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PhD THESIS

Factors affecting Gut Microbiome in Healthy Dog

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie Sklodowska

Abstract

The field of microbiome is a research topic in expansion, especially regarding the study of the gut microbiome in companion animals, in relation to diet and health status of the subject. Recently, several factors have been study to understand their influence on the variation of the microbial composition of the gastrointestinal (GI) tract. Unfortunately, there are a large amount of research with a few numbers of dogs per study. In particular, when it comes for nutritional investigation, the change in the diet corresponds to a modulation of one nutritional element per time, and a few researches take into account the effect of different type of diet – such as dry extruded diet *Vs* home-made diet – on the gut microbiome.

The purpose of this doctoral thesis was to complement the information in the literature, by implementing a standardized method of analysis, from the collection of feces to the final taxonomic annotation, for all the studies carried out. Furthermore, the main aim was to form a sufficiently large database of data collected from healthy subjects. With this purpose, an attempt has been made to characterize the intestinal microbiome of healthy dogs; in the first place, we tried to understand if there were factors that influences the microbiome even in the absence of disease, in order to divide the subjects into macro-categories and to facilitate subsequent analyzes. The two factors initially identified were the type of diet administered and the sex of the animals, together with their hormonal status. It has been observed that there were substantial shifts, in terms of taxa, depending on the category analyzed. These variations, however, did not affect the health status of the animals. In fact, the dogs included in the database come from numerous dietary studies performed by the same research group. This means that the dogs, initially recruited as healthy, were followed for the entire duration of the experiments, i.e. from 30 to 45 days. If they did not maintain the requirement of being healthy during the course of the experiments, they would have been excluded from the study. These results have led to think that there are key bacteria that distinguish each subject based on both environmental and genetic factors. The presence and abundance of these bacteria could lead to the formation of the concept of enterotype even in dogs.

Following these results, several studies have been conducted to confirm this hypothesis. Initially, a classic diet modulation study was carried out to verify whether the addition of a certain class of polyphenols could influence the intestinal microbial composition. Together with the analysis of the fecal microbiome, the level of serotonin and cortisol in saliva was also analyzed. Some changes were observed at the level of a single taxa during the course of the experiment, but not to induce a shift in the entire gut microbial composition. The level of serotonin, on the other hand, was strongly influenced by the addition of polyphenols in the diet, so further studies will be necessary to understand what is the action mechanism of polyphenols in GI tract. Subsequently, an attempt was made to analyze the metabolome of healthy dogs, using a new method that could determine, in addition to the specific metabolites, also the generic metabolic profile, and that did not destroy the samples after being analyzed. The implementation of this method has allowed us to appreciate how the metabolic profile also changes based on the type of diet that is administered to the dog. This result provides further confirmation of the possibility of creating macro-categories of healthy subjects. An innovative study, on the other hand, was the comparison of the fecal microbiome with that of the blood in healthy animals. Firstly, the primary objective was the verification of the

presence of bacterial DNA in the blood of healthy dogs. Subsequently, another aim was the correlation of the intestinal microbiome with those of the blood. Unfortunately, the annotations were not overlapping, but in terms of beta diversity, both the fecal and the blood microbiome divided the subjects on the basis of the type of diet given to the animals. Finally, to conclude the results obtained so far, we wanted to do a network analysis on the database of healthy dogs analyzed at the beginning. This allowed a definition of the relationships existing between the microorganisms that make up the microbiome, and which relationships are significant based on the type of diet administered to dogs and their sex. This study provides a turning point in studies on the intestinal microbiome in dogs, establishing which relationships are useful for the definition of macro-categories of healthy subjects, and which are the threshold values of abundance of these key microorganisms for the definition of enterotype.

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List of Acronyms

AA: Absolute Abundance

AD: Acute uncomplicated Diarrhea

AHDS: Acute Hemorragic Diarrhea Syndrom

BARF: Bones and Raw Food

BCFA: Branched Chain Fatty Acids

CE: chronic enteropathies

CKD: Chronic Kidney Disease

CNS: Central Nervous System

DNA: Deoxyribonucleic acid

ENS: Enteric Nervous System

EPI: Exocrine Pancreatic Insufficiency

FOS: Fructooligosaccharides

GI: Gastrointestinal

IBD: Inflammatory Bowel Disease

LDA: Linear Discriminant Analysis

LEfSe: Linear Discriminant analysis Effect Size

NGS: Next Generation Sequencing

NMR: Nuclear Magnetic Resonance

PCA: Principal Component Analysis

PCoA: Principal Coordinate Analysis

PCR: Polymerase Chain Reaction

QIIME: Quantitative Insights Into Microbial Ecology

RA: Relative Abundance

RNA: Ribonucleic acid

SCFA: Short Chain Fatty Acids

SparCC: Sparse Correlations for Compositional data

CHAPTER 1

GENERAL INTRODUCTION

1.1 General concepts of pet food and nutrition

Cats and dogs belong to the Mammals class and Carnivora order. Even though, dogs are included in the superfamily of Canidae where it is possible to find animals with very different food habits, such as bears and racoons that are omnivores, and pandas that are herbivores. Cats belong to the huge family of Felidae, where all the species are strict carnivores. Although dogs are considered carnivores as well as cats, their evolution indicates a predilection on a natural omnivore diet, while the history of cats reveals a carnivore diet in all the evolutionary pathway of this animal.

The highly specialized diet of cats induced some specific adaptations on their metabolism, that are translate as peculiar nutritional needs. For this reason, cats cannot satisfy the nutritional requirements with a vegetable diet, but they need the consumption of animal proteins. Moreover, cats need to include the taurine in their diet, they are sensitive to the arginine lack and they are not able to transform beta-carotene into active vitamin A, and lastly, to convert tryptophane into niacin.

The firsts evidence of the differences between dogs and cats are visible along all the gastrointestinal (GI) tract, beginning from the mouth and ending with the anus, together with the physiology of digestion and absorption. In all the species, the mouth has the role of taking the food inside the organism, thus starting physical chewing and mixing food with saliva. The saliva acts as a lubricant, making easier the chewing and the swallowing. Dogs and cats often swallow large boluses of food without almost chewing them, thus they act differently than herbivores and ruminants, which completely chew the food. the examination of the teeth of dogs and cats reveals some substantial differences between the two animal species. They both have the same number of incisors and canines – six incisors and two canines in the upper and lower arch – but dog has a higher number of premolars (16 vs 10) and molars (10 vs 4) than the cat, and this allow the dog a more powerful ability to chew and crush food. This fact is an indicator that dogs can also chew feeds richer in vegetable fibers and are more omnivore than cats, since the cats' teeth are characteristics of animals that are strict carnivores (Robertson, Feldman, e Polonsky 1989). Dogs evolved differently than cats and they are able to eat a diet more omnivore.

Food passes from the mouth to the stomach via the esophagus. The mucosal cells lining the esophagus secrete mucus, which helps lubricate the bolus carried to the stomach. The stomach acts as a reservoir for food and allows for fractional rather than continuous feeding. Moreover, the beginning of the chemical digestion of the proteins is in the stomach, that also allows the mixing of the food with the gastric secretions and regulates the entry of food into the small intestine. Most digestive processes in dogs and cats that happened upstream of the small intestine are of mechanic and chemical nature. Proteins, carbohydrates, and digestible lipids are hydrolyzed to amino acids and dipeptides, monosaccharides and glycerol, free fatty acids, monoglycerides and diglycerides. These small units are adsorbed from the organism, together with dietary vitamins and minerals. The adsorption consists in the transfer of the nutritive substances from the intestinal lumen to the blood or lymphatic circulation, where they will be moved to all the tissues of the organism.

The dimension and the functional capability of the large intestine vary between the mammalian species. The cecum of the non-ruminant herbivores, such as horse and rabbit, is relatively bigger than the cecum of other animals, and it has a higher digestive ability. The large intestine and cecum of swine, omnivores animals, are of bigger dimension than those ones of strict carnivores. For example, cats have only a trace of the cecum and a shorter large intestine. Regarding dogs, their cecum is shorter than the one of swine, but is longer than the one of cats. This is concordant with the fact that dog is adapted at the consume of a more omnivore diet that the diet of cat.

The feces characteristics of cats and dogs can be influenced by the quantity and the type of indigestible substances that are included in the animal's diet. The bacterial "digestion" of those substances – due to the fermentative and metabolic activity of the taxa – produced a variety of gas, volatile fatty acids and other secondary products. In the end, when the indigestible proteins reach the large intestine, the microbial activity gives rise to the production of amino acid compound such as indole and scatole.

1.2 Gut microbiome of companion animals: characteristics and general description

Although "Microbiome" and "Microbiota" are nowadays used interchangeably, there is a slight difference between the meaning of these two terms. Microbiota is usually defined as the assemblage of living microorganisms present in a defined environment (Marchesi e Ravel 2015). Instead, the term microbiome includes the activity behind the community of microorganisms, that involves the whole spectrum of molecules produced by microorganisms, including the structural elements such as nucleic acids, proteins, lipids, and polysaccharides, and molecules produced by coexisting hosts and structured by surrounding environmental conditions (Burge 1988).

The microbiota, nowadays identified as a part of the gastrointestinal tract, is a complex microbial ecosystem consisting of all microorganisms that live in association with the organism (Ley et al. 2005; Marteau et al. 2004). The number of cells of the intestinal microbiota is much higher than that of the organism's prokaryotic cells and their genetic makeup is also significantly higher than the human and animal genome (Finegold, Sutter, e Mathisen 1983). It is estimated that the intestine of mammals contains 10^{10}-10^{14} microbial cells. An important number if we consider that it is equivalent to about 10 times the total number of host cells (Honneffer, Minamoto, e Suchodolski 2014).

In the last 30 years, numerous studies have focused on the qualitative and quantitative characterization of the microbiota and recently the development of sophisticated sequencing techniques has offered new information on the genetic heritage possessed by the microbiome, i.e., the set of genes (intended as coding sequences of DNA) that intestinal bacteria are able to express. Considering the profound impact of the microbiome on health, most of the studies available on the gastrointestinal microbiota and/or the functionality of the microbiome have focused primarily on humans and laboratory animals. The adoption of Next Generation Sequencing (NGS) technologies, instead of the classic plate culture methods, has improved the scientific knowledge on the bacterial diversity of the gastrointestinal tract in monogastric farm animals, allowing to effectively profile even anaerobic microorganisms or with different growth needs.

The study of the canine microbiome has also highlighted its similarities with humans, greater than those with rodents or pigs, and even greater than those with the wolf (Swanson et al., 2011; Lyu et al., 2018; Coelho et al., al., 2018). The sharing of environments is believed to have influenced the evolution of both species, with the canine microbiome co-evolving with the human one (Huang et al., 2019). Environmental factors also seem to have put a positive pressure on numerous genes in both species, mainly linked to digestive aspects (adaptability to dietary changes due to the consumption of common foods, genetic sets responsible for the digestion of starches and other complex carbohydrates), and neurological (overlapping of genes involved in neurological processes, which in humans show significant correlations with neurological pathologies) (Lyu et al., 2018; Saulnier et al., 2013).

Recent research has in fact shown that microbial populations change along the enteric tract also as a function of the environment and the available oxygen gradient, which decreases along the gastrointestinal tract. Thus, the small intestine is populated by aerobic and facultative anaerobic microorganisms, while the populations most represented in the cecum and in the descending colon are facultative or obligate anaerobic (Yadav et al., 2018; Pilla and Suchodolsky, 2020). Microbiome composition variations can occur along the GI tract, for physiological and environmental reasons, however it is important to note that key bacterial taxa are constantly present in the fecal samples of healthy dogs, indicating a bacterial core. Three phyla are always present in the gut microbiome of healthy dogs: Fusobacterium, Bacteroidetes and Firmicutes. Within this latter, many taxa are included. Bacterial class Clostridia is one of the major taxa that compose Firmicutes phylum, and it is dominated by Clostridium cluster IV, where we can find the family of Ruminococcaceae and specie of *Faecalibacterium prausnitzii*; Clostridium cluster XI, composed by the family of Peptostreptococcaceae; and Clostridium cluster XIVa, with taxa from Lachnospiraceae family and *Blautia* spp. (Vázquez-Baeza et al. 2016; Handl et al. 2011; Garcia-Mazcorro et al. 2012). Additional prevalent classes in the Firmicutes phylum are Bacilli and Erysipelotrichi. The former class is composed prevalently of Lactobacillaceae order, dominated by *Streptococcus* and *Lactobacillus* genera, whilst Erysipelotrichi comprises *Turicibacter*, *Catenibacterium* and *Coprobacillus* genera (Garcia-Mazcorro et al. 2012; 2011). In the Bacteroidetes phylum the bacteria *Prevotella*, *Bacteroides* and *Megamonas* genera (Hand et al. 2013; Garcia-Mazcorro et al. 2012) can be found. Within phylum Fusobacteria, genus *Fusobacterium* is the most present and it is associated with a healthy GI tract in dogs. An interesting fact is that in humans *Fusobacterium* is usually associated to gastrointestinal diseases, indicating a different role of this taxa in the GI tract of dogs (Vázquez-Baeza et al. 2016). In smaller amounts, it is possible to identify also bacteria from Proteobacteria and Actinobacteria phyla, especially colonizing the small intestine where a little amount of oxygen is available. The major components of the Proteobacteria phylum are taxa from the family Enterobacteriaceae (e.g., *Escherichia coli*); an increase of these bacteria is associated with the incidence of many diseases. Actinobacteria phylum includes families Corynebacteriaceae, where *Corynebacterium* spp. is part, and Coriobacteriaceae, that includes *Collinsella* spp. (J. B. Honneffer et al. 2017).

The research on the intestinal microbiota in pets (such as dogs and cats), shares more similarities with the human field, as these animals are now considered real members of the home family and consequently all the factors that potentially promote their longevity (including the microbiome) are topics of great interest. In livestock species, on the other hand, research on the intestinal

microbiota is a direct consequence of the primary objective of breeding, which is the optimization of food efficiency and the growth performance of animals.

Although a clear scientific definition of "intestinal health" is still lacking, some major components related to the identification of gastrointestinal well-being and function have recently been proposed, which include optimal digestion and absorption, immune status, intestinal mucosa, and neuroendocrine and motor function. A normal and stable microbiota plays a key role in animal health because the symbiotic balance between the intestinal tract and the microbiota is essential for maintaining their well-being (Hanning et al., 2015). One of the few recognized fundamental factors for a "good" microbiota is biodiversity. Biodiversity is a very well-known index borrowed from biology and microbiology. Alpha diversity is a measure of microbiome diversity applicable to a single sample, that defines the "wealth" of organisms in an ecosystem (in this case the enteric tract of the dog) in terms of number (richness) and also of distribution (evenness) of the organisms themselves. Beta diversity is a measure of similarity or dissimilarity between two microbial communities. Basically, it is important not only that they are very numerous (in order not to leave empty ecological niches), but also that they belong to many taxa (to prevent dysbiosis, often caused by the excessive growth of some over others) (Alcock et al., 2016; Vazques -Baeza et al., 2016).

This factor has proved important starting from several studies that have observed a decrease in biodiversity in dogs suffering from enteropathy, both idiopathic and on a dietary basis (Minamoto et al., 2015). The biodiversity data of the enteric microbiome therefore seems to be directly related to intestinal health.

1.3 The effect of diet on gut microbiome

The main effects that diet and food can have on the microbiome concern the richness of its composition (biodiversity), and the production of postbiotics more or less favorable to intestinal health, as short chain fatty acids (SCFA) and other compounds deriving from the bacterial digestion of different nutrients.

The main growth substrates of intestinal bacteria are nutrients that by-pass the digestion of the host because they are indigestible or not absorbed, which are digested by microorganisms into simpler molecules (mono and di-saccharides, amino acids and free fatty acids) intended for absorption by the microbiome itself, and nutrients that exceed the needs of the host and are not digested by bacteria. Once they reach the lower part of the gastrointestinal tract (cecum and colon), they undergo microbial fermentation with the production of postbiotics (Wernimont et al., 2020), which as previously described can have an important role both locally for the health of the intestinal environment and for the health of the host. The interaction between pet food, the GI microbiome, and the host is represented in Figure 1.

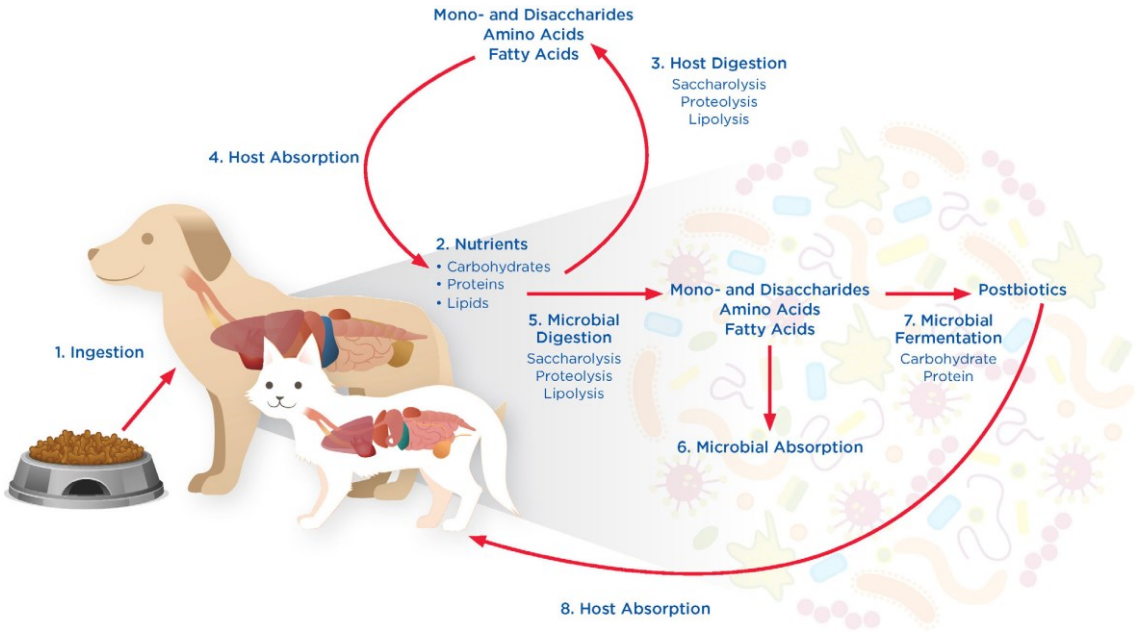

Figure 1. Summarization of the interactions between the diet, the gut microbiome and the host (Wernimont et al., 2020).

Nutrition clearly represents an important factor in influencing the composition of the microbiome and its metabolism, even if today there is no clear understanding of the effects caused by different food choices. The research, also stimulated by the growing interest in pet food other than extruded food, has therefore been oriented towards understanding the effect on the microbiome of dietary macronutrients such as proteins, carbohydrates, and fats but also of compounds that may have a direct activity on its profile and function, and therefore on the health of the host.

The composition of the gut microbiome is similar between cats and dogs, but some differences are noted, since the relationship between the bacterial community of GI tract and the host is certainly influenced by the anatomy and physiology of each species. In a study of Hall et al. (2018) dogs and cats were left in a situation where they could choose from a variety of foods of similar palatability, but with different macronutrient content. The results showed that the microbial postbiotics detected in cats were different from the microbial postbiotics detected in dogs. A specific example from this study was the production of equol in dogs and cats. Equol is an isoflavone-derived metabolite produced from the microbial metabolism of daidzein, and it is known that a high carbohydrate diet increases the production of equol (Vazquez et al., 2020). From the study described previously (Hall et al., 2018), it was observed an elevated level of circulating daidzein sulfate in cats, while equol sulfate level in plasma was not different between cats and dogs. This latter finding was interpreted as evidence of the different capacity that cat gut microbiome has in the production of equol, compared to that of the dog's microbiome.

The dietary factors that to date seem to have the greatest influence on the ecology of the intestinal microbiota of dogs and cats are the physical form of the diet (extruded or wet industrial feed, fresh feed, BARF or raw meat diet, etc.) and the overall digestibility of the diet, including the proportion between macronutrients, the qualities of the protein component (digestibility and

amino acid profile) and the characteristics of complex carbohydrates, especially the indigestible ones (fiber).

With the use of fresh diets or a share of fresh ingredients (in particular the component of animal origin, the main source of protein in the diet of carnivores such as dogs and cats), an increase in the biodiversity of the intestinal microbiome has been observed. Also, the profile of microbial metabolites (volatile fatty acids, branched volatile fatty acids, products of putrefactive metabolism) changes between moist or extruded industrial foods (Kim et al., 2017; Sandri et al., 2017; Scarsella et al., 2020). It is interesting to note that these effects seem to be only partially dependent on the type of meat used, but rather related to the levels of macro and micronutrients, and that they are reversible when the period of use is short with the return to an industrial food (Herstad et al., 2017; Sandri et al., 2017).

The degree of digestibility of the diet determines both the amount of digested and absorbed nutrients of the dog and cat's and the amount undigested or in excess reaches the large bowel colonized by a large microbial population that can use it as nutritional substrate.

In the study by Mori et al. (2019) a comparison of the gut microbial population of diets with different levels of protein, carbohydrates and fats in healthy and obese dogs has been performed, observing how the most significant differences derived from the level of fiber and the live weight of the subject (obese *Vs* normal weight). In particular, dogs that ate a diet for body weight loss (high level of protein and fiber, medium digestible carbohydrates and low-fat content) showed a significant reduction in Actinobacteria and Firmicutes phylum and an increase in Fusobacteria phyla compared to those fed a hydrolyzed diet (medium level of hydrolyzed protein and digestible carbohydrates, high level of fat and reduced fiber). However, dietary interventions do not always have significant effects on the microbiome (Bresciani et al., 2018; Pilla et al., 2019). In fact, it seems that diets with different compositions but with similar chemical analysis have minimal effects on the composition of its gut microbiome in dogs (Huang et al., 2019).

Complex carbohydrates in the diets of dogs and cats provide energy (digestible starch) but also an amount of indigestible fiber. These fibers, considering their effect in the intestinal environment of monogastrics, are normally classified on the basis of solubility/insolubility and fermentability. Beet pulp (mixture of soluble and insoluble fiber) and cellulose (insoluble, non-fermentable, non-viscous) are often found as sources of fiber, but also vegetable macro-ingredients such as corn, rice, potatoes, oats, and barley, which also contain amounts of resistant starch and soluble fiber available for the growth of intestinal bacteria (De Godoy et al., 2013). These characteristics make the fibrous and indigestible compounds of great interest due to their potential for modulating the microbiome, even independently of the other components of the diet.

For instance, Yang et al. (2017) evaluated the effect of resistant or non-absorbed starch in the small intestine; in another study, the increase of the potato fiber inclusion (containing both digestible and resistant starch) resulted in an increase of Faecalibacterium in the microbiome of healthy dogs (Panasevich et al., 2015), that is a SCFA producing genus, member of the Ruminococcoaceae family (Phylum Firmicutes). Faecalibacterium is considered positive for intestinal health, being its presence reduced in dogs suffering from inflammatory pathology of the GI tract (Suchodolsky et al., 2015).

A diet based on fresh raw meat with different starch sources (rice vs potato) and different levels of rapidly digestible starch and resistant starch in healthy dogs showed no obvious shifts in bacterial populations, but differences in the production of postbiotics important for intestinal health, such as the increase in the production of lactic acid with a reduction of fecal pH and concentration of ammonia in the feces, confirming that there is also an individual factor in the gut microbiome and, especially in elderly subjects, a certain resilience of the microbiome itself against moderate changes in the diet (Sandri et al., 2020).

The addition of a mixture of soluble and insoluble fibers to diets with different compositions (high content of cereals vs hydrolyzed meat) has shown the production of very similar postbiotics, albeit conditioned by the starting microbial profile, the improvement of the quality of the feces with decrease of pH and the increase of microbial populations considered positive for intestinal health (Jackson and Jewell, 2018).

There are now numerous studies in dogs on the effect of fibers to the gut microbiome, but in the cat the information is still rather limited and the feline gut microbiome, also given the food ecology as a strict carnivore status, seems to be more stable to dietary supplements. Indeed, Barry et al. (2012), albeit with a very limited sample (4 cats), did not report relevant differences in response to 3 different diets supplemented with cellulose, fructooligosaccharides (FOS) or pectin. As for the protein component of the diet, its quality (digestibility and completeness of the amino acid profile with respect to the needs of dogs and cats), quantity and weight in the energy distribution (ratio compared to other foods that provide energy, such as carbohydrates) are important factors for host metabolism as well as for its microbiome, as undigested and absorbed nutrients are available for microbial metabolism in the lower digestive tract (Wernimont et al., 2020). There is various evidence that diets based on fresh raw meat, often characterized by higher levels of protein and fat than extruded foods contribute in both dogs and cats to modify the composition of intestinal bacterial populations (Herstad et al., 2017; Kim et al., 2017; Sandri et al., 2017; Algya et al., 2018; Butowsky et al., 2019), which are even more rich and diversified (α and β biodiversity) (Kim et al., 2017; Sandri et al., 2017), although fresh meat represents a potential source of pathogens and therefore an infectious risk (Freeman et al. 2013)

The products of bacterial metabolism of proteins are mostly putrefactive compounds (polyamines such as putrescine and cadaverine, short-chain branched fatty acids, hydrogen sulphide and uremic toxins) deriving from the decomposition of amino acids (Wernimont et al., 2020). Some of these compounds have been associated in humans with the pathogenesis and progression of some inflammatory diseases such as atopy (Nylund et al., 2015), chronic renal failure (Niwa et al., 1997), and chronic enteritis (Nikolaus et al., 2017). What emerges from these observations is that the amount of protein that bypasses the digestion of the host, in the form of nitrogenous residues (e.g. ammonia and urea) available for the metabolism of the microbiome basically depends on the amount of total protein, on its weight in the energy distribution and its quality, understood as digestibility and biological value (amino acid profile) (Wernimont et al., 2020).

The effect of fats, and their concentration in the diet, on the gut microbial population is still partially known in dogs and cats. In humans and mice, diets high in fat (45-60% of the daily energy breakdown) have led to a profound and rapid shift of the populations of the microbiome, a reduction in the concentration of beneficial postbiotics (SCFA) and an increase in putrefactive

and inflammatory compounds, particularly in obese subjects (Martinez et al., 2017; Shen et al., 2014).

In this sense, it is conceivable that the pro-inflammatory nature of dietary lipids can influence the composition of the microbiome through the alteration of the host-microbiome immune-mediated homeostasis (Wernimont et al., 2020) rather than through a direct action on bacterial metabolism. Further studies are needed to better understand these mechanisms, as well as the potential prebiotic effects of various fatty acids derived from dietary lipids, which seem to positively influence the production of postbiotics and therefore the health of the intestinal environment.

1.4 The role of microbiome on the health status of the host

The gut microbiome, when it is in equilibrium, beneficially influenced the host health, by modulation of the immune system, defense against intestinal pathogens, and providing vitamins and nutrients that are necessary to a good maintenance of the host health (Suchodolski 2021).

The alterations that could happened at compositional level in the gut microbiota are defined as gut dysbiosis. These modifications usually result in functional changes in the microbial transcriptome, proteome or metabolome (Zeng, Inohara, e Nuñez 2017). Studies in companion animals showed that these changes, both in microbial composition and/or function (Janeczko et al. 2008; Blake et al. 2019), are also associated with pathologies not related to the GI tract, such as chronic kidney disease (CKD) (Summers et al. 2019), heart disease (Li, Larouche-Lebel, et al. 2021; Seo et al. 2020; Li, Larouche-Lebel, et al. 2021), neurological disorders (Jeffery et al. 2017), diabetes mellitus (Kieler et al. 2019) and obesity (Sanchez et al. 2020).

Most research on gut microbiome of companion animals focused on the fecal microbiome, since feces are easy to collect, and the sampling is not invasive and do not bring suffer to the animals. Although these positive aspects on the analysis of the fecal microbiome, feces do not provide a complete information regarding the presence of mucosa-adherent or entero-invasive bacteria, or the composition of the small intestine microbiota. This latter can give signs of a pathological status of the host, even with a normal composition. The cause is due several reasons, such as feeding diets with poor digestibility, inflammatory condition that leads to a damage of the transporters in the epithelial brush border (Honneffer et al. 2015; Giaretta et al. 2018), and in the end, the lack of digestive enzymes in subjects with exocrine pancreatic insufficiency (EPI) (Westermarck e Wiberg 2006).

One of the responsible for the changes in the microbiota composition might be oxygen (Rigottier-Gois 2013). The availability of this element can increase in the intestinal lumen in situations where there is an increase of the gut permeability; one of those situations could be the inflammation (Rivera-Chávez, Lopez, e Bäumler 2017). The presence of a high level of free oxygen can impact the microbial population of the strict anaerobe, inducing an uncontrolled growth of the facultative anaerobes, like bacteria from the Enterobacteriaceae family (Zeng, Inohara, e Nuñez 2017).

The gut microbiota is highly diverse in terms of composition and phylogeny. Moreover, similar bacteria could express different genes, based on the gut environment, and thus have different metabolic function. One of the factors that can influence the potential virulence of bacteria is the

concentration of metabolites in the intestinal lumen. For instance, norepinephrine, a stress hormone, can induce *Salmonella* to express genes that cause enteritis (Pullinger et al. 2010), as well as the change in the ratios of the SCFAs butyrate, propionate, and acetate (Lawhon et al. 2002). The differences in the SCFA ratio between ileum and colon can influence the growth rate of *Escherichia coli* (Zhang et al. 2020). The metabolism of bile acids is also an important bacterial-derived metabolic pathway, and when it undergoes to a disruption, an overgrowth of potential enteropathogens could happen. A dysbiotic microbiome means also that there could be a decrease in bacteria that are able to convert primary to secondary acids, that leads to an overgrowth of *Clostridium difficile* in the colon tract (Weingarden et al. 2014). On the other hand, *Clostridium hiranonis*, one of the main converters of primary to secondary bile acids in dogs, decreased when a dysbiosis occurs. This situation could be induced by broad-spectrum antibiotics (Chaitman et al. 2020; Pilla et al. 2020) and it is often present during chronic inflammatory enteropathies (Blake et al. 2019; Giaretta et al. 2018; Wang et al. 2019). Another key player of the gut dysbiosis is *Faecalibacterium prausnitzii*, a SCFA and anti-inflammatory peptides producer; indeed, it is usually decreased during an ongoing of canine and feline diseases (Giaretta et al. 2018; Pilla et al. 2020; Suchodolski et al. 2015).

Acute uncomplicated diarrhea (AD) is a disease the leads to a strong dysbiosis of the gut microbiome. The alterations interest not only the GI microbial population, but also blood and urine metabolites, that means that this condition has an impact on the overall metabolic profile of the host. Dogs with acute hemorrhagic diarrhea syndrome (AHDS) showed similar alteration at the GI microbial population level and metabolites presence. Compared to healthy dogs, both subjects having AD and AHDS have a low level of Ruminococcaceae and *Faecalibacterium* spp. Some studies highlighted a correlation between the presence of the netF gene in fecal samples of dogs with AHDS and Clostridium perfringens, since the netF toxin gene has been found in the genome of C. perfringens isolated from intestinal biopsies of AHDS dogs (Leipig-Rudolph et al. 2018; Sarwar et al. 2018). Moreover, the recovery from AHDS leads to a decrease on netF gene and abundance of C. perfringens (Ziese et al. 2018).

Another pathology that has been found to induce similar alterations in gut microbiome, like for the case of AD and AHDS, is the development of chronic enteropathies (CE). Examples of the induced modification include dysbiosis and the decrease of abundances of bacteria that are SCFA producers (Suchodolski et al. 2015; Guard et al. 2015; Minamoto et al. 2019). Dogs CE are classified in different ways, such as food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), and immunosuppressant-responsive diarrhea, also known as idiopathic inflammatory bowel disease (IBD). Dogs with CE, even if derived from different responses, have a similar dysbiotic microbiome (Suchodolski et al. 2012), and a significantly decreased fecal bacterial diversity (Minamoto et al. 2019; 2015).

Weaning is a critical moment in the puppy's life and is often associated with the development of intestinal dysbiosis and diarrhea often treated with antibiotics. Early exposure to antibiotics in puppies has been shown to influence the intestinal microbiota (in terms of decreased *Lactobacillus* and selection of opportunistic pathogens such as *Streptococcus*, and *Corynebacterium*) and the metabolism of carbohydrates and proteins, in terms of decreased lactate and SCFA production and increased concentrations of branched-chain fatty acids (BCFA), such as putrescine, cadaverine, spermidine and spermine (Vàsquez-Baeza et al., 2016; Minamoto

et al., 2019; German et al., 2003 ; Honneffer et al., 2014). Manipulation of the gut microbiota through probiotics and prebiotics has shown great potential for treating a wide range of gut diseases or for partially reversing antibiotic-related dysbiosis. The growing volume of genomic information is accelerating the discovery of new probiotic strains, as well as the development of food-derived postbiotics useful for the treatment of dysbiosis.

1.5 The brain-gut-microbiome axis

Recently, several researches in the human and veterinary fields have shown that the intestinal microbiota can influence the physiological and cognitive functions of the brain by producing a broad range of active substances such as hormones, immune factors and metabolites. Close communication between intestinal bacteria and the central nervous system is facilitated by communication via the enteric nervous system (ENS) (Wernimont et al., 2020). The ENS is part of the autonomic nervous system and it is represented by neurons (grouped in ganglia) that are embedded in the lining of the gastrointestinal system wall. The ENS is connected to the central nervous system through a two-way communication: the gut-brain axis. More precisely, the communication between the intestine and the brain occurs through different pathways: neuronal, endocrine, immune and metabolic.

Stress, processed by the brain, modulates the gastrointestinal function and, vice versa, the signals coming from the enteric nervous system regulate the behavior and emotional responses of the animal (Baj et al., 2019). Studies conducted over the past decade have shown that gut bacteria can influence neuronal development and affect behavior.

The microbiota uses many channels to communicate with the central nervous system (CNS), including:

- The sympathetic nervous system and the hypothalamus-pituitary-adrenal axis (HPA). Recent studies (O'Mahony et al. 2015; Kennedy et al. 2014; Forsythe, Kunze, e Bienenstock 2016)have shown that the gut microbiota can influence the development of systems that govern the endocrine response to stress. The release of adrenaline and noradrenaline from the neuroendocrine portion of the adrenal glands leads to fight or fly behavioral responses and can also impair intestinal motility. Same goes for the secretion of cortisol.

- The immune signal: scientific evidence (Shi et al. 2017) shows that the intestinal microbiota influences the immune cells located in the intestinal mucosa. These immune cells release some mediating molecules such as cytokines, molecules important in the host's responses to inflammatory and infectious phenomena.

- The production of intestinal bacterial metabolites: through the fermentation and metabolism of indigestible fibers (such as some substances with prebiotic action), the intestinal microbiota produces SCFAs, that are particularly important metabolites capable of influencing brain function (Forsythe, Kunze, e Bienenstock 2016)

- The production of hormones and neurotransmitters, including dopamine and serotonin: as previously described, most of the data on microbiota-gut-brain interactions have been recently acquired, using laboratory animals, more specifically, germ-free mice and rats (McVey Neufeld

et al. 2015; Torres et al. 2020; Turnbaugh et al. 2009). On the other hand, intestinal dysbiosis is often associated with gastrointestinal and metabolic diseases and inflammatory phenomena limited - and not - to the intestinal mucosa (Kirchoff et al., 2019).

Tryptophan is an essential amino acid and is usually found in two forms: bound to albumin or in free form. Moreover, tryptophan is a precursor of other 2 metabolic pathways, the kynurenine and the serotonin pathway (Fernstrom 1983; Marsh et al. 2002). About 90% of the catabolism happened through the kynurenine pathway (Dougherty et al. 2008), meanwhile only the 3% of tryptophan from diet is used for serotonin synthesis (Richard et al. 2009). In addition to protein, kynurenine, and serotonin synthesis, tryptophan is also used for the production of tryptamine, which is a neuromodulator of serotonin (R. S. G. Jones 1982).

Interesting studies point out that many of the effects of the dysbiotic gut microbiota can be restored by exposing germ free mice to normal microbiota development during their early life, but not later. This fact confirms the existence of a critical time window at the beginning of life. for regular development. of the gut-brain axis (Wallis et al., 2020). In another study (Qu, Liu, e Miao 2021), offspring from mothers exposed to antibiotics during pregnancy show a reduction in social interactions, a reduced exploration of the environment and atypical behaviors.

Furthermore, a recent study in adult rats found that antibiotic-induced intestinal dysbiosis leads to deficits in spatial memory, increased visceral sensitivity, depressive behaviors, and alterations in serotonin in the central nervous system and in the receptors of the hypothalamus-pituitary-adrenal axis. Probiotics, on the other hand, are able to influence the hypothalamus-pituitary-adrenal axis and the behavior of animals. Diets supplemented with *Lactobacillus* spp or *Bifidobacterium* spp resulted in lesser memory and learning deficits, less intestinal pain and decreased behavior related to anxiety and depression (Durack et al., 2019).

1.6 The importance of Metabolome related to gut microbiome

The evaluation of the microbiome as in a "normal" status has been assessed with a variety of metrics, including analysis that highlighted both the composition and the functionality of the microbiome (Bäckhed et al. 2012). The use of metrics to analyze the microbiome function in order to characterize a healthy status of the host is increasing, even if these types of analysis do not come without limitations. One examples of these metrics is the predicted metabolic capacity, which utilizes both the imputed gene makeup of the bacteria composing the microbiome and metabolite profiles (Langille et al. 2013).

Since dogs and cats are becoming always more a fundamental family member, the expectation of a high quality veterinary care has become a standard (Holbrook 2008). The importance of the metabolomics approaches in veterinary and animal production research is increasing, and this evidence is particularly true when these methods are used to evaluate the chemical composition of different diets and to answer questions related to different functional responses based on dietary intervention (Kirwan 2013; Jones e Cheung 2007; Whitfield, German, e Noble 2004).

Metabolomics is a branch of the "omics" sciences that directly connects genomics and proteomics to the phenotype of the organism and its biochemical information (Jones et al. 2014). Metabolome analysis includes two main definitions, namely metabolomics and metabonomics.

Metabolomics is defined as the study of the total metabolites (small molecules < 1500 Da) in a sample (Lindon, Holmes, e Nicholson 2001). Metabonomics is the quantitative measure of metabolites in response to changing factors such as environment, diet, diseases, therapeutic intervention, or toxicity (Nicholson, Lindon, e Holmes 1999).

The microbial population of the GI tract produces bacterial metabolites, called "postbiotics", that can affect the host in several ways (Tsilingiri et al. 2012; Ojeda et al. 2016). Postbiotics are the products derived from the metabolic activity of bacteria. Regarding the nutritional field of study, postbiotics are generated from undigested food that bypass the colon tract to become available to the microorganisms (Wernimont et al. 2020). Rather than be only compounds derived from carbohydrates, fat, and protein, postbiotics are also microbial metabolites derived, for example, from secondary plant compounds. These metabolites may have a beneficial or negative effect on the host.

1.7 Tools and techniques for microbiome analysis

Metagenomic studies on the gut microbiome of any mammal species can be summarized in four steps:

1. Sampling collection, mostly fecal material, since it is easy to collect and manage.
2. DNA extraction for metagenomic.
3. DNA sequencing.
4. Bioinformatics analysis of the data.

These steps are visualized graphically in Figure 2.

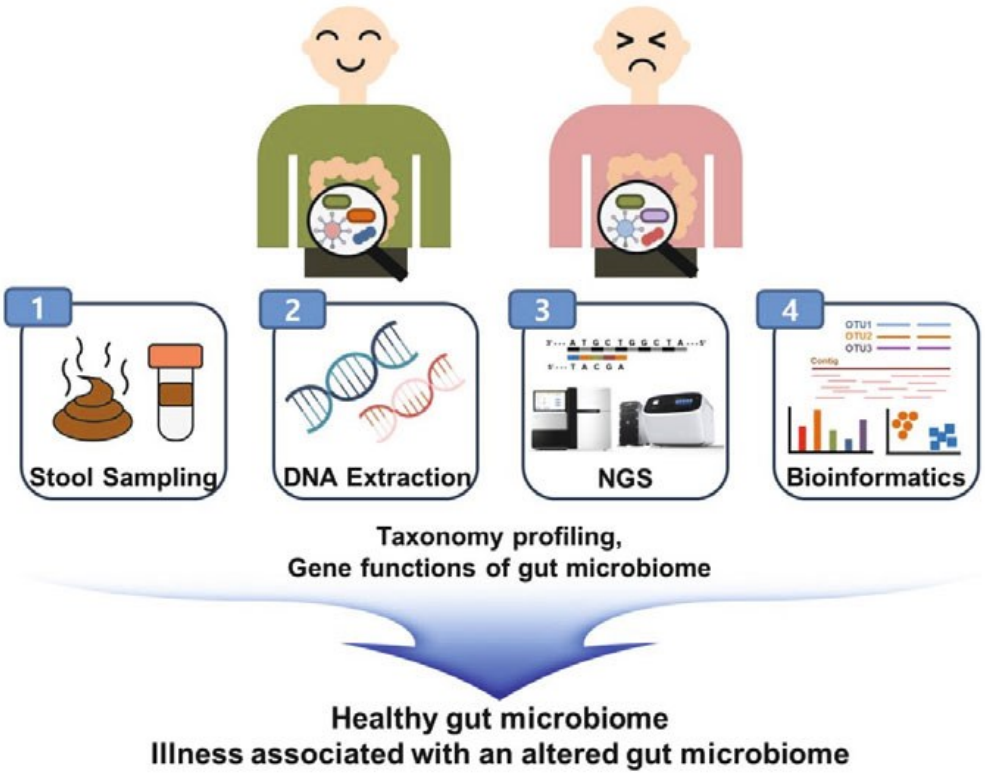

Figure 2. Overview of the gut microbiome analysis (Song, Lee, e Nam 2018).

Each step of a metagenomic study can bring errors and bias due to different methods that can be applied. The lack of a methodology standardization arises to a variety of results, putting studies one against each other since most of them are not concordant.

The yield and the quality of microbial DNA extracted from fecal samples are consistently affected by the method of the fecal sampling used. Environmental enzymes such as DNase can affect the genomic stability of the bacteria composing the microbial population in the sample; this evidence can induce a bias during the identification of the gut microbiota profile (Bag et al., 2016). The golden way is to extract metagenomic DNA as soon as the fecal material arrives in the laboratory; otherwise, the sample should be frozen immediately and stored until analysis (Rochelle et al., 1994). The most recommended protocol is to immediately freeze the sample in dry ice or in liquid nitrogen and storing at -80°C until DNA extraction (The HMP consortium, 2012). Attempts have been made to find a system to store fecal material at room temperature. One study demonstrated that stool samples can be collected with sterile cotton swabs and stored for two weeks in a range of temperature from -80°C to 20°C (Lauber et al., 2010). However, this method includes a high risk of skin bacteria contamination and the small amount of fecal material taken is not sufficient for analyzing the metagenome (Budding et al., 2014). Other methods involved the use of preservatives such as ethanol, EDTA buffers, commercial reagents such as RNA-later, OMNIgene GUT, FTA cards and DNA/RNA shield.

The next critical point in the gut microbiome analysis is the DNA extraction from the fecal material. Since there are several differences among the diverse bacterial species, especially those regarding the cell membrane and the cell wall structure, the several DNA extraction methods existing can bring to different outputs, thus producing erroneous profiles of the microbial community (Smith et al., 2011; Maukonen et al., 2012). Recent investigations highlighted an improvement of the extraction efficiency with the use of bead beating DNA extraction method, since the cell walls of Gram-positive bacteria is effectively disrupted (Bag et al., 2016; Lim et al., 2018).

The DNA sequencing is usually performed with Next Generation Sequencing (NGS) techniques. One of the most known first-generation techniques known from all the scientific community is the Sanger technique, that used chemically modified nucleotides called dideoxynucleotides (dNTPs). The Sanger sequencing was largely used for numerous bug sequencing projects, such as the Human Genome Project, that is also the most famous one (International Human Genome Sequencing Consortium, 2001), but has several limitations of speed and cost, since it is a low-throughput technique. NGS methods have the advantage of being high-throughput, having high speed of process, and relatively low cost (Kchouk et al., 2017). The main characteristic of these techniques is the use of massive parallel sequencing systems. The most common platforms used are from Roche, ABI, Illumina and Ion Torrent. The differences of these platform are compared in Table 1.

Table 1. Comparison of different sequencing platform.

Platform	*Reads per run*	*Reads length*	*Read type*	*Advantages*	*Disadvantages*	*Application*
454 GS FLX	1 M	600	SE, PE	Long read length; short run time	Expensive; Low throughput; high error rate (1%); no longer available	16S amplicon
SOLiD	3 B	75	SE	High accuracy	Expensive; long run time	16 amplicon
Ion Torrent PGM/ Proton	4 M – 5.5 M / 60 M – 80 M	200, 400 / 200	SE	Short run time; low cost; different chips available	High error rate (1%)	16S amplicon / Shotgun
Illumina MiSeq	250 M	300	Se, PE	High throughput; low cost	Short read length; long run time	16S amplicon; Targeted gene
Pacbio RS II (P6-C4)	50 K	10-15 K		Single molecule real-time sequencer; short run time; long read length	High error rate (10-15%); lack of application to meta-analysis	16S amplicon; Shotgun; transcriptome
MinION	Variable	Variable		Single molecule real-time sequencer; small size instrument; low capital cost	High error rate (5-15%)	16S amplicon; targeted gene; shotgun; transcriptome

During these years, specific bioinformatic tools have been developed for the computational analysis of the microbiome. Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Bolyen et al. 2019), Mothur (Schloss et al., 2009), and the Metagenomic Rapid Annotation using Subsystem Technology (MG-RAST) server (Meyer et al., 2008) are the most popular open-source bioinformatics pipelines available to perform microbiome analysis from raw sequencing data. QIIME2 and MG-RAST usually adopt UCLUST algorithm to perform the Operational Taxonomic Units (OTUs) clustering (Edgar, 2010), while Mothur use its own algorithm based on nearest neighbor, furthest neighbor, and average neighbor distances (Schloss et al., 2009). QIIME2 has the advantage to minimize the loss of information during the OUT picking, by

clustering several times reads that have not been assigned to the reference database. QIIME2 and Mothur use the command line usage, whilst MG-RAST has its own website with a graphical user interface.

The investigation of the significant differences in the microbial composition of different groups is performed with numerous techniques. One of the most known and used method is UniFrac (Lozupone et al., 2006), that is a distance metric, and the Bray-Curtis dissimilarity measure (Bray and Curtis, 1957), that estimates the phylogenetic distance between taxa. Other statistical methods used regard the multidimensional scaling; in this category are included the Principal Co-ordinates Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS) (Gower, 1966; Shepard, 1966). Another widely used tool is the Linear discriminant analysis Effect Size (LEfSe) that detects the differential abundances of taxa related to the features with a non-parametric Kruskal-Wallis rank sum test (Segata et al., 2011). Information about the biological functions of the microbiome is possible to gain also with 16S data thanks to a recent tool called Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013).

1.8 References

Alcock, Joe, Carlo C. Maley, e C. Athena Aktipis. 2014. «Is Eating Behavior Manipulated by the Gastrointestinal Microbiota? Evolutionary Pressures and Potential Mechanisms». *BioEssays* 36 (10): 940–49. https://doi.org/10.1002/bies.201400071.

Algya, Kiley M, Tzu-Wen L Cross, Kristen N Leuck, Megan E Kastner, Toshiro Baba, Lynn Lye, Maria R C de Godoy, e Kelly S Swanson. 2018. «Apparent total-tract macronutrient digestibility, serum chemistry, urinalysis, and fecal characteristics, metabolites and microbiota of adult dogs fed extruded, mildly cooked, and raw diets1». *Journal of Animal Science* 96 (9): 3670–83. https://doi.org/10.1093/jas/sky235.

Bäckhed, Fredrik, Claire M. Fraser, Yehuda Ringel, Mary Ellen Sanders, R. Balfour Sartor, Philip M. Sherman, James Versalovic, Vincent Young, e B. Brett Finlay. 2012. «Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications». *Cell Host & Microbe* 12 (5): 611–22. https://doi.org/10.1016/j.chom.2012.10.012.

Bag, Satyabrata, Bipasa Saha, Ojasvi Mehta, D. Anbumani, Naveen Kumar, Mayanka Dayal, Archana Pant, et al. 2016. «An Improved Method for High Quality Metagenomics DNA Extraction from Human and Environmental Samples». *Scientific Reports* 6 (1): 26775. https://doi.org/10.1038/srep26775.

Baj, Andreina, Elisabetta Moro, Michela Bistoletti, Viviana Orlandi, Francesca Crema, e Cristina Giaroni. 2019. «Glutamatergic Signaling Along The Microbiota-Gut-Brain Axis». *International Journal of Molecular Sciences* 20 (6): 1482. https://doi.org/10.3390/ijms20061482.

Barry, Kathleen A., Ingmar S. Middelbos, Brittany M. Vester Boler, Scot E. Dowd, Jan S. Suchodolski, Bernard Henrissat, Pedro M. Coutinho, Bryan A. White, George C. Fahey, e Kelly S. Swanson. 2012. «Effects of Dietary Fiber on the Feline Gastrointestinal Metagenome». *Journal of Proteome Research* 11 (12): 5924–33. https://doi.org/10.1021/pr3006809.

Beckonert, Olaf, Hector C. Keun, Timothy M. D. Ebbels, Jacob Bundy, Elaine Holmes, John C. Lindon, e Jeremy K. Nicholson. 2007. «Metabolic Profiling, Metabolomic and Metabonomic Procedures for NMR Spectroscopy of Urine, Plasma, Serum and Tissue Extracts». *Nature Protocols* 2 (11): 2692–2703. https://doi.org/10.1038/nprot.2007.376.

Blake, Amanda B., Blake C. Guard, Julia B. Honneffer, Jonathan A. Lidbury, Jörg M. Steiner, e Jan S. Suchodolski. 2019. «Altered Microbiota, Fecal Lactate, and Fecal Bile Acids in Dogs with Gastrointestinal Disease». *PLOS ONE* 14 (10): e0224454. https://doi.org/10.1371/journal.pone.0224454.

Bolyen, Evan, Jai Ram Rideout, Matthew R. Dillon, Nicholas A. Bokulich, Christian C. Abnet, Gabriel A. Al-Ghalith, Harriet Alexander, et al. 2019. «Reproducible, Interactive,

Scalable and Extensible Microbiome Data Science Using QIIME 2». *Nature Biotechnology* 37 (8): 852–57. https://doi.org/10.1038/s41587-019-0209-9.

Bray, J Roger, e John T Curtis. 1957. «An ordination of the upland forest communities of southern Wisconsin». *Ecological monographs* 27 (4): 326–349.

Bresciani, Francesca, Yasushi Minamoto, Jan S. Suchodolski, Giorgia Galiazzo, Carla G. Vecchiato, Carlo Pinna, Giacomo Biagi, e Marco Pietra. 2018. «Effect of an Extruded Animal Protein-Free Diet on Fecal Microbiota of Dogs with Food-Responsive Enteropathy». *Journal of Veterinary Internal Medicine* 32 (6): 1903–10. https://doi.org/10.1111/jvim.15227.

Budding, Andries E., Matthijs E. Grasman, Anat Eck, Johannes A. Bogaards, Christina M. J. E. Vandenbroucke-Grauls, Adriaan A. van Bodegraven, e Paul H. M. Savelkoul. 2014. «Rectal Swabs for Analysis of the Intestinal Microbiota». *PLOS ONE* 9 (7): e101344. https://doi.org/10.1371/journal.pone.0101344.

Burge, MN. 1988. *Fungi in biological control systems*. Manchester University Press.

Butowski, Christina F., David G. Thomas, Wayne Young, Nick J. Cave, Catherine M. McKenzie, Douglas I. Rosendale, e Emma N. Bermingham. 2019. «Addition of Plant Dietary Fibre to a Raw Red Meat High Protein, High Fat Diet, Alters the Faecal Bacteriome and Organic Acid Profiles of the Domestic Cat (Felis Catus)». *PLOS ONE* 14 (5): e0216072. https://doi.org/10.1371/journal.pone.0216072.

Chaitman, Jennifer, Anna-Lena Ziese, Rachel Pilla, Yasushi Minamoto, Amanda B. Blake, Blake C. Guard, Anitha Isaiah, et al. 2020. «Fecal Microbial and Metabolic Profiles in Dogs With Acute Diarrhea Receiving Either Fecal Microbiota Transplantation or Oral Metronidazole». *Frontiers in Veterinary Science* 7: 192. https://doi.org/10.3389/fvets.2020.00192.

Coelho, Luis Pedro, Jens Roat Kultima, Paul Igor Costea, Coralie Fournier, Yuanlong Pan, Gail Czarnecki-Maulden, Matthew Robert Hayward, et al. 2018. «Similarity of the dog and human gut microbiomes in gene content and response to diet». *Microbiome* 6 (1): 72. https://doi.org/10.1186/s40168-018-0450-3.

De Godoy, Maria R. C., Katherine R. Kerr, e Jr Fahey. 2013. «Alternative Dietary Fiber Sources in Companion Animal Nutrition». *Nutrients* 5 (8): 3099–3117. https://doi.org/10.3390/nu5083099.

Dougherty, Donald M., Dawn M. Marsh-Richard, Charles W. Mathias, Ashley J. Hood, Merideth A. Addicott, F. Gerard Moeller, Christopher J. Morgan, e Abdulla A.-B. Badawy. 2008. «Comparison of 50- and 100-g l-Tryptophan Depletion and Loading Formulations for Altering 5-HT Synthesis: Pharmacokinetics, Side Effects, and Mood States». *Psychopharmacology* 198 (3): 431. https://doi.org/10.1007/s00213-008-1163-2.

Durack, Juliana, e Susan V. Lynch. 2018. «The gut microbiome: Relationships with disease and opportunities for therapy». *Journal of Experimental Medicine* 216 (1): 20–40. https://doi.org/10.1084/jem.20180448.

Edgar, Robert C. 2010. «Search and clustering orders of magnitude faster than BLAST». *Bioinformatics* 26 (19): 2460–61. https://doi.org/10.1093/bioinformatics/btq461.

Emwas, Abdul-Hamid, Claudio Luchinat, Paola Turano, Leonardo Tenori, Raja Roy, Reza M. Salek, Danielle Ryan, et al. 2015. «Standardizing the Experimental Conditions for Using Urine in NMR-Based Metabolomic Studies with a Particular Focus on Diagnostic Studies: A Review». *Metabolomics* 11 (4): 872–94. https://doi.org/10.1007/s11306-014-0746-7.

Fernstrom, J D. 1983. «Role of precursor availability in control of monoamine biosynthesis in brain.» *Physiological Reviews* 63 (2): 484–546. https://doi.org/10.1152/physrev.1983.63.2.484.

Finegold, S.M., V.L. Sutter, e G.E. Mathisen. 1983. «Normal indigenous intestinal flora». In *Human intestinal microflora in health and disease*, 1:3–31.

Forsythe, Paul, Wolfgang Kunze, e John Bienenstock. 2016. «Moody microbes or fecal phrenology: what do we know about the microbiota-gut-brain axis?» *BMC Medicine* 14 (1): 58. https://doi.org/10.1186/s12916-016-0604-8.

