SUMMARY

of the doctoral thesis entitled:

RESEARCH ON THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL IMPLICATIONS OF THE MICROBIOME IN THE DIGESTION OF THE DOG

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The doctoral thesis, titled "***Research on the physiological and pathophysiological implications of the microbiome in the digestion of the dog,***" is structured according to current regulations, comprising two main parts:

Part I corresponds to the bibliographic study and includes 42 pages, representing 23% of the thesis volume.

Part II presents the original research and includes 141 pages, representing 73% of the doctoral thesis.

The microbiome is considered an integral part of the mammalian organism and has been referred to as the "hidden organ" (*Soontararak et al., 2019*). Since the neonatal period represents a critical stage for the establishment and development of the intestinal microbiota, it profoundly influences the future health trajectory of individuals. Dysbiosis, an imbalance or disturbance of the intestinal microbial community, has been associated with various conditions such as inflammatory bowel diseases, obesity, and even neurological disorders (*Beretta et al., 2023*).

Furthermore, the pediatric stage of life is essential for a healthy start in puppies lives. Throughout their lives, many dogs suffer from chronic conditions that affect their quality and life expectancy as well as their bond with their owners. Many of these chronic conditions, refractory to treatment, originate from early life risk factors. Therefore, improving preventive veterinary care and ensuring the health of puppies in kennels and subsequently at owners' homes are factors that will contribute to improving quality of life and preventing late-onset conditions.

Chapter I: Summarizes recent bibliographic data and clinical studies on the canine microbiome, similarities with the human microbiome, as found in the latest publications, considering this is the latest approach in human medicine.

Chapter II: Presents basic notions about the microbiome and its functions in adult dogs and puppies, the roles of different bacterial taxa played in canine health.

Chapter III: Discusses the roles of the microbiota on various systems and organs, including digestion, interactions with dietary nutrients, and the impact of different food formulas on the optimal development of the microbiome or aberrant development of certain taxa. It also covers bile acid metabolism, the microbiome's relationship with immunity/enteritis/obesity, dermatological conditions, and the gut-brain axis.

The primary goal of the study was to identify the moment of microbiota maturation in growing puppies and the threshold of similarity to that of adults in terms of the composition of the main bacteria describing a healthy normobiotic microbiota. Additionally, the study aimed to identify an initial functional parameter from the microbiota metabolomics, specifically the level of secondary bile acids in feces.

Part II, titled "***Personal Research***," constitutes approximately 80% of the thesis and is organized into 4 chapters, general conclusions, and bibliography. The results are illustrated through tables, graphs, and figures.

Chapter IV: "***Research on the context of the digestive microbiome in growing puppies in relation to age***" was conducted on a cohort of 78 puppies of the American Akita, American Bully, French Bulldog, Rottweiler, and German Shorthaired Pointer breeds, aged 7-56 weeks. A total of 112 unique individual samples were collected, from which have resulted 20 composite samples were created for testing, collected at 2-4 week intervals. The fecal samples were initially refrigerated, then transferred to -20 °C until final testing by qPCR method in a laboratory in Germany, using Roche Diagnostics equipment and analyzed with statistical interpretation tools such as MedCalc® Statistical Software version 22.021, JMP 5.0 Professional Edition English Academic, and SAS Institute GmbH. Statistical data confirmation was done using the Student's T-test and ANOVA.

Group I: Puppies aged 7-14 weeks

Included 7 composite samples from all 5 breeds. The bacteriological load results showed a total bacterial count log/DNA/gram, with an average of 9.33 in the analyzed samples. *Fecalibacterium spp* was identified with an average of 6.33 log DNA/g feces, below the adult mean value. *Blautia spp* had a value of 7.09 log DNA/g feces, below the minimum adult value. *C. hiranonis* averaged 8.51 log DNA/g feces, slightly above the adult maximum. *E. coli* averaged 6.46 log DNA/g feces, within the normal adult range. *Fusobacteria spp* averaged 6.02 log DNA/g feces, below the adult minimum. *Turicibacter spp* averaged 8.07 log DNA/g feces, within the normal adult range. The total bile acids average was 2.12 [µmol/g], significantly below the adult maximum, due to the very young age.

