EFFECTS OF A HIGH-PROTEIN, HIGH-FIBER DIET IN ADULT FEMALE DOGS UNDERGOING WEIGHT GAIN AND WEIGHT LOSS CONDITIONS

BY

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DISSERTATION

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ABSTRACT

Obesity is a complex multifactorial disease with increasing incidence and economic burden to humans and pets worldwide. Understanding the pathophysiological changes during the process of weight gain and weight loss with the use of nutritional therapy, and advanced scientific techniques could potentially help develop preventive or management strategies that can alleviate adverse effects. Numerous studies have been conducted in humans and rodents, however, there is limited knowledge regarding the effects of dietary modifications on metabolic responses, gut microbial structure, and microbial-derived metabolites of adult dogs. Therefore, we aimed to test the effects of high-protein, high-fiber (HPHF) diets in adult dogs during weight gain and weight loss conditions. We hypothesized that these diets would minimize detrimental impacts (e.g., reduce blood lipids and inflammatory marker concentrations, and alter obesity-induced gut dysbiosis), maximize positive effects (e.g., alter body composition and blood hormone concentrations, and modulate fecal metabolite concentrations and fecal microbiota populations), and provide beneficial outcomes (e.g., decrease the risk of obesity-associated comorbidities and increase longevity) which could be the key to remedies of obesity.

The first aim was to determine the effects of specially formulated dry diets on apparent total tract macronutrient digestibility, fecal bile acid concentrations, fecal fermentative-end products concentrations, and fecal microbiota populations of adult female dogs after spay surgery. Twenty-eight adult intact female beagles (age: 3.02 ± 0.71 yr, BW: 10.28 ± 0.77 kg; BCS: 4.98 ± 0.57) were used in a longitudinal spay study. After a 5-wk baseline phase whereby all dogs were fed a control diet at a rate to maintain BW, 24 dogs were spayed using standard procedures. Four dogs were sham-operated and fed the control diet to serve as lean controls (COSH). Spayed dogs were randomly allotted to one of three dry diets (n=8/group): 1) Control

diet (CO) contain a moderate amount of crude protein and a low amount of dietary fiber; 2) HPHF diet; and 3) HPHF diet containing additional omega-3 and medium-chain fatty acids (HPHFO). After the first 12 wk, dogs were fed an amount that exceeded needs [up to 200% the energy needed to maintain body weight (BW)] for an additional 12 wk. Apparent organic matter and energy digestibilities had greater decreases ($P < 0.05$) in HPHF and HPHFO than COSH and COSP. Increases in fecal acetate ($P = 0.0001$) and total short-chain fatty acid (SCFA) concentrations were greater ($P < 0.05$) in HPHF and HPHFO than COSH and COSP. Fecal secondary bile acid percentages tended to be greater ($P = 0.08$) and decreases in primary bile acid percentages tended to be greater ($P = 0.08$) in HPHFO than other treatments. Principal coordinates analysis (PCoA) plots of weighted UniFrac distances revealed that HPHF and HPHFO clustered together and separated from COSH and COSP at wks 12 and 24 ($P < 0.05$), with relative abundances of *Faecalibacterium*, *Romboutsia*, and *Fusobacterium* increasing to a greater ($P < 0.01$) extent and *Catenibacterium, Bifidobacterium, Prevotella 9, Eubacterium, and Megamonas* decreasing to a greater ($P < 0.05$) extent in HPHF or HPHFO.

The second aim was to determine the effects of restricted feeding of a HPHF diet and weight loss on body composition, voluntary physical activity, complete blood cell count, serum chemistry profile, serum hormone concentrations, and serum inflammatory marker concentrations of overweight dogs. Twelve overweight adult spayed female beagle dogs (age: 5.5±1.1 yr; BW: 14.8±2.0 kg, BCS: 7.9±0.8) were fed a high-protein (42.0% dry matter), high-fiber (26.8% dry matter) diet during a 4-wk baseline phase to maintain BW. A 24-wk weight loss phase then followed. After baseline (wk 0), dogs were initially fed at 80% of that needed to maintain BW and then adjusted weekly to target 1.5% weekly weight loss. After 24 wk, dogs lost 31.2% of initial BW and had 1.43±0.73% weight loss per wk. BCS decreased ($P < 0.0001$) by

2.7 units, lean mass percentage increased ($P < 0.0001$) by 11.3%, and fat mass and percentage decreased ($P < 0.0001$) by 3.1 kg and 11.7%, respectively, with weight loss. Many serum metabolites and hormones were altered, with triglycerides, leptin, insulin, C-reactive protein, and interleukin-6 decreasing ($P < 0.05$) with weight loss.

The third aim was to evaluate the effects of restricted feeding of a HPHF diet and weight loss on fecal characteristics, fermentative metabolite concentrations, and fecal bile acid concentrations of overweight dogs. The experimental design and animals used were described in **Aim #2.** Fecal dry matter percentage increased ($P < 0.05$), while fecal scores decreased (firmer stool; $P < 0.001$) with restricted feeding and weight loss. Fecal acetate concentrations tended to decrease ($P = 0.051$) and fecal ammonia concentrations decreased ($P < 0.05$), while fecal valerate concentrations increased ($P < 0.01$) with restricted feeding and weight loss. Fecal deoxycholic acid concentrations decreased ($P < 0.05$), fecal secondary bile acid concentrations tended to decrease ($P = 0.058$), and fecal ursodeoxycholic acid concentrations increased ($P < 0.01$) with restricted feeding and weight loss.

The fourth aim was to evaluate the effects of restricted feeding of a HPHF diet and weight loss on the fecal microbiota of overweight dogs. The experimental design and animals used were described in **Aim #2.** Principal components analysis (PCoA) plots of weighted and unweighted UniFrac distances revealed that fecal microbial populations tended to shift away from that measured at baseline (wk 0) with restricted feeding and weight loss ($P < 0.05$). Relative abundances of fecal *Bifidobacterium*, Coriobacteriaceae UCG-002, undefined Muribaculaceae, *Allobaculum, Eubacterium, Lachnospira, Negativivibacillus, Ruminococcus gauvreauii group,* uncultured Erysipelotrichaceae, and *Parasutterella* increased ($P < 0.05$), whereas Prevotelaceae Ga6A1 group, *Catenibacterium, Erysipelatoclostridium, Fusobacterium, Holdemanella,*

Lachnoclostridium, Lactobacillus, Megamonas, Peptoclostridium, Ruminococcus gnavus group, and *Streptococcus* decreased ($P < 0.01$) with restricted feeding and weight loss.

