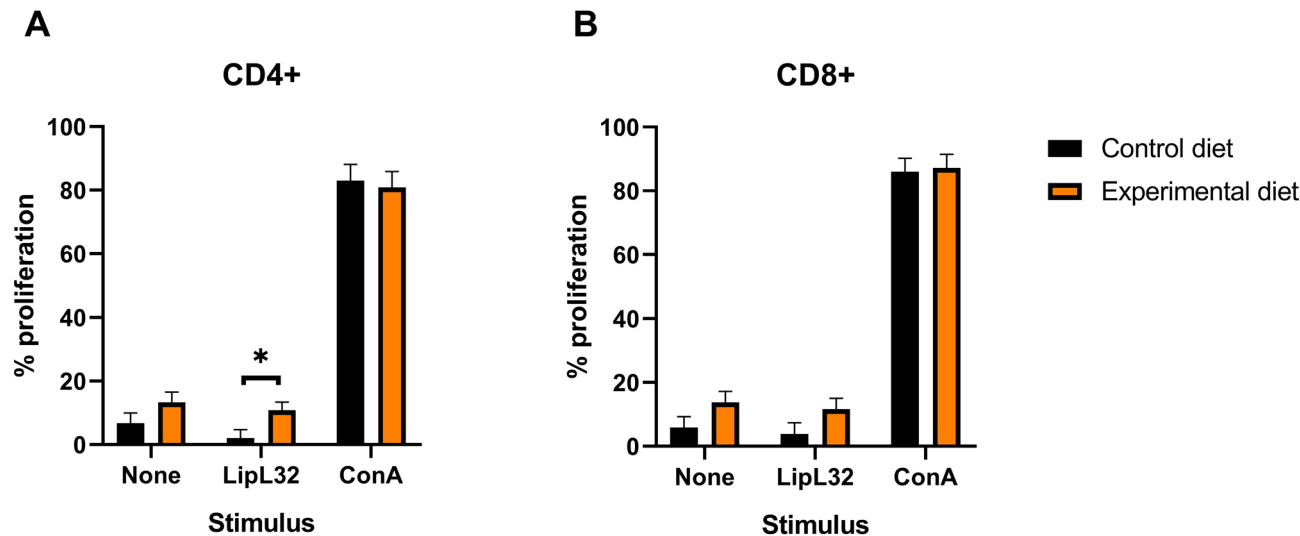


Fig. 2. T lymphocyte proliferation evaluated by flow cytometry. **(A)** Percentage of CD3⁺CD4⁺ cells; **(B)** Percentage of CD3⁺CD8⁺ cells, from dogs fed control diet and experimental diet, that proliferated at least once when non-stimulated (None) or in response to recombinant antigen from *Leptospira interrogans* (LipL32) and concanavalin A (ConA). Bars correspond to mean plus standard error of the mean. * $P < 0.05$.

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

Table 3. Concentration of cytokines after lymphocyte stimulation with Concanavalin A in weeks 4, 8 and 12 of dogs fed the control and experimental diets. ¹Control: complete diet without the inclusion of shrimp hydrolysate; Experimental: control diet with 5% of shrimp hydrolysate in replacement of wheat gluten. ²SEM: Standard error of the mean. Abbreviations: IFN-γ: interferon-gamma; IL, interleukin; TNF-α: tumor necrosis factor alpha.

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 ($P = 0.002$; Fig. 1B). The interaction between diet and week affected the production of total ROS ($P = 0.030$) and of superoxide ($P = 0.030$) after 60 min of PMA stimulation, with values being significantly higher at week 12 regardless of the diet (Table S3).

Lymphocyte proliferation and cytokine production

The percentages of proliferation of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells stimulated with concanavalin A (ConA) were not affected by the diet. The experimental diet induced more extensive proliferation of CD3⁺CD4⁺ cells in response to recombinant antigen 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 (Fig. 2).

The levels of IL-17, IFN-γ, TNF-α and IL-10 in culture supernatants of non-stimulated peripheral blood mononuclear cells (PBMC) or stimulated with LipL32 were below the detection limits (62.5 pg/mL, 31.3 pg/mL, 31.3 pg/mL and 15.6 pg/mL, respectively). Regarding the time effect, 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 S4) in week 12. 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 3).

