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Characterization of the fecal microbiota in dogs with obesity and after weight loss

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Declarations

I declare that I myself have composed this thesis and that this work has not been submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except the work that form part of jointly authored publication. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

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ABSTRACT

The prevalence of obesity is rapidly increasing worldwide, constituting an important health problem. Similarly, as occurs in human, the number of pets with obesity is increasing notably, being the most common metabolic disorder in companion animals. Apart from genetic susceptibility, sedentary lifestyle and increased food consumption, environment factors such as changes in the gut microbiota seem to play a role in the development of this metabolic disease.

In addition, studies in humans, animal models and dogs have revealed that the fecal microbiota of subjects with obesity is different from that of lean subjects, and changes after weight loss. However, the impact of weight loss on the fecal microbiota in dogs with obesity has not been fully characterized, existing discrepancies between different studies that aimed to investigate the effect of weight loss on the fecal microbiota of dogs.

This study reviews the current knowledge about the role of the gut microbiota in the maintenance of energy homeostasis in mammalian hosts. Focusing on dogs and humans, and describing the mechanism purposed to explain how the gut bacteria can contribute to the development of obesity.

Furthermore, to evaluate the possible changes in the gut microbiota of obese dogs associated to weight loss, 16S rRNA gene sequencing was performed in fecal samples of 20 dogs with obesity and after weight loss with a high-fiber-high-protein diet. The endpoint of the weight loss program was individually tailored to the ideal body weight of each dog.

The results obtained showed that after weight loss, the fecal microbiota of dogs with obesity changed significantly. This shift in the fecal microbiota composition was characterized

by an increase in bacterial diversity, a decrease in Firmicutes, and increase in Bacteroidetes and Fusobacteria.

Taxonomic analysis of the gut microbial communities is the first approach to understand the composition of gut microbiota in obese individuals and detect gut microbiota signatures in the obese phenotype. However, methodological approaches such as metagenomic and metabolomic analyses are needed to elucidate the functions of these bacteria, which will allow to understand their interaction with the host, hence their possible role in obesity.

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Acc1	Acetyl-CoA carboxylase
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1
AEA	Arachidonoyl ethanolamide/Anandamide
AF	As fed
AMPk	Adenosine monophosphate kinase
ANOSIM	Analysis of Similarity
ATP	Adenosine triphosphate
BCS	Body condition score
CB	Cannabinoid receptor
ChREBP	Carbohydrate responsive element-binding protein
CPT	Carnitine-palmitoyltransferase
CY-7A1	Cholesterol 7a-hydroxylase
DC	Dendritic cells
DEXA	Dual-energy x-ray absorptiometry
DI	Dysbiosis index
DM	Dry Matter
DNA	Deoxyribonucleic acid
eCBs	Endocannabinoid system
Fad	Fatty acid synthase
FOS	Fructo-oligosaccharides
F:B	Firmicutes/Bacteroidetes
FDR	False discovery rate
FIAF	Fasting induced adipocyte factor
FXR	Farnesoid X Receptor
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GPCRs	G-protein-coupled receptors
FMT	Fecal microbiota transplantation
IBD	Inflammatory bowel disease
INF-γ	Interferon gamma
IL	Interleukin
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide

LTK	Lipoteichoic acids
LogSQ	Logarithm of starting quantity or logarithm of relative DNA copy number
MAMPs	Microbe-associated molecular patterns
ME	Metabolizable energy content
MOS	Manna-oligosaccharides
NAPE-PLD	N-acylphophatidylethanolamine phospholipase D
OTU	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PRRs	Pattern recognition receptors
PYY	Peptide YY
qRT-PCR	Quantitative real-time reverse transcriptase-polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acids
SD	Standard deviation
SIgA	Secretory immunoglobulin A
SREBP1	Sterol regulatory element binding transcription factor 1
TNF-α	Tumor necrosis factor alpha
T5KO mice	TLR5 genetically deficient T mice
TLRs	Tool-like receptors
16S	16 ribosomal subunit
2-AG	2-arachidonyl glycerol

1.   To provide information about the role of gut microbiota in the etiology of obesity

2.   To describe the current knowledge about gut microbiota signatures in obese dogs

3.   To characterize the fecal microbiota composition of client-owned dogs with obesity before and after weight loss with a high-protein-high-fiber weight loss diet

4.   To test the effect of the weight loss diet on the fecal microbiota composition of a small cohort of obese dogs without weight loss

CHAPTER I: LITERATURE REVIEW

Gut microbiota: functions in mammalian hosts and its role in obesity

1.1. The obesity problem

Obesity is a major medical concern in terms of its impact on the quality of life of our society. Shared by both humans and small companion animals (i.e. pet dogs and cats), obesity is a complex disorder, defined as an accumulation of excessive amounts of adipose tissue in the body (Bartges et al. 2017). Although the pathology of obesity remains unclear, some factors are associated with the development of this pathology including diet, level of physical activity, behavioral factors, socioeconomic factors, environmental exposures, genetics, metabolism and lately, the microbiome composition (Day 2017). Obesity is considered a pandemic in most developed countries because of its contribution to the development of comorbidities, as well as of its impact on decreased lifespan in both companion animals (German 2006) and humans (Kopelman 2000).

Despite treatment options, such as dietary management and increasing physical activity, the prevalence rises every year in humans as well as in pets. The prevalence of obesity has been estimated between 19.7 and 59.3 % in dogs (McGreevy et al. 2005; Hill 2009; Courcier et al. 2010; Corbee 2013; Mao et al. 2013). In humans, it is expected that by 2030, more than 2.16 billion people will be overweight and 1.12 billion obese (Kolahi, Moghisi, and Soleiman Ekhtiari 2018). Beyond the health consequences associated with the disease, obesity carries social disadvantages, reducing socio-economic productivity and creating an economic burden (Blüher 2019).

Among all the factors contributing to obesity, the gut microbiota is receiving renewed attention due to its role in gut homeostasis. The mammalian intestinal tract accommodates various microorganisms such as viruses, bacteria, fungi, archaea, and protozoa, which shape the gut microbiota (Hillman et al. 2017). This ecosystem has a symbiotic interaction with the intestine of the host (Bäckhed et al. 2005). Commensal gut bacteria have been the subject of

study during recent decades since many of their metabolic products affect host physiology (Jandhyala et al. 2015). Therefore, some microbial functions such as enhancement of gut barrier integrity (Natividad and Verdu 2013), energy harvesting (den Besten, van Eunen, et al. 2013), protection against pathogens (Bäumler and Sperandio 2016), regulation of host immunity and development of the normal intestinal epithelium (Gensollen et al. 2016) are mediated by bacteria in the intestine. The disruption of the normal bacterial ecology within the gut may lead to functional changes in host-microbial interactions and homeostasis. Gut microbiota alterations have been reported in different gastrointestinal and metabolic diseases such as inflammatory bowel disease (IBD), cancer, obesity, metabolic syndrome, liver disease, and diabetes, in both humans (Durack and Lynch 2019) and companion animals (Redfern, Suchodolski, and Jergens 2017). However, the mechanisms as how microbes and their metabolites can contribute to the development of disease (McFall-Ngai et al. 2013) are still under investigation.

This literature review aims to summarize the current knowledge on the interactions of intestinal bacteria with the host, with special emphasis on the role of intestinal microbiota in obesity. A summary of the possible mechanisms proposed to date, in which the gut microbiota can contribute to the development of obesity will be discussed. In addition, an overview of the studies that mention possible microbiota modulators will be reviewed.

1.2 Gut microbiota in humans and dogs

Gut microbiota composition is affected by the physiological conditions within the region of the gastrointestinal tract (Donaldson, Lee, and Mazmanian 2016). Although the intestinal microbiota composition is variable in each segment of the gut (Hillman et al. 2017), strict anaerobic bacteria are the most abundant in the human gut. The major phyla in the gut are Firmicutes, Bacteroidetes, and Proteobacteria, with lower abundances of Actinobacteria, Verrumicrobia and Fusobacteria (Sommer and Bäckhed 2013). The lower gastrointestinal tract has a greater abundance of Firmicutes and Bacteroidetes, whilst the upper part is more enriched in Proteobacteria and Firmicutes (Vuik et al. 2019). The large intestine harbors the majority of the microbes found in the body, since the colonic conditions allow for the dense growth of diverse species of bacteria (Sears 2005).

There are also differences between the lumen and the mucosal surface of the intestine. The fecal, luminal and mucosal bacterial composition seems to be significantly different in humans (Eckburg et al. 2005; Li et al. 2015). The predominant genera found in the lumen are *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Lactobacillus* and *Ruminococcus*. Conversely, *Clostridium*, *Lactobacillus*, *Enterococcus* and *Akkermansia* are the predominant genera found in the mucosa and mucus layers of the small intestine (Swidsinski et al. 2005). Despite these differences in microbiota composition between stool samples and the cross-section of the intestine, fecal samples are used in most studies because they are easy to collect (Dieterich, Schink, and Zopf 2018).

In dogs, the gut microbiota also differs along the intestinal tract, increasing gradually in diversity and abundance along the small and large intestine (Suchodolski et al. 2005; Suchodolski, Camacho, and Steiner 2008). Most of the bacteria belong to the phyla Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria and Actinobacteria (Suchodolski, Camacho, and Steiner 2008; Honneffer, Lidbury, and Suchodolski 2017). Within the mentioned phyla, the most abundant taxa that have been reported comprise Ruminococcaceae, *Faecalibacterium*, Peptostreptococcaceae, Lachnospiraceae, *Blautia*, *Streptococcus*, *Lactobacillus*, *Turicibacter*, *Catenibacterium* and *Coprobacillus*. The majority of bacteria from the phylum Bacteroidetes consists of the genera *Prevotella*, *Bacteroides* and *Megamonas*. The genus *Fusobacterium* is also highly represented, and its abundance, conversely to humans, is associated with a healthy status in dogs (Vázquez-Baeza et al. 2016). The phyla Actinobacteria and Proteobacteria are

also detected in fecal samples of dogs but with less abundance. Contrastingly to humans, stool samples have been demonstrated to provide accurate information about the intestinal microbiota composition in dogs (Vázquez-Baeza et al. 2016).

Bacterial diversity is also a commonly used parameter for characterizing microbiota. Many gastrointestinal diseases are associated with lower bacterial diversity (Durbán et al. 2012) and therefore, the identification of the core microbiota is important to define the profile of a "healthy" or "normal" gut microbiota (Rinninella et al. 2019). However, this aim is difficult because of the enormous number of factors influencing gut microbiota composition (Hasan and Yang 2019).

1.3 Factors affecting gut microbiota composition

One of the difficulties commonly found when analyzing gut microbiota is variability between different subjects (Suchodolski et al. 2005; Ursell et al. 2012). This is explained by the host and environmental pressures, which influence the gut microbiota composition (Rothschild et al. 2018). Host factors include biological conditions within the gastrointestinal tract, such as an intact mucus layer, digestive enzymes and antimicrobial proteins, and immunological factors like secretory immunoglobulin A (SIgA). In addition, life factors which can affect the diversity of the gut microbiota, are age, health status and delivery mode. Some external environmental factors that modulate the gut microbiota are antibiotics, diet and pre- and probiotics (Hasan and Yang 2019).

1.3.1 Age

It was until recently believed that gut microbiota colonization in mammals is initiated at birth, however, recent studies have reported traces of microbiota in the amniotic fluid and placenta (Collado et al. 2016; Aagaard et al. 2014). Differences in the fecal microbiota of vaginally born infants *versus* those delivered *via* cesarean section have been reported (Grönlund et al. 1999). From birth through the first year of life, the human gut microbiota undergoes major transitions, becoming more diverse over time (Bäckhed et al. 2015). These transitions in the gut microbiota are probably induced by interactions with the developing intestinal environment and diet, which are known to modulate the early gut microbiota composition (Guaraldi and Salvatori 2012). After infancy, the composition of gut bacteria becomes more similar to an adult gut microbiota (Yatsunenko et al. 2012). Studies reported that monozygotic twins have a significantly more similar microbiota composition than dizygotic twins, suggesting that the microbiome composition is in part influenced by host genetics (Goodrich et al. 2014).

A study in German Shepherd dogs, revealed different clustering of the fecal microbiota composition from 7 weeks to 18 months, in addition, an increase of diversity was observed from pregnancy to end of lactation (Vilson et al. 2018). Similarly, the study carried out by Guard and colleagues revealed significant differences between puppies from parturition to 2, 21, 42 and 56 days of life, and showing a gradual increase in bacterial diversity (Guard et al. 2017). In addition, a change in the gut bacterial composition across age has also been

demonstrated, showing a decrease in bacterial diversity associated with age, in both humans and dogs, from an adult microbiota to an elderly microbiota (Mizukami et al. 2019; Odamaki et al. 2016), which also correlates with a decline in health status (Claesson et al. 2012).

1.3.2 Geographical location and diet

Gut microbiota changes associated with dietary factors can be classified as either short-term or long-term. In humans, a different microbial composition was reported between individuals from Malawi, Venezuela and the United States (Yatsunenko et al. 2012), also in children from Bangladesh and the United States (Lin et al. 2013) and between subjects from rural areas of Africa compared to African Americans (Ou et al. 2013). However, sometimes it is difficult to separate the factor of long-term dietary habit from the environmental factor associated with location. Geographical location has been proposed to influence the intestinal microbiota composition in humans (Yatsunenko et al. 2012). However, these differences remain unclear when different geographical locations do not implicate different dietary habits (Lay et al. 2005).

The factor of geographical location affecting gut bacteria profile seems to not be as relevant in dogs. A recent study showed differences in the fecal microbiota Shannon diversity index between Western United States and Midwestern dogs, however, no differences in gut microbiota were reported between dogs from the other regions of United States (Jha et al. 2020). This could be explained because diet macronutrient composition in dogs is more uniform across developed countries, since dogs are usually fed commercial diets manufactured to adhere to specific standards.

Short term dietary interventions have been studied in humans to evaluate the effect on intestinal microbiota (Thaiss et al. 2014). The results obtained are variable, several studies have reported no significant effect of diet change on gut microbiota composition (Xu and Knight 2015; Wu et al. 2011). Extreme dietary macronutrient shifts, such as a change to animal-based diets or plant-based diet, have shown slight effects in the intestinal microbiota, reporting changes in the relative abundance of several bacterial groups and in beta diversity. However, fecal microbiota composition returned to baseline 2 days after the diet intervention (David et al. 2014). Several studies tagged fiber content and type as crucial modulators of intestinal microbiota composition (Chassaing, Vijay-Kumar, and Gewirtz 2017). Some bacterial

populations such as *Lactobacillus* and *Bifidobacterium* are increased after dietary fiber consumption (Liu et al. 2017), which is accompanied with an increase of butyrate. However, no significant differences were observed in humans, in terms of alpha bacterial diversity when reviewing a high number of studies evaluating fiber effect on intestinal microbiota (So et al. 2018). However, these changes are only maintained during the time that the dietary fiber was consumed. Concluding that short-term changes in fecal microbiota due to diet shift are transient (Albenberg and Wu 2014).

Similarly, in healthy dogs, only minor dietary effects on fecal microbiota have been observed when the macronutrient composition of the dietary changes remained within a reasonable range (Sandri et al. 2017; Schauf et al. 2018). Moreover, diets with similar macronutrient content but substituting the traditional mixed plant and animal source of protein for plant-based protein exclusively, did not lead to significant differences in the fecal microbiota composition (Bresciani et al. 2018). Consistently in dogs as in humans, large shifts in microbiome composition have been observed only with drastic dietary changes, such as animal-based raw food diets, which include significantly more protein and less fiber and carbohydrates than kibble diets (Schmidt et al. 2018; Bermingham et al. 2017).

1.3.3 Antibiotics

Antibiotics have a dual role in the modulation of the gut microbiota. They eliminate microbial pathogens but also beneficial bacteria that contribute to the homeostasis of intestinal health (Klingensmith and Coopersmith 2016). Studies in mouse models revealed that microbial depletion with antibiotics affected host serotonin biosynthesis, secondary bile acids metabolism, and induced a delayed intestinal motility (Ge et al. 2017). More evidence of the important role of gut microbiota in obesity is demonstrated by several studies in which low doses of antibiotics administrated in early life altered the intestinal microbiota and adiposity in humans and mice (Cox et al. 2014; Stark et al. 2019). Cho and colleagues developed a study in which administration of penicillin, vancomycin or chlortetracycline in young mice induced a significant change in the gut microbiota composition at family level, and a consequent increase in percent of body fat from 22.9% in the control group to 32% (Cho et al. 2012).

Gut microbiota disruption due to antibiotics promotes the invasion of pathogens in the intestine, such as *Clostridium difficile* (Ramnani et al. 2012). Moreover, it has been shown that

the use of antibiotics such as clindamycin, clarithromycin, metronidazole and ciproflaxin affects gut microbiota composition by alteration of the relative abundance of some intestinal bacteria taxa and by the expression of resistance genes up to 2 years in humans (Dethlefsen and Relman 2011; Jakobsson et al. 2010; Jernberg et al. 2007).