Freeman, Lisa M., Marjorie L. Chandler, Beth A. Hamper, e Lisa P. Weeth. 2013. «Current knowledge about the risks and benefits of raw meat–based diets for dogs and cats». *Journal of the American Veterinary Medical Association* 243 (11): 1549–58. https://doi.org/10.2460/javma.243.11.1549.

Garcia-Mazcorro, Jose F., Scot E. Dowd, Jeffrey Poulsen, Jörg M. Steiner, e Jan S. Suchodolski. 2012. «Abundance and Short-Term Temporal Variability of Fecal Microbiota in Healthy Dogs». *MicrobiologyOpen* 1 (3): 340–47. https://doi.org/10.1002/mbo3.36.

Garcia-Mazcorro, Jose F., David J. Lanerie, Scot E. Dowd, Casey G. Paddock, Niels Grützner, Jörg M. Steiner, Renata Ivanek, e Jan S. Suchodolski. 2011. «Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing». *FEMS Microbiology Ecology* 78 (3): 542–54. https://doi.org/10.1111/j.1574-6941.2011.01185.x.

German, A.j., E.j. Hall, e M.j. Day. 2003. «Chronic Intestinal Inflammation and Intestinal Disease in Dogs». *Journal of Veterinary Internal Medicine* 17 (1): 8–20. https://doi.org/10.1111/j.1939-1676.2003.tb01318.x.

Giaretta, Paula R., Raquel R. Rech, Blake C. Guard, Amanda B. Blake, Anna K. Blick, Jörg M. Steiner, Jonathan A. Lidbury, et al. 2018. «Comparison of Intestinal Expression of the Apical Sodium-Dependent Bile Acid Transporter between Dogs with and without Chronic Inflammatory Enteropathy». *Journal of Veterinary Internal Medicine* 32 (6): 1918–26. https://doi.org/10.1111/jvim.15332.

Gower, John C. 1966. «Some distance properties of latent root and vector methods used in multivariate analysis». *Biometrika* 53 (3–4): 325–338.

Guard, Blake C., James W. Barr, Lavanya Reddivari, Cory Klemashevich, Arul Jayaraman, Jörg M. Steiner, Jairam Vanamala, e Jan S. Suchodolski. 2015. «Characterization of Microbial Dysbiosis and Metabolomic Changes in Dogs with Acute Diarrhea». *PLOS ONE* 10 (5): e0127259. https://doi.org/10.1371/journal.pone.0127259.

Hall, Jean A., Matthew I. Jackson, Jodi C. Vondran, Melissa A. Vanchina, e Dennis E. Jewell. 2018. «Comparison of circulating metabolite concentrations in dogs and cats when allowed to freely choose macronutrient intake». *Biology Open* 7 (11). https://doi.org/10.1242/bio.036228.

Hand, Daniel, Corrin Wallis, Alison Colyer, e Charles W. Penn. 2013. «Pyrosequencing the Canine Faecal Microbiota: Breadth and Depth of Biodiversity». *PLOS ONE* 8 (1): e53115. https://doi.org/10.1371/journal.pone.0053115.

Handl, Stefanie, Scot E. Dowd, Jose F. Garcia-Mazcorro, Jörg M. Steiner, e Jan S. Suchodolski. 2011. «Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats». *FEMS Microbiology Ecology* 76 (2): 301–10. https://doi.org/10.1111/j.1574-6941.2011.01058.x.

Hanning, Irene, e Sandra Diaz-Sanchez. 2015. «The Functionality of the Gastrointestinal Microbiome in Non-Human Animals». *Microbiome* 3 (1): 51. https://doi.org/10.1186/s40168-015-0113-6.

Herstad, Kristin M. V., Karina Gajardo, Anne Marie Bakke, Lars Moe, Jane Ludvigsen, Knut Rudi, Ida Rud, Monika Sekelja, e Ellen Skancke. 2017. «A Diet Change from Dry Food to Beef Induces Reversible Changes on the Faecal Microbiota in Healthy, Adult Client-Owned Dogs». *BMC Veterinary Research* 13 (1): 147. https://doi.org/10.1186/s12917-017-1073-9.

Holbrook, Morris B. 2008. «Pets and People: Companions in Commerce?» *Journal of Business Research*, Animal Companions, Consumption Experiences, and the Marketing of Pets: Transcending Boundaries in the Animal-Human Distinction, 61 (5): 546–52. https://doi.org/10.1016/j.jbusres.2007.07.010.

Honneffer, Julia B, Yasushi Minamoto, e Jan S Suchodolski. 2014. «Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs». *World Journal of Gastroenterology : WJG* 20 (44): 16489–97. https://doi.org/10.3748/wjg.v20.i44.16489.

Honneffer, Julia B., Jörg M. Steiner, Jonathan A. Lidbury, e Jan S. Suchodolski. 2017. «Variation of the Microbiota and Metabolome along the Canine Gastrointestinal Tract». *Metabolomics* 13 (3): 26. https://doi.org/10.1007/s11306-017-1165-3.

Honneffer, Julia, Blake Guard, Joerg M Steiner, e Jan S Suchodolski. 2015. «Mo1805 untargeted metabolomics reveals disruption within bile acid, cholesterol, and tryptophan

metabolic pathways in dogs with idiopathic inflammatory bowel disease». *Gastroenterology* 148 (4): S–715.

Huang, Zongyu, Zhiyuan Pan, Ruifu Yang, Yujing Bi, e Xiaohui Xiong. 2020. «The canine gastrointestinal microbiota: early studies and research frontiers». *Gut Microbes* 11 (4): 635–54. https://doi.org/10.1080/19490976.2019.1704142.

International Human Genome Sequencing Consortium, Eric S. Lander, Lauren M. Linton, Bruce Birren, Chad Nusbaum, Michael C. Zody, Jennifer Baldwin, et al. 2001. «Initial Sequencing and Analysis of the Human Genome». *Nature* 409 (6822): 860–921. https://doi.org/10.1038/35057062.

Jackson, Matthew I., e Dennis E. Jewell. 2019. «Balance of saccharolysis and proteolysis underpins improvements in stool quality induced by adding a fiber bundle containing bound polyphenols to either hydrolyzed meat or grain-rich foods». *Gut Microbes* 10 (3): 298–320. https://doi.org/10.1080/19490976.2018.1526580.

Janeczko, S., D. Atwater, E. Bogel, A. Greiter-Wilke, A. Gerold, M. Baumgart, H. Bender, et al. 2008. «The Relationship of Mucosal Bacteria to Duodenal Histopathology, Cytokine MRNA, and Clinical Disease Activity in Cats with Inflammatory Bowel Disease». *Veterinary Microbiology* 128 (1): 178–93. https://doi.org/10.1016/j.vetmic.2007.10.014.

Jeffery, Nick D., Andrew K. Barker, Cody J. Alcott, Jon M. Levine, Ilyssa Meren, Jane Wengert, Albert E. Jergens, e Jan S. Suchodolski. 2017. «The Association of Specific Constituents of the Fecal Microbiota with Immune-Mediated Brain Disease in Dogs». *PLOS ONE* 12 (1): e0170589. https://doi.org/10.1371/journal.pone.0170589.

Jobard, Elodie, Olivier Trédan, Déborah Postoly, Fabrice André, Anne-Laure Martin, Bénédicte Elena-Herrmann, e Sandrine Boyault. 2016. «A Systematic Evaluation of Blood Serum and Plasma Pre-Analytics for Metabolomics Cohort Studies». *International Journal of Molecular Sciences* 17 (12): 2035. https://doi.org/10.3390/ijms17122035.

Jones, Michael D., Paul D. Rainville, Giorgis Isaac, Ian D. Wilson, Norman W. Smith, e Robert S. Plumb. 2014. «Ultra High Resolution SFC–MS as a High Throughput Platform for Metabolic Phenotyping: Application to Metabolic Profiling of Rat and Dog Bile». *Journal of Chromatography B*, Metabolomics II, 966 (settembre): 200–207. https://doi.org/10.1016/j.jchromb.2014.04.017.

Jones, Oliver AH, e Victoria L Cheung. 2007. «An Introduction to Metabolomics and its Potential Application in Veterinary Science». *Comparative Medicine* 57 (5): 436–42.

Jones, R. S. G. 1982. «Tryptamine: A Neuromodulator or Neurotransmitter in Mammalian Brain?» *Progress in Neurobiology* 19 (1): 117–39. https://doi.org/10.1016/0301-0082(82)90023-5

Kchouk, Mehdi, Jean-Francois Gibrat, e Mourad Elloumi. 2017. «Generations of sequencing technologies: from first to next generation». *Biology and Medicine* 9 (3).

Kennedy, Paul J, John F Cryan, Timothy G Dinan, e Gerard Clarke. 2014. «Irritable bowel syndrome: A microbiome-gut-brain axis disorder?» *World Journal of Gastroenterology : WJG* 20 (39): 14105–25. https://doi.org/10.3748/wjg.v20.i39.14105.

Kieler, Ida Nordang, Melania Osto, Leoni Hugentobler, Lara Puetz, M. Thomas P. Gilbert, Torben Hansen, Oluf Pedersen, et al. 2019. «Diabetic Cats Have Decreased Gut Microbial Diversity and a Lack of Butyrate Producing Bacteria». *Scientific Reports* 9 (1): 4822. https://doi.org/10.1038/s41598-019-41195-0.

Kim, Junhyung, Jae-Uk An, Woohyun Kim, Soomin Lee, e Seongbeom Cho. 2017. «Differences in the Gut Microbiota of Dogs (Canis Lupus Familiaris) Fed a Natural Diet or a Commercial Feed Revealed by the Illumina MiSeq Platform». *Gut Pathogens* 9 (1): 68. https://doi.org/10.1186/s13099-017-0218-5.

Kirchoff, Nicole S., Monique A. R. Udell, e Thomas J. Sharpton. 2019. «The Gut Microbiome Correlates with Conspecific Aggression in a Small Population of Rescued Dogs (Canis Familiaris)». *PeerJ* 7 (gennaio): e6103. https://doi.org/10.7717/peerj.6103.

Kirwan, Jennifer. 2013. «Metabolomics for the Practising Vet». *In Practice* 35 (8): 438–45. https://doi.org/10.1136/inp.f5259.

Lamichhane, Santosh, Christian C. Yde, Mette S. Schmedes, Henrik Max Jensen, Sebastian Meier, e Hanne Christine Bertram. 2015. «Strategy for Nuclear-Magnetic-Resonance-Based Metabolomics of Human Feces». *Analytical Chemistry* 87 (12): 5930–37. https://doi.org/10.1021/acs.analchem.5b00977.

Langille, Morgan G. I., Jesse Zaneveld, J. Gregory Caporaso, Daniel McDonald, Dan Knights, Joshua A. Reyes, Jose C. Clemente, et al. 2013. «Predictive Functional Profiling of Microbial Communities Using 16S RRNA Marker Gene Sequences». *Nature Biotechnology* 31 (9): 814–21. https://doi.org/10.1038/nbt.2676.

Lauber, Christian L., Nicholas Zhou, Jeffrey I. Gordon, Rob Knight, e Noah Fierer. 2010. «Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples». *FEMS Microbiology Letters* 307 (1): 80–86. https://doi.org/10.1111/j.1574-6968.2010.01965.x.

Lawhon, Sara D., Russell Maurer, Mitsu Suyemoto, e Craig Altier. 2002. «Intestinal Short-Chain Fatty Acids Alter Salmonella Typhimurium Invasion Gene Expression and Virulence through BarA/SirA». *Molecular Microbiology* 46 (5): 1451–64. https://doi.org/10.1046/j.1365-2958.2002.03268.x.

Leipig-Rudolph, Miriam, Kathrin Busch, John F. Prescott, Iman Mehdizadeh Gohari, Christian M. Leutenegger, Walter Hermanns, Georg Wolf, Katrin Hartmann, Jutta Verspohl, e Stefan Unterer. 2018. «Intestinal Lesions in Dogs with Acute Hemorrhagic Diarrhea Syndrome Associated with NetF-Positive Clostridium Perfringens Type A». *Journal of Veterinary Diagnostic Investigation* 30 (4): 495–503. https://doi.org/10.1177/1040638718766983.

Ley, Ruth E., Fredrik Bäckhed, Peter Turnbaugh, Catherine A. Lozupone, Robin D. Knight, e Jeffrey I. Gordon. 2005. «Obesity Alters Gut Microbial Ecology». *Proceedings of the National Academy of Sciences* 102 (31): 11070–75. https://doi.org/10.1073/pnas.0504978102.

Li, Qinghong, Éva Larouche-Lebel, Kerry A. Loughran, Terry P. Huh, Jan S. Suchodolski, e Mark A. Oyama. 2021. «Gut Dysbiosis and Its Associations with Gut Microbiota-Derived Metabolites in Dogs with Myxomatous Mitral Valve Disease». *mSystems* 6 (2): e00111-21. https://doi.org/10.1128/mSystems.00111-21.

Li, Qinghong, Éva Larouche-Lebel, Kerry A. Loughran, Terry P. Huh, Jan S. Suchodolski, e Mark A. Oyama. 2021. «Metabolomics Analysis Reveals Deranged Energy Metabolism and Amino Acid Metabolic Reprogramming in Dogs With Myxomatous Mitral Valve Disease». *Journal of the American Heart Association*, maggio. https://doi.org/10.1161/JAHA.120.018923.

Lim, Mi Young, Eun-Ji Song, Sang Ho Kim, Jangwon Lee, e Young-Do Nam. 2018. «Comparison of DNA Extraction Methods for Human Gut Microbial Community Profiling». *Systematic and Applied Microbiology* 41 (2): 151–57. https://doi.org/10.1016/j.syapm.2017.11.008.

Lindon, J.C., E. Holmes, e J.K. Nicholson. 2001. «Pattern recognition methods and applications in biomedical magnetic resonance». *Progress in Nuclear Magnetic Resonance Spectroscopy*, 2001.

Lozupone, Catherine, Micah Hamady, e Rob Knight. 2006. «UniFrac – An online tool for comparing microbial community diversity in a phylogenetic context». *BMC Bioinformatics* 7 (1): 371. https://doi.org/10.1186/1471-2105-7-371.

Lyu, Tianshu, Guangshuai Liu, Huanxin Zhang, Lidong Wang, Shengyang Zhou, Huashan Dou, Bo Pang, Weilai Sha, e Honghai Zhang. 2018. «Changes in feeding habits promoted the differentiation of the composition and function of gut microbiotas between domestic dogs (Canis lupus familiaris) and gray wolves (Canis lupus)». *AMB Express* 8 (1): 123. https://doi.org/10.1186/s13568-018-0652-x.

Marchesi, Julian R., e Jacques Ravel. 2015. «The Vocabulary of Microbiome Research: A Proposal». *Microbiome* 3 (1): 31. https://doi.org/10.1186/s40168-015-0094-5.

Marsh, Dawn M, Donald M Dougherty, F. Gerard Moeller, Alan C Swann, e Ralph Spiga. 2002. «Laboratory-Measured Aggressive Behavior of Women: Acute Tryptophan Depletion and Augmentation». *Neuropsychopharmacology* 26 (5): 660–71. https://doi.org/10.1016/S0893-133X(01)00369-4.

Marteau, P., P. Lepage, I. Mangin, A. Suau, J. Doré, P. Pochart, e P. Seksik. 2004. «Gut Flora and Inflammatory Bowel Disease». *Alimentary Pharmacology & Therapeutics* 20 (s4): 18–23. https://doi.org/10.1111/j.1365-2036.2004.02062.x.

Martinez, Kristina B., Vanessa Leone, e Eugene B. Chang. 2017. «Western diets, gut dysbiosis, and metabolic diseases: Are they linked?» *Gut Microbes* 8 (2): 130–42. https://doi.org/10.1080/19490976.2016.1270811.

Maukonen, Johanna, Catarina Simões, e Maria Saarela. 2012. «The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples». *FEMS Microbiology Ecology* 79 (3): 697–708. https://doi.org/10.1111/j.1574-6941.2011.01257.x.

McVey Neufeld, K. A., A. Perez-Burgos, Y. K. Mao, J. Bienenstock, e W. A. Kunze. 2015. «The Gut Microbiome Restores Intrinsic and Extrinsic Nerve Function in Germ-Free Mice Accompanied by Changes in Calbindin». *Neurogastroenterology & Motility* 27 (5): 627–36. https://doi.org/10.1111/nmo.12534.

Meyer, F., D. Paarmann, M. D'Souza, R. Olson, EM Glass, M. Kubal, T. Paczian, et al. 2008. «The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes». *BMC Bioinformatics* 9 (1): 386. https://doi.org/10.1186/1471-2105-9-386.

Minamoto, Yasushi, Tomomi Minamoto, Anitha Isaiah, Panpicha Sattasathuchana, Agostino Buono, Venkat R. Rangachari, Isaac H. McNeely, Jonathan Lidbury, Jörg M. Steiner, e Jan S. Suchodolski. 2019. «Fecal Short-Chain Fatty Acid Concentrations and Dysbiosis in Dogs with Chronic Enteropathy». *Journal of Veterinary Internal Medicine* 33 (4): 1608–18. https://doi.org/10.1111/jvim.15520.

Minamoto, Yasushi, Cristiane C Otoni, Samantha M Steelman, Olga Büyükleblebici, Jörg M Steiner, Albert E Jergens, e Jan S Suchodolski. 2015. «Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease». *Gut Microbes* 6 (1): 33–47. https://doi.org/10.1080/19490976.2014.997612.

Mori, Akihiro, Ai Goto, Ryoko Kibe, Hitomi Oda, Yasushi Kataoka, e Toshinori Sako. 2019. «Comparison of the effects of four commercially available prescription diet regimens on the fecal microbiome in healthy dogs». *Journal of Veterinary Medical Science* 81 (12): 1783–90. https://doi.org/10.1292/jvms.19-0055.

Nicholson, J. K., J. C. Lindon, e E. Holmes. 1999. «"Metabonomics": understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data». *Xenobiotica* 29 (11): 1181–89. https://doi.org/10.1080/004982599238047.

Nikolaus, Susanna, Berenice Schulte, Natalie Al-Massad, Florian Thieme, Dominik M. Schulte, Johannes Bethge, Ateequr Rehman, et al. 2017. «Increased Tryptophan Metabolism Is Associated With Activity of Inflammatory Bowel Diseases». *Gastroenterology* 153 (6): 1504-1516.e2. https://doi.org/10.1053/j.gastro.2017.08.028.

Niwa, Toshimitsu, Tomohide Nomura, Satoshi Sugiyama, Takashi Miyazaki, Saori Tsukushi, e Shuichi Tsutsui. 1997. «The protein metabolite hypothesis, a model for the

progression of renal failure: An oral adsorbent lowers indoxyl sulfate levels in undialyzed uremic patients.» 1997.

Nylund, L., M. Nermes, E. Isolauri, S. Salminen, W. M. de Vos, e R. Satokari. 2015. «Severity of Atopic Disease Inversely Correlates with Intestinal Microbiota Diversity and Butyrate-Producing Bacteria». *Allergy* 70 (2): 241–44. https://doi.org/10.1111/all.12549.

Ojeda, Patricia, Alexandria Bobe, Kyle Dolan, Vanessa Leone, e Kristina Martinez. 2016. «Nutritional Modulation of Gut Microbiota — the Impact on Metabolic Disease Pathophysiology». *The Journal of Nutritional Biochemistry* 28 (February): 191–200. https://doi.org/10.1016/j.jnutbio.2015.08.013.

O'Mahony, S. M., G. Clarke, Y. E. Borre, T. G. Dinan, e J. F. Cryan. 2015. «Serotonin, Tryptophan Metabolism and the Brain-Gut-Microbiome Axis». *Behavioural Brain Research*, Special Issue: Serotonin, 277 (January): 32–48. https://doi.org/10.1016/j.bbr.2014.07.027.

Panasevich, Matthew R., Katherine R. Kerr, Ryan N. Dilger, George C. Fahey, Laetitia Guérin-Deremaux, Gary L. Lynch, Daniel Wils, et al. 2015. «Modulation of the Faecal Microbiome of Healthy Adult Dogs by Inclusion of Potato Fibre in the Diet». *British Journal of Nutrition* 113 (1): 125–33. https://doi.org/10.1017/S0007114514003274.

Pilla, Rachel, Frederic P. Gaschen, James W. Barr, Erin Olson, Julia Honneffer, Blake C. Guard, Amanda B. Blake, et al. 2020. «Effects of Metronidazole on the Fecal Microbiome and Metabolome in Healthy Dogs». *Journal of Veterinary Internal Medicine* 34 (5): 1853–66. https://doi.org/10.1111/jvim.15871.

Pilla, Rachel, Blake C. Guard, Joerg M. Steiner, Frederic P. Gaschen, Erin Olson, Dirk Werling, Karin Allenspach, Silke Salavati Schmitz, e Jan S. Suchodolski. 2019. «Administration of a Synbiotic Containing Enterococcus faecium Does Not Significantly Alter Fecal Microbiota Richness or Diversity in Dogs With and Without Food-Responsive Chronic Enteropathy». *Frontiers in Veterinary Science* 6: 277. https://doi.org/10.3389/fvets.2019.00277.

Pilla, Rachel, e Jan S. Suchodolski. 2020. «The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease». *Frontiers in Veterinary Science* 6. https://doi.org/10.3389/fvets.2019.00498.

Pullinger, Gillian D., Sonya C. Carnell, Fathima F. Sharaff, Pauline M. van Diemen, Francis Dziva, Eirwen Morgan, Mark Lyte, Primrose P. E. Freestone, e Mark P. Stevens. 2010. «Norepinephrine Augments Salmonella enterica-Induced Enteritis in a Manner Associated with Increased Net Replication but Independent of the Putative Adrenergic Sensor Kinases QseC and QseE». *Infection and Immunity* 78 (1): 372–80. https://doi.org/10.1128/IAI.01203-09.

Richard, Dawn M, Michael A Dawes, Charles W Mathias, Ashley Acheson, Nathalie Hill-Kapturczak, e Donald M Dougherty. 2009. «L-Tryptophan: Basic Metabolic Functions,

Behavioral Research and Therapeutic Indications». *International Journal of Tryptophan Research* 2 (gennaio): IJTR.S2129. https://doi.org/10.4137/IJTR.S2129.

Rigottier-Gois, Lionel. 2013. «Dysbiosis in Inflammatory Bowel Diseases: The Oxygen Hypothesis». *The ISME Journal* 7 (7): 1256–61. https://doi.org/10.1038/ismej.2013.80.

Rivera-Chávez, Fabian, Christopher A. Lopez, e Andreas J. Bäumler. 2017. «Oxygen as a Driver of Gut Dysbiosis». *Free Radical Biology and Medicine*, Redox relationships in gut-microbiome interactions, 105 (aprile): 93–101. https://doi.org/10.1016/j.freeradbiomed.2016.09.022.

Robertson, K.A., E.C. Feldman, e K. Polonsky. 1989. «Spontaneous diabetes mellitus in 24 dogs: incidence of type I versus type II disease». *Journal of Veterinary Internal Medicine*, 1989.

Rochelle, Paul A., Barry A. Cragg, John C. Fry, R. John Parkes, e Andrew J. Weightman. 1994. «Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S rRNA gene sequence analysis». *FEMS Microbiology Ecology* 15 (1–2): 215–25. https://doi.org/10.1111/j.1574-6941.1994.tb00245.x.

Sanchez, Sandra Bermudez, Rachel Pilla, Benjamin Sarawichitr, Alessandro Gramenzi, Fulvio Marsilio, Joerg M. Steiner, Jonathan A. Lidbury, Georgiana R. T. Woods, Alexander J. German, e Jan S. Suchodolski. 2020. «Fecal Microbiota in Client-Owned Obese Dogs Changes after Weight Loss with a High-Fiber-High-Protein Diet». *PeerJ* 8 (ottobre): e9706. https://doi.org/10.7717/peerj.9706.

Sandri, Misa, Simeone Dal Monego, Giuseppe Conte, Sandy Sgorlon, e Bruno Stefanon. 2017. «Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs». *BMC Veterinary Research* 13 (1): 65. https://doi.org/10.1186/s12917-017-0981-z.

Sandri, Misa, Sandy Sgorlon, Elisa Scarsella, e Bruno Stefanon. 2020. «Effect of Different Starch Sources in a Raw Meat-Based Diet on Fecal Microbiome in Dogs Housed in a Shelter». *Animal Nutrition* 6 (3): 353–61. https://doi.org/10.1016/j.aninu.2020.03.003.

Sarwar, F, M Werner, A Ziese, K Busch, Y Minamoto, A Blake, e others. 2018. «Prevalence of Clostridium perfringens encoding netF gene in dogs with acute and chronic gastrointestinal diseases». *Seattle: American College of Veterinary Internal Medicine*.

Saulnier, Delphine M., Yehuda Ringel, Melvin B. Heyman, Jane A. Foster, Premysl Bercik, Robert J. Shulman, James Versalovic, et al. 2013. «The intestinal microbiome, probiotics and prebiotics in neurogastroenterology». *Gut Microbes* 4 (1): 17–27. https://doi.org/10.4161/gmic.22973.

Scarsella, Elisa, Bruno Stefanon, Michela Cintio, Danilo Licastro, Sandy Sgorlon, Simeone Dal Monego, e Misa Sandri. 2020. «Learning Machine Approach Reveals Microbial

Signatures of Diet and Sex in Dog». *PLOS ONE* 15 (8): e0237874. https://doi.org/10.1371/journal.pone.0237874.

Schloss, Patrick D., Sarah L. Westcott, Thomas Ryabin, Justine R. Hall, Martin Hartmann, Emily B. Hollister, Ryan A. Lesniewski, et al. 2009. «Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities». *Applied and Environmental Microbiology* 75 (23): 7537–41. https://doi.org/10.1128/AEM.01541-09.

Segata, Nicola, Jacques Izard, Levi Waldron, Dirk Gevers, Larisa Miropolsky, Wendy S. Garrett, e Curtis Huttenhower. 2011. «Metagenomic Biomarker Discovery and Explanation». *Genome Biology* 12 (6): R60. https://doi.org/10.1186/gb-2011-12-6-r60.

Seo, Joonbum, Linda Matthewman, Dong Xia, Jenny Wilshaw, Yu-Mei Chang, e David J. Connolly. 2020. «The Gut Microbiome in Dogs with Congestive Heart Failure: A Pilot Study». *Scientific Reports* 10 (1): 1–9. https://doi.org/10.1038/s41598-020-70826-0.

Shen, Wan, H. Rex Gaskins, e Michael K. McIntosh. 2014. «Influence of Dietary Fat on Intestinal Microbes, Inflammation, Barrier Function and Metabolic Outcomes». *The Journal of Nutritional Biochemistry* 25 (3): 270–80. https://doi.org/10.1016/j.jnutbio.2013.09.009.

Shepard, Roger N. 1966. «Metric structures in ordinal data». *Journal of Mathematical Psychology* 3 (2): 287–315.

Shi, Na, Na Li, Xinwang Duan, e Haitao Niu. 2017. «Interaction between the Gut Microbiome and Mucosal Immune System». *Military Medical Research* 4 (1): 14. https://doi.org/10.1186/s40779-017-0122-9.

Smith, Birgitte, Nan Li, Anders Schou Andersen, Hans Christian Slotved, e Karen Angeliki Krogfelt. 2011. «Optimising Bacterial DNA Extraction from Faecal Samples: Comparison of Three Methods». *The Open Microbiology Journal* 5 (aprile): 14–17. https://doi.org/10.2174/1874285801105010014.

Song, Eun-Ji, Eun-Sook Lee, e Young-Do Nam. 2018. «Progress of Analytical Tools and Techniques for Human Gut Microbiome Research». *Journal of Microbiology* 56 (10): 693–705. https://doi.org/10.1007/s12275-018-8238-5.

Suchodolski, Jan S. 2016. «Diagnosis and Interpretation of Intestinal Dysbiosis in Dogs and Cats». *The Veterinary Journal*, Special Issue: Recent Developments in Veterinary Diagnostics, 215 (settembre): 30–37. https://doi.org/10.1016/j.tvjl.2016.04.011.

———. 2021. «Analysis of the Gut Microbiome in Dogs and Cats». *Veterinary Clinical Pathology* n/a (n/a). https://doi.org/10.1111/vcp.13031.

Suchodolski, Jan S., Scot E. Dowd, Vicky Wilke, Jörg M. Steiner, e Albert E. Jergens. 2012. «16S RRNA Gene Pyrosequencing Reveals Bacterial Dysbiosis in the Duodenum of Dogs

with Idiopathic Inflammatory Bowel Disease». *PLOS ONE* 7 (6): e39333. https://doi.org/10.1371/journal.pone.0039333.

Suchodolski, Jan S., Mary L. Foster, Muhammad U. Sohail, Christian Leutenegger, Erica V. Queen, Jörg M. Steiner, e Stanley L. Marks. 2015. «The Fecal Microbiome in Cats with Diarrhea». *PLOS ONE* 10 (5): e0127378. https://doi.org/10.1371/journal.pone.0127378.

Suchodolski, Jan S., Melissa E. Markel, Jose F. Garcia-Mazcorro, Stefan Unterer, Romy M. Heilmann, Scot E. Dowd, Priyanka Kachroo, et al. 2012. «The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease». *PLOS ONE* 7 (12): e51907. https://doi.org/10.1371/journal.pone.0051907.

Summers, Stacie C., Jessica M. Quimby, Anitha Isaiah, Jan S. Suchodolski, Paul J. Lunghofer, e Daniel L. Gustafson. 2019. «The Fecal Microbiome and Serum Concentrations of Indoxyl Sulfate and P-Cresol Sulfate in Cats with Chronic Kidney Disease». *Journal of Veterinary Internal Medicine* 33 (2): 662–69. https://doi.org/10.1111/jvim.15389.

Swanson, Kelly S., Scot E. Dowd, Jan S. Suchodolski, Ingmar S. Middelbos, Brittany M. Vester, Kathleen A. Barry, Karen E. Nelson, et al. 2011. «Phylogenetic and Gene-Centric Metagenomics of the Canine Intestinal Microbiome Reveals Similarities with Humans and Mice». *The ISME Journal* 5 (4): 639–49. https://doi.org/10.1038/ismej.2010.162.

The HMP consortium, Barbara A. Methé, Karen E. Nelson, Mihai Pop, Heather H. Creasy, Michelle G. Giglio, Curtis Huttenhower, et al. 2012. «A Framework for Human Microbiome Research». *Nature* 486 (7402): 215–21. https://doi.org/10.1038/nature11209.

Torres, Joana, Jianzhong Hu, Akihiro Seki, Caroline Eisele, Nilendra Nair, Ruiqi Huang, Leonid Tarassishin, et al. 2020. «Infants Born to Mothers with IBD Present with Altered Gut Microbiome That Transfers Abnormalities of the Adaptive Immune System to Germ-Free Mice». *Gut* 69 (1): 42–51. https://doi.org/10.1136/gutjnl-2018-317855.

Tsilingiri, Katerina, Theolis Barbosa, Giuseppe Penna, Flavio Caprioli, Angelica Sonzogni, Giuseppe Viale, e Maria Rescigno. 2012. «Probiotic and Postbiotic Activity in Health and Disease: Comparison on a Novel Polarised Ex-Vivo Organ Culture Model». *Gut* 61 (7): 1007–15. https://doi.org/10.1136/gutjnl-2011-300971.

Turnbaugh, Peter J., Vanessa K. Ridaura, Jeremiah J. Faith, Federico E. Rey, Rob Knight, e Jeffrey I. Gordon. 2009. «The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice». *Science Translational Medicine* 1 (6): 6ra14-6ra14. https://doi.org/10.1126/scitranslmed.3000322.

Vázquez, Lucía, Ana Belén Flórez, Sanne Verbruggen, Begoña Redruello, Jessica Verhoeven, Koen Venema, e Baltasar Mayo. 2020. «Modulation of Equol Production via Different Dietary Regimens in an Artificial Model of the Human Colon». *Journal of Functional Foods* 66 (marzo): 103819. https://doi.org/10.1016/j.jff.2020.103819.

Vázquez-Baeza, Yoshiki, Embriette R. Hyde, Jan S. Suchodolski, e Rob Knight. 2016. «Dog and Human Inflammatory Bowel Disease Rely on Overlapping yet Distinct Dysbiosis Networks». *Nature Microbiology* 1 (12): 1–5. https://doi.org/10.1038/nmicrobiol.2016.177.

Wallis, Natalie, e Eleanor Raffan. 2020. «The Genetic Basis of Obesity and Related Metabolic Diseases in Humans and Companion Animals». *Genes* 11 (11): 1378. https://doi.org/10.3390/genes11111378.

Wang, Shuai, Rene Martins, Megan C. Sullivan, Elliot S. Friedman, Ana M. Misic, Ayah El-Fahmawi, Elaine Cristina Pereira De Martinis, et al. 2019. «Diet-Induced Remission in Chronic Enteropathy Is Associated with Altered Microbial Community Structure and Synthesis of Secondary Bile Acids». *Microbiome* 7 (1): 126. https://doi.org/10.1186/s40168-019-0740-4.

Weingarden, Alexa R., Chi Chen, Aleh Bobr, Dan Yao, Yuwei Lu, Valerie M. Nelson, Michael J. Sadowsky, e Alexander Khoruts. 2014. «Microbiota transplantation restores normal fecal bile acid composition in recurrent Clostridium difficile infection». *American Journal of Physiology-Gastrointestinal and Liver Physiology* 306 (4): G310–19. https://doi.org/10.1152/ajpgi.00282.2013.

Wernimont, Susan M., Jennifer Radosevich, Matthew I. Jackson, Eden Ephraim, Dayakar V. Badri, Jennifer M. MacLeay, Dennis E. Jewell, e Jan S. Suchodolski. 2020. «The Effects of Nutrition on the Gastrointestinal Microbiome of Cats and Dogs: Impact on Health and Disease». *Frontiers in Microbiology* 11: 1266. https://doi.org/10.3389/fmicb.2020.01266.

Westermarck, Elias, e Maria E. Wiberg. 2006. «Effects of diet on clinical signs of exocrine pancreatic insufficiency in dogs». *Journal of the American Veterinary Medical Association* 228 (2): 225–29. https://doi.org/10.2460/javma.228.2.225.

Whitfield, Phillip D., Alexander J. German, e Peter-John M. Noble. 2004. «Metabolomics: An Emerging Post-Genomic Tool for Nutrition». *British Journal of Nutrition* 92 (4): 549–55. https://doi.org/10.1079/BJN20041243.

Yadav, Monika, Manoj Kumar Verma, e Nar Singh Chauhan. 2018. «A Review of Metabolic Potential of Human Gut Microbiome in Human Nutrition». *Archives of Microbiology* 200 (2): 203–17. https://doi.org/10.1007/s00203-017-1459-x.

Yang, Xiaoping, Kwame Oteng Darko, Yanjun Huang, Caimei He, Huansheng Yang, Shanping He, Jianzhong Li, Jian Li, Berthold Hocher, e Yulong Yin. 2017. «Resistant Starch Regulates Gut Microbiota: Structure, Biochemistry and Cell Signalling». *Cellular Physiology and Biochemistry* 42 (1): 306–18. https://doi.org/10.1159/000477386.

Yin, Peiyuan, Rainer Lehmann, e Guowang Xu. 2015. «Effects of Pre-Analytical Processes on Blood Samples Used in Metabolomics Studies». *Analytical and Bioanalytical Chemistry* 407 (17): 4879–92. https://doi.org/10.1007/s00216-015-8565-x.

Zeng, M. Y., N. Inohara, e G. Nuñez. 2017. «Mechanisms of Inflammation-Driven Bacterial Dysbiosis in the Gut». *Mucosal Immunology* 10 (1): 18–26. https://doi.org/10.1038/mi.2016.75.

Zhang, Shiying, Belgin Dogan, Cindy Guo, Deepali Herlekar, Katrina Stewart, Ellen J. Scherl, e Kenneth W. Simpson. 2020. «Short Chain Fatty Acids Modulate the Growth and Virulence of Pathosymbiont Escherichia Coli and Host Response». *Antibiotics* 9 (8): 462. https://doi.org/10.3390/antibiotics9080462.

Ziese, Anna-Lena, Jan S. Suchodolski, Katrin Hartmann, Kathrin Busch, Alexandra Anderson, Fatima Sarwar, Natalie Sindern, e Stefan Unterer. 2018. «Effect of Probiotic Treatment on the Clinical Course, Intestinal Microbiome, and Toxigenic Clostridium Perfringens in Dogs with Acute Hemorrhagic Diarrhea». *PLOS ONE* 13 (9): e0204691. https://doi.org/10.1371/journal.pone.0204691.

CHAPTER 2

AIM OF THE RESEARCH

The study of the dog's gut microbiome has characterized the entire course of research carried out during the doctorate. Many efforts have been made so far to characterize the gut microbial composition, in order to understand the relationship with the health conditions of companion animals. Specifically, many studies have focused on the role of diet in influencing the microbial population. To date, however, only specific changes to the diet have been studied, such as the addition or differences between one nutrient rather than another, and therefore on the chemical composition of the diet itself. Unfortunately, these studies did not take into account the effect that different types of diets, such as extruded diet or home-made diet, have on the gut microbiome, as well as other factors, such as sex. Moreover, these studies considered only few subjects recruited.

The main purpose of this thesis is to give a broader interpretation to the relationships existing between the microorganisms that are part of the gut microbiome of the dog, which can be influenced by various factors, both environmental and genetic. Specifically, the ultimate goal is to characterize the microbiome of healthy subjects, establish threshold values and establish what is defined as the "core" of the microbiome. To do this, it was also necessary to study the metabolome, or the set of metabolites produced by the microbiome, which can characterize the function of the microbial population in each group examined. In addition to this, an innovative and ambitious goal was to characterize the blood microbiome in healthy subjects in order to perform a relationship to the gut microbiome.

In Chapter 3, the microbial profiles of fecal samples from more than 130 healthy dogs, analyzed following a standardized pipeline. Dogs were classified based on Diet and Gender factors, applying a discriminant analysis with size reduction, followed by a random forest analysis on the relative abundances of bacterial genera.

In chapter 4, the activity of proanthocyanidins extracted from grapevine, added to the diet, on the gut microbiome and the relationship with the endocrine response measured in saliva was evaluated.

In chapter 5, the main purpose of the study was to determine the presence of specific metabolites in fecal samples from dogs fed with three different types of diet, using an NMR method, never used before in dog studies. Another goal was to be able to distinguish dogs fed with different diets based on their metabolic profile.

In chapter 6, one of the objectives was to confirm the evidence, demonstrated in chapter 3, regarding the relationship of the fecal microbiome with the diet of dogs; another purpose was to investigate the presence of bacterial DNA in the bloodstream of healthy dogs, and finally, to look for a possible correlation between the gut and the blood microbiome.

In chapter 7, the main objective was to complete the results obtained previously and shown in the previous chapters. The aim of the study was to analyze co-occurrence patterns of microorganisms, related to the diet and sex of dogs. The proposed method helps to investigate the intestinal microbiome of healthy subjects and clearly highlights the interactions existing between bacteria and between bacteria and the host.

CHAPTER 3

3.1 Introduction to the study

The gut microbiome of humans and mammals is an increasingly studied research topic, due to the many aspects that can influence it and, consequently, modify it. Over the years, the techniques have been refined, and increasingly specific results have been obtained. Unfortunately, we are a long way off in obtaining an analysis of the microbiome that we can consider a gold standard, and for this reason we are also very far in characterizing the intestinal microbiome of mammals, and in this case of dogs, considered healthy. The definition of reference values that characterize the microbial composition of a healthy subject could help in the search for easily evaluable molecular markers for the characterization of a subject with different pathologies, and above all gastrointestinal ones. The different types of diets that can be administered to dogs and cats can influence the bacterial population that inhabits the gastrointestinal tract in such a way as to make the microbiome significantly different for each category considered. The need to identify a valid tool to deal with these problems arises spontaneously, from the moment in which the countless studies on the modulation of the microbiome based on the changes of some macronutrients, but more often concerning the addition of some additives, rely on a few subjects for study, over a short period of time, characterizing the variations at the level of a single taxonomic level and not considering the microbiome as a single core.

What makes the gut microbiome so interesting is its use to differentiate animals and humans into "enterotypes". An enterotype is a group of subjects that fall into a classification based on their bacteriological ecosystem in the intestine. Differentiating animals by enterotypes is equivalent to carrying out a process of size reduction, in order to concentrate the variations of the microbial population in a few categories. Enterotypes can be caused or influenced by various environmental and genetic factors. Despite countless studies in companion animals, there is still not enough data to classify dogs and cats into enterotypes.

This study wants to highlight that, depending on the variations in terms of gut microbial population based on the type of diet, dogs can be divided into macro groups; this also applies on sex, including the spayed/neuter dogs. The investigation included the analysis of 340 fecal samples collected serially from 132 subjects, recruited for diet modulation experiments. In addition to the categorization based on sex, the subjects were categorized based on the form of diet administered: extruded industrial dry diet, industrial wet diet, home diet and B.A.S.E.™ (Nutrigene; www.nutrigenefood.com) diet.

Initially, substantial differences were observed at the Phylum level: the bacteria belonging to the Firmicutes group are the most represented in all the diets studied, but they appear to have a higher prevalence in the home diet than in the B.A.S.E.™; the latter, in turn, detects a higher abundance of Firmicutes even with respect to the dry and wet extruded industrial diet. The second most abundant Phylum is Bacteroidetes, with higher prevalence in B.A.S.E.™ and wet industrial diets compared to the home-made and dry extruded industrial diets.

In terms of genera, the groups of bacteria most represented in the four diets are essentially *Fusobacterium*, *Lactobacillus*, *Blautia*, *Megamonas*, *Prevotella*, *Ruminococcus*, *Streptococcus*, *Collinsella*, an unassigned genus belonging to the Bacteriaceae family and finally *Clostridium*, which constitutes the genus most represented among the diets of this study. From these

taxonomic annotations, the genera that showed a higher degree of differentiation based on the diets were subsequently used to highlight any subjects clustering: the dogs fed with an extruded dry and wet industrial diet formed two well separated and distinct clusters. On the contrary, dogs characterized by a home-made diet or B.A.S.E.™ overlapped, making it difficult to distinguish between one group and the other. This last consideration has allowed us to hypothesize that, beyond the chemical composition, even the physical form of the diet itself (extruded dry or wet) can influence the structure of the intestinal microbiome. Consequently, by repeating the grouping of dogs based on the form of the diets (therefore on three types) it was possible to appreciate three distinct and separate groups. The genera with a higher discriminatory power, in this case, were: *Anaerobiospirillum*, *Bacteroides*, *Clostridium*, *Collinsella*, *Escherichia*, *Fusobacterium*, *Oscillospira*, *p-75-a5*, *Peptococcus*, *Epulopiscium*, *Eubacterium* and an unassigned genus of the Bacteriaceae family.

Regarding the sex factor, the results were comparable. Not only both phyla and genera were influenced by sex but, through the gut microbiome, it was possible to distinguish female subjects from male ones, while less clear was the separation between neutered male dogs and spayed females, which in turn formed a single separate group.

Also in this case, the genera that showed a higher degree of differentiation based on sex were used to highlight any clustering of dogs: males and females are grouped into two distinct clusters, while this was not the case for neutered subjects, both females and males (similarly to what has been observed with the diet factor). It can be concluded that the physiological and hormonal state of the dog, or of the animal in a more general sense, influenced the gut microbial composition. In addition, by repeating the clustering based on three categories (whole males, whole females and neutered subjects, without distinction of sex), it was possible to distinguish the three clusters to which they belong. The genera that allow this clear distinction between the three groups are: *Blautia*, *Dorea*, *Clostridium*, *Fusobacterium*, *Oscillospora*, *Phascolarctobacterium*, *Slackia*, *Streptococcus*, *Ruminococcus*, *Sutterella* and an unassigned genus of the Bacteriaceae family.

This type of analysis highlighted consistent differences in beta diversity, despite the enormous variability existing between the subjects, which made it difficult to compare the individual taxa among the factors considered. These results bring us back to the concept of enterotype, confirming the existence of a possible classification, based on the microbiome, which would distinguish different groups of animals. At this level, beta diversity becomes more important than alpha diversity, since, outside the enormous individual variability, there are specific bacteria that belong to one rather than the other group, and therefore are highly discriminatory for different factors considered. Ultimately, the presence, as well as the abundance, of some bacteria determines what is called "core microbiome".

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3.2 Learning Machine Approach Reveals Microbial Signatures Of Diet And Sex In Dog

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3.2.1 Abstract

The characterization of the microbial population of many niches of the organism, as the gastrointestinal tract, is now possible thanks to the use of high-throughput DNA sequencing technique. Several studies in the companion animals field already investigated faecal microbiome in healthy or affected subjects, although the methodologies used in the different laboratories and the limited number of animals recruited in each experiment does not allow a straight comparison among published results. In the present study, we report data collected from several in house researches carried out in healthy dogs, with the aim to seek for a variability of microbial taxa in the faeces, caused by factors such as diet and sex. The database contains 340 samples from 132 dogs, collected serially during dietary intervention studies. The procedure of samples collection, storage, DNA extraction and sequencing, bioinformatic and statistical analysis followed a standardized pipeline. Microbial profiles of faecal samples have been analyzed applying dimensional reduction discriminant analysis followed by random forest analysis to the relative abundances of genera in the feces as variables. The results supported the responsiveness of microbiota at a genera taxonomic level to dietary factor and allowed to cluster dogs according this factor with high accuracy. Also sex factor clustered dogs, with castrated males and spayed females forming a separated group in comparison to intact dogs, strengthening the hypothesis of a bidirectional interaction between microbiota and endocrine status of the host. The findings of the present analysis are promising for a better comprehension of the mechanisms that regulate the connection of the microorganisms living the gastrointestinal tract with the diet and the host. This preliminary study deserves further investigation for the identification of the factors affecting faecal microbiome in dogs.

3.2.2 Introduction

A growing number of researches investigated the composition and the variation of gut microbiome in relation to healthy conditions and environmental factors for companion animals and livestock (Sandri et al. 2014; Deng & Swanson 2015). The microbiota that composes the gastrointestinal (GI) tract of humans and animals has been indicated to be responsible of very important basic functions contribution of metabolic activities, protection against pathogens, sending signals to the immune system and the direct or not affection of most of the physiologic functions (Pilla & Suchodolski 2019).

Several studies, both using bacterial culture or molecular methods, aimed to demonstrate that the abundance and the biodiversity of the microbiota increase along the tract (Suchodolski, Camacho & Steiner 2008). Moreover, the advent of innovative technologies allowed a more frequent utilization of molecular methods to identify the non-culturable bacteria within the canine GI tract. It is estimated that the total microbial load is about 10 times the number of cells present in the host (Suchodolski 2011).

Dietary intervention studies with clinically healthy dogs have underpinned a high individual variability, which reduced the possibility to find modifications of faecal microbiome in relation to the experimental factor. Moreover, the methodology and the techniques applied in these studies largely vary, limiting the comparison of data obtained from different researches. Upstream methodological issues are the sampling and the DNA isolation procedures using internal protocols or commercial kits, which can affect the yield and purity of the DNA, the integrity and the presence of inhibitors of PCR and lead to different results (Videnska et al. 2019). The sequencing platforms, the selection of the amplification regions, the depth of sequencing are other upstream choices that affect the final results. According to Allali et al. (2017) (Allali et al. 2017), the results obtained by 3 sequencing platforms are different in terms of diversity and abundance, even though lead to the comparable biological considerations. Downstream to sequencing, the use of different bioinformatics pipelines (Peker et al. 2019) is another methodological factor affecting the results of microbial communities. The Human Microbiome Project, launched at the National Institute of Health, aims at the characterization of the microbiome in healthy human subjects in 5 major body sites, namely gut, nasal passage, oral cavity, skin, urogenital tract (The HMP consortium 2012). In the web site https://www.hmpdacc.org, the common repository for diverse human microbiome datasets, a minimum reporting standards is implemented. Specifically, the 16S rRNA DNA barcode technique aims at investigating whether there is a core healthy bacterial microbiota in these sites. For livestock and companion animals, minimum standards for sample collections and processing are not agreed yet.

Even though a high variability of microbial composition among studies is found, there are some key bacterial species consistently present in fecal samples of healthy subjects, regardless of the method used, suggesting the presence of a core faecal bacterial community (Hand et al. 2013). Nevertheless, the growing knowledge in this topic denotes the existence of a strong variability between microbiome profiles of individuals (Guard & Suchodolski 2016), that has to be taken into consideration when a comparison between several experiments with a limited number of dogs is reported.

Here, we report on an analysis of a dataset of faecal microbiome which supplied 340 bacterial profiles of healthy dogs. These studies were performed following the same protocol, starting from reagents to sequencing platforms and bioinformatics pipeline, to limit the variability associated with the methodology and the technology. Microbial profiles of faecal samples were classified for the factors diets and sex, applying dimensional reduction discriminant analysis followed by random forest analysis to the relative abundances of genera in the faeces.

3.2.3 Material and methods

3.2.3.1 Sample population

The dataset is composed of individual records of dogs obtained from 8 dietary intervention studies (DIS) conducted in the past 5 years, for a total of 340 samples. All the dogs were recruited with the same inclusion criteria, which consisted of healthy conditions, as ascertain by a clinical examination, freedom from external and internal parasites, no pharmacological treatments since at least 3 months. A summary of the studies is reported in the S1 Table. Briefly, dogs were recruited from different living environment for every DIS and they were undergone through diet modulation. The DIS 1 (Sandri et al. 2017), 2 (Sandri et al. 2019), 3 and 8 (Scarsella et al. 2020) were private kennels, DIS 4 (Sandri et al. 2020) and 7 were shelters and DIS 5 and 6 were dogs privately owned. For every DIS, the first faecal sample (T0) was collected from dogs that were fed the usual diet they received since at least 6 months (control diet, CTR). Starting from day 1 (T1), dogs were divided in groups and the diet has been changed, as reported in the S1 Table. According to the experimental design, a second faecal sample was collected after 14 (T14) and 28 days (T28) for seven of the eight DIS; only in DIS 6 the second faecal sample was collected after 45 days from the beginning of the study.

The diets involved in the database were grouped in four categories, namely commercial extruded complete diet (K), commercial moist complete diet (W), home-made diet (H) and a raw meat diet with the addition of a complementary food, from here on called Base (B) (www.nutrigenefood.com). A detailed description of the nutritive value of the diets for each experiment is reported in the S2 Table. For the variable sex, the dogs were grouped in males (M), castrated males (MC), females (F) and spayed females (FC). The age and the breed of the dogs were also imputed in the database.

Stool samples were collected with the same protocol for every DIS, from the ground soon after the evacuation, using sterile gloves, introduced into a sterile plastic bag, immediately frozen at -20° C and delivered to the laboratory for storage at -20° C until analysis. Time elapsed from the sample collection to DNA extraction was lower than 30 days.

3.2.3.2 Faecal DNA extraction, sequencing and bioinformatic analysis

The entire procedure, starting from the microbial DNA extraction method and ending with taxonomic annotation with a bioinformatic analysis, was standardized and utilized for all the samples. Frozen stools were cleaned from external contaminations with soil with a sterile blade and successively thawed at room temperature. Total DNA was extracted from 150 mg of faeces using a Faecal DNA MiniPrep kit (Zymo research; Irvine, CA, US), following the manufacturer's instruction. A ZymoBIOMICS™ Microbial Community Standard (Zymo Research, Irvine, CA, USA) was used to assess the efficiency of the entire pipeline, from DNA extraction method to taxonomic annotation. The mock community contains eight bacterial species: *Pseudomonas aeruginosa* (4.2%), *Escherichia coli* (10.1%), *Salmonella enterica* (10.4%), *Lactobacillus fermentum* (18.4%), *Enterococcus faecalis* (9.9%), *Staphylococcus aureus* (15.5%), *Listeria monocytogenes* (14.1%) and *Bacillus subtilis* (17.4%); expected composition of the mock community was given by the manufacturer.

The total amount of DNA extracted and the quality were measured with a QubitTM 3 Fluorometer (Thermo Scientific; Waltham, MA, USA) and libraries were prepared with the amplification of the V3 and V4 regions from the 16S rRNA gene, adding Indexes for sequencing, using a Nextera DNA Library Prep kit (Illumina; San Diego, CA, USA), following manufacturer's instruction and primers (Klindworth et al. 2013). The resulting amplicons were sequenced with a MiSeq (Illumina; San Diego, CA, USA) in 2x300 paired-end mode, following the standard procedures, for a depth of 50,000 reads.

Raw sequences (FASTQ) of the 8 DIS were collated and processed using the bioinformatic program called Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (Bolyen et al. 2019), and uploaded to NCBI Sequence Read Archive (S1 Table). After demultiplexing, sequenced reads that passed the quality check (Phred score 30) were annotated for 16S rRNA against the Greengenes database. Chimeras were also detected and then filtered from the reads and the remaining sequences were clustered into Operational Taxonomic Units (OTUs) by using an open reference approach in QIIME 2.

3.2.3.3 Computation and statistical analysis

Annotated OTU were imputed on a spreadsheet together with age, sex, breed and the number of the study to allow and facilitate further statistical analysis. The annotates sequences from each sample and each taxonomic level were normalized to ‰ abundance profiles, already known as Relative Abundance (RA). Within each DIS, taxa with RA lower than 1‰ in more than half of the samples were excluded from the statistical analysis (Sandri et al. 2019). Beta diversity was evaluated with the phylogeny based on UniFrac (Lozupone & Knight 2005) distance metric and visualized using Principal Coordinate Analysis (PCoA) plots. Permutational multivariate analysis of variance (PERMANOVA) was performed using UniFrac distances by including diets or sex as one of the factors to assess the differences in community composition. For each of the different taxonomic levels, RAs were initially analysed with descriptive statistics to show their distributions, by means of the graphical appraisal of boxplots, reporting maximum, minimum, first and third quartile, mean and median.

Linear discriminant analysis (LDA) was applied for dimensional reduction of variables with the aim to classify dogs for diets (H, B, K, W) or sex (M, F, MC and FC) classes. The technique applied was class dependent with the step-wise method. After this step, we looked at the discriminant functions, to see which one was not linear, and thus which one described and predicted better the class of each observation with the greatest value. Random Forest (RF) classification was built only with the selected variables from LDA that had a non-linear trend: the prediction performance of the classifier is based on the Out-Of-Bag (OOB) data; these data can also evaluate the variable importance, shown with the decrease in prediction rule accuracy by a random permutation of the values in each feature. The ranking parameter mentioned is called Mean Decreased Accuracy (MDA). To confirm what LDA and RF results, a nonparametric Kruskal-Wallis test was applied at the genera level, with Bonferroni correction for multiple comparison. A p-value below 0.05 was considered statistically significant (Addinsoft 2020).

3.2.4 Results

The partitioning of the 340 dogs for the categorical variables diets and sex are reported in the contingency table (Table 1). The age was not used for classification purposes, since is not straight to compare the age of dogs behaving to different breeds. For the factor diets, the number of observations for the H group represented 30 samples out of 340 (8.82%), whilst dogs fed with K diet accounted for more than 50% of the samples, followed by W and B diets.