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 (Fig. 3A), IFN-γ and TNF-α double producers (Fig. 3B), and the CD4⁺/CD8⁺ T cell ratio (Fig. 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, $P < 0.001$; Fig. 3A and B). Regardless

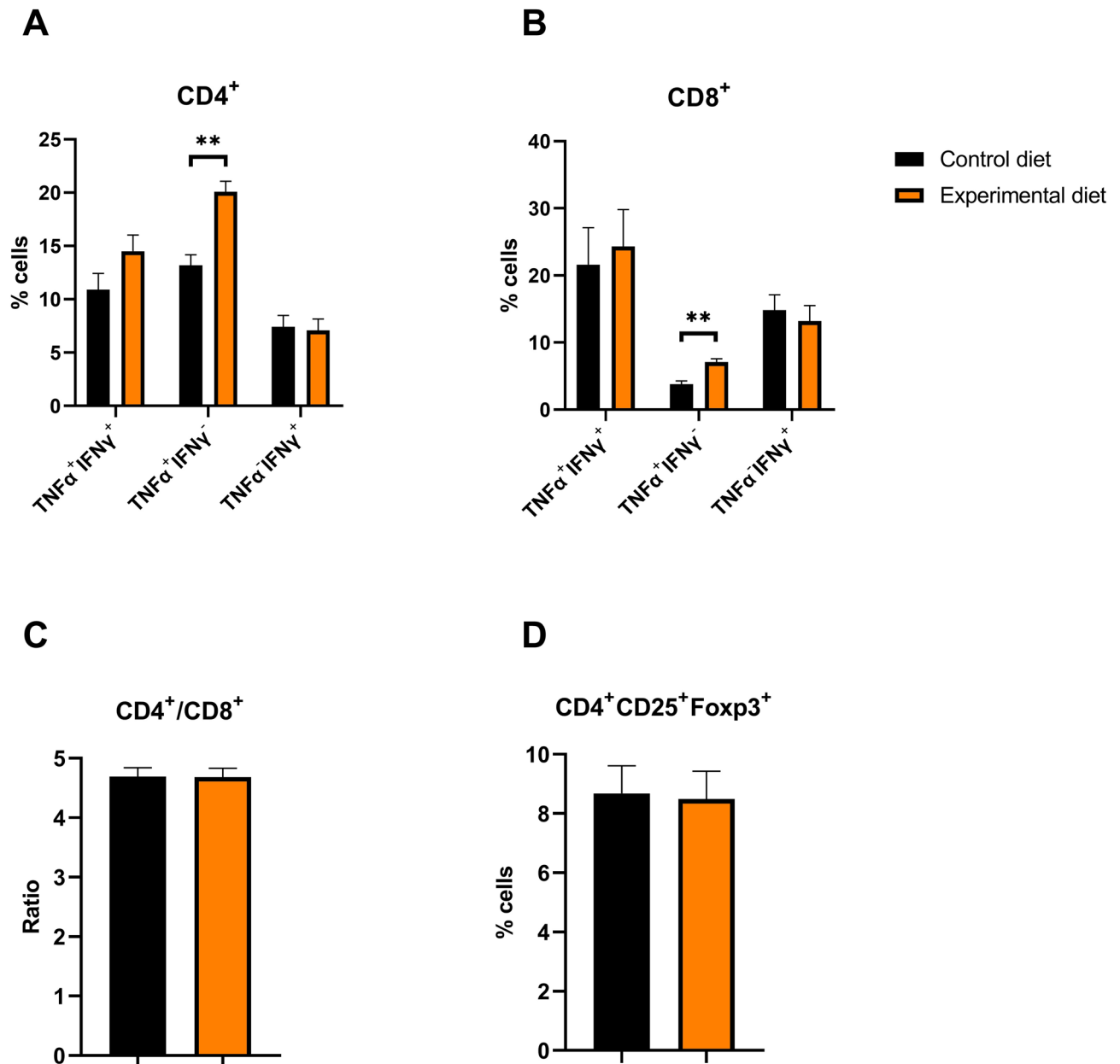


Fig. 3. Intracellular cytokine measurement by flow cytometry. (A) Percentage of CD3⁺CD4⁺ cells expressing interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and both cytokines; (B) Percentage of CD3⁺CD8⁺ cells expressing IFN-γ, TNF-α and both cytokines; (C) Ratio of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells calculated from the percentage of CD4⁺ and CD8⁺ T cells; (D) Percentage of CD3⁺CD4⁺CD25⁺ cells expressing Foxp3 from dogs fed control diet and experimental diet. Bars correspond to mean plus standard error of the mean. ** $P < 0.01$.

of the diet, the percentage of CD4⁺ T cells was greatest in week 4 (63.9%, $P = 0.050$; Table S5). 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 eight (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 (Fig. 3D), over time and in the interaction of diet and week.

Fecal IgA and microbiota

Fecal IgA concentration was not affected by diet, week, and their interaction (Fig. 4). Regardless of the diet, *Fusobacterium* was the most abundant genus, followed by genus pertaining to *Muribaculaceae* and genus *Bacteroides* (Fig. 5A). Across all the samples, most of the features were assigned to the phylum Bacteroidota (formerly Bacteroidetes), followed by Bacillota (Fig. 5B). Beta diversity metrics indicate a clear separation of