Overall, our results suggest that consumption of HPHF diets alone or when fed during restricted feeding may help ameliorate obesity and its comorbidities by positively altering body composition, serum chemistry profile, and blood hormones, and reducing inflammatory markers, modulating fecal fermentative metabolites, fecal bile acids, and fecal microbiota. Spay surgery appeared to have minor effects on these outcomes. Because the diets tested had high fiber content, their consumption decreased apparent nutrient and energy digestibilities, but did not initiate deleterious consequences.

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CHAPTER 1: INTRODUCTION

Obesity prevalence in pet dogs has been growing worldwide and becoming one of the most serious nutrition and health concerns. Chronic obesity causes secondary diseases (e.g., type 2 diabetes, cardiovascular disorders, intestinal dysbiosis, gastrointestinal diseases, and cancers) and can complicate existing health conditions (German, 2006; Weiss and Hennet, 2017; Upadhyay et al., 2018; Emerenziani et al., 2019), leading to poor quality of life and shortened life span. Furthermore, it is also an economic burden on pet parents (Bomberg et al., 2017; Pet Product News, 2021).

Genetic and environmental factors are predisposing factors of obesity (German, 2006; Albuqerque et al., 2017). Additionally, surgical sterilization, a permanent contraceptive option that is commonly used for controlling pet overpopulation is also linked to increasing canine obesity risk (Lefebvre et al., 2013). Successful prevention and management of obesity require multidimensional approaches. Among many strategies, nutritional intervention is not only an effective and non-invasive method but is also well-accepted by pet owners (German, 2016; Linder and Parker, 2016). A high-protein and/or high-fiber diet has been efficiently incorporated into the weight management plan for dogs by improving body composition and blood lipid profile, altering serum hormone leptin and insulin productions, reducing circulating inflammatory marker concentrations (Jeusette et al., 2005; German et al., 2009; German et al., 2010; Warren et al., 2011; Bastien et al., 2015; Floerchinger et al., 2015; Starr et al., 2019), and consequently improving quality of life and life expectancy of dogs (German, 2006; German, 2016). However, the knowledge of how these diets influence gut microbiota and their metabolites of dogs undergoing weight gain and/or weight loss conditions are limited (Salas-Mani et al., 2018; Bermudez Sanchez et al., 2020). Moreover, such research has lacked a full investigation on the alterations that result

from dietary change, including changes to physiological outcomes, fecal microbiota, and fecal microbial-derived metabolites, especially in dogs undergoing spay surgery. Therefore, the main focus of this dissertation may provide a better understanding of how each factor plays a role in the management and treatment of canine obesity.

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CHAPTER 2: LITERATURE REVIEW

PREVALENCE OF CANINE OBESITY

Obesity is one of the most common nutrition-associated health problems in dogs and cats. The prevalence of canine obesity has been evaluated in many studies around the world. Mason (1970) reported that 28% (n=1,000) dogs in England were obese. According to the Association for Pet Obesity Prevention (APOP), 36.9% and 18.9% of the dog population in the US were considered overweight (BCS 6 to 7 out of 9) and obese (BCS 8 to 9 out of 9), respectively (APOP, 2018). In 2005, 428 veterinary practices across Australia collaborated and surveyed obesity rates in 2,661 dogs, reporting that 33.5% were overweight and 7.6% were obese (McGreevy et al., 2005). Obesity is not a concern limited to England, US, and Australia, but is also prevalent in China, Japan, and Spain. Mao et al. (2013) conducted the first epidemiological survey of canine obesity in China, reporting that 44.4% of the 2,391 cases evaluated were obese. A recent study conducted in private Japanese veterinary clinics reported that of the 9,120 dogs evaluated, 15.1% and 39.8% were classified as obese and overweight, respectively (Usui et al., 2015). Another study evaluated 93 dogs in 10 Spanish veterinary practices, with 40.9% of dogs considered to be obese (Montoya-Alonso et al., 2017). Based on the research that has been conducted in several countries around the world over the past few decades, it appears that the number of overweight and obese dogs is high and continues to increase.

DEFINITION OF OBESITY

By definition, obesity is the accumulation of excessive fat in the body as a result of an imbalance between increased energy consumption and decreased energy expenditure. In humans, there are standard criteria to identify overweight and obese conditions. The Centers for Disease

Control and Prevention (CDC) and the World Health Organization (WHO) use body mass index [BMI; weight (kg) divided by height (m^2)] to assess weight status because it is correlated with the degree of body fatness, has a practical use and low cost, and is reproducible. However, this tool does not account for gender, ethnicity, muscle mass, and frame size. Based on its disadvantages, BMI cannot be the only indicator used for diagnosing health problems. A similar limitation is present in companion animals. In dogs and cats, the current body weight relative to ideal body weight has been used as a classifier, with an overweight animal exceeding optimal (ideal) body weight by 15% and an obese animal exceeding optimal weight by 30% (German, 2006). Because limited information exists regarding the optimal body weight for many dog breeds, especially mixed breeds, such measures have limited value.

Another measurement of obesity is indirectly and directly measured fat mass. Body condition scoring (BCS), typically using a 5-point or 9-point scoring system has been applied in practice (Laflamme, 1997). They are used as an indirect assessment of percentage body fat. A score of 3.0 (5-point system) or 5.0 (9-point system) serves as an ideal body condition score, equating to approximately 15-20% body fat for dogs and cats. For the scores below or above the ideal body fat percentage is estimated to change by roughly 10% for each BCS unit on a 5-point scale (or 5% on a 9-point scale) (Toll et al., 2010).

Unlike BMI in humans, BCS of dogs requires observation and palpation skills, and has subjectivity involved in assigning a score. Therefore, using the same evaluator to increase precision, accuracy, and repeatability of the score is recommended. To avoid the subjectivity of BCS, advanced techniques or machines have been incorporated into research and shown to be highly effective at identifying the degrees of fat mass. These include chemical (carcass) analysis (the analysis of total water, fat, and ash content of whole-body homogenate), densitometry, total

body water measurement, absorptiometry [e.g., dual-energy X-ray absorptiometry (DEXA)], ultrasonography (US), electrical conductance, and advanced imaging techniques [e.g., computed tomography (CT) and magnetic resonance imaging (MRI)] (German, 2006; Kovner et al., 2010).