The average crude protein content (%/DM) was 27.2%, 27.6%, 36.3% and 30.1% for K, W, H and B diet respectively (Table 2; S2 Table). Indeed, the average lipid content (%/DM) was more variable between diets, being 15.3%, 20.0%, 27.2% and 22.8% for K, W, H and B diets, respectively. For the variable sex, F was the most populated group (145 dogs) and FC the least represented group (28 samples out of 340, 8.24%). The number of records for M and MC in the database was quite similar (78 males and 89 castrated males).

The phylogenetic distance among the samples was investigated using the PCoA of the UniFrac distances of the OTU and in Fig 1A the beta diversity for the factors diets and sex was reported. Two separated clusters of samples from dogs fed with K and W diets were shown, whilst the samples of the H and B groups were more scattered and tended to overlap. The beta diversity with UniFrac distances was also calculated for the factor sex and plotted as PCoA (Fig 1B). Castrated dogs, MC and FC, clustered closer showed a lower beta diversity in comparison to M and F subjects. To evaluate these differences highlighted with the PCoA, we performed a PERMANOVA with the two variables taking into account–diets and sex. A significant contribution of the diets ($P < 0.05$) and sex ($P < 0.05$) was detected. Regarding the sex variable, no differences were observed between FC and MC groups.

3.2.4.1 Diet

Firmicutes were on average the most abundant phylum (mean RA of 713.9 ‰) and a higher mean value ($P < 0.001$) was observed for H diet in comparison to B diet. The mean RA of this latter group was higher ($P < 0.001$) than RAs of K and W diets (Fig 2). The second more abundant phylum was Bacteroidetes (mean RA of 103.4‰), which was highest in B (119.8‰) and W (mean RA 123.6‰) diets in comparison to H and K diets ($P < 0.001$). Significant differences of mean RA of for Bacteroidetes was also observed between H (58.6‰) and K diets (mean RA 96.0 ‰). The RAs of Fusobacteria (mean RA 57.5‰) were higher in W diets in comparison to B diet ($P < 0.05$) and this higher ($P < 0.001$) than H and K diets.

At a genus levels, taxonomic annotations identified 37 features, with *Clostridia* being the most abundant (mean RA 238.5 ‰), having the maximum value (mean RA 334.1 ‰) in H diet and the minimum in K diet (214.1 ‰). Other highly represented bacteria were the *unassigned* genus of Bacteriaceae, *Fusobacterium*, *Lactobacillus*, *Blautia*, *Megamonas*, *Prevotella*, *Ruminococcus*, *Streptococcus* and *Collinsella*. These 37 genera were used in LDA to identify input variables which significantly varied between diets.

The results of the LDA for the factor diets showed that faecal samples of dogs fed with K and W diets were grouped in two distinct clusters, whilst the samples of dogs fed B and H overlapped, making difficult to separate the two groups (Fig 3A). For the genera *Megamonas*, *Allobaculum*, *Slackia*, *Butyricicoccus*, *Anaerobiospirillum*, *Bacteroides*, *Clostridium*, *Collinsella*, *Escherichia*,

Fusobacterium, *Oscillospira*, *p-75-a5*, *Peptococcus* and *Roseburia* and for an *unassigned* genus of the family Bacteriaceae, the highest significant coefficients ($P < 0.0001$) were found (S3 Table). Considering the overlapping of the dogs fed B and H diets, the data of these two groups of diets were merged in a new group (named H-B) and the LDA was rerun.

The results of the second LDA showed 3 distinct groups (Fig 3B) and, again, for an *unassigned* genus of Bacteriaceae and for the genera *Anaerobiospirillum*, *Bacteroides*, *Clostridium*, *Collinsella*, *Escherichia*, *Fusobacterium*, *Oscillospira*, *p-75-a5* and *Peptococcus* the highest significant coefficients ($P < 0.0001$) were observed. However, significant coefficients were found for other two genera, *Epulopiscium* and *Eubacterium*, and the levels of significance of the coefficients of *Megamonas*, *Allobaculum*, *Slackia* and *Butyricicoccus* were lower than those observed for the LDA with 4 dietary groups.

A RF classifier was built using the variables having significant linear functions in the LDA for diets. For the factor diets and considering 3 groups (H-B, K, W), the percentage of correctly classified samples was 71.56%, 88.24% and 77.47% for K, W and H-B diets, respectively (Fig 4A; S4 Table). Overall, the RF model correctly classified 75.29% of the total samples. The MDA values indicated the highest discriminatory power of the RF for the *unassigned* genus of Bacteroidaceae and for the genus *Peptococcus* (Fig 4B).

A non -parametric Kruskal-Wallis test was applied to the three groups at the genera level, confirming results of LDA and RF analysis and highlighting some differences between taxa. Results are shown on S5 Table. The main relevant differences were between bacteria that were highly represented in all the three diets: the *unassigned* genus of Bacteriaceae, highly represented in W diet (176.4‰), *Fusobacterium*, having the maximum value in the W diet (98.8‰), *Lactobacillus*, more represented in K diet (66.1‰), *Blautia*, higher in W diet (92.2‰), *Megamonas*, with higher abundance in H-B diet (31.9‰), *Prevotella*, higher in K diet (35.9‰), *Ruminococcus*, more represented in H-B diet (35.3‰), *Streptococcus*, with the maximum value in K diet (71.4‰) and *Collinsella*, higher in H-B diet than in K diet (14.6‰ vs 5.9‰).

3.2.4.2 Sex

The most abundant phyla were also affected by sex (Fig 5). The RA of Firmicutes was similar between FC and MC and significantly higher ($P < 0.001$) than F and M dogs. Moreover, the RA of M was higher than that of F ($P < 0.001$). Conversely, the RA of Bacteroidetes was lower in M than in F ($P < 0.001$), but higher ($P < 0.001$) than castrated dogs (FC and MC). Significant differences for the RA of Fusobacterium were observed between entire (F and M) and castrated (FC and MC) dogs.

The LDA using sex as a factor showed that that M and F constituted two distinct groups and separated from to the castrated dogs (MC and FC), which clustered together (Fig 6A). The coefficients for an *unassigned* genus of Bacteriaceae and for the genera *Blautia*, *Dorea*, *Clostridium*, *Fusobacterium*, *Oscillospira*, *Phascolarctobacterium*, *Slackia* and *Streptococcus* were highly significant ($P < 0.0001$, S6 Table). Considering the overlapping of the FC and MC dogs, the data of these two groups were merge in new group (named C) and the LDA was rerun. In the new analysis, significant coefficients were found for the same genera, but other two bacteria resulted highly significant (*Ruminococcus* and *Sutterella*).

The RF was used for the classification of data according to the sex factor, with 3 levels (F, M and C), using the variables that had significant linear functions in the LDA. Overall, the percentage of correctly classified total subjects in all categories were 67.35%, meanwhile, and for F and C groups the classification was correct with values of 65.6% and 71.3%, respectively (Fig 7A; S7 Table). The percentages of correctly classified subjects for M was very low. For the sex category, the curve of the MDA of the genera was smoother than for diets, and the higher discriminatory power for the RF classification was shown for *Dorea*, *Sutterella* and *Oscillospira* (Fig 7B).

3.2.5 Discussion

The PCoAs of Fig 1 indicated that the factors diets and sex had an influence on the beta diversity of gut microbiome, even though a large individual variability was observed. Several studies have pointed out the high variations of faecal microbial communities in healthy dogs (Sandri et al. 2019), suggesting that gut microbiome can be considered an individual fingerprint (Garcia-Mazcorro et al. 2017). However, is not clear if this depend upon the genetic background of the host (nature) or if the environment in a broad sense (nurture) has a prevailing role in shaping the gut microbiome (Gupta et al. 2017). Apart from the different methodological approaches among the reported studies, it is likely that several factors other than diet could have affected the RA of the phyla. For humans, geographical or ethnic variations, host genetic, immunity, lifestyle and diet or dietary habits have been reported as factors affecting gut microbiota (Gupta et al. 2017). Sex (Le Sciellour et al. 2019), genetic performances (Lu et al. 2018), phase of growth, other than diet (Niu et al. 2019) have been reported to influence gut microbiota in pigs. In healthy dogs, variations of microbiome with age, from weaning to adulthood, were reported (Ribeiro et al. 2019; Vilson et al. 2018), with a stabilization of the core gut microbiota at maturity. Since only adult dogs were recruited in the present study, the factor age was not considered.

3.2.5.1 Diet

Human microbiome can be split in enterotypes, meaning that individuals can be clustered on the basis of the abundance of microbial taxa of the gut (Arumugam et al. 2011), which correspond to specific functional and metabolic activities. The main microbial phyla in the gut of healthy humans are Firmicutes and Bacteroidetes, and their ratio is important for the classification in enterotypes. Mobeen et al. (2018) report that a value of the ratio higher than 1 is prevailing in Asian Countries, while very low values are observed in Burkina Faso and Malaysia and variable ratios were found for Western Countries. Also in dog, the main phyla of faecal microbiota belong to Firmicutes and Bacteroidetes, but their RAs are not been used yet to identify enterotypes. Although a similarity of microbiota between dogs and humans are reported in relation to inflammatory bowel diseases (Vazquez-Baeza et al. 2016) and to the diet (Coelho et al. 2018), contradictory results may suggest that anatomical and physiological differences among species, and the related differences in nutrients and dietary requirements, have to be taken into account. For instance, according to Turnbaugh (2006), obesity in humans and in mice is associated to an increase of Firmicutes and a reduction of Bacteroidetes; indeed obese dogs have a significant decrease of Firmicutes and increase of Protebacteria (Park et al. 2015), whilst Bacteroidetes and Fusobacteria are unchanged. Furthermore, contradictory results in the literature make difficult the identification of a gut microbiome core in dogs. The shift on RA of Bacteroidetes and

Firmicutes in relation to the diets (Fig 2) did not correspond with that reported in other researches in dogs. In the study of Mori et al. (2019), a low fat high protein diet caused a reduction of Bacteroidetes and an increase of Fusobacteria. Algya et al. (2018) reported a significant increase of Bacteroidetes and Fusobacteria and a decrease of Firmicutes in raw diet, based on chicken and sweet potatoes, in comparison to kibble diet. Indeed, the increase of Fusobacteria RA in the W diet was in agreement with these authors, but not for Bacteroidetes, which showed a very low RA in the H diet, that contains the highest lipid content (Table 2). Bacteroidetes, Gram negative either aerobic and anaerobic bacteria, originated from ancestors living in environment and possess high ability to utilize different carbohydrates, thanks to a large number of carbohydrate-active enzymes that enable them to ferment dietary glycans either from the diet of from the host (El Kaoutari et al. 2013). For this reason, Bacteroidetes easily adapt to diverse environmental conditions (Backhed et al. 2005), which determine their RA across the hosts (Goodrich et al. 2016; Goodrich et al. 2014).

Modification of gut microbiota in response to dietary treatments have been observed also at other taxonomic levels in healthy dogs. Algya et al (2018) reported an increase of genus *Clostridium* in dogs fed with a commercial extruded diet, in comparison to dogs fed with a raw meat diet and a commercial moist diet. On the contrary, Bermingham et al (2017) observed a decrease of *Clostridium* in dogs fed with a commercial extruded diet in comparison to the raw meat diet. Other variations were observed for *Lactobacillus* and *Dorea*, meanwhile *Prevotella*, *Turicibacter*, *Ruminococcus* and *Megamonas* increased in kibble diet group rather than raw meat diet group. A similar result has been reported by Herstad et al. (2017), where *Clostridium* and *Dorea* increased when dogs were fed with a high content of boiled minced beef compare to the control diet based on a commercial extruded diet. Kim et al (2017) confirmed a lower abundance of the family Clostridiaceae in dogs fed with a commercial extruded diet in comparison to a home made diet. Also in a study of Sandri et al (2017), the RAs of *Clostridium* and *Prevotella* were higher in kibble based diet, but *Lactobacillus* and *Megamonas* decreased in dog fed with a meat based diet. *Megamonas* was also significantly higher in another study (Algya et al 2018), where the same comparison was reported. In this study, also the abundances of *Lactococcus*, *Clostridium* and *Fusobacterium* genera increased after the administration of a H diet. Interestingly, *Fusobacterium* showed the highest significant difference in W diet, incomparison to H-B and K diet. Also the Ras of *Prevotella*, *Slackia* and *Collinsella* were similar in W and H-B diets and differed from K diet. At the best of our knowledge, straight comparison on faecal microbiome between a complete moist diet and a complete extruded diet is not reported in literature. These findings require to be confirmed with further studies.

These contradictory results can be due to the variability of the environments in which the studies were carried out, together with the methodological issues previously reported. Moreover, the gut microbiome is an ecosystem that strongly interacts with the host and the variations of the abundances are probably better depicted with a multivariate approach. The prevalence of specific taxa can be effective to identify dysbiosis events, but probably not enough to characterize the conditions of the dogs, as the overall assessment of several, in not all, taxa remains necessary (AlShawaqfeh et al. 2017). Vazquez-Baeza et al (2016) reported that the diversity and structure of microbial community, more than the variation of single taxa, could be used as a signature of the faecal microbiota to separate dogs with IBD from healthy dogs.

The relationship between a dependent variable and several independent variables was investigated with LDA, which identified significant features to separate the database according to the diet factor (Fig 4). Interestingly, the data of H and the B diets were merged in a cluster, even though the composition of these two diets was different (Table 2). It is likely that the presence of raw meat and the physical form of these diets had a similar impact on shaping gut microbiome. To classify dog data on the basis of diet, a RF was used, an approach that was successfully applied to create a urban microbial fingerprint based on microbioma (Ryan 2019) and to identify healthy dogs or affected by inflammatory bowel disease using faecal microbial profile (Vazquez-Baeza et al. 2016). The results showed an excellent classification accuracy (Fig 4) and indicated that some genera are more responsive to dietary factors. A limitation of this classification could be that while 71 dogs changed diet from the T0 sample and the following sampling times, 64 dogs at T14 or T28 received the same diet. For these latter subjects, it would be possible that the two fecal samples from the same dog are very if not completely similar, thus ending up including the same sample twice. However, it should be considered that 19 of these 64 dogs were misclassified by RF, suggesting that other factors rather than diet can shape the faecal microbiome of dogs, also in a relatively short time. Although we are aware that this can be a limitation for the current classification, the aim of this study was to develop a model more than assessing a precise identification of diet responsive and core faecal microbiome in dogs, which indeed deserves further investigations.

3.2.5.2 Sex

Very limited information is available for the variation of gut microbiome in relation to sex in dogs. Coelho et al (2018) did not find significant differences in microbiome composition between male and female and the results are partly in agreement with our study. As can be seen in Fig 5, the main differences were found between whole (F and M) and castrated (FC and MC) dogs, while F and M were more similar. These differences were confirmed by the multivariate approach of LDA (Fig 6) and by the learning machine classifier (Fig 7). Sexdependent effects on the microbiome have been reported in animal models (Markle et al. 2013; Bolnick et al. 2014). In mice, male and female microbiota diverge after puberty, reflecting the sex bias in expression of autoimmune diseases, such as type 1 diabetes. Although the mechanism of sexual influence remains unclear, a bidirectional interaction of microbiota with endocrine status of host is likely, considering that the divergence between male and female microbiota can be reversed by male castration (Markle et al. 2013). Our findings on the effect of sex on gut microbiome are promising and confirm what already emerged for human and other animal models. Despite this, it is still difficult to draw definitive considerations, as the relationship between intestinal microbiome and sex is still poorly studied and not clear.

The study by Markle et al (2013) shows that there is a well-defined structure of the intestinal microbial community that develops and diversifies during sexual maturation, indicating this process as a determining factor. The results of this study are based on animals raised in the same conditions: our results are obtained through a very standardized sample collection, processing and data analysis pipeline, but the subjects were not in the same environment. Despite this, our results agree with that highlighted by other studies (Markle et al. 2013; Bolnick et al. 2014), confirming and strengthening the hypothesis that there is a bidirectional interaction between

microbiota and hormone levels of the host. In the literature it has also been highlighted how castration can reverse the divergence of the microbiome between males and females (Markle et al. 2013). Indeed, although the FC and MC variables have a limited number of subjects, we managed to highlight how much these two variables can actually be superimposed, not discriminating each other; by joining them, we managed to obtain a single class extremely discriminatory towards subjects F and M. Although F and M subjects were underrepresented in H diet and that no MC and only 2 FC dogs were fed with W diet, the strong classification power obtained with RF suggested that sex can play a role in shaping the gut microbiome and thus is a factor that requires to be unraveled with appropriated and deeper investigation.

3.2.6 Conclusion

In this study, a collection of 16S rRNA data were used to investigate if diet composition and sex affected fecal microbial community in healthy dogs. The association of discriminant analysis with learning machine indicated that diet and sex are factors inferring fecal microbiome and that dogs can be clustered on the basis of them. However, the study has some limitations, due to the underrepresentation of dogs in the H diet and in the entire female and male categories. To generalize these preliminary results, a larger dataset is required with the contribution of the scientific community, which can be possible if a basic standardization of the protocols among laboratories will be agreed.

3.2.7 TABLES AND FIGURES

Table 1. Diets X sex contingency table reporting the number of observations.

Diets	Sex				
	F	**FC**	**M**	**MC**	**Total**
B	21	7	10	18	56
H	1	10	3	16	30
K	70	9	37	55	171
W	53	2	28	0	83
Total	145	28	78	89	**340**

F = whole female; FC = spayed female; M = whole male; MC = castrated male; B = Base diet; H = home-made diet; K = commercial extruded complete diet; W = commercial moist complete diet.

Table 2. Mean chemical composition and energy content of the diets fed to the dogs. Details of the diet of each dietary intervention study are reported in the Supplementary Table S2.

Item	Unit	Diet			
		Kibble	Moist	Home made	Base
Crude protein	%/DM	27.2	27.6	36.3	30.1
Lipids	%/DM	15.3	20.0	27.2	22.8
Crude fiber	%/DM	3.3	1.0	1.9	1.2
Ash	%/DM	7.2	4.5	11.0	4.2
NFE	%/DM	47.0	47.0	23.7	41.8
ME	kcal/kg DM	4124	4530	4589	4667

ME = Metabolizable energy; K = commercial extruded complete diet; W = commercial moist complete diet; H = home-made diet; B = Base diet.

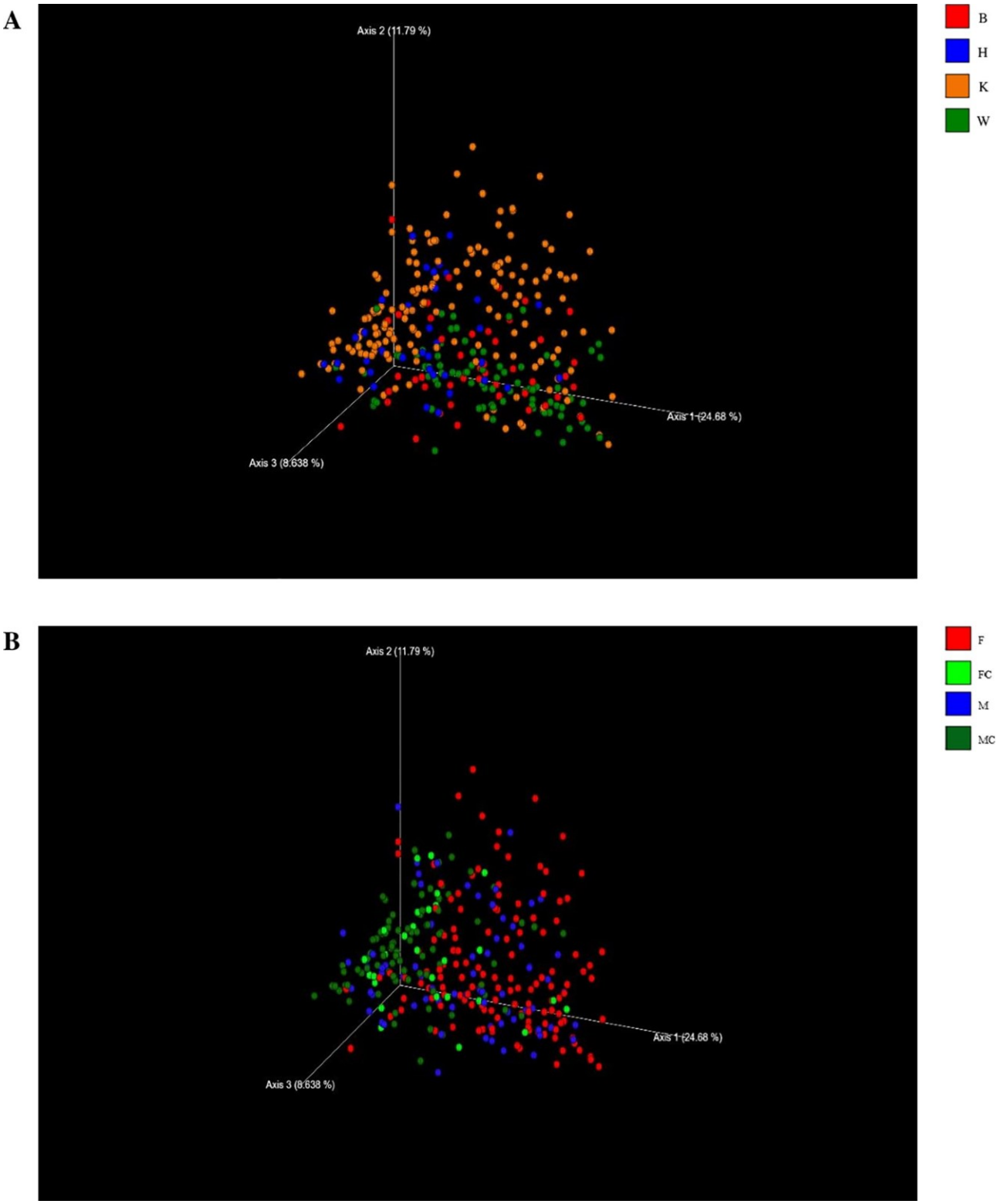

Fig 1. Principal Coordinate Analysis (PCoA) of microbial community from the fecal samples of dogs included in the database; each dot represents a different subject. The 3D PCoA plot was based on weighted UniFrac distances of 16 r RNA gene. (**A**) Subjects are divided in classes based on their different diets. W (green dots) = commercial moist complete diet; K (orange dots): commercial extruded complete diet; H (blue dots) = home-made diet; B (red dots) = Base diet. PERMANOVA confirmed the differences between the four groups of diets for $P < 0.05$. (**B**) Dogs are divided into groups of different sex. F (red dots) = whole females subjects; M (blue dots) = whole males subjects; FC (light green dots) = spayed females subjects; MC (dark green dots) = neutered males subjects. PERMANOVA confirmed the differences between F, M and castrated subjects for $P < 0.05$, but not between FC and MC.

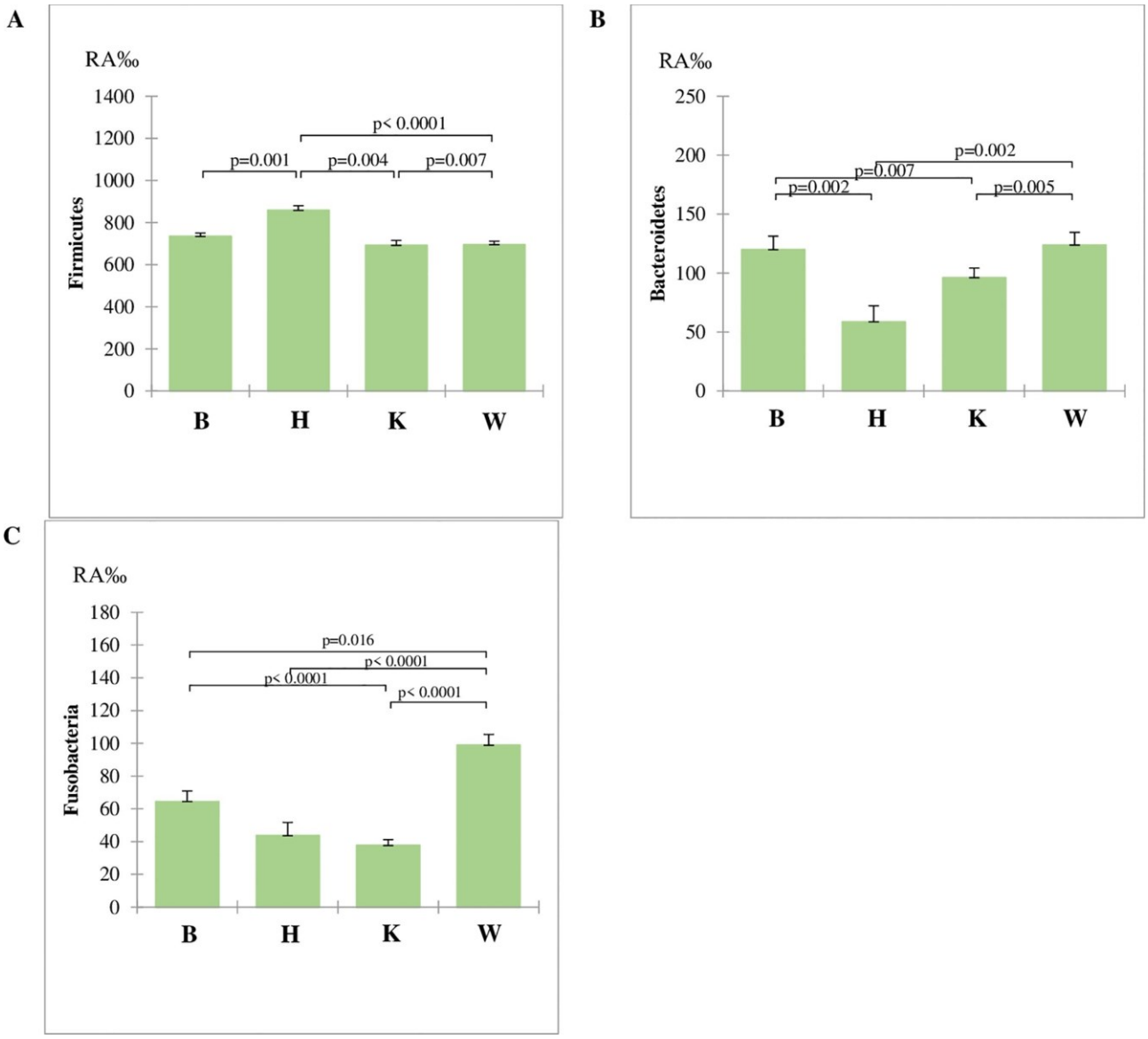

Fig 2. Relative Abundances (RA) for the factor diets of the three represented phyla in the fecal microbiota. RA were compared with the Kruskal-Wallis nonparametric test: (**A**) Firmicutes; (**B**) Bacteroidetes; (**C**) Fusobacteria. Data are reported as mean and standard error. W = Commercial moist complete diet; K = Commercial extruded complete diet; H = Home-made diet; B = Base diet.

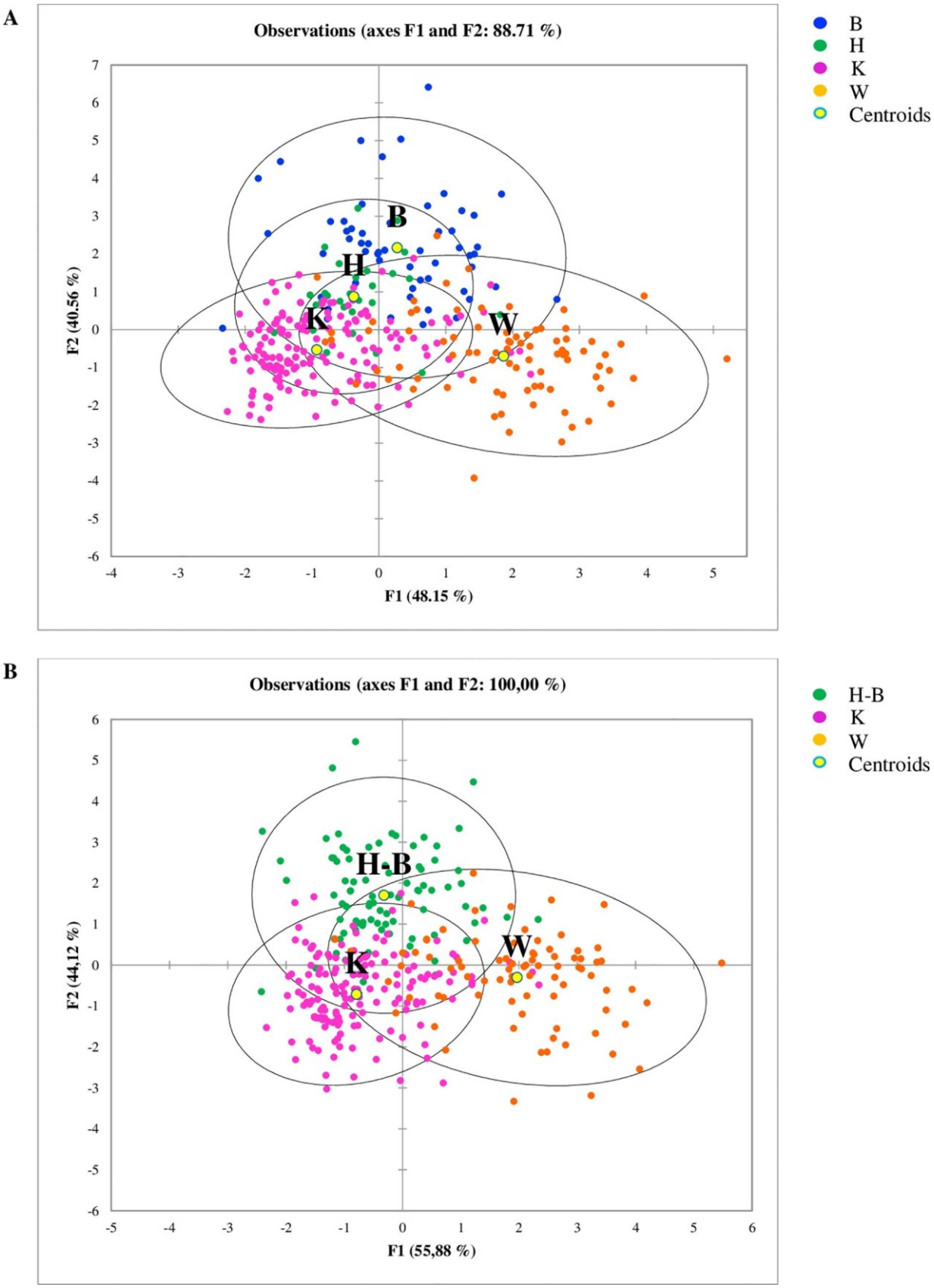

Fig 3. Linear Discriminant Analysis (LDA) based on relative abundances of bacteria genera, showing the clustering of the samples according to the diets. (**A**) result of the LDA using four categories diets, showing that H and B diets are close to each other; (**B**) result of the LDA where H and B diets were collapsed in one group (H-B). W =

Commercial moist complete diet; K = Commercial extruded complete diet; H = Home-made diet; B = Base diet; H-B = home-made diet and Base diet collapsed together.

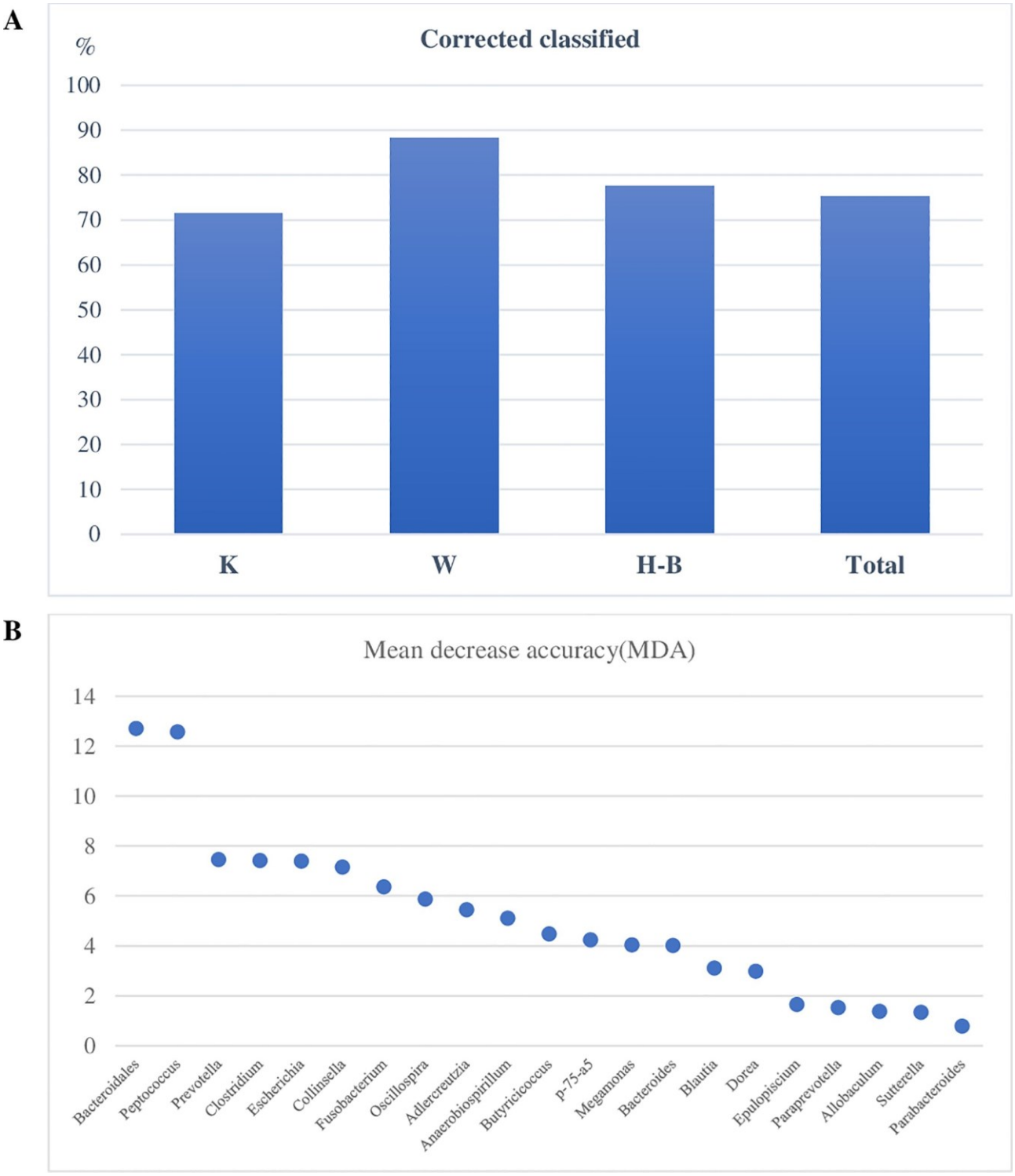

Fig 4. Random Forest (RF) classification of dogs according to the diets based on relative abundances of bacteria genera in the feces. (**A**) percentage of dogs corrected classified based on Out Of Bag data. (**B**) discriminatory power of the genera important for the RF classification of dogs within diets. W = commercial moist complete diet; K = commercial extruded complete diet; H-B = home-made diet and Base diet collapsed together.

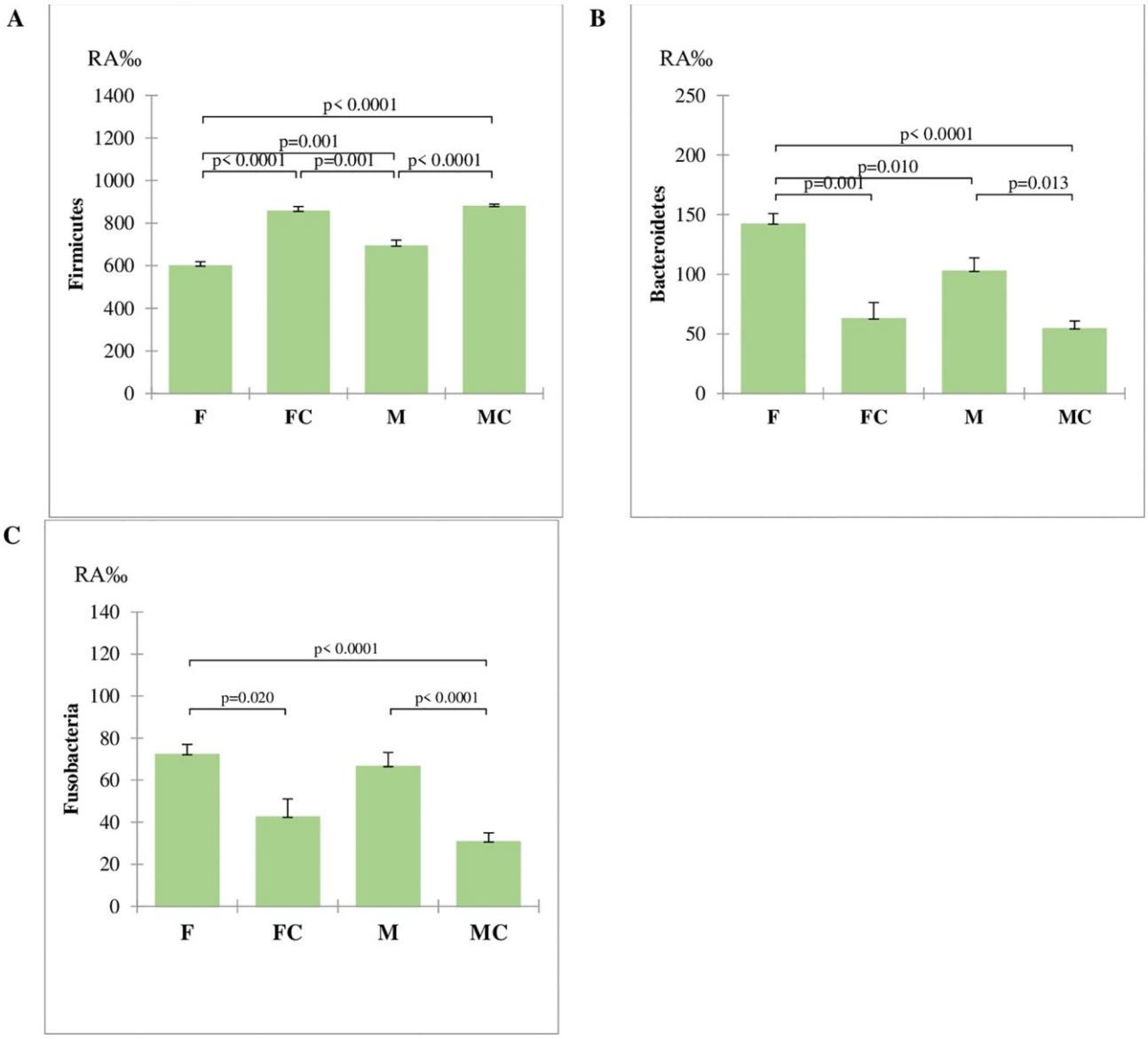

Fig 5. Relative Abundances (RA) for the category sex of the three represented phyla in the fecal microbiota. RA were compared with the Kruskal-Wallis nonparametric test: (**A**) Firmicutes; (**B**) Bacteroidetes; (**C**) Fusobacteria. Data are reported as mean and standard error. F = whole females subjects; M = whole males subjects; FC = spayed females subjects; MC = neutered males subjects.

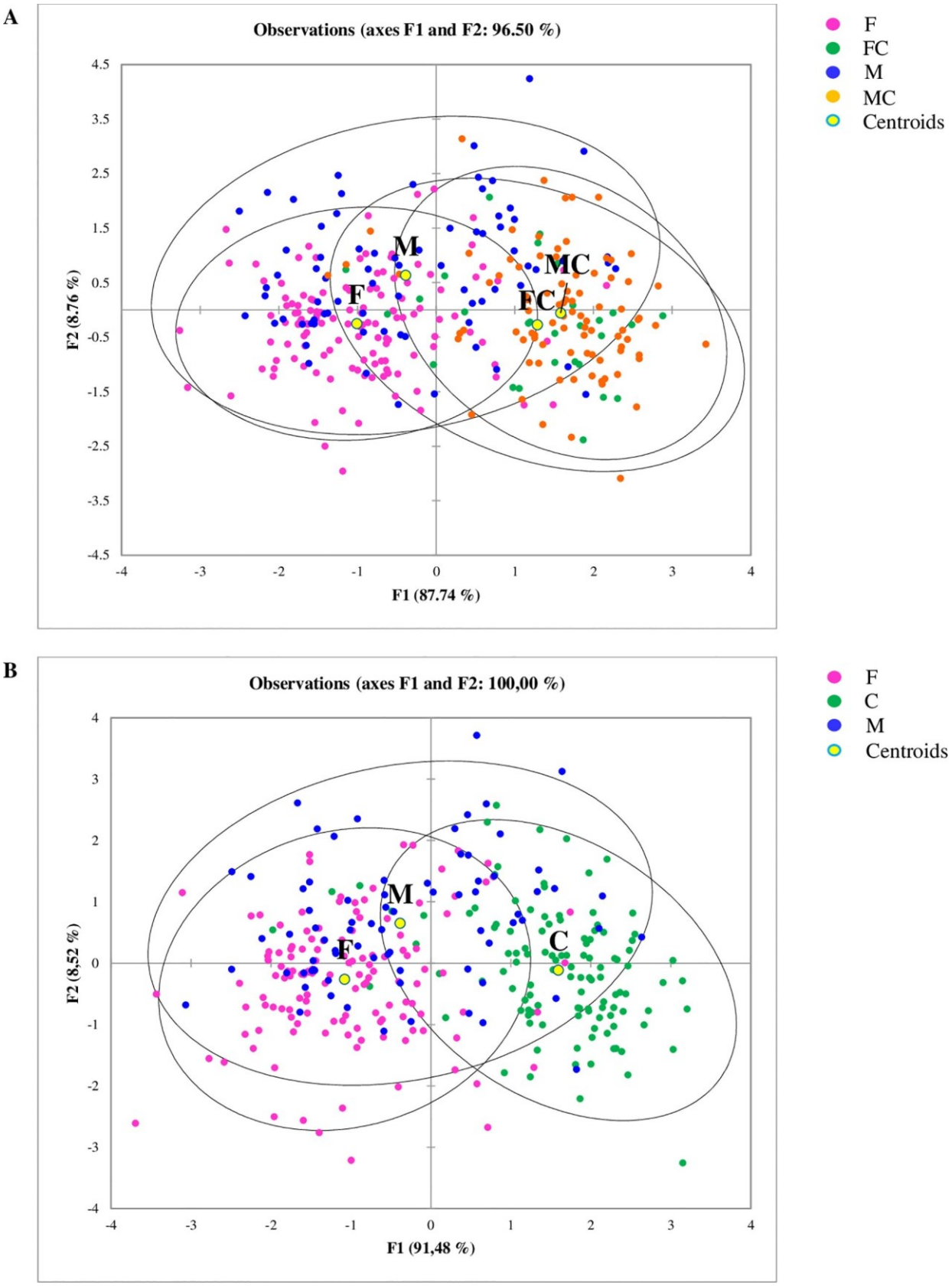

Fig 6. Linear Discriminant Analysis (LDA) based on relative abundances of bacteria genera, showing the clustering of the samples according to the sex. **(A)** result of the LDA using four categories of sex, showing that FC and MC sex are close to each other; **(B)** result of the LDA where FC and MC sex were collapsed in one group. F = whole females

subjects; M = whole males subjects; FC = spayed females subjects; MC = neutered males subjects; C = spayed female subjects and neutered male subjects collapsed together.

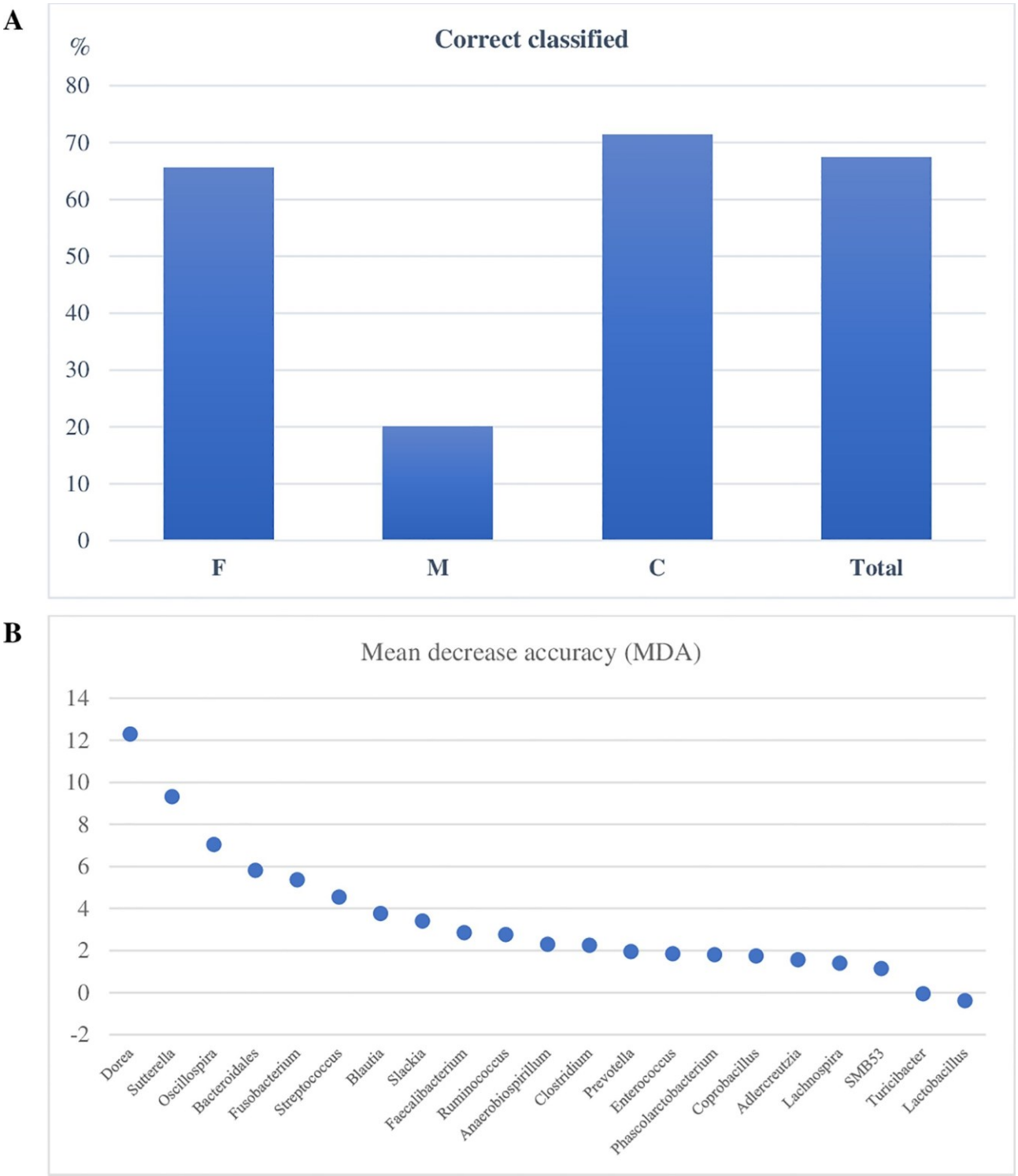

Fig 7. Random Forest (RF) classification of dogs according to the sex based on relative abundances of bacteria genera in the feces. (**A**) percentage of dogs corrected classified based on Out Of Bag data; (**B**) discriminatory power of the genera important for the RF classification of dogs within sex. F = whole females subjects; M = whole males subjects; C = spayed female subjects and neutered male subjects collapsed together.

3.2.8 References

Addinsoft. 2020. "XLSTAT statistical and data analysis solution". Boston, USA.

Algya KM, Cross TL, Leuck KN, Kastner ME, Baba T, Lye L, et al. 2018. "Apparent total-tract macronutrient digestibility, serum chemistry, urinalysis, and fecal characteristics, metabolites and microbiota of adult dogs fed extruded, mildly cooked, and raw diets". *J Anim Sci.* 2018; 96(9):3670–83. https://doi.org/10.1093/jas/sky235

Allali I, Arnold JW, Roach J, Cadenas MB, Butz N, Hassan HM, et al. 2017. "A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome". *BMC Microbiol.* 17(1):194. https://doi.org/10.1186/s12866-017-1101-8.

AlShawaqfeh MK, Wajid B, Minamoto Y, Markel M, Lidbury JA, Steiner JM, et al. 2017. "A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy". *FEMS Microbiol Ecol.* 93(11). https://doi.org/10.1093/femsec/fix136

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. 2011. "Enterotypes of the human gut microbiome". *Nature.* 473(7346):174–80. https://doi.org/10.1038/nature09944

Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. 2005. "Host-bacterial mutualism in the human intestine". *Science.* 307(5717):1915–20. https://doi.org/10.1126/science.1104816

Bermingham EN, Maclean P, Thomas DG, Cave NJ, Young W. 2017. "Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs". *PeerJ.* 5:e3019. https://doi.org/10.7717/peerj.3019

Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, Parks B, et al. 2014. "Individual diet has sex-dependent effects on vertebrate gut microbiota". *Nat Commun.* 5:4500. https://doi.org/10.1038/ncomms5500

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. 2019. "Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2". *Nat Biotechnol.* 37(8):852– 7. https://doi.org/10.1038/s41587-019-0209-9

Coelho LP, Kultima JR, Costea PI, Fournier C, Pan Y, Czarnecki-Maulden G, et al. 2018. "Similarity of the dog and human gut microbiomes in gene content and response to diet". *Microbiome.* 6(1):72. https:// doi.org/10.1186/s40168-018-0450-3

Deng P, Swanson KS. 2015. "Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges". *Br J Nutr.* 113 Suppl:S6–17. https://doi.org/10.1017/S0007114514002943.

El Kaoutari A, Armougom F, Gordon JI, Raoult D, Henrissat B. 2013. "The abundance and variety of carbohydrate-active enzymes in the human gut microbiota". *Nat Rev Microbiol.* 11(7):497–504. https://doi. org/10.1038/nrmicro3050

Garcia-Mazcorro JF, Barcenas-Walls JR, Suchodolski JS, Steiner JM. 2017. "Molecular assessment of the fecal microbiota in healthy cats and dogs before and during supplementation with fructo-oligosaccharides (FOS) and inulin using high-throughput 454-pyrosequencing". *PeerJ*. 5:e3184. https://doi. org/10.7717/peerj.3184

Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. 2016. "Genetic Determinants of the Gut Microbiome in UK Twins". *Cell Host Microbe*. 19(5):731–43. https://doi.org/10.1016/j. chom.2016.04.017

Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. 2014. "Human genetics shape the gut microbiome". *Cell*. 159(4):789–99. https://doi.org/10.1016/j.cell.2014.09.053

Guard BC, Suchodolski JS. 2016. "HORSE SPECIES SYMPOSIUM: Canine intestinal microbiology and metagenomics: From phylogeny to function". *J Anim Sci*. 94(6):2247–61. https://doi.org/10.2527/jas. 2015-0029

Gupta VK, Paul S, Dutta C. 2017. "Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity". *Front Microbiol*. 8:1162. https://doi.org/10.3389/fmicb.2017. 01162

Hand D, Wallis C, Colyer A, Penn CW. 2013. "Pyrosequencing the canine faecal microbiota: breadth and depth of biodiversity". *PLoS One*. 8(1):e53115. https://doi.org/10.1371/journal.pone.0053115

Herstad KM, Gajardo K, Bakke AM, Moe L, Ludvigsen J, Rudi K et al. 2017. "A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs". *BMC Vet Res*. 13(1):147. https://doi.org/10.1186/s12917-017-1073-9

Kim J, An JU, Kim W, Lee S, Cho S. 2017. "Differences in the gut microbiota of dogs (Canis lupus familiaris) fed a natural diet or a commercial feed revealed by the Illumina MiSeq platform". *Gut Pathog*. 9:68. https://doi.org/10.1186/s13099-017-0218-5

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. 2013. "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies". *Nucleic Acids Res*. 41(1):e1. https://doi.org/10.1093/nar/gks808

Le Sciellour M, Renaudeau D, Zemb O. 2019. "Longitudinal Analysis of the Microbiota Composition and Enterotypes of Pigs from Post-Weaning to Finishing". *Microorganisms*. 7(12). https://doi.org/10.3390/ microorganisms7120622

Lozupone C, Knight R. 2005. "UniFrac: a new phylogenetic method for comparing microbial communities". *Appl Environ Microbiol*. 71(12):8228–35. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Lu D, Tiezzi F, Schillebeeckx C, McNulty NP, Schwab C, Shull C, et al. 2018. "Host contributes to longitudinal diversity of fecal microbiota in swine selected for lean growth". *Microbiome*. 6(1):4. https://doi.org/ 10.1186/s40168-017-0384-1

Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. 2013. "Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity". *Science*. 339 (6123):1084–8. https://doi.org/10.1126/science.1233521

Mobeen F, Sharma V, Tulika P. 2018. "Enterotype Variations of the Healthy Human Gut Microbiome in Different Geographical Regions". *Bioinformation*. 14(9):560–73. https://doi.org/10.6026/ 97320630014560

Mori A, Goto A, Kibe R, Oda H, Kataoka Y, Sako T. 2019. "Comparison of the effects of four commercially available prescription diet regimens on the fecal microbiome in healthy dogs". *J Vet Med Sci*. 81 (12):1783–90. https://doi.org/10.1292/jvms.19-0055

Niu Q, Li P, Hao S, Kim SW, Du T, Hua J, et al. 2019. "Characteristics of Gut Microbiota in Sows and Their Relationship with Apparent Nutrient Digestibility". *Int J Mol Sci*. 20(4). https://doi.org/10.3390/ ijms20040870

Park HJ, Lee SE, Kim HB, Isaacson RE, Seo KW, Song KH. 2015. "Association of obesity with serum leptin, adiponectin, and serotonin and gut microflora in beagle dogs". *J Vet Intern Med*. 29(1):43–50 https://doi.org/10.1111/jvim.12455

Peker N, Garcia-Croes S, Dijkhuizen B, Wiersma HH, van Zanten E, Wisselink G, et al. 2019. "A Comparison of Three Different Bioinformatics Analyses of the 16S-23S rRNA Encoding Region for Bacterial Identification". *Front Microbiol*. 10:620. https://doi.org/10.3389/fmicb.2019.00620

Pilla R, Suchodolski JS. 2019. "The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease". *Front Vet Sci*. 6:498. https://doi.org/10.3389/fvets.2019.00498

Ribeiro EM, Peixoto MC, Putarov TC, Monti M, Pacheco PDG, Loureiro BA, et al. 2019. "The effects of age and dietary resistant starch on digestibility, fermentation end products in faeces and postprandial glucose and insulin responses of dogs". *Arch Anim Nutr*. 73(6):485–504. https://doi.org/10.1080/ 1745039X.2019.1652516

Ryan FJ. 2019. "Application of machine learning techniques for creating urban microbial fingerprints". *Biol Direct*. 14(1):13. https://doi.org/10.1186/s13062-019-0245-x

Sandri M, Dal Monego S, Conte G, Sgorlon S, Stefanon B. 2017. "Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs". *BMC Vet Res*. 13(1):65. https:// doi.org/10.1186/s12917-017-0981-z

Sandri M, Manfrin C, Pallavicini A, Stefanon B. 2014. "Microbial biodiversity of the liquid fraction of rumen content from lactating cows". *Animal*. 8(4):572–9. https://doi.org/10.1017/S1751731114000056

Sandri M, Sgorlon S, Conte G, Serra A, Dal Monego S, Stefanon B. 2019. "Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs". *Ital J Anim Sci*. 18(1):1205–14. https://doi.org/10.1080/1828051X.2019.1645624

Sandri M, Sgorlon S, Scarsella E, Stefanon B. 2020. "Effect of different starch sources in a raw meat-based diet on fecal microbiome in dogs housed in a shelter". *Anim Nutr*. 6(3): 353-361. https://doi.org/10.1016/j.aninu.2020.03.003.