Group II: Puppies aged 15-34 weeks

Included 7 composite samples from the 5 studied breeds. The bacteriological load results showed a total bacterial count log/DNA/gram, with an average of 9.76 log DNA/g feces, below the adult mean value. *Fecalibacterium spp* was identified with an

average of 6.88 log/DNA/g feces, within the adult range. *Blautia spp* had an average value of 7.55 log/DNA/g feces, below the healthy adult minimum. *C. hiranonis* averaged 8.85 log DNA/g feces, above the adult maximum. *E. coli* averaged 6.6 log DNA/g feces, within the normal adult range. *Fusobacteria spp* averaged 6.86 log DNA/g feces, slightly below the adult minimum. *Turicibacter spp* averaged 7.83 log DNA/g feces, within the normal adult range. The total bile acids average was 2.44 [µmol/g], significantly below the healthy adult maximum.

Group III: Puppies aged 39-57 weeks

Included 6 composite samples from 3 studied breeds. The bacteriological load results showed a total bacterial count log/DNA/gram, with an average of 9.35 log DNA/g feces, significantly below the adult mean value. *Fecalibacterium spp* was identified with an average of 6.99 log/DNA/g feces, within the adult range. *Blautia spp* had an average value of 7.11 log/DNA/g feces, below the healthy adult minimum. *C. hiranonis* averaged 8.02 log DNA/g feces, above the adult maximum. *E. coli* averaged 5.6 log DNA/g feces, within the normal adult range. *Fusobacteria spp* averaged 6.87 log DNA/g feces, slightly below the adult minimum. *Turicibacter spp* averaged 7.51 log DNA/g feces, within the normal adult range. The total bile acids average was 4.2 [µmol/g], significantly below the healthy adult maximum.

For this group, we expected the individual parameter values to be closer to those of healthy adults. However, an disbalance in the ratio of healthy microbiota types was observed, likely due to unknown external factors, such as parasitic contaminations, which may have disrupted microbiota development, as indicated by intestinal scores in the table.

In the kennels studied, we were able to conduct temporal analyses for 4 different litters: American Akita, German Shorthaired Pointer, American Bully, and French Bulldog, to identify a correlation between bacterial evolution and the animal's age.

The centralized results indicate diversity due to multiple factors influencing microbiome development, such as breed, birth method, and highly diverse food composition in terms of macronutrients and micronutrients, particularly fibers, and corelation with the weeks age.

Tracking the evolution of all 6 bacterial species and the total number of bacteria compared to healthy adults, the following graph shows the differences between samples. These differences, however, cannot be attributed exclusively to age, as there are at least 5 other variables. If all dogs were fed the same food, belonged to the same breed, and were born identically, this differentiation would be possible.

Two samples, 39 and 49, resemble the healthy adult microbiota the most, except for the level of *Turicibacter spp*, which is significantly below adult minimum values. *Turicibacter spp* plays a significant role in interacting with host lipid levels: triglycerides, cholesterol, and bile acids, and helps limit excessive weight by modulating lipid biology.

Another observed aspect suggests that all samples have *C. hiranonis* levels significantly above healthy adult values. *C. hiranonis* has been shown to convert primary bile acids into secondary bile acids. Its deficiency leads to limited secondary bile acids, dysbiosis, and potential diarrhea.

We did not identify related aspects in the literature concerning elevated C. hiranonis levels, especially in healthy junior patients.

The final data analysis indicates clear similarities in the microbiota of some samples compared to the reference limits for healthy adult dogs.

Considering all 6 bacteria tested and the total bacteria in each sample as a parameter of microbiota health, two samples showed similar healthy bacteria diversity and a microbiota most similar to healthy adults: samples 39 (30-weeks-old French Bulldog) and 49 (32-week-sold American Bully), as shown in the graph. Both litters with marked results on the diagram came from puppies delivered by cesarean section.

However, even the samples from puppies aged 40-56 weeks did not show optimal *Blautia* levels compared to the adult control group from the literature.