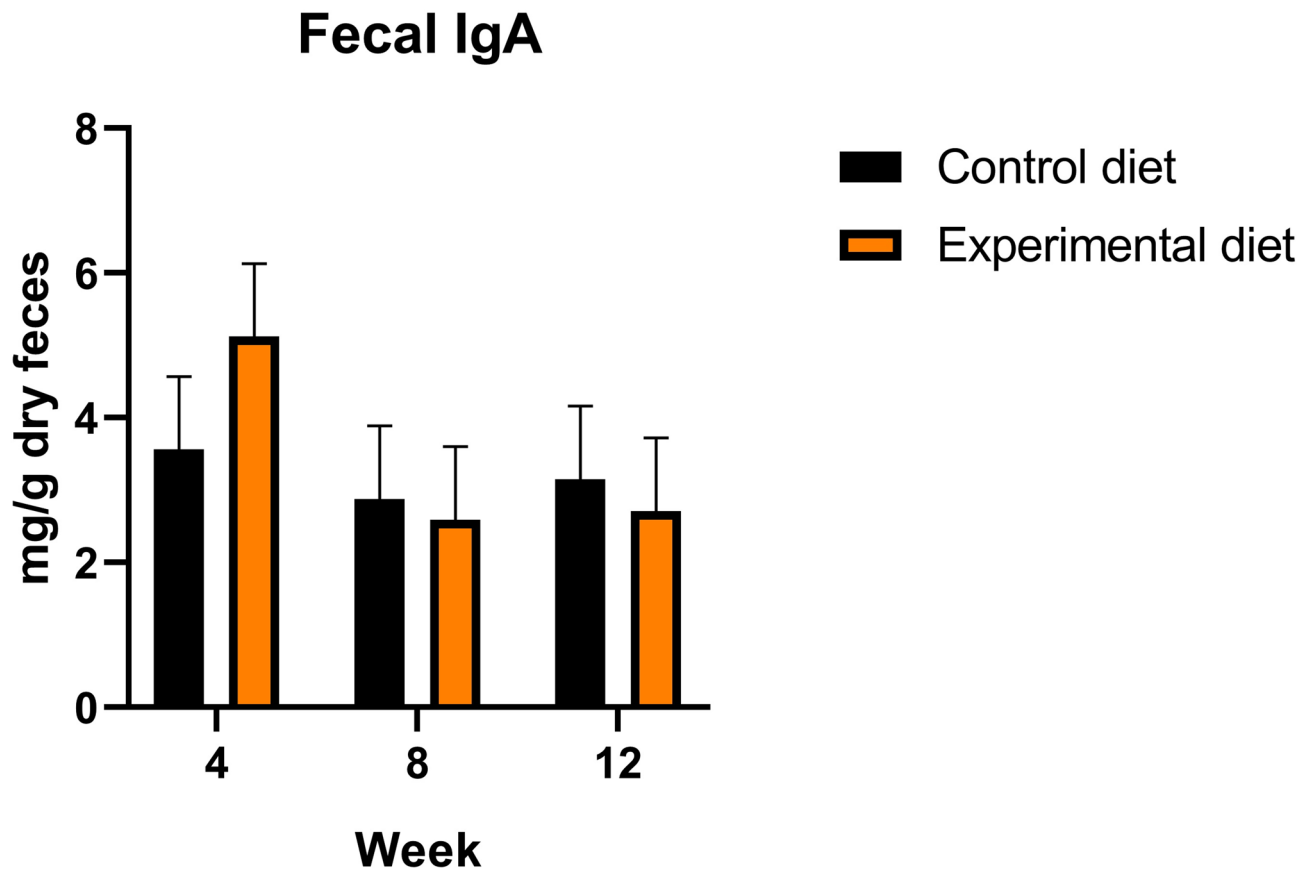


Fig. 4. Fecal immunoglobulin A (IgA) concentrations in weeks 4, 8 and 12 in dogs fed control diet and experimental diet. Bars correspond to mean plus standard error of the mean.

bacterial communities between diets, by using Compositional Tensor Factorization (CTF) distance (Fig. 5C). However, when Bray-Curtis distances were compared at each time point, no differences between the diets were detected. At the same time, 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 ($P=0.033$, $r^2=0.215$; Fig. 5E). Regarding alpha diversity, no differences were observed between diets on Shannon entropy, Faith's phylogenetic diversity and Fisher's Alpha through Kruskal-Wallis and Wilcoxon tests between diets within weeks (Fig. 5D). The experimental diet led to an increased abundance of genera pertaining to *Oscillospiraceae* and *Clostridia*, while the abundance of *Sellimonas* decreased ($P<0.05$; Fig. 5F).

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 sustainability¹⁹. 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' health²⁰. 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^{21,22}. 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 function²³. 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 pathway²⁴. The levels of eosinophils observed in the present study were within the normal range values for dogs (0–9%²¹), likewise the levels of IgE in blood (25–410 $\mu\text{g/mL}$ ²⁵). 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