Scarsella E, Cintio M, Iacumin L, Ginaldi F, Stefanon B. 2020. "Interplay between Neuroendocrine Biomarkers and Gut Microbiota in Dogs Supplemented with Grape Proanthocyanidins: Results of Dietary Intervention Study". *Animals*. 10:531. https://doi.org/10.3390/ani10030531

Suchodolski JS, Camacho J, Steiner JM. 2008. "Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis". *FEMS Microbiol Ecol*. 66(3):567–78. https://doi.org/10.1111/j.1574-6941.2008.00521.x

Suchodolski JS. 2011. "Intestinal microbiota of dogs and cats: a bigger world than we thought". *Vet Clin North Am Small Anim Pract*. 41(2):261–72. https://doi.org/10.1016/j.cvsm.2010.12.006

The HMP Project Consortium. 2012. "Function and diversity of the healthy human microbiome". *Nature*. 486(7402):207–14. https://doi.org/10.1038/nature11234

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. "An obesity-associated gut microbiome with increased capacity for energy harvest". *Nature*. 444(7122):1027–31. https://doi. org/10.1038/nature05414

Vazquez-Baeza Y, Hyde ER, Suchodolski JS, Knight R. 2016. "Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks". *Nat Microbiol*. 1:16177. https://doi.org/10. 1038/nmicrobiol.2016.177

Videnska P, Smerkova K, Zwinsova B, Popovici V, Micenkova L, Sedlar K, et al. 2019. "Stool sampling and DNA isolation kits affect DNA quality and bacterial composition following 16S rRNA gene sequencing using MiSeq Illumina platform". *Sci Rep*. 9(1):13837. https://doi.org/10.1038/s41598-019-49520-3

Vilson A, Ramadan Z, Li Q, Hedhammar A, Reynolds A, Spears J, et al. 2018. "Disentangling factors that shape the gut microbiota in German Shepherd dogs". *PLoS One*. 13(3):e0193507. https://doi.org/ 10.1371/journal.pone.0193507

CHAPTER 4

4.1 Introduction to the study

The use of prebiotics and probiotics in the diet of humans is now well established for the treatment of intestinal dysbiosis and, consequently, gastrointestinal diseases. Unfortunately, currently there is still not enough information regarding the use of prebiotics and probiotics in the diet of pets and livestock; even less information is available regarding the use of proanthocyanidins deriving from grapevine, which are part of the polyphenols category. This category of molecules passes almost completely unabsorbed in the small intestine, thus remaining accessible to the metabolic activity of the gut microbial population.

The gut microbiome interacts with the tryptophan metabolism of the host, and therefore influences its availability for the synthesis of serotonin. Serotonin is a compound involved in the connection of the emotional and cognitive centers of the brain to the intestine and its microbiome. Serotonin is also involved in the regulation of intestinal motility, as it is secreted by enterochromaffin cells of the intestinal epithelium. In this way it regulates the rate of food transit and, indirectly, the bacterial metabolic activity.

In addition to the interaction of the microbiome with the metabolism of tryptophan, the microbial population of the GI tract also produces other metabolites that affect the central nervous system. For example, the microbiome can synthesize molecules such as gamma acetyl butyric acid (GABA) and short-chain fatty acids (SCFA), which have direct effects, not only on the intestinal epithelium, but also on the local mucosal immune system and on the vagus nerve.

This study aims to verify the effect of the addition of proanthocyanidins in different doses in the diet of dogs, on the gut microbiome and on the metabolites produced by the metabolic activity of the microbial population, with particular regard to the measurement of serotonin and salivary cortisol. In fact, the determination of these two compounds in saliva could increase the adoption of new molecular markers for the determination of intestinal dysbiosis through simpler and faster analyzes.

The study involved the recruitment of 24 healthy dogs, equally distributed between males and females, who lived in the same environment, and therefore easily controlled from an experimental point of view. The dogs were divided into three groups, depending on the diet administered. The first group received a complete diet with all the nutrients necessary for the dog's energy maintenance, but without polyphenols. The second group received the same diet as the control group, with the addition of proanthocyanidins according to the dose of 1 mg/kg of live body weight. The third group received the diet of the control group, with polyphenols added according to the dose of 3 mg/kg of live body weight. The study lasted 28 days, where dog feces were collected at the beginning, middle and end of the study. The saliva, on the other hand, was collected at the beginning and end of the study.

A shift in the microbiome was observed, with an increase in abundance of the genera *Escherichia* and *Eubacterium* in dogs fed with the diet containing the lowest dose of polyphenols, and of the genera *Fusobacterium* and *Phascolarctobacterium* in dogs fed with the diet containing the highest dose of polyphenols. Regarding saliva serotonin and cortisol levels, a significant increase in serotonin was observed at the end of the study in both groups of dogs that were fed the basic diet with added polyphenols, while no significant changes were observed in salivary cortisol levels.

Only in a few bacterial species the fermentative and metabolic action against polyphenols has been studied. This activity has been observed to depend on the daily dose of administration and on the individual variability of the gut microbial community. This fact leads us to think that individual differences also bring to differences in the bioavailability and bioactivity of the metabolites deriving from polyphenols. In fact, this study reported that the addition of proanthocyanidins influenced the intestinal microbiome of healthy dogs, even though the differences were not substantial, and this was probably due to a low dosage of polyphenols in experimental diets. In addition, the results obtained also highlighted a high individual variability in terms of relative abundances of the microorganisms that constitute the intestinal microbiome. On the other hand, salivary molecular biomarkers were more influenced by the variation in diet, and further studies are needed to establish whether this variation is due to the gut microbiome.

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4.2 Interplay between Neuroendocrine Biomarkers and Gut Microbiota in Dogs Supplemented with Grape Proanthocyanidins: Results of Dietary Intervention Study

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4.2.1 Abstract

Several studies on the interaction between gut microbiota and diets, including prebiotics, have been reported in dogs, but no data are available about the effects of dietary administration of grape proanthocyanidins. In the study, 24 healthy adult dogs of different breeds were recruited and divided in 3 groups of 8 subjects each. A group was fed with a control diet (D0), whilst the others were supplemented with 1 (D1) or 3 (D3) mg/kg live weight of grape proanthocyanidins. Samples of feces were collected at the beginning and after 14 and 28 days for microbiota, short chain fatty acid, and lactic acid analysis. Serotonin and cortisol were measured in saliva, collected at the beginning of the study and after 28 days. A significantly higher abundance ($p < 0.01$) of *Enterococcus* and *Adlercreutzia* were observed in D0, whilst *Escherichia* and *Eubacterium* were higher in D1. *Fusobacterium* and *Phascolarctobacterium* were higher ($p < 0.01$) in D3. Salivary serotonin increased ($p < 0.01$) at T28 for D1 and D3 groups but cortisol did not vary. Proanthocyanidins administration influenced the fecal microbiota and neuroendocrine response of dogs, but a high variability of taxa was observed, suggesting a uniqueness and stability of fecal microbiota related to the individual.

4.2.2 Introduction

The interaction of intestinal microbiota with the host has attracted the scientific community, and a large body of research has been published to highlight the coevolution of anatomical, physiological, immunological, and developmental functions of host and microbiota (Gilbert, Sapp &Tauber 2012). Other studies have underpinned the interplay between gut microbes and their products of fermentation with the host, not only from a nutritional point of view (LeBlanc et al. 2013) but also for the modulation of immunological, endocrinal, and neurological functions (Round & Mazmanian 2009).

The bidirectional link between the host brain and the gut microbiota relies mainly on the neural communication of the central nervous system (CNS) with the periphery and on the humeral communications (Jenkins et al. 2016). Neural communications involve the vagus nerve and the dorsal root ganglia of the small and large intestine, through projections from the enteric nervous system to sympathetic ganglia and parasympathetic innervation of the gut. Humeral communications also depend on products of microbial activity, cytokines, and hormones.

Among the compounds that are involved in the connection of the emotional and cognitive centers of the brain with the gut and its resident microbiota, serotonin plays a paramount role. Other than being a neurotransmitter within the CNS, serotonin is secreted by the enterochromaffin cells of the intestinal epithelium and stimulates gut motility (Jenkins et al. 2016), regulating the transit time of the food and then the extent of bacterial fermentation and the amount of end products of fermentation.

Gut microbiota interacts with tryptophan metabolism and influences its amount available for serotonin synthesis, thus interacting with the serotonergic system (O'Mahony et al. 2015). The modification of tryptophan metabolism, reported for instance for irritable bowel syndrome, implies a serotonin deficiency,

which leads to a depressive mood, or to the production of neurotoxic/neuroprotective metabolites that have the CNS as target (Kennedy et al. 2014). Furthermore, microbial populations synthesize other signal molecules, such as GABA and short-chain fatty acids (SCFAs), which have effects on the gut epithelium, on the local mucosal immune system, and on the vagus nerve (Forsythe, Kunze & Bienenstock 2016). Perturbation of gut microbiota also induce the modulation of other neurotransmitters and signal molecules (e.g., dopamine, cytokines, interleukins, and Tumor Necrosis Factor (TNF) from the gastrointestinal tract, which can in turn activate a neuroendocrine response, as the hypothalamus adrenal pituitary axis (HPA), or can modulate the metabolism of tryptophan, inferring with the serotonergic system (Forsythe, Kunze & Bienenstock 2016).

The use of food additives as prebiotic, probiotic, and synbiotic offers a therapeutic approach to restore the gastrointestinal and microbial balance (de Souza et al. 2019), but less information is available for other bioactive compounds, as polyphenols. In plants, polyphenols are in a glycosylate form and after ingestion by the organism they are recognized as xenobiotics; therefore, their catabolized bioavailability is reduced in comparison with the common nutrients (Monagas et al. 2010). It has been estimated that only 5%–10% of total polyphenol intake is absorbed in the small intestine, while the remaining 90%–95% may be accumulated in the colon lumen where it is consequently processed by the enzymatic activities of the gut microbial population (Cardona et al. 2013). Therefore, it is likely that microorganisms populating the gut can be involved in the absorption of polyphenolic structure, thanks to the conversion in low-molecular-weight compounds. Consequently, evidence suggests that the health benefits of polyphenols are related not only to the original molecules found in plants but also to their intermediates and end-products, given the greater bioavailability compared to the parent molecules (Russell et al. 2009). However, the metabolism of polyphenols has been reported only for few microbial species of the gut (Kutschera et al. 2011), which depends on the daily dose and on the individual variability of the gut microbiota community. Hence, inter individual differences in the composition of the gut microbiota may lead to variations of bioavailability and bioactivity of polyphenols metabolites (Gross et al. 2010). Nevertheless, recent studies have shown that both polyphenols and polyphenols-derived molecules are able to shape hindgut microbiota through selective prebiotic effects and antimicrobial activities against pathogenic microorganisms (Cardona et al. 2013).

The aim of the study is to evaluate the activity of titrated proanthocyanidins extracted from grape on the gut microbiota of dogs and the relationship with endocrine responses measured in saliva.

The study was performed with resident dogs housed in a shelter and fed a standard diet supplemented with two doses of grape proanthocyanidins.

4.2.3 Materials and Methods

4.2.3.1 Animals and Housing

Twenty-four healthy adult dogs of different breeds were recruited for the study (Table S1). The group consisted of 18 castrated and 3 intact males and 3 spayed females. Dogs were housed in the same shelter and allocated in pairs in different boxes of 6 × 3 m enclosures, with a 2 × 3 m roof covering the paved portion of the pen and equipped with beds. Dogs were fed in the box at around 16:00 p.m., and water was always available. The study started in June in Northeast Italy (46.029051 N; 13.231521 E), with an average temperature during the period of 20–30 °C and 60%–70% relative humidity. At the beginning of the study, dogs were weighed and the average live weight was 28 ± 9 kg.

All protocols, procedures, and the care of the animals complied with the Italian legislation on animal care (DL n.116, 27/1/1992) and the study was approved by the ethical committee of the University of Udine.

4.2.3.2 Experimental Design

From at least 90 days from the beginning of the study and during the period, dogs were fed commercial extruded diet, formulated with beef meat, cereals and potato, chicken fat, beet pulp, flax seeds, salmon oil, yeast, minerals, and vitamins. The chemical composition was 90% dry matter, 26.2% crude protein, 15.6% crude lipid, 2.3% crude fiber, and 9.1% ash.

The recruited dogs did not receive antibiotic treatments or probiotic supplementations, at least two months before the experiment started. Moreover, before the beginning of the study, dogs were divided in 3 groups of 8 individuals each, matched for live weight and including 1 female per group. During the study, dogs were housed in the usual boxes. The first group received placebo tablets without polyphenols (D0), whilst the second group and the third group were supplemented with tablets containing 1 mg/kg of live weight (D1) or 3 mg/kg of live weight (D3) of dried extract of grape polyphenols (ARDA Natura, Fiorenzuola D'Arda, PC, Italy), standardized to >95% of proanthocyanidins. The D0, D1, and D3 tablets were produced by Tecnozoo s.r.l. (Torreselle di Piombino Dese, Padova Italy), with barley malt extract, dextrose, sucrose, magnesium stearate, and E554 (sodium silicate and synthetic aluminum) as additives.

3.2.3.3 Collection of Samples

For both the studies, stool and saliva samples were collected before the meal at the beginning of treatment (T0), after 14 days (T14), and at the end of the trial, after 28 days (T28). The feces were collected after evacuation from the ground using sterile gloves and were placed in 50 mL sterile tubes and immediately frozen at −20 °C until analysis. Salivary samples were collected with SalivaBio swabs (Salimetrics, LLC 101 Innovation Boulevard, State College, PA, US) following the procedure previously described (Sandri et al. 2015). After sampling, the swabs were introduced into tubes specifically designed to avoid cortisol sequestration (Salivette; no. 51.1534,

Sarstedt, Nümbrecht, Germany), temporally stored in an iced box before the final storage at −20 °C, until analysis. The hair samples were collected from the neck of the dogs at the beginning of the study and the regrowth was sampled after 28 days. Hair samples were introduced in a paper envelop to avoid condensation and stored at room temperature until analysis.

4.2.3.4 Short Chain Fatty Acids and Lactic Acid Analysis in Feces

The analysis of lactic acid and short chain fatty acids (SCFAs) (acetic; propionic; butyric) were performed by HPLC according to the following procedure: 1 g of fecal material was diluted with 50 mL of 0.1 N H_2SO_4 aqueous solution and homogenized for 15 min by a mechanical stirrer (Instruments Srl, Milano, Italia). The mix was centrifuged (20000× *g* for 20 min at 4 °C) to separate the liquid phase from the solid residuals, and the liquid phase was subsequently microfiltered with 0.45 µm syringe filter of polypore (Alltech, Casalecchio di Reno BO, Italy). A total of 20 µL of the resulting sample was directly injected in the HPLC instrument using an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm, 9 µm) and a precolumn (Bio-Rad, Hercules, CA, USA) kept at 40 °C. The isocratic elution flux was 0.6 mL/min and using 0.008 N H_2SO_4 solution as a mobile phase the detection length was 220 nm. The concentration of SCFAs (acetic, propionic, butyric, isobutyric, valeric and isovaleric) and lactic acid of fecal samples was measured by HPLC according to the protocol described by Sandri et al. (2017). SCFAs and lactic acid concentrations were calculated with reference to a standard solution of 4.50 mg/mL of lactic acid, 5.40 mg/mL of acetic acid, 5.76 mg/mL of propionic acid, 7.02 mg/mL of butyric acid and isobutyric acid, 8.28 mg/mL of valeric acid, and isovaleric acid in 0.1 N H_2SO_4 (Sigma–Aldrich® Co., Milan, Italy). Quantifications were calculated using an external calibration curve based on these standards. The sum of SCFAs and lactic acid was calculated and the single acid was expressed as molar percentage of the total acids (TA).

4.2.3.5 Fecal DNA Extraction, Sequencing, and Taxonomic Annotation

Microbial DNA from fecal samples was extracted from 150 mg of starting material using a Fecal DNA MiniPrep kit (Zymo Research; Irvine, CA, US), following the manufacturer's instruction, including a bead beating step. DNA concentration was measured with a QubitTM 3 Fluorometere (Thermo Scientific; Waltham, MA, USA), then DNA was fragmented and the 16S rRNA of V3 and V4 regions amplified for library preparation, adding also the Indexes for sequencing, using a Nextera DNA Library Prep kit (Illumina; San Diego, CA, USA), following manufacturer's instruction and primers (Klindworth et al. 2013). The resulting amplicons were sequenced with a MiSeq (Illumina; San Diego, CA, USA) in 2 × 300 paired-end mode, following the standard procedures.

The Quantitative Insights into Microbial Ecology (QIIME 2) (Bolyen et al. 2019) was used to process the raw sequences, which were uploaded to NCBI Sequence Read Archive (Bioproject ID PRJNA564012). After demultiplexing, sequenced reads that passed the quality check (Phred score ≥30) were annotated for 16S rRNA against the most recent Greengenes database (version gg.13_8.otus.tar.gz), with 99% identifying with reference sequences. Chimeras were also detected and then filtered from the reads, and the remaining sequences were clustered into exact sequence variants by using an open reference approach in QIIME 2. This procedure is the preferred

strategy for exact sequence variants picking in QIIME2, which includes taxonomy assignment, sequence alignment, and tree-building steps.

4.2.3.6 Quantitative Real-time PCR (qPCR)

Quantification of total bacteria and of taxa used to describe dysbiosis index in feces, namely, *Faecalibacterium* spp., *Fusobacteria*, *Blautia* spp., *Turicibacter* spp., *Escherichia coli*, *Clostridium hiranonis*, and *Streptococcus* spp., were evaluated by qPCR using the oligonucleotides tested by AlShawaqfeh et al. (2017).

The qPCR data for *Faecalibacterium* spp., *Fusobacteria*, *Blautia* spp., *Turicibacter* spp., *Escherichia coli*, *Clostridium hiranonis*, and *Streptococcus* spp were normalized to the qPCR data for total bacteria and therefore expressed as percentage of the total bacteria. All samples were run in triplicate. SYBR-based qPCR assays were performed following the run protocol reported by AlShawaqfeh et al. (2017) [18] with some modifications. Briefly, SYBR-based reaction mixtures (total 10 µL) contained 5 µL of SsoFast™ EvaGreen® supermix (BioradLaboratories, US), 1.6 µL of water, 0.4 µL of each primer (final concentration: 400 nM), and 2 µL of DNA previously standardized at 25 ng/µL. PCR conditions were 95 °C for 2 min, and 40 cycles at 95 °C for 5 and 10 s at the optimized annealing temperature. A melt curve analysis was performed for SYBR-based qPCR assays under the following conditions: 1 min at 95 °C, 1 min at 55 °C, and 80 cycles of 0.5 °C increments (10 sec each). A RotorGene Q thermal cycler (Qiagen, Germany) was used for all qPCR assays. Data are expressed as average values and standard deviations.

4.2.3.7 Endocrine Analysis of Saliva and Hair Samples

For cortisol analysis in the hair, the method described by Accorsi et al. (2008) was used, with minor modifications (Sgorlon et al. 2019). Briefly, 150 mg of hair were weighted from each sample and placed into 15 mL glass vial. Samples were washed three times with 2.5 mL of isopropanol (2-propanol 99.5%, Sigma Aldrich, Milan, Italy) and 3 min of vortex. Isopropanol was discarded after each wash, and after the final wash, hair samples were placed on a plastic disk and let dry for 48 hours at room temperature. Dried hair samples were trimmed with a blade, and 50 mg of trimmed hair were weighed and placed into a 15 mL glass centrifuge tube with 5 ml of methanol. Samples were incubated in water bath at 45 °C for 18 h under moderate shaking. At the end of incubation, tubes were centrifuged at 5000 g for 10 min, and 2 mL of supernatant was transferred to a 1.5 mL Eppendorf tube and centrifuged in a spin-vacuum (Centrifugal System, RC 10.10, Jouan, Cologno Monzese, Italy) at 40 °C until complete evaporation of methanol. Dried samples were then reconstituted with 0.6 mL of PBS, with 0.1 % bovine serum albumin (Sigma Aldrich, Milan, Italy). Salivary swabs were thawed and centrifuged at room temperature at 1500 g for 15 min to obtain clear saliva for the analysis.

Cortisol concentrations in saliva (HCS) and hair (HCH) samples were measured according to the RIA procedure, as described by Sgorlon et al. (2015). Samples were assayed in duplicate, the sensitivity of the assay was 3.125 pg/well, and the intra-assay and inter-assay coefficients of variation in high and low cortisol reference samples were 5.9% and 9.1% and 13.5 % and 15.1 %, respectively.

Serotonin was determined in salivary samples (SES) with an ultrasensitive enzyme immunoassay commercial kit (Serotonin Research ELISA DEE5900; Demeditec Diagnostic Gmbh Germany), designed to measure serotonin in various biological samples. Samples were assayed in duplicate, and the sensitivity of the test was 0.005 ng/mL and specificity (cross reactivity) was 100% for serotonin, 0.19% for tryptamine, and lower than 0.03% for other related compounds. The intra-assay and inter-assay coefficients of variation in high and low serotonin reference samples were 7.0% and 9.9% and 16.9 % and 18.1%, respectively.

4.2.3.8 Computation and Statistical Analysis

Data were imputed on a spreadsheet for analysis. The 16S rRNA annotated sequences were normalized to % abundance profiles for each sample and each taxonomic level. Taxa with relative abundance lower than 10% (Sandri et al. 2019; Fuhrman 2009; Nogueira et al. 2019) in more than half of the samples were excluded from the statistical analysis. The average percentage of reads excluded was 1.9%. Shannon α-biodiversity index was calculated at the genus level including all taxa according to the equation H' = −sum(Pi × ln Pi), where Pi = frequency of every genus within the sample. Evenness index was calculated as J' = H' / ln S, where S = total number of genera within each sample. Beta diversity was evaluated with the phylogeny based UniFrac distance metric (Lozupone et al. 2007) and visualized using principal coordinate analysis (PCoA) plots.

Analysis of similarity (ANOSIM) was then performed to test whether the microbial communities differed significantly between D0, D1, and D3 diets at T0, T14, and T28 times of sampling, using the 'Vegan' package in R (Version 3.2.1). A linear discriminant analysis (LDA) effect size (LEfSe) was applied to detect taxa that differed between D0, D1, and D3 groups at T0, T14, and T28 (Segata et al. 2011).

Before statistical analysis, normality of distribution of the independent variables was checked with the nonparametric Kolmogorov–Smirnoff test. Linear Mixed Model was used to analyze the SCFA and lactic acid concentrations, their molar proportions, and H' and J' indexes. The model included the fixed effect of time of sampling (3 levels, T0, T14, and T28), treatments (3 levels D0, D1, and D3), and the interaction of time of sampling with treatment, with the subject (dog) as random factor repeated over the time of sampling. For cortisol (HCS) and serotonin (SES) in saliva and cortisol in hair (HCH), the same model was used, using two levels for the fixed effect of time of sampling (T0 and T28). Data obtained by qPCR analysis were subjected to two-way ANOVA to test the data obtained during the time for each microbial group. If appropriate, means were compared by Tukey's multiple range test for $p < 0.05$. Statistical analysis were performed with XLSTAT (Addinsoft 2020).

4.2.4 Results

The mean concentration of lactate and SCFAs in the fecal samples of the dogs are reported in Table 1. Molar content of acetate, butyrate, isobutyrate, and isovalerate did not significantly differ between diets and time of sampling, whilst for propionate a significant increase ($p < 0.05$) was observed at T28 for the D3 group, and a significant ($p < 0.05$) interaction of diet with time of sampling was calculated for lactate. The latter was related to an exceptional high concentration in

the sample of a single dog at T0 of the D3 diet, even though no gastrointestinal disease or other symptoms were observed. Looking at the molar proportion of SCFAs and lactate, a significant effect was observed also for isobutyrate, which was higher ($p < 0.05$) at T28 for D0 diet.

The effect of proanthocyanidins administration to the dogs on fecal microbiota was initially evaluated in terms of biodiversity. The Shannon index of alpha biodiversity (H') and the derived Evenness index (J'), calculated on the relative abundances of microbial genera in the feces, did not significantly differ between the times of sampling (T0, T14, and T28) and the treatments (D0, D1, and D3) (Figure 1). The principal coordinate analysis, calculated on the weighted UniFrac distance matrices, was employed to assess the beta diversity of the microbial community (Figure 2) between dietary treatments (D0, D1, and D3) and times of sampling (T0, T14, and T28). The analysis of data with ANOSIM, analyzed for each of the three times of sampling or all together, did not significantly differ between dietary treatments, and it was not possible to identify cluster of dogs on the basis of dietary treatments or times of sampling.

The effect of proanthocyanidins on fecal microbiota is depicted in Figure 3, which reports the results from LEfSe analysis. The cladogram (Figure 3) highlights taxa that were significantly affected by dietary treatments (Figure 3A) and the increase of significant relative abundance is also reported (Figure 3B). The relative abundance of the family Enterococcaceae and its genus *Enterococcus*, together with genus *Adlercreutzia*, were significantly higher in subjects fed with D0 diet. The relative abundance of family Enterobacteriaceae representing genus *Escherichia* and genus *Eubacterium* were the most abundant taxa in dogs with D1 treatment. Finally, for the D3 diet, the relative abundances of families Paraprevotellaceae, Mogibacteriaceae and Fusobacteriaceae and of genera *Fusobacterium* and *Phascolarctobacterium* were significantly higher. The relative abundance of genera, which significantly differed between the three treatments (Figure 3C-D) showed a high individual variability. Genus *Fusobacterium* had a significant increase at T14 and T28 for the subjects fed with D3 diet, and genus *Escherichia* showed a higher relative abundance at T28 of D1 dieSerotonin (SES) and cortisol (HCS) were measured in saliva and the latter also in hair (HCH) at the beginning and at the end of the study. The mean value of cortisol in hair and saliva (HCS and HCH, respectively) and of SES in saliva at T0 and T28 are reported in Table 2, together with HCS:SES and HCS:HCH ratios. Time of sampling caused a significant increase of HCS and SES, whilst HCH did not vary. However, a consistent increase ($p < 0.01$) of SES was observed at T28 for the D1 and D3 diets. Diet and time of sampling significantly ($p < 0.05$) affected the ratio of HCS:SES, and time of sampling significantly influenced the ratio of HCS:HCH ($p < 0.01$).

The results of the qPCR analysis are reported in Figure 4. The panels A, B, and C represent the percentage of *Faecalibacterium* spp., *Fusobacteria*, *Blautia* spp., *Turicibacter* spp., *Escherichia coli*, *Clostridiumhiranonis*, and*Streptococcus*spp. duringtimeincomparisonoftotalbacteria. Atthebeginning of the experiment (Figure 4A), the amount of the monitored microorganisms was similar in group D0 and D3, while in group D1, a higher amount of *Faecalibacterium* spp., *Fusobacterium*, and *Clostridium hiranonis* was found, corresponding to the 8.8%, 3.8%, and 11.2%, respectively. After 14 days of the treatment (Figure 4B), several changes have been observed. For the D0 group, the concentration of *Streptococcus* spp. increased considerably, as in the other groups, reaching a concentration of about 15% of the total quantified species (D0 and D1) and 7.9% in D3 samples. Compared to T0, the amount of *Faecalibacterium* spp.,

Fusobacterium spp., and *Clostridium hiranonis* decreased significantly ($p < 0.05$). In group D3, there was an increase in the amount of the analyzed microorganisms. The last sampling point is characterized by a slight decrease of *Streptococcus* spp. in all the treatments, but only in group D3 this reduction was significant. Moreover, the other microorganisms decreased among groups except for *Blautia* spp. in group D1, where a significant increase was observed. *Escherichia coli* was detected in a very low amount in all the tested groups. Despite this, it is possible to observe that, at T28, for D0 and D1 treatments, the percentage of *Escherichia coli* was higher than in T0, while in the D3 group, it was lower. In fact, at the beginning of the experiment (T0), the abundance of *Escherichia coli* was equal to 0.023%, 0.007%, and 0.003% for the groups D0, D1, and D3, respectively, whereas, after 28 days, it was 0.117%; 0.933%; and 0.002% for the groups D0, D1, and D3, respectively.

4.2.5 Discussion

Recently, many researchers have focused their attention on studying the effects that prebiotics, probiotics, or synbiotics can have on the gut microbiota (Nogueira et al. 2019; Redfern, Suchodolski & Jergens 2017; Pinna et al. 2018). Among the compounds that could have the ability to affect intestinal microbial communities, polyphenols have gained popularity. However, there are still relatively few researchers who have tested the potential effects of different polyphenols sources on mice (Wang et al. 2018) and on humans (Edwards et al. 2017). To the best of our knowledge, limited studies have analyzed the influence that bioactive compounds can exert on the gut-brain-microbiota axis in dogs. The purpose of this study was to investigate the relationship between polyphenols administration with gut microbial community, end products of fermentations, and endocrine biomarkers, validating the brain-gut-microbiota axis also in dogs.

In the present study, proanthocyanidins were supplemented with the tablets, which corresponded to a concentration of 71 and 203 mg/kg of kibble. In a study of Fragua et al. (2017) (Fragua et al. 2017), the effect of dietary supplementations of 240 and 480 mg/kg kibble of a grape and blueberry extract on working memory in aged dogs was investigated. These authors reported that, after 75 days of supplementation, a significant improvement of cognitive response of dogs was observed for both the amounts in comparison to the control group. Considering that the extract contained 27% of polyphenols, the concentration of these active compounds was 65 and 130 mg/kg kibble.

The average concentration of SCFAs in the fecal samples (Table 1) was 272.4 μmol/g and was higher than the values reported for dogs fed diets with low fiber content (Beloshapka et al. 2012), which were in a range of 195.5–216.9 μmol/g. In adult dogs, SCFAs supply only 2% to 7% of the maintenance energy requirements (Stevens & Hume 1998). Therefore, despite the fact that they do not represent the major source of energy, as for ruminants, it has been demonstrated that SCFAs can improve gut health by reducing the production of cytokine or other inflammatory molecules within the intestinal mucosa (Forsythe, Kunze & Bienenstock 2016).

Considering the data of SCFAs and lactic acid (Table 1), it was interesting to note that for the D3 samples, the mean molar proportions of propionate and isobutyrate were higher and lower, respectively, for the D3 diet at T28. The concentration of acetate and propionate are positively related to the amount of fiber in the diet (Panasevich et al. 2013) and that of branched chain fatty

acids (isobutyrate and isovalerate) are more related to amino acids metabolism in the gut (Sgorlon et al. 2019). In the present study, the diet was the same for all the dogs and the only variation was the administration of proanthocyanidins and, accordingly, the observed change of SCFAs could be related to a shift of microbial community.

At first sight, the microbiota were not modified by the administration of proanthocyanidins, since the biodiversity index H' and Evenness J' (Figure 1) and beta diversity (Figure 2) did not significantly change. As reported by Suchodolski et al. (2012), a significant decrease of biodiversity is associated to inflammatory bowel disease in dogs. Moreover, it has been reported that there is a relationship between the low biodiversity of intestinal microbiota and high microbial fitness, with subsequent unhealthy eating behavior and obesity of the subjects (Alcock, MAley & Aktipis 2014). In healthy dogs, as those recruited for this study, changes of biodiversity can be probably detected when the variation of nutrients supplied with the diet is relevant (Sandri et al. 2019).

The comparison of relative abundances between groups showed for the D3 group a significant shift of some taxa (Figure 3). Since the diet was the same for all the dogs, the observed changes of relative abundance could be attributed to the administration of proanthocyanidins. Polyphenols are considered safe for dog nutrition (Martineau et al. 2016) and were studied for their antioxidant properties, to prevent or support therapy for arthritis (Comblain et al. 2017; Colitti et al. 2012; Sgorlon et al. 2016) or to increase cognitive ability (Fragueet al. 2017). However, a literature search did not produce any results reporting the effect of proanthocyanidins on fecal microbiota of dogs. Jose et al. (2017) showed that the administration of polyphenols from pomegranate peel to dogs caused a shift of fecal pH and ammonia and lactate concentrations, suggesting a positive impact on gut fermentation, but no data on microbial population was reported. In another study, the dietary supplementation of eugenol to dogs led to a reduction of pH and ammonia in the feces, a decrease of *Parabacteroides*, and an increase of *Megamonas*. In humans, the consumption of red wine polyphenols (Queipo-Ortuno et al. 2012) caused an increase of Fusobacteria in the gut, and in rats, the administration of grape seed proanthocyanidins influenced gut microbiota and caused an increase of *Phascolarctobacterium*, other than other bacteria genera. In swine (Choy et al. 2014), the correlations between microbial taxa and phenolic acids end products were investigated using network analysis. The authors reported that epicatechin catechin, the monomeric units of proanthocyanidins, were positively correlated with the Mogibacteriaceae family. These data would corroborate our findings and, thus, some bioactivity of proanthocyanidins on fecal microbiota of dogs. However, these results must be considered with caution since the taxa significantly different for the D1 did not correspond to those found for the D3 group or, in any case, no dose response effect was observed. Moreover, in Figure 3, the individual relative abundances of 2 significant taxa at T0, T14, and T28 indicated a high variability, which can be attributed to age, sex, breed, and other factors related to the environment, as already highlighted by previous research (Garcia-Mazcorro et al. 2017; Reddy et al. 2019). Nonetheless, the influence exerted by the genetics of the host, reported in humans (Goodrich et al. 2017) and livestock (Roehe et al. 2016), should also considered among the factors affecting the gut microbiota.

Several studies have evaluated the variation of salivary cortisol in relation to environment and breed (Sandri et al. 2015) and physical activity (Colussi et al. 2018), but less information is available for the salivary serotonin. Actually, it is known that serotonin in saliva is related to

peripheral levels, reflecting circulating plasma concentration and not central serotonin turnover, at least in adult phenylketonuria patients (Leung et al. 2018). The results of Table 2 indicate the significant increase of SES in D1 and D3 groups at T28 in comparison to T0. Accordingly, the higher SES concentrations reported above suggest that the serotonin detected in salivary samples derives from host–microbiota interaction. Other factors that stimulate the serotoninergic system and serotonine receptors affect the concentration of SES, but in the study the dogs were under the same environmental conditions and the food administered was the same, apart from the addition of prebiotic and it is likely that the observed differences of SES were related to the microbial metabolism. It is unlike that the small concentration of proanthocyanidins caused a bitter or astringent taste, also considering that no food refusal was detected.

The activation of the serotonergic nervous system has been reported to decrease the concentration of salivary cortisol in pigs under stressful conditions (Koopmans et al. 2006) and, on this basis, a negative correlation between HCS and SES would have been expected. Indeed, SES was significantly higher for D3 and D1 groups at T28 in comparison to the D1 group, even though HCS concentrations were significantly higher at T28 for D0 and D1 groups, suggesting that serotonin in saliva does not probably reflect central serotonergic activity. Bacteria can use tryptophan to produce serotonin (Clarke et al. 2014), and *Escherichia coli*, other than *Lactobacillus* spp., have been reported to be very active. The relative abundance of *Escherichia coli* at T28 was higher in D1 group, (Figure 3), suggesting that the increase of salivary serotonin was related to the presence of this genus, which was almost absent in the D3 group. The increase of salivary cortisol found in D0 and D1 groups could be due to the activation of the HPA from the presence of this gut commensal aero-anaerobic bacillus (Clarke et al. 2014). However, this consideration deserves further evaluations. According to Mondo et al. (2020), aggressive dogs show a shift of fecal microbiota, with a reduction of Paraprevotellaceae and Mogibacteriaceae. Aggressive dogs also showed an increase of Catenibacterium and Megamonas, but not significant differences in fecal cortisol and testosterone was reported. These results agree with the higher abundances of Paraprevotellaceae and Mogibacteriaceae found in the D3 group, which displayed also the higher SES concentration. Furthermore, the higher abundance of *Fusobacterium* in the D3 group would agree with the results reported in the study of Kirchoff et al. (2019), which reports lower abundance of Fusobacteriaceae in aggressive dogs. Although the results of these two published studies are contradictory for the Paraprevotellaceae, which increased in the latter research, the data would validate a microbiota-gut-brain axis also in dogs.

Metagenomic analysis is a very useful tool to study the dynamics of the microbial populations, but often they do not allow one to quantify microorganisms at genus or species level. For this reason, qPCR analyses were performed in order to focus on some specific genera and species, such as *Faecalibacterium* spp., *Fusobacteria*, *Blautia* spp., *Turicibacter* spp., *Escherichia coli*, *Clostridium hiranonis*, and *Streptococcus* spp., which are some of the most common species and genera normally found in the gut of dogs. Diseases, metabolic disorders, changes in diet, and other factors can interfere with the abundance of these microorganisms in feces (AlShawaqfeh et al. 2017). The data obtained from the qPCR analysis indicated that the supplementation of proanthocyanidins in the diet did not determine substantial changes in the composition of the microbial populations analyzed. However, after the administration of the tablets, in all the groups, a high increase in the population of *Streptococcus* spp. was observed. As previously reported, the

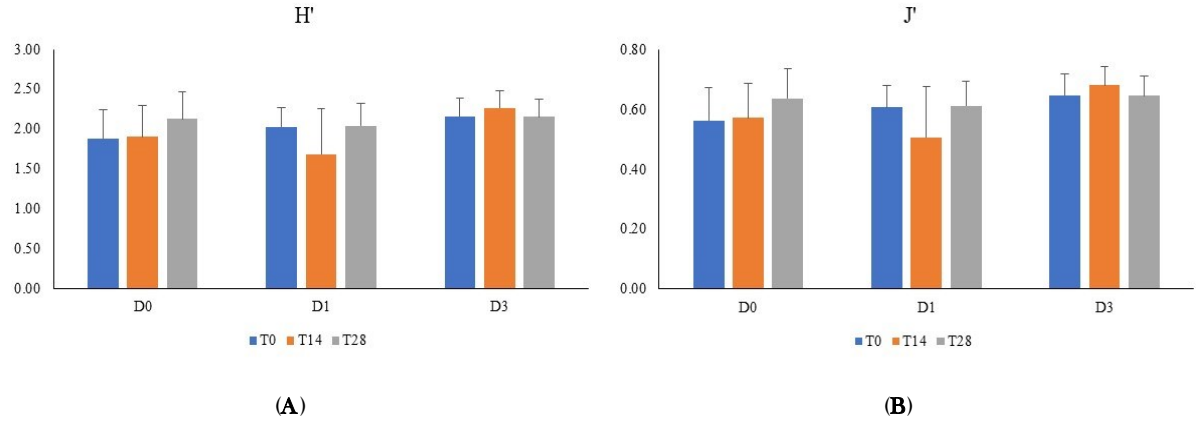
tablets included barley malt extract, thus, it is possible to speculate that this observed change in the microbiota composition is caused by the inclusion of barley malt extract in the tablets (Teixera et al. 2018).

Several studies investigated the effects of the integration in the diet of polyphenols, finding that some of them are able to promote the adhesion of beneficial bacteria (such as probiotic strains), by inhibiting the colonization of pathogenic microorganisms, such as *Salmonella* spp. and *Escherichia coli*, *S. aureus*, *L. monocytogenes* and *C. albicans* (Wilson & Situ 2017). This could explain the slight reduction in the number of *Escherichia coli* in group D3, after 28 days of treatment, confirmed also by the results obtained from metagenomic analysis. However, there are some parameters that would have been taken into consideration, such as the presence of polyphenols and the products of their microbial metabolism in feces, urine, and blood to better explain their role in microbial modulation.

4.2.6 Conclusions

The inclusion of proanthocyanidins in the diet of dogs influenced fecal microbiota, and the modifications of relative abundances of the taxa differed between the low and high doses of proanthocyanidins. It is likely that higher doses of proanthocyanidins would be required to induce detectable modifications of fecal microbial community. However, the results highlighted a great variability of relative abundances for all the taxonomic levels among the dogs, suggesting a uniqueness and stability over time of fecal microbiota, which probably responds differently to dietary intervention. Interestingly, salivary biomarkers varied after the inclusion in the diet of proanthocyanidins. Whether the observed variations of salivary serotonin and cortisol are related to the modifications of gut microbiota or to other factors deserves further study.

4.2.7 TABLES AND FIGURES



(**A**) (**B**)

Figure 1. (**A**) Shannon index of biodiversity (H') and (**B**) Evenness (J') of the microbial communities for dietary treatments with increasing dose of grape proanthocyanidins. H' and J' were calculated on the relative abundances of genera in the faeces of dogs fed a basal diet supplemented with increasing amount of grape proanthocyanidins (D0, D1 and D3). D0: Dogs without supplementation of proanthocyanidins; D1: Dogs supplemented with 1 mg/kg live weight of proanthocyanidins; D3: Dogs supplemented with 3 mg/kg live weight of proanthocyanidins. T0: beginning of the study; T14: after 14 days of the study; T28: after 28 days of the study.

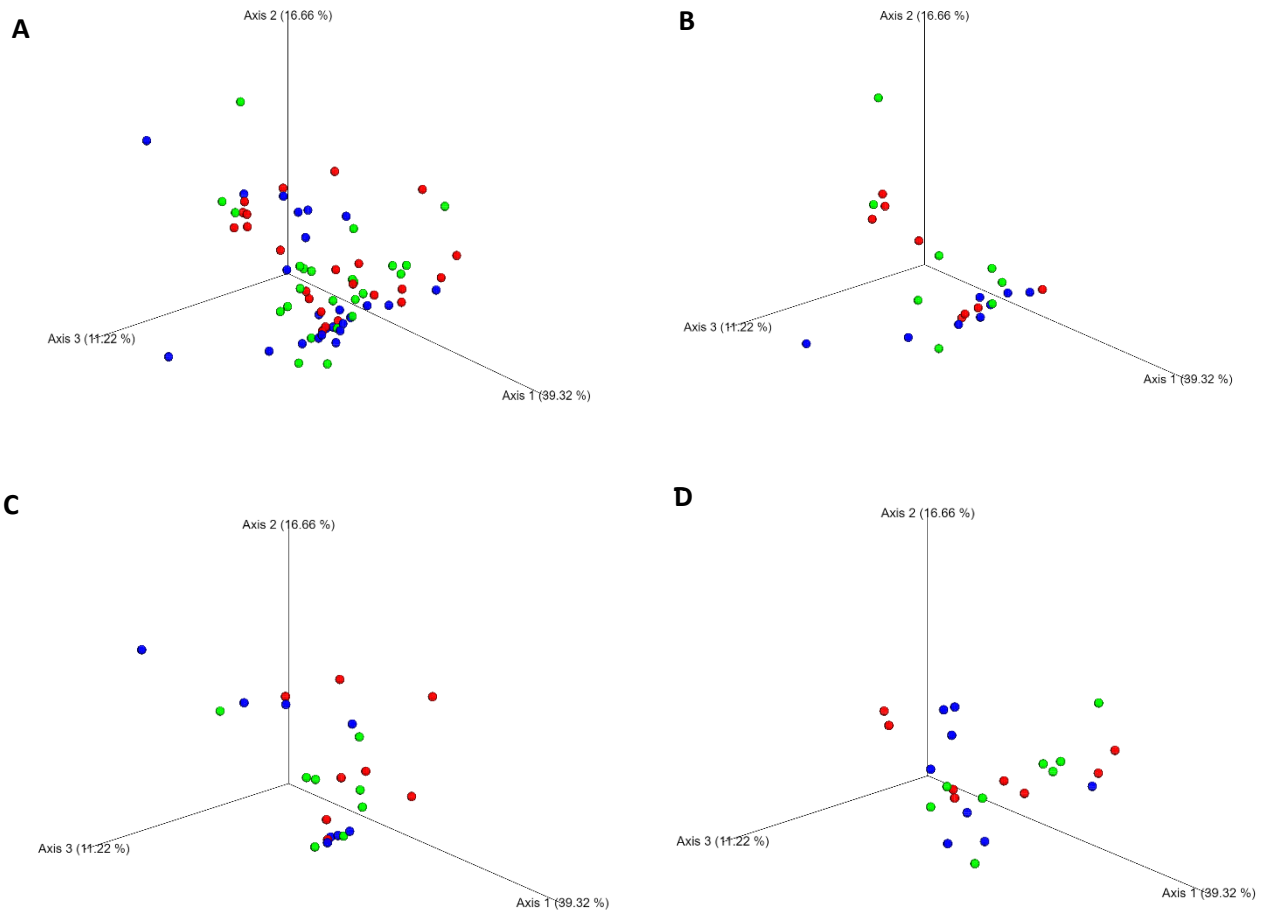


Figure 2. Principal coordinate analysis (PCoA) plot representing the beta diversity of the microbial community between dietary treatments with increasing dose of grape proanthocyanidins (D0, D1 and D3). PCoA was calculated on the weighted UniFrac distance matrices. (**A**) Beta diversity for the 3 dietary treatments and the 3 time of sampling; (**B**) Beta diversity for the 3 dietary treatments and at the beginning of the study (T0); (**C**) Beta diversity for the 3 dietary treatments after 14 days (T14); (**D**) Beta diversity for the 3 dietary treatments after 14 days (T14).

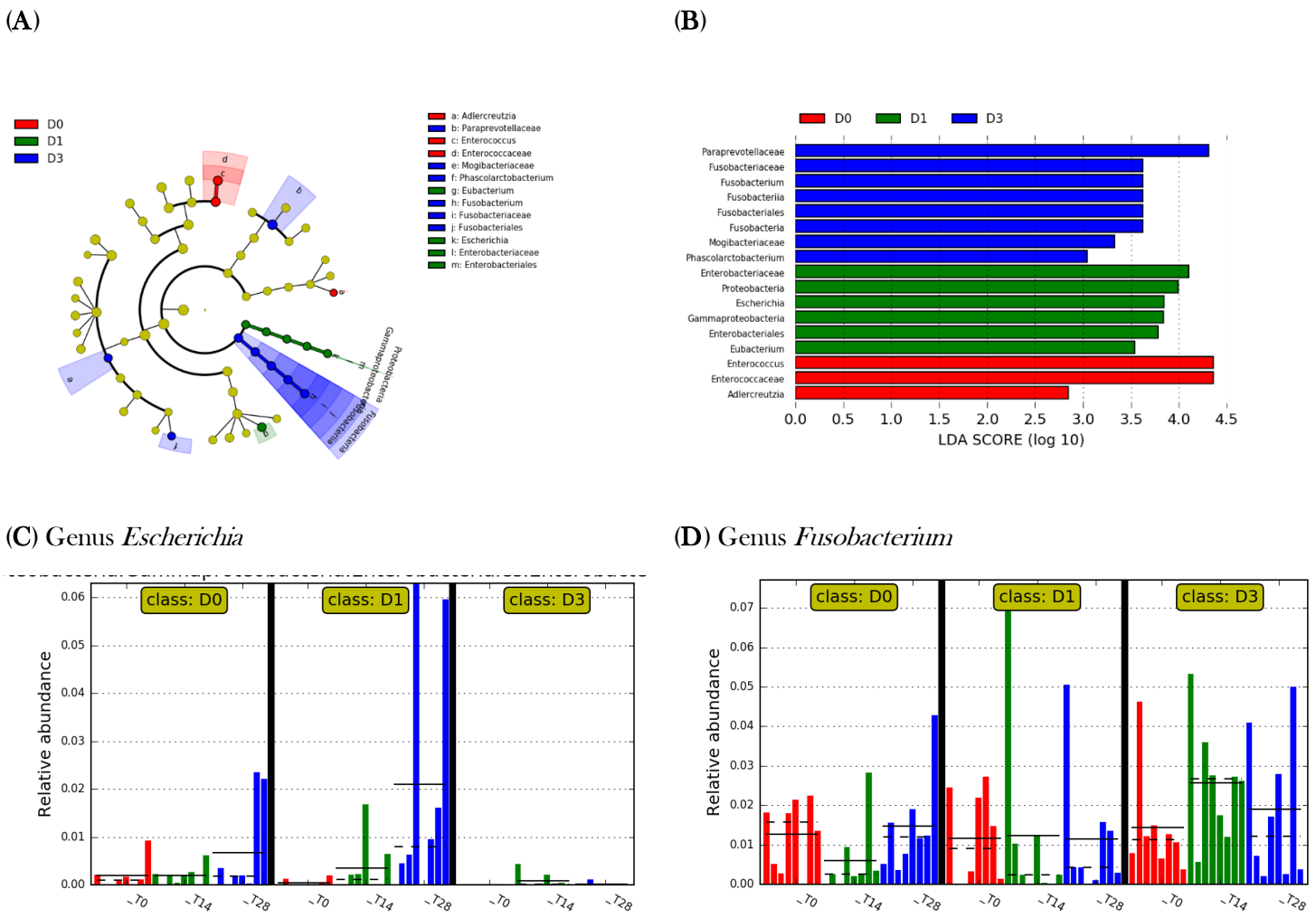


Figure 3. Bacterial taxa differentially abundant in the feces of the dogs without administration of proanthocyanidins (D0), receiving a supplementation of 1 mg/kg live weight (D1) or 3 mg/kg live weight (D3) of proanthocyanidins. The cladogram in (**A**) highlights impactful communities within each treatment and (**B**) shows the score of the linear discriminant analysis (LDA, significant threshold > 2). (**C**) and (**D**) show the individual data for two of significant genera, where dotted line denotes the median and solid line the mean of each subgroup.

Table 1. Mean concentrations and molar proportions of lactate and volatile fatty acids in the feces of the dogs fed diet without supplementation of grape proanthocyanidins (D0) or supplemented with 1 mg/kg live weight (D1) or 3 mg/kg live weight (D3) of grape proanthocyanidins at the beginning of the study (T0) and after 14 (T14) and 28 (T28) days of administration.

Item		D0			D1			D3				Effects		
		T0	T14	T28	T0	T14	T28	T0	T14	T28	SEM [1]	Diet	Time	D x T
Lactate	µmol/g	4.7 ^{ab}	3.8 ^{ab}	1.4 ^{ab}	0.9 ^{b}	7.5 ^{ab}	2.1 ^{ab}	15.3 ^{a}	2.5 ^{ab}	2.9 ^{ab}	1.02	NS	NS	NS
Acetate		143.3	128.8	113.7	137.9	123.4	124.4	143.7	139.6	150.5	4.09	NS	NS	NS
Propionate		39.3 ^{b}	35.9 ^{b}	38.0 ^{b}	39.0 ^{b}	49.5 ^{ab}	45.5 ^{ab}	38.9 ^{b}	48.0 ^{ab}	61.0 ^{a}	1.67	*	*	*
Isobutirate		96.8	61.9	90.5	82.7	58.4	71.0	55.6	72.5	82.2	4.12	NS	NS	NS
Butirate		8.1	8.0	9.8	8.7	11.6	10.6	11.3	11.3	11.1	0.46	NS	NS	NS
Isovalerate		4.2	12.4	4.2	4.5	4.2	4.0	5.3	3.7	5.7	1.06	NS	NS	NS
Total		296.3	250.7	257.6	273.7	254.6	257.6	270.1	277.6	313.3	6.63	NS	NS	NS
Lactate	%	1.8 ^{ab}	2.5 ^{ab}	0.6 ^{b}	0.3 ^{b}	6.4 ^{ab}	0.8 ^{ab}	7.9 ^{a}	0.9 ^{ab}	1.0 ^{ab}	0.75	NS	NS	*
Acetate		48.3	50.8	44.8	50.5	48.4	48.7	52.2	50.2	47.9	0.89	NS	NS	NS
Propionate		13.2 ^{b}	14.4 ^{ab}	15.0 ^{ab}	14.2 ^{ab}	18.6 ^{ab}	17.8 ^{ab}	14.4 ^{ab}	17.4 ^{ab}	19.6 ^{a}	0.52	NS	* *	NS
Isobutirate		32.4 ^{ab}	24.5 ^{abc}	34.0 ^{ab}	30.3 ^{abc}	19.8 ^{bc}	26.9 ^{abc}	19.1 ^{c}	26.2 ^{abc}	26.2 ^{abc}	1.19	NS	NS	NS
Butirate		2.8	3.2	3.9	3.1	4.3	4.2	4.4	4.0	3.5	0.17	NS	NS	NS
Isovalerate		1.5	4.7	1.7	1.6	2.5	1.6	2.1	1.3	1.8	0.42	NS	NS	NS

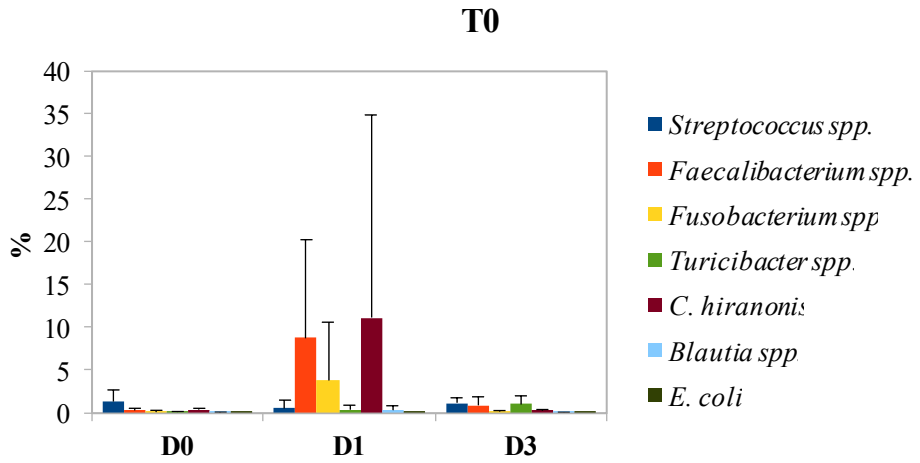
[1] SEM: standard error of the means. ^{a,b,c}: means with different superscripts are significantly different for $p < 0.05$. *: $p < 0.05$; **: $p < 0.01$; NS: Not Significant.

Table 2. Mean concentrations of cortisol (HCS) and serotonin (SES) in saliva and cortisol in hair (HCH) of dogs fed diet without supplementation of grape proanthocyanidins (D0) or supplemented with 1 mg/kg live weight (D1) or 3 mg/kg live weight (D3) of grape proanthocyanidins at the beginning of the study (T0) and after 28 (T28) days of administration.