In healthy dogs that were administered metronidazole for 14 days, the fecal microbiota composition showed lower bacterial diversity (Igarashi et al. 2014). The changes reverted after 28 days, while after 42 days of antimicrobial intake, the fecal microbiota composition returned to the initial microbiota profile detected before antibiotic intake. However, the same results were not observed with prednisolone intake, showing no significant differences in terms of bacterial diversity after antimicrobial use (Igarashi et al. 2014). In the case of tylosin, after 7 days healthy dogs showed a significantly different microbiota composition compared to the baseline day, before antimicrobial intake. After 63 days, however, these differences were no longer significant (Manchester et al. 2019).

1.3.4 Probiotics

"*Probiotics are living microorganisms that when administrated in adequate amounts provide benefits to the host*" (Gibson et al. 2017). High tolerance to gastric acid and bile, capability to adhere to intestinal surfaces, low pH and gastric juice withstand, antibiotic resistance, exopolysaccharides production, and inhibition of potentially pathogenic species by its antimicrobial activity, are some essential characteristics of probiotics. Due to the demonstrated beneficial effect on the host (Fijan 2014), the most commonly used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium* (Marco, Pavan, and Kleerebezem 2006). The proposed mechanisms by which probiotics may improve host health are growth promotion of beneficial bacteria, protection against adhesion of harmful bacteria, and exclusion or inhibition of pathogens directly producing antimicrobial compounds or inducing an immune response from the host (Cleusix et al. 2007; Servin 2004). Probiotics may also enhance the intestinal epithelial barrier and modulate the immune system and commensal microbiota (Mohan et al. 2008).

Due to the ability of some probiotics and ingested bacteria to modulate bacterial colonization in the gut, probiotics have been used as a therapeutic approach in the treatment of several gastrointestinal diseases (Alagón Fernández Del Campo et al. 2019). In patients with

IBD, ingestion of a probiotic preparation (VSL#3) showed a decrease of inflammatory cytokines and helped to maintain remission status (Miele et al. 2009; Fedorak et al. 2015). There is also evidence that commensal bacteria may regulate epithelial permeability in the gut (Plöger et al. 2012), which is an obesity associated parameter. Probiotic bacteria such as VSL3 and *Lactobacillus rhamnosus* showed decrease of gut permeability in addition to preventing apoptosis of colonic cells in mouse models of colitis (Mennigen et al. 2009; Miyauchi, Morita, and Tanabe 2009).

In animal models, convincing evidence suggest that probiotics may have a potential anti-obesity activity (Kobyliak et al. 2016). *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum*, suppressed body weight gain and reduced fat accumulation in liver tissue, as well as cholesterol in plasma in a diet-induced obesity model. In addition, in the liver, it was observed an up-regulation of the fatty acid oxidation-related genes (such as the peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PGC1α, the carnitine-palmitoyltransferase I and II (CPT1, CPT2) and peroxisomal acyl-coenzyme A oxidase 1, ACOX1 genes) in mice receiving probiotic treatment (Yoo et al. 2013; Park et al. 2013).

Although there is evidence that probiotics may have a positive effect on health, especially in individuals with a risk of developing gastrointestinal disease, further studies must be done in order to clarify the dose, the strain-dependence and the mechanisms underlying their beneficial effects (Markowiak and Śliżewska 2017). Related to metabolic disorders in humans, one study evaluating the effect of the probiotics *Lactobacillus acidophilus*, *L. bulgaricus*, *L. bifidum*, and *L. casei* in individuals with type 2 diabetes reported a reduction of insulin resistance and the levels of triglyceride after 6 weeks of daily intake of probiotic supplementation, however, these differences were not significant (Mazloom, Yousefinejad, and Dabbaghmanesh 2013) In addition, studies that examine the effect of probiotic supplementation in obese individuals reported a limited influence on weight loss, highlighting the necessity of additional studies to prove that probiotic supplementation could become an effective strategy for the prevention and treatment of obesity (Wang et al. 2019).

The effect of probiotics on the intestinal microbiota in dogs is controversial. Commercial probiotics include *Enterococcus faecium*, *Lactobacillus acidophilus*, and *Bifidobacterium sp. animalis* and a mix of different strains (multi-strain probiotics). Although, most of the studies that showed their efficacy are *ex vivo* (Schmitz et al. 2014), there is one

study that has demonstrated an improvement in gastrointestinal disease signs with the use of a mix of probiotic strains (Rossi et al. 2014; White et al. 2017).

Although, the beneficial effect of probiotic in obese dogs has not been yet investigated, in healthy dogs, the effect of probiotics such as *Enterococcus faecium*, *Bifidobacterium animalis*, *Lactobacillus casei*, and *Lactobacillus fermentum* is very limited. Hence, no differences in the fecal microbiota composition or serum biochemical parameters after probiotic supplementation have been reported (Strompfová et al. 2006; Strompfová et al. 2014; Chung et al. 2009; Lucena et al. 2019). Despite a modulation of the gut microbiota (increase in bifidobacterial and lactobacilli) and greater levels of butyrate and lactate were observed after Fructooligosaccharides and *Lactobacillus acidophilus* supplementation in healthy dogs (Swanson et al. 2002), its beneficial effect for the host was attributed to the fructo-oligosaccharides incorporated.

1.3.5 Prebiotics

Prebiotics are defined as fermentable products that have an impact on the composition and/or activity of the gastrointestinal microbiota, providing a benefit for host health (Roberfroid et al. 2010). Usually, prebiotics are classified as fibers and complex polysaccharides that are not able to be digested by host enzymes (Gibson, et al 2010). The main beneficial effect of dietary fiber for the host is the ability to maintain an intact intestinal mucosal barrier (Ray 2018). Some examples of prebiotics are the disaccharides lactulose, tagatose, the oligo and polysaccharides fructo-oligosaccharides (FOS), manna-oligosaccharides (MOS) xylo-oligosaccharides, polydextrose, galacto-oligosaccharides or inulin (Hughes and Rowland 2001; Koh et al. 2013; Ogué-Bon et al. 2010). In animal studies, administration of the fiber inulin prevented increased mucus penetrability in Western style diet -fed mice (Schroeder et al. 2018).

In healthy dogs, several fibers have been associated with changes in gut microbiota composition (Spears, Karr-Lilienthal, and Fahey 2005; Middelbos et al. 2010; Beloshapka et al. 2013; Myint et al. 2017; Panasevich et al. 2015). In addition, studies in obese mice treated with prebiotics, resulted in a lower concentration of LPS, cytokines and a decreased hepatic expression of inflammatory and oxidative stress markers than those without prebiotic intake *via* glucagon-like peptide 2 (GLP-2). This was associated with a decrease in intestinal permeability

(Cani et al. 2009). However, further studies are necessary to demonstrate a significant beneficial effect of probiotics and prebiotics in gastrointestinal diseases (Schmitz and Suchodolski 2016).

1.4   Gut microbiota and obesity

The first association between gut microbiota and obesity was observed when fecal content from normal mice was transplanted into germ-free mice, with the consequence of weight gain despite a reduction in food intake (Bäckhed et al. 2004). This study suggested that gut microbiota was involved in harvesting energy from the diet. Subsequently, it was revealed that lean individuals have different fecal microbiota composition than obese individuals, both in mice (Ley et al. 2005) and humans (Ley et al. 2006). In addition, when fecal microbiota from obese mice donors was transplanted to germ-free mice, they developed the obese phenotype (Turnbaugh et al. 2008; Turnbaugh et al. 2006). Similar results were observed when the donors were twins discordant for obesity (Ridaura et al. 2013), suggesting that the gut microbiota composition contributes to energy metabolism. This was also supported by another study in which germ-free mice were protected against diet-induced obesity even when the diet was low in complex carbohydrates, suggesting that germ-free mice are resistant to developing obesity by having elevated levels of Fiaf (factor induce adiposity) and increased AMPk activity which led to an increased fatty acid metabolism (Bäckhed et al. 2007).

Initially, a significantly higher abundance of Firmicutes and lower abundance of Bacteroidetes was reported in the obese phenotype and the proportion of Bacteroidetes increased after a weight loss diet (Ley et al. 2006). It was hypothesized that Firmicutes help to incorporate more calories from the diet, caused by a higher proportion of genes encoding enzymes involved in energy extraction from complex carbohydrate (Turnbaugh et al. 2006). However, the postulated higher ratio of Firmicutes/Bacteroidetes in obesity was not always observed in studies (Schwiertz et al. 2010), and a decrease in the abundance of Firmicutes has also been observed after weight loss (Duncan et al. 2007). Despite the discrepancies between studies, the gut microbiota of obese individuals shows in general a lower bacterial diversity (Le Chatelier et al. 2013), and calorie restriction has been shown to increase gut bacteria richness in obese and overweight individuals (Cotillard et al. 2013; Le Chatelier et al. 2013). However, the discrepancies in the results may be due to other factors that influence the gut bacteria composition, such as age, genetic background, geographical location, diet and other environmental factors.

1.5   Fecal microbiota alterations in obese dogs

The first study to evaluate the fecal microbiota composition of obese and lean dogs reported minor differences between the groups (Handl et al. 2013). Similar results were observed in a study with beagle dogs, however, the study showed lower microbial diversity in the obese group (Park et al. 2015).

A further study that compared fecal microbiota between normal weight, overweight and obese companion dogs, showed few differences in some bacteria populations, such as family Bifidobacteriaceae, being increased in obese dogs, and the genus *Eubacterium* being decreased. A trend towards lower bacterial richness in obese dogs compared to normal weight dogs has also been reported but did not reach statistical significance (Forster et al. 2018). The results were similar when the fecal microbiota of overweight dogs was evaluated before and after a 12-week weight loss program, revealing minor changes in fecal microbiota composition that did not reach significance (Kieler et al. 2017). However, an actual change in the microbial composition was observed after a 17-week weight loss program in a recent study of obese beagle dogs (Salas-Mani et al. 2018). It was the first study to report similar results as in obese humans (Ley et al. 2006) showing a greater abundance of Firmicutes in obese dogs and lesser of Bacteroidetes which changed after weight loss.

To find an explanation as to why the intestinal microbiota of dogs with obesity was different from that of ideal weight dogs, it is important to explore the gut microbiota functions and its effect on host physiology, therefore on host health.

1.6 Host-microbial relationship and possible mechanisms to link gut microbiota and obesity

The importance of exploring the gut microbiota functions resides in its effect on host physiology and, therefore, on host health. The symbiotic relationship between the gut microbiota and the host is represented by the interactions with the intestinal cells, the immune system, the enteric nervous system, and the endocrine system (Cani, Everard, and Duparc 2013; Bengmark 2013; Sittipo et al. 2018). These functions are mediated by the metabolic interconversions and the exchange of metabolites between the host and bacteria (Rowland et al. 2018; Jandhyala et al. 2015). Gut microbiota processes contribute to epithelial development, mucosal immunity, defense against pathogens, xenobiotic degradation, nutrient metabolism, biosynthesis of bioactive compounds, and bile acid metabolism among others (Nicholson et al. 2012).

Bacterial metabolism, including *de novo* synthesis and the molecular transformations of dietary compounds and intestinal metabolites, has a crucial role in the maintenance of intestinal health. Therefore, a disruption of the intestinal microbiota may contribute to intestinal pathologies or metabolic disorders. For example, alterations in the metabolism of bile acids, branched fatty acids, and choline, has been associated with the development of metabolic diseases such as obesity and type 2 diabetes (Palau-Rodriguez et al. 2015). Different mechanisms have been proposed to explain the link between gut microbiota and obesity. The conversion of undigested nutrients and host products by commensal bacteria in the gut produces different metabolites that have crucial roles in different physiological functions of the host, but are also targeted by the gut microbiota (Sittipo, Shim, and Lee 2019). Control of the energy homeostasis in the host by increased energy harvest from the diet, regulation of lipid metabolism, as well as the release of hormones implicated in satiety, and the intestinal low-grade inflammation related with an increase in gut permeability of the intestine, have been some of the mechanisms through which gut microbiota can contribute to the etiology of obesity (Figure 1.1).

1.6.1 SCFAs increase the energy harvest from the diet

The digestive system of humans and companion animals, such as dogs and cats, is not capable of digesting a high number of polysaccharides from the diet, such as cellulose, xylan and pectin. These food compounds reach the intestine where they are fermented by the intestinal microbiota, generating ATP (adenosine triphosphate) and simple carbon molecules that include SCFAs. The three most abundant SCFAs detected in feces are acetate, propionate and butyrate (Macfarlane and Macfarlane 2003). These volatile compounds perform different roles in the mammalian body such as regulation of gene expression, chemotaxis, differentiation, proliferation and apoptosis (Corrêa-Oliveira et al. 2016). Butyrate has also been proposed as an energy source for human colonocytes (den Besten, Lange, et al. 2013), moreover, butyrate and propionate have the ability to regulate gene expression by inhibiting histone deacetylases (Steliou et al. 2012). It has been hypothesized that the composition of the gut microbiota could influence the amount of energy harvested from the SCFAs (Bäckhed et al. 2005), since a greater content of SCFA in fecal samples and higher expression of genes involved in polysaccharides metabolism have been associated with an obese phenotype in mice (Turnbaugh et al. 2006). However, this is not supported by evidence in humans where high-fiber diets, which would be expected to increase SCFA production, protect against weight gain (Du et al. 2010). In addition, *in vitro* fermentations using fecal samples from obese and ideal weight individuals showed no difference in total SCFA production (Yang, Keshavarzian, and Rose 2013).

1.6.2 Lipid metabolism and gut microbiota

The first studies that aimed to elucidate the mechanism in which gut microbiota contributes to obesity, revealed that SCFAs might induce *de novo* lipogenesis. This conclusion was made after observing an excess in body fat mass in germ-free mice which received the fecal microbiota of conventionally raised mice (conventionalized mice) (Bäckhed et al. 2004). Accordingly, an increase of lipogenic genes expression such as acetyl-CoA carboxylase (Acc1) and fatty acid synthase (Fas) was reported after conventionalization (Bäckhed et al. 2004). Interestingly, Acc1 and Fas are transcriptional sites of two transcription factors involved in lipogenesis in the liver in response to insulin and carbohydrates, sterol response element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) (Bäckhed et al. 2004). Accordingly, to investigate the effect of gut microbiota on host energy

and lipid metabolism, another study evaluated the serum, adipose tissue and liver metabolomes of germ-free mice and conventionalized mice. The results showed increased levels of energy metabolites, such as pyruvic acid, citric acid, fumaric acid, and malic acid in conventionalized mice, however, a reduction of serum cholesterol and fatty acids was also detected in comparison with germ-free mice (Velagapudi et al. 2010). These results were also in agreement with a subsequent *in vitro* study with HepG2 cells treated with gut microbiota-producing lipids. These compounds activated *de novo* lipogenesis and triglyceride synthesis *via* the mTOR/SREBP1 pathway, with consequent fat accumulation in HepG2 cells (Go et al. 2013).

Fasting induced adiposity factor, also known as angiopoietin-like protein 4 (Fiaf/ANGPTL4), produced by large intestinal epithelial cells and the liver, plays a crucial role in triglyceride metabolism by inhibiting lipoprotein lipase (LPL). Inhibition of Fiaf expression causes accumulation of fat in peripheral tissues (Dutton and Trayhurn 2008). Seeking for a possible hypothesis to explain the increase in fat storage in conventionalized mice, Backhed and colleagues analyzed the role of Fiaf as a gut microbiota-host signaling pathway. Total body fat content in germ-free and conventionalized, normal and Fiaf knockout mice was measured. The results showed that Fiaf suppression is essential for the microbiota-induced deposition of triglycerides in adipocytes (Bäckhed et al. 2004; Bäckhed et al. 2007) In addition, it was suggested that expression of Fiaf may be mediated by the composition of the gut microbiota (Aronsson et al. 2010; Bäckhed et al. 2004). However, the association between Fiaf expression and fat storage is still under discussion. The study conducted by Fleissner and colleagues found that different diets modulated the intestinal microbiota in germ-free mice and conventionally raised mice did not result in changes in the level of Fiaf, as suggested in the previous studies (Fleissner et al. 2010).

Another proposed mechanism in which gut microbiota may interact in obesity is by suppression of the activity of the enzyme adenosine monophosphate kinase (AMPk). AMPk is a crucial enzyme in the regulation of energy homeostasis in cells. AMPk activates enzymes including acetyl-CoA carboxylase (Acc1) and carnitine-palmitoyltransferase I (CPT-1), both involved in energy expenditure by fatty acid oxidation in the mitochondria (Kim et al. 2016). In addition to favoring catabolic pathways, AMPk inhibits anabolic pathways such as lipogenesis, glycogenolysis, and protein synthesis, improving insulin sensitivity (Angin et al. 2016). Its association with gut microbiota was first studied by an experiment in which higher levels of AMPk, Acc1 and CPT-1 in the liver and skeletal muscle were observed in germ-free

mice in comparison with conventional raised mice when fed a Western type diet (Bäckhed et al. 2007). Inhibition of AMPk by gut microbiota leads to a decrease in fatty acid oxidation and consequently results in increased fat accumulation (Hardie 2008; Boulangé et al. 2016).

It has also been proposed that gut bacteria may contribute indirectly to the high fat diet induced obesity phenotype through the regulation of Farnesoid X Receptor (FXR) *via* bile acids metabolism (Wahlström et al. 2017). Bile acids are physiological molecules synthetized in the liver and secreted into the intestinal lumen with the bile. The functions of bile acids include facilitating fat digestion and absorption. In addition, they play a crucial role in removing toxic metabolites with the feces. Bile acids are reabsorbed from the intestine and transported back to the liver *via* blood circulation regulating its feedback, synthesis and secretion (Liu et al. 2018).