FACTORS CONTRIBUTING TO OBESITY

Obesity is considered to be one of the most serious public health concerns in humans. Several studies have been conducted to identify the primary causes of obesity so that effective prevention strategies may be developed. Similar to humans, risk factors for pet obesity have also been studied. Because pets are considered members of the family nowadays, many similarities exist between human and pet obesity. Not surprisingly, for dogs, owner-related factors play an essential role in obesity risk, including owner age, socioeconomic status, education level, feeding habits (e.g., frequency of giving treats, price of diet fed), amount of exercise, owner misperception of their dog's body condition, awareness of pet nutrition, and health consciousness of both their pet and themselves (German, 2006; Courcier et al., 2010; Linder and Mueller, 2014).

Additional factors that increase obesity risk include dog breed (e.g., Labrador Retriever, Cairn Terrier, Cavalier King Charles Spaniel, Scottish Terrier, and Cocker Spaniel breeds have greater risk), age (middle age has a higher risk than younger animals), sex (incidence in females more than males), indoor lifestyle, presence of other diseases (e.g., hypothyroidism, hyperadrenocorticism, or canine distemper infection) and pharmaceutical use (e.g., glucocorticoids or anticonvulsant drugs) (German, 2006; Laflamme, 2006).

Although there are many factors that contribute to its development, neutering is one of the most well-known causes of obesity in both dogs and cats, with excessive energy intake and lower energy expenditure resulting from the lack of sex hormones.

BENEFITS AND DETRIMENTS OF NEUTERING

The overpopulation of domestic pets increases the risk of zoonosis exposure to humans, especially rabies. Additionally, millions of unwanted healthy dogs and cats in shelters are euthanized in the US every year, creating a moral and ethical problem for the society (McNeil and Constandy, 2006; Kustritz, 2007). Several contraceptive methods are used for controlling the population of unwanted pets, such as gonadotropin-releasing hormone (GnRH) agonists implantation and gonadectomy. GnRH agonist implantation is a non-surgical sterilization used to induce long-term suppression of fertility, as a result of continued activation of GnRH receptors, it causes desensitization and therefore, suppression of gonadotropin secretion (Kumar and Sharma, 2014). The functions of GnRH analogs continue for approximately 6 months to one year in dogs and cats. However, this method is not practical for the large pet populations in shelters in terms of economics, and because its efficacy is questionable given the variation in time to onset and duration of action (Goericke-Pesch, 2016; Rhodes, 2017). Gonadectomy is the irreversible contraceptive of choice and is a widely accepted method in the US and many other countries around the world. Ovariectomy (OVE) or ovariohysterectomy (OVH) are surgical sterilization methods that remove the ovaries resulting in permanent termination of reproductive function. The primary purpose for its use is to minimize the pet overpopulation issue. It may also be used as a treatment for some diseases, such as ovarian tumors and cysts, vaginal hyperplasia, uterine diseases, perineal adenomas, and perineal hernias in dogs and cats (Reichler, 2009). It may also be used to eliminate the risk of mammary tumors (MT), which have a lower prevalence in spayed dogs and cats compared to intact animals (Schneider et al., 1969; Overley et al., 2005). The timing of spay surgery plays a crucial role in reducing the chance of MT. Dogs spayed prior to their first estrus and cat spayed prior to 6 months of age have a 99.5% and 91% reduced chance, respectively,

of developing MT. In addition to increasing the life expectancy of the animals, gonadectomy results in a decrease in undesirable behaviors (e.g., urine spraying; aggression). There are not only benefits, however, because spayed animals may have complications from the surgery or have an increased risk of certain diseases, including urinary incontinence and urinary tract infection (Spain et al., 2004; Kustritz, 2007). Lastly, neutering is a significant risk factor for obesity in both dogs and cats (Lefebvre et al., 2013).

REPRODUCTIVE HORMONES AND EATING BEHAVIOR

There has been extensive research conducted to evaluate the effects of estrogen on eating behaviors in rodents and humans, but very little has been done in dogs or cats. The physiology of the reproductive system of female dogs is quite similar to both rodents and humans. Therefore, the information generated from those research studies can be used to understand the physiological functions of reproductive hormones and their impact on eating behavior and metabolism of dogs.

Reproductive hormones (e.g., estrogens, progesterone and testosterone) play essential roles in controlling energy intake. Estrogen, in particular, suppresses food intake and prevents body weight gain. Permanently removing sex steroid hormones by gonadectomy in female animals leads to increased food intake by increasing meal size, and consequently weight gain and increased adiposity. The same physiological changes are presented in women undergoing menopause, the stage of life when gonadal hormones production decline, ultimately leading to their loss completely. Notably, these responses can be prevented by estradiol treatment. In normal situations, women have a significant decrease in food intake during the peri-ovulatory phase when compared to the other phases of the ovarian cycle. This occurs because of the influence of estradiol, which is secreted at the highest level during this phase (Asarian and Geary, 2006).

The effects of estrogen on eating behaviors are mediated through estrogen receptors located in the brain, although their specific locations are unknown (Asarian and Geary, 2006). Asarian and Geary (2013) believed that estradiol controls normal eating behaviors by acting on estrogen receptor alpha (ERα), also called Esr 1, in the caudal brain stem. Additionally, estrogen not only directly modulates physiological control of food consumption in the central nervous system, but also interacts with other hormonal and neuronal signals that regulate feeding behaviors in the central and peripheral systems.