Item	D0		D1		D3		Effects			
	T0	T28	T0	T28	T0	T28	SEM [1]	Diet	Time	D x T
HCS (ng/mL)	1.23 ^{a}	4.80 ^{a}	1.88 ^{ab}	6.26 ^{b}	1.37 ^{a}	2.88 ^{b}	0.44	NS	**	*
HCH (ng/g)	6.80	6.89	6.96	6.56	6.91	7.10	0.15	NS	NS	NS
SES (ng/mL)	32.47 ^{b}	34.97 ^{b}	42.37 ^{ab}	77.64 ^{a}	44.31 ^{ab}	75.41 ^{a}	5.77	NS	**	*
HCS:SES	0.21 ^{ab}	0.31 ^{a}	0.08 ^{b}	0.09 ^{ab}	0.07 ^{b}	0.05 ^{b}	0.03	*	*	NS
HCS:HCH	0.18 ^{b}	0.68 ^{a}	0.27 ^{b}	0.96 ^{a}	0.20 ^{b}	0.44 ^{ab}	0.06	NS	**	NS

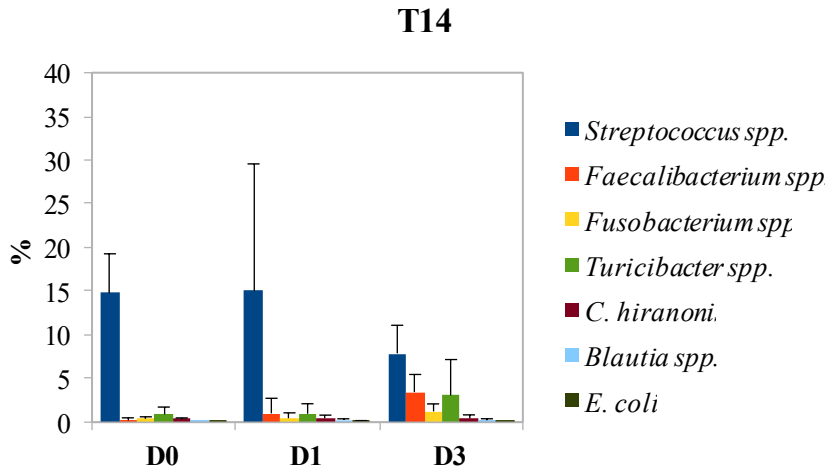
[1] SEM: standard error of the means. ^{a,b,c}: means with different superscripts are significantly different for $p < 0.05$. *: $p < 0.05$; **: $p < 0.01$; NS: Not Significant

(A)



(a)

(B)



(C)

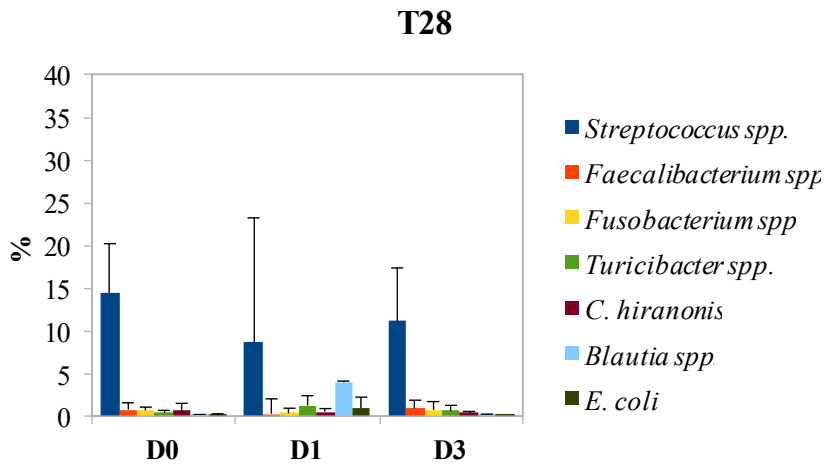


Figure 4. Results obtained from the analysis of the qPCR data. Each panel contains the quantification of the microbial communities researched in the feces of the dogs fed with increasing amounts of proanthocyanidins (D0, D1, and D3). D0: 0 mg/kg live weigh of grape proanthocyanidins; D1: 1 mg/kg live weight of grape proanthocyanidins; and D3: 3 mg/kg live weight of grape proanthocyanidins. (**A**) T0: beginning of the study; (**B**) T14 after 14 days of the study; and (**C**) T28: after 28 days of the study.

4.2.8 References

Accorsi, P.A.; Carloni, E.; Valsecchi, P.; Viggiani, R.; Gamberoni, M.; Tamanini, C.; Seren, E. 2008. "Cortisol determination in hair and faeces from domestic cats and dogs". *Gen. Comp. Endocrinol.* 155, 398–402. http://dx.doi.org/10.1016/j.ygcen.2007.07.002

Addinsoft. 2020. "XLSTAT Statistical and Data Analysis Solution". Boston, MA, USA.

Alcock, J.; Maley, C.C.; Aktipis, C.A. 2014. "Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms". *Bioessays* 36, 940–949. http://dx.doi.org/10.1002/bies.201400071

AlShawaqfeh, M.K.; Wajid, B.; Minamoto, Y.; Markel, M.; Lidbury, J.A.; Steiner, J.M.; Serpedin, E.; Suchodolski, J.S. 2017. "A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy". *FEMS Microbiol. Ecol.* 93. http://dx.doi.org/10.1093/femsec/fix136

Beloshapka, A.N.; Duclos, L.M.; Vester Boler, B.M.; Swanson, K.S. 2012. "Effects of inulin or yeast cell-wall extract on nutrient digestibility, fecal fermentative end-product concentrations, and blood metabolite concentrations in adult dogs fed raw meat-based diets". *Am. J. Vet. Res.* 73, 1016–1023. http://dx.doi.org/10.2460/ajvr.73.7.1016

Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. 2019. "Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2". *Nat. Biotechnol.* 37, 852–857. http://dx.doi.org/10.1038/s41587-019-0209-9

Cardona, F.; Andres-Lacueva, C.; Tulipani, S.; Tinahones, F.J.; Queipo-Ortuno, M.I. 2013. "Benefits of polyphenols on gut microbiota and implications in human health". *J. Nutr. Biochem. 24*, 1415–1422. http://dx.doi.org/10.1016/j.jnutbio.2013.05.001

Choy, Y.Y.; Quifer-Rada, P.; Holstege, D.M.; Frese, S.A.; Calvert, C.C.; Mills, D.A.; Lamuela-Raventos, R.M.; Waterhouse, A.L. 2014. "Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins". *Food Funct.* 5,2298–2308. http://dx.doi.org/10.1039/C4FO00325J

Clarke, G.; Stilling, R.M.; Kennedy, P.J.; Stanton, C.; Cryan, J.F.; Dinan, T.G. 2014. "Minireview: Gut microbiota: the neglected endocrine organ". *Mol. Endocrinol.* 28, 1221–1238. http://dx.doi.org/10.1210/me.2014-1108

Colitti, M.; Gaspardo, B.; Della Pria, A.; Scaini, C.; Stefanon, B. 2012. "Transcriptome modification of white blood cells after dietary administration of curcumin and non-steroidal anti-inflammatory drug in osteoarthritic affected dogs". *Vet. Immunol. Immunopathol.* 147, 136–146. http://dx.doi.org/10.1016/j.vetimm.2012.04.001

Colussi, A.; Stefanon, B.; Adorini, C.; Sandri, M. 2018. "Variations of salivary cortisol in dogs exposed to different cognitive and physical activities". *Ital. J. Anim. Sci.* 17, 1030–1037. http://dx.doi.org/10.1080/1828051X.2018.1453756

Comblain, F.; Barthélémy, N.; Lefèbvre, M.; Schwartz, C.; Lesponne, I.; Serisier, S.; Feugier, A.; Balligand, M.; Henrotin, Y. 2017. "A randomized, double-blind, prospective, placebo-controlled study of the efficacy of a diet supplemented with curcuminoids extract, hydrolyzed collagen and green tea extract in owner's dogs with osteoarthritis". *BMC Vet. Res.* 13, 395. http://dx.doi.org/10.1186/s12917-017-1317-8

de Souza, E.L.; de Albuquerque, T.M.R.; dos Santos, A.S.; Massa, N.M.L.; de Brito Alves, J.L. 2019. "Potential interactions among phenolic compounds and probiotics for mutual boosting of their health-promoting properties and food functionalities—A review". *Crit. Rev. Food Sci. Nutr.* 59, 1645–1659. http://dx.doi.org/10.1080/10408398.2018.1425285

Edwards, C.A.; Havlik, J.; Cong, W.; Mullen, W.; Preston, T.; Morrison, D.J.; Combet, E. 2017. "Polyphenols and health: Interactions between fibre, plant polyphenols and the gut microbiota". *Nutr. Bull.* 42, 356–360. http://dx.doi.org/10.1111/nbu.12296

Forsythe, P.; Kunze, W.; Bienenstock, J. 2016. "Moody microbes or fecal phrenology: What do we know about the microbiota-gut-brain axis?" *BMC Med.* 14, 58. http://dx.doi.org/10.1186/s12916-016-0604-8

Fragua, V.; Lepoudère, A.; Leray, V.; Baron, C.; Araujo, J.A.; Nguyen, P.; Milgram, N.W. 2017. "Effects of dietary supplementation with a mixed blueberry and grape extract on working memory in aged beagle dogs". *Nutr. Sci. 6*,e35. http://dx.doi.org/10.1017/jns.2017.33

Fuhrman, J.A. 2009. "Microbial community structure and its functional implications". *Nature* 459, 193–199. http://dx.doi.org/10.1038/nature08058

Garcia-Mazcorro, J.F.; Barcenas-Walls, J.R.; Suchodolski, J.S.; Steiner, J.M. 2017. "Molecular assessment of the fecal microbiota in healthy cats and dogs before and during supplementation with fructo-oligosaccharides (FOS) and inulin using high-throughput 454-pyrosequencing". *PeerJ* 5, e3184. http://dx.doi.org/10.7717/peerj.3184

Gilbert, S.F.; Sapp, J.; Tauber, A.I. 2012. "A symbiotic view of life: We have never been individuals". *Q. Rev. Biol.* 87, 325–341. http://dx.doi.org/10.1086/668166

Goodrich, J.K.; Davenport, E.R.; Clark, A.G.; Ley, R.E. 2017. "The Relationship Between the Human Genome and Microbiome Comes into View". *Annu. Rev. Genet.* 51, 413–433. http://dx.doi.org/10.1146/annurev-genet-110711-155532

Gross, G.; Jacobs, D.M.; Peters, S.; Possemiers, S.; van Duynhoven, J.; Vaughan, E.E.; van de Wiele, T. 2010. "In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability". *J. Agric. Food Chem.* 58, 10236–10246. http://dx.doi.org/10.1021/jf101475m

Jenkins, T.A.; Nguyen, J.C.; Polglaze, K.E.; Bertrand, P.P. 2016. "Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis". *Nutrients* 8, 56. http://dx.doi.org/10.3390/nu8010056

Jose, T.; Pattanaik, A.K.; Jadhav, S.E.; Dutta, N.; Sharma, S. 2017. "Nutrient digestibility, hindgut metabolites and antioxidant status of dogs supplemented with pomegranate peel extract". *J. Nutr. Sci.* 6, e36. http://dx.doi.org/10.1017/jns.2017.34

Kennedy, P.J.; Cryan, J.F.; Dinan, T.G.; Clarke, G. 2014. "Irritable bowel syndrome: A microbiome-gut-brain axis disorder?" *World J. Gastroentero.* 20, 14105–14125. http://dx.doi.org/10.3748/wjg.v20.i39.14105

Kirchoff, N.S.; Udell, M.A.R.; Sharpton, T.J. The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (Canis familiaris). *PeerJ* 2019, *7*, e6103.

Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glockner, F.O. 2013. "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies". *Nucleic. Acids Res.* 41, e1. http://dx.doi.org/10.1093/nar/gks808

Koopmans, S.J.; Guzik, A.C.; van der Meulen, J.; Dekker, R.; Kogut, J.; Kerr, B.J.; Southem, L.L. 2006. "Effects of supplemental L-tryptophan on serotonin, cortisol, intestinal integrity, and behavior in weanling piglets". *Anim. Sci.* 84, 963–971. http://dx.doi.org/10.2527/2006.844963x

Kutschera, M.; Engst, W.; Blaut, M.; Braune, A. 2011. "Isolation of catechin-converting human intestinal bacteria." *J. Appl. Microbiol.* 111, 165–175. http://dx.doi.org/10.1111/j.1365-2672.2011.05025.x

LeBlanc, J.G.; Milani, C.; de Giori, G.S.; Sesma, F.; van Sinderen, D.; Ventura, M. 2013. "Bacteria as vitamin suppliers to their host: A gut microbiota perspective." *Curr. Opin. Biotechnol.* 24, 160–168. http://dx.doi.org/10.1016/j.copbio.2012.08.005

Leung, J.; Selvage, C.; Bosdet, T.; Branov, J.; Rosen-Heath, A.; Bishop, C.; Sirrs, S.; Horvath, G. 2018. "Salivary serotonin does not correlate with central serotonin turnover in adult phenylketonuria (PKU) patients." *Mol. Genet. Metab. Rep.* 15, 100–105. http://dx.doi.org/10.1016/j.ymgmr.2018.03.008

Lozupone, C.A.; Hamady, M.; Kelley, S.T.; Knigh, R. 2007. "Quantitative and qualitative diversity measures lead to different insights into factors that structure microbial communities." *Appl. Environ. Microbiol.* 73, 1576–1585. http://dx.doi.org/10.1128/AEM.01996-06

Martineau, A.S.; Leray, V.; Lepoudere, A.; Blanchard, G.; Bensalem, J.; Gaudout, D.; Ouguerram, K.; Nguyen, P. Neurophenols Consortium. 2016. "A mixed grape and blueberry extract is safe for dogs to consume". *BMC Vet. Res.* 12, 162. http://dx.doi.org/10.1186/s12917-016-0786-5

Monagas, M.; Urpi-Sarda, M.; Sánchez-Patán, F.; Llorach, R.; Garrido, I.; Gómez-Cordovés, C.; Andres-Lacueva, C.; Bartolomé, B. 2010. "Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites." *Food Funct.* 1, 233–253. http://dx.doi.org/10.1039/c0fo00132e

Mondo, E.; Barone, M.; Soverini, M.; D'Amico, F.; Cocchi, M.; Petrulli, C.; Mattioli, M.; Marliani, G.; Candela, M.; Accorsi, P.A. 2020. "Gut microbiome structure and adrenocortical activity in dogs with aggressive and phobic behavioral disorders." *Heliyon* 1, e03311. http://dx.doi.org/10.1016/j.heliyon.2020.e03311

Nogueira, J.P.S.; He, F.; Mangian, H.F.; Oba, P.M.; De Godoy, M.R.C. 2019. "Dietary supplementation of a fiber-prebiotic and saccharin-eugenol blend in extruded diets fed to dogs." *J. Anim. Sci.* 97, 4519–4531. http://dx.doi.org/10.1093/jas/skz293

O'Mahony, S.M.; Clarke, G.; Borre, Y.E.; Dinan, T.G.; Cryan, J.F. 2015. "Serotonin, tryptophan metabolism and the brain-gut-microbiome axis." *Behav. Brain Res.* 277, 32–48. http://dx.doi.org/10.1016/j.bbr.2014.07.027

Panasevich, M.R.; Rossoni Serao, M.C.; de Godoy, M.R.; Swanson, K.S.; Guerin-Deremaux, L.; Lynch, G.L.; Wils, D.; Fahey, G.C., Jr.; Dilger, R.N. 2013. "Potato fiber as a dietary fiber source in dog foods." *J. Anim. Sci.* 91, 5344–5352. http://dx.doi.org/10.2527/jas.2013-6842

Pinna, C.; Vecchiato, C.G.; Bolduan, C.; Grandi, M.; Stefanelli, C.; Windisch, W.; Zaghini, G.; Biagi, G. 2018. "Influence of dietary protein and fructooligosaccharides on fecal fermentative end-products, fecal bacterial populations and apparent total tract digestibility in dogs." *BMC Vet. Res.* 14, 106. http://dx.doi.org/10.1186/s12917-018-1436-x

Queipo-Ortuno, M.I.; Boto-Ordonez, M.; Murri, M.; Gomez-Zumaquero, J.M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andres-Lacueva, C.; Tinahones, F.J. 2012. "Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers." *Am. J. Clin. Nutr.* 95, 1323–1334. http://dx.doi.org/10.3945/ajcn.111.027847

Reddy, K.E.; Kim, H.R.; Jeong, J.Y.; So, K.M.; Lee, S.; Ji, S.Y.; Kim, M.; Lee, H.J.; Lee, S.; Kim, K.H.; et al. 2019. "Impact of Breed on the Fecal Microbiome of Dogs under the Same Dietary Condition." *J. Microbiol. Biotechnol.* 29, 1947–1956. http://dx.doi.org/10.4014/jmb.1906.06048

Redfern, A.; Suchodolski, J.; Jergens, A. 2017. "Role of the gastrointestinal microbiota in small animal health and disease." *Vet. Rec.* 181, 370. http://dx.doi.org/10.1136/vr.103826

Roehe, R.; Dewhurst, R.J.; Duthie, C.A.; Rooke, J.A.; McKain, N.; Ross, D.W.; Hyslop, J.J.; Waterhouse, A.; Freeman, T.C.; Watson, M.; et al. 2016. "Bovine Host Genetic Variation Influences Rumen Microbial Methane Production with Best Selection Criterion for Low Methane Emitting and Efficiently Feed Converting Hosts Based on Metagenomic Gene Abundance." *PLoS Genet.* 12, e1005846. http://dx.doi.org/10.1371/journal.pgen.1005846

Round, J.L.; Mazmanian, S.K. 2009. "The gut microbiota shapes intestinal immune responses during health and disease." *Nat. Rev. Immunol.* 9, 313–323. http://dx.doi.org/10.1038/nri2515

Russell, W.R.; Scobbie, L.; Labat, A.; Duthie, G.G. 2009. "Selective bio-availability of phenolic acids from Scottish strawberries." *Mol. Nutr. Food Res.* 53, S85–S91. http://dx.doi.org/10.1002/mnfr.200800302

Sandri, M.; Colussi, A.; Perrotta, M.G.; Stefanon, B. 2015. "Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context." *J. Vet. Behav.* 10, 302–306. http://dx.doi.org/10.1016/j.jveb.2015.03.011

Sandri, M.; Dal Monego, S.; Conte, G.; Sgorlon, S.; Stefanon, B. 2017. "Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs." *BMC Vet. Res.* 13, 65. http://dx.doi.org/10.1186/s12917-017-0981-z

Sandri, M.; Sgorlon, S.; Conte, G.; Serra, A.; Dal Monego, S.; Stefanon, B. 2019. "Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs." *Ital. J. Anim. Sci.* 18, 1205–1214. http://dx.doi.org/10.1080/1828051X.2019.1645624

Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W.S.; Huttenhower, C. 2011. "Metagenomic biomarker discovery and explanation." *Genome. Biol.* 12, R60. http://dx.doi.org/10.1186/gb-2011-12-6-r60

Sgorlon, S.; Fanzago, M.; Guiatti, D.; Gabai, G.; Stradaioli, G.; Stefanon, B. 2015. "Factors affecting milk cortisol in mid lactating dairy cows." *BMC Vet. Res.* 11, 259. http://dx.doi.org/10.1186/s12917-015-0572-9

Sgorlon, S.; Mattiello, A.; Ronutti, L.; Sandri, M.; Stefanon, B. 2019. "Concentration of elements in the hair of growing and adult dogs." *It. J. Anim. Sci.* 18, 1126–1134. http://dx.doi.org/10.1080/1828051X.2019.1621687

Sgorlon, S.; Stefanon, B.; Sandri, M.; Colitti, M. 2016. "Nutrigenomic activity of plant derived compounds in health and disease: Results of a dietary intervention study in dog." *Res. Vet. Sci.* 109, 142–148. http://dx.doi.org/10.1016/j.rvsc.2016.10.005

Stevens, C.E.; Hume, I.D. 1998. "Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients." *Physiol. Rev.* 78, 393–427. http://dx.doi.org/10.1152/physrev.1998.78.2.393

Suchodolski, J.S.; Dowd, S.E.; Wilke, V.; Steiner, J.M.; Jergens, A.E. 2012. "16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease." *PLoS ONE* 7, e39333. http://dx.doi.org/10.1371/journal.pone.0039333

Teixeira, C.; Prykhodko, O.; Alminger, M.; Fak Hallenius, F.; Nyman, M. 2018. "Barley Products of Different Fiber Composition Selectively Change Microbiota Composition in Rats." *Mol. Nutr. Food Res.* 62, e1701023. http://dx.doi.org/10.1002/mnfr.201701023

Wang, J.; Tang, L.; Zhou, H.; Zhou, J.; Glenn, T.C.; Shen, C.L.; Wang, J.S. 2018. "Long-term treatment with green tea polyphenols modifies the gut microbiome of female sprague-dawley rats." *J. Nutr. Biochem.* 56, 55–64. http://dx.doi.org/10.1016/j.jnutbio.2018.01.005

Wilson, K.; Situ, C. 2017. "Systematic review on effects of diet on gut microbiota in relation to metabolic syndromes." *J. Clin. Nutr. Metab.* 1, 1–12.

CHAPTER 5

5.1 Introduction to the study

The study of metabolites resulting from the fermentation and metabolic action of the gut microbial population is important in order to understand the health of the host subject. The metabolites of the microbiome, also called postbiotics, have an important energy reserve function for the host, regulate intestinal motility and constitute anti-inflammatory compounds. Metabolomics is widely used nowadays, especially in the human medical field and in the field of animal sciences. It can improve the conditions of well-being, both human and animal, and identify mechanisms of action in diseases, constituting a tool to improve the prediction and the effectiveness of treatments. Specifically, nutritional metabolomics can be a safe method for finding the explanations underlying the different responses obtained from animals fed with different types of diet.

At the moment, methods that destroy the sample are often applied for the research and quantification of metabolites in biological matrices such as feces and other fluids, as they allow, in addition to the determination of the metabolite, the precise quantification of the same. Unfortunately, the determination of all metabolites present in feces and other biological fluids is still a long way off, but non-targeted metabolomics may be the key to a better understanding of the GI tract. One of the most effective methods is the proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), which is able to determine the smallest metabolites and it is a simple method that does not destroy the sample.

The main purpose of this study is the use of NMR analysis for the determination of specific metabolites in fecal samples from dogs fed with different diets. The second goal was to be able to distinguish dogs based on their diet through their metabolic profile resulting from NMR analysis. For this study, 48 healthy adult dogs were recruited, equally divided between males and females. The recruitment took place thanks to the help of various veterinary clinics, so the dogs come from different backgrounds and lifestyles, depending on the owners. Although the recruitment of dogs can be a limit, due to the lack of standardization of the study, the variability has allowed us to evaluate the true routine of dogs, making a substantial contribution to veterinary science. The dog's feces were collected at the time of recruitment and stored at -20°C until the time of analysis.

Eighteen metabolites were found out of 21 metabolites selected for determination in feces. Given the power of the instrument used, it was only possible to do an analysis on the frequency of the presence of these metabolites in the entire group of dogs examined. Through a chi-square analysis, only two metabolites were found to be significant, namely valeric acid and L-threonine. Although only these two metabolites were found to be significant in terms of presence, it was also possible to speculate on the frequency of other metabolites, which were shown to be more or less present in different groups of dogs based on their diet.

The analysis of the metabolic profile deriving from the NMR method made it possible to group the dogs into different clusters, which distinguished them based on their diet. The analysis was done using Principal Component Analysis (PCA) where the first 14 PCs explained more than 90% of the variability of the spectra. The first 3 PCs constituted 27.98%, 27.05% and 12.78% of the variability.

Over the years, considerable efforts have been made to implement this method in the human sphere, while much less effort has been made for the veterinary sciences. The results obtained with this study are preliminary and indicate the need to continue along this line in order to improve the quality of the final result. For example, the determination of metabolites is strongly influenced by the pH at which samples are prepared and measured. In order to be able to perform the determination of the 21 metabolites, an internal library has been prepared, which will need to be expanded with further metabolites in the future. Despite everything, this study proceeded to lay the foundations for implementing the use of NMR in order to study the metabolic profile of the gut microbiome of dogs.

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5.2 An application of nuclear magnetic resonance spectroscopy to study faecal canine metabolome

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5.2.1 Abstract

Metabolomics provides a description of the phenotype of an organism and complementary biochemical information to genomics and proteomics. The purpose of this research was to depict the metabolite profile of faecal samples from dogs fed three different diets through NMR spectroscopy analysis. Samples were collected from 14 dogs fed a commercial extruded diet, 18 dogs fed a homemade diet and 16 dogs fed a raw meat-based diet. The average BCS for all dogs was 4–5 and the average Faecal Score was 2–3. Only healthy animals were considered, as assessed from the clinical evaluation of the veterinarians. Faecal samples were prepared using phosphate buffer (pH 7.1) combined with deuterated water and analysed with NMR spectroscopy using a Bruker Avance III HD 400 MHz spectrometer. Principal component analysis of the spectra signals demonstrated clustering of dogs according to diet, with 57.8% of the variance explained by the first three components. Targeted metabolome analysis was also performed on 56 metabolites of interest, selected from a database of 558 metabolites. Our data suggest that metabolome analysis using NMR is a promising approach to describe the phenotypic variation that occurs among dogs fed different diets.

5.2.2 Introduction

Microbiota of the gastrointestinal tract and the consequent metabolites are important to health. Bacteriaderived metabolites, including short-chain fatty acids (SCFAs), act as energy sources, regulate intestinal mobility and are anti-inflammatory (Machiels et al. 2014). Other metabolites, such as indole and other byproducts of tryptophan degradation, have an immunomodulatory role and may strengthen the intestinal barrier (Pavlidis et al. 2015; Whittemore et al. 2019).

The study of metabolomics provides a direct link to the phenotype of an organism and complementary biochemical information to genomics and proteomics (Jones et al. 2014). Nowadays, metabolomics may be used to improve human and canine health and welfare because it may be used to identify molecular mechanisms of action, help classify diseases, and serve as a tool to improve patient diagnosis, prognosis and treatment efficacy (Carlos et al. 2020). The complete catalogue of the faecal metabolites is far from being known. Targeted and non-targeted metabolomics as well as metabolite profiling could offer another key to the understanding of the gastrointestinal tract. Many techniques, such as proton nuclear magnetic resonance (^{1}H-NMR)

spectroscopy, are able to characterise the small metabolites that are present in biological samples (Wishart 2008).

In veterinary science, nutritional metabolomics may evaluate biofluids and other biological matrices to help explain the functional responses between animals fed different diets and to identify candidate dietary biomarkers for specific food and dietary patterns; in the end, it may describe the connections that exist between diet and disease incidence (Gibney et al. 2005; Forster et al. 2015; O'Gorman and Brennan 2015). To the best of our knowledge, only specific studies related to changes in a few dietary ingredients or the comparison of two different types of diets have been conducted until now. Moreover, the faecal metabolome of dogs has never been analysed using an NMR spectroscopy approach.

The primary purpose of this research was to determine the presence of specific metabolites in faecal samples of dogs fed three substantially different diets using NMR analysis. A secondary objective was to distinguish dogs fed different diets based on their faecal metabolomic profile.

5.2.3 Materials and methods

5.2.3.1 Animals and samples collection

A total of 48 healthy privately owned dogs were enrolled from three veterinary clinics located in NorthEast of Italy. All the characteristics of the animals are reported in Table S1. Both females (11 whole females and 19 spayed females) and males (11 whole males and 7 castrated males) were present in this study and subjects were of different breed and in the adult phase (more than 2 years old, less than 10 years old). Dogs were divided based on the type of diet administered, including a commercial extruded dry food (KIBBLE, 14 dogs), raw meat-based diet (BARF, 16 dogs) and home-made diet (HOME, 18 dogs). The main ingredients of kibbles were chicken meat and fat, rice, and beet pulp, with an average crude protein content of 26.5% and fat content of 15.5% on a drymatter basis. The BARF diet was made of a mix of meats (about 60% of beef or turkey or chicken, as fed), offal (about 20% as fed), bones (about 10% as fed), vegetables and oils (about 10% as fed). The HOME diet was formulated by a nutritionist, with an average of 45% raw beef meat, 40% boiled rice, 10% vegetables as fed and 5% mineral vitamins supplement. The daily intake for all the three diets was under the supervision of the veterinarians that recommended to follow the prescribed dietary regimen to cover nutrition requirements. The owners had to follow tables with the corresponded daily dose. The selection of the dogs was based on the information given by the veterinarians and the owners. A detailed protocol was provided to clinicians, that were asked to recruit dogs with body condition score (BCS) between 4 and 5 and faecal score (FS) between 2 and 3.

Subjects with poor information were discharged. Moreover, we considered only healthy animals, free from internal and external parasites and with no antibiotic therapy since at least three months before the recruitment. This information was possible thanks to the clinical observations of the veterinarians. Dogs were housed in their usual home and living conditions followed by the owners, and informed consent was obtained from them prior to the study. Owners were also instructed to strictly follow the scheduled diet and time of administration and to restrain food

rewords at least 30 d before the collection of samples. All protocols, procedures, and care of the animals complied with the Italian legislation on animal care and were approved by the ethical committee of the University of Udine (28 June 2019, protocol n. 7/2019). During the visit to the veterinary clinics, samples of faecal material were taken with sterile gloves, placed into a sterile plastic bag and immediately stored at 20 C until analysis.

5.2.3.2 Sample preparation for ^{1}H-NMR spectroscopy

The sample preparation for ^{1}H-NMR spectroscopy followed the procedure of Lamichhane et al. (2015) study. Faecal water was extracted using 1:2 weight of fresh faeces-to-buffer ratio with 0.75 M phosphate-buffered saline (PBS, pH 7.4), by whirl mixing for 2 min with Ultra Turrex (IKA, Staufen, Germany). Aliquots were centrifuged at 10.000 g for 15 min at 4 C and the supernatants were carefully removed and stored in Eppendorf tubes at 80 C until analysis. For the ^{1}H-NMR spectroscopic analysis, the extracted samples were thawed and centrifuged again at 10.000 g for 15 min at 4 C. If the supernatant resulted still turbid, an ulterior centrifugation at 10.000 g for 15 min at 4 C was applied. This step was important for the final acquisition of the data: if the samples were not completely clear, the small particles remaining interfered with the analysis, thus not allowing a good interpretation of the spectra. Afterword, a 500 mL sample of clear supernatant was taken and placed into a 1.5 mL Eppendorf tube, adding 100 mL of deuterium oxide (D_2O) containing 0.025 mg/mL of 3-(trimethylsilyl) propionic acid-d4 sodium salt (TSP) and 3 mL of sodium azide (NaN_3) 10%. Finally, after mixing well each preparation, the samples were transferred into a 5 mm ^{1}HNMR tube. One-dimensional ^{1}H-NMR experiments were carried out using a Bruker Avance III HD 400 MHz spectrometer (Bruker, Rheinstetten, Germany) equipped with a 5 mm triple resonance (TXI) probe at 298 K. A standard Bruker noesygppr1d sequence was used to suppress signals from water molecules (most important acquisition parameter; time domain, 65536; dummy scan, 4; number of scans, 64; sweep amplitude, 14 ppm; time delay, 5 s; mixing time, 0.01 s). The spectra were referenced to TSP (chemical shift 0 ppm), phased, and baseline corrected in Topspin 4.0 software (Bruker, Rheinstetten, Germany).

5.2.3.3 Creation of the standards database

For the identification of the metabolites, an in-house library of pure molecules was developed. The selected metabolites (Sigma–Aldrich^{VR} Co., Milan, Italy) were 2phenylethylamine, gamma-aminobutyric acid (GABA), L-threonine, acetic acid, butyric acid, iso-valeric acid, iso-butyric acid, lactic acid, propionic acid, valeric acid, cortisol, diisopropylamine, dopamine, indole, kynurenine, putrescine, serotonin, tyramine, tyrosine, tryptamine, and tryptophan. Each metabolite was placed in a NMR tube and a 600 mL solution, containing D_2O with 0.025 mg/mL of TSP, to arrive at the final concentration of 0.002 M, was added. Identification of metabolites in the faecal samples was achieved comparing NMR spectra with those of pure metabolites taking advantages of standard routine present in AssureNMR 2.2 software (tolerance at 0.1 ppm, coupling difference at 0.8 Hz, minimum intensity of 75% and signal noise ratio at 5).

5.2.3.4 Computational and statistical analysis

^{1}H-NMR spectra were processed with Topspin 4.0 software and statistical analysed with AssureNMR 2.2 software. The NMR spectra of faecal samples were further divided into 0.05

ppm integral region and integrated. The values were than normalised to pareto-scaled. Before multivariate statistical analysis, the parts of d 4.55–5.50 ppm were removed to eradicate the baseline effect of imperfect water suppression (Table S2). PCA was used to analyse the whole spectra within faecal samples. After this step, a contingency table with the observed frequencies for each metabolite was created and analysed by a chi-square test to highlight the metabolites that significantly differed among groups. A p-value below .05 was considered statistically significant. All analyses were performed with XLSTAT (Addinsoft 2020).

5.2.4 Results

All dogs included in the study did not show clinical signs of disease at the sampling collection and from their previous clinical history. The faecal collection method used herein was totally non-invasive.

Data analysis was performed using Principal component analysis (PCA). The first 14 PCs explained more than 90% of the variation of the spectra and the first three PCs accounted for 27.98%, 17.05% and 12.78% of variation, respectively. The bi-dimensional plot (Figure 1(a)) showed a clustering of faecal samples based on the diet fed to dogs. Subjects fed a raw meat-based (BARF) diet showed a better clustering than those fed a commercial extruded dry food (KIBBLE) or a home-made (HOME) diet. Of greatest interest was the observation that some dogs did not fit into the clustering of its diet group, especially those dogs fed a KIBBLE or HOME diet. No clustering was observed for sex and size of the dogs (Figure 1(b,c)).

^{1}H-NMR spectroscopy allowed the detection of 18 of the 21 targeted metabolites in faecal samples of every dog analysed. The metabolites were identified by comparison with a small database of standard molecules created for this study. The frequencies, expressed as percentage of the metabolites detected in faecal samples, are shown in Table 1. The chi-square test did not detect any significant differences in the presence/absence among the three groups of dogs except for two metabolites. Acetic acid was not different among the 3 diets because it was detected in faecal samples of all dogs, whilst cortisol, kynurenine and putrescine were not detected in any, and thus, the chi-square test could not be applied. L-threonine and valeric acid were identified as being significantly different in terms of presence or absence among groups; L-threonine was not detected in all dogs fed a HOME diet, although the two other groups (dogs fed a BARF diet or a KIBBLE diet) had the presence of this metabolite in all samples. On the contrary, valeric acid had the opposite trend; this metabolite was detected in 27.8% of dogs fed a HOME diet, whilst it was not observed at all in dogs fed a BARF or KIBBLE diet. The frequencies table of the metabolites presence for sex and size did not show any relevant results (Tables S3 and S4). Figure 2 shows a representative spectrum of a faecal sample extracted in PBS buffer. The obtained ^{1}H-NMR spectra contained resonances from SCFAs (predominantly acetate, propionate, and butyrate), branched-chain fatty acids (isovalerate, iso-butyrate), biogenic amines (2-phenylethylamine, diisopropylamine, putrescine, tyramine, tryptamine, dopamine), bioproducts of the tryptophan pathway (indole, kynurenine and serotonin) and amino acids (tyrosine).

Figure 3 shows a typical spectrum derived from a faecal sample, divided in portions of a chemical shift for highlighting the signals of each metabolite. In general, the 1-dimensional (1D) NMR

spectra show very good reproducibility of the chemical shift due to the maintenance of a uniform pH by adding phosphate buffer to the sample.

5.2.5 Discussion

To the best of the authors' knowledge, this is the first study that investigates the impact of different diets on the faecal metabolome of clinically healthy dogs using ^{1}H-NMR spectroscopy.

Pre-analytical handling and processing of samples have been demonstrated to considerably affect the results of human metabolome studies (Yin, Lehmann, e Xu 2015; Lamichhane et al. 2015) and a standardization of the method and protocols used for metabolomic analysis was already implemented in the human area (Beckonert et al. 2007; Emwas et al. 2015; Jobard et al. 2016). Conversely, the standardization of protocols for companion animals still requires effort, especially when relatively large numbers of samples are collected at once and when evaluating samples coming from an un-controlled environment, as is the case for client-owned dogs.

Also, when pet owners are required to collect samples, it is critical to have proper storage and handling of samples prior to being received in the laboratory for the analysis. Since this was the first time that the faecal metabolome of dogs was investigated using an ^{1}H-NMR spectroscopy approach, considerable efforts were made to develop and apply specific protocols for the handling and processing of samples.

The influence of diet on the faecal metabolome is poorly investigated in a general way. These preliminary results indicated that the first 3 PCs together explain about 60% of the variance and this could be due to the complexity of the matrix under study and to the several factors that may affect the variability. Indeed, some studies revealed certain correlation between the variability of the microbiome and consequently, the metabolome, and different type of breeds, (Alessandri et al. 2019; Reddy et al. 2019), age of dogs (Mizukami et al. 2019) and sex (Scarsella et al. 2020).

Considering that the portion from d 10 to 6.8 ppm of the ^{1}H-NMR spectra dominated the PCA loadings, this would indicate that the metabolites belonging to this portion are characteristic of the faecal metabolome and represent the end-products of digestion and microbial fermentation in the gut, which largely depend upon diet composition (Table S1). Figures 1(a) demonstrate the clustering that occurred between the groups of dogs based on their dietary intake. Beyond this result, it is interesting to observe that some subjects remain outside the dietary group they belong to. Although the owners were informed to follow the diet and the time of administration, we cannot exclude a discontinuous administration of foods and that some food rewards were offered. Then, the owners could have fed the dogs with rewards but not necessarily every day. We are aware that the decision of recruiting dogs from owners did not guarantee any standardization of the results, but this apported a substantial contribution in the veterinary field, reporting a picture of what a dog routine life is. Further investigations are required to understand the role of diet on metabolome and the connection between microbiome and metabolome.

Although untargeted metabolomics does not provide a quantitative metabolite measurement, the normalised regions of spectra for each specific metabolite across all samples showed exhaustive information about the individual metabolome and the factors affecting it. In this study, it was

decided to measure 21 selected metabolites by the creation of an in-house library of pure molecules. This approach was needed to confirm the presence of compounds that are normally detected, quantified and studied in faecal samples with other methods and instruments, but are often destroyed in samples.

The prevalence of valeric acid and L-threonine were found to be significant different in the chi-squared test, as valeric acid was present in some HOME-fed dogs, but was missing in all BARF- and KIBBLE-fed dogs. On the contrary, L-threonine was less prevalent in dogs fed HOME diet while it was present in all remaining dogs. SCFAs are the major products of the bacteria fermentation of carbohydrates and proteins in the gut (Kovatcheva-Datchary and Arora 2013). An increased utilisation of carbohydrates from nondigestible plant polysaccharides often results in higher production of SCFAs. Furthermore, valeric acid is known to have histone deacetylase (HDACs) inhibitory properties as well as butyric acid, thus it may have beneficial effects regarding the prevention of epigenetic aberration in the host (Yuille et al. 2018). The detection of valeric acid in dogs fed a HOME diet could be due to the contribution of plant fibre and products of the dairy industry present in that diet. Another explanation may be due to the fact that SCFAs are volatile compounds, making it difficult to detect them alone in the metabolomic screen. Contrary, L-threonine is an essential amino acid that is normally consumed with the proteins and peptides present in the diet. It is possible to speculate that some dogs fed a HOME diet either received a lower amount of protein or were able to digest the proteins at a higher rate, resulting in a lower detection of this metabolite within the faecal samples of this group.

The presence or absence of other metabolites was interesting even if they were not shown to be different according to the chi-square test. For instance, indole was detected only in dogs fed the BARF diet, while the presence of tryptophan was minor in this group of dogs when compared to HOME- and KIBBLEfed dogs. These results are consistent with what has already been highlighted in the study by Ephraim et al. (2020), where the impact of long- term consumption of foods containing low, medium, and high levels of protein in dogs was evaluated. The BARF diet, by definition, is characterised by a high protein intake. Recent discoveries revealed that circulating concentration of tryptophan appears to be under the influence of the gut microbiota. The mechanisms regarding the regulation of this compound is still unclear, but may involve the physiologically dominant route of the kynurenic pathway (Schwarcz et al. 2012; Stone et al. 2013). Although the limitation of the analysis due to the instrument, this may explain why it was not possible to detect kynurenine, whilst on the contrary we detected indole, tryptophan and serotonin, with differences regarding the three considered diets. Moreover, tryptophan is also required by a bacteria-specific tryptophanase enzyme for indole production (Lee and Lee 2010; Li and Young 2013). Furthermore, certain bacteria are able to produce tryptophan via enzymes such as tryptophan synthase (Yanofsky 2007; Raboni et al. 2009) whilst other strains can produce serotonin from tryptophan (Lyte 2011; Jimenez et al. 2013).

Gamma-aminobutyric acid (GABA) also highlighted some differences among the three groups of dogs. GABA is an important neurotransmitter with inhibitory effects on the central nervous system. It seems that GABA can also be influenced by diet. In a study by Olson et al. (2018), feeding a ketogenic diet led to an alteration in the intestinal microbiota and an increase of the hippocampal GABA/glutamate ratio. Schmidt et al. (2018) observed that BARF-fed dogs had a higher abundance of GABA and 4-hydroxybutyric acid (GHB) in their faeces; considering the

presence or absence of GABA of the present study, those observations are in contrast, but because it was not possible to quantify the metabolites, further investigation is needed to understand the mechanisms behind their release.

There are several limitations to this study. Firstly, the current study evaluated a small number of standards. It is necessary to use a larger database of standards in the future to identify with greater accuracy as many metabolites as possible. Secondly, further investigations are needed to understand the pathways contributing to the detection of some metabolites rather than others and to determine whether this result was due to the volatile structure of certain compounds. Thirdly, the inclusion of a wider number of dogs in future studies could help to identify which metabolites are most affected by the diet type.

5.2.6 Conclusions

Despite these limitations, the NMR approach seems to be a valid approach. This technique does not destroy the sample and is very simple to implement, in terms of cost and in order to standardise the procedures. Nevertheless, it has been possible to recognise clusters of dogs based on their diet. This may indicate the importance of diet on the well-being and health of dogs when it comes to the metabolome and microbiome. The importance of this study is due to the implementation of a methodological aspect in terms of handling of the samples and protocol. In the future, new investigations will be conducted to proceed with the quantification of these metabolites and to amplify the database of standards created specifically for the study of the dog faecal metabolome.

5.2.7 TABLES AND FIGURES

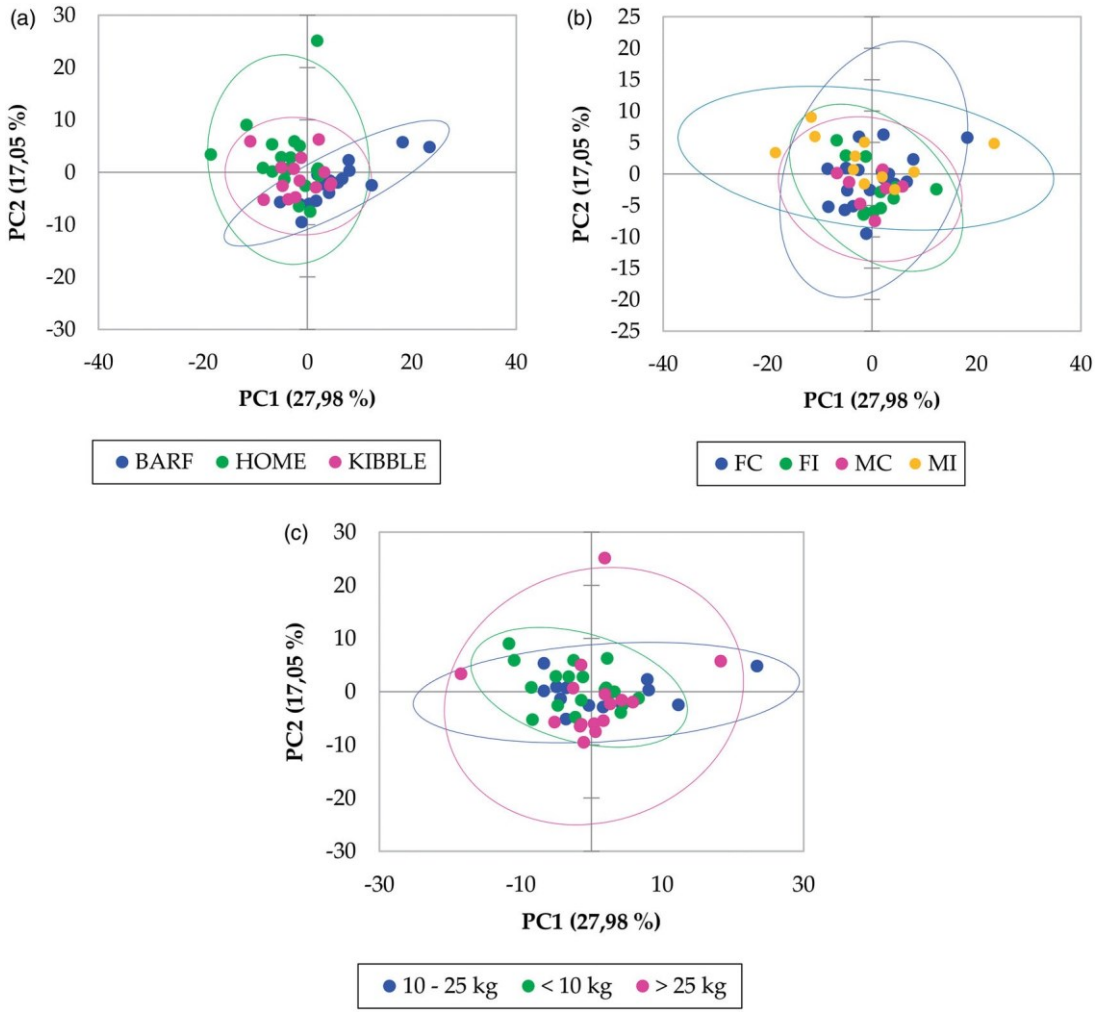


Figure 1. Results of principal component analysis (PCA) of 1H-NMR spectra of faeces from dogs. (a) fed with a commercial extruded complete diet (KIBBLE), a home-made diet (HOME) or a raw meat-based diet (BARF); (b) divided in spayed females (FC), whole females (FI), neutered males (MC) and whole males (MI) and (c) classified in under 10 kg of live weight (<10 kg), between 10 and 25 kg of live weight (10–25 kg) and over 25 kg of live weight (>25 kg).

Table 1. Percentage of presence of metabolites analysed in each study group of dogs based on their diet. For each metabolite, a chi-square test was performed and the relative *p*-value is reported. A *p*-value below 0.05 was considered significant.

% of presence	BARF	HOME	KIBBLE	*p*-value
2-phenylethylamine	50.0	44.4	35.7	0.732
GABA	6.3	11.1	28.6	0.195
L-threonine	100.0	77.8	100.0	0.026
acetic acid	100.0	100.0	100.0	n.d
butyric acid	75.0	77.8	92.9	0.408
iso-valeric acid	37.5	44.4	64.3	0.319
iso-butyric acid	25.0	38.9	42.9	0.551
lactic acid	100.0	94.4	100.0	0.427
propionic acid	100.0	88.9	100.0	0.176
valeric acid	0.0	27.8	0.0	0.010
diisopropylamine	18.8	5.6	7.1	0.405
indole	6.3	0.0	0.0	0.360
dopamine	0.0	5.6	14.3	0.269
serotonin	37.5	16.7	7.1	0.107
tyramine	68.8	55.6	57.1	0.703
tyrosine	62.5	77.8	85.7	0.322
tryptamine	43.8	44.4	64.3	0.446
tryptophan	62.5	66.7	71.4	0.875

BARF: raw meat-based diet; HOME: home-made diet; KIBBLE: complete extruded diet. n.d.: not determined.

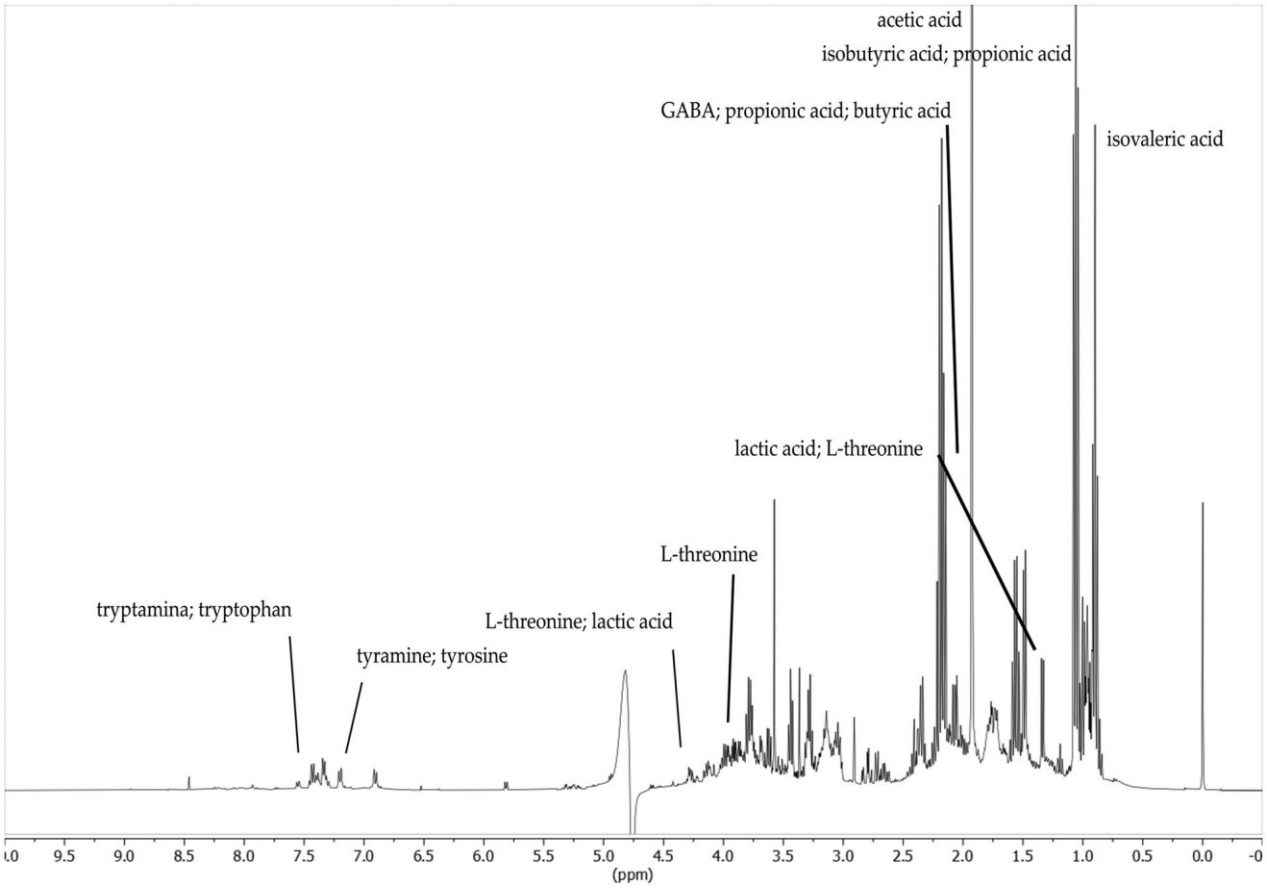
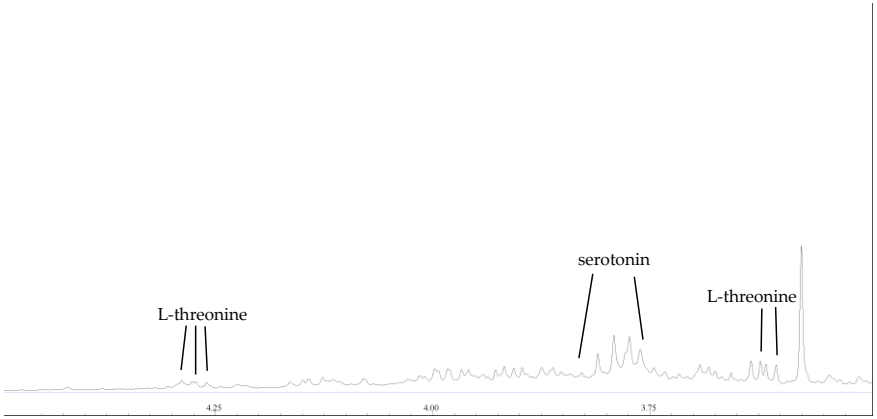
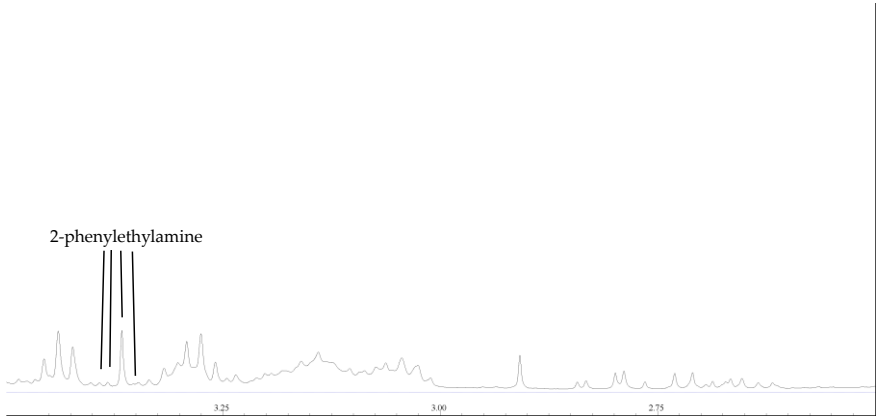


Figure 2. ^{1}H-NMR spectra from faecal sample. Assignments appear on the signals used for molecule determination. The vertical scale of each portion is conveniently set to ease the signals observation.



(a)



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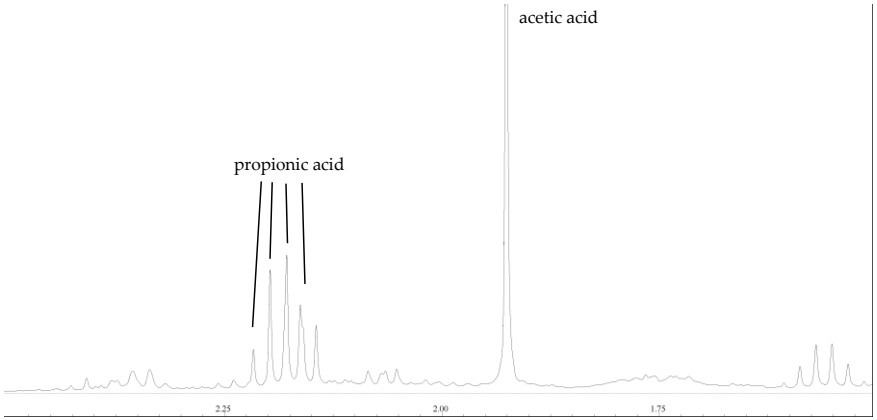

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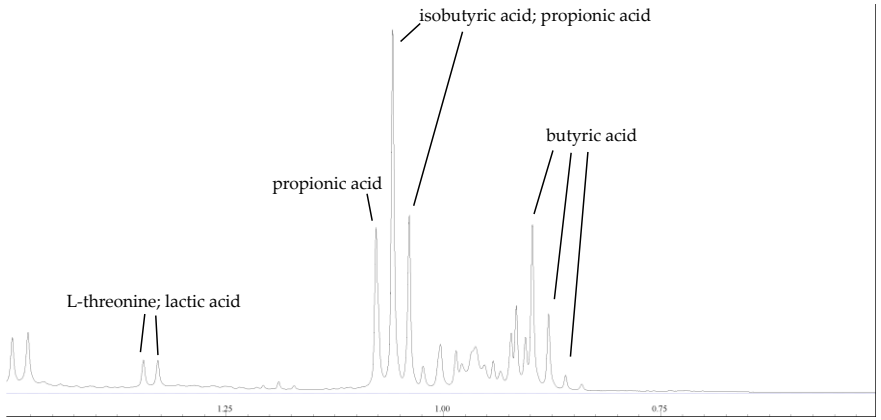

(d)

Figure 3. Portions of ^{1}H-NMR spectra from a faecal sample, highlighted with green boxes. Assignments appear on the signals used for molecule determina tion. The vertical scale of each portion is conveniently set to ease the signals observation. (a) Portion from d 3.5 to 4.5 ppm; (b) Portion from d 2.5 to 3.5 ppm; (c) Portion from d 1.5 to 2.5 ppm and (d) Portion from d 0.5 to 1.5 ppm.