Gut microbiota plays a crucial role in bile acid metabolism by the conversion of primary bile acids (cholic acid and chenodeoxycholic acid) to secondary bile acids (deoxycholic acid and lithocholic acid). In humans, 95% of the bile acids secreted into the gut are reabsorbed, and the rest 5% are converted to secondary bile acids by gut bacteria such as some species of the genera *Clostridium* (Gopal-Srivastava and Hylemon 1988; Sorg and Sonenshein 2008).

Both primary and secondary bile acids activate FXR signaling, which regulates bile acid production, lipid and glucose homeostasis (Zhang and Edwards 2008). FXR regulates expression of cholesterol 7a-hydroxylase (CYP7A1) and CYP27A1, by its inhibition, which is required for the beginning of bile acid synthesis, regulating in this way the production of bile acids (Chiang 2009). In addition, Swann and colleagues suggested a contribution of gut microbiota in the diversity of bile acids (Swann et al. 2011). A subsequent study confirmed that gut microbiota can modulate the synthesis of bile acids by regulating the enzymatic activity of CYP7A1 and CYP27A1 (Sayin et al. 2013).

In addition to the interaction of the gut microbiota with FXR *via* bile acid metabolism, secondary bile acids are also ligands for the G protein coupled receptor (GPCR) TGR5, which is expressed in the brown adipose tissue and muscle. Stimulation of the TGR5 signaling pathway confers to bile acids the ability to modulate energy expenditure by regulating the activity of type 2 deiodinase and the subsequent activation of thyroid hormone (Watanabe et al. 2006). Moreover, it has been suggested that TGR5 stimulates the expression of glucagon-like peptide 1 (GLP-1) (Thomas et al. 2009), presenting a possible role in glucose homeostasis

(Aron-Wisnewsky et al. 2013). These findings highlight the possible contribution of gut microbiota to host metabolism homeostasis *via* TGR5 stimulation by secondary bile acids.

1.6.3 Gut microbiota and its role in satiety

SCFAs may also regulate host metabolism *via* G-protein-coupled receptors (GPCRs), expressed by immune cells, adipocytes, enteroendocrine cells and the gut epithelium. These mediate signaling to secrete GLP-1 and leptin production (Xiong et al. 2004; Samuel et al. 2008; Tolhurst et al. 2012), which regulates metabolic functions such as appetite and satiety.

The production of SCFAs by commensal microbiota also has been proposed to have a beneficial role in systemic glucagon and insulin regulation (satiety and host metabolism). SCFAs can act as signaling molecules, for example binding to GPR43 and GRP41. GRP43 is expressed in immune cells and in adipocytes, GRP41 by its side is expressed also in adipose tissue and in a subset of enteroendocrine cells in the gut epithelium (Brown et al. 2003) and both have been suggested to have a role in energy homeostasis. Mice deficient in GRP43 show an obese phenotype, and when overexpressing GRP43 in the adipose tissue present resistance to diet induced obesity, even when receiving a high fat diet (HFD) (Kimura et al. 2013). GPCRs stimulate peptide YY (PYY) which reduce appetite, GLP-1, which inhibit glucagon release and promotes insulin secretion, decreasing the glucosyl levels in blood (Kasubuchi et al. 2015). Studies in mice showed that acetate may have a role in the central appetite regulation (Frost et al. 2014). In fact, there are several studies in humans and animal models that confirm that the intake of indigestible polysaccharides upregulates the GLP-1 and PYY levels *via* SCFAs (Zhou et al. 2008; Tarini and Wolever 2010). GPR41 and GPR43 knockout mice had reduced levels of GLP-1 and impaired glucose tolerance (Tolhurst et al. 2012). In addition, a role of the gut microbiota in the suppression of food intake has been demonstrated with *in vitro* studies in which it has been shown that SCFAs stimulate leptin production through the interaction with the GRP41/43 (Xiong et al. 2004; Zaibi et al. 2010).

1.6.4 Gut microbiota and the innate immunity

Intestinal microbiota plays a crucial role in maintaining immune homeostasis in the gut (Kurilshikov et al. 2017). In the intestinal epithelium, enterocytes and dendritic cells (DCs)

express PRRs, such as TLRs, which are activated by contact with microbe-associated molecular patterns (MAMPs), and pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoteichoic acids (LTK) of bacterial cell walls, flagellin and double- or single-stranded RNA and DNA. This interaction is crucial to promote innate immunity, contributing to homeostatic balance of the gut microbiota by mediating host-microbe interaction (Clevers and Bevins 2013).

Considering the important role of TLRs and MAMPs, in the immune homeostasis, the function of one of the most expressed TLRs in the intestinal mucosa was evaluated. TLR5 genetically deficient T mice (T5KO mice), apart from exhibiting elevated proinflammatory gene expression, surprisingly had an increase in body mass, which was associated with a greater secretion of proinflammatory factors, such as IL-1β and INF-γ (Vijay-Kumar et al. 2007). Mice deficient in TLR5 expression also showed hyperphagia, hyperlipidemia, hypertension, insulin resistance, and increased fat deposition in comparison with conventionally raised mice, suggesting a role of TLR5 with metabolic syndrome, by its association with increased adiposity (Vijay-Kumar et al. 2010). In addition, they showed that antibiotic use improved the metabolic syndrome. The role of gut microbiota was also confirmed after observing that wild type and T5KO mice had different microbiota compositions. In fact, when T5KO microbiota was transplanted to wild-type germ free mice, a similar inflammatory phenotype as exhibited by the T5KO mice was observed. However, a subsequent study did not reproduce the same results with different animal colonies (Letran et al. 2011), concluding that the suggested inflammatory phenotype is not a consistent feature of TLR5-deficient mice.

1.6.5 Alteration in intestinal permeability and low-grade chronic inflammation

Obesity is associated with low-grade inflammation due to the release of signaling molecules from the adipose tissue (adipokines) such as TNF-alpha, IL-1 and IL-6, which have been associated with increased insulin resistance (Ouchi et al. 2011) and risk of cardiovascular diseases (Lau et al. 2005). In addition, one of the consequences of insulin resistance is the excessive fat storage in the haptic and adipose tissue, increasing inflammation and contributing to the fat accumulation loop.

Seeking an inflammatory factor in high fat diet induced obesity in mice, Cani and his group reported that low-grade inflammation in obesity and diabetes could be mediated by

lipopolysaccharide (LPS) from Gram-negative bacteria *via* interactions with the receptors CD14 and TLR4 (Cani, Amar, et al. 2007; Davis et al. 2008). This increase in plasma LPS in mice fed with a HFD was denominated "metabolic endotoxemia", and was accompanied with significantly decreased populations of *Bacteroides*/*Prevotella* spp, *Bifidocbacterium* spp, and *Lactobacillus* spp. (Cani, Amar, et al. 2007). Interestingly, metabolic endotoxemia improved after supplementation with Bifidobacteria (Cani, Neyrinck, et al. 2007).

Consistent with the previous hypothesis of LPS triggering low grade inflammation, an increase in intestinal permeability in obese mice during HFD was observed by reduction of the expression of genes that encode for tight junction proteins ZO-1 and occludin (Cani et al. 2008). Consequently, the same research group reported enhancement of the gut barrier integrity after *Bifidobacteirum* spp. supplementation by an increase of glucagon like peptide-2 (GLP-2) production (Cani et al. 2009). Subsequently, a possible role of the endocannabinoid (eCBs) system was proposed to link gut microbiota with gut barrier integrity and obesity. The cannabinoid receptors 1 (CB1) and 2 (CB2) are G proteins activated by the eCBs. Arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonyl glycerol (2-AG) together with their synthetizing and degrading enzymes, are two eCBs that play an important role in eating stimulation, adipogenesis and glucose uptake (Matias and Di Marzo 2007; Di Marzo et al. 2009).

Considering all the above, and that gut LPS has been demonstrated to regulate eCBs synthesis (Maccarrone et al. 2001), the role of gut microbiota in obesity was evaluated *via* eCBs activation in mice models of obesity. The results confirmed an increase of the expression of CB1 and the enzyme implicated in AEA synthesis, N-acylphophatidylethanolamine phospholipase D (NAPE-PLD) in the intestine and adipose tissue of obese mice. Same study also reported a modulation of the intestinal permeability *via* CB1 activation. In addition, it was demonstrated an *in vivo* and *in vitro* modulation of eCBs by the gut microbiota, *via* LPS by blocking the cannabinoid driven adipogenesis (Muccioli et al. 2010). Despite authors reported a decrease in fat mass and CB1 expression in obese mice by modulation of the gut microbiota composition using prebiotic, the composition of the microbiota was not evaluated to confirm this statement. In addition, the mechanisms by which the gut microbiota participate in the regulation of eCBs is not fully understood and requires further investigation (Cani 2012).
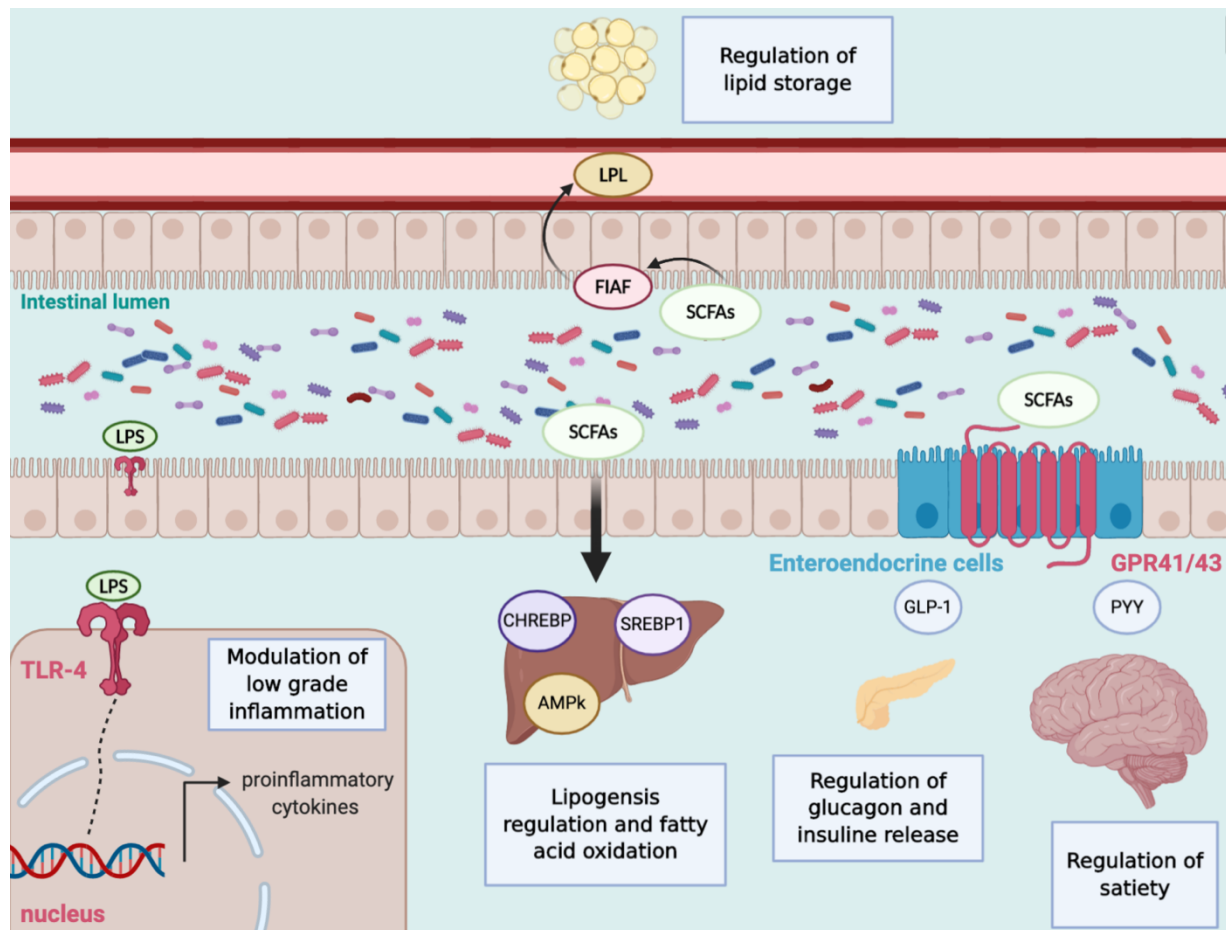
Figure 1.1 **Suggested mechanisms by which the gut microbiota could contribute to the pathogenesis of obesity.**

1.7   Conclusion

In summary, the gut microbiota lives in a mutualistic relationship with their mammalian host, affecting many important functions that maintain the health status of the host, and regulating energy balance. According to the studies described, the gut microbiota increases energy uptake and modulates energy homeostasis through the production of metabolites that act as signaling molecules, mediating host functions such as satiety, gut motility, energy storage and energy expenditure. Obese individuals have an altered fecal microbiota composition, and fecal microbiota of obese individuals transferred the obese phenotype in mice, suggesting an important role of gut microbiota in the development of obesity.

However, additional studies are necessaries in humans and companion animals to understand if gut microbiota manipulation could control the development of obesity, improve its prognosis or contribute to a faster weight loss rate.

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CHAPTER II

Fecal microbiota in client-owned obese dogs changes after weight loss with a high-fiber-high-protein diet

2.1   Abstract

Background. The fecal microbiota from obese individuals can induce obesity in animal models. In addition, studies in humans, animal models and dogs have revealed that the fecal microbiota of subjects with obesity is different from that of lean subjects and changes after weight loss. However, the impact of weight loss on the fecal microbiota in dogs with obesity has not been fully characterized.

Methods. In this study, we used 16S rRNA gene sequencing to investigate the differences in the fecal microbiota of 20 pet dogs with obesity that underwent a weight loss program. The endpoint of the weight loss program was individually tailored to the ideal body weight of each dog. In addition, we evaluated the qPCR based Dysbiosis Index before and after weight loss.

Results. After weight loss, the fecal microbiota structure of dogs with obesity changed significantly ($_{weighted}$ ANOSIM; p=0.016, R=0.073), showing an increase in bacterial richness (p=0.007), evenness (p=0.007) and the number of bacterial species (p=0.007). The fecal microbiota composition of obese dogs after weight loss was characterized by a decrease in Firmicutes (92.3% to 78.2%, q=0.001), and increase in Bacteroidetes (1.4% to 10.1%, q=0.002) and Fusobacteria (1.6% to 6.2%, q=0.040). The qPCR results revealed an overall decrease in the Dysbiosis Index, driven mostly due to a significant decrease in E. coli (p=0.030), and increase in *Fusobacterium* spp. (p=0.017).

Conclusion. The changes observed in the fecal microbiota of dogs with obesity after weight loss with a weight loss diet rich in fiber and protein were in agreement with previous studies in humans, that reported an increase of bacterial biodiversity and a decrease of the ratio Firmicutes/Bacteroidetes.

2.1 Introduction

Canine obesity is a serious metabolic disease that affects the quality of life and decreases life span (Salt et al. 2019; German et al. 2012). The prevalence of obesity has been increasing in the past years in small animals (Courcier et al. 2010), and it is a major healthcare problem in veterinary practice (Chandler et al. 2017; German 2006). Obesity is associated with a greater risk of developing other diseases such as diabetes mellitus, cardiovascular and orthopedic diseases, and even some types of cancer (Kopelman 2000; Tropf et al. 2017; German, Ryan, et al. 2010). Diet restriction increased life span in dogs and weight loss regimen based on weight loss diet and exercise decreased plasma insulin concentrations and insulin:glucose ratio (Kealy et al. 2002; German et al. 2009). Due to the detrimental effect of obesity on the welfare of both dogs and their owners, investigating new approaches to prevent obesity and promote weight loss in small animals is of crucial interest in veterinary research (Day 2017; Bartges et al. 2017).

In the past years, there has been interest on investigating a possible role for gut microbiota in obesity in humans, mouse models (Zhao 2013; Turnbaugh et al. 2008; Ridaura et al. 2013), and also in dogs (Forster et al. 2018; Handl et al. 2013; Park et al. 2015; Kieler et al. 2017; Salas-Mani et al. 2018). Studies have found that obesity is associated with alterations, disruption, and decreased biodiversity of the intestinal microbiota (Durack and Lynch 2019; Ley et al. 2005; Ley et al. 2006; Cotillard et al. 2013). In addition, colonization of germ-free mice with the fecal microbiota of obese humans lead to significant weight gain when compared to mice that received fecal microbiota from lean controls (Ridaura et al. 2013), suggesting that gut microbiota impacts host physiology and metabolism.