Yong (2017) reported the interactions between estradiol and other appetite regulatory signals, including leptin, insulin, ghrelin, cholecystokinin (CCK) and serotonin. Estradiol increases the eating-inhibitory effect of acute leptin administration, but this effect disappears when leptin is given long-term (Chen and Heiman, 2000). In contrast, Asarian and Geary (2006) reported that the effects of insulin on eating-inhibitory potency are decreased after estradiol treatment. Interestingly, insulin alone limits food intake in ovariectomized rats, with this effect being more potent than in intact rats (Leeners et al., 2017). The small intestinal hormone, CCK, is released during meals and acts on CCK-A receptors to produce a satiation effect. Several studies have demonstrated that estradiol increases the satiating potency of CCK in rats. Unlike the others hormone mentioned ghrelin is a hormone secreted by the stomach. It stimulates food consumption and its secretion increases during fasting (Asarian and Geary, 2006). Clegg et al. (2007) reported that estradiol-treated, ovariectomized rats had lower food consumption than the untreated group, suggesting that estradiol decreases the appetite-stimulating effect of ghrelin. Brain serotonin or 5-hydroxytryptamine (5-HT) also affects food intake regulation. The secretion of 5-HT is increased during meals and decreased during fasting, again with estradiol enhancing its effects (Rivera et al., 2012).

It is certain that neutering or spaying changes the physiology of dogs and cats, with increased food consumption leading to an imbalance between energy intake and energy expenditure, with obesity being the consequence. Although spaying dogs may have some adverse effects, it provides numerous benefits. Therefore, it is important to understand the pathophysiological changes that occur after spay surgery so that strategies to manage homeostatic controls of energy balance can be developed, minimizing any adverse effects and ensuring a good quality and length of life.

CLINICAL CONSIDERATIONS OF OBESITY

Clinically obese pets are prone to having several clinical conditions that can reduce the quality of life and life expectancy of dogs and cats. Rafaj et al. (2017) reported that an accumulation of excess body fat in 37 overweight and obese dogs was associated with a chronic low degree of inflammation, which was likely due to elevated concentrations of serum interleukin-6 (increased from 227 to 261 pg/mL) and C-reactive protein (CRP) (increased from 3.7 to 4.2 µg/mL). In addition, one study reported that the inflammatory cytokines, tumor necrosis factor-α (TNF-α) and CRP, were decreased from the range of ≤ 0.9 to 6.6 to ≤ 0.9 to 3.8 pg/mL (TNF-α), and from 2.18 to 1.4 mg/L (CRP) following weight loss in dogs (German et al., 2009).

Dyslipidemia or hyperlipidemia refers to an abnormality in lipid metabolism, and is characterized by excess cholesterol and/or triglycerides in fasted blood samples. This condition is considered one of the most common comorbidities associated with or exacerbated by obesity. Jeusette et al. (2005) experimentally induced obesity in dogs by feeding a maintenance diet in large quantities for a period of 10 to 15 months, and maintained them in a grossly obese state for at least 1 year. They then collected plasma samples, reporting increased total plasma cholesterol (+41%), very-low-density lipoprotein (VLDL) cholesterol (+125%), high-density lipoprotein (HDL)

cholesterol (+45%), and low-density lipoprotein (LDL) cholesterol (+58%) fractions, total plasma triglycerides (+75%), and in VLDL triglyceride (+118%) fraction compared with lean dogs. After switching to a high-protein, low-energy diet, the overall plasma lipid values were decreased even before weight loss was observed.

Many studies have indicated that obesity leads to endocrine and metabolic diseases in dogs and cats [e.g., type 2 diabetes mellitus (DM), hypothyroidism, hyperadrenocorticism, insulinoma, and dyslipidemia] (German, 2006; Tvarijonaviciute et al., 2012; Clark and Hoenig, 2016; Weeth, 2016). In humans and cats, type 2 DM is the most common endocrinopathy caused by obesity and is related to beta cell compromise. The mechanisms involved include: 1) excess adiposity and increased circulating non-esterified fatty acid concentrations that interfere with insulin signaling in muscle and liver, leading to hypersecretion of insulin and insulin resistance, and 2) beta cell dysfunction, cell apoptosis, and decreased beta cell mass (Clark and Hoenig, 2016; Franz and Evert, 2017). Type 2 DM is not common in obese dogs, but a metabolic syndrome (MS) similar to that in humans is observed. Metabolic syndrome is an important health issue in human medicine because it is not only associated with the development of DM, but also hypertension, hypertriglyceridemia, and reduced HDL. Tvarijonaviciute et al. (2012) modified the term of MS for use in dogs and used a new approach for assessment. Based on their research, canine obesity-related metabolic dysfunction criteria include a BCS of 7-9 on a 9-point scale, blood triglycerides > 200 mg/dL, total blood cholesterol > 300 mg/dL, systolic blood pressure > 160 mmHg, fasting plasma glucose > 100 mg/dL or previous diagnosis of type 2 DM. These criterion are believed to help monitor and identify MS cases and provide treatments that will address the obesity-related metabolic problems.

In humans, symmetric or asymmetric left ventricular hypertrophy secondary to systemic hypertension has been described as an obesity-related cardiac structural change (Murdolo et al., 2015). However, the effect of obesity on hypertension is controversial in dogs. Mehlman et al. (2013) reported similar results in obese dogs, while another study found that obesity altered cardiac structure and function in dogs without abnormal findings, perhaps because of the small sample size (Trof et al., 2017). Obesity is also a substantial risk for the development of pulmonary dysfunction (Garcia-Guasch et al., 2015) and tracheal collapse (White and Williams, 1994). Furthermore, previous studies have demonstrated that obesity is a major risk factor for orthopedic disorders, including osteoarthritis, ligament rupture, and hip dysplasia (Edney and Smith, 1986; Brown et al., 1996; Smith et al., 2001; van Hagen et al., 2005). In one study, a weight reduction of 8.6% of the initial body weights improved the degree of lameness in dogs (Marshall et al., 2010).

Obesity is considered a significant risk factor in the development of chronic kidney disease (CKD) and urinary tract disorders. Several clinical and histopathological changes have been found in obese dogs with CKD, such as an expansion of the Bowman capsule, cell proliferation in glomeruli, thickening of glomerular and tubular basement membrane, proteinuria, and an increased urine protein: creatinine ratio (Heneger et al., 2001; Tvarijonaviciute et al., 2013). An increased incidence of calcium oxalate uroliths has also been reported in obese dogs (Lekcharoensuk et al., 2000). Finally, obese pets have a higher incidence of certain dermatologic disorders (e.g., atopic dermatitis) (Zang and Silverberg, 2015) and cancers (e.g., transitional cell carcinoma, mammary tumor) (Glickman et al., 1989), and have increased anesthetic risk (German, 2006).