5.2.8 References

Addinsoft 2020. "XLSTAT statistical and data analysis solution." Boston, USA.

Alessandri G, Milani C, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, Sinderen Dv, et al. 2019. "Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features." *Environ Microbiol.* 21(4):1331–1343. https://doi.org/10.1111/1462-2920.14540

Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC, Nicholson JK. 2007. "Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts." *Nat Protoc.* 2(11):2692–2703. https://doi.org/10.1038/nprot.2007.376

Carlos G, dos Santos FP, Froehlich PE. 2020. "Canine metabolomics advances." *Metabolomics.* 16(2):16. https://doi.org/10.1007/s11306-020-1638-7

Emwas A-H, Luchinat C, Turano P, Tenori L, Roy R, Salek RM, Ryan D, Merzaban JS, Kaddurah-Daouk R, Zeri AC, et al. 2015. "Standardizing the experimental conditions for using urine in NMR-based metabolomic studies with a particular focus on diagnostic studies: a review." *Metabolomics.* 11(4):872–894. https://doi.org/10.1007/s11306-014-0746-7

Ephraim E, Cochrane C-Y, Jewell DE. 2020. "Varying protein levels influence metabolomics and the gut microbiome in healthy adult dogs." *Toxins.* 12(8):517. https://doi.org/10.3390/toxins12080517

Forster GM, Heuberger AL, Broeckling CD, Bauer JE, Ryan EP. 2015. "Consumption of cooked navy bean powders modulate the canine fecal and urine metabolome." *CMB.* 3(2): 90–101.

Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B. 2005. "Metabolomics in human nutrition: opportunities and challenges." *Am J Clin Nutr.* 82(3): 497–503. https://doi.org/10.1093/ajcn/82.3.497

Jimenez E, Ladero V, Chico I, Maldonado-Barrag A, Lopez M, Martın V, Fernandez L, Fernandez M, Alvarez MA, Torres C, et al. 2013. "Antibiotic resistance, virulence determinants and production of biogenic amines among enterococci from ovine, feline, canine, porcine and human milk." *BMC Microbiol.* 13(1):288. https://doi.org/10.1186/1471-2180-13-288

Jobard E, Tredan O, Postoly D, Andre F, Martin A-L, ElenaHerrmann B, Boyault S. 2016. "A systematic evaluation of blood serum and plasma pre-analytics for metabolomics cohort studies." *IJMS.* 17(12):2035. https://doi.org/10.3390/ijms17122035

Jones MD, Rainville PD, Isaac G, Wilson ID, Smith NW, Plumb RS. 2014. "Ultra high resolution SFC–MS as a high throughput platform for metabolic phenotyping: application to metabolic profiling of rat and dog bile." *J Chromatogr B.* 966:200–207. https://doi.org/10.1016/j.jchromb.2014.04.017

Kovatcheva-Datchary P, Arora T. 2013. "Nutrition, the gut microbiome and the metabolic syndrome." *Best Pract Res Clin Gastroenterol.* 27(1):59–72. https://doi.org/10.1016/j.bpg.2013.03.017

Lamichhane S, Yde CC, Schmedes MS, Jensen HM, Meier S, Bertram HC. 2015. "Strategy for nuclear-magnetic-resonance-based metabolomics of human feces." *Anal Chem.* 87(12):5930–5937. https://doi.org/10.1021/acs.analchem.5b00977

Lee J-H, Lee J. 2010. "Indole as an intercellular signal in microbial communities." *FEMS Microbiol Rev.* 34(4): 426–444. https://doi.org/10.1111/j.1574-6976.2009.00204.x

Li G, Young KD. 2013. "Indole production by the tryptophanase TnaA in Escherichia coli is determined by the amount of exogenous tryptophan." *Microbiology.* 159(Pt_2): 402–410. https://doi.org/10.1099/mic.0.064139-0

Lyte M. 2011. "Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics." *BioEssays.* 33(8):574–581. https://doi.org/10.1002/bies.201100024

Machiels K, Joossens M, Sabino J, Preter VD, Arijs I, Eeckhaut V, Ballet V, Claes K, Immerseel FV, Verbeke K, et al. 2014. "A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis." *Gut.* 63(8):1275–1283. http://dx.doi.org/10.1136/gutjnl-2013-304833

Mizukami K, Uchiyama J, Igarashi H, Murakami H, Osumi T, Shima A, Ishiahra G, Nasukawa T, Une Y, Sakaguchi M. 2019. "Age-related analysis of the gut microbiome in a purebred dog colony." *FEMS Microbiol Lett.* 366(8):fnz095 https://doi.org/10.1093/femsle/fnz095

O'Gorman A, Brennan L. 2015. "Metabolomic applications in nutritional research: a perspective." *J Sci Food Agric.* 95(13):2567–2570. https://doi.org/10.1002/jsfa.7070

Olson CA, Vuong HE, Yano JM, Liang QY, Nusbaum DJ, Hsiao EY. 2018. "The gut microbiota mediates the anti-seizure effects of the ketogenic diet." *Cell.* 173(7):1728–1741. https://doi.org/10.1016/j.cell.2018.04.027

Pavlidis P, Powell N, Vincent RP, Ehrlich D, Bjarnason I, Hayee B. 2015. "Systematic review: bile acids and intestinal inflammation-luminal aggressors or regulators of mucosal defence?" *Aliment Pharmacol Ther.* 42(7):802–817. https://doi.org/10.1111/apt.13333

Raboni S, Bettati S, Mozzarelli A. 2009. "Tryptophan synthase: a mine for enzymologists." *Cell* Mol Life Sci. 66(14):2391–2403. https://doi.org/10.1007/s00018-009-0028-0

Reddy KE, Kim H-R, Jeong JY, So K-M, Lee S, Ji SY, Kim M, Lee H-J, Lee S, Kim K-H, et al. 2019. "Impact of breed on the fecal microbiome of dogs under the same dietary condition." *J Microbiol Biotechnol.* 29(12):1947–1956. https://doi.org/10.4014/jmb.1906.06048

Scarsella E, Stefanon B, Cintio M, Licastro D, Sgorlon S, Monego SD, Sandri M. 2020. "Learning machine approach reveals microbial signatures of diet and sex in dog." *PLoS One*. 15(8):e0237874. https://doi.org/10.1371/journal.pone.0237874

Schmidt M, Unterer S, Suchodolski JS, Honneffer JB, Guard BC, Lidbury JA, Steiner JM, Fritz J, Kolle P. 2018. "The fecal microbiome and metabolome differs between dogs fed bones and raw food (BARF) diets and dogs fed commercial diets." *PLoS One*. 13(8):e0201279. https://doi.org/10.1371/journal.pone.0201279

Schwarcz R, Bruno JP, Muchowski PJ, Wu H-Q. 2012. "Kynurenines in the mammalian brain: when physiology meets pathology". *Nat Rev Neurosci*. 13(7):465–477. https://doi.org/10.1038/nrn3257

Stone TW, Stoy N, Darlington LG. 2013. "An expanding range of targets for kynurenine metabolites of tryptophan." *Trends Pharmacol Sci*. 34(2):136–143. https://doi.org/10.1016/j.tips.2012.09.006

Whittemore JC, Stokes JE, Price JM, Suchodolski JS. 2019. "Effects of a synbiotic on the fecal microbiome and metabolomic profiles of healthy research cats administered clindamycin: a randomized, controlled trial." *Gut Microbes*. 10(4):521–539. https://doi.org/10.1080/19490976.2018.1560754

Wishart DS. 2008. "Metabolomics: applications to food science and nutrition research." *Trends Food Sci Technol.* 19(9): 482–493. https://doi.org/10.1016/j.tifs.2008.03.003

Yanofsky C. 2007. "RNA-based regulation of genes of tryptophan synthesis and degradation, in bacteria." *RNA*. 13(8): 1141–1154. https://doi.org/10.1261/rna.620507

Yin P, Lehmann R, Xu G. 2015. "Effects of pre-analytical processes on blood samples used in metabolomics studies." *Anal Bioanal Chem*. 407(17):4879–4892. https://doi.org/10.1007/s00216-015-8565-x

Yuille S, Reichardt N, Panda S, Dunbar H, Mulder IE. 2018. "Human gut bacteria as potent class I histone deacetylase inhibitors in vitro through production of butyric acid and valeric acid." *PLoS One*. 13(7):e0201073. https://doi.org/10.1371/journal.pone.0201073

CHAPTER 6

6.1 Introduction to the study

The intestinal microbiome has been extensively studied over the years, both for humans and for dogs. This is due not only to a strong interest in improving the welfare conditions of pets, but also to the fact that the dog's intestinal microbiome is very similar to that of humans. This has allowed a continuous exchange of information and results, which has led to a better characterization of the microbial population and the definition of the ideal condition for the host in good health.

However, the improvement of the techniques for the analysis of the intestinal microbiome has led to an even greater characterization of the microbial population at the individual level, highlighting the existence of a high variability among the hosts, especially in cases of diet modulation studies. Despite this evidence, it is clear that certain taxa are always present, and that these characterize the host's intestinal microbiome on the basis of environmental and genetic factors. In fact, these taxa constitute the core of the microbiome in healthy subjects.

The actual presence of these taxa can be decisive to identify dysbiotic events. The term dysbiosis refers to a change in the gut microbial composition. These events can be caused or can cause the onset of a pathological inflammatory condition, such as irritable bowel syndrome, which consequently can cause the condition of the leaky gut. This condition is extensively studied in humans, less in dogs, but it should not present in healthy subjects. If in the case of the study of intestinal dysbiosis there are already numerous researches, there is a shortage of literature regarding dysbiosis related to the blood microbiome. Blood has always been considered a sterile environment, where the presence of microorganisms is essentially observed only in the case of serious infections. However, some recent evidence suggests that this is not the case, and the origin of some blood-dwelling bacteria may be due to a translocation from the intestine to the blood due to the condition of the leaky gut.

This study made it possible to study three aspects, which concern the confirm that dogs can be divided based on their diet, in terms of physical form of the diet, only by studying the fecal microbiome; the search for the presence of a microbial population in the bloodstream; finally, the study of a possible link between intestinal and blood microbiome. For this reason, 36 dogs were recruited, in adulthood and in good health, equally divided between males and females. The recruitment of the dogs took place with the help of some veterinary clinics, which means that the dogs were heterogeneous in terms of lifestyle. The dogs were also divided into groups based on the type of diet taken. Sampling of stool and blood took place at the time of recruitment, during a normal visit to the vet.

Both fecal and blood microbiome allowed dogs to be separated based on their diet. Not many overlaps have been seen in terms of microbial composition, but the fact that both microbiomes lead to the same result is a sign of an existing link between gut and blood. The difference between the microbial population of the intestine and that of the blood can be explained by the filtering role played by immune and intestinal cells, which may have limited the translocation of some bacteria.

The hypothesis underlying the presence of microorganisms in the blood is that these are in a "dormant" phase; occasionally, they "wake up" and reproduce, and thus they are transported by the bloodstream to various compartments and tissues of the host, inducing a state of chronic

disease. This would explain the reason for their presence even in subjects apparently without any pathology in progress, at the time of the sampling collection.

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6.2 Blood Microbiome: A New Marker of Gut Microbial Population in Dogs?

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6.2.1 Abstract

The characterization of the microbial population in different compartments of the organism, such as the gastrointestinal tract, is now possible thanks to the use of high-throughput DNA sequencing technique. Several studies in the companion animals field have already investigated the fecal microbiome in healthy or sick subjects; however, the methodologies used in the different laboratories and the limited number of animals recruited in each experiment do not allow a straight comparison among published results. Previously, our research focused on the characterization of the microbial taxa variability in 340 fecal samples from 132 healthy dogs, collected serially from several in-house experiments. The results supported the responsiveness of microbiota to dietary and sex factors and allowed us to cluster dogs with high accuracy. For the present study, intestinal and blood microbiota of healthy dogs from different breeds, genders, ages and food habits were collected, with three principal aims: firstly, to confirm the results of our previous study regarding the fecal microbiome affected by the different type of diet; secondly, to investigate the existence of a blood microbial population, even in heathy subjects; and thirdly, to seek for a possible connection between the fecal and the blood microbiota. Limited researches have been published on blood microbiota in humans, and this is the first evidence of the presence of a bacterial population in the blood of dogs. Moreover, gut and blood microbiota can discriminate the animals by factors such as diet, suggesting some relationship between them. These preliminary results make us believe in the use of the blood microbiome for diagnostic purposes, such as researching and preventing gut inflammatory diseases.

6.2.2 Introduction

The numerous studies that investigated the composition and the variation of gut microbiome in relation to healthy conditions and environmental factors for companion animals and livestock have attracted the scientific community and are growing exponentially (Sandri et al. 2014; Deng & Swanson 2015). The microbial population that inhabits the gastrointestinal tract of both humans and animals has been considered responsible of very important basic functions, from the regulation of metabolic activities to protection against pathogens and modulation of immune system and then physiologic functions (Pilla & Suchodolski 2020). The advent of innovative technologies allows for a more frequent utilization of molecular methodologies to identify non-culturable bacteria within the canine gut, highlighting the high individual variability of microbial population, especially in dietary intervention studies (Jha et al. 2020; Forster et al. 2018). Despite

these evidences, it appears clear that some key bacterial species are consistently present in fecal samples of healthy subjects, suggesting the presence of a core fecal bacterial community (Hand et al. 2013).

In a recent study on healthy dogs, the paramount role of diet and sex on the gut microbiome has been reported (Scarsella et al. 2020), confirming the idea that the microbes inhabiting the gut can be considered as an individual fingerprint (Garcia-Mazcorro et al. 2017). Human microbiome can be split in enterotypes, meaning that individuals can be clustered on the basis of the abundance of microbial taxa of the gut (Arumugam et al. 2011). The categorization in enterotypes has not yet been applied to dogs, also because the contradictory results that can be found in the literature make the identification of a gut microbiome core in dogs difficult. Interestingly, Scarsella et al. (2020) showed that dogs fed with a homemade diet (H) cluster together with dogs fed with a raw-based diet supplemented with a complementary food (B.A.S.E., Italy), even though the composition of these two diets was different. On the opposite, dogs fed with a commercial extruded complete diet (K) and a commercial moist complete diet (W) formed two distinctly clusters. It is likely that the presence of raw meat in the former diets and the physical form of the diets had a similar impact on shaping gut microbiome. Similar results have been obtained analyzing the gut microbiome in relation to the sex factor.

The prevalence of specific taxa can be effective to identify dysbiosis events. The concept of "dysbiosis" refers to a change in the composition of symbiotic or commensal microbial communities (Petersen et al. 2014). Considerable research has been dedicated to address the relationship between the gut microbiome and the health status of the subjects, both human and animals, but there is still a limited number of studies, only in the human field, that explored dysbiosis related to the blood microbiome and its potential role in pathogenesis. Blood has been traditionally considered to be a sterile environment, but some evidences in various domesticated mammals and birds (Mandal et al. 2016; Vientòs-Plotts et al. 2017) and in humans (Li et al. 2018; Whittle et al. 2019; Qiu et al. 2019) suggest that it is populated by microbes. The origin of these bacteria is mainly attributed to the translocation from the gastro-intestinal tract (Païssé et al. 2016), but it has been suggested that also a part of the microbial population of the oral cavity and the skin can diffuse into the blood (Cogen, Nizet & Gallo 2008). The hypothesis is that many bacteria found in healthy human blood may be in a dormant state (Potgieter et al. 2015), or they are present in their L-forms (Mercier, Kawai & Errington 2014).

The present study has three principal aims: firstly, to confirm the results of our previous study regarding the role of diet on fecal microbiome; secondly, to investigate the presence of a microbial population in the bloodstream in heathy subjects' and thirdly, to seek for a possible connection between the fecal and the blood microbiota.

6.2.3 Materials and Methods

6.2.3.1 Animals and Housing

Thirty-six healthy dogs were enrolled from three veterinary clinics. Both females (20 dogs, 10 of which were spayed) and males (16 intact dogs) were present in this study, and the dogs were of different breeds (small- to medium-size) and in the adult phase (more than two years old, less than 10). Dogs were divided on the basis of the type of diet consumed regularly since at least three months before the visit. The first group (10 dogs) was fed with commercial extruded complete dry pet foods (KIBBLE), the second group (16 dogs) with a raw meat diet (BARF) and the third group (10 dogs) with a homemade diet (HOME). The main ingredients of kibbles were chicken meat and fat, rice, and beet pulp, with an average crude protein content of 26.5% and fat content of 15.5% on a dry-matter basis. The BARF diet was made of a mix of meats (about 60% of beef or turkey or chicken, as fed), offal (about 20% as fed), bones (about 10% as fed), vegetables and oils (about 10% as fed). The HOME diet was formulated by a nutritionist, with an average of 45% raw beef meat, 40% boiled rice, 10% vegetables as fed and 5% mineral vitamins supplement. The dogs were presented to the clinics for normal routine visit, and the owners were asked to take the leftover feces and blood samples collected for the screening. Two clinics were situated in the city and were more generalist, and one was specialized and situated in the countryside. For each dog, one sample of blood and one sample of feces were collected the same day, to minimize the variability between these two types of biological matrices. An informed consent was obtained by the owners, and the dogs were housed in their usual home and condition. All protocols and procedures for the animals complied with the Italian legislation on animal care and were approved by the ethical committee of the University of Udine (OPBA, Prot. N. 7/2019, issued on 28 June 2019). Fecal material was transferred, using sterile gloves, into a sterile plastic bag and immediately stored at −20 °C until the analysis. Whole blood was collected in a K_3-EDTA tube with venipuncture from the radial vein after shaving the coat and careful disinfected with chlorhexidine alcohol solution. The samples were immediately stored at −20 °C until the analysis.

6.2.3.2 Fecal and Blood DNA Extraction, Sequencing, and Taxonomic Annotation

Microbial DNA was extracted by following the instructions of two commercial kit, based on the starting material. DNA from fecal samples was extracted from 150 mg of starting material, using a Fecal DNA MiniPrep kit with a bead beating step (Zymo Research; Irvine, CA, USA), whilst DNA from blood samples was extracted from 200 µL of starting material, using a Exgene™ Clinic SV kit (GenAll Biotechnology, Seoul, Korea). A ZymoBIOMICS™ Microbial Community Standard (Zymo Research, Irvine, CA, USA) was used to assess the efficiency of the entire pipeline, from DNA extraction method to taxonomic annotation. The mock community contains eight bacterial species: *Pseudomonas aeruginosa* (4.2%), *Escherichia coli* (10.1%), *Salmonella enterica* (10.4%), *Lactobacillus fermentum* (18.4%), *Enterococcus faecalis* (9.9%), *Staphylococcus aureus* (15.5%), *Listeria monocytogenes* (14.1%) and *Bacillus subtilis* (17.4%); expected composition of the mock community was given by the manufacturer.

DNA concentration was measured with a QubitTM 3 Fluorometer (Thermo Scientific; Waltham, MA, USA), and then the 16S rRNA of V3 and V4 regions were amplified for library preparation, adding also the indexes for sequencing, using a Nextera DNA Library Prep kit

(Illumina; San Diego, CA, USA), following the manufacturer's instruction and primers (Klindworth et al. 2013). The resulting amplicons were sequenced with a NovaSeq 6000 (Illumina; San Diego, CA, USA) in 2 × 300 paired-end mode, following the standard procedures. The Quantitative Insights into Microbial Ecology (QIIME 2) (Bolyen et al. 2019) was used to process the raw sequences, which were uploaded to NCBI Sequence Read Archive (Bioproject ID PRJNA668368). After demultiplexing, sequenced reads that passed the quality check (Phred score ≥ 30) were annotated for 16S rRNA against the most recent Greengenes database (version gg.13_8.otus.tar.gz), with 99%identifying with reference sequences. Chimeras were also detected and then filtered from the reads, and the remaining sequences were clustered into exact sequence variants by using an open-reference approach in QIIME 2. This procedure is the preferred strategy for exact sequence variants picking in QIIME2, which includes taxonomy assignment, sequence alignment and tree-building steps.

6.2.3.3 Quantitative Real-Time PCR (qPCR)

Quantifications of total bacteria were evaluated by qPCR, using the oligonucleotides tested by AlShawaqfeh et al. (2017). All samples were run in triplicate. The DNA extracted from the ZymoBIOMICS™ Microbial Community Standard (Zymo Research, Irvine, CA, USA) was used as a positive control and for the quantification of the total 16S copies DNA/g bacteria.

SYBR-based qPCR assays were performed by following the run protocol reported by AlShawaqfeh et al. (2017), with some modifications. Briefly, SYBR-based reaction mixtures (total 12.5 µL) contained 6.25 µL of Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), 3.25 µL of water, 0.25 µL of each primer (final concentration: 300 nM), and 2.5 µL of DNA previously standardized at 1 ng/µL. PCR conditions were 95 °C for 2 min, and 40 cycles at 95 °C for 5 and 10 s at the optimized annealing temperature. A melt curve analysis was performed for SYBR-based qPCR assays under the following conditions: 1 min at 95 °C, 1 min at 55 °C, and 80 cycles of 0.5 °C increments (10 s each). A CFX96 Touch System (Bio-Rad Laboratories, Hercules, CA, USA) was used for all qPCR assays. Data are expressed as average values and standard deviations.

6.2.3.4 Computation and Statistical Analysis

Annotated Operational Taxonomic Units (OTUs) were imputed on a spreadsheet, together with diets, to allow and facilitate further statistical analysis. The annotates sequences from each sample and each taxonomic level were normalized to ‰ abundance profiles, already known as Relative Abundance (RA). Taxa with RA lower than 1‰ in more than half of the samples were excluded from the statistical analysis (Sandri et al. 2019). RAs were transformed into Absolute Abundances (AA), multiplying each datum with the quantification of total bacteria revealed by the qPCR for each sample. A non-parametric Kruskal–Wallis test was applied at the phylum, family and genera level of fecal and blood matrices, with Bonferroni correction for multiple comparison. A p-value below 0.05 was considered statistically significant, and below 0.1 was considered a trend. Principal Component Analysis (PCA) was used to analyze phylum, family and genera level of taxa within fecal and blood samples. Shannon and Evenness biodiversity indexes were calculated at the genus level of blood and gut microbiome (Sandri et al. 2020). Beta diversity was assessed with the Brian Curtis dissimilarity matrix and visualized by using principal coordinate analysis (PCoA) plot.

Analysis of similarity (ANOSIM) was performed with the "Vegan" package in R (Version 3.2.1), to test whether the gut and the blood microbiome significantly differed between the three diets. All of these analyses were performed with XLSTAT (Addinsoft 2020).

6.2.4 Results

6.2.4.1 General Description of Blood Microbiome Related to Gut Microbiome

The collection of blood and fecal samples on the same day was conducted to investigate the possible relationship between blood microbiome and gut microbiome. Venn diagrams (Figure 1) show the results of the annotation comparison between blood and feces on three different taxa levels. As expected, the amount of bacteria in the blood was very low in comparison to feces; however, in the former matrix, the number of annotated taxa was much higher. Nevertheless, it is also notable that the blood and gut microbiomes share 9, 19 and 13 annotations at a phylum, family and genus level, respectively.

At the phylum level, gut and blood microbiome shared Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria (Table 1). Furthermore, the blood microbiome was characterized by four additional taxa, which were not shown in Table 1 because they were not relevant at the statistical analysis, due to the very low abundances detected for them. More interestingly, the blood microbiome is characterized by high quantities—in some cases even more than gut the microbiome—of Proteobacteria and Actinobacteria, in every diet considered in this study. Furthermore, Fusobacteria phylum on blood samples resulted in being significative different in BARF diet compared to the HOME diet, whilst dogs fed with a KIBBLE diet have intermediate values.

6.2.4.2 Characterization of Fecal Microbiome Related to Diets

At the family level, only the AA of Clostridiaceae, Coriobacteriaceae and Fusobacteriaceae resulted in significantly different copies between diets. In each of the thee families mentioned above, the higher abundance was detected in dogs fed with a BARF diet in comparison to dogs fed with a KIBBLE diet, whilst subjects that received a HOME diet had AA not significantly different from the other two diets. (Table 2). Lachnospiraceae family had very high AA in all dogs, and, although not significantly different between diets, these taxa showed a trend, with higher abundances in fecal samples of dogs fed with a KIBBLE diet, compared to subjects receiving a BARF or a HOME diet.

At the genus level (Table 3), again, *Clostridium* was significantly different in dogs fed with different diets. In particular, this genus had a higher AA in dogs fed with a BARF diet, compared to subjects receiving a HOME and a KIBBLE diet. The same trend was observed in the AA of *Collinsella*, which was significative higher in fecal samples of dogs fed the BARF diet. Although statistical analysis of the gut microbiome highlighted only these two genera, the other two genera showed a trend of AA between diets. The genus *Catenibacterium*, that is part of the Erysipelotrichaceae family (phylum Firmicutes), had higher AA in dogs fed with HOME diet, whilst it was decreased in fecal samples of dogs fed with KIBBLE diet, and those fed a BARF diet had the lowest abundances. Besides these taxa, also *Slackia* genus, part of the

Coriobacteriaceae family (phylum Actinobacteria), showed a trend, having the highest AA in dogs fed with a BARF diet, whilst dogs of the KIBBLE-diet group had the lowest values.

The PCA, performed with AA of phylum, family and genus level of the gut microbiome (Figure 2), showed a picture of the clusterization between the three groups of dogs based on their diets. In particular, the clearer clusterization was obtained for dogs fed with the BARF diet, at all the levels analyzed. Of greatest interest is the observation of some subjects that did not fit into the clusterization of their diet group.

6.2.4.3 Characterization of Blood Microbiome Related to Diets

A significant difference on blood microbiome in relation to different administered diet was observed. At the family level, the AA of Corynebacteriaceae, Fusobacteriaceae, Phyllobacteriaceae, Ruminococcaceae and Sphingomonadaceae resulted in being significantly different between the three diets. In particular, Fusobacteriaceae, Ruminococcaceae and Sphingomonadaceae showed the highest abundances in dogs fed with a BARF diet, compared to dogs fed with a KIBBLE and HOME diets (Table 4). The other taxa reported highlighted a trend with a *p*-value below 0.1. Moreover, in blood microbiome, Lachnospiraceae family was detected, and, as for gut microbiome, it had a higher AA in dogs fed with KIBBLE; on the contrary, intermediate values were shown in subjects receiving a BARF diet, whilst dogs of the HOME group diet had the lowest abundances.

At the genus level (Table 5), only *Corynebacterium*, belonging to the Corynebacteriaceae family (phylum Actinobacteria), resulted in significantly different amounts in dogs fed with different diets. In particular, this genus had higher AA in dogs fed with a BARF diet in comparison to subjects receiving a HOME diet and a KIBBLE diet. The other taxa reported in Table 5 highlighted a trend with a *p*-value below 0.1. Of interest were *Propionibacterium*, *Sphingomonas* and *Turicibacter* genera, which had higher AA compared to the other bacteria. *Propionibacterium*, part of the Propionibacteriaceae family (phylum Actinobacteria), was consistently higher in blood microbiome of subjects receiving a HOME diet, while it was detected in lower amounts in dogs fed with a BARF diet. In contrast, *Sphingomonas*, a genus of the Sphingomonadaceae family (phylum Proteobacteria), appeared to be higher in this latter group of dogs, compared to the HOME diet group. In the end, *Turicibacter*, belonging to the family of the Turicibacteriaceae (phylum Firmicutes), resulted in being higher in dogs fed with a KIBBLE diet, whilst the BARF group showed the lowest values.

Although gut and blood microbiome showed differences in terms of taxa that can discriminate between dogs fed with different diets, a multivariate approach highlights that both types of microbiomes almost equally cluster the subjects on the base of what they eat. The PCA (Figure 3), performed with AA on phylum, family and genus level of the blood microbiome detected a clusterization of the three groups of dogs, based on their diets. The clearest clusterization was obtained with data from dogs fed with the BARF diet, and this result is better shown at the phylum level. Furthermore, in the blood microbiome, it was also observed that some dogs do not fit in the clusterization of their diet group.

6.2.4.4 Alpha and Beta Diversity of Gut and Blood Microbiome Related to Diets

Alpha and beta diversity levels were analyzed at the genus level of both gut and blood microbiome. Alpha diversity was calculated through the Shannon and Evenness indexes, and no significative differences were found in dogs fed with the three diets, in the microbial population of fecal and blood samples. Results are shown in Table 6.

Beta diversity was computed through the Bray–Curtis dissimilarities distance matrix, at the genus level of both gut and blood microbiome. The PCoA (Figure 4) highlighted clear differences on gut microbiome of subjects fed with the three different diets. In particular, the dogs in the BARF group are very separated from the rest of the population, meaning that their microbial population is different from dogs fed a different type of diet. Although the separation of dogs is not distinct for gut microbiome case, in the blood microbiome, the clustering is appreciable.

6.2.5 Discussion

Several studies have pointed out that the gut microbiome is highly variable among healthy dogs (Sandri et al. 2019) and can represent an individual fingerprint (Garcia-Mazcorro et al. 2017). Several factors, other than different methodological approaches, affect the abundances of organisms in the gut microbiome, such as diet, sex, age and disease (Cintio et al. 2020; Vilson et al. 2018). In humans, geographical variations, ethnicity, host genetic, immunity, lifestyle and dietary habits have been reported to affect gut microbiota (Gupta, Paul & Dutta 2017). In healthy dogs, variations of microbiome with age, from weaning to adulthood, were reported (Vilson et al. 2018; Ribeiro et al. 2019), with a stabilization of the core gut microbiota at maturity. A strong similarity between human and dog microbiota has been reported, suggesting that the same factors affecting gut microbial community in the former can act also in the latter (Coelho et al. 2018).

Figure 2 showed a picture of the clustering between the three groups of dogs based on their diets. The more distinct clustering was appreciable for dogs fed with BARF diet, at phyla, family and genus level. Recently, a study on healthy dogs confirmed the role of diet on the gut microbiome (Scarsella et al. 2020), but also several other studies indicated the influence of diet on intestinal microbiome (Deng & Swanson 2015; Jha et al. 2020; Sandri et al. 2019; Beloshapka et al. 2013). Beyond this result, it is interesting to observe that some subjects did not fit in the cluster of their dietary group. This evidence could be due to an unpredictable and discontinuous administration of foods not foreseen in the daily diet of the dogs, since they were not recruited from a kennel or a shelter, but from local veterinary clinics. The owners could have fed the dogs with rewards or fresh foods but not necessarily every day. For instance, the subjects receiving a KIBBLE diet, marked with green squares, were fed also with some homemade or some raw-meat-based diet, and this could also be possible for some dogs fed with a BARF or a HOME diet, where kibble could have been fed.

Moreover, the gut microbiome ecosystem has strong interactions with the environment and the genetics of the host. Vazquez-Baeza et al. (2016) reported that the diversity and structure of microbial community, more than the variation of single taxon, could be used as a signature of the fecal microbiota to separate dogs with IBD from healthy dogs. The better characterization of the gut microbiome has been obtained, in the past, thanks to studies with subjects that lived in the

same controlled environment, such as a shelter, kennel or university facilities, where the dogs received the same diet under strict control, either for a short or long period of time (Forster et al. 2018; Ribeiro et al. 2019; Beloshapka et al. 2013). In this study, although the potential bias due to the effect of the different environments and of the owners can be claimed, it was still possible to separate dogs based on their diet. Moreover, the characterization of gut microbiome with a multivariate approach allowed the identification of those subjects for which diet was mixed in terms of administered type.

Another aim of the study was to investigate if gut microbes can translocate to the blood crossing the gut wall. The so called "leaky gut" condition is widely studied in humans and dogs and often is related to inflammatory bowel diseases and related enteropathies (Ridyard et al. 2007; Suchodolski 2016; Stewart, Pratt-Phillips & Gonzales 2017; Tizard & Jones 2018), but this functional deficiency should not happen in healthy animals. In the present study, we analyzed the presence of bacterial DNA in blood of healthy animals, and, surprisingly, several taxa were detected. Blood has been traditionally considered to be a sterile environment, but some evidences for bacterial presence in various domesticated mammals and birds (Mandal et al. 2016; Vientòs-Plotts et al. 2017) and in humans (Li et al. 2018; Whittle et al. 2019; Qiu et al. 2019) do exist. Despite the difficulties to cultivate blood bacteria, several studies reported the successful growth of numerous bacteria from blood of healthy individuals confirmed by microscopic observation (Potgieter et al. 2015; Damgaard et al. 2015). Even if the majority of taxa annotated in blood was present at a very low abundances, they were detectable. One of the hypotheses is that the bacteria populating the blood are in a "dormient phase" (Kell et al. 1998), and this would explain why there is a microbial population even in healthy subjects and also why this condition is not pathogenic. The hypothesis is that, occasionally, some of these bacteria "wake up" and reproduce, becoming active again and being transported to various tissues and organs of the body, thus inducing a state of chronic disease (Kell & Pretorius 2015). However, cultural-independent methods do not provide evidence of whether a blood microbial signature is from transient nucleic acids or from live bacteria (Lagier et al. 2018). To the best of our knowledge, this is the first study characterizing canine blood microbiome. External factors, such as contamination of reagents and blood with external bacteria during the sampling, could have led to an artifactual appearance of microbiome into the blood. For what the reagents and the sequencing pipeline is concerned, a mock bacterial community was used as internal standard to validate the methodology, and the results confirmed the lack of contamination.

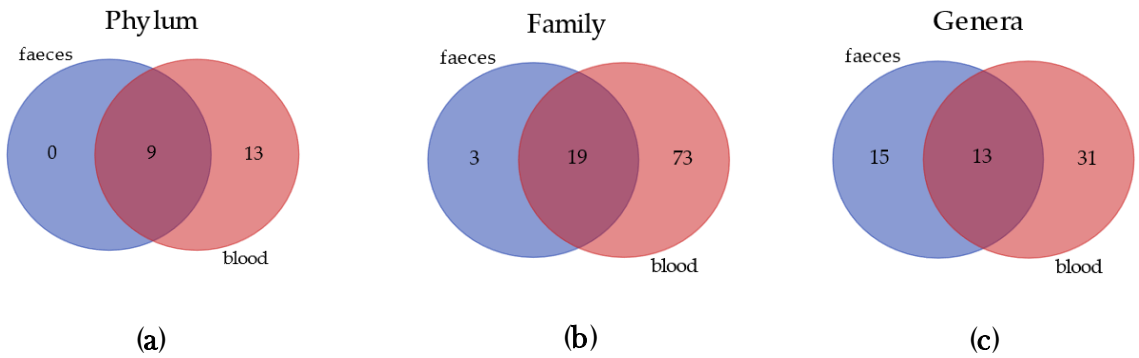
Interestingly, the number of bacterial taxa annotated for blood samples was much higher compared to fecal samples, especially at a family level, with 92 taxa annotated in blood and 22 in feces (Figure 1). The blood bacterial taxa found in this study are in line with the literature found for humans (Païssé et al. 2016). The most detected bacteria in blood (Table 1) were Proteobacteria and Actinobacteria, which differed consistently from the predominant phyla of the gut microbiome, that were Firmicutes and Bacteroidetes (Deng & Swanson 2015; Turnbaugh et al. 2006). This difference between blood and feces could be explained by the role of filter played by intestinal and immune cells, which could have limited the translocation of certain bacteria. Moreover, it has been reported that mesenteric lymph nodes (MLN) are actively involved in the translocation of certain pathogens, filtering the microbes to control the downstream colonization of fluids and organs (Runyon et al. 1994).

The recognition of "good" or "bad" microorganisms is due to the presence of dendritic cells in the gut (Rimoldi et al. 2005), although environmental factors can shift commensal bacteria to pathogenic bacteria, causing a disruption at a variable extent of tight junction and a leaky gut. Furthermore, tissues and organs, such as skin, oral cavity and nasal or vaginal mucosa, can probably contribute to the bacterial DNA present in the blood (Costello et al. 2009). Although the microbial composition in the feces was not equal to those in the blood, in terms of abundances and presence of certain taxa, the clustering of dogs in the three diets was comparable. The PCA multivariate analysis allowed to separate the dogs by using the gut microbiome (Figure 2), and almost the same results were obtained by using the blood microbial community (Figure 3).

6.2.6 Conclusions

This study confirmed that diet is a factor driving the shift of gut microbial population in dogs, and researches in this direction are still needed to better clarify the association between this factor and gut microbiome. A limited number of research studies have been published on blood microbiota in humans, and this is the first evidence of the presence of a bacterial population in the blood of healthy dogs. From our results, we can speculate that blood microbiome, or at least a part of it, can derive from the translocation of some gut bacteria, and it consequently could be associated with a shift of the intestinal microbial population. These preliminary results deserve further studies, including also dogs suffering of gastrointestinal diseases; however, if confirmed, the results pave the way for the use of blood microbiome for diagnostic purposes.

6.2.7 TABLES AND FIGURES



(a) (b) (c)

Figure 1. Similarity in bacterial community composition at phylum, family and genus levels in blood and feces of healthy dogs. (**a**) Venn diagrams showing the number of core phylum in blood and feces. Core phylum is defined as a phylum that is found in all dogs. (**b**) Venn diagrams showing the number of core families in blood and feces. Core family is defined as a phylum that is found in all dogs. (**c**) Venn diagrams showing the number of core genera in blood and feces. Core genera are defined as phyla that are found in all dogs.

Table 1. Mean and standard deviation of phylum Absolute Abundances (AA), expressed in 16S copies DNA/g bacteria, characterizing feces and blood of subjects fed with three different diets. [a,b] on the same row denotes differences between means for p-value < 0.05.

		BARF		HOME		KIBBLE	
		mean	st.dev.	mean	st.dev.	mean	st.dev.
Actinobacteria	faeces	65.0	83.9	18.2	30.8	21.6	32.2
	blood	8.8	10.2	21.2	17.2	12.7	7.2
Bacteroidetes	faeces	97.3	119.8	69.7	97.2	106.1	89.1
	blood	2.8	5.1	3.1	3.9	10.9	8.2
Firmicutes	faeces	1062.3	368.0	1064.2	474.6	896.3	345.7
	blood	24.6	11.9	30.7	25.9	31.0	12.8
Fusobacteria	faeces	100.8	99.9	93.7	126.2	75.9	141.0
	blood	0.2 [b]	0.2	0.0 [a]	0.0	0.1 [ab]	0.4
Proteobacteria	faeces	10.4	10.9	60.1	83.7	57.4	128.8
	blood	18.2	14.5	13.3	21.5	17.6	14.8
OD1	faeces	n.a.		n.a.		n.a.	
	blood	1.9	2.6	0.8	2.6	0.0	0.1

BARF, raw-meat-based diet; HOME, homemade diet; KIBBLE, complete extruded diet.

Table 2. Kruskal–Wallis non-parametric test results of the family Absolute Abundances (AA), expressed in 16S copies DNA/g bacteria, characterizing fecal samples of subjects fed with three different diets. Mean and standard deviation (SD) are reported; [a,b] on the same row denotes differences between means for p-value < 0.05.

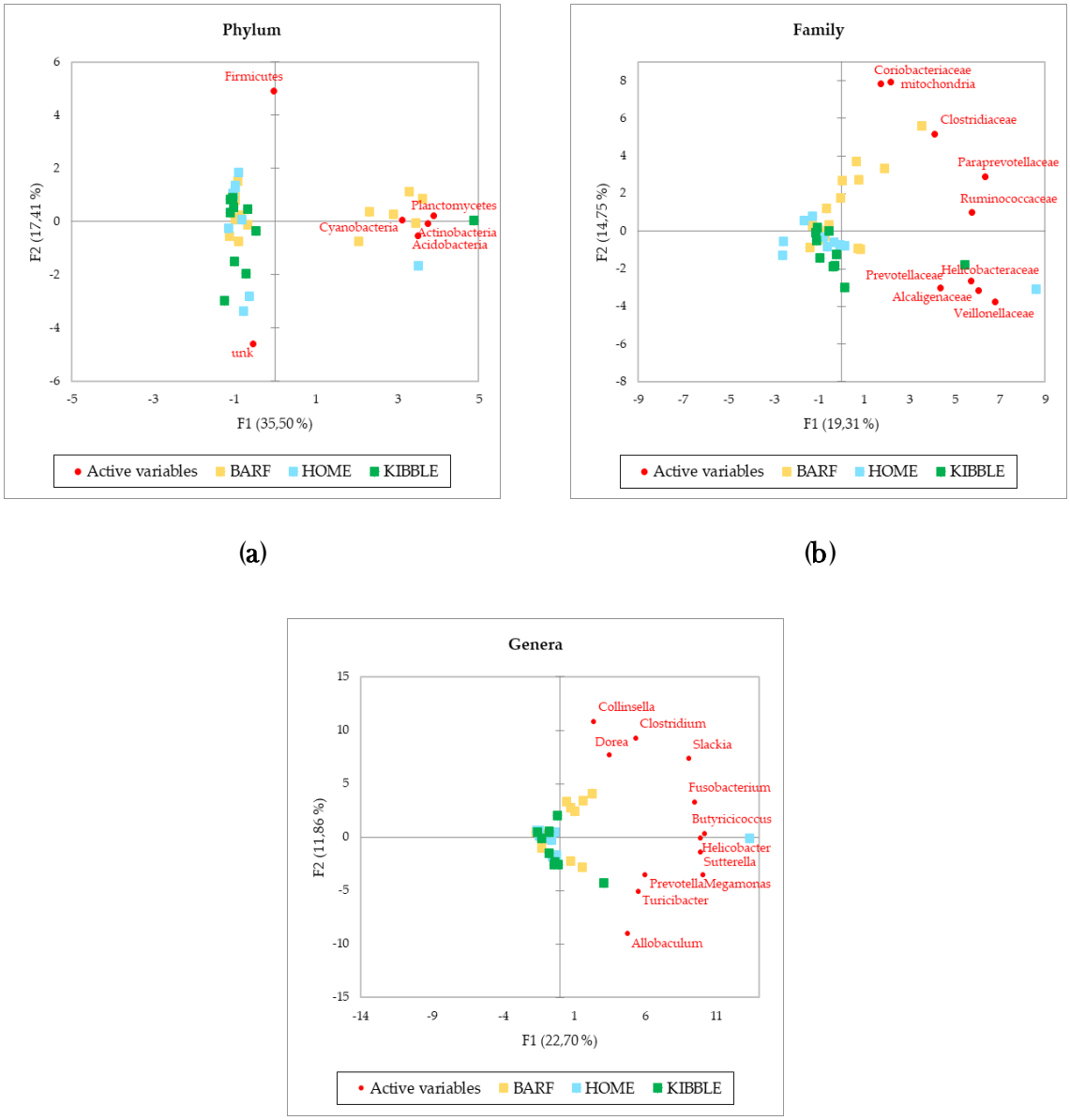
	BARF		**HOME**		**KIBBLE**		
	mean	st.dev.	mean	st.dev.	mean	st.dev.	p-value
Clostridiaceae	446.6 [b]	364.5	229.5 [ab]	268.3	175.4 [a]	195.7	0.045
Coriobacteriaceae	75.5 [b]	75.7	29.1 [ab]	37.1	19.6 [a]	35.7	0.021
Fusobacteriaceae	132.5 [b]	165.1	75.0 [ab]	79.8	16.0 [a]	20.2	0.015
Lachnospiraceae	304.8	173.3	338.2	291.5	428.3	247.9	0.469

BARF, raw-meat diet; HOME, homemade diet; KIBBLE, complete extruded diet.

Table 3. Kruskal–Wallis non-parametric test results of the genus Absolute Abundances (AA), expressed in 16S copies DNA/g bacteria, characterizing fecal samples of subjects fed with three different diets' mean and standard deviation (SD) are reported; [a,b] on the same row denotes differences between means for p-value < 0.05.

	BARF		**HOME**		**KIBBLE**		
	mean	st.dev.	mean	st.dev.	mean	st.dev.	p-value
Catenibacterium	2.1	8.3	18.5	31.7	6.9	15.1	0.069
Clostridium	389.0 [b]	389.4	139.9 [a]	227.8	122.7 [ab]	119.7	0.030
Collinsella	56.4 [b]	74.6	11.8 [ab]	10.8	2.5 [a]	4.9	0.022
Slackia	4.7	7.0	3.3	10.4	0.1	0.1	0.071

BARF, raw-meat diet; HOME, homemade diet; KIBBLE, complete extruded diet.



(a)

(b)

(c)

Figure 2. Principal Component Analysis (PCA) of bacteria absolute abundancies (AA) regarding (a) Phylum level, (b) Family level and (c) Genus level on faecal samples of dogs fed with a raw meat mased diet (BARF), a home-made based diet (HOME) and a commercial complete extruded diet (KIBBLE).

Table 4. Kruskal–Wallis non-parametric test results of the family Absolute Abundances (AA), expressed in 16S copies DNA/g bacteria, characterizing blood samples of subjects fed with three different diets mean and standard deviation (SD) are reported; [a,b] on the same row denotes differences between means for p-value < 0.05.

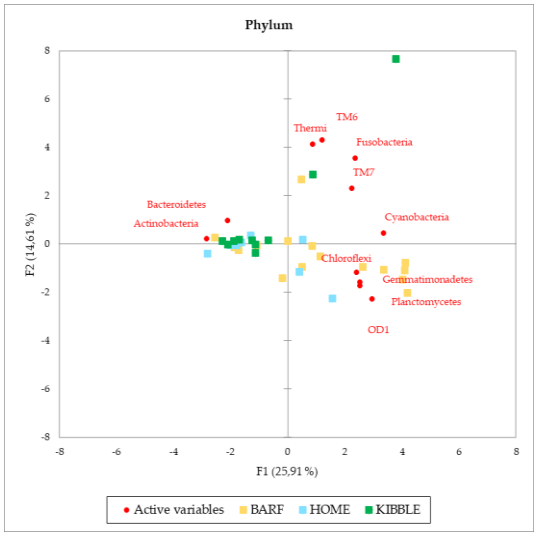
	BARF		HOME		KIBBLE		
	mean	st.dev.	mean	st.dev.	mean	st.dev.	p-value
Bifidobacteriaceae	0.2	0.2	0.0	0.0	0.3	0.7	0.066
Coriobacteriaceae	0.7	2.0	0.0	0.0	0.6	1.7	0.070
Corynebacteriaceae	0.4 [ab]	0.6	0.7 [b]	0.6	0.0 [a]	0.0	0.003
Fusobacteriaceae	0.2 [b]	0.2	0.0 [a]	0.0	0.1 [ab]	0.4	0.027
Lachnospiraceae	3.5	3.6	0.3	0.5	3.8	6.5	0.054
Phyllobacteriaceae	0.0 [a]	0.0	0.4 [b]	0.8	0.0 [ab]	0.0	0.016
Propionibacteriaceae	6.0	10.0	19.5	16.0	9.9	9.7	0.057
Ruminococcaceae	4.9 [b]	5.8	0.1 [a]	0.2	1.6 [ab]	2.8	0.031
Sphingomonadaceae	10.2 [b]	11.3	0.6 [a]	1.6	1.9 [ab]	2.9	0.045
Turicibacteraceae	0.5	1.5	1.4	1.9	2.8	4.0	0.054

BARF: raw meat diet; HOME: home-made diet; KIBBLE: complete extruded diet.

Table 5. Kruskal–Wallis non-parametric test results of the genus Absolute Abundances (AA), expressed in 16S copies/g, characterizing blood samples of subjects fed with three different diets. Mean and standard deviation (SD) are reported; [a,b] on the same row denotes differences between means for *p*-value < 0.05.

	BARF		**HOME**		**KIBBLE**		
	mean	st.dev.	mean	st.dev.	mean	st.dev.	*p*-value
Corynebacterium	0.4 [ab]	0.6	0.7 [b]	0.6	0.0 [a]	0.0	0.003
Delftia	0.5	1.6	1.1	1.3	0.8	0.8	0.058
Propionibacterium	6.0	10.0	19.5	16.0	9.9	9.7	0.057
Sedimentibacter	0.0	0.0	0.3	0.9	0.7	1.2	0.069
Sphingomonas	9.7	11.5	0.3	0.8	1.5	1.9	0.071
Turicibacter	0.5	1.5	1.4	1.9	2.8	4.0	0.054

BARF, raw-meat diet; HOME, homemade diet; KIBBLE, complete extruded diet.



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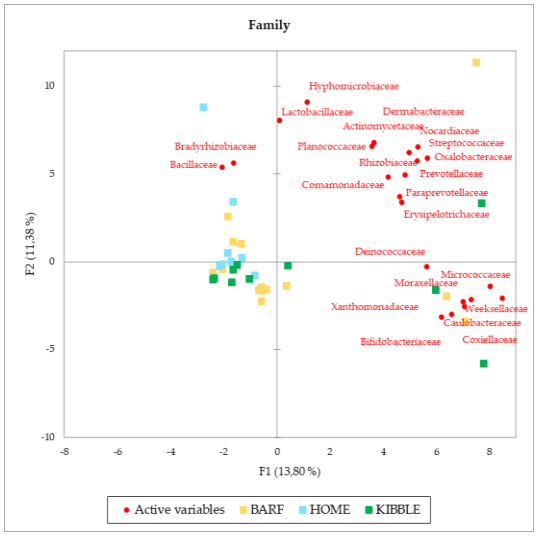

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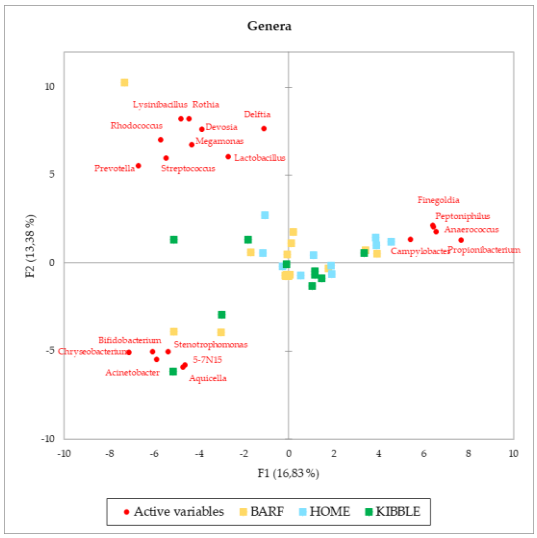

(c)

Figure 3. Principal Component Analysis (PCA) of bacteria absolute abundancies (AA) regarding (**a**) Phylum level, (**b**) Family level and (**c**) Genera level on blood samples of dogs fed with a raw meat mased diet (BARF), a home-made based diet (HOME) and a commercial complete extruded diet (KIBBLE).

6.2.8 References

Addinsoft. 2020. "XLSTAT Statistical and Data Analysis Solution." Boston, MA, USA.