While a relationship between the gut microbiome and obesity has been observed, it remains unclear as to how the gut microbiome contributes to the development of obesity, but proposed mechanisms include the production of short chained fatty acids (SCFAs), monosaccharides, and other bioactive molecules. These bacterial products may lead to an increase in dietary energy harvest (Turnbaugh et al. 2006), changes in lipid metabolism (Ghazalpour et al. 2016), changes in fat storage regulation (Bäckhed et al. 2004; Bäckhed et al. 2007), altered satiety (Arora, Sharma, and Frost 2011), and an increase in systemic low-grade inflammation via the interaction with either the enteric nervous system (Schwartz 2000; Tehrani et al. 2012; de Lartigue, de La Serre, and Raybould 2011), the endocrine system (Mondo et al. 2020; Kirchoff, Udell, and Sharpton 2019; Scarsella et al. 2020), or the immune system (Cani et al. 2007; Cani et al. 2012).

In human and animal models of obesity, a greater abundance of the phylum Firmicutes and lesser abundance of Bacteroidetes have been reported (Turnbaugh et al. 2009; Vrieze et al. 2012), and the Firmicutes/Bacteroidetes (F:B) ratio is commonly used as a marker of gut microbial dysbiosis in obesity. The F:B ratio is greater in individuals with obesity and, interestingly, decreases after weight loss (Ley et al. 2005; Ley et al. 2006). Previous data have shown similarities in the gut microbiota of humans and dogs (Swanson et al. 2011; Coelho et al. 2018), but it is not clear whether results from human and animal models can be translated to canine obesity.

One study in research Beagles evaluated the fecal microbiota of lean dogs and dogs that developed obesity after overfeeding for 6 months. Analysis of fecal microbiota demonstrated differences in microbial communities between dogs in the obese and lean groups, with a lesser diversity in the obese group. In particular, there was a lesser abundance of Firmicutes and Fusobacteria in the obese group, and the abundance of Proteobacteria was significantly greater in the obese group compared to the lean group (Park et al. 2015).

In one study of client-owned dogs, a dominance of the phylum Firmicutes has been seen, with significant differences at the taxonomic level, between dogs with obesity and those in ideal body condition, but no significant differences in the overall composition of fecal microbiota or bacterial diversity (Handl et al. 2013). However, in a second study, a trend towards lower fecal microbial diversity was seen in dogs with obesity, compared with dogs in ideal bodyweight (Forster et al. 2018).

Two studies have evaluated the impact of weight loss on the fecal microbiota of dogs with obesity. In one study, the fecal microbiota was assessed before, during, and after 12 weeks of a weight loss program that consisted of diet and exercise or diet alone. Despite the short follow-up period, differences in bacterial abundance were identified after 6 weeks and 12 weeks of the weight loss program. While not all the dogs lost as much weight as expected, a decrease in *Megamonas* and an unknown genus of the family Ruminococcaceae was observed in the dogs with a higher weight loss rate (Kieler et al. 2017). The fecal microbiota composition of research Beagles with obesity has also been assessed before and after a 17-week weight loss program with a hypocaloric diet (Salas-Mani et al. 2018). Despite all dogs reaching ideal body weight, no significant impact on diversity was seen and microbial communities remained similar to baseline values after 17 weeks. At the genus level, significant differences were found only in the abundances of the Firmicutes genera *Lactobacillus*, *Clostridium*, and *Dorea*, which

decreased after the weight loss program, and *Allobaculum,* which increased (Salas-Mani et al. 2018).

A number of limitations need to be considered in these studies. Microbiome analysis evaluates a large number of variables, which limits the statistical power, especially in small cohorts (Falony et al. 2016). In addition, studies with healthy client-owned dogs have identified large individual variations, which need to be taken into account (Garcia-Mazcorro et al. 2012). Given that obesity develops over time, it is reasonable to expect that significant changes will be seen only when follow-up focuses on the long-term improvement. Therefore, the aim of this study was to use 16S rRNA sequencing to evaluate the differences in fecal microbiota composition of client-owned dogs with obesity before and after weight loss. We also performed quantitative PCR to calculate the Dysbiosis Index in obese dogs before and after weight loss and to compare the values obtained with the established reference intervals from healthy dogs (AlShawaqfeh et al. 2017). Moreover, we evaluated the fecal microbiota of obese client-owned dogs enrolled in the weight loss program that did not reach ideal body weight to quantify the effect of the diet alone on the fecal microbiota.

2.2 Materials and methods

2.2.1 Study animals, eligibility criteria and ethical considerations

Client-owned dogs with obesity were referred to the Royal Canin Weight Management Clinic, University of Liverpool, UK. All were presented between June 2009 and August 2017, and completed their weight loss regimens between November 2009 and August 2018. To be included in the study, dogs had to be clinically healthy with no signs of gastrointestinal disease, a BCS of ≥6, and no antimicrobial usage in the past month (Igarashi et al. 2014). None of the dogs had antimicrobials throughout their weight loss program. No fecal analyses were performed on the fecal samples and so occult infection with intestinal parasites could not be excluded. However, no dog showed signs consisted with parasitic infection.

Historical data and fecal samples, before and after participation in the weight loss program, had to be available for the analysis. The final number of dogs with obesity that met the inclusion criteria was 25. At time point zero (T0), fecal samples of all 25 dogs were collected. Twenty dogs completed the weight loss program and reached their target weight, whilst five dogs stopped their program early as request of the owners for undeclared reasons. From dogs that completed the weight loss, a fecal sample from the visit in which they reached their target body weight, was collected to include in the analysis as time point two (T2). Dogs that did not complete the weight loss program but had a fecal sample from the first follow-up visit (time point one (T1)) were included in the analysis to account for the effect of the new diet on the fecal microbiota. The study protocol was reviewed and approved by the University of Liverpool Veterinary Research Ethics Committee (Approval reference: RETH000353 and VREC793), the Royal Canin ethical review committee, and the WALTHAM ethical review committee. Owners of dogs with obesity gave informed consent in writing.

2.2.2 Weight loss regimen

Prior to commencing weight reduction, all dogs were considered to be healthy apart from their obesity. All dogs were screened for overall health by performing complete blood count, serum biochemical analysis, serum free thyroxine concentration (by equilibrium dialysis) and urinalysis. Dogs were weighed at admission, and the body condition score (BCS) was estimated using a 9-integer scale by the attending clinician (AJG). Percentage body fat was measured by dual-energy x-ray absorptiometry (DEXA) as previously described (Raffan et al.

2006). For weight reduction, all dogs were fed a dry therapeutic diet (Canine Satiety® diet, Royal Canin), with the exception of one dog (OBE16) that was fed a combination of wet and dry food (Canine Satiety® diet, Royal Canin). Moreover, the formulation of Satiety changed in 2010 (**Table 2.1**, **Data S2.1**). The endpoint of the study was achievement of ideal body weight which, given differing degrees of adiposity, was individually set for each dog using the results of body composition analysis from before weight loss, as previously reported (German et al 2012). Briefly, pre-weight-loss body composition data were entered into a computer spreadsheet which contained a bespoke mathematical formula to predict ideal bodyweight. The formula was based upon typical changes in body composition seen from previous weight loss studies at the same clinic (German et al. 2007; German, Holden, et al. 2010).

Table 2.1   Average composition of diets for weight loss

Criterion	**Dry food**[1]		**Wet food**[2]	
ME content	2900 / 2865 Kcal/kg		602 Kcal/kg	
	Per 100g AF	g/1000 Kcal (ME)	Per 100g AF	g/1000 Kcal (ME)
Moisture	8 / 10	28 / 33	83	1379
Crude protein	30 / 30	103 / 105	8.5	141
Crude fat	10 / 10	33 / 33	2.0	33
Starch	19 / 18	66 / 61	1.8	30
NFE	30 / 29	102 / 100	3.0	50
Crude fiber	18 / 16	60 / 58	2.0	33
Total dietary fiber	28 / 28	97 / 97	3.2	53
Ash	5.3 / 5.7	18 / 20	1.5	25

[1] Satiety Support Canine Dry (Royal Canin); [2]Satiety Support Canine Wet (Royal Canin); [3] Diet formulation changed in 2010; figures in column refer to pre-2010 and post-2010 diets, respectively. ME= Metabolizable energy content, as measured by animal trials according to the American Association of Feed Control Officials protocol (AAFCO, 2010); AF= as fed; DM= dry matter.

2.2.3 Fecal collection and DNA extraction

Fecal samples from dogs with obesity were collected after spontaneous defecation and stored at -20°C being shipped to the Gastrointestinal Laboratory at Texas A&M University in February 2019. DNA was extracted from approximately 100 mg of stool using the Mo Bio PowerSoil® DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions.

2.2.4 Quantitative real-time PCR (qPCR) and Dysbiosis Index (DI)

Quantitative PCR was performed using universal bacteria primers and primers for the following bacterial groups: *Blautia* spp., *Clostridium hiranonis* (*C. hiranonis*), *Escherichia coli* (*E. coli*), *Faecalibacterium* spp., *Fusobacterium* spp., *Streptococcus* spp., and *Turicibacter* spp. The analysis was performed using a CFX 96 Touch TM Real-Time PCR Detection system (Biorad Laboratories). Ten µL SYBR-based reaction mixtures: 5 µL of SsoFast™ EvaGreen® Supermix (Biorad Laboratories), 2.2 µL of water, 0.4 µL of each primer (final concentration: 400 nM), and 2 µL of DNA (1: 10 or 1: 100 dilution) were used for a protocol of 95°C for 2 min, and 40 cycles at 95°C 5 s and 10 s at the optimized annealing temperature for each primer set. Afterwards, a melt curve analysis was completed (AlShawaqfeh et al. 2017).

Results from the qPCR analysis for *Blautia* spp., *C. hiranonis*, *E. coli*, *Faecalibacterium* spp., *Fusobacterium* spp., *Streptococcus* spp., and *Turicibacter* spp. are expressed as the abundance of DNA for each bacterial group (logarithm of starting quantity or logarithm of relative DNA copy number). Relative DNA copy number for the mentioned bacteria were used to calculate a single numerical value known as the Dysbiosis Index (AlShawaqfeh et al. 2017). A value <0 is indicative of a normal microbiota, numbers between 0 and 2 are considered equivocal, while numbers greater than 2 indicate fecal dysbiosis. The dysbiosis index is a commercially available assay, and the reference intervals have been validated with dogs from various countries, including the UK.

2.2.5 16S rRNA gene sequencing

The V4 variable region of the 16S rRNA gene was sequenced at the MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA). Primers 515F (5'-GTGYCAGCMGCCGCGGTAA) (Parada, Needham, and Fuhrman 2016) to 806RB (5'-GGACTACNVGGGTWTCTAAT) (Apprill et al. 2015) and HotStarTaq Plus Master Mix (Qiagen, USA) were used to amplify samples and perform the Illumina MiSeq protocol following the manufacturer's guidelines. Raw sequences were uploaded into Sequence Read Archive of the NCBI GenBank database under the accession number PRJNA580258.

2.2.6 Analysis of sequences

Quantitative Insights into Microbial Ecology 2 (QIIME 2.0) was used for analysis of the 16S rRNA amplicon sequences (Bolyen et al. 2019). Sequences were demultiplexed and

the OTU table was created using DADA2 (Callahan et al. 2016). Operational taxonomic units (OTUs) were defined as sequences with at least 97% similarity within the Greengenes v 13.8 database (DeSantis et al. 2006). Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance OTUs, containing less than 0.01% of the total reads in the dataset were removed (McDonald et al. 2012). All samples were rarefied to an even depth of 36,775 sequence reads, based on the lowest read depth of samples.

Alpha diversity was evaluated with Chao1, Shannon diversity, and observed OTUs. Beta-diversity metric was estimated by unweighted and weighted phylogeny-based UniFrac distances and visualized using PCoA (Principal Coordinate Analysis) plots.

2.2.7 Statistical analysis

Normality was tested for all continuous variables using the Shapiro-Wilk test. Results were reported as mean (standard deviation [SD]) or median (range), when data were normally- or not normally-distributed, respectively. Differences in dog characteristics (i.e., age) between groups at baseline were compared using either the t-test. The ANOSIM (Analysis of Similarity) test within PRIMER 6 software package (PRIMER-E Ltd., Luton, UK) was used to analyze significant differences in microbial communities between groups.

Alpha diversity indices (Shannon, Chao1, and Observed OTUs), Dysbiosis Index, and quantitative PCR results were compared between groups using Wilcoxon test. A statistical software package (Prism version 8.0; GraphPad Software, San Diego, CA, USA), was used for the described analyses. To minimize false discoveries in univariate statistics, OTUs not present (0%) in at least 50% of the samples of at least one of the compared groups were considered rare and excluded from analysis. Filtered taxa were tested with Wilcoxon test for paired analysis using statistical software (R Studio Software version 1.2.1335 ©, R Studio, Boston, MA, USA; and JMP Pro 14; SAS, Durham, NC, USA). P-values were adjusted using the Benjamini–Hochberg Step-up method with a false discovery rate (FDR) of 0.05. For all statistical analyses, significance was set at $p < 0.05$.

2.3 Results

2.3.1 Animal population characteristics

After a mean of 330.9 (SD 203.4) days on weight loss diet, BCS changed significatively in dogs with obesity when compared before (BCS 8; range 6-9) and after weight loss (median BCS 5; range 4-7; p<0.001). However, BCS was not significantly different at baseline between the group of dogs that lost weight and those that did not complete the weight loss program (p=0.551). At baseline, the mean age in dogs with obesity that lost weight was 69.4 (SD 32.3) months, and 75.0 (SD 23.6) months for those that did not lose weight (p=0.730), (**Table 2.2**, **Data S2.1**). The breeds included in the obese group were: Labrador Retriever (n=7), Golden Retriever (n=2), Cavalier King Charles spaniel (n=2), Border Collie (n=2), Pug (n=2), Lhasa Apso (n=1), American Bulldog (n=1), Dachshund (n=1), Rottweiler (n=1), Newfoundland (n=1), Bichon Frise (n=1), Rough Collie (n=1), and mixed breed (n=3).

Body composition measurements were available for 19 out of the 20 dogs with obesity that lost weight (n=20) and mean body fat mass was 44.8% (SD 5.0%) before weight loss (T0) and 30.4% (SD 6.5%) after weight loss (T2, p<0.001). Mean rate of weight loss of starting body weight was 0.68% (SD 0.29%) per week, while the energy intake during the weight loss period was 60.8 (SD 5.5) kcal per $kg^{0.75}$ of ideal body weight per day (**Table 2.2**, **Data S2.1**).

Dogs were classified as having discontinued prematurely (n=5) when their body weight at the time that they dropped out of the study (T2) were still significantly above of their ideal weight. The time between enrollment (T0) and the first follow-up (T1) was 15 (14-37) days, during which they had lost a median of 2.7% (0.0%-4.8%) of their starting body weight. At the time they dropped out of the program, after 414 (414-781) days on weight loss, they lost 9.9% (-3.6%-21.3%) of body weight. Although one dog did eventually lose 21.3% of its body weight, it was still significantly (~13%) above its ideal weight at the time it discontinued and had also not lost any weight when the follow-up fecal sample was taken. All other dogs in this group lost <11% of their starting weight and were also above their ideal weights at the point that their weight loss was ended. The median rate of weight loss of starting body weight was 0.10% (-0.06%-0.39%) per week and the energy intake was 54.0 (51.8%-60.9%) kcal per $kg^{0.75}$ of ideal body weight per day. Body composition measurements were available in 4 out of 5 of the dogs at baseline, and median body fat was 44.7% (44.0%-47.5%). Body condition score did not change significatively during the period of weight loss (**Data S2.1**). No significant differences

were identified for alpha and beta diversity when sex, age and neutered status were investigated for association with fecal microbiota in all dogs at baseline (Data not shown).

Table 2.2   Demographics for obese dogs enrolled in the study.

	Days on weight loss diet, Mean (SD)	Age in months, Mean (SD)	Sex	Sexual status	BCS Baseline, median (range)	BCS after weight loss, median (range)	BCS during weight loss, median (range)
Obese dogs, completed study (n=20)	330.9 (SD 203.4)	69.4 (SD 32.3)	10F/10M	18N/2I	8 (6-9)	5 (4-7)	N/A
Obese dogs, did not complete study (n=5)	536.4 (SD 154.8)	75 (SD 23.6)	3F/2M	5N	8 (8-9)	N/A	8 (8-9)

Unpaired *T*-test was used to compare age between the groups of obese dogs that completed the study vs. obese dogs that did not complete the study ($p = 0.730$). Mann-Whitney for unpaired analysis and Wilcoxon for paired analysis were used to test significance of differences in BCS between groups: obese before vs. after weight loss ($p < 0.001$); obese dogs that completed the program at baseline vs. obese dogs that did not complete the study ($p = 0.551$).