Based on the records of Nationwide Mutual Insurance Company (Columbus, OH), a pet insurance company in the US, approximately 20 percent of the claims filed in 2017 were related to conditions or diseases associated with obesity, resulting in more than $69 million in veterinary

expenses (Pet Product News, 2019). Pet-owners should be aware of the increased costs associated with having overweight or obese pets, whether they are due to veterinary care, medications or specialized diets.

DUAL-ENERGY X-RAY ABSORPTIOMETRY (DEXA)

Body composition is used in clinical practice as a criterion for monitoring nutritional status and several clinical conditions such as osteoporosis, sarcopenia and obesity (Andreoli et al., 2016). Most research articles, especially those pertaining to animals, divide and discuss body composition into three parts, including bone mineral content (BMC), lean muscle mass, and fat mass. Lysen and Israel (2017), however, divided body composition into two compartments: fat mass and fat-free mass (water, protein, mineral components). In some articles, fat-free mass is used interchangeably with the term "lean body mass". In the past, invasive measurements (e.g., dissection or chemical analysis) were used for grading quality and quantity of products intended for human consumption or accessing companion animal obesity. Nowadays, however, non-invasive procedures are preferred. Several non-invasive and precise techniques for measuring body composition have been developed and applied to humans and animals. These preferred methods include DEXA, US, CT, and MRI (Scholz et al., 2015).

DEXA is a technique that was initially used for the measurement of BMC for age-associated bone loss in human patients (Toll et al., 1994), but is now commonly used for body composition research in veterinary and human medicine. The principle of this instrument is based on the attenuation of two different X-ray photon energies (40 kV and 70 kV) that apply to different tissue types or parts of the body. The machine then uses computerized algorithms to analyze and categorize each body compartment and reports the data in grams. The advantages of this technique are that it is easy to use, is not expensive (once equipment is available), has low radiation, and

allows for quick analysis of the data. However, one disadvantage of DEXA is that the machine provides only 2-dimensional information.

The precision and accuracy of DEXA technology had been evaluated in several studies. The error in DEXA scanning was affected by several factors related to the machine, operator, and subject variability (Morgan and Prater, 2017). Johnson and Dawson-Hughes (1991) reported that the used of DEXA for body composition measurement in humans had coefficients of variation (CV; %) of approximately 1% to 7%. Similar results have been reported in animal research. Munday et al. (1994) tested the repeatability and precision of DEXA in four Labrador retrievers and five domestic short-hair cats. Those researchers reported a CV of less than 6% for total mass, lean mass, BMC, bone mineral density, and fat mass, with the fat mass having the poorest CV. Lauten et al. (2001) tested the accuracy of DEXA by comparing body composition data generated from DEXA with the data from chemical analyses of whole carcass homogenates from the same dogs. The chemical or carcass analysis was a quantitative measurement of water, lipid, and ash content of the entire body homogenate. The results from that experiment showed no significant differences in fat and lean components, but the percentage of BMC estimated by DEXA was different from the percentage of ash content measured by chemical analyses. This discrepancy was thought to be due to the measurement of minerals from tissues that contributed to ash, while DEXA measured only minerals present within bone. Nonetheless, DEXA is considered to be the gold standard for measuring body composition of dogs and cats.

OBESITY AND CHRONIC INFLAMMATORY CONDITIONS

Obesity is known as a cause of persistent low-grade inflammation that contributes to systemic metabolic dysfunction and is associated with an increase in the amount of body fat mass. White adipose tissue (WAT) is recognized as an active endocrine organ that produces and secretes

a variety of adipocyte-derived proteins with endocrine functions [e.g., leptin, adiponectin, TNF-α, IL-6, CRP, monocyte chemotactic protein-1 (MCP-1), visfatin, resistin, and angiotensinogen], all of which are called adipokines. These substances are involved in glucose and lipid metabolism, hemostasis, fluid balance, immune response, and inflammation (Kershaw and Flier, 2004; Radin et al., 2009; German et al., 2010). The initial cause of inflammatory reactions in obese individuals is thought to be due to an expansion of WAT, leading to decreased blood perfusion and tissue hypoxia and an increased production of reactive oxygen species (ROS), which then triggers the release of hypoxia-inducible factor-1 alpha (HIF-1α), pro-inflammatory cytokines (e.g., IL-6) and acute phase protein (APP) (e.g., CRP). Then, the secretion of HIF-1α decreases adiponectin production but increases leptin, macrophage inhibitory factor (MIF), vascular endothelial growth factor (VEGF), and plasminogen activator inhibitor (PAI-1) concentrations in adipocytes. Additionally, an accumulation of macrophages in WAT is also found during obesity, and is associated with an increased of MCP-1 and MIF in WAT (Chen et al., 2006; Trayhurn et al., 2008). Furthermore, adipokines that are secreted by visceral adipose tissue directly circulate via the portal vein into the liver, leading to the release of CRP from the liver. Generally, serum CRP concentrations are increased in obese humans, with this phenomenon expected to be similar in dogs and cats. Interestingly, Veiga et al. (2008) reported that CRP concentrations in obese dogs were lower than those of lean dogs (obese group: 0.76±0.1; lean group: 2.72±0.7 μg/mL) while serum insulin, insulin:glucose ratio, triglycerides, cholesterol, and fructosamine concentrations were increased in the obese group.

Malondialdehyde (MDA) is an end-product of lipid peroxidation and is accepted as a biomarker of oxidative stress, resulting from an excess of ROS. Additionally, MDA can interact with DNA and protein, potentially leading to mutations and cancer (Del Rio et al., 2005). In

humans, MDA is used for investigating several health problems, including cancers, cardiovascular, pulmonary, psychiatric, liver, Alzheimer's, or infectious diseases, and DM (Delibas et al., 2002; Dierckx et al., 2003; Azizi et al., 2016). In dogs, MDA is also used as an indicator of increased oxidative stress in various pathological conditions. Li et al. (2014) evaluated plasma MDA concentrations from 3 groups of dogs: 1) healthy control group, 2) dogs with treated hyperlipidemia, and 3) dogs with untreated hyperlipidemia. They reported that MDA concentrations were the highest in the untreated group, and that a positive correlation existed among MDA, triglycerides, and VLDL triglyceride concentrations in untreated hyperlipidemic dogs. Another study was conducted by Macotpet et al. (2013). They compared serum MDA concentrations of 213 clinically normal dogs against 107 dogs with malignant tumors. Indeed, cancer-bearing dogs had greater MDA concentrations (4.68±1.32 μmol/L) than clinically normal dogs (2.95±0.61 μmol/L). Although MDA measurement has been studied and used widely in humans and animals, some limitations exist [e.g., effects of diet and physical activity (PA), effects of sampling time and sample storage conditions, variation in the production of MDA and high reactivity of MDA and cross-reactions with biochemicals present in the biological samples, different analytical methods]. Also, uncertain results have been observed, such as increased MDA concentrations in healthy controls (Azizi et al., 2016). Therefore, further studies are needed to obtain more reliable MDA results.