AlShawaqfeh, M.K.; Wajid, B.; Minamoto, Y.; Markel, M.; Lidbury, J.A.; Steiner, J.M.; Serpedin, E.; Suchodolski, J.S. 2017. "A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy." *FEMS Microbiol. Ecol.* 93, fix136. http://dx.doi.org/10.1093/femsec/fix136

Arumugam, M.; Raes, J.; Pelletier, E.; Le Paslier, D.; Yamada, T.; Mende, D.R.; Fernandes, G.R.; Tap, J.; Bruls, T.; Batto, J.-M.; et al. 2011. "Enterotypes of the human gut microbiome." *Nature* 473, 174–180. http://dx.doi.org/10.1038/nature09944

Beloshapka, A.N.; Dowd, S.E.; Suchodolski, J.S.; Steiner, J.M.; Duclos, L.; Swanson, K.S. 2013. "Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing." *FEMS Microbiol. Ecol.* 84, 532–541. http://dx.doi.org/10.1111/1574-6941.12081

Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. 2019. "Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2." *Nat. Biotechnol.* 37, 852–857. http://dx.doi.org/10.1038/s41587-019-0209-9

Cintio, M.; Scarsella, E.; Sgorlon, S.; Sandri, M.; Stefanon, B. 2020. "Gut Microbiome of Healthy and Arthritic Dogs." *Vet. Sci.* 7, 92. http://dx.doi.org/10.3390/vetsci7030092

Coelho, L.P.; Kultima, J.R.; Costea, P.I.; Fournier, C.; Pan, Y.; Czarnecki-Maulden, G.; Hayward, M.R.; Forslund, S.K.; Schmidt, T.S.B.; Descombes, P.; et al. 2018. "Similarity of the dog and human gut microbiomes in gene content and response to diet." *Microbiome* 6, 72. http://dx.doi.org/10.1186/s40168-018-0450-3

Cogen, A.L.; Nizet, V.; Gallo, R.L. 2008. "Skin microbiota: A source of disease or defence?" *Br. J. Dermatol.* 158, 442–455. http://dx.doi.org/10.1111/j.1365-2133.2008.08437.x

Costello, E.K.; Lauber, C.L.; Hamady, M.; Fierer, N.; Gordon, J.I.; Knight, R. 2009. "Bacterial Community Variation in Human Body Habitats Across Space and Time." *Science* 326, 1694–1697. http://dx.doi.org/10.1126/science.1177486

Damgaard, C.; Magnussen, K.; Enevold, C.; Nilsson, M.; Tolker-Nielsen, T.; Holmstrup, P.; Nielsen, C.H. Viable 2015. "Bacteria Associated with Red Blood Cells and Plasma in Freshly Drawn Blood Donations." *PLoS ONE* 10, e0120826. http://dx.doi.org/10.1371/journal.pone.0120826

Deng,P.; Swanson,K.S. 2015. "Gut microbiota of humans, dogs and cats: Current knowledge and future opportunities and challenges." *Br. J. Nutr.* 113, S6–S17. http://dx.doi.org/10.1017/S0007114514002943

Forster, G.M.; Stockman, J.; Noyes, N.; Heuberger, A.L.; Broeckling, C.D.; Bantle, C.M.; Ryan, E.P. 2018. "A Comparative Study of Serum Biochemistry, Metabolome and Microbiome Parameters of Clinically Healthy, Normal Weight, Overweight, and Obese Companion Dogs." *Top. Companion Anim. Med.* 33, 126–135. http://dx.doi.org/10.1053/j.tcam.2018.08.003

Garcia-Mazcorro, J.F.; Barcenas-Walls, J.R.; Suchodolski, J.S.; Steiner, J.M. 2017. "Molecular assessment of the fecal microbiota in healthy cats and dogs before and during supplementation with fructo-oligosaccharides (FOS) and inulin using high-throughput 454-pyrosequencing." *PeerJ* 5, e3184. http://dx.doi.org/10.7717/peerj.3184

Gupta, V.K.; Paul, S.; Dutta, C. 2017. "Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity." *Front. Microbiol.* 8, 1162. http://dx.doi.org/10.3389/fmicb.2017.01162

Hand, D.; Wallis, C.; Colyer, A.; Penn, C.W. 2013. "Pyrosequencing the Canine Faecal Microbiota: Breadth and Depth of Biodiversity." *PLoS ONE* 8, e53115. http://dx.doi.org/10.1371/journal.pone.0053115

Jha, A.R.; Shmalberg, J.; Tanprasertsuk, J.; Perry, L.; Massey, D.; Honaker, R.W. 2020. "Characterization of gut microbiomes of household pets in the United States using a direct-to-consumer approach." *PLoS ONE* 15, e0227289. http://dx.doi.org/10.1371/journal.pone.0227289

Kell, D.B.; Kaprelyants, A.S.; Weichart, D.H.; Harwood, C.R.; Barer, M.R. 1998. "Viability and activity in readily culturable bacteria: A review and discussion of the practical issues." *Antonie Van Leeuwenhoek* 73, 169–187. http://dx.doi.org/10.1023/A:1000664013047

Kell, D.B.; Pretorius, E. 2015. "On the translocation of bacteria and their lipopolysaccharides between blood and peripheral locations in chronic, inflammatory diseases: The central roles of LPS and LPS-induced cell death." *Int. Biol.* 7, 1339–1377. http://dx.doi.org/10.1039/c5ib00158g

Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. 2013. "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies." *Nucleic Acids Res.* 41, e1. http://dx.doi.org/10.1093/nar/gks808

Lagier, JC.; Dubourg, G.; Million, M.; Cadoret F.; Bilen M.; Fenollar F.; Levasseur A.; Rolain J.; Fournier P.E.; Raoult D. 2018. "Culturing the human microbiota and culturomics." *Nat Rev Microbiol* 16, 540–550. https://doi.org/10.1038/s41579-018-0041-0

Li, Q.; Wang, C.; Tang, C.; Zhao, X.; He, Q.; Li, J. 2018. "Identification and Characterization of Blood and Neutrophil-Associated Microbiomes in Patients with Severe Acute Pancreatitis Using Next-Generation Sequencing." *Front. Cell. Infect. Microbiol.* 8, 5. http://dx.doi.org/10.3389/fcimb.2018.00005

Mandal, R.K.; Jiang, T.; Al-Rubaye, A.A.; Rhoads, D.D.; Wideman, R.F.; Zhao, J.; Pevzner, I.; Kwon, Y.M. 2016. "An investigation into blood microbiota and its potential association with Bacterial Chondronecrosis with Osteomyelitis (BCO) in Broilers." *Sci. Rep.* 6, 25882. http://dx.doi.org/10.1038/srep25882

Mercier, R.; Kawai, Y.; Errington, J. 2014. "General principles for the formation and proliferation of a wall-free (L-form) state in bacteria." *eLife* 3, e04629. http://dx.doi.org/10.7554/eLife.04629

Païssé, S.; Valle, C.; Servant, F.; Courtney, M.; Burcelin, R.; Amar, J.; Lelouvier, B. 2016. "Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing." *Transfusion* 56, 1138–1147. http://dx.doi.org/10.1111/trf.13477

Petersen, C.; Round, J.L. 2014. "Defining dysbiosis and its influence on host immunity and disease." *Cell. Microbiol.* 16, 1024–1033. http://dx.doi.org/10.1111/cmi.12308

Pilla, R.; Suchodolski, J.S. 2020. "The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease." *Front. Vet. Sci.* 6, 498. http://dx.doi.org/10.3389/fvets.2019.00498

Potgieter, M.; Bester, J.; Kell, D.B.; Pretorius, E. 2015. "The dormant blood microbiome in chronic, inflammatory diseases." *FEMS Microbiol. Rev.* 39, 567–591. http://dx.doi.org/10.1093/femsre/fuv013

Qiu, J.; Zhou, H.; Jing, Y.; Dong, C. 2019. "Association between blood microbiome and type 2 diabetes mellitus: A nested case-control study." *J. Clin. Lab. Anal.* 33, e22842. http://dx.doi.org/10.1002/jcla.22842

Ribeiro, É.D.M.; Peixoto, M.C.; Putarov, T.C.; Monti, M.; Pacheco, P.D.G.; Loureiro, B.A.; Pereira, G.T.; Carciofi, A.C. 2019. "The effects of age and dietary resistant starch on digestibility, fermentation end products in faeces and postprandial glucose and insulin responses of dogs." *Arch. Anim. Nutr.* 73, 485–504. http://dx.doi.org/10.1080/1745039X.2019.1652516

Ridyard, A.E.; Brown, J.K.; Rhind, S.M.; Else, R.W.; Simpson, J.W.; Miller, H.R.P. 2007. "Apical Junction Complex Protein Expression in the Canine Colon: Differential Expression of Claudin-2 in the Colonic Mucosa in Dogs with Idiopathic Colitis." *J. Histochem. Cytochem.* 55, 1049–1058. http://dx.doi.org/10.1369/jhc.7A7211.2007

Rimoldi, M.; Chieppa, M.; Salucci, V.; Avogadri, F.; Sonzogni, A.; Sampietro, G.M.; Nespoli, A.; Viale, G.; Allavena, P.; Rescigno, M. 2005. "Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells." *Nat. Immunol.* 6, 507–514. http://dx.doi.org/10.1038/ni1192

Runyon, B.A.; Squier, S.; Borzio, M. 1994. "Translocation of gut bacteria in rats with cirrhosis to mesenteric lymph nodes partially explains the pathogenesis of spontaneous bacterial peritonitis." *J. Hepat.* 21:791-796. http://doi.org/10.1016/s0168-8278(94)80241-6

Sandri, M.; Manfrin, C.; Pallavicini, A.; Stefanon, B. 2014. "Microbial biodiversity of the liquid fraction of rumen content from lactating cows." *Animal* 8, 572–579. http://dx.doi.org/10.1017/S1751731114000056

Sandri, M.; Sgorlon, S.; Conte, G.; Serra, A.; Monego, S.D.; Stefanon, B. 2019. "Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs." *Ital. J. Anim. Sci.* 18, 1205–1214. http://dx.doi.org/10.1080/1828051X.2019.1645624

Sandri, M.; Sgorlon, S.; Scarsella, E.; Stefanon, B. 2020. "Effect of different starch sources in a raw meat-based diet on fecal microbiome in dogs housed in a shelter." *Anim. Nutr.* 6, 353–361. http://dx.doi.org/10.1016/j.aninu.2020.03.003

Scarsella, E.; Stefanon, B.; Cintio, M.; Licastro, D.; Sgorlon, S.; Monego, S.D.; Sandri, M. 2020. "Learning machine approach reveals microbial signatures of diet and sex in dog." *PLoS ONE* 15, e0237874. http://dx.doi.org/10.1371/journal.pone.0237874

Stewart, A.S.; Pratt-Phillips, S.; Gonzalez, L.M. 2017. "Alterations in Intestinal Permeability: The Role of the "Leaky Gut" in Health and Disease." *J. Equine Vet. Sci.* 52, 10–22. http://dx.doi.org/10.1016/j.jevs.2017.02.009

Suchodolski, J.S. 2016. "Diagnosis and interpretation of intestinal dysbiosis in dogs and cats." *Vet. J.* 215, 30–37. http://dx.doi.org/10.1016/j.tvjl.2016.04.011

Tizard, I.R.; Jones, S.W. 2018. "The Microbiota Regulates Immunity and Immunologic Diseases in Dogs and Cats." *Vet. Clin. Small Anim. Pract.* 48, 307–322. http://dx.doi.org/10.1016/j.cvsm.2017.10.008

Turnbaugh, P.J.; Ley, R.E.; Mahowald, M.A.; Magrini, V.; Mardis, E.R.; Gordon, J.I. 2006. "An obesity-associated gut microbiome with increased capacity for energy harvest." *Nature* 444, 1027–1031. http://dx.doi.org/10.1038/nature05414

Vázquez-Baeza, Y.; Hyde, E.R.; Suchodolski, J.S.; Knight, R. 2016. "Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks." *Nat. Microbiol.* 1, 1–5. http://dx.doi.org/10.1038/nmicrobiol.2016.177

Vientós-Plotts, A.I.; Ericsson, A.C.; Rindt, H.; Grobman, M.E.; Graham, A.; Bishop, K.; Cohn, L.A.; Reinero, C.R. 2017. "Dynamic changes of the respiratory microbiota and its relationship to fecal and blood microbiota in healthy young cats." *PLoS ONE* 12, e0173818. http://dx.doi.org/10.1371/journal.pone.0173818

Vilson, Å.; Ramadan, Z.; Li, Q.; Hedhammar, Å.; Reynolds, A.; Spears, J.; Labuda, J.; Pelker, R.; Björkstén, B.; Dicksved, J.; et al. 2018. "Disentangling factors that shape the gut

microbiota in German Shepherd dogs." *PLoS ONE* 13, e0193507. http://dx.doi.org/10.1371/journal.pone.0193507

Whittle, E.; Leonard, M.O.; Harrison, R.; Gant, T.W.; Tonge, D.P. 2019. "Multi-Method Characterization of the Human Circulating Microbiome." *Front. Microbiol.* 9, 3266. http://dx.doi.org/10.3389/fmicb.2018.03266

CHAPTER 7

7.1 Introduction to the study

The previous chapters have one thing in common. The dog's gut microbiome is made up of key microorganisms, which make up what is called the "core" of the microbiome. These taxa are essential for animal welfare and allow us to distinguish subjects on the basis of environmental and genetic factors, such as diet and sex. If in general the microbial population makes it possible to distinguish subjects on the basis of these factors, a still unresolved question is what are the relationships that exist between the taxa that characterize these categories of healthy subjects.

Recent microbiome analysis techniques have allowed us to go into more detail in the description of the intestinal microbiome. In fact, these methods allow to describe the single taxa, highlighting one property at a time of the microbiome, and thus not allowing to have an overview of what interactions exist within what can be considered a true and own organ. One of the most popular methods for studying the relationships between microorganisms is the creation of a network through the determination of correlations between taxa. In the past, Pearson's or Spearman's correlation coefficient has often been used; however, the use of these pairwise correlations presented some issued. The first is represented by the presence of compositional errors, while an even greater problem is caused by the sparse data. During the analysis of the microbial composition, there may be many zeroes which, for one thing, suggest the absence of that taxa, but it can also mean that the taxa is present in quantities that are too low to be detected. For this reason, other computational approaches have been developed, and one of the most used is the SparCC algorithm. SparCC uses iterative approximations and a log transformation of the microbial composition data to estimate pairwise correlations between taxa.

For this study, the data from the research from Chapter 3 were used. The sequences collected come from the analysis of fecal samples of 132 dogs in good health, subjected to different diet modulation experiments, all carried out by the same group of research. The samples were all analyzed in the same way, thus following a standardized analysis pipeline. The dogs were divided based on their diet and their sex, just to complete the results highlighted in chapter 3. The relative abundances of microorganisms that are part of the intestinal microbiome of dogs were analyzed through a new bioinformatics tool called SCNIC, which uses the SparCC algorithm to obtain correlations between bacteria. The taxa, subsequently grouped into modules, were analyzed for Diet and Sex factors through microbial composition analysis (ANCOM) and Kruskal-Wallis non-parametric test.

The analysis of the bacterial network showed that there were strong correlations between some taxa, which for this reason can be grouped into modules. Some of these modules, subsequently analyzed in terms of microbial composition, were significant for the Diet and Sex factors, and were positioned grouped in the network, depending on whether they had significative higher abundance in one diet over another, or in a sex than another. This helped us to understand that there were certain bacteria that co-exist together on the basis of well-defined factors, and that their abundances can be used as threshold values for determining a microbiome corresponding to an optimal health condition. This result is a further step towards the definition of "enterotype" also in the dog, a concept widely used in humans. Furthermore, the microorganisms belonging to the significant modules for Diet and Sex can help to modulate diets according to need, and to modify

any therapies for the treatment of enteropathies or diseases related to the GI tract, which are related to a state of intestinal dysbiosis.

This work will be submitted to a proper journal soon.

7.2 Network-based gut microbiome analysis in dogs

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7.2.1 Abstract

A growing number of studies in the last decade described the microbial population in different niches of the organism, and especially the gut microbiome, thank to High-throughput DNA sequencing techniques, that are easily accessible to researchers. Furthermore, network analysis allows the characterization of bacteria with indirect associations with outcomes such as diseases, diet and sex of the host, via their association with other taxa. The present study is a follow up of a previous one, that reported data collected from several in house dietary intervention studies carried out in healthy dogs. The dataset represented 132 dogs with 334 faecal samples collected serially from 8 studies. The animals were divided according to diet (commercial extruded diet, 171; commercial moist diet, 83; home-made diet, 30; BASE™ diet, 56) and sex (whole males, 78; whole females, 145; neutered males, 89; spayed females, 28). The procedure of samples collection, storage, DNA extraction and sequencing, bioinformatic and statistical analysis followed a defined pipeline. The extracted DNA was prepared for the sequencing of the V3 and V4 regions of the 16 rRNA gene with a MiSeq (illumina, San Diego, CA, USA) in a 2x300 paired-end mode. Raw sequences were processed using the bioinformatic program QIIME 2 and annotated to greengene database. Here, the results of the 16S rRNA sequencing of the V3 and V4 regions, was used to systematically analyze the structure of the gut microbiome of dogs fed with the above-mentioned different diets. The correlation network analysis was performed calculating pairwise relationship between taxa with the SparCC (Sparse Correlations for Compositional data) algorithm, that was designed to solve the problem of spurious correlations given by the compositional nature of microbiome data. Firstly, we identified candidate bacteria highly abundant in the microbial community, and secondly, we looked at taxa with direct and indirect associations with the factors diet and sex, that were the same considered in the previous study. In conclusion, this study demonstrated an approach to interpret the network structure of the gut microbiome on dogs categorized by diet and sex, giving a better explanation of the interactions between bacteria that resulted in a clustering of the dogs based on environmental or genetic influences. In particular, this study paved the bases to understand how the gut community of bacteria was interconnected and operated in relation to dietary composition and sex.

7.2.2 Introduction

The human and animal gut harbors a great community of microorganisms, extremely diverse one from another but that have the power of closely interact with each other and with the host. The field of microbiome in relation to healthy condition and environmental factors is continuously expanding for companion animals and livestock (Sandri et al. 2014; Deng e Swanson 2015). During these years, researchers have focused on the role of gut microorganisms found to be involved in metabolic activities, protection against pathogens, sending signals to the immune system and directly and indirectly affecting most of the physiologic functions (Pilla e Suchodolski 2020).

Several associations have been discovered between the composition of gut microbiome and the simultaneous appearance of diseases related or not to the gastrointestinal tract but correlated to a dysbiosis state of the animal. The definition of gut dysbiosis is related to an alteration in the composition of the microbial population that lives in the gastrointestinal tract. The modification of the balance existing between the abundance of bacteria inhabiting the intestine, leads to functional changes in microbial transcriptome, proteome or metabolome (Zeng, Inohara, e Nuñez 2017). These changes can induce important consequences for the host, like the beginning of conditions such as obesity, cardiovascular disease, inflammatory bowel disease and diabetes (David et al. 2014; Muegge et al. 2011; Thorburn, Macia, e Mackay 2014).

The composition of the gut microbiome has been showed to be influenced by several factors, both genetic and environmental. Improving health of the host through dietary management or selecting therapies to adopt by some genetic factor responses are the goal of the recent researches. Several experiments have shown that diet composition is reflected in different gut microbiome profiles (Pilla e Suchodolski 2020). In the previous published research on healthy dogs (Scarsella, Stefanon, et al. 2020), the role that diet and sex have on the gastrointestinal microbiota has been reported, showing that the composition of microbial population inhabiting the gut can be considered as an individual fingerprint (Garcia-Mazcorro et al. 2017). Dogs gut microbiome are not yet categorized into enterotypes, as it is for human. An enterotype is a clusterization of individuals having the same abundance of certain microbial taxa of the gut (Arumugam et al. 2011). This categorization has not yet applied to dogs mainly for the contradictory results that can be found in literature, due to small sample size of the experiments. Previously, we reported an analysis of a dataset of 334 faecal microbiome from healthy dogs. Microbial profiles were classified for the factors diet and sex, applying dimensional reduction discriminant analysis followed by Random Forest analysis. Interestingly, dogs fed with a home-made diet cluster together with dogs fed with a raw meat-based diet supplemented with a complementary food (BASE™), whilst dogs fed a commercial extruded complete diet and a commercial moist complete diet formed two groups aside. Comparable results have been detected analyzing the gut microbiome in relation to sex of the dogs.

Recently, studies regarding the microbiome complexity have advanced considerably, especially due high-throughput DNA sequencing technologies. These highly performing techniques allowed the identification of the non-culturable bacteria within the gastrointestinal tract (Suchodolski, Camacho, e Steiner 2008). The sequences obtained can be analyzed with a variety of data techniques in order to describe the microbial composition, diversity and how the bacterial

population can change according to several factors. Until now, most of these techniques performed better for a singular taxa discussion, highlighting one properties per time (Barberán et al. 2012). Thus, one of the big issues related to the generation of large datasets of sequences is to answer, for example, how these microbial communities are organized. Deciphering these microbial interactions has become one of the primary aim of most researchers, because it could lead to a detection of key bacteria in health and disease (Bäumler e Sperandio 2016; Eickhoff e Bassler 2018; Schirmer et al. 2016). The creation of co-occurrence network based on correlation is one of the preferred methods to explore these microbial interactions. Accordingly, microorganisms are joined with and edge and those bacteria that are highly correlated can be grouped in sets, referred as modules. The combination of taxa in modules can be useful for fully understanding mutual relationships, such as cross-feeding or shared environmental niches (Ban, An, e Jiang 2015; C. Lozupone et al. 2012).

Here we present a network analysis for bacterial species in 334 fecal samples of 132 healthy dogs. The aim is to analyze co-occurrence patterns of microorganisms in dogs related to their diet and sex. The proposed method is helpful to investigate the gut microbiome of healthy dogs and to highlight better interactions between bacteria and between bacteria and host. In particular, this study paved the bases to understand how the gut community of bacteria was interconnected and operated in relation to dietary composition and sex.

7.2.3 Materials And Methods

7.2.3.1 Sample Population

The dataset is composed of individual records of dogs obtained from 8 dietary intervention studies (DIS) conducted in the past 5 years, for a total of 340 samples. All the dogs were recruited with the same inclusion criteria, which consisted of healthy conditions, as ascertain by a clinical examination, freedom from external and internal parasites, no pharmacological treatments since at least 3 months. A summary of the studies is reported in the S1 Table. Briefly, dogs were recruited from different living environment for every DIS and they were undergone through diet modulation. The description of the different DIS and the collection and analytical procedures was already reported (Scarsella et al., 2020). The factors considered for this study were diet and sex, whose were already discussed in this research. Briefly, the diets considered in this database were four, called commercial extruded complete diet (K), commercial moist complete diet (W), home-made diet (H) and a raw meat diet with the addition of a complementary food, called BASE™ (B) (www.nutrigenefood.com). Moreover, dogs were grouped based on their sex, in males (M), castrated males (MC), females (F) and spayed females (FC).

7.2.3.2 Faecal Dna Extraction, Sequencing And Bioinformatic Analysis

The entire procedure, starting from the microbial DNA extraction method and ending with taxonomic annotation with a bioinformatic analysis, was standardized and utilized for all the samples. The protocols used form the DNA extraction to MiSeq sequencing (Illumina; San Diego, CA, USA) are described on the previous research from Scarsella et al. (2020).

The resulting raw sequences (FASTQ) were processed using the bioinformatic tool called Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Bolyen et al. 2019). After demultiplexing, sequenced reads that passed the quality check (Phred score ≥30) were annotated for 16S rRNA against the Greengenes database. Chimeras were also detected and then filtered from the reads and the remaining sequences were clustered into Amplicon Sequence Variants (ASVs) by using an open reference approach in QIIME 2. Sequences can be found in NCBI, uploaded to Sequence Read Archive (Supplementary Table 1).

We used Sparse Cooccurrence Network Investigation for Compositional data (SCNIC) (Shaffer, Thurimella, and Lozupone 2020) in QIIME2 (q2-SCNIC) to perform the network analysis. The correlation network was built using Sparse Correlations for Compositional data (SparCC) algorithm; the network was built using edges with the default correlation coefficient of at least 0.35 using SparCC method (Friedman e Alm 2012), and network was visualized by Cytoscape. By eliminating or summarizing highly correlated features, dependence between features is decreased and this will increase the accuracy of methods, that assume the independence of features such as false discovery rate technique (FDR) measurement (Benjamini and Hochberg 2000), and statistical power is increased by reducing the number of feature comparisons.

7.2.3.3 Computation And Statistical Analysis

Annotated ASVs were imputed on a spreadsheet together with age, sex, breed and the number of the study to allow and facilitate further statistical analysis. The annotates sequences from each sample and each taxonomic level were normalized to ‰ abundance profiles, already known as Relative Abundance (RA). Taxa were attributed at the corresponding module, found with SCNIC. To confirm the results of the SparCC correlation analysis regarding taxa belonging at the same module and groups of diets and sex, a non-parametric Kruskal-Wallis test was applied at the genus level, with Bonferroni correction for multiple comparison. A p-value < 0.05 was considered statistically significant (Addinsoft 2020). ASVs and modules that differed in terms of diet and sex were also identified using Analysis of Composition of Microbiomes (ANCOM) (Mandal et al. 2015).

7.2.4 Results

7.2.4.1 Microbial Co-Abundance Network Modules

Before the building of the microbial network and the differentiation of the taxa based on environmental and genetical factor such as diet and sex, the gut microbiome has been analyzed through SparCC algorithm, to find existing correlation between bacteria. Afterwards, graphical network has been constructed with only taxa that has an R-value below 0.35, and modules were created from the aggregation of bacteria based on the network analysis. This threshold has been chosen based on results from a comparative analysis of Shaffer et al. (2020). Eighteen modules were detected, with a total bacterial taxa number of 55 (Supplementary Table 2). Module 0 contains 8 taxa, 4 belonging to the phylum Firmicutes, 2 bacteria from the Bacteroidetes phylum, one belonging to the phylum Proteobacteria and the last one from Fusobacteria. Module 1 has 5

taxa, all of them belonging to the Firmicutes phylum. These two modules are the largest in terms of taxa number.

7.2.4.2 Taxa And Modules Associated To Diet

Microbial composition analysis (ANCOM) was performed to study the effect of factor Diet on the gut microbiome network. 50 taxa were found significant to Diet, and they are shown on Figure 1. 28 bacteria out of 50 were aggregated into modules through SCNIC analysis. From the 18 modules found after the correlation analysis, 11 were found to be statistically significative at the factor Diet. As shown in the network graph (Figure 2), taxa from the modules 5, 18, 7, 8 and 11 were close from each other, and in the same way it was possible to find taxa from the modules 10, 6 and 15, and lastly, bacteria from modules 9, 12 and 3. Bacteria belonging to the significant modules related to Diet factor are summarized in Table 1. Regarding the first group of modules, most bacteria were part of the phylum Bacteroidetes, although in the module 18 there were bacteria belonging to the Order of Clostridiales (phylum Firmicutes), and in the module 7 was present *Sporobacter termitidis* (phylum Firmicutes). In the second group, in modules 6 and 15 there were bacteria of the phylum Firmicutes, whilst taxa in module 10 belonged to phylum Actinobacteria. The last group of modules showed only taxa belonging to phylum Firmicutes.

The relative abundances of each of the significant module for Diet factor were analyzed with a non-parametric Kruskal-Wallis test, in order to verify the difference between the categories exanimated. Modules 5 and 9 contained bacteria with higher significative RAs in BASE diet than the other three diets. Modules 18 and 11 showed higher RAs in MOIST diet whilst the modules 8 and 6 was significantly higher in KIBBLE diet than to the other classes. Interestingly, RAs in module 3 were almost at the same level in BASE, HOME and MOIST diet, and were significantly higher than in KIBBLE diet (Table 2).

7.2.4.3 Taxa And Modules Associated To Sex

ANCOM was also performed to study the effect of factor Sex on the gut microbiome network. 43 taxa were found significant for the factor Sex (Figure 3). 25 bacteria of these 43 belonged to modules found through SCNIC network analysis. 9 out of 18 modules discriminated the microbiome for the factor Sex. Figure 4 shows the network with the taxa highlighted that belonged to the modules significant for Sex. As in the case of Diet, modules 5, 7 and 11 were close together. Also the taxa belonging to the remaining significant modules appeared to be close, but it was still possible to distinguish a group of bacteria from modules 1, 15, 6 and 10, and a second group with bacteria from modules 14 and 2. Taxa from significant modules for the factor Sex are summarized in Table 3. In the first group there were almost all taxa belonging to the Bacteroidetes phylum, apart from module 7, where *Sporobacter termitidis* (Firmicutes phylum) was also present. In the second group all bacteria belonged to the Firmicutes phylum, while in the last group there were taxa belonging to Firmicutes in module 14, while in module 2 there was the genus *Pseudoramibacteri_Eubacterium* (Firmicutes phylum), *Bifidobacterium breve* (Actinobacteria phylum) and S24-7 family (Bacteroidetes phylum).

Also in this case, the RAs of the modules were subjected to a non-parametric Kruskal-Wallis test, to verify the significance of the factor Sex. The modules belonging to the first group contained taxa with significantly higher abundances in the entire female category, compared to entire male

dogs and the remaining neutered dogs. The second group of modules appeared to have higher relative abundances in castrated subjects, both male and female, compared to entire male and female dogs.

7.2.5 Discussion

Network analysis could be of help in better understanding the gut microbiome of dogs is an integrative view of the gut microbial ecology based on the microbial module. To the best of our knowledge, this is the first network analysis approach applied to the gut microbiome of dogs.

As already stated in other studies, the analysis of co-occurring microorganisms together with a correlation network analysis allows the summary visualization of many information (Chaffron et al. 2010). This method was successfully applied to distinguished the associations between marine microorganisms and their environment (Ruan et al. 2006). For this study, most of the bacteria composing the gut microbiome of dogs were grouped in modules. The graphically view of the network showed that significant modules in Diet and Sex were grouped close based on the degree of significance with each category of Diet and Sex. These structural properties allowed an easy comparison among gut microbiomes deriving from a complex dataset, in order to highlight how environmental and genetic factors such as diet and sex may influence the composition and the function of the microbial communities.

Only positive associations are calculated with SCNIC bioinformatic tool, that use SparCC algorithm. It may seem unnatural at first attempt, but these findings resulted to be logical when the research is focused on the gut microbiome. Within the anoxic environment of the gut, the microbial energy production is limited, and this would make positive associations, like mutual cross-feeding, that transform the production and the utilization of energy more efficient (Pacheco, Moel, e Segrè 2019). Moreover, microbial associations, if positive, alleviate potential stresses on the ecosystem, making even greater the diversity of a healthy gut microbiome (Stachowicz 2001; Lozupone et al. 2012).

After the network analysis with SCNIC, 18 modules were found and 11 of them were significant for the factor Diet. For the factor Sex the number of significant modules were 9 out of 18. It is well known that diet can alter the composition and the activity of the microbial population (Scarsella, Cintio, et al. 2020), in the way of introducing new bacteria, providing nutrients, selecting for enrichment or depletion of certain taxa via nutrient surplus or starvation and finally, shifting the expression profiles of some bacteria (David et al. 2014). Several studies showed that diet has a greater impact on microbiome than the host genotype. For example, some studies on fruit fly showed as *Lactobacillus plantarum* promotes a variety of fitness phenotypes at various stages of the fruit fly development. Since *Drosophila melanogaster* larvae are attracted to *L. plantarum* metabolites (Venu et al. 2014), they promote the replication of this bacteria on their food (Storelli et al. 2011). Furthermore, *L. plantarum* does not stably colonize the gut, thus it has to be repeatedly ingested; this fact indicates the microbiome need of an external support, that is valuable to the host physiology.

Several investigation highlighted a high variability in terms of gut microbial composition between subjects (Scarsella, Cintio, et al. 2020; Sandri et al. 2020; Cintio et al. 2020). It is likely that each

dog presented a unique fecal microbiome, that is also resilient to slight dietary modification. Cintio et al. (2020) found that there is a variation of the gut microbiome in arthritic dogs, although a large inter-variability among dogs existed. They suggested that different bacteria strains could contribute in its own way in order to modify the inflammatory status of the subjects. Thus, it was not possible to attribute a role to each taxa composing the microbiome related to the arthritic disease, but the hypothesis is that the variation of the total gut microbial composition could reshape the entire physiology of the host.

The most significative modules related to factor Diet were module 3, module 9 and module 15. All bacteria including in these modules are part of Firmicutes phylum. Firmicutes has been suggested to play a role in modulating the immune system (Ling et al. 2014). Moreover, obesity in humans and mice showed an association with the increase of Firmicutes and the decrease of Bacteroidetes (Turnbaugh et al. 2006). Module 3 is composed of only Firmicutes bacteria, in particular taxa from the genus *Clostridium*, both from Clostridiceae and Peptostreptococcaceae families, and *Clostridium baratii*. This module highlighted that the sum of the RAs of the bacteria that are part of it, are significantly higher in all diets that have a "wet" form, that are BASE diet, Home-made diet and complete wet commercial diet. This evidence confirms the idea that the presence of raw meat and the physical form of these diets had a similar impact on shaping the gut microbiome (Scarsella, Stefanon, et al. 2020). The increase of *Clostridium* in dogs fed with a BASE and Home-made diet are in agreement with another study, where this taxa was decreased in dogs fed with a commercial extruded diet in comparison to dogs fed with a raw meat diet (Bermingham et al. 2017).

Very limited information is available for the variation of gut microbiome in relation to sex in dogs. In mice, the gut microbiota of males and females become different after puberty; the mechanism of sexual influence remains unclear, but it appears that a bidirectional interaction between microbiota and the endocrine status of the host, also because the microbial composition difference between males and females are reversed by male castration (Markle et al. 2013). The module 1 appeared to be the most significant at the ANCOM analysis for the Sex factor. All bacteria in this module are part of the phylum Firmicutes and the sum of the RAs showed a higher significative value for the castrated males and females categories than the entire males and females subjects. This is in accordance with the results of a previous study (Scarsella, Stefanon, et al. 2020). Moreover, in this module are included *Dorea longicatena* and *Ruminococcus lactaris*. The genera of these two taxa resulted to be discriminant for whole subjects in comparison of castrated subjects in the study of Scarsella et al. (2020).

The clustering of bacteria in these modules could be indicative of factors that influenced the healthy gut microbiome of dogs. We found modules that were not significant for either factor Diet or Sex. This evidence could explain the existence of a potential further stabilizing force for the ecosystem, as suggested by Loftus et al. (2021) (Loftus, Hassouneh, e Yooseph 2021).

7.2.6 Conclusion

This study helped to better understand the ecology of the gut microbiome of dogs related to environmental and genetic factors such as diet and sex and put the basis on the definition of enterotypes also in dogs, a concept already applied in human. With these results, individuals could be clustered on the basis of the abundance of microbial taxa of the gut. Moreover, taxa belonging to modules could help on modulate diets and therapies for the treatment of enteropathies or gut related pathologies linked to a dysbiosis status of the host. Further studies are needed in order to highlight new associations between bacteria related to other factors that could influence the gut microbiome.

7.2.7 TABLES AND FIGURES

Figure 1. Volcano plot of the significative taxa and modules on factor Diet after the ANCOM analysis

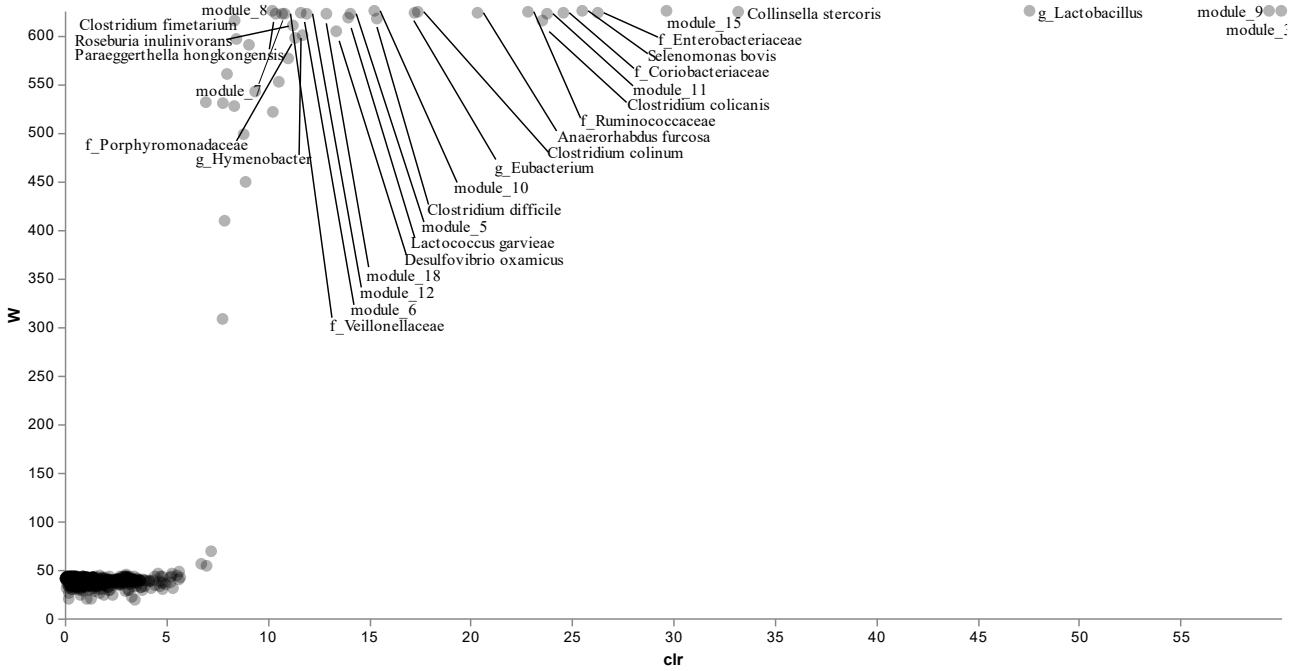

Figure 2. Graphical view of the gut microbiome network resulted from SCNIC analysis; highlighted with different colors are bacteria from significative modules for factor Diet after ANCOM analysis.

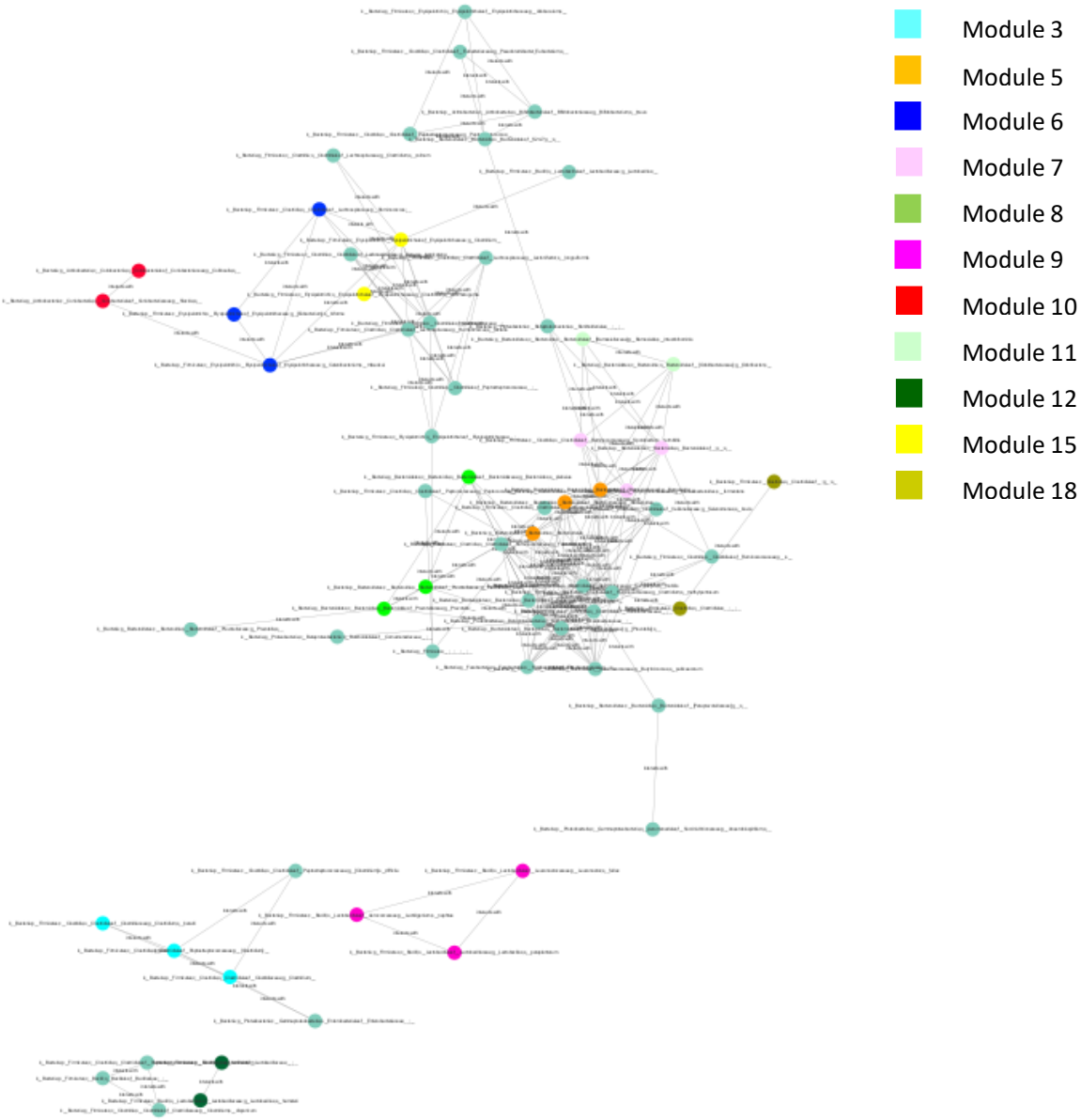

Figure 3. Volcano plot of the significative taxa and modules on factor Sex after the ANCOM analysis

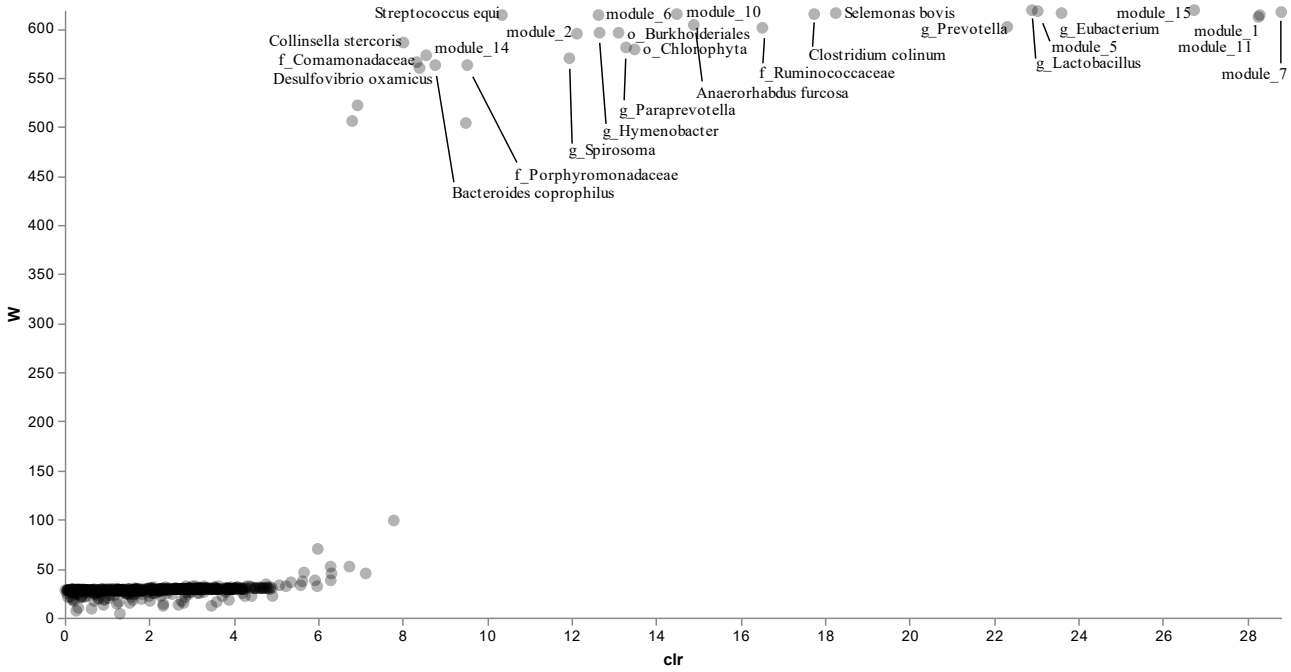

Figure 4. Graphical view of the gut microbiome network resulted from SCNIC analysis; highlighted with different colors are bacteria from significative modules for factor Sex after ANCOM analysis.

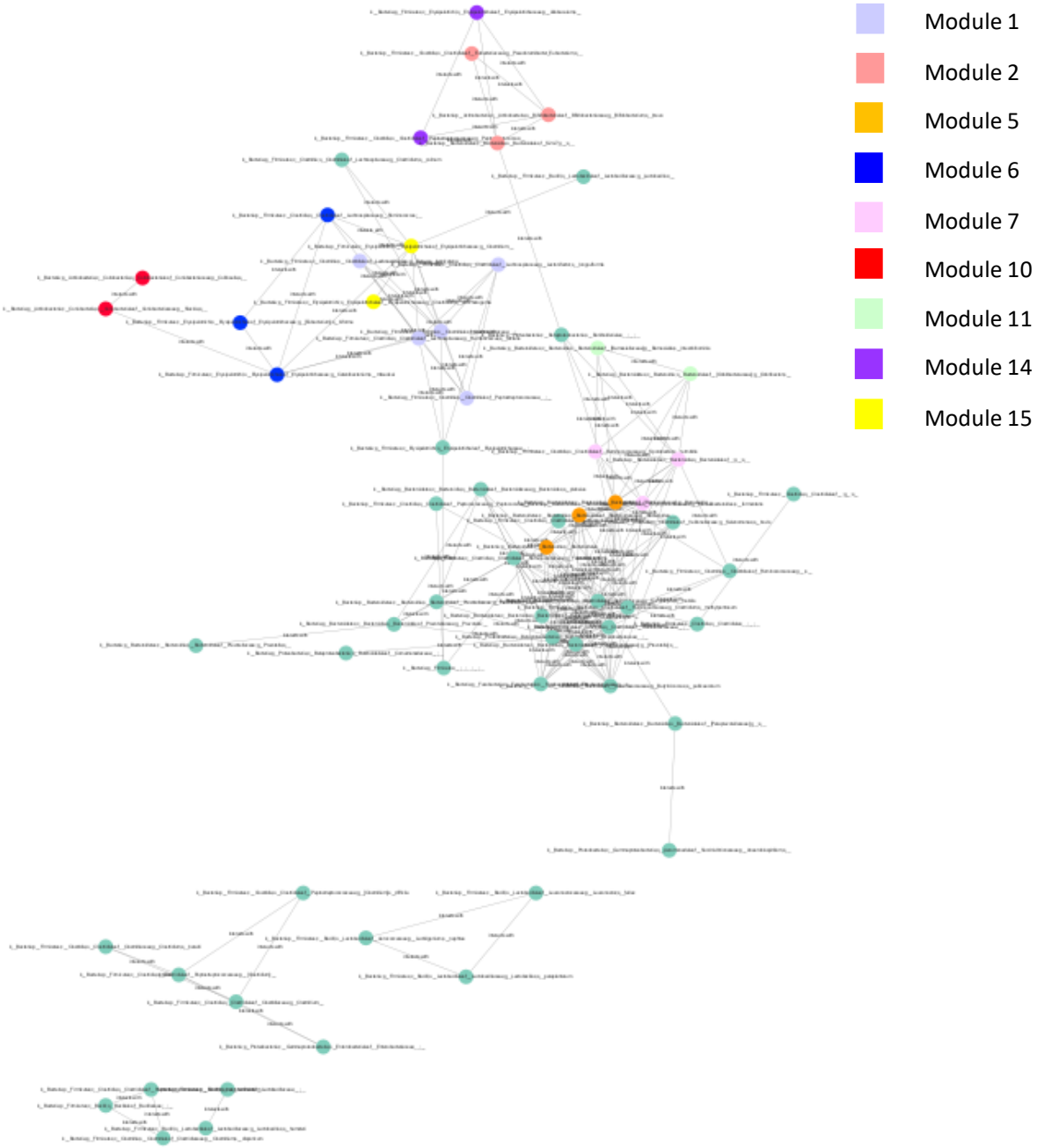

Table 1. Taxa belonging to modules significative to factor Diet at the ANCOM analysis.

Colour	Module n.	Taxa
Light Blue	Module 3	Clostridium baratii; g_Clostridium
Orange	Module 5	g_Bacteroides; o_Bacteroidales; g_Barnesiella
Blue	Module 6	Eubacterium biforme; Catenibacterium mitsuokai; g_Ruminococcus
Pink	Module 7	Macellibacteroides fermentans; Sporobacter termitidis; o_Bacteroidales
Green	Module 8	Bacteroides plebeius; Prevotella copri
Magenta	Module 9	Lacticigenium naphtae; Leuconostoc fallax; Lactobacillus paraplantarum
Red	Module 10	g_Collinsella; g_Slackia
Light Green	Module 11	g_Odoribacter; Barnesiella intestinihominis
Dark Green	Module 12	Lactobacillus hamsteri; f_Lactobacillaceae
Yellow	Module 15	Clostridium saccharogumia; f_Erysipelotrichaeae, g_Clostridium
Ocher	Module 18	o_Clostridiales

Table 2. Results of the Kruskal-Wallis non parametric test applied to the sum of RAs of the taxa belonging to significative modules to factor Diet after ANCOM analysis.

	BASE		HOME		KIBBLE		MOIST		Sig of
	mean	sd	mean	sd	mean	sd	mean	sd	p-value
module 15	2.2 [a]	5.3	14.9 [c]	12.1	8.4 [b]	12.0	5.6 [a]	13.2	<0.0001
module 10	2.3 [a]	4.4	7.5 [c]	5.1	2.1 [a]	2.7	4.3 [b]	5.1	<0.0001
module 9	3.1 [b]	5.3	0.1 [a]	0.2	0.2 [a]	2.6	0.3 [a]	2.7	<0.0001
module 8	22.0 [ab]	23.4	15.5 [a]	24.1	52.8 [b]	69.7	19.2 [a]	37.7	<0.0001
module 3	90.1 [b]	87.2	72.2 [b]	95.9	13.2 [a]	40.8	92.7 [b]	71.5	<0.0001
module 6	10.8 [a]	22.4	27.9 [bc]	30.4	50.8 [c]	67.6	21.9 [b]	27.2	<0.0001
module 5	18.0 [b]	20.3	2.9 [a]	5.1	5.0 [a]	9.1	8.6 [b]	9.0	<0.0001
module 7	3.2 [bc]	5.7	0.5 [a]	1.5	2.9 [b]	6.7	5.3 [c]	7.5	<0.0001
module 11	0.4 [a]	0.9	0.1 [a]	0.5	0.3 [a]	1.0	1.2 [b]	1.7	<0.0001
module 12	5.1	16.5	3.1	8.5	23.9	57.4	1.1	3.3	N.S.
module 18	1.9 [a]	3.1	3.4 [ab]	5.6	6.1 [b]	7.9	11.2 [c]	11.3	<0.0001

Table 3. Taxa belonging to modules significative to factor Sex at the ANCOM analysis.

Colour	Module n.	Taxa
Orange	Module 5	g_Bacteroides; o_Bacteroidales; g_Barnesiella
Blue	Module 6	Eubacterium biforme; Catenibacterium mitsuokai; g_Ruminococcus
Pink	Module 7	Macellibacteroides fermentans; Sporobacter termitidis; o_Bacteroidales
Red	Module 10	g_Collinsella; g_Slackia
Light Green	Module 11	g_Odoribacter; Barnesiella intestinihominis
Lilac	Module 1	Lactonifactor longoviformis; Dorea longicatena; f_Lachnospiraceae; f_Peptostreptococcaceae; Ruminococcus lactaris
Yellow	Module 15	Clostridium saccharogumia; f_Erysipelotrichaeae, g_Clostridium
Salmon	Module 2	Bifidobacterium breve; g_Pseudoramibacter_Eubacterium; f_S24-7
Violet	Module 14	g_Peptostreptococcus; g_Allobaculum

Table 4. Results of the Kruskal-Wallis non parametric test applied to the sum of RAs of the taxa belonging to significative modules to factor Sex after ANCOM analysis.

	F		FC		M		MC		Sig of
	mean	sd	mean	sd	mean	sd	mean	sd	p-value
module 15	5.9 [a]	11.0	9.3 [bc]	12.2	8.7 [ab]	16.7	8.6 [c]	8.0	< 0,0001
module 10	2.8 [a]	4.2	4.9 [b]	4.9	2.4 [a]	3.6	4.3 [b]	4.5	< 0,0001
module 6	20.5 [a]	27.0	37.9 [bc]	32.7	34.2 [ab]	80.7	58.9 [c]	53.1	< 0,0001
module 5	11.4 [c]	12.7	5.2 [ab]	10.3	6.1 [b]	11.2	3.3 [a]	10.1	< 0,0001
module 7	5.9 [c]	8.6	0.3 [ab]	0.6	2.5 [b]	4.4	0.5 [a]	2.4	< 0,0001
module 11	1.0 [c]	1.7	0.1 [ab]	0.5	0.4 [b]	0.8	0.0 [a]	0.0	< 0,0001
module 1	282.0 [a]	126.7	455.9 [bc]	145.8	372.4 [b]	191.3	503.0 [c]	153.1	< 0,0001
module 2	5.7 [b]	14.1	0.2 [a]	0.9	2.3 [a]	8.5	1.3 [a]	6.3	< 0,0001
module 14	0.6 [b]	2.3	0.0 [a]	0.0	0.4 [b]	2.2	0.0 [a]	0.0	0.005

7.2.8 References

Addinsoft. 2020. *XLSTAT statistical and data analysis solution*. Boston, USA.

Arumugam, Manimozhiyan, Jeroen Raes, Eric Pelletier, Denis Le Paslier, Takuji Yamada, Daniel R. Mende, Gabriel R. Fernandes, et al. 2011. «Enterotypes of the Human Gut Microbiome». *Nature* 473 (7346): 174–80. https://doi.org/10.1038/nature09944.

Ban, Yuguang, Lingling An, e Hongmei Jiang. 2015. «Investigating microbial co-occurrence patterns based on metagenomic compositional data». *Bioinformatics* 31 (20): 3322–29. https://doi.org/10.1093/bioinformatics/btv364.

Barberán, Albert, Scott T. Bates, Emilio O. Casamayor, e Noah Fierer. 2012. «Using Network Analysis to Explore Co-Occurrence Patterns in Soil Microbial Communities». *The ISME Journal* 6 (2): 343–51. https://doi.org/10.1038/ismej.2011.119.

Bäumler, Andreas J., e Vanessa Sperandio. 2016. «Interactions between the Microbiota and Pathogenic Bacteria in the Gut». *Nature* 535 (7610): 85–93. https://doi.org/10.1038/nature18849.

Benjamini, Y., and Hochberg, Y. 2000. "On the Adaptive Control of the False Discovery Rate in Multiple Testing With Independent Statistics." *J Educ Behav Stat.* 25 (1), 60–83.

Bermingham, Emma N., Paul Maclean, David G. Thomas, Nicholas J. Cave, e Wayne Young. 2017. «Key Bacterial Families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) Are Related to the Digestion of Protein and Energy in Dogs». *PeerJ* 5 (marzo): e3019. https://doi.org/10.7717/peerj.3019.

Bolyen, Evan, Jai Ram Rideout, Matthew R. Dillon, Nicholas A. Bokulich, Christian C. Abnet, Gabriel A. Al-Ghalith, Harriet Alexander, et al. 2019. «Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2». *Nature Biotechnology* 37 (8): 852–57. https://doi.org/10.1038/s41587-019-0209-9.

Chaffron, Samuel, Hubert Rehrauer, Jakob Pernthaler, e Christian von Mering. 2010. «A Global Network of Coexisting Microbes from Environmental and Whole-Genome Sequence Data». *Genome Research* 20 (7): 947–59. https://doi.org/10.1101/gr.104521.109.

Cintio, Michela, Elisa Scarsella, Sandy Sgorlon, Misa Sandri, e Bruno Stefanon. 2020. «Gut Microbiome of Healthy and Arthritic Dogs». *Veterinary Sciences* 7 (3): 92. https://doi.org/10.3390/vetsci7030092.

David, Lawrence A., Arne C. Materna, Jonathan Friedman, Maria I. Campos-Baptista, Matthew C. Blackburn, Allison Perrotta, Susan E. Erdman, e Eric J. Alm. 2014. «Host

lifestyle affects human microbiota on daily timescales». *Genome Biology* 15 (7): R89. https://doi.org/10.1186/gb-2014-15-7-r89.

Deng, Ping, e Kelly S. Swanson. 2015. «Gut Microbiota of Humans, Dogs and Cats: Current Knowledge and Future Opportunities and Challenges». *British Journal of Nutrition* 113 (S1): S6–17. https://doi.org/10.1017/S0007114514002943.

Eickhoff, Michaela J., e Bonnie L. Bassler. 2018. «SnapShot: Bacterial Quorum Sensing». *Cell* 174 (5): 1328-1328.e1. https://doi.org/10.1016/j.cell.2018.08.003.

Friedman, Jonathan, e Eric J. Alm. 2012. «Inferring Correlation Networks from Genomic Survey Data». *PLOS Computational Biology* 8 (9): e1002687. https://doi.org/10.1371/journal.pcbi.1002687.

Garcia-Mazcorro, Jose F., Jose R. Barcenas-Walls, Jan S. Suchodolski, e Jörg M. Steiner. 2017. «Molecular Assessment of the Fecal Microbiota in Healthy Cats and Dogs before and during Supplementation with Fructo-Oligosaccharides (FOS) and Inulin Using High-Throughput 454-Pyrosequencing». *PeerJ* 5 (aprile): e3184. https://doi.org/10.7717/peerj.3184.

Ling, Zongxin, Zailing Li, Xia Liu, Yiwen Cheng, Yueqiu Luo, Xiaojuan Tong, Li Yuan, et al. 2014. «Altered Fecal Microbiota Composition Associated with Food Allergy in Infants». *Applied and Environmental Microbiology* 80 (8): 2546–54. https://doi.org/10.1128/AEM.00003-14.