The correlation between the total bacteria number and age is not significantly different from zero ($p = 53.58$; $R2 = 0.02295$). For the available data, no linear regression or correlation between bacteria and age could be established. Due to the limited and diverse sample number, a clear correlation could not be determined.

Chapter V: "***Research on the Context of the Digestive Microbiome in Growing Puppies Related to Nutritional Formula***" analyzes the nutritional aspects of the 78 puppies of the American Akita, American Bully, French Bulldog, Rottweiler, and German Shorthaired Pointer breeds, aged 7-56 weeks, with statistical nutritional determinations using Roche Diagnostics equipment and analyzed with statistical interpretation tools such as MedCalc® Statistical Software version 22.021, JMP 5.0 Professional Edition English Academic, and SAS Institute GmbH. Statistical data confirmation was done using the Student's T-test and ANOVA.

The groups were divided into premium industrial food for growing puppies, meeting the FEDIAF standards and requirements for growth, and BARF food.

Group A - Included Rottweiler (5 puppies, 1 sample), American Akita (1 litter, 5 puppies aged 10 weeks), American Bully (2 litters, 13 puppies from different kennels). Initially fed with a super-premium extruded formula for growth, then with pet shop products labeled for the growth period and/or homemade food. Pet shop products were complete and balanced, intended for the growth stage, alone or in combination with other foods specified for each puppy/litter.

Group B -Included German Shorthaired Pointer (3 litters, 22 puppies), American Bully (2 litters, 13 puppies), Rottweiler (5 puppies), American Akita (1 litter, 5 puppies from different kennels), fed with a super-premium brand adapted to age after weaning (up to 14 weeks), then transitioned to another complete and balanced formula for the next growth stage (after 14 weeks), enriched with prebiotic fibers, DHA

for brain development and anti-inflammatory properties, and an immune support complex, adapted to each age and size.

Group C: Fed mixed with pet shop products labeled for the growth period, which didn't meet the FEDIAF nutritional requirements for growth stages, combined with BARF in equal parts from weaning (4 weeks) onwards. This group included French Bulldog (5 litters, 27 puppies from the same kennel) fed with incomplete BARF and growth-specific kibbles. This group wasn't confirmed negative for any intestinal parasites.

All three groups underwent a 5-7 day dietary transition at weeks 3-5, then 8-10, to avoid multiple stressors and ensure acceptance of the new food and digestive adaptation to the new age and size-appropriate formulas.

Statistical data shows an increase in bacterial diversity with age, confirming scientific aspects published in other studies, influenced by dietary factors such as fermentable dietary fibers, protein levels, and macronutrient ratio.

In the food-dependent evaluation, we also measured particular interferences of each of the nutrients previously validated as contributors to optimal microbiota development: proteins, lipids, fibers.

Statistically analyzing the nutrient factor: lipids resulted that only 24.1% of the variation in total bacterial concentration can be explained by a linear relationship with respect to lipid concentration. Among the factors influencing the level of total bacteria is further shown to be the concentration of protein and fiber in the diet.

Analyzing the nutritional factor : protein on microbiota growth, although the regression equation is highly significant (P=0.0117), it manages to explain 30.43% of the variance of total bacteria concentration in relation to variations in protein level in the feed formulas.

The correlation between concentrations of total bacteria and the nutritional influence factor: dietary fiber is significant (p = 0.03), having an acceptable degree of association. This is surprising because it is known from other studies that fiber has a major contribution to the development of the digestive microbiota. The applied statistical analysis could give these conflicting values, due to the limited number of samples and the diversity of interrelated factors.

It is considered that the nutrition factor is essential through all the nutrients monitored and the contribution to the development of the microbiota is all the more important as the puppies follow a complete, balanced diet adapted to the growth and management of the food is consistent and constant over the long term.

Chapter VI: "***Research on the context of the digestive microbiome in growing puppies related to birth method, breed, and kennel***" analyzes the microbiome in relation to birth method, breed, and kennel for the same cohort from previous chapters.