Superoxide dismutase (SOD) is an efficient enzymatic antioxidant and works synergistically with cofactors. There are three forms of SOD; in the cytosol, on extracellular surfaces (both forms require copper and zinc), and in mitochondria (requires manganese). The mitochondrial SOD plays an essential role in defense mechanisms and scavenges superoxide anion, one of the major ROS produced during physiologic and pathophysiologic states. The

measurement of SOD concentration in biological samples can be used as an indicator of oxidative stress when the amount of SOD is low (Rahman, 2007). For example, one study reported that the plasma SOD concentrations of 15 diabetic cats were lower (211.4±143.5 U/mL) than that of 20 healthy cats (352.2±200.4 U/mL) (Webb and Falkowski, 2009).

HORMONAL DISTURBANCES ASSOCIATED WITH OBESITY IN DOGS

Several obesity-related hormones have been studied, including leptin, adiponectin, and insulin. Leptin is a satiety hormone, synthesized and secreted predominantly by white adipocytes, and with circulating leptin concentrations being positively correlated with body fat mass (Ricci and Bevilacqua, 2012). This hormone plays an important role in the regulation of food intake and energy expenditure (Myers et al., 2008). Klok et al. (2007) reported that leptin secretion was affected by age, gender, PA, and caloric intake of humans, in contrast with the information in dogs reported by Ishioka et al. (2007). They found that BCS had a positive correlation with plasma leptin concentration, but was not affected by age, gender or gonadectomy. Some additional factors that modulate leptin secretion include insulin, fasting and refeeding state, food intake (e.g., glucose, a large amount of fat and carbohydrates in the diet), circadian rhythm, feeding rate, medications (e.g., corticosteroids), and thyroid hormone concentrations (Ricci and Bevilacqua, 2012; Kuryszko et al., 2016). Based on the clinical surveys of eight veterinary practices in Japan from 1999 to 2003, dogs categorized as being lean (BCS 3 out of 5) or obese (BCS 5 out of 5) had plasma leptin concentrations of 3.0±0.4 and 12.8±0.8 ng/mL, respectively (Ishioka et al., 2007). Similar results were reported by Park et al. (2014). They measured serum leptin concentrations of dogs in two different age groups (age: > 8- or < 8-year old), with lean or obese conditions. Those results showed that leptin concentrations of lean dogs [2.64±0.44 ng/mL (< 8-year old) or 3.02±0.66 ng/mL (> 8-year old)] were lower than those of obese dogs [10.29±1.87 ng/mL (< 8-

year old) or 10.24±1.47 ng/mL (> 8-year old)]. Therefore, obese dogs have higher serum or plasma leptin concentrations than lean individuals, and this condition may develop into leptin resistance. Leptin resistance is the failure of elevated leptin concentration to suppress food consumption and maintain body mass. The potential mechanisms explaining leptin resistance include inadequate amounts of leptin being transported through the blood-brain barrier or alterations in cellular leptin receptor signaling in the arcuate nucleus of the hypothalamus (Myers et al., 2008).

Adiponectin is an adipocyte-derived hormone, which has insulin-sensitizing and lipid-reducing effects. In contrast to leptin, circulating adiponectin concentrations are negatively correlated with adiposity and feeding conditions, but are positively correlated with fasting condition in humans, rodents, and dogs (Ishioka et al., 2006; Lee and Shao, 2014). Ishioka et al. (2006) reported that of the 71 dogs that were sampled during their visit to an animal hospital in Japan, the mean adiponectin concentration of optimal weight dogs (33.4±2.9 μg/mL) was higher that that of obese dogs (16.8±3.0 μg/mL). Recent studies have shown similar trends, with lower adiponectin concentrations being observed in obese individuals. Park et al. (2014) tested 100 dog serum samples of 2 different age groups and reported adiponectin concentrations of 12.28±1.19 (< 8-year old) or 11.47±2.66 μg/mL (> 8-year old) for lean dogs, and 5.83±0.90 (< 8-year old) or 6.68±1.39 μg/mL for obese dogs. In humans and rodents, adiponectin has been categorized into low, middle and high molecular weight forms, with each form having different biological properties (Wang and Scherer, 2016). For example, a lower ratio of high-molecular weight (HMW) and total adiponectin concentration was present in animals with insulin resistance, indicating that HMW adiponectin correlates with insulin sensitivity and has a glucose-lowering effect (Pajvani et al., 2004). The effects of different adiponectin complexes are unknown in dogs and needs further study.

Insulin is an endocrine hormone synthesized and secreted by pancreatic β cells, and its function is to stimulate uptake, utilization, and storage of glucose. Furthermore, Chaudhuri et al. (2004) reported that insulin infusion reduces serum CRP and serum amyloid A (SAA) in human patients with acute myocardial infarction by 40% within 24 hours, supporting an anti-inflammatory property of insulin. The normal range of insulin concentration in dogs is not yet established, but one study reported mean serum insulin concentrations of 12 healthy female dogs (age: 2-8 years old) being 10.7±0.7 μU/mL (Cardinali et al., 2017). Obesity is a well-known cause of type 2 DM in humans and cats, but this fact is unclear for dogs because type 1 DM is the majority of canine DM cases, which is caused by an autoimmune destruction of pancreatic β cells (Clark and Hoenig, 2016). However, the relationship between obesity and insulin resistance has been described in dogs. Insulin resistance is characterized by elevated fasting plasma insulin concentrations and poor response by insulin-responsive tissues. Several studies have been conducted on this topic, including a study of seven adult beagles that were overfed with a high-energy diet for 7 months (Gayet et al., 2004). After body weight increased by 43±5%, in that study, insulin sensitivity was decreased by approximately 44±0.5%. German et al. (2009) also reported that the median fasting plasma insulin concentrations of 26 obese dogs (27 μU/mL) were reduced after weight loss (18 μU/mL).