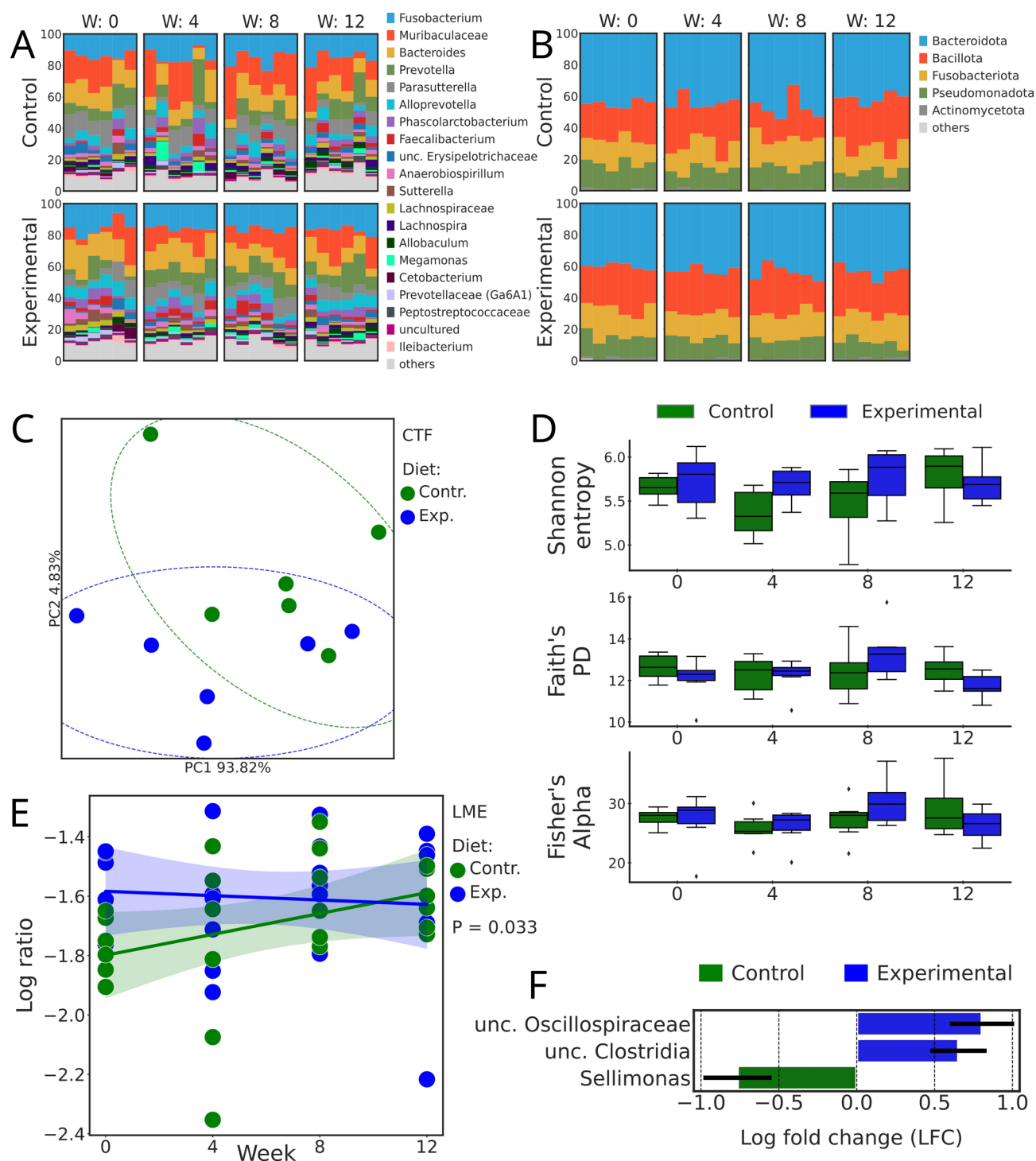


Fig. 5. Bacterial relative abundances, composition and diversity. **(A)** Taxonomy barplots at the genus level of dogs fed control and experimental diets in weeks 0, 4, 8, and 12. If genus level was not assigned, the last available taxonomy rank was used for the label; **(B)** Taxonomy barplots at the phylum level of dogs fed control and experimental diets in weeks 0, 4, 8, and 12; **(C)** 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; **(D)** Alpha diversity metrics. Boxplots of Shannon entropy Faith's PD and Fisher's Alpha indices of fecal bacteria of dogs fed control and experimental diets; **(E)** Linear mixed-effects analysis based on log-ratios of fecal bacteria of dogs fed control and experimental diets; **(F)** 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.

trial¹⁰. The IgE levels decreased in week 12 in dogs fed either diet, whereas the eosinophils levels were lower in weeks four 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/dL²¹). Fish hydrolysates have been demonstrated to lower glucose levels and regulate hyperglycemia in vitro, in murine models, and in human subjects²⁶. Furthermore, reduced glucose levels were observed in red seabream fed diets containing shrimp hydrolysate from *L. vannamei*¹⁵. Little is known about 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-1²⁷. 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 ROS²⁸. The excessive production of superoxide radicals is considered the main mechanism responsible for tissue damage in diabetes mellitus²⁹ and superoxide production may stimulate insulin release through the metabolism of branched-chain keto-acids in mitochondria³⁰. Additionally, high blood glucose levels increase the generation of ROS in both mitochondria and cytosol, which contributes to the development of various diabetes-related pathologies³¹. 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 hydrolysate⁸ 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 eight 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 activation³². 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 differentiation³³. The increase of both IL-8 and SCF could be linked, as previously demonstrated³⁴ potentially resulting from the production of IL-8 by hematopoietic progenitors cells³⁵.