Birth Method Impact: Measured through the correlation of total bacteria with birth method as a single parameter, revealing statistically significant data by

dividing puppies into two groups. Limited differences in total bacterial concentration based on birth method were observed. Although presumed significant differences in the microbiota between vaginally and cesarean-born puppies, the limited sample representation might have influenced statistical results.

Breed Impact: Analyzed by structuring according to brachycephalic and non-brachycephalic breeds. The statistical analysis was performed with ANOVA of the differentiation factor - Total bacteria log DNA/g feces by breed type, brachycephalic (nr=11) and non-brachycephalic (nr=9) breeds, resulting in minor significant differences. The Shapiro-Wilk test for the normal distribution of the 2 groups of brachycephalic (nr=11) and non-brachycephalic (nr=9) breeds was applied to confirm the findings identified with ANOVA. The Shapiro-Wilk test for the normal distribution revealed statistically minor significant differences, based on a limited number of samples.

Impact - Kennel: To study potential significant differences in mean bacterial concentration between kennels, ANOVA analysis was applied when data were normally distributed. In this case the equality of variances was also tested. If the variances were not equal, but the data were normally distributed, Welch-ANOVA analysis was applied, which allows the analysis of this type of data. In cases where data were not normally distributed, the Kruskal-Wallis test was applied.

The Welch-Anova analysis generated a significant result ($p = 0.084$). The result can be accepted, indicating a 91.6% probability that the differences in the mean values of total bacteria according to the KENNEL factor are due to this factor, thus the concentration of total bacteria for the French Bulldog breed are at the significance level different from the concentration of total bacteria of the other breeds.

The American Akita litter shows a delay in average total bacteria growth, possibly due to lack of dietary fiber. The German Pointer and American Bully litters show similarities and normality in the development of the mean total bacteria relative to the adults, but also between them, due to the prebiotic fiber enriched diets. The French Bulldog breed has a higher total bacteria count, possibly correlated with the BARF feed factor, which may contain pathogens.

Also, all mean values of total bacterial concentrations recorded for the breeds studied are outside the range of 10.9 log DNA/g feces - 15.1 log DNA/g feces, and below the minimum value of the adult range of 10.9 log DNA/g feces.

This was expected and is mainly due to the fact that the microbiota of puppies is still developing. In addition, it is well known that the type of food, and more specifically the fermentable fibers in the total dietary fiber composition, contributes to an increase in microbiota diversity and bacterial numbers. This is a second factor contributing to the population of healthy bacteria and their increased number.

As the Welch-ANOVA analysis was at the limit of significance, it was opted to perform the Tukey-Kramer HSD post-hoc test to compare the mean values of total bacterial concentrations. From the Tukey-Kramer HSD test it can be seen that there is a

difference, at the limit of significance, between the total bacterial concentrations for the French Bulldog and American Akita breeds. The American Bulldog and German Pointer did not differ significantly in mean total bacteria values.

The mean values of total bacteria for American Bully and German Pointer differ at the limit of significance from those for American Akita and French Bulldog.

These results can be further clarified by performing the T-test for each pair of breeds to highlight any significant differences

If one accepts a probability of 93.6% (instead of 95%) to consider that there is a significant difference between the American Akita and French Bulldog breeds in terms of total bacteria concentration, then these two breeds differ significantly in this respect.

Between American Akita and French Bulldog kennels there is a significant difference in the mean values of total bacterial concentrations. When one obtains a probability of P = 0.0646 when comparing the two means, one can say that there is a 93.6% probability that this difference is due to the factor considered, i.e. the kennel (which includes other factors in our case).

Most of the differences could be observed by comparing similar kennel factors, which are similar in bacterial growth rate and food type.

The ANOVA for comparing mean concentrations of *Fecalibacterium prausnitzii* as a function of kennel ranged from 3.4 log DNA/g feces to 8.0 log DNA/g feces. *Fecalibacterium prausnitzii* being one of the dominant digestive bacteria.