Loftus, Mark, Sayf Al-Deen Hassouneh, e Shibu Yooseph. 2021. «Bacterial Associations in the Healthy Human Gut Microbiome across Populations». *Scientific Reports* 11 (1): 2828. https://doi.org/10.1038/s41598-021-82449-0.

Lozupone, Catherine A., Jesse I. Stombaugh, Jeffrey I. Gordon, Janet K. Jansson, e Rob Knight. 2012. «Diversity, Stability and Resilience of the Human Gut Microbiota». *Nature* 489 (7415): 220–30. https://doi.org/10.1038/nature11550.

Lozupone, Catherine, Karoline Faust, Jeroen Raes, Jeremiah J. Faith, Daniel N. Frank, Jesse Zaneveld, Jeffrey I. Gordon, e Rob Knight. 2012. «Identifying Genomic and Metabolic Features That Can Underlie Early Successional and Opportunistic Lifestyles of Human Gut Symbionts». *Genome Research* 22 (10): 1974–84. https://doi.org/10.1101/gr.138198.112.

Mandal, Siddhartha, Will Van Treuren, Richard A. White, Merete Eggesbø, Rob Knight, e Shyamal D. Peddada. 2015. «Analysis of composition of microbiomes: a novel method for studying microbial composition». *Microbial Ecology in Health and Disease* 26 (1): 27663. https://doi.org/10.3402/mehd.v26.27663.

Markle, Janet G. M., Daniel N. Frank, Steven Mortin-Toth, Charles E. Robertson, Leah M. Feazel, Ulrike Rolle-Kampczyk, Martin von Bergen, Kathy D. McCoy, Andrew J.

Macpherson, e Jayne S. Danska. 2013. «Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity». *Science* 339 (6123): 1084–88. https://doi.org/10.1126/science.1233521.

Muegge, Brian D., Justin Kuczynski, Dan Knights, Jose C. Clemente, Antonio González, Luigi Fontana, Bernard Henrissat, Rob Knight, e Jeffrey I. Gordon. 2011. «Diet Drives Convergence in Gut Microbiome Functions Across Mammalian Phylogeny and Within Humans». *Science* 332 (6032): 970–74. https://doi.org/10.1126/science.1198719.

Pacheco, Alan R., Mauricio Moel, e Daniel Segrè. 2019. «Costless Metabolic Secretions as Drivers of Interspecies Interactions in Microbial Ecosystems». *Nature Communications* 10 (1): 103. https://doi.org/10.1038/s41467-018-07946-9.

Pilla, Rachel, e Jan S. Suchodolski. 2020. «The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease». *Frontiers in Veterinary Science* 6. https://doi.org/10.3389/fvets.2019.00498.

Ruan, Quansong, Debojyoti Dutta, Michael S. Schwalbach, Joshua A. Steele, Jed A. Fuhrman, e Fengzhu Sun. 2006. «Local similarity analysis reveals unique associations among marine bacterioplankton species and environmental factors». *Bioinformatics* 22 (20): 2532–38. https://doi.org/10.1093/bioinformatics/btl417.

Sandri, M., C. Manfrin, A. Pallavicini, e B. Stefanon. 2014. «Microbial Biodiversity of the Liquid Fraction of Rumen Content from Lactating Cows». *Animal* 8 (4): 572–79. https://doi.org/10.1017/S1751731114000056.

Sandri, Misa, Sandy Sgorlon, Elisa Scarsella, e Bruno Stefanon. 2020. «Effect of Different Starch Sources in a Raw Meat-Based Diet on Fecal Microbiome in Dogs Housed in a Shelter». *Animal Nutrition* 6 (3): 353–61. https://doi.org/10.1016/j.aninu.2020.03.003.

Scarsella, Elisa, Michela Cintio, Lucilla Iacumin, Federica Ginaldi, e Bruno Stefanon. 2020. «Interplay between Neuroendocrine Biomarkers and Gut Microbiota in Dogs Supplemented with Grape Proanthocyanidins: Results of Dietary Intervention Study». *Animals* 10 (3): 531. https://doi.org/10.3390/ani10030531.

Scarsella, Elisa, Bruno Stefanon, Michela Cintio, Danilo Licastro, Sandy Sgorlon, Simeone Dal Monego, e Misa Sandri. 2020. «Learning Machine Approach Reveals Microbial Signatures of Diet and Sex in Dog». *PLOS ONE* 15 (8): e0237874. https://doi.org/10.1371/journal.pone.0237874.

Schirmer, Melanie, Sanne P. Smeekens, Hera Vlamakis, Martin Jaeger, Marije Oosting, Eric A. Franzosa, Rob ter Horst, et al. 2016. «Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity». *Cell* 167 (4): 1125-1136.e8. https://doi.org/10.1016/j.cell.2016.10.020.

Shaffer, Michael, Kumar Thurimella, e Catherine A. Lozupone. 2020. «SCNIC: Sparse Correlation Network Investigation for Compositional Data». https://www.biorxiv.org/content/10.1101/2020.11.13.380733v1.

Stachowicz, John J. 2001. «Mutualism, Facilitation, and the Structure of Ecological Communities: Positive interactions play a critical, but underappreciated, role in ecological communities by reducing physical or biotic stresses in existing habitats and by creating new habitats on which many species depend». *BioScience* 51 (3): 235–46. https://doi.org/10.1641/0006-3568(2001)051[0235:MFATSO]2.0.CO;2.

Storelli, Gilles, Arnaud Defaye, Berra Erkosar, Pascal Hols, Julien Royet, e François Leulier. 2011. «Lactobacillus Plantarum Promotes Drosophila Systemic Growth by Modulating Hormonal Signals through TOR-Dependent Nutrient Sensing». *Cell Metabolism* 14 (3): 403–14. https://doi.org/10.1016/j.cmet.2011.07.012.

Suchodolski, Jan S., Jennifer Camacho, e Jörg M. Steiner. 2008. «Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis». *FEMS Microbiology Ecology* 66 (3): 567–78. https://doi.org/10.1111/j.1574-6941.2008.00521.x.

Thorburn, Alison N., Laurence Macia, e Charles R. Mackay. 2014. «Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases». *Immunity* 40 (6): 833–42. https://doi.org/10.1016/j.immuni.2014.05.014.

Turnbaugh, Peter J., Ruth E. Ley, Michael A. Mahowald, Vincent Magrini, Elaine R. Mardis, e Jeffrey I. Gordon. 2006. «An Obesity-Associated Gut Microbiome with Increased Capacity for Energy Harvest». *Nature* 444 (7122): 1027–31. https://doi.org/10.1038/nature05414.

Venu, Isvarya, Zachary Durisko, Jianping Xu, e Reuven Dukas. 2014. «Social attraction mediated by fruit flies' microbiome». *Journal of Experimental Biology* 217 (8): 1346–52. https://doi.org/10.1242/jeb.099648.

Zeng, M. Y., N. Inohara, e G. Nuñez. 2017. «Mechanisms of Inflammation-Driven Bacterial Dysbiosis in the Gut». *Mucosal Immunology* 10 (1): 18–26. https://doi.org/10.1038/mi.2016.75.

CHAPTER 8

Final Considerations

Although great progress has been made in studying the gut microbiome of livestock and companion animals, especially in dogs, there are still many questions that remain open and not fully clarified. For example, it is still not entirely clear what is the role of the intestinal microbiome in the onset of diseases, gastrointestinal and not, and what is its involvement in the case of chronic diseases.

The aim of the research conducted during my PhD course was to better define the composition of the intestinal microbiome in healthy subjects, not affected by any pathology. To do this, it was necessary to analyze a substantial number of samples, since nowadays, one of the major shortcomings in the literature is the presence of studies with a large number of subjects. Given the high variability existing at the individual level, it is necessary, if not almost mandatory, to increase the canine population analyzed, in order to characterize the interactions between microorganisms in detail and to define the factors that influence these relationships. However, the results obtained are based mainly on fecal specimen, and do not consider the interaction with the physiological response of the host at a tissue and organ levels. Future researches will need, including the measurements of other parameters at a whole organism level together with fecal microbiome.

In chapter 3 it was observed that there were some specific taxa that were significant for the Diet and Sex factor. This means that their abundances were significantly different between the types of Diet examined (dry extruded, wet extruded, home-made and B.A.S.E.™, www.nutrigenefood.com), and between subjects differentiated on the basis of their sex and hormonal status (whole male, whole female, neutered male, spayed female). In fact, the linear discriminant analysis of the microbial composition led to a separation of the subjects based on their Diet and Sex. This result was confirmed by applying a Random Forest analysis which classified the subjects correctly with a high success rate. All this leads us to think that there are key taxa that characterize the gut microbiome of subjects divided into macro categories, which could be defined in the future as enterotypes.

In chapter 4 the aim was to verify if the addition of proanthocinidins extracted from grapevine to the dog's diet could influence the gut microbiome and consequently, the endocrine status of the animals, through the measurement of some salivary molecular markers, such as serotonin and cortisol. Some variations at the single taxa level were observed in the gut microbiome of dogs fed with the addition of polyphenols, but the alpha diversity did not show significant differences between the three groups examined since the variations at the microbiome were found to be minimum. Among the salivary markers, serotonin was found to be the one most affected by the addition of polyphenols in the diet. These results suggested that polyphenols, as prebiotics, play an important role in the modulation of the intestinal microbiome, especially in terms of the function performed by the microbiome for the well-being and health of the host. Since large variations in the microbiome have not been noticed, in the future it will be necessary to try to better understand what microbial interactions occur with the addition of prebiotics or probiotics, and what influence they have on the production of metabolites.

In chapter 5, the goal was to implement a new method for the analysis of the intestinal metabolome, which could be easier and faster, and which did not completely destroy the sample once analyzed. For this reason, it was decided to use a proton magnetic resonance (NMR) analysis, never implemented until now for intestinal metabolomics studies in dogs. The metabolites selected for the determination of their presence were 21. By further developing the method, in the future it will also be possible to determine their quantity, as well as their presence, making the analysis of fecal metabolites easier. Furthermore, through an untargeted approach, that is the analysis of the general metabolic profile, it was possible to divide the subjects on the basis of the type of diet taken. This allows us to ascertain that the relationships between the key bacteria, that are part of the gut microbiome, produce a series of metabolites - also called postbiotics - which are characteristic of each macrocategory and can be used for the definition of "enterotype".

In chapter 6 we wanted to explore the microbial composition of the intestine in correlation with that of the blood. Blood has always been thought of as a sterile fluid, where the presence of microorganisms is due solely and exclusively to serious infections. Recently, some discoveries in humans have led to a re-evaluation of this idea. Unfortunately, this area of research is still too little explored, and almost nothing is known about the blood microbiome in companion animals. Despite the few overlaps in terms of annotation of the fecal microbiome and the blood microbiome, the PCA showed a similar result for both types of microbiomes; the subjects were, in both cases, grouped on the basis of the type of diet administered to them. This result gives a starting point for reflection on what really is the meaning of the presence of microorganisms in the blood. A first question is if the taxa identified are really alive in the blood, or they are in a "dormant" phase, which could explain the reason for some chronic diseases. Secondly, the reflection is why the blood microbiome is influenced by the diet in a similar way to the gut microbiome. In the future, this could be an increasingly investigated research topic, and could provide fundamental help in identifying the cause of some diseases. For this reason, more studies will be needed with the inclusion of dogs subject with diseases, primarily gastrointestinal, and subsequently also other types of diseases.

In chapter 7, the final goal was to conclude and complete the results obtained during the PhD course. The key to these studies has always been to find out what relationships exist between the components of the gut microbiome, and what effect these relationships have in the fermentation and metabolic activity of microorganisms, and consequently, in the microbial composition of other host compartments. For this reason, it has been decided to investigate the correlations, especially positive, through a network analysis. It was possible to appreciate which taxa co-exist thanks to the formation of bacterial modules. Subsequently these modules were analyzed for the Diet and Sex factors, in order to complete the results of chapter 3. By doing so, it was easy to appreciate which were the significant modules affected by these considered factors, as it was possible to look in the whole of the gut microbiome of healthy subjects and establish threshold values within macro-categories. This allows us to get even closer to the definition of enterotypes in dogs, as well for humans.

The research work conducted during these three years allowed to reach a standardization of the methodology used for the study of the gut microbiome. The very high number of samples analyzed through all the duration of the PhD helped in the understanding of the relationships

existing between the microorganisms that habit the gastrointestinal tract, and the host. Specifically, this research work allowed to reach three fundamental conclusions.

Firstly, the obtained results, together with their considerations, paved the basis for future studies, where more parameters should be considered to unravel the complex links between the microbiome and the host.

Secondly, each experiment performed during these years permitted an overall assessment of the bacterial RAs composing the gut microbiome of healthy dogs.

Lastly, this research explored dietetic factors affecting the gut microbiome, but also factors linked to the physiology of the animals, such as the sex.

The microbiome study field is in a growing phase, with several methodologies used until now, in order to obtain reliable results. The issue arising from the use of different methods is the difficulty on making comparisons between studies, therefore, to find a common line among the results of similar studies. The experiments reported in this thesis were obtained with the use of the same wet and dry laboratory pipeline, and for this reason the data showed consistent results with each other. There are several other factors that play a role in the relationship between gut microbiome and the host, that remain to understand better, such as the breed, the size, the age, the maternal imprinting and so on. All these aspects will be hard to solve, since there are several experimental and economic limitations about finding fundings to cover the costs regarding research on dogs, but also the recruitment of the dogs for the experiments.

Possible future perspectives may concern the ultimate definition of enterotypes based on the intestinal microbiome of healthy dogs, or in the absence of pathologies. To do this, it will be necessary to carry out more studies with the inclusion of subjects with different diseases, and to expand the database consisting of healthy subjects, in order to investigate any other macro-categories influencing the microbial population. Furthermore, an interesting point of reflection is the study of the blood microbiome, which could give a turning point in understanding the onset of some chronic diseases. It could be interesting to carry out studies of bacterial cultures from blood.

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APPENDIX A

Supplementary materials of Chapter 3

S1 Table. Summary of the dietary intervention studies that were included in the dataset.

Study	Source	N. Subjects	Time of sampling for each dog *				Faecal samples	Sequence Read Archive repository
			T0	T1 - T14	T15 - T28	T1 - T45		
								SRP150679
1	Sandri et al. (2017)	4	Kibble	Kibble	Base-1			
		4	Kibble	Base-1	Kibble		24	
2	Sandri et al. (2019)	4	Kibble	Base-1	Base-3			SRP150679
		4	Kibble	Base-3	Base-1		24	
3	Doctoral Thesis	4	Kibble	Kibble-P	Kibble			PRJNA611632
		4	Kibble	Kibble	Kibble-P		24	
4	Sandri et al. (2020)	10	Home	Home				PRJNA529651
		9	Home	Base-1				SUB5371799
		9	Home	Base-2			56	
5	Master Thesis	5	Kibble	Moist-S	Moist-S			PRJNA611632
		5	Kibble	Moist-H	Moist-H		21	
6	Cintio et al. (2020)	6	Kibble			Moist-S		PRJNA611632
		17	Kibble			Moist-H	46	
7	Unpublished	9	Kibble	Moist-D0	Moist-D0			PRJNA611632
	Unpublished	8	Kibble	Moist-D1	Moist-D1			
	Unpublished	9	Kibble	Moist-D3	Moist-D3		77	
8	Scarsella et al. (2020)	8	Kibble	Kibble-D0	Kibble-D0			PRJNA564012
		8	Kibble	Kibble-D1	Kibble-D1			
		8	Kibble	Kibble-D3	Kibble-D3		72	

* The columns report the food administered to the group of dogs during the interval

Kibble = Complete extruded food
Kibble-P = Complete extruded food + probiotic
Kibble-D0 = complete extruded food without polyphenols
Kibble-D1 = complete extruded food with polyphenols, 1 mg/kg
Kibble-D3 = complete extruded food with polyphenols, 3 mg/kg
Moist-S = complete moist food, with sunflower oil and salmon oil
Moist-H = complete moist food, with hemp oil
Moist-D0 = complete moist food without polyphenols
Moist-D1 = complete moist food with polyphenols, 1 mg/kg
Moist-D3 = complete moist food with polyphenols, 3 mg/kg
Home = home based diet, with raw meat
Base-1 = mixed diet, beef raw meat and complementray vegetable food
Base-2 = mixed diet, beef raw meat and complementray vegetable food
Base-3 = mixed diet, beef raw meat and complementray vegetable food

S2 Table. Chemical compositions and nutritive values of the diets of the Dietray Intervention Studies (DIS)

		Dry Matter	Crude Protein	Crude Lipids	Crude fiber	Ash	NFE	Metabolizable Energy
		%	%DM	%DM	%DM	%DM	%DM	kcal/kg DM
DIS 1	Kibble	90.0	26.7	10.6	2.8	10.0	49.9	3812
	Base-1	57.6	26.2	18.2	0.7	4.3	50.6	4465
DIS 2	Kibble	92.0	23.9	15.2	2.2	7.3	51.4	4153
	Base-1	44.2	27.2	19.2	0.7	4.2	48.7	4516
	Base-3	42.6	26.0	19.0	0.8	3.8	50.4	4518
DIS 3	Kibble	91.0	27.6	15.3	4.8	8.4	43.9	4015
DIS 4	Home	52.0	30.1	22.8	1.2	4.2	41.8	4667
	Base-1	53.0	29.7	21.7	0.9	4.8	42.9	4600
	Base-2	52.0	28.9	21.7	1.1	5.2	43.1	4582
DIS 5, 6	Kibble	90.0	26.6	18.3	4.2	6.6	44.3	4250
	Moist-S	19.8	36.2	27.0	1.8	10.7	24.4	4595
	Moist-H	20.1	35.8	27.4	2.8	10.6	23.4	4580
DIS 7	Kibble	91.0	27.6	15.3	4.8	8.4	43.9	4015
	Moist-D0	20.1	36.0	26.9	1.5	10.8	24.9	4596
	Moist-D1	20.5	37.0	27.4	1.7	11.6	22.3	4584
	Moist-D3	20.3	36.5	27.1	1.6	11.2	23.6	4590
DIS 8	Kibble-D0	91.0	28.5	16.0	2.5	5.5	47.5	4248
	Kibble-D1	91.0	28.5	16.0	2.5	5.5	47.5	4248
	Kibble-D3	91.0	28.5	16.0	2.5	5.5	47.5	4248

Kibble = Complete extruded food
Kibble-P = Complete extruded food + porbiotic
Kibble-D0 = complete extruded food without polyphenols
Kibble-D1 = complete extruded food with polyphenols, 1 mg/kg
Kibble-D3 = complete extruded food with polyphenols, 3 mg/kg
Moist-S = complete moist food, with sunflower oil and salmon oil
Moist-H = complete moist food, with hemp oil
Moist-D0 = complete moist food without polyphenols
Moist-D1 = complete moist food with polyphenols, 1 mg/kg
Moist-D3 = complete moist food with polyphenols, 3 mg/kg

Home = home based diet, with raw meat
Base-1 = mixed diet, beef raw meat and complementray vegetable food
Base-2 = mixed diet, beef raw meat and complementray vegetable food
Base-3 = mixed diet, beef raw meat and complementray vegetable food

	KW non-parametric test					LDA1					LDA2			
	B	H	K	W	*P*value	B	H	K	W	*P*value	H+B	K	W	*P*value
Sutterella	7.556	0.637	6.690	10.828	**< 0.0001**	-0.078	-0.083	0.029	-0.010	0.000	-0.085	0.025	-0.016	0.002
Prevotella	19.775	11.441	35.884	15.006	**< 0.0001**	-0.021	-0.038	0.026	-0.013	0.000	-0.040	0.025	-0.019	0.000
Epulopiscium	0.510	0.501	0.870	2.198	**< 0.0001**	0.073	0.116	0.075	0.301	0.000	0.084	0.081	0.328	< 0,0001
Paraprevotella	17.594	9.393	14.936	23.647	**0.005**	0.013	0.013	0.016	0.001	0.001	0.008	0.017	-0.001	0.000
Blautia	78.045	92.550	66.339	92.242	**0.002**	0.023	0.017	0.013	0.041	0.001	0.020	0.013	0.041	0.001
Parabacteroides	4.181	0.237	2.000	2.892	**0.000**	0.223	0.078	-0.010	-0.075	0.001	0.169	-0.012	-0.070	0.265
Adlercreutzia	0.359	0.883	0.831	0.400	**0.000**	-0.652	-0.003	0.123	-0.395	0.005	-0.272	0.151	-0.379	0.012
Dorea	26.085	31.304	25.502	25.053	0.079	-0.007	-0.009	0.019	-0.008	0.509	-0.010	0.019	-0.005	0.596
Megamonas	47.240	3.178	10.928	18.451	**< 0.0001**	0.041	0.007	0.012	0.021	< 0.0001	0.026	0.011	0.018	0.001
Allobaculum	27.880	12.965	13.129	12.831	**0.002**	0.070	0.014	0.013	0.007	< 0.0001	0.043	0.015	0.005	0.001
Slackia	2.592	6.545	2.528	3.696	**< 0.0001**	0.071	0.449	0.046	0.045	< 0.0001	0.241	0.058	0.062	0.001
Butyricicoccus	2.015	0.306	0.999	0.544	0.105	0.455	0.203	0.144	-0.041	< 0.0001	0.367	0.160	-0.079	0.018
Bacteroidales	106.019	107.525	107.192	176.357	**< 0.0001**	0.005	0.016	0.012	0.024	< 0.0001	0.013	0.013	0.023	< 0,0001
Anaerobiospirillum	6.415	5.458	1.558	1.949	**< 0.0001**	0.185	0.152	0.014	-0.093	< 0.0001	0.169	0.011	-0.091	< 0,0001
Bacteroides	54.855	32.449	36.247	67.092	**< 0.0001**	0.023	0.040	0.011	0.019	< 0.0001	0.032	0.012	0.021	< 0,0001
Clostridium	289.849	334.082	214.091	219.455	**< 0.0001**	0.020	0.020	0.013	0.011	< 0.0001	0.021	0.013	0.011	< 0,0001
Collinsella	15.796	15.368	5.863	14.515	**< 0.0001**	0.054	0.006	0.002	0.059	< 0.0001	0.036	0.000	0.058	< 0,0001
Escherichia	11.553	4.206	4.631	21.221	**< 0.0001**	0.038	0.011	0.016	0.067	< 0.0001	0.023	0.013	0.064	< 0,0001
Fusobacterium	63.304	43.552	37.936	98.781	**< 0.0001**	0.019	0.013	0.003	0.034	< 0.0001	0.013	0.003	0.034	< 0,0001
Oscillospira	2.348	0.457	1.711	6.635	**< 0.0001**	-0.008	-0.188	-0.009	0.372	< 0.0001	-0.134	-0.040	0.304	< 0,0001
p-75-a5	1.393	3.345	4.070	5.942	**< 0.0001**	-0.217	-0.227	0.046	-0.055	< 0.0001	-0.220	0.037	-0.088	< 0,0001
Peptococcus	7.966	7.355	1.963	7.265	**< 0.0001**	0.275	0.275	0.013	0.111	< 0.0001	0.279	0.009	0.115	< 0,0001
Roseburia	1.711	0.152	0.226	0.659	**< 0.0001**	0.522	-0.235	-0.031	0.072	< 0.0001	0.000	0.000	0.000	NS
Turicibacter	26.746	19.594	22.877	8.835	0.114	0.020	0.001	0.008	-0.003	0.002	0.000	0.000	0.000	NS
Phascolarctobacterium	6.933	4.383	5.637	9.630	**0.001**	0.000	0.000	0.000	0.000	NS	0.071	-0.013	0.007	0.001
Coprococcus	1.400	1.597	3.340	7.197	**< 0.0001**	0.000	0.000	0.000	0.000	NS	-0.019	0.059	0.135	< 0,0001
Eubacterium	7.914	11.689	11.579	8.650	0.575	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Ruminococcus	35.737	34.544	24.624	33.630	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Catenibacterium	10.350	17.400	17.074	6.797	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Coprobacillus	1.640	3.293	1.840	2.875	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Enterococcus	4.780	2.110	9.457	6.167	0.123	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Faecalibacterium	9.633	11.374	13.038	13.063	0.555	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS

Helicobacter	0.961	0.441	0.449	0.635	0.093	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Lachnospira	2.724	1.293	2.086	1.115	**0.000**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Lactobacillus	44.828	74.372	66.072	6.958	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
SMB53	2.970	2.380	2.160	5.021	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Streptococcus	15.13322	85.23303	71.45458	32.681	**0.001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS

S3 Table. Comparison of the mean relative abundances (RA) and coefficients of the linear discriminant analysis (LDA) using genera as input variables and considering 4 diet categories (LDA1) or 3 diet categories (LDA2). For this latter, B and H diets were collapsed together.

W: Commercial moist complete diet;
K: Commercial extruded complete diet;
H: Home-made diet;
B: Base diet;
H-B: home-made diet and Base diet collapsed together.

S4 Table. Confusion matrix of the linear discriminant analysis (LDA), considering 4 diet categories (LDA1) or 3 diet categories (LDA2). For this latter, H and B diets were collapsed together.

LDA1

from \ to	B	H	K	W	Total	% correct
B	38	5	9	4	56	67.86%
H	2	17	9	2	30	56.67%
K	3	3	153	12	171	89.47%
W	4	1	15	63	83	75.90%
Total	47	26	186	81	340	79.71%

LDA2

from \ to	H-B	K	W	Total	% correct
H-B	68	14	4	86	79.07%
K	7	154	10	171	90.06%
W	6	14	63	83	75.90%
Total	81	182	77	340	83.82%

W: Commercial moist complete diet;
K: Commercial extruded complete diet;
H: Home-made diet;
B: Base diet;
H-B: home-made diet and Base diet collapsed together.

	KW non-parametric test					LDA1					LDA2			
	F	FC	M	MC	*P*value	F	FC	M	MC	*P*value	C	F	M	*P*value
Prevotella	36.540	15.415	23.220	14.508	**0.033**	0.015	-0.004	0.011	-0.008	0.002	-0.004	0.014	0.011	0.001
Coprobacillus	2.028	2.854	1.516	2.828	**0.001**	-0.035	0.008	-0.159	0.009	0.043	0.020	0.020	-0.127	0.017
Adlercreutzia	0.797	0.738	0.368	0.639	0.051	0.240	0.395	-0.126	0.264	0.068	0.293	0.284	-0.072	0.031
Faecalibacterium	12.014	14.767	9.688	14.418	**0.023**	0.029	0.099	0.029	0.106	0.169	0.081	0.018	0.013	0.081
Turicibacter	15.990	20.278	22.064	23.860	0.943	0.012	0.028	0.025	0.033	0.264	0.035	0.012	0.027	0.157
Lactobacillus	43.948	73.669	37.650	58.938	**0.001**	0.013	0.019	0.014	0.018	0.347	0.019	0.014	0.015	0.232
Anaerobiospirillum	2.545	2.919	2.181	3.711	0.285	0.033	0.107	0.027	0.158	0.352	0.151	0.032	0.028	0.236
Enterococcus	4.220	29.580	4.968	7.104	0.742	0.013	0.034	0.015	0.021	0.038	0.026	0.014	0.017	0.269
Bacteroidales	143.404	98.559	129.155	95.538	**< 0.0001**	0.016	0.004	0.016	0.006	< 0.0001	0.006	0.016	0.017	< 0,0001
Blautia	57.069	90.348	86.966	96.169	**< 0.0001**	0.011	0.015	0.022	0.017	< 0.0001	0.014	0.012	0.022	< 0,0001
Clostridium	176.473	308.886	235.470	319.935	**< 0.0001**	0.014	0.021	0.016	0.022	< 0.0001	0.020	0.014	0.016	< 0,0001
Dorea	17.563	34.400	25.993	37.111	**< 0.0001**	0.024	0.071	0.035	0.073	< 0.0001	0.055	0.011	0.029	< 0,0001
Fusobacterium	72.096	42.306	66.393	30.567	**< 0.0001**	0.027	0.017	0.030	0.009	< 0.0001	0.011	0.020	0.025	< 0,0001
Oscillospira	4.955	0.553	2.846	0.366	**< 0.0001**	0.079	-0.163	0.011	-0.134	< 0.0001	-0.113	0.093	0.016	< 0,0001
Phascolarctobacterium	9.299	4.460	5.934	3.898	**< 0.0001**	0.028	0.091	0.009	0.105	< 0.0001	0.092	0.035	0.007	< 0,0001
Slackia	2.502	4.587	2.523	4.409	**< 0.0001**	-0.046	0.207	0.021	0.198	< 0.0001	0.253	0.007	0.051	< 0,0001
Streptococcus	29.356	67.437	35.251	106.081	**< 0.0001**	0.013	0.021	0.014	0.026	< 0.0001	0.027	0.014	0.015	< 0,0001
Peptococcus	5.865	6.078	4.098	2.981	**0.002**	0.031	0.087	-0.014	0.002	0.007	0.000	0.000	0.000	NS
Ruminococcus	22.395	37.765	29.394	38.676	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.044	0.020	0.022	< 0,0001
Sutterella	11.998	1.245	7.907	1.053	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.004	0.061	0.040	< 0,0001
SMB53	4.285	1.376	2.700	1.722	**0.001**	0.000	0.000	0.000	0.000	NS	-0.043	0.049	-0.023	0.003
Lachnospira	1.550	1.976	1.226	2.977	**0.001**	0.000	0.000	0.000	0.000	NS	0.169	0.036	0.073	0.014
Megamonas	22.800	16.735	22.314	7.030	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.008	0.020	0.016	0.019
Eubacterium	7.005	11.196	10.629	14.984	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Paraprevotella	21.449	11.797	19.151	9.546	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Allobaculum	17.500	14.110	14.195	13.711	0.263	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Bacteroides	62.174	30.273	46.613	25.994	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Butyricicoccus	1.189	0.585	1.069	0.740	**0.016**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Catenibacterium	7.225	17.583	10.861	24.700	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS

Collinsella	10.222	9.871	10.161	11.256	0.641	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Coprococcus	4.997	2.098	3.780	2.435	**0.001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Epulopiscium	1.435	0.197	1.216	0.747	**0.003**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Escherichia	11.374	9.727	12.084	5.195	**0.050**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Helicobacter	0.507	0.405	0.769	0.582	0.477	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
p-75-a5	3.816	4.499	3.834	4.372	0.445	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Parabacteroides	3.945	0.467	2.448	0.530	**< 0.0001**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS
Roseburia	0.697	0.120	0.636	0.447	**0.006**	0.000	0.000	0.000	0.000	NS	0.000	0.000	0.000	NS

S6 Table. Comparison of the mean relative abundances (RA) and coefficients of the linear discriminant analysis (LDA) using genera as input variables and considering 4 sex categories (LDA1) or 3 sex categories (LDA2). For this latter, FC and MC sex were collapsed together.

F: whole females subjects;
M: whole males subjects;
FC: spayed females subjects;
MC: neutered males subjects;
C: spayed females subjects and neutered males subjects collapsed together.

S7 Table. Confusion matrix of the linear discriminant analysis (LDA) considering 4 sex categories (LDA1) or 3 sex categories (LDA2). For this latter, FC and MC were collapsed together.

LDA1

from \ to	F	FC	M	MC	Total	% correct
MC	5	4	5	75	89	84.27%
FC	5	3	2	18	28	10.71%
F	123	1	11	10	145	84.83%
M	37	0	28	13	78	35.90%
Total	170	8	46	116	340	67.35%

LDA2

from \ to	C	F	M	Total	% correct
C	102	6	9	117	87.18%
F	7	124	14	145	85.52%
M	18	39	21	78	26.92%
Total	127	169	44	340	72.65%

F: whole females subjects;
M: whole males subjects;
FC: spayed females subjects;
MC: neutered males subjects;
C: spayed females subjects and neutered males subjects collapsed together.

Supplementary Materials Chapter 4

Table S1. Breed, sex and weight of the dogs recruited for the study.

Dietary group	*Breed*	*Sex*	*Live weight, kg*
D0	Amstaff	CM	33
D0	Mongrel shepherd	CM	30
D0	Pitbull Terrier	CM	32
D0	Mongrel Rottweiler	CM	35
D0	Border Collie	CM	24
D0	American Pit Bull Terrier	CM	34
D0	Mongrel Shepherd	M	27
D0	Mongrel Rottweiler	SF	30
D1	Maremmano Sheperd	CM	51
D1	Mongrel German Sheperd	CM	35
D1	Mongrel Sheperd	SF	15
D1	Mongrel German Sheperd	M	45
D1	Mongrel Spinone	CM	25
D1	Mongrel Lupoid	CM	19
D1	Mongrel Maremmano Sheperd	CM	26
D1	Mongrel	CM	20
D3	Mongrel	M	20
D3	Mongrel Hound	CM	25
D3	Mongrel German Sheperd	CM	31
D3	Mongrel Lupoid	CM	29
D3	Mongrel	CM	29
D3	Mongrel	CM	24
D3	Mongrel Lupoid	CM	21
D3	Mongrel Sheperd	SF	21

D0: Dogs without dietary supplementation of proanthocyanidins
D1: Dogs receiving a dietary supplementation of 1 mg/kg live weight of proanthocyanidins
D3: Dogs receiving a dietary supplementation of 3 mg/kg live weight of proanthocyanidins
MC: Castrated Male
FS: Spayed Female
M: Male

Supplementary Materials Chapter 5

Table S1. description of dogs recruited for the study.

Diet	LW (kg)	Sex	Age	Breed
HOME	4.4	FC	10	Mixed breed
BARF	19.2	FC	7	Mixed breed
HOME	6.5	FC	3	Mixed breed
HOME	22.7	MC	5	English Pointer
BARF	3.5	FC	10	Mixed breed
HOME	12.2	MC	7	Mixed breed
BARF	42	FI	4	Flat coated retriever
BARF	40	FI	10	Flat coated retriever
KIBBLE	22	FI	4	Labrador
BARF	25.3	MI	5	Australian Shepherd
HOME	31.4	MC	10	Mixed breed
KIBBLE	26.3	FC	10	Gordon setter
BARF	24.6	MI	4	Belgian Shepherd Dog
HOME	18	FC	9	Mixed breed
BARF	21.3	MI	6	Border Collie
HOME	26	FI	2	Mixed breed
KIBBLE	9	MI	3	Cavalier King Charles Spaniel
BARF	29	FI	3	Labrador
HOME	37	MI	2	Czechoslovakian Wolfdog
HOME	10	FI	10	Dachshund
BARF	9.5	FI	10	Mixed breed
HOME	5	FC	9	Maltese
KIBBLE	19.7	FC	2	Mixed breed
BARF	23	FI	5	Flat coated retriever
HOME	49	FC	7	Berner Sennenhund
KIBBLE	5.7	FC	3	Jack Russel Terrier
BARF	28	FC	7	Flat coated retriever
HOME	3	MC	4	Maltese
BARF	32	FC	9	Weimaraner
BARF	30	FC	4	Mixed breed
KIBBLE	5.6	FC	10	Dachshund
BARF	29	MC	4	English Bulldog
KIBBLE	20	MI	5	Schnauzer
HOME	7.9	MI	5	Poodle
KIBBLE	12	FC	6	Mixed breed
KIBBLE	7	FC	7	Mixed breed
HOME	37.3	MI	3	Weimaraner
KIBBLE	9	FI	10	Mixed breed
KIBBLE	6	MC	5	Japan Chil

KIBBLE	8	FI	6	Jack Russel Terrier
BARF	30	MC	7	Siberian Husky
BARF	30	FC	4	Saarlooswolfhond
KIBBLE	3	MI	4	Maltese
HOME	7.2	FC	3	Jack Russel Terrier
HOME	11	MI	2	Bouledogue français
KIBBLE	8	FC	6	Mixed breed
HOME	4.3	FI	9	Maltese
HOME	6.3	MI	8	Pinscher

MC: castrated male; FC: spayed female; MI: intact male; FC: intact female.
BARF: raw meat-based diet; HOME: home-made diet; KIBBLE: complete extruded diet.

Table S2. PCA loadings reported in squared cosine and metabolites associated with the integrated regions of the spectra.

	PC1	PC2	PC3	Metabolites
10.000 - 9.950	**0.695**	0.025	0.134	
9.950 - 9.900	**0.692**	0.008	0.155	
9.900 - 9.850	**0.681**	0.018	0.196	
9.850 - 9.800	**0.692**	0.011	0.194	
9.800 - 9.750	**0.706**	0.025	0.186	
9.750 - 9.700	**0.687**	0.024	0.201	
9.700 - 9.650	**0.709**	0.021	0.188	
9.650 - 9.600	**0.736**	0.021	0.145	
9.600 - 9.550	**0.714**	0.012	0.176	
9.550 - 9.500	**0.723**	0.015	0.170	
9.500 - 9.450	**0.741**	0.019	0.158	
9.450 - 9.400	**0.600**	0.032	0.072	
9.400 - 9.350	**0.747**	0.044	0.047	
9.350 - 9.300	**0.744**	0.017	0.154	
9.300 - 9.250	**0.776**	0.016	0.141	
9.250 - 9.200	**0.757**	0.032	0.125	
9.200 - 9.150	**0.767**	0.035	0.118	
9.150 - 9.100	**0.726**	0.047	0.133	
9.100 - 9.050	**0.720**	0.047	0.072	
9.050 - 9.000	**0.758**	0.050	0.083	
9.000 - 8.950	**0.746**	0.055	0.086	Serotonin, Tryptophan, Tryptamine
8.950 - 8.900	**0.842**	0.007	0.045	
8.900 - 8.850	**0.788**	0.069	0.059	
8.850 - 8.800	**0.740**	0.119	0.035	
8.800 - 8.750	**0.452**	0.096	0.003	
8.750 - 8.700	**0.274**	0.099	0.017	
8.700 - 8.650	**0.525**	0.297	0.008	
8.650 - 8.600	**0.544**	0.186	0.000	
8.600 - 8.550	0.260	**0.344**	0.000	
8.550 - 8.500	0.016	**0.242**	0.027	
8.500 - 8.450	0.007	0.030	0.000	
8.450 - 8.400	0.026	**0.337**	0.166	
8.400 - 8.350	0.012	**0.322**	0.207	
8.350 - 8.300	0.006	**0.373**	0.305	
8.300 - 8.250	0.000	**0.349**	0.239	
8.250 - 8.200	0.033	**0.301**	0.045	
8.200 - 8.150	0.002	0.187	0.155	
8.150 - 8.100	0.006	0.189	**0.236**	
8.100 - 8.050	0.001	**0.313**	0.274	
8.050 - 8.000	0.001	0.128	**0.343**	
8.000 - 7.950	0.001	0.020	**0.323**	

7.950 - 7.900	**0.253**	0.002	0.177	
7.900 - 7.850	0.000	0.123	0.120	
7.850 - 7.800	0.003	0.029	0.039	
7.800 - 7.750	0.077	0.001	0.058	
7.750 - 7.700	0.097	0.003	**0.226**	
7.700 - 7.650	**0.371**	0.121	0.249	
7.650 - 7.600	0.301	0.098	**0.352**	
7.600 - 7.550	0.130	0.000	0.012	
7.550 - 7.500	0.142	0.001	0.068	
7.500 - 7.450	**0.297**	0.093	0.031	
7.450 - 7.400	0.083	0.016	0.001	
7.400 - 7.350	**0.272**	0.096	0.155	
7.350 - 7.300	0.089	0.234	0.065	Tyramine, Tyrosine
7.300 - 7.250	0.060	0.004	**0.491**	
7.250 - 7.200	0.000	0.000	0.088	
7.200 - 7.150	0.075	0.056	**0.180**	
7.150 - 7.100	0.291	0.000	**0.349**	
7.100 - 7.050	0.192	0.000	0.029	
7.050 - 7.000	**0.224**	0.048	0.152	
7.000 - 6.950	0.018	**0.427**	0.047	
6.950 - 6.900	0.007	0.028	0.067	
6.900 - 6.850	0.015	0.052	**0.326**	
6.850 - 6.800	0.112	0.007	**0.370**	
6.800 - 6.750	**0.321**	0.038	0.026	
6.750 - 6.700	**0.522**	0.040	0.004	
6.700 - 6.650	**0.515**	0.241	0.031	
6.650 - 6.600	**0.577**	0.275	0.011	
6.600 - 6.550	**0.482**	0.347	0.014	
6.550 - 6.500	**0.180**	0.131	0.006	
6.500 - 6.450	0.335	**0.404**	0.025	
6.450 - 6.400	**0.372**	0.194	0.007	
6.400 - 6.350	0.281	0.054	0.031	
6.350 - 6.300	0.211	0.334	0.022	
6.300 - 6.250	0.189	0.368	0.023	
6.250 - 6.200	0.203	**0.378**	0.011	
6.200 - 6.150	0.150	**0.446**	0.010	
6.150 - 6.100	0.078	**0.481**	0.007	
6.100 - 6.050	0.056	**0.479**	0.009	
6.050 - 6.000	0.053	**0.528**	0.006	
6.000 - 5.950	0.035	**0.510**	0.013	
5.950 - 5.900	0.000	**0.396**	0.000	
5.900 - 5.850	0.028	**0.487**	0.015	
5.850 - 5.800	0.062	0.073	0.040	
5.800 - 5.750	0.070	**0.440**	0.022	

5.750 - 5.700	0.039	0.449	0.011	
5.700 - 5.650	0.033	0.451	0.006	
5.650 - 5.600	0.039	0.393	0.006	
5.600 - 5.550	0.029	0.420	0.010	
5.550 - 5.500	0.021	0.452	0.011	
4.550 - 4.500	0.005	**0.413**	0.016	
4.500 - 4.450	0.073	**0.465**	0.108	
4.450 - 4.400	0.007	0.180	**0.574**	
4.400 - 4.350	0.003	0.044	**0.637**	
4.350 - 4.300	0.025	0.241	**0.387**	
4.300 - 4.250	0.077	0.154	**0.408**	
4.250 - 4.200	0.104	**0.286**	0.286	
4.200 - 4.150	0.294	**0.508**	0.013	
4.150 - 4.100	**0.362**	0.160	0.146	
4.100 - 4.050	0.251	**0.338**	0.125	
4.050 - 4.000	0.256	**0.290**	0.081	L-threonine, Serotonine, Lactic acid
4.000 - 3.950	0.221	**0.299**	0.061	
3.950 - 3.900	0.384	**0.413**	0.003	
3.900 - 3.850	**0.439**	0.328	0.013	
3.850 - 3.800	0.364	**0.425**	0.011	
3.800 - 3.750	**0.347**	0.300	0.002	
3.750 - 3.700	0.226	**0.253**	0.103	
3.700 - 3.650	0.243	**0.459**	0.001	
3.650 - 3.600	0.108	**0.338**	0.000	
3.600 - 3.550	0.181	0.140	0.023	
3.550 - 3.500	**0.343**	0.223	0.169	
3.500 - 3.450	**0.325**	0.187	0.208	
3.450 - 3.400	0.310	0.072	**0.435**	
3.400 - 3.350	0.188	**0.209**	0.027	
3.350 - 3.300	0.023	0.093	0.029	
3.300 - 3.250	0.168	0.000	**0.386**	
3.250 - 3.200	0.073	0.090	0.078	
3.200 - 3.150	**0.358**	0.176	0.018	
3.150 - 3.100	**0.433**	0.173	0.002	
3.100 - 3.050	**0.200**	0.085	0.137	L-threonine, 2-phenylethylamine, Dopamine
3.050 - 3.000	0.002	0.013	**0.468**	
3.000 - 2.950	0.135	0.019	**0.477**	
2.950 - 2.900	0.118	0.182	**0.353**	
2.900 - 2.850	0.074	0.057	**0.611**	
2.850 - 2.800	0.097	0.069	**0.546**	
2.800 - 2.750	0.184	0.186	**0.246**	
2.750 - 2.700	0.225	0.133	**0.329**	
2.700 - 2.650	**0.444**	0.119	0.200	
2.650 - 2.600	**0.590**	0.063	0.149	

2.600 - 2.550	**0.598**	0.004	0.110	
2.550 - 2.500	**0.507**	0.001	0.154	
2.500 - 2.450	**0.438**	0.018	0.213	
2.450 - 2.400	0.012	0.015	0.008	
2.400 - 2.350	**0.265**	0.198	0.107	
2.350 - 2.300	0.261	**0.349**	0.099	
2.300 - 2.250	0.206	0.204	**0.309**	
2.250 - 2.200	0.068	0.022	0.012	
2.200 - 2.150	**0.278**	0.196	0.024	
2.150 - 2.100	0.184	**0.249**	0.102	
2.100 - 2.050	0.006	0.186	0.067	
2.050 - 2.000	0.002	0.128	0.203	Propionic acid, Butyric acid, Valeric acid, GABA, Acetic acid
2.000 - 1.950	**0.348**	0.096	0.268	
1.950 - 1.900	0.089	**0.382**	0.004	
1.900 - 1.850	0.241	**0.256**	0.241	
1.850 - 1.800	**0.278**	0.109	0.180	
1.800 - 1.750	**0.316**	0.299	0.075	
1.750 - 1.700	0.115	0.204	0.148	
1.700 - 1.650	0.005	0.034	**0.367**	
1.650 - 1.600	0.048	0.046	**0.307**	
1.600 - 1.550	0.124	**0.324**	0.011	
1.550 - 1.500	**0.369**	0.359	0.001	
1.500 - 1.450	0.186	0.211	0.031	
1.450 - 1.400	0.168	0.039	**0.530**	
1.400 - 1.350	0.014	0.052	0.063	
1.350 - 1.300	**0.265**	0.027	0.255	
1.300 - 1.250	0.165	0.101	0.042	
1.250 - 1.200	0.007	**0.309**	0.054	
1.200 - 1.150	0.045	**0.158**	0.039	
1.150 - 1.100	0.078	0.036	0.071	
1.100 - 1.050	0.247	0.118	0.007	
1.050 - 1.000	**0.326**	0.253	0.018	L-threonine, Lactic acid, Propionic acid, Iso-butyric acid, Butyric acid
1.000 - 0.950	0.004	0.178	0.005	
0.950 - 0.900	0.144	**0.509**	0.013	
0.900 - 0.850	0.109	**0.389**	0.025	
0.850 - 0.800	**0.752**	0.030	0.008	
0.800 - 0.750	**0.798**	0.019	0.008	
0.750 - 0.700	0.022	0.028	0.029	
0.700 - 0.650	**0.235**	0.079	0.006	
0.650 - 0.600	**0.670**	0.066	0.090	
0.600 - 0.550	**0.697**	0.068	0.118	
0.550 - 0.500	0.078	**0.429**	0.001	
0.500 - 0.450	**0.627**	0.039	0.156	
0.450 - 0.400	**0.665**	0.057	0.147	

0.400 - 0.350	**0.670**	0.082	0.127
0.350 - 0.300	**0.653**	0.069	0.140
0.300 - 0.250	**0.632**	0.058	0.150
0.250 - 0.200	**0.609**	0.079	0.165

Values in bold correspond for each variable to the factor for which the squared cosine is the largest

Table S3. Percentage of presence of metabolites analyzed in each study group of dogs based on their sex. For each metabolite, a chi-square test was performed and the relative p-value is reported. A p-value below 0.05 was considered significant.

% of presence	FC	FI	MC	MI	p-value
2-phenylethylamine	47.4	45.5	42.9	36.4	0.948
GABA	0.0	9.1	14.3	45.5	0.008
L-threonine	94.7	90.9	100.0	81.8	0.517
acetic acid	100.0	100.0	100.0	100.0	N.D.
butyric acid	89.5	63.6	100.0	72.7	0.156
iso-valeric acid	42.1	45.5	42.9	63.6	0.695
iso-butyric acid	26.3	45.5	14.3	54.5	0.231
lactic acid	100.0	100.0	85.7	100.0	0.113
propionic acid	100.0	90.9	100.0	90.9	0.481
valeric acid	5.3	9.1	0.0	27.3	0.193
diisopropylamine	0.0	18.2	0.0	27.3	0.069
dopamine	5.3	9.1	0.0	9.1	0.849
indole	0.0	0.0	0.0	9.1	0.329
serotonin	15.8	27.3	28.6	18.2	0.833
tyramine	63.2	81.8	28.6	54.5	0.152
tyrosine	84.2	72.7	85.7	54.5	0.287
tryptamine	57.9	72.7	14.3	36.4	0.068
tryptophan	63.2	72.7	57.1	72.7	0.860

FC: spayed females
FI: whole females
MC: neutered males
MI: whole males

Table S4. Percentage of presence of metabolites analyzed in each study group of dogs based on their live weight. For each metabolite, a chi-square test was performed and the relative p-value is reported. A p-value below 0.05 was considered significant.

% of presence	< 10 kg	10 - 25 kg	> 25 kg	P-value
2-phenylethylamine	31.6	38.5	62.5	0.167
GABA	21.1	15.4	6.3	0.464
L-threonine	89.5	89.5	93.8	0.897
acetic acid	100.0	100.0	100.0	N.D.
butyric acid	89.5	84.6	68.8	0.275
iso-valeric acid	47.4	30.8	62.5	0.235
iso-butyric acid	36.8	53.8	18.8	0.143
lactic acid	100.0	100.0	93.8	0.360
propionic acid	100.0	84.6	100.0	0.060
valeric acid	10.5	15.4	6.3	0.726
diisopropylamine	10.5	15.4	6.3	0.726
dopamine	0.0	15.4	6.3	0.210
indole	0.0	7.7	0.0	0.253
serotonin	10.5	38.5	18.8	0.156
tyramine	63.2	46.2	68.8	0.443
tyrosine	84.2	46.2	87.5	0.019
tryptamine	63.2	38.5	43.8	0.323
tryptophan	68.4	61.5	68.8	0.900

Supplementary Materials Chapter 7

S1 Table. Summary of the dietary intervention studies that were included in the dataset. (Same as in Chapter 3)

S2 Table. Membership of taxa into modules

Taxa	Module
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Butyricicoccus;s__pullicaecorum	module_0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;__;__	module_0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__[Prevotella];s__	module_0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__	module_0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;__;__	module_0
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__;s__	module_0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Succinispira;s__mobilis	module_0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium;s__methylpentosum	module_0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lactonifactor;s__longoviformis	module_1
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea;s__longicatena	module_1
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;__;__	module_1
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;__;__	module_1
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Ruminococcus;s__lactaris	module_1
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Pseudoramibacter_Eubacterium;s__	module_2
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium;s__breve	module_2
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7;g__;s__	module_2
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__baratii	module_3
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__[Clostridium];__	module_3
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;__	module_3
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__disporicum	module_4
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Clostridium;s__ruminantium	module_4
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;__;__	module_4
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;__	module_5
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;__;__;__	module_5
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];g__Barnesiella;__	module_5
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__[Eubacterium];s__biforme	module_6

k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Catenibacterium;s__mitsuokai	module_6
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Ruminococcus;__	module_6
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Macellibacteroides;s__fermentans	module_7
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Sporobacter;s__termitidis	module_7
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__;s__	module_7
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__plebeius	module_8
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__copri	module_8
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;__	module_8
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Lacticigenium;s__naphtae	module_9
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Leuconostoc;s__fallax	module_9
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__paraplantarum	module_9
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella;s__	module_10
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Slackia;s__	module_10
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Odoribacter;s__	module_11
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];g__Barnesiella;s__intestinihominis	module_11
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__hamsteri	module_12
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;__;__	module_12
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium;__	module_13
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium;s__	module_13
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Peptostreptococcus;__	module_14
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Allobaculum;s__	module_14
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium;s__saccharogumia	module_15
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium;__	module_15
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;g__Anaerobiospirillum;s__	module_16
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__;s__	module_16
k__Bacteria;p__Firmicutes;__;__;__;__;__	module_17
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__Peptococcus;s__	module_17
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__;s__	module_18
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;__;__;__	module_18

APPENDIX B

List of Publications

Journal Publications:

Scarsella, E., Segato, J., Zuccaccia, D., Swanson, K. S., & Stefanon, B. (2021). An application of nuclear magnetic resonance spectroscopy to study faecal canine metabolome. *Italian Journal of Animal Science*, *20*(1), 887-895.

Scarsella, E., Zecconi, A., Cintio, M., & Stefanon, B. (2021). Characterization of Microbiome on Feces, Blood and Milk in Dairy Cows with Different Milk Leucocyte Pattern. *Animals*, *11*(5), 1463.

Scarsella, E., Sandri, M., Monego, S. D., Licastro, D., & Stefanon, B. (2020). Blood Microbiome: A New Marker of Gut Microbial Population in Dogs?. *Veterinary Sciences*, *7*(4), 198.

Cintio, M., **Scarsella, E.**, Sgorlon, S., Sandri, M., & Stefanon, B. (2020). Gut Microbiome of Healthy and Arthritic Dogs. *Veterinary sciences*, *7*(3), 92.

Sandri, M., Sgorlon, S., **Scarsella, E.**, & Stefanon, B. (2020). Effect of different starch sources in a raw meat-based diet on fecal microbiome in dogs housed in a shelter. *Animal Nutrition*, *6*(3), 353-361.

Scarsella, E., Stefanon, B., Cintio, M., Licastro, D., Sgorlon, S., Dal Monego, S., & Sandri, M. (2020). Learning machine approach reveals microbial signatures of diet and sex in dog. *Plos one*, *15*(8), e0237874.

Cintio, M., Polacchini, G., **Scarsella, E.**, Montanari, T., Stefanon, B., & Colitti, M. (2020). MicroRNA Milk Exosomes: From Cellular Regulator to Genomic Marker. *Animals*, *10*(7), 1126.

Scarsella, E., Cintio, M., Iacumin, L., Ginaldi, F., & Stefanon, B. (2020). Interplay between neuroendocrine biomarkers and gut microbiota in dogs supplemented with grape proanthocyanidins: results of dietary intervention study. *Animals*, *10*(3), 531.

Contributing Author:

Scarsella, E., Sandri, M., Cintio, M., Sgorlon, S., Conte, G., & Stefanon, B. (2019). DogBiome: the gut microbiome project for dog. *ASPA 23rd Congress Book of Abstract, Ital J Anim Sci, 18*, S1.

Stefanon, B., Cintio, M., **Scarsella, E.**, & Iacumin, L. (2019). Interplay between salivary biomarkers and gut microbiome in dogs. *ASPA 23rd Congress Book of Abstract, Ital J Anim Sci, 18*, S1.

Scarsella, E., Cintio, M., Zecconi, A., & Stefanon, B. (2020). Milk microbiome: evaluation study on the differences among cows with a different health status classified by leukocyte pattern. *28th Animal Science Days International Symposium, Acta Fytotechnica et Zootechnica, 23*(5).

Scarsella, E., Cintio, M., Zecconi, A., & Stefanon, B. (2021). MiRNAs in milk exosomes are differentially expressed based on the health status of the mammary gland. *EAAP - 72nd Annual Meeting, Davos, Switzerland.*

Stefanon, B., Cintio, M., Sgorlon, S., Zecconi, A., Colitti, M., & **Scarsella, E.** (2021). MiRNAs cargo of milk exosomes are related to somatic cell count in dairy cows. *ASPA 24th Congress Book of Abstract, Ital J Anim Sci, 20,* S1.

Scarsella, E., Jha, A., & Stefanon, B. (2021). Network-based gut microbiome analysis in dogs. *ASPA 24th Congress Book of Abstract, Ital J Anim Sci, 20,* S1.