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 hydrolysate⁹. 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 decreased⁵. 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^{36,37} and diet might influence their regulation with important effects in autoimmune diseases³⁸. Moreover, TNF- α can promote a reduction of Th1 cells producing IFN- γ and an increase of Th17 cells secreting IL-17³⁹, and an increment of the anti-inflammatory cytokine IL-10⁴⁰. 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 ⁴¹. It has also been shown to play an important role in the homeostasis of intestinal mucosal⁴².

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 cells⁴³. 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 vaccination⁴⁴. 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 strategies⁴⁵. 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 diets⁴⁶. 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^{47–50}.

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 inclusion⁷. 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 pathogens⁵¹ high circulating levels in blood have been associated with the development of insulin resistance, diabetes and cardiovascular disease⁵². However, experiments conducted in ob/ob mice and TNFR1/R2 double knockout mice have demonstrated that increasing TNF- α levels can enhance glucose homeostasis⁵³. 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 required 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 diet⁵⁴.

Immunoglobulin A is a secretory immunoglobulin present, among others, in the intestinal mucosa, protecting it from pathogens that can be used as an inflammatory biomarker⁵⁵. The levels of IgA might be influenced by diet^{56,57}. 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 diets⁵⁸ whereas feeding mice with fish protein hydrolysate led to an increase in IgA concentrations in the small intestine⁵⁹. Additionally, IgA helps to regulate the intestinal microbiota, such as their colonization, invasion, growth, and motility⁶⁰. 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 species⁶¹. 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 dogs⁶².

The greatest relative abundance of genus found in both diets pertained to phyla Bacteroidota and Bacillota. These phyla abundance is in accordance with data reported in dog feces⁶³. The four most predominant genera found (*Fusobacterium*, *Muribaculaceae*, *Bacteroides*, *Prevotella*) were previously observed in Beagle dogs fed diets with comparable macronutrients composition⁶⁴ suggesting that microbiota composition is mainly a consequence of the amount of macronutrients that comprise diets than of the ingredients¹⁰. *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 hydrolysate¹⁰. According to our previous findings⁸ 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 inflammation⁶⁵. Increased production of short-chain fatty acids beneficial to animal health^{66–68} such as acetate, butyrate, propionate and valerate have been associated with the presence of *Oscillospira* in the gut^{65,69}. However, a decrease in fecal butyrate levels was observed in dogs fed shrimp hydrolysate, compared to a diet without hydrolysate inclusion¹⁸. Clostridia, a class of Bacillota, 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 dogs⁷⁰. Previous research has suggested an association between improved digestibility of protein and increased abundance of *Clostridiaceae* in dogs⁷¹ being bacteria of the *Clostridium* genus crucial in the fermentation of amino acids, such as lysine and proline⁷². 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 diets¹⁸. 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 humans⁷³. However, in dogs diagnosed with inflammatory bowel disease, a decrease in the relative abundance of *Sellimonas* and *Oscillospiraceae* UCG-005 has been observed⁷⁴. 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 petfood 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 should 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.

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 N° 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.

Animals, diet and experimental design

Details on animals, diets and experimental design of the trial were earlier reported¹⁸. Briefly, 12 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 9⁷⁵, 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 BW⁷⁶ following the equation $ME \text{ (kcal/d)} = 110 \times BW^{0.75}$. Fresh water was provided *ad libitum*.

Two extruded isoproteic diets were formulated to meet the nutritional requirements of adult medium dogs⁷⁶ using the same ingredients, except for the inclusion of 5% (w/w) shrimp hydrolysate (experimental diet) in replacement of wheat gluten (control diet; Table S6), a high-level protein source¹⁸ commonly used in petfood. The level of inclusion was chosen based on results previously published⁸ and due to practical and economic considerations for the petfood industry. The shrimp hydrolysate (Symrise Aqua Feed, Elven, France) was obtained by enzymatic hydrolysis of heads and cephalothoraxes of *L. vannamei*⁷⁷. A detailed characterization of the diets was earlier reported¹⁸. 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.

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 $500 \times g$ 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 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 chamber⁷⁸. The PBMC were re-suspended in PBS for ROS assay, and in fetal bovine serum (FBS, heat inactivated South America origin, S181H, BioWest, Nuaille, 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 assays⁷⁹. 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.