The availability of nutrients essential for sustaining *F. prausnitzii* may influence the distribution of this species in the gut. A recent study based on a functional metabolic functional map of the *F. prausnitzii* strain predicted its inability to synthesize the amino acids alanine, cysteine, methionine, serine and tryptophan. Auxotrophy for vitamins and cofactors, such as biotin, folate, niacin, pantothenate, pyridoxine and thiamine, was observed by further analysis of *F. prausnitzii* genomes. In contrast, this species was predicted to produce cobalamin, and in IBD patients are known to experience cobalamin deficiency.

ANOVA for comparing mean concentrations of *Blautia spp*. by kennel with the Kruska - Wallis test for comparing mean concentrations of *Blautia spp*. by kennel indicates a significant difference at the borderline of significance (p = 0.073097). If a statistical threshold of 0.08 > 0.05 is accepted then it can be considered that there are significant differences between kennels in terms of *Blautia spp*. concentrations.

Based on the available data, significant differences in mean *Blautia spp*. concentrations are found only between French Bulldog and American Akita kennels (p = 0.0357). It is difficult to define the causality of the large differences between the two kennels.

It is known for *Blautia spp*. that, during flavonoid conversion, Blautia-catalyzed reactions include demethylation, dehydroxylation and deglycosylation, due to corresponding enzymes such as β-glucosidases and O-glycosidases.

By analyzing the key metabolic pathways and enzymes related to biotransformation, it is possible to predict whether the bacterium can biotransform specific bioactive substances

ANOVA for comparing the mean concentrations of *C. hiranonis* by kennel reveals that statistically significant differences between kennels are statistically limited. The SD values show variability in bacterial concentrations, and the kennel with the highest variability in values of this bacterium is the American Akita, due to wide variability between times of sampling of nestlings and large variations in feed between times. This could be an inconsistent factor when compared to the other kennels.

The kennel with the greatest variability of *C. hiranonis* concentration values was American Akita (SD = 2.55476 log DNA/g faeces) (Table 6.18). This is also reflected in the 95% confidence interval within which *C. hiranonis* concentrations can be expected for animals developed under conditions identical to those of the American Akita kennel. It can be stated that, the conditions under which the puppies were grown, with 95% probability, the concentrations of *C. hiranonis* in the American Akita kennel nest will be in the range 0.5936 log DNA/g faeces - 13.286 log DNA/g faeces.

The variability in the *C. hiranonis* in this litter of puppies could be due to the varying type of food received. Formula feed 2 months after weaning is nutritionally inadequate for the growth period. Another possible cause for the variation of *C. hiranonis* in this kennel could be the genetic predisposing factor for IBD, as Akita are known to be predisposed to IBD and generally allergy-prone immune-mediated pathologies. In adults with IBD, *C. hiranonis* has a low proportion in the microbiota of those investigated

On the other hand, the variability in *C. hiranonis* values between kennels could be due to feeding. American Bully and German Pointer were feed a complete and balanced age-appropriate diet containing sufficient prebiotic fiber which contributes to the development of *C. hiranonis*. All other kennels have a normal variability in the values of *C. hiranonis* concentrations.

ANOVA analysis to identify the existence of significant differences in *E. coli* concentrations among the kennels studied showed for the American Bully kennel only one value of the mean *E. coli* concentration exceeding the maximum accetable value, the maximum value of the confidence interval of the mean *E. coli* concentration for this kennel does not exceed the maximum accetable value.

For the American Akita kennel, although all nest values were below the upper limit of the mean *E. coli* concentration, the very high variability of the recorded values resulted in a confidence interval of the mean *E. coli* concentration that has an upper limit (9.2917 log DNA/g feces) well above the maximum allowed (8 log DNA/g feces). Here it can be suspected that there is a risk that in this kennel there is a risk of values above the accetable limit, because the average per kennel can always be above 8 log DNA/g feces.

This risk is associated with bacterial overpopulation with pathogenic *E. coli* and long-term IBD risks, correlated also with the lower level of *C. hiranonis*. High *E. coli* levels may also influence a poor vaccine response.