PREVENTION AND MANAGEMENT OF OBESITY

Several obesity prevention and management strategies have evolved over the past few decades in human medicine, such as lifestyle modifications, increased PA, psychological therapy, pharmacotherapy, surgery, and dietary interventions. Many of these options are available for companion animals.

A recent study assessed PA levels in dogs with ideal weight, overweight or obesity using accelerometers. The total PA was expressed as means of counts per minute (cpm), and PA levels were classified into 3 categories: 1) sedentary behavior (<1351 cpm), 2) light-moderate intensity PA (1352-5695 cpm), 3) vigorous intensity (>5696 cpm). In that study, obese dogs spent less time in vigorous PA than healthy weight dogs (20 vs. 6 minutes/day) (Morrison et al., 2013). Warren et al. (2011) also reported the inverse correlation between low average daily steps and high BCS of dogs. Additionally, Chauvet et al. (2011) combined exercise regimen (walking on an underwater treadmill) in a weight loss study of eight obese dogs. Over a 3-month weight loss program, the dogs lost weight (18.9±5.44%), and the duration and distance of exercise had increased from 8 to 37 minutes and 0.05 to 2.7 kilometers per day, respectively. All evidence suggests that not only does low PA lead to obesity, but obesity may also promote low PA. Therefore, an exercise regimen should be added to both preventive and management programs for inactive or obese dogs.

Controlling food intake so that caloric intake does not exceed the daily maintenance energy requirements (MER) is a crucial component of weight management. The total MER of animals with similar body weights may differ depending on activity level, physiological status (e.g., growth, maintenance, gestation, lactation), and neuter status. Equations are used to estimate the energy requirement of dogs. Gross et al. (2010) developed an equation using a constant × the resting energy requirement (RER), which is suggested to be 70 x $BWkg^{0.75}$. The constants used are between 1.0 and 1.8 depending on the status of the animal. The National Research Council (2006) suggests calculating the MER of dogs based on breed, age, housing, PA, and physiological status (e.g., MER for inactive pet dogs = 95 × $BWkg^{0.75}$). Generally, neutering results in a reduction in resting metabolic rate of animals but increases appetite. Therefore, neutered pets require lower energy consumption compared to those that are intact. Mitsuhashi et al. (2011) observed that the

MER of neutered cats was less than the NRC recommendation for lean adult cats to maintain their body weight after spaying by 25% and lower than the cat's requirement before surgery by 26%. Nevertheless, MER of dogs after spay surgery have also been described, but further investigation is needed to increase the accuracy of energy intake.

Key dietary factors used to prevent and manage obesity include use of low-fat, high-fiber, and/or high-protein diets. Dietary fat is required for supporting normal physiologic functions and absorption of fat-soluble vitamins. It is a supplier of essential fatty acids and is a highly digested and metabolized source of energy in pet food, generating 2.25 times the metabolizable energy of proteins and carbohydrates. However, animals will gain weight and have increased retention of body fat when consuming food with more calories supplied from fat even, if the total calories consumed are reduced (Toll et al., 2010).

Not only is the concentration of fat in the diet important, but the type of fat also plays a role in the management of obesity. Polyunsaturated omega-3 fatty acids (FA) are well-known nutrients used in the management of several clinical problems including neoplasia, dermatological disease, hyperlipidemia, cardiovascular disease, renal disease, gastrointestinal disorders, and orthopedic disease. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are currently believed to be the most bioactive omega-3 FA and are rich in marine animals. The common functionalities of omega-3 FA are their anti-inflammatory properties, lipid-lowering effects, ability to increase serum adiponectin concentrations, and anti-tumor effects. Burri et al. (2018) tested a diet supplemented with 8% krill meal, a rich source of omega-3 FA, in a group of dogs competing in the 2016 Iditarod dog sled race, with 10 Iditarod dogs being fed with an experimental diet and another 11 dogs fed a control diet. Blood samples were collected and measured for plasma CRP and creatine kinase (CK) concentrations because they are indicators of inflammation and muscle

damage caused by an extreme exercise challenge. Plasma CRP and CK were increased to a lower extent in the supplemented group (CRP: from 4.26 ± 0.69 to 16.56 ± 3.03 μg/mL; CK: from 99.55 ± 12.15 to 515.69 ± 98.98 IU/L) compared to control group (CRP: from 7.05 ± 2.27 to 37.04 ± 9.16 μg/mL; CK: from 90.75 ± 8.15 to 715.90 ± 218.9 IU/L).

A recent study conducted by de Godoy et al. (2018) reported that the inclusion of 2% fish oil in the diet helped reduce the plasma cholesterol concentrations of overweight dogs after 30, 60, and 69 days of consumption compared to baseline (day 0). Another study reported that the mean adiponectin concentrations of 20 healthy dogs supplemented with 220 mg/kg of fish oil (offering a dose of 66 mg/kg DHA and EPA, once daily) for 30 days were 3.4 μg/mL higher than baseline, and 5.3 μg/mL higher than for the control group, with no change in body weight, body conditon score, or % body fat in either group (Mazaki-Tovi et al., 2014).

The effects of omega-3 FA on skeletal muscle metabolism have been investigated, and this property might also support the management of obesity. Skeletal muscle is an insulin-sensitive tissue that is important in metabolic homeostasis and energy expenditure. Recent evidence suggested that EPA is responsible for anabolic effects in skeletal muscle, increasing muscle protein synthesis by 25% compared to control cells. EPA also appears to play a role in attenuating muscle protein breakdown through the inhibition of the NF-κB pathway. Additionally, the treated cells had protein degradation 22% lower than the control cells (Kamolrat and Grey, 2013). Wang et al. (2013) observed that incubated cells with 400 μM DHA for 24 hours performed similar to 400 μM EPA by attenuating protein degradation, and with even greater efficiency (29% vs. 17% reduction).