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.

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), MCP-1/CCL2, NGF- β , SCF, TNF- α and 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).

Reactive oxygen species production

For the evaluation of ROS production, PBMC (1×10^6 cells/well) were stimulated with 100 nM PMA for 30 and 60 min at 37 °C and 5% CO₂. 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 was acquired by flow cytometry using a FACSCanto II system (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the FlowJo v10 software (BD Biosciences; Figure S1).

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% CO₂. 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 2.5×10^4 cells/well in 96-well plates and incubated for 4 days at 37 °C and 5% CO₂, with 1 μ g/mL of ConA from *Canavalia ensiformis* (C0412, Sigma Aldrich) or 10 μ g/mL 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 $300 \times g$ for 5 min, and supernatants were collected and stored at -80 °C 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 °C. 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, Franklin Lakes, NJ, USA; Figure

S2). Culture supernatants were later thawed to determine the concentration of TNF- α , IFN- γ , IL-10, and IL-17 A, 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).

Intracellular staining

Peripheral blood mononuclear cells were thawed, washed in complete RPMI at 37 °C, and left resting overnight at 37 °C and 5% CO₂. Cells were plated at 1 × 10⁶ cells/well and incubated at 37 °C and 5% CO₂ for 4 h in the presence of 1 × eBioscience Cell Stimulation Cocktail (Invitrogen) and 3 µg/mL 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 °C. 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 NaN₃, 2% FBS) and incubated, protected from light, for 25 min at 4 °C. Cells were then washed with PBS and fixed with 2% formaldehyde. For Fc γ 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 , respectively⁸⁰. 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; Figure S3).

For intracellular staining of the transcription factor Foxp3, PBMC (1 × 10⁶ cells/well) were stained with FVD eFluor 780 (Invitrogen) at 1:1000 in PBS and incubated, protected from light, for 20 min, at 4 °C. 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 °C. 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; Figure S4). UltraComp eBeads were used for antibody-fluorescence compensation.

Fecal collection and analyses

During two consecutive days at weeks 0, 4, 8, and 12, individual fresh feces were collected immediately after defecation and stored at -20 °C per dog and per week until further analysis. Feces were later thawed and homogenized.

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 °C 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 15,000 × g at 5 °C for 15 min. Supernatant was stored at -20 °C 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.

Fecal microbiota analyses

Fecal DNA was extracted with E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc., Georgia), following manufacturer's instructions. Primers targeting the V4 region of the 16 S rRNA gene (forward: GTGYCAGCMGCCGCGGTAA, reverse: GGACTACNVGGGTWTCTAAT) with attached adapters and barcodes were then used for amplification⁸². 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 pipeline⁸³. Primers and adapters were removed from the sequences by the cutadapt⁸⁴. After trimming, reads were denoised and merged by the dada2⁸⁵. Resulted amplicon sequence variants were classified by VSEARCH-based consensus⁸⁶ and pre-fitted sklearn-based classifiers⁸⁷ against the Silva database (v138.1, 16 S 99%)⁸⁸. The reference reads were preprocessed by RESCRIPt⁸⁹. The taxonomic classification of phyla has been updated to reflect the most recent nomenclature.

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). When significant ($P \leq 0.05$), multiple comparison of means was conducted by Tukey's post hoc test. For alpha diversity estimation of fecal microbiota, Shannon's entropy⁹⁰, Faith's phylogenetic diversity⁹¹ and Fisher's Alpha⁹² indices were calculated, and for beta diversity, CTF⁹³ and Bray-Curtis⁹⁴ 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 Qurro⁹⁵. Bray-Curtis distances were compared by PERMANOVA test⁹⁶. Shannon differences and log ratios were then analyzed with linear mixed-effects models (LME)⁹⁷. Differentially abundant genera (only for counts of genera with relative abundance $\geq 0.1\%$ and prevalence $\geq 10\%$) were detected by Ancom-BC⁹⁸.

Data availability

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.

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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. ARJC: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

Declarations

Competing interests

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Additional information

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