2.3.2 qPCR and Dysbiosis Index

On analysis by qPCR, there was a decreased abundance of *E. coli* (T0: 4.8 vs. T2: 3.5; p=0.030), and an increased abundance of *Fusobacterium* spp. (T0: 7.4 vs. T2: 8.0; p=0.017) after weight loss (**Figure 2.1**). The values for the abundances of the evaluated bacteria by qPCR and for the Dysbiosis Index showed that the changes observed in the fecal microbiota before and after weight loss were mostly within the established reference intervals for clinically healthy dogs.
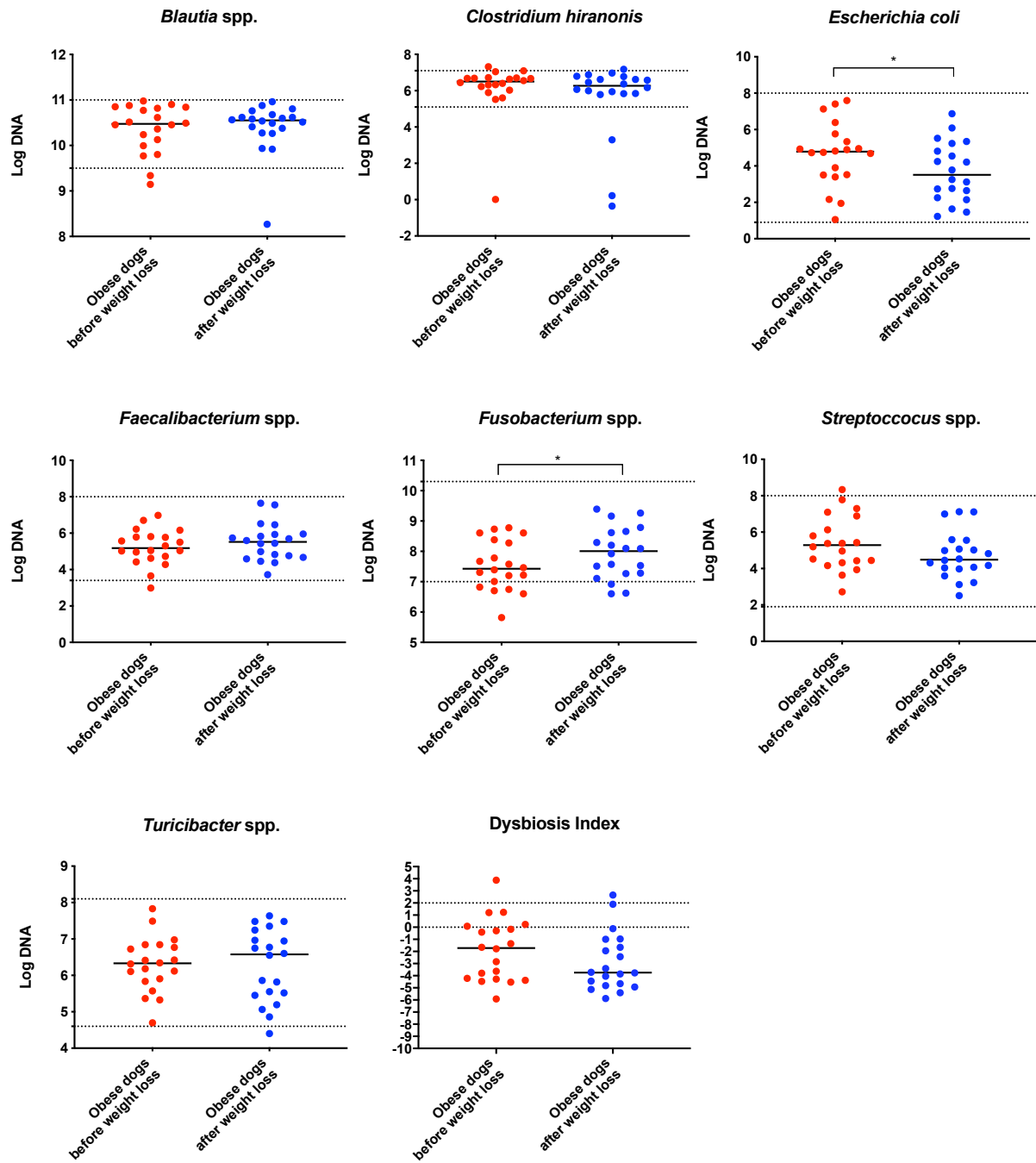
Figure 2.1   Dysbiosis Index and quantitative PCR results for *Blautia* spp., *C. hiranonis*, *E. coli*, *Faecalibacterium* spp., *Fusobacterium* spp., *Streptoccocus* spp., and *Turicibacter* spp.

Bacterial concentrations are expressed in Log DNA (Log of the starting quantity, which is the relative DNA copy number). The dotted lines indicate the established reference intervals for each bacterial group for clinically healthy dogs. The Dysbiosis Index is a mathematical algorithm that summarizes the results in one number. A negative value is indicative of a normal microbiota, numbers between 0 and 2 are considered equivocal, and values greater than 2 indicate dysbiosis. Wilcoxon test was used to compare bacterial abundance and Dysbiosis Index between dogs with obesity before and after weight loss. Significance *p < 0.05, **p < 0.01, ***p < 0.001

2.3.4 Changes in the fecal microbiota with weight loss in dogs with obesity

Weighted UniFrac analysis of similarities revealed significant clustering of the microbial communities in dogs with obesity before and after weight loss ($_{weighted}$ ANOSIM; p=0.016, R=0.073; **Figure 2.2A**). Alpha diversity evenness and richness, as indicated by Shannon (p=0.007), Chao1 (p=0.007), and Observed OTUs (p=0.007) indices were significantly increased after weight loss (**Figure 2.2B**).

A

B **C** **D**
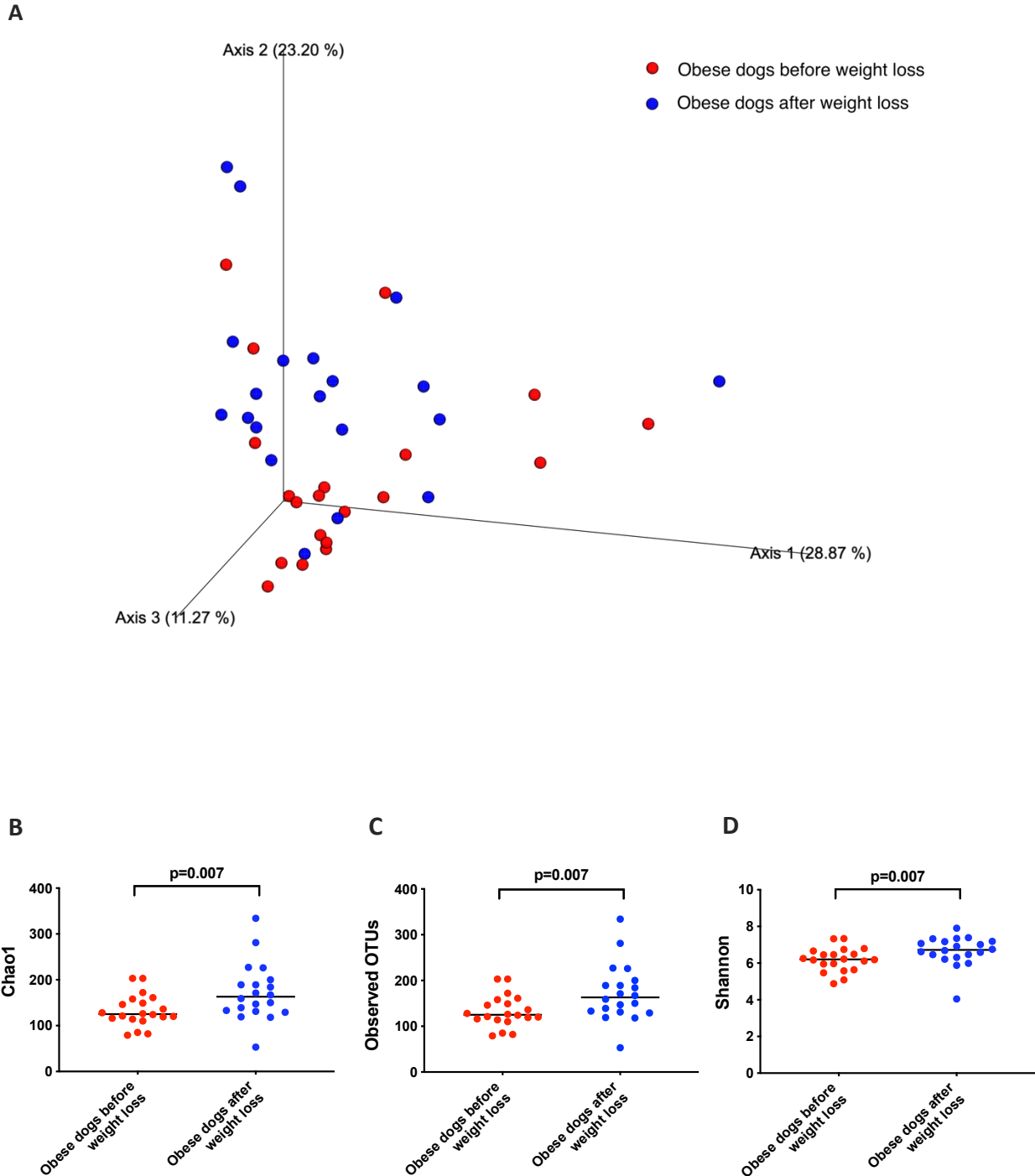
Figure 2.2 Principal coordinate analysis of beta and alpha diversity of dogs with obesity before and after weight loss.

(**A**) PCoA plot based on weighted UniFrac distances of 16S rRNA genes. Visible clustering was confirmed by ANOSIM, showing that fecal microbiota of obese dogs changed significatively after weight loss (p=0.016, R=0.073). (**B**) Observed OTUs, an indicator of species richness, and (**C**) Chao1, indicator of rare bacterial species abundance showed an increase after weight loss (p=0.007). (**D**) Shannon index, indicator of bacterial evenness, also increased significatively when dogs lost weight (p=0.007).

When the bacterial abundance was investigated at the taxa level, significant differences were found for the phyla Bacteroidetes, Firmicutes and Fusobacteria (**Figure 2.3A**). The median Firmicutes/Bacteroidetes ratio decreased from 0.123 to 0.014 (p=0.004) in dogs with obesity after weight loss (**Figure 2.3B**).
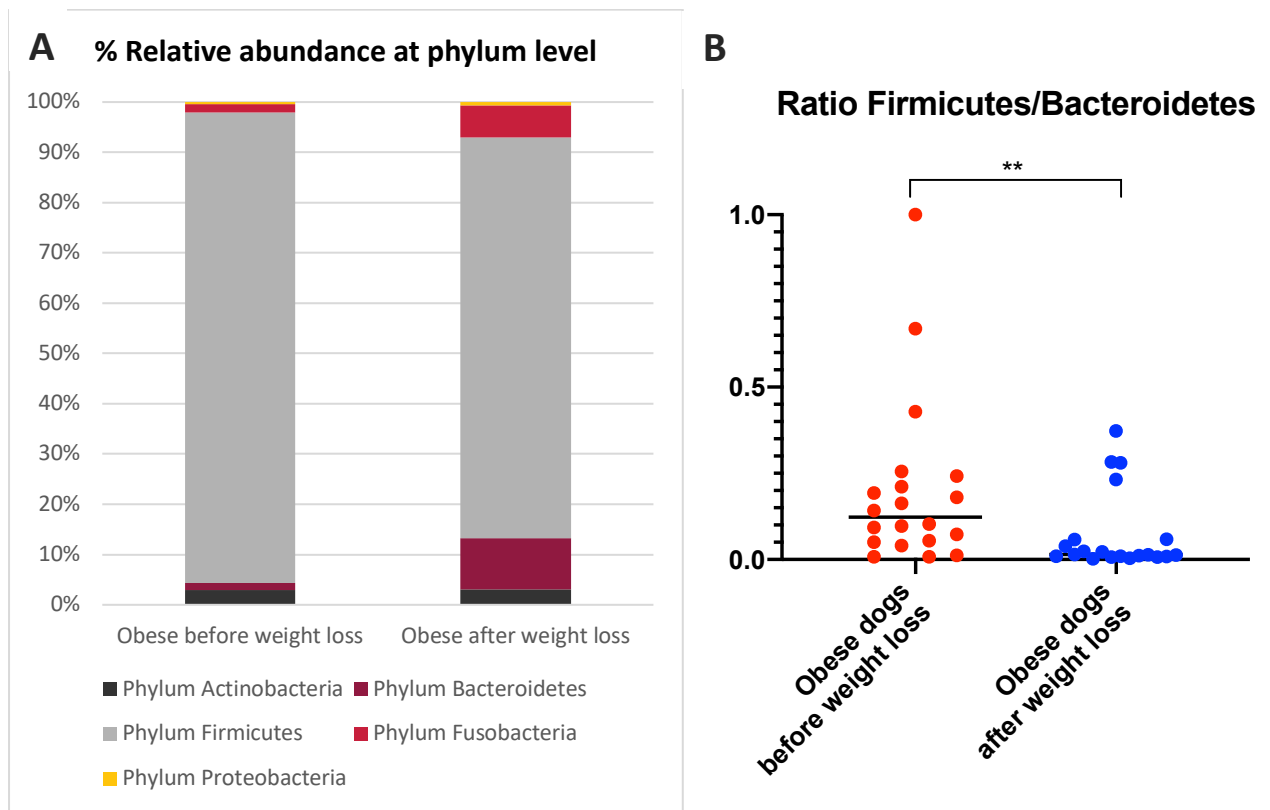
Figure 2.3 **Abundance of fecal bacteria at phylum level found in obese dogs before and after weight loss.**

(**A**) Relative abundance of the phyla detected in fecal samples of obese dogs before weight loss and after weight loss. Increases of the abundance of the phyla Bacteroidetes (q=0.002) and Fusobacteria (q=0.040), and decreases of the abundance of the phylum Firmicutes (q=0.001) were observed after weight loss. (B) Firmicutes/Bacteroidetes ratio values for each dog. After weight loss, Firmicutes/Bacteroidetes ratio decreased significantly as a consequence of a greater abundance of Bacteroidetes and lesser of Firmicutes (p=0.004).

The relative abundance of Bacteroidetes (T0: 1.4% vs. T2: 10.1%; q=0.002; **Figure 2.4A**) and Fusobacteria (T0: 1.6% vs. T2: 6.2%; q=0.040; **Figure 2.5A**) increased significantly after weight loss, whilst the abundance of the phylum Firmicutes, instead decreased (T0: 92.3% vs. T2: 78.2%; q=0.001; **Figure 2.6A**).

Figure 2.4 **Relative abundance of bacterial populations belonging to the phylum Bacteroidetes detected in fecal samples of obese dogs that changed after weight loss.**

Individual values for each dog before (red) and after weight loss (blue) of the relative abundance for each bacterial population as indicated: **A**) phylum Bacteroidetes, **B**) family Bacteroidaceae, **C**) genus *Bacteroides*, **D**) *Bacteroides plebeius*, **E**) family Paraprevotellaceae, **F**) and genus *Paraprevotella*. Significance *p < 0.05, **p < 0.01, ***p < 0.001. Red significance lines indicate p-values that did not pass multiple comparison correction.

The increase in Bacteroidetes (**Figure 2.4C**) was driven by the genera Bacteroides (T0: 0.7% vs. T2: 7.9%; q=0.017; **Figure 2.4F**) and *Paraprevotella* (T0: 0% vs. T2: 0.1%; q=0.033).

From the phylum Fusobacteria (**Figure 2.5C**), the genus *Fusobacterium* (T0: 1.6% vs. T2: 6.2%; q=0.099) increased after weight loss. However, the corrected q-value did not reach significance.
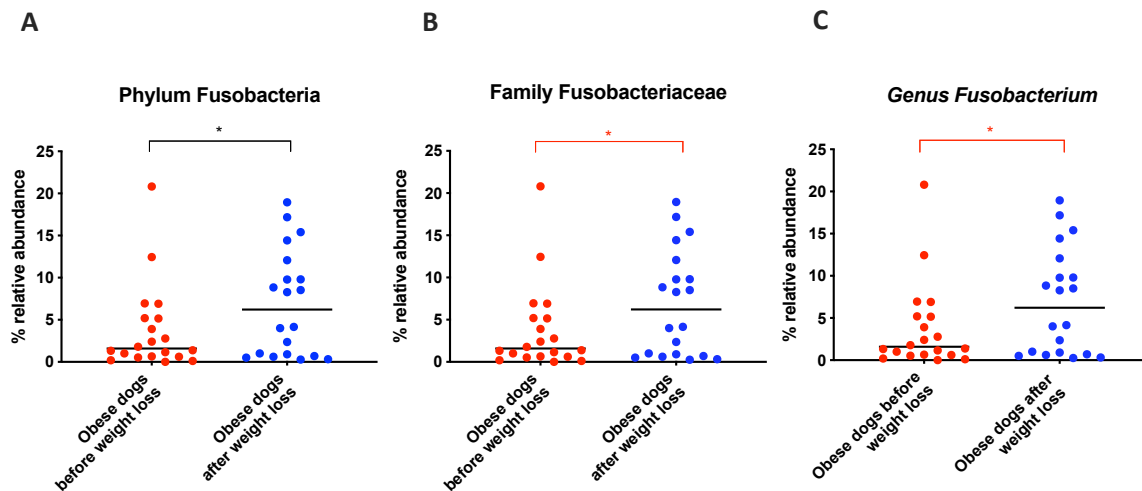
Figure 2.5 **Relative abundance of bacterial populations belonging to the phylum Fusobacteria detected in fecal samples of obese dogs that changed after weight loss.**

Individual values for each dog before (red) and after weight loss (blue) of the relative abundance for each bacterial population as indicated: **A**) phylum Fusobacteria, **B**) family Fusobacteriaceae, **C**) and genus *Fusobacterium*. Significance *p < 0.05, **p < 0.01, ***p < 0.001. Red significance lines indicate p-values that did not pass multiple comparison correction.