ANOVA analysis of the mean *Fusobacteria spp.* values by kennel, observes that the confidence intervals for American Akita and French Bulldog are clearly disjoint and for this reason it is suspected that there is a significant difference between the mean *Fusobacteria* levels of the two kennels.

To test the hypothesis a T-test was performed to compare the concentration of *Fusobacteria spp*. between American Akita and French Bulldog kennels.

Fusobacteria spp., being a bacterium with probiotic benefits, has found its resources in the diets of American Bully and German Pointer. In the case of the American Akita kennel, the feed did not contain sufficient prebiotic fiber to contribute to the optimal growth of Fusobacteria in this breed. On the other hand, French Bulldog kennel litters, being fed 1/2 BARF and 1/2 industrial junior feed, had a limited fiber intake, insufficient compared to the recommended level.

ANOVA analysis of the relationship between the total concentration of *Turicibacter spp* as a function of kennel yielded a weakly significant result, bordering on significance ($p = 0.0588$). Due to the way in which the ANOVA analysis and the Tukey-Kramer HSD test are mathematically constructed, the result obtained for the Tukey-Kramer test is insignificant in such situations.

Maintaining a significance level of 0.05, the mean concentrations of Turicibacter spp. for the German Pointer and French Bulldog breeds do not differ significantly. If we accept a significance level of 0.1, we consider that there are significant differences between the two breeds in the mean concentrations of *Turicibacter spp*.

A significance level of 0.05 indicates that there is a 95% probability that the difference between the mean values of *Turicibacter spp*. is due to the kennel factor and only 5% of this difference is due to chance. It is possible, for clear medical reasons, to accept a significance level of 0.1, i.e. to accept a 90% probability that the difference in the mean values is due to the kennel factor with their complementary factors.

There is a very good correlation between the concentrations of *Fecalibacterium prausnitzii* and *Fusobacteria spp*. The Pearson correlation coefficient is highly significant ($p<0.0001$). The 2 species are in a cross-feeding relationship, the optimum levels of *Fecalibacterium prausnitzii* will influence the optimum levels of *Fusobacteria spp.*

Chapter VII of the second part of the PhD thesis, entitled "***Research on the context of the digestive microbiome of growing puppies in relation to bile acid levels*** " involves the analysis of bile acids in the animals by gas or liquid chromatography. For the extraction of secondary bile acids a defined amount of feces was weighed. The bile acids were extracted in the extraction buffer included in the overnight bile acid analysis kit.

According to the statistical analysis, there is a significant relationship between the level of *C. hiranonis* colony count and the level of bile acids (p=0.0338), thus the magnitudes correlate inversely proportionally.

The inverse correlation between *C. hiranonis* and total bile acids is not surprising. This may also be attributable to the fact that in the average samples we also have puppies under 16 weeks of age, in which the bile acid value is reduced below the adult maximum. The increased development of *C. hiranonis* resulted in low total bile acid values. On the other hand, the low bile acid values correlated with optimal levels of *C. hiranonis* in the digestive tract confirm the lack of risk of liver shunts in the healthy puppies included in the study.

Since the data corresponding to bile acid concentrations are not normally distributed compared to adults, comparison of the means of the values of this parameter was made using the non-parametric Kruskal-Wallis test.

It can be seen that there are no statistically significant differences by kennel between the mean bile acid concentration values (p = 0.835). The low bile acid values may be primarily due to the age of the puppies, which are healthy juniors with no breed predisposition to liver shunts.

The very high variability in bile acid concentration values for the American Akita litter influenced the outcome of the analysis. For this reason, a comparative analysis of each pair was chosen. After checking the normality of the data and the equality of variances, it was decided to perform parametric or non-parametric tests, as appropriate.

Statistical analysis performed using independent samples, such as T-test, Welch's test and non-parametric Mann-Whitney test, confirmed the lack of significant differences between the mean values of bile acid concentration and breed or kennel.

We conclude that the relationship between *C. hiranonis* and total bile acids in the studied litters confirms the normality of lipid metabolization in the puppies of those litters, regardless of other puppy parameters or external factors.

Chapter VIII contains 194 bibliographical sources cited in the text.