Belonging to the phylum Firmicutes, the family Clostridiaceae decreased in abundance after weight loss (T0: 37.3% vs. T2: 24.6%; q=0.068), but this difference did not reach significance after Benjamini correction (**Figure 2.6B**). The same was noticed for the genus *Clostridium* (T0: 0.7% vs. T2: 0.6%; q=0.119; **Figure 2.6C**). The genus *Megamonas* (T0: 0.2% vs. T2: 0.0%; q=0.027; **Figure 2.6E**) and the genus *Catenibacterium* (T0: 2.3% vs. T2: 0.5%; q=0.017) decreased significantly after weight loss (**Figure 2.6F**), and the genus *Coprobacillus* increased in abundance (T0: 0% vs. T2: 0.5%, q=0.033; **Figure 2.6G**).
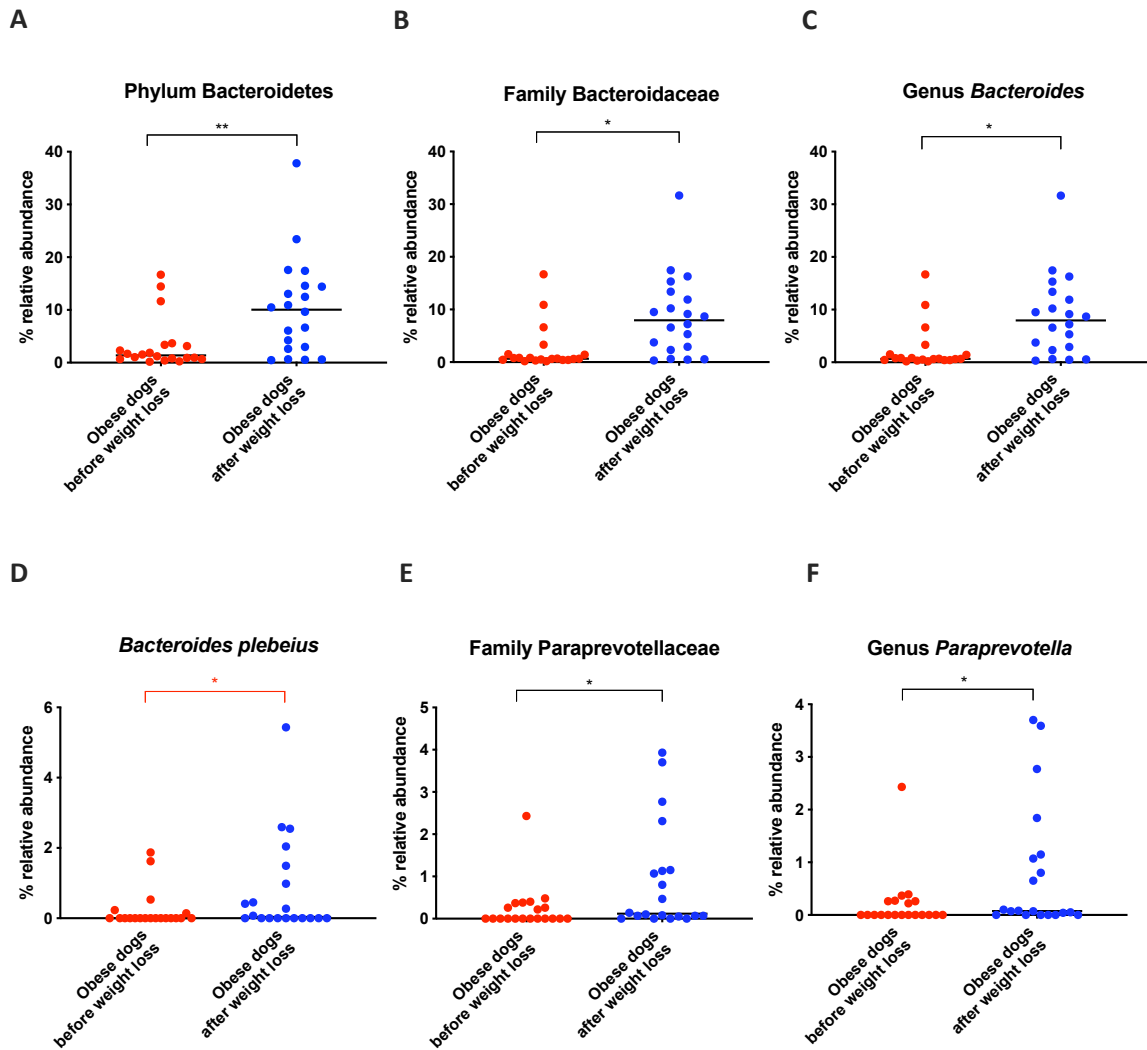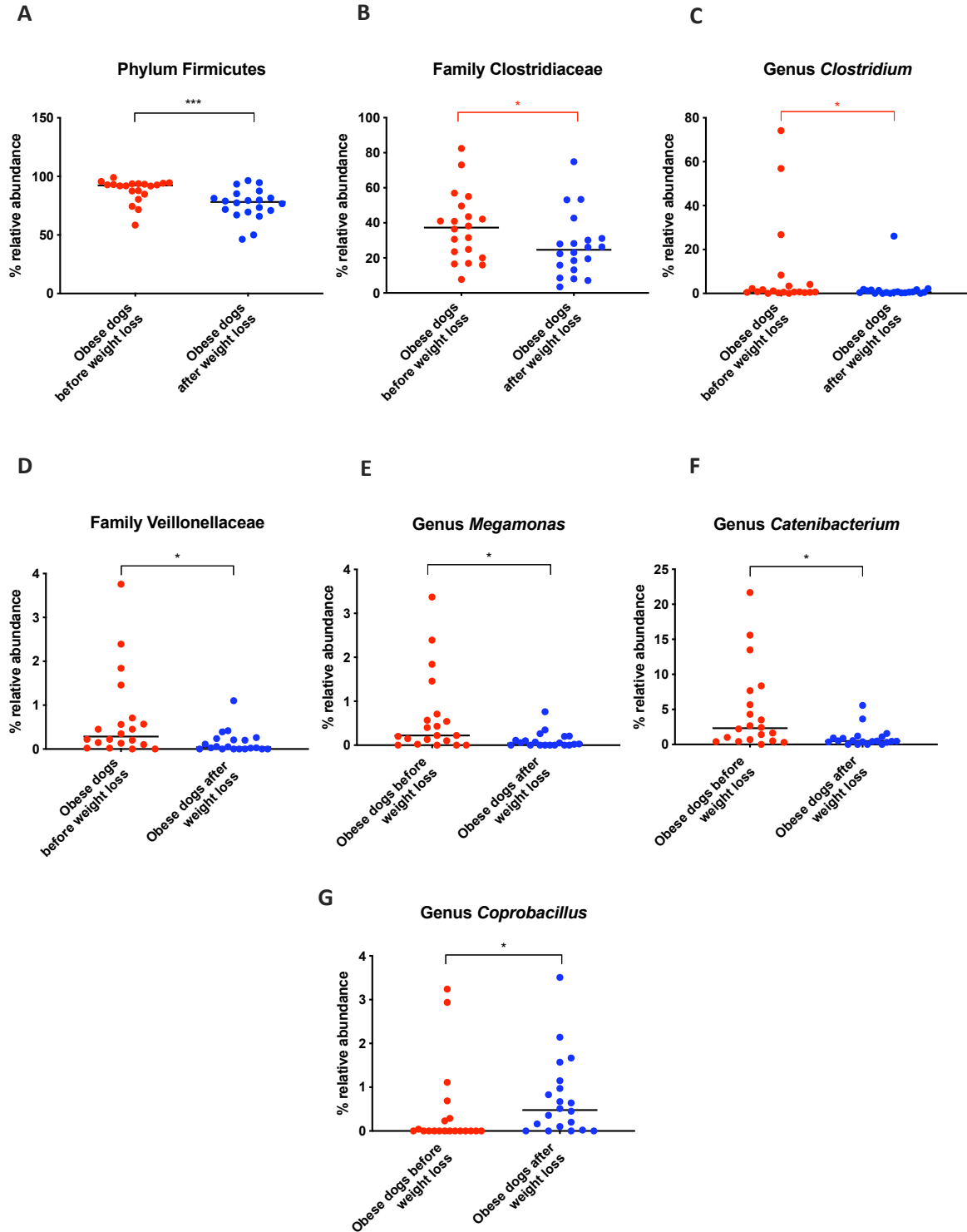
Figure 2.6   Relative abundance of bacterial populations belonging to the phylum Firmicutes detected in fecal samples of obese dogs that changed after weight loss.

Individual values for each dog before (red) and after weight loss (blue) of the relative abundance for each bacterial population as indicated: **A**) phylum Firmicutes, **B**) family Clostridiaceae, **C**) genus *Clostridium*, **D**) family Veillonellaceae, **E**) genus *Megamonas*, **F**) genus *Catenibacterium*, **G**) and genus *Coprobacillus*.

Significance *p < 0.05, **p < 0.01, ***p < 0.001. Red significance lines indicate p-values that did not pass multiple comparison correction.

2.3.6 A short-term change in diet does not alter fecal microbiota.

The fecal microbiota beta diversity of the dogs with obesity that stopped the weight loss program before reaching the endpoint (n=5) was analyzed before and during the weight loss program. No significant differences were evident between the two time points ($_{Weighted}$ ANOSIM; p=0.778 R=-0.080; **Figure 2.7A**). Alpha diversity evenness and richness, as indicated by Shannon (p=0.313), Chao1 (p=0.438), and Observed OTUs (p=0.438) indices did not show significant differences after a median period of 15 days on weight loss diet.

A

B   **C**   **D**
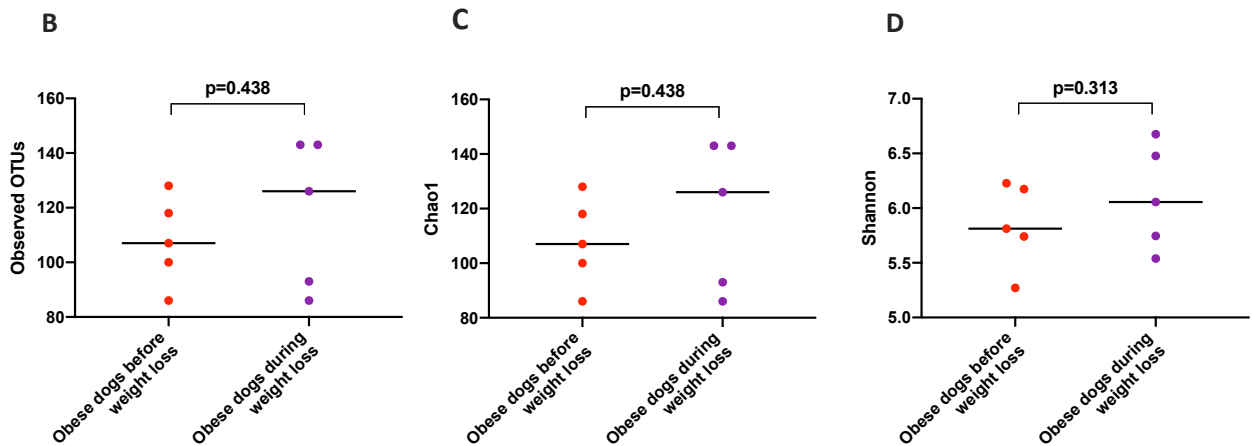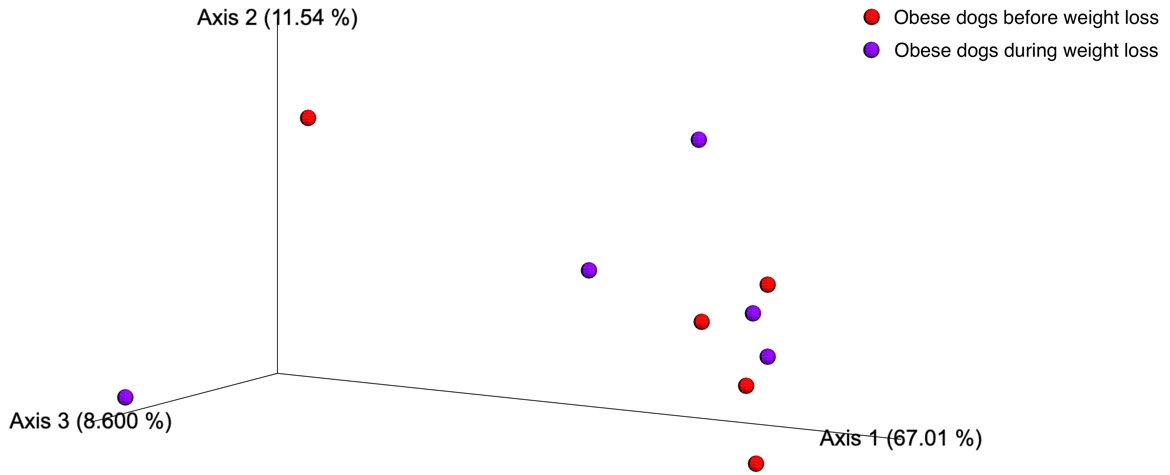
Figure 2.7   Principal coordinate analysis of beta diversity and alpha diversity indices of obese dogs that did not complete the weight loss program.

A) PCoA plot based on weighted UniFrac distances of 16S rRNA gene shows no clustering of microbial communities from feces of obese dogs before weight loss (red) and during weight loss (purple). Fecal microbiota profile of obese dogs did not change after a median period of 15 days (weighted ANOSIM; p=0.778, R=-0.080). **B**) Observed OTUs, an indicator of species richness, **C**) Chao1, indicator of rare bacterial species abundance (p=0.438), and D) Shannon index, indicator of bacterial evenness (p=0.313), were not different in obese dogs after a median period of 15 days on weight loss program.

2.4   Discussion

In this study, we report significant differences in the fecal microbiota in a population of 20 obese client-owned dogs after weight loss. Dogs with obesity were enrolled in a weight loss program with the endpoint set as the achievement of target body weight (German et al. 2007; German, Holden, et al. 2010).

We observed that the fecal microbiota richness and evenness of dogs increased significantly after weight loss, which is consistent with previous studies for dogs and humans with obesity, where a lesser richness and evenness of the fecal microbiota was reported in obese individuals (Park et al. 2015; Peters et al. 2018). At the phylum level, our results showed a decrease of the abundance of Firmicutes (92.3% vs. 78.2%) and an increase of the abundance of Bacteroidetes (1.4% vs. 10.1%) after weight loss, as a result, we observed a decrease of the F:B ratio in dogs with obesity after weight loss (**Figure 2.3B**). This is consistent with the literature (Ley et al. 2006), since the F:B ratio of obese individuals has been reported to be greater in studies that analyzed the fecal microbiota of obese humans, dogs, and animal models of obesity, that also decreased after weight loss (Ley et al. 2005; Ley et al. 2006; Turnbaugh et al. 2006; Turnbaugh et al. 2009; Salas-Mani et al. 2018).

An important difference of the core microbiota between humans and dogs is the abundance of the phylum Fusobacteria. In human studies, Fusobacteria is not as abundant in the fecal microbiota compared to dogs (Swanson et al. 2011; Coelho et al. 2018). In fact, in humans, a high abundance of Fusobacteria is associated with colon cancer (Kelly, Yang, and Pei 2018). In contrast, in dogs, Fusobacteria appears to play an important role in the maintenance of health, and has been reported to be decreased in dogs with gastrointestinal diseases (AlShawaqfeh et al. 2017; Minamoto et al. 2014). Previous studies have demonstrated that a greater abundance of Fusobacteria is associated with leanness and it increases after weight loss in dogs (Park et al. 2015; Handl et al. 2013). Our results by 16S rRNA gene sequencing confirm that the abundance of Fusobacteria increases after weight loss (**Figure 2.5**).

In agreement with this, results from qPCR (**Figure 2.1**) showed also a significant increase in *Fusobacterium* spp. and a significant decrease in *E. coli,* with a numerical decrease in the Dysbiosis index, although this was not significant. Most dogs remained within the established reference interval for clinically healthy dogs.

A greater abundance of the class Clostridia has been associated with an obese phenotype and it is reported to decrease after weight loss in humans (Nadal et al. 2009). In a similar study in dogs, the genus *Clostridium* decreased after a weight loss program of 17 weeks (Salas-Mani et al. 2018). Our results also showed a slight decrease of *Clostridium* after weight loss. However, this difference was not statistically significant (**Figure 2.6**).

One of the factors to consider may be the variability between diets. In order to evaluate the effect of the weight loss diet in the gut microbiota, fecal microbiota of dogs with obesity before and after an initial period on the weight loss diet was analyzed in a small cohort of dogs with obesity that did not complete the weight loss program. The fecal microbiota analysis before and after this period did not show significant differences (**Figure 2.7**). Despite the short-term on weight loss diet and the small sample size, similar results were shown in the study carried out by Kieler and colleagues (Kieler et al. 2017), that evaluated the fecal microbiota of overweight pet dogs after a weight loss program. In addition, the same weight loss diet used in our study was used with or without exercise in the mentioned study, and researchers observed only minor changes in the microbiome composition. In that particular study, the dogs were followed for 12 weeks, and it is not clear how many dogs reached an ideal body weight. The main finding was that a decrease in abundance of the genus *Megamonas* correlated with a greater weight loss rate during 12-week weight loss program (Kieler et al. 2017). We also observed a decrease in the genus *Megamonas* after weight loss, which could be attributed to an effect of the weight loss diet. However, the role of *Megamonas* in obesity is unclear and merits further investigation.

There is significant interest in body weight management by modifying macronutrient distribution in diets. Fiber promotes digestive health and weight control and has been demonstrated to improve satiety in dogs (Weber et al. 2007), however, the effect of diet on fecal microbiota in dogs is controversial. Whilst diet has been shown to modulate the gut microbiota in humans (David et al. 2014), in dogs this correlation is not always clear. Gut microbiota seems to be modulated by diet only when its formulation changes significantly in macronutrient content from the previous diet (Schmidt et al. 2018; Kim et al. 2017) or the intestinal microbiota is compromised due to a gastrointestinal disease (Bresciani et al. 2018). Consistently, in healthy dogs, small variations in diets seem not to have substantial effects on the composition of the fecal microbiota (Sandri et al. 2017; Schauf et al. 2018; Bresciani et al. 2018). The diet used in our study is considered a high-protein, high-fiber diet (**Table 2.1**).

Although we cannot exclude that changes observed in the fecal microbiota of dogs with obesity after weight loss are associated to the new diet, minor changes have been reported when an increase of fiber is included in the diet of healthy dogs (Middelbos et al. 2010).

It has been hypothesized that increased satiety, an important factor to aid weight loss, could be mediated by SCFAs (Arora, Sharma, and Frost 2011). Our results confirm significant differences in SCFAs-producing bacteria, as Clostridiaceae, Veillonellaceae and *Blautia* between obese before and after weight loss (**Data S2.2**). However, study of SCFAs in obese dogs and after weight loss it is necessary to confirm its role in satiety and hence, in weight modulation.

2.5   Conclusion

In summary, this study shows that the fecal microbiota of dogs with obesity significantly changes after weight loss. In addition, our results by qPCR show that after weight loss with a high-fiber and high-protein diet, the abundance of the bacterial population analyzed are mostly within the reference intervals for clinically healthy dogs.

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OBESE DOGS (completed the study)

#SAMPLEID	Days on diet (T2)	Age in months (T0)	Sex	Breed	Neutered status	BCS (T0)	BCS (T2)	Weight loss diet (Satiety Royal Canin)	Diet before weight loss diet (Satiety Royal Canin)	Body weight loss rate per week (T2)	Percentage weight loss of starting body weight (T2)	Percentage starting body fat (T0)	Percentage ending body fat (T2)	Change in lean tissue mass	Energy intake during weight loss of ideal body weight (kcal per kg0.75)
OBE01	250	33	F	Lhasa Apso	N	9	5	dry diet pre-2010	Royal Canin Hepatic dry	0,86	30,6%	36,9%	24,8%	-17,6	73,6
OBE02	748	26	M	Golden Retriever	N	8	5	dry diet pre-2010	Royal Canin Obesity dry	0,23	24,9%	50,2%	40,7%	-11,6	61,4
OBE03	139	16	M	Labrador	N	6	5	dry diet post-2010	Royal Canin Urinary mod cal	0,80	12,0%	38,7%	28,5%	2,7	64,5
OBE04	349	43	M	Golden Retriever	N	7	5	dry diet post-2010	Chappie dry	0,38	19,0%	47,7%	37,0%	-3,6	63,6
OBE05	504	59	F	Labrador	N	9	5	dry diet post-2010	JWB Natural, wet and dry	0,45	32,3%	51,9%	38,5%	-14,4	57,1
OBE06	204	67	M	American Bulldog	N	7	5	dry diet post-2010	Wainrights dry	0,75	21,8%	39,9%	18,4%	0,3	55,8
OBE07	434	108	F	Mix breed	N	9	5	dry diet post-2010	Royal Canin Hypoallergenic dry	0,57	35,6%	50,5%	32,6%	-12,4	56,7
OBE08	280	57	M	Labrador	N	8	6	dry diet post-2010	Wainrights dry	0,54	21,4%	47,3%	31,0%	3,8	55,4
OBE09	223	91	F	Labrador	N	7	5	dry diet post-2010	Bakers Weight Control dry and Royal Canin Obesity dry	0,71	22,5%	46,4%	31,6%	-0,3	57,1
OBE10	188	126	M	Labrador	I	7	5	dry diet post-2010	Chappie dry	0,81	14,6%	39,2%	29,4%	-0,8	55,4
OBE11	126	41	M	Cavalier King Charles Spaniel	N	9	?	dry diet post-2010	Wagg dry, Hero Light wet	1,22	35,3%	44,6%	23,0%	-5,6	60,2
OBE12	768	73	F	Mix breed	N	9	5	dry diet post-2010	Royal Canin Satiety/ Obesity, Burns dry	0,43	47,1%	48,9%	23,4%	-19,8	60,2
OBE13	263	98	F	Labrador	N	9	5	dry diet post-2010	Pedigree Light dry	0,89	33,3%	48,5%	33,1%	-7,4	68,3
OBE14	349	57	M	Dachshund	N	8	5	dry diet post-2010	Wainrights Light	0,61	30,4%	48,0%	28,6%	-3,9	55,0
OBE15	702	53	M	Mix breed	N	9	6	dry diet post-2010	Bakers Weight Control dry	0,27	27,4%	50,1%	39,4%	-12,2	58,9
OBE16	366	79	F	Rottweiler	N	8	5	dry diet post-2010/ wet diet post-2010	Pedigree dry	0,59	28,4%	45,4%	31,4%	-6,9	53,0
OBE17	119	41	M	Newfoundland	I	6	4	dry diet post-2010	Gilpa and JWB dry	0,60	10,1%	36,0%	21,9%	13,2	71,8
OBE18	116	105	F	Cavalier King Charles Spaniel	N	9	6	dry diet post-2010	JW Light dry	1,27	21,1%	NO DEXA	NO DEXA	NO DEXA	62,2
OBE19	112	80	F	Bichon Frise	N	7	4	dry diet post-2010	Butchers Lean and Tasty tinned wet	1,18	23,6%	37,5%	24,3%	-6,2	63,3
OBE20	378	135	F	Border Collie	N	9	7	dry diet post-2010	Morrisons dry	0,49	22,0%	44,3%	40,2%	-20,7	61,8
MEAN (SD) or MEDIAN (RANGE)	330.9 (SD 203.4)	69.4 (SD 32.3)	10F/10 M	N/A	18N/2I	8 (6-9)	5 (4-7)	N/A	N/A	0.68 (SD 0.29)	25.7% (SD 8.6%)	44.8% (SD 5.0%)	30.4% (SD 6.5%)	-6.5 (SD 8.5)	60.8 (SD 5.5)

OBESE DOGS (did not complete the study)

#SAMPLEID	Days on diet (T1)	Days on diet (T2)	Age in months (T0)	Sex	Breed	Neutered status	BCS (T0)	BCS (T1)	Subgroup	Weight loss diet (Satiety Royal Canin)	Diet before weight loss diet (Satiety Royal Canin)	Body weight loss rate per week (T2)	Percentage weight loss of starting body weight (T1)	Percentage weight loss of starting body weight (T2)	Percentage starting body fat (T0)	Percentage ending body fat (T2)	Change in lean tissue mass	Energy intake during weight loss of ideal body weight (kcal per kg0.75)
OBE21	15	659	102	F	Rough Collie	N	8	8	OBESE	dry diet post-2010	Royal Canin Obesity dry	0,10	1,6%	9,9%	47,5%	NO DEXA	NO DEXA	54,0
OBE22	37	414	85	F	Border Collie	N	8	8	OBESE	dry diet post-2010	Harringtons dry and Mero wet	0,39	0,0%	21.3%	44,0%	NO DEXA	NO DEXA	60,9
OBE23	14	414	68	M	Pug	N	9	9	OBESE	dry diet post-2010	Royal Canin Pug dry	0,11	4,8%	6,7%	NO DEXA	NO DEXA	NO DEXA	58,0
OBE24	14	414	33	F	Pug	N	9	9	OBESE	dry diet post-2010	Royal Canin Pug dry	-0,06	2,7%	-3,6%	44,4%	NO DEXA	NO DEXA	54,0
OBE25	25	781	87	M	Labrador	N	8	8	OBESE	dry diet post-2010	Burns Weight Control dry	0,10	3,3%	10,8%	44,9%	NO DEXA	NO DEXA	51,8
MEAN (SD) or MEDIAN (RANGE)	15 (14-37)	414 (414-781)	85 (33-102)	3F/2M	N/A	5N	8 (8-9)	8 (8-9)	5OB	N/A	N/A	0.10 (-0.06-0.39)	2.7% (0.0%-4.8%)	9.9% (-3.6%-21.3%)	44.7% (44.0%-47.5%)	N/A	N/A	54.0% (51.8%-60.9%)
*MEAN (SD) or MEDIAN (RANGE)	N/A	N/A	70.5 (SD 30.8)	13F/12M	N/A	23N/2I	8 (6-9)	N/A	18OB/7OW	N/A	N/A	N/A	N/A	N/A	44.9% (SD 4.6%)	N/A	N/A	59.8 (SD 5.5)

* Mean (SD) or median (range) calculated for all obese dogs at baseline, including the ones that completed the study and lost weight and the ones that did not complete the study

T0; time point zero, before weight loss program

T1; time point one, interim follow-up only available for the group of obese dogs that stopped the weight loss program

T2; time point two, when the group of obese dogs that completed the study reached target body weight and the group of dogs that did not complete the study dropped out the weight loss program

BCS; Body Condition Score (0-9 scale)

DEXA; Dual-energy X-ray absorptiometry

F female

M male

N neutered

I intact

Supplementary data 2.2   Median percentage of relative abundance of the different bacterial populations detected in fecal samples of obese dogs before and after weight loss

Obese dogs before weight loss vs. obese dogs after weight loss

LEVEL: 2 Phylum		Obese dogs before weight loss			Obese dogs after weight loss			Obese dogs before weight loss vs. obese dogs after weight loss	
		Median	Range		Median	Range		P value	Q value
Actinobacteria		2,9	0-9.2		3	0.2-18.5		0,729	0,729
Bacteroidetes		1,4	0.2-16.7		10,1	0.5-37.8		<0,001	0,002
Firmicutes		92,3	58.4-99.1		78,2	46.4-96.5		<0,001	0,001
Fusobacteria		1,6	0-20.8		6,2	0.3-18.9		0,024	0,040
Proteobacteria		0,5	0-6.3		0,7	0.1-17.3		0,294	0,368

LEVEL 3: Class		Obese dogs before weight loss			Obese dogs after weight loss			Obese dogs before weight loss vs. obese dogs after weight loss	
		Median	Range		Median	Range		P value	Q value
Actinobacteria		0,1	0-1.6		0,1	0-1.5		0,375	0,469
Coriobacteriia		2,6	0-9.2		2,4	0.2-18.5		0,522	0,580
Bacteroidia		1,4	0.2-16.7		10,1	0.4-37.8		0,001	0,010
Bacilli		1,4	0-31		0,9	0.2-10.7		0,105	0,298
Clostridia		68,9	45.6-97.1		64,1	41-87.7		0,133	0,298
Erysipelotrichi		9,4	1.1-30.8		7,4	1.4-17.7		0,165	0,298
Fusobacteriia		1,6	0-20.8		6,2	0.3-18.9		0,024	0,120
Betaproteobacteria		0,1	0-1.9		0,1	0-3.8		0,179	0,298
Epsilonproteobacteria		0	0-0.2		0	0-0.5		0,223	0,318
Gammaproteobacteria		0,2	0-6.3		0,2	0-17.2		0,644	0,644

LEVEL 4: Order		Obese dogs before weight loss			Obese dogs after weight loss			Obese dogs before weight loss vs. obese dogs after weight loss	
		Median	Range		Median	Range		P value	Q value
Actinomycetales		0	0-1		0,1	0-1.1		0,731	0,731
Coriobacteriales		2,6	0-9.2		2,4	0.2-18.5		0,522	0,574
Bacteroidales		1,4	0.2-16.7		10,1	0.4-37.8		0,001	0,011
Lactobacillales		0,6	0-31		0,3	0-5.9		0,165	0,327
Turicibacterales		0,4	0-20.2		0,3	0-9.6		0,404	0,494
Clostridiales		68,9	45.6-97.1		64,1	41-87.7		0,133	0,327
Erysipelotrichales		9,4	1.1-30.8		7,4	1.4-17.7		0,165	0,327
Fusobacteriales		1,6	0-20.8		6,2	0.3-18.9		0,024	0,132
Burkholderiales		0,1	0-1.9		0,1	0-3.8		0,179	0,327
Campylobacterales		0	0-0.2		0	0-0.5		0,223	0,350
Enterobacteriales		0,2	0-6.3		0,1	0-17.2		0,298	0,500

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LEVEL 5: Family		Obese dogs before weight loss			Obese dogs after weight loss			Obese dogs before weight loss vs. obese dogs after weight loss	
		Median	Range		Median	Range		P value	Q value
Coriobacteriaceae		2,6	0-9.2		2,4	0.2-18.5		0,522	0,554
Bacteroidaceae		0,7	0.2-16.7		7,9	0.3-31.6		0,001	0,010
Prevotellaceae		0,1	0-5.4		0,1	0-2.7		0,954	0,954
Paraprevotellaceae		0	0-2.4		0,1	0-3.9		0,006	0,036
Streptococcaceae		0,3	0-30.9		0,3	0-5.9		0,468	0,531
Turicibacteraceae		0,4	0-20.2		0,3	0-9.6		0,404	0,490
Unknown family of order Clostridiales		0,3	0-4.4		0	0-1.5		0,117	0,251
Unknown family of order Clostridiales		0,1	0-1.4		0,5	0-3		0,012	0,051
Clostridiaceae		37,3	7.7-82.5		24,6	3.5-74.9		0,024	0,068
Lachnospiraceae		27,6	6.6-45		31,9	4.8-66.6		0,330	0,468
Peptostreptococcaceae		0,8	0-9.7		0,4	0-13.4		0,121	0,251
Ruminococcaceae		0,6	0.1-5.5		1,2	0-17.1		0,133	0,251
Veillonellaceae		0,3	0-3.8		0	0-1.1		0,001	0,010
Erysipelotrichaceae		9,4	1.1-30.8		7,4	1.4-17.7		0,165	0,281
Fusobacteriaceae		1,6	0-20.8		6,2	0.3-18.9		0,024	0,068
Alcaligenaceae		0,1	0-1.9		0,1	0-3.8		0,369	0,483
Enterobacteriaceae		0,2	0-6.3		0,1	0-17.2		0,298	0,460

LEVEL 6: Genus		Obese dogs before weight loss			Obese dogs after weight loss			Obese dogs before weight loss vs. obese dogs after weight loss	
		Median	Range		Median	Range		P value	Q value
Collinsella		2,4	0-9.1		2	0.2-18.5		0,870	0,901
Slackia		0,1	0-0.6		0,2	0-1.6		0,073	0,226
Bacteroides		0,7	0.2-16.7		7,9	0.3-31.6		0,001	0,017
Prevotella		0,1	0-5.4		0,1	0-2.7		0,954	0,954
Paraprevotella		0	0-2.4		0,1	0-3.7		0,005	0,033
Streptococcus		0,3	0-30.9		0,2	0-5.9		0,229	0,369
Turicibacter		0,4	0-20.2		0,3	0-9.6		0,404	0,509
Unknown genus of order Clostridiales		0,3	0-4.4		0	0-1.5		0,117	0,226
Unknown genus of order Clostridiales		0,1	0-1.4		0,5	0-3		0,012	0,058
Unknown genus of family Clostridiaceae		19,6	3.5-48.6		19,3	2.9-46.4		0,133	0,226
Unknown genus of family Clostridiaceae		4,6	0.9-8.6		3	0-65		0,083	0,226
Clostridium		0,7	0-74.2		0,6	0-26.1		0,033	0,119
Unknown genus of family Lachnospiraceae		9,7	2.2-19.5		11,1	1.4-20		0,133	0,226
Unknown genus of family Lachnospiraceae		1	0-4.2		1,4	0-7.1		0,475	0,551
Blautia		12,5	2.8-37.2		13,7	3.2-36		0,756	0,812
Dorea		0,7	0-2.3		1,1	0-2.7		0,123	0,226
Ruminococcus		2,6	0-7.2		2,9	0.1-6.3		0,349	0,482
Unknown genus of family Peptostreptococcaceae		0,8	0-9.7		0,4	0-13.4		0,121	0,226
Unknown genus of family Ruminococcaceae		0,1	0-2.1		0,4	0-4.9		0,119	0,226
Faecalibacterium		0,2	0-3.4		0,5	0-11.1		0,332	0,482
Megamonas		0,2	0-3.4		0	0-0.8		0,003	0,027
Unknown genus of family Erysipelotrichaceae		1,6	0-12.4		3,6	0.1-12.8		0,123	0,226
Allobaculum		1,4	0.1-11.3		1,9	0-5.4		0,430	0,520
Catenibacterium		2,3	0-21.7		0,5	0-5.6		0,001	0,017
Coprobacillus		0	0-3.2		0,5	0-3.5		0,006	0,033
Eubacterium		0,7	0-16.9		1	0.1-3.1		0,622	0,693
Fusobacterium		1,6	0-20.8		6,2	0.3-18.9		0,024	0,099
Sutterella		0,1	0-1.9		0,1	0-3.8		0,370	0,487
Unknown genus of family Enterobacteriaceae		0,2	0-6.3		0,1	0-17.2		0,298	0,455

LEVEL 7: Species		Obese dogs before weight loss			Obese dogs after weight loss			Obese dogs before weight loss vs. obese dogs after weight loss	
		Median	Range		Median	Range		P value	Q value
Collinsella stercoris		2,4	0-8.9		1,8	0.2-16.9		0,927	0,954
Unknown species of genus Slackia		0,1	0-0.6		0,2	0-1.6		0,073	0,197
Unknown species of genus Bacteroides		0,3	0-7.8		2,1	0-12		0,006	0,033
Unknown species of genus Bacteroides		0,3	0.1-8.7		4	0-19.7		0,001	0,021
Bacteroides plebeius		0	0-1.9		0	0-5.4		0,043	0,149
Prevotella copri		0,1	0-5.4		0,1	0-2.7		0,954	0,954
Unknown species of genus Paraprevotella		0	0-2.4		0,1	0-3.7		0,005	0,033
Unknown species of genus Streptococcus		0,3	0-30.9		0,2	0-5.9		0,229	0,348
Unknown species of genus Turicibacter		0,4	0-20.2		0,3	0-9.6		0,404	0,494
Unknoww species of order Clostridiales		0,3	0-4.4		0	0-1.5		0,117	0,211
Unknoww species of order Clostridiales		0,1	0-1.4		0,5	0-3		0,012	0,053
Unknown species of family Clostridiaceae		19,6	3.5-48.6		19,3	3-46.4		0,133	0,211
Unknown species of family Clostridiaceae		4,6	0.9-8.6		3	0-65		0,083	0,207
Unknown species of genus Clostridium		0,6	0-61.8		0,4	0-20.5		0,049	0,157
Unknonw species of family Lachnospiraceae		9,7	2.2-19.5		11,1	1.4-20		0,133	0,211
Unknonw species of family Lachnospiraceae		1	0-4.2		1,4	0-7.1		0,475	0,536
Unknown species of genus Blautia		4,9	0.6-34.5		6,5	0.8-25.7		0,729	0,797
Blautia producta		4,9	2.2-10.8		6,3	2.4-14.9		0,409	0,494
Unknown species of genus Dorea		0,7	0-2.3		1,1	0-2.7		0,123	0,211
Unknonw species of genus Ruminococcus		0	0-1.5		0,4	0-3.5		0,068	0,197
Unknonw species of genus Ruminococcus		0,3	0-1		0,4	0-1.7		0,13	0,211
Ruminococcus gnavus		1,6	0-7.2		1,6	0-4.4		0,841	0,892
Unknown species of family Peptostreptococcaceae		0,8	0-9.7		0,4	0-13.4		0,121	0,211
Unknown species of family Ruminococcaceae		0,1	0-2.1		0,4	0-4.9		0,119	0,211
Faecalibacterium prausnitzii		0,2	0-3.4		0,5	0-11.1		0,332	0,465
Unknown species of genus Megamonas		0,2	0-3.4		0	0-0.8		0,003	0,032
Unknown species of family Erysipelotrichaceae		1,6	0-12.4		3,6	0.1-12.8		0,123	0,211
Unknonw species of genus Allobaculum		1,4	0.1-11.3		1,9	0-5.4		0,430	0,502
Unknown species of genus Catenibacterium		2,3	0-21.7		0,5	0-5.6		0,001	0,021
Unknown species of genus Coprobacillus		0	0-3.2		0,5	0-3.5		0,006	0,033
Eubacterium biforme		0,4	0-16.9		0,3	0-3.1		0,347	0,467
Eubacterium dolichum		0,1	0-0.8		0,1	0-2.4		0,011	0,053
Unknown species of genus Fusobacterium		1,6	0-20.8		6,2	0.3-18.9		0,024	0,093
Unknonw species of genus Sutterella		0,1	0-1.9		0,1	0-3.8		0,369	0,479
Unknown species of family Enterobacteriaceae		0,2	0-6.3		0,1	0-17.2		0,298	0,434

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The overall goal of the study was to provide information about the role of gut microbiota in the etiology of obesity, focusing on the mechanisms linking gut microbiota and obese-related conditions. A description of current knowledge on gut microbiota alterations in obese dogs has also been included. In addition, valuable data on fecal microbiota in obese dogs before and after weight loss has been reported based on experimental analysis.

Chapter I is presented as a literature review, which provides an overview of the composition of gut microbiota in mammals, and the importance of gut microbiota to maintain host homeostasis. This chapter covers the most recent advances of ongoing studies with particular emphasis on obesity, and focuses on elucidating 1) the role of gut microbiota in the health status of the host, 2) the association between gut microbiota and the development of obesity, 3) the gut microbiota features in dogs with obesity and after weight loss.

Chapter II consists of an interventional study in which the fecal microbiota composition of client-owned dogs with obesity has been evaluated by 16S rRNA sequencing and quantitative PCR before and after weight loss. The aim was to elucidate if weight loss in obese dogs with a high-protein high-fiber diet modulates the composition of the gut microbiota. The results showed that the fecal microbiota of dogs with obesity changed significantly after weight loss.

The strength of this research is the intervention study with an individual tailored weight loss program. The dogs underwent a real weight loss program and were followed until their

ideal body weight was achieved. In addition, as discussed in chapter II, the present study supports previous results in dogs and humans, in which a decrease of the phyla Firmicutes and an increase of Bacteroidetes was reached after weight loss. In addition, an increase in alpha diversity, and a shift in beta diversity was also observed.

Seeking a hypothesis to explain the results obtained in Chapter II, in which a change in gut microbiota after weight loss is shown, special attention was focused on diet. As previously discussed, it is well known that gut microbiota composition can be affected by diet. Despite this, no significant differences in gut microbiota were observed in the group of dogs under the same weight loss diet which did not lose weight. However, these results must be taken cautiously when evaluating only the effect of diet on gut microbiota composition, as the study included a small number of dogs under a weight loss diet over a reasonably short time period.

Another hypothesis to explain the observed changes in fecal microbiota, is its attribution to energy restriction. It has been observed that caloric restriction apart from influencing weight loss, reducing fat mass, and producing changes in the host metabolism, can also influence the composition of the gut microbiota in obese (Santacruz et al. 2009; Zheng, Wang, and Jia 2018) and non-obese individuals (Zou et al. 2020). In addition, similar results to those obtained in chapter II were described in an *in vivo* study in which mice fed a calorie restricted diet for 3-6 weeks showed an increase in Bacteroidetes and a reduction in the abundance of Firmicutes (Fabbiano et al. 2018).

The study carried out by Le Roy and colleagues aimed to elucidate the role of gut microbiota in visceral fat mass in mice with different microbiota enterotypes. Their results concluded that gut microbiota may have a greater contribution to shaping host fat than diet alone (Le Roy et al. 2019). Considering all of the above, a plausible explanation of the changes observed in fecal microbiota would be that a high-fiber, high-protein diet together with energy

restriction modulates the gut microbiota towards a "lean microbiota phenotype", mainly characterized by a greater amount of Bacteroidetes and a reduction of Firmicutes. As a consequence of these three factors, weight loss is achieved, with consequent reduction of fat mass.

Further studies are necessary to unravel the causes of the change in the composition of the fecal microbiota after weight loss with a high-fiber high-protein diet in obese dogs. However, healthy dogs with ideal body weight did not show changes in their body weight after 21 days consuming the same high-fiber high-protein diet that the dogs enrolled in this study. No significant difference in fecal microbiota composition was observed when compared to the dogs on the other different diets evaluated. However, the fecal microbiota was not evaluated at baseline, making it not possible to evaluate changes in fecal microbiota associated with weight loss diet (Mori et al. 2019).

Nevertheless, according to the material reviewed in chapter I, it is expected that a combination of events influences the changes observed in the fecal microbiota composition. For example, a greater content in fiber and protein in the diet has been demonstrated to improve satiety in healthy dogs (Weber et al. 2007), which could be mediated by a higher SCFAs production due to an increase of SCFAs-producing bacteria. Supporting this theory, an increase in the fiber content in the diet led into an increase in butyrate and SCFAs-producing bacteria in healthy dogs (Swanson et al. 2002). Moreover, SCFAs are proposed as a ligand to several receptors involved in many host metabolic functions, such as lipogenesis, bile acid metabolism and glucose homeostasis. Therefore, analysis of fecal SCFAs would be useful to observe if changes in its concentration are consistent with the results obtained from the fecal microbiota analysis. However, limitations of the data extracted from fecal SCFAs analysis must be also considered, which include the absorption of SCFAs by the host and cross-feeding between the gut bacteria.

As previously mentioned, a greater level of plasma LPS was found in mice fed a high fat diet, which was associated with a reduction of the expression of genes that encode for tight junctions and a subsequent increase of gut permeability (Cani et al. 2007; Cani et al. 2008). Thus, the analysis of LPS in the serum samples of obese dogs before and after weight loss would be interesting to evaluate the possible effect of a weight loss intervention in dogs with a high-fiber and high-protein diet on intestinal permeability. In addition, LPS serum levels can also be associated with the relative abundance of gram-negative bacteria found in fecal samples, since the percentage of gram-negative bacteria can be estimated with the study presented in chapter II.

Taxonomic analysis is the first step towards understanding if the microbiota has a possible role in the pathology of obesity in dogs, however, further work would be necessary to evaluate the functions of these microorganisms. Considering this, to complete the characterization of the fecal microbiota in obese dogs and after weight loss, HPLC-MS untargeted fecal metabolome analysis is an ongoing study which aims to support the results obtained from the fecal microbiota analysis.

Apart from this untargeted fecal metabolome study, subsequent studies including meta-genomics and meta-transcriptomics analyses could be useful in providing information about what metabolic functions the bacteria are able to carry out in the intestine. Just as the host environment and external factors can contribute to a change in the fecal microbiota composition, a shift in the population of intestinal bacteria may modulate the crosstalk with the host, exposing the importance of evaluating functionality of the gut bacteria after weight loss.

When considering the role of gut microbiota in the regulation of the health host status, the discovery of new tools that can modulate the composition of the gut microbiota presents great potential for research. Recent studies have been focused on evaluating the metabolic

consequences of supplementation with probiotics, prebiotics as well as fecal microbiota transplantation (FMT) in weight loss interventions.

As detailed in chapter I, studies that aim to evaluate the effect of probiotic and prebiotic supplementation in obese individuals suggest that the mechanisms in which probiotics and prebiotic might have a positive effect on weight loss are through an increasing of satiety, decrease of low-grade inflammation and improvement of plasma lipid profiles as well as glucose tolerance (Cerdó et al. 2019). However, no effects were observed in a considerable number of clinical studies in overweight and obese individuals when prebiotics or probiotics were administrated (Wang et al. 2019). Interestingly, better outcomes were reported in obese and overweight children and adolescents. Moreover, weight reduction was increased when physical activity or diet was accompanied with a supplementation of a mix of probiotics and prebiotics (Wiciński et al. 2020).

Considering the initial studies in mice in which the obese phenotype of the donor was conferred to the recipient germ-free mice (Ridaura et al. 2013), gut microbiota transplantation has been proposed as a possible alternative to help weight loss or to maintain an ideal body weight. Consistently, recent studies have been carried out on this topic. Although fecal microbiota changes towards the fecal microbiota composition of the lean donors were observed after FMT with oral capsules in obese patients, no differences in body weight were reported (Allegretti et al. 2020). In contrast, in other study with overweight sedentary adults, autologous FMT (aFMT) with fecal capsules of the weight loss period with a green-Mediterranean diet induced a change in the gut microbiota, and showed a significantly attenuated weight re-gain post-weight loss diet period (Rinott et al. 2020). However, these results were not observed with other type of diets, suggesting that gut microbiota modulation must be considered from an individualized point of view and taken into account possible influential factors, such as, diet, lifestyles and history of antibiotic intake.

Despite the existing evidence of the role of gut microbiota in obesity, its associated mechanisms are still under discussion and this area merits further investigation. Nevertheless, it is an interesting research topic that provides promising alternative approaches in the field of therapy directed to improve metabolic and gastrointestinal diseases.

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LIST OF PAPERS

List of manuscript related with the thesis as published/submitted/drafted

Paper I. **Bermudez-Sanchez S.,** Pilla R., Sarawichitr B., Gramenzi A., Marsilio F., Steiner J.M., Lidbury J.A., Woods G.R.T., German J.A., Suchodolski J.S. (2020) Fecal microbiota in client-owned obese dogs changes after weight loss with a high-fiber-high-protein diet. *PeerJ*. 8:e9706, doi: 10.7717/peerj.9706.

Paper II. **Bermudez-Sanchez S.,** Pilla R., Sarawichitr B., Gramenzi A., Marsilio F., Steiner J.M., Lidbury J.A., Woods G.R.T., Suchodolski J.S., German J.A. (2020) Untargeted fecal metabolome analysis in obese dogs after weight loss (Manuscript in preparation).

List of related published manuscripts by the author, not included in the thesis

Paper I. Melegari I., Sarchese V. Di Profio F., Robertto S., **Bermudez Sanchez S**., Orusa R., Marsilio F., Di Martino B. (2018) First molecular identification of kobuviruses in wolves (Canis lupus) in Italy. *Arch.Virol*. 163(2):509-513, doi: 10.1007/s00705-017-3637-1.

Paper II. Di Profio F., Melegari I., Sarchese V., Robetto S., **Bermudez Sanchez S**., Carella E., Orusa R., Cavadini P., Lavazza A., Marsilio F., Martella V., Di Martino B (2018) Potential role of wolf (Canis lupus) as passive carrier of European Brown Hare Syndrome Virus (EBHSV). *Res. Vet, Sci*., 117:81-84, doi: 10.3389/fmicb.2018.01287.

Paper III Di Profio F., Sarchese V., Melegari I., Palombieri A., Massirio I., **Bermudez Sanchez S**., Friedrich KG., Coccia F., Marsilio F., Martella V., Di Martino B (2019) Seroprevalence for norovirus genogroups GII and GIV in captive non-human primates. *Zoonoses Public Health,* 66, 310-315, doi: 10.1111/zph.12566.

Paper IV.   Sarchese V., Di Profio F., Melegari I., Palombieri A., **Sanchez SB.,** Arbuatti A., Ciuffetelli M., Marsilio F., Martella V., Di Martino B. (2019) Hepatitis E virus in sheep in Italy. *Transbound Emerg. Dis.*, 66(3): 1120-1125, doi: 10.1111/tbed.13157.

Proceeding I.   **Bermudez Sanchez S.,** Pilla R., Gramenzi A., Marsilio F., Steiner J.M., Lidbury J.A., Suchodolski J.S. (2019) Fecal microbial metabolism is altered in dogs with chronic enteropathy. *J. Vet. Inter. Med.,* 34:340, doi: 10.1111/jvim.15658.

LIST OF CONFERENCES

ORAL COMMUNICATIONS

➢ **ECVIM-CA (European College of Veterinary Internal Medicine Companion Animals) (2019) Bermudez-Sanchez, S.**, Pilla R., Gramenzi A., Marsilio F., Steiner M., Lidbury A., Suchodolski S. "Fecal microbial metabolism is altered in dogs with chronic enteropathy". September 2019, Milan (Italy).

POSTERS

➢ **Keystone Symposia. Microbiome: its therapeutic implications (2019) Bermudez-Sanchez S.,** Pilla R., Gramenzi A., Marsilio F., Steiner J.M., Lidbury J.A., German J.A., Suchodolski J.S. "Obese dog fecal microbiota shifts towards lean dog fecal microbiota after weight loss intervention". October 2019, Killarney (Ireland).

➢ **American College of Veterinary International Medicine (ACVIM) (2019) Bermudez-Sanchez, S.**, Pilla R., Steiner M., Lidbury A., Suchodolski J. "Prevalence of methanogens in fecal samples of dogs with chronic enteropathy". June 2019, Phoenix, Arizona (USA).

➢ **2nd National congress of the Italian Society of Virology (2018) Sarchese V.**, Di Profio F., Melegari I., Palombieri A., Bermudez Sanchez S., Marsilio F., Martella V., Di Martino B."Hepatitis E virus in sheep in Italy" November 2018, Rome (Italy).

➢ **XVIII Convegno S. I. Di. L. V. (2018) Di Profio F.**, Sarchese V., Melegari I., Palombieri A., Massirio I., Bermudez Sanchez S., Friedrich KG., Coccia F., Marsilio F., Martella V., Di Martino B Bermudez-Sanchez, Sandra. "Indagine sierologica per norovirus gii e giv in primati non umani in cattività". November 2018, Perugia (Italy).

- **64° CONVEGNO GEI (Società italiana di biologia dello sviluppo e della cellula) (2018) Bermudez-Sanchez, S.**, Suchodolski J., Gramenzi A., Marsilio F. "Evaluation of the fecal microbiota and its metabolites in obese pet owners and their obese pets". June 2018, L'Aquila (Italy).

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