Medium-chain triglycerides (MCT) are dietary triglycerides containing intermediate chain length FA (C6-12). In general, MCT are more rapidly hydrolyzed and absorbed than long-chain triglycerides (LCT) because of their low MW, and are transported directly in the portal venous

system to the liver, without being incorporated into chylomicrons. They also do not rely on carnitine for transport through the mitochondrial membrane. This makes MCT a readily utilized energy source. Coconut oil is known as a rich source of MCT (approximately 66% MCT), and lauric acid (C12) is the major medium-chain FA present (47.7% of the total fat), followed by caprylic acid (C8; 7.6%), capric acid (C10; 5.5%), and caproic acid (C6; 0.52%). In addition, only 20-30% of lauric acid from coconut oil is taken up by the intestine and is used in the same pathway as other MCT, which means that coconut oil contains only 23.16% MCT that are absorbed and metabolized the same way as pure MCT oil. Therefore, the functionality of coconut oil and MCT oil may not be the same (Hall and Jewell, 2012; Clegg, 2017).

The effects of MCT on satiety and weight loss have been investigated in several human studies, but only a few studies have been conducted in dogs. Potential mechanisms that affect satiety include the production of ketones and the rapid and complete absorption of MCT. Additionally, Kinsella et al. (2017) reported that coconut oil promotes a less satiating effect when compared with MCT oil. In a recent study, researchers compared BW and fat mass changes in dogs over a 14-week weight loss period. Three diets supplemented with 8% fat were fed to dogs with fat being supplied by the following sources: C0 [8% of soya bean-canola esterified fatty acid oil (EAO)], C20 (6.4% of soya bean-canola and 1.6% of coconut EAO), or C40 (4.8% of soya bean-canola and 3.2% of coconut EAO). At the end of the study, the C0 group lost more weight and fat mass compared to C20 and C40 groups that were fed a mixture of coconut oil (Fragua et al., 2015). It appeared that the results conflicted with the known benefits of coconut oil. To date, there is lack of research and a lot still unknown as regards the functionality of MCT oil and coconut oil, especially in dogs. Further work is needed to confirm the beneficial effects of both substances.

The quantity and type of carbohydrate also play crucial roles in the management of obesity. Generally, an excess of ingested digestible carbohydrates from the body's energy needs is stored as glycogen or converted to fat. Because the liver has a limited ability to store glycogen, excess glucose is often stored in the form of fat (Fung, 2017). Dietary fiber is nondigestible soluble and insoluble carbohydrates (≥ 3 monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (≥ 3 monomeric units), determined by FDA to have physiological effects that are beneficial to human health (U.S. Food and Drug Administration, 2016). Fiber is well recognized for its many health benefits, particularly in the prevention and treatment of obesity.

Short-chain fructooligosaccharides (scFOS) are an inulin-type fructan fiber that is completely fermented by colonic bacteria and results in the production of short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate. scFOS has been shown to alter glucose and lipid metabolism. Respondek et al. (2007) tested scFOS (1% wt:wt dry matter) in the diet fed to eight obese dogs in a cross-over design study. They reported that the glucose infusion rate was increased, and insulin sensitivity index tended to be greater in the scFOS-supplemented group compared with controls (7.77 vs. 4.72 mg/kg/min, and 0.047 vs. 0.026 mg/kg/min, respectively). These data suggest that scFOS improve peripheral insulin sensitivity in obese dogs. Additionally, some data showed that the blend of FOS and sugar beet fiber at the 10% inclusion level (DMB; 8% FOS and 2% sugar beet pulp) was able to lower postprandial blood glucose, triglyceride and cholesterol concentrations (Diez et al., 1997). The decreases in plasma cholesterol may have been due to an increase in bile acid synthesis corresponding to the excretion of bile acids in the feces (Surampudi et al., 2016). Additionally, scFOS reduced cholesterol absorption, enhanced loss of cholesterol via

the feces, or inhibited cholesterol synthesis by propionate. The primary mechanism of lowering plasma triglycerides may be due to decreases in the activity of hepatic lipogenesis (Baylot, 2005).

β-glucans are soluble fibers that are presented primarily in oats and barley. β-glucans have gained interest in their use in human and pet nutrition. They are commonly used in diets for patients with high serum cholesterol concentrations (Reyna-Villasmil et al., 2007; Shimizu et al., 2008; Cloetens et al., 2012; Smith et al., 2013). The reduction in serum cholesterol concentrations by β-glucans is primarily due to its viscosity and fermentation capabilities (Nakashima et al., 2018). An increased digesta viscosity impairs the emulsification of lipids by bile salts in the small intestine, and as a result, disturbs lipid digestion and absorption. This may result in lower circulating total cholesterol (TC) and LDL-C concentrations, without affecting HDL-C. Moreover, β-glucans can bind bile acids, decreasing bile acid reabsorption in the small intestine, and increasing elimination via feces. Another possible mechanism by which β-glucans exhibit cholesterol-lowering effects was demonstrated by Wang et al. (2017). They reported a greater increase in 7 α-hydroxylase (7 α-HC) concentrations (p = 0.049) in subjects consuming 3 grams of high-molecular-weight β-glucan compared to controls. The results from that study suggested that β-glucans modulate cholesterol metabolism by increasing bile acid synthesis without affecting the absorption or synthesis of cholesterol. This action promotes the utilization of cholesterol for synthesis of bile acids, thus lowering cholesterol in the bloodstream (Anderson et al., 2009; Gunness and Gidley, 2010). Whole grain barley also contains phytochemical compounds (e.g., phenolic acids, flavonoids, lignans, tocols, phytosterols, and folate) that may provide benefits and protection from certain diseases (Idehen et al., 2017). Furthermore, increased consumption of fiber may reduce the amount of energy absorbed from the diet by reducing fat and protein digestibility (Weber et al., 2007; Adam et al., 2014; Kröger et al., 2017; Davis, 2018; Hervik and Svihus, 2019).

The fermentation of dietary fibers by microbiota in the large intestine produce SCFA in a molar ratio of approximately 4 acetate: 1 propionate: 1 butyrate. The SCFA are absorbed and used as an energy source, but fermentation involves microbial and heat losses so caloric density is low. Moreover, dietary fibers affect satiety through several physiological mechanisms in the body. Their physiochemical properties, including physical structure, water-holding capacity, and viscosity (Hervik and Svihus, 2019) may all impact satiety. Weber et al. (2007) studied the satiation of a HPHF diet compared with high-protein (HP) or high-fiber (HF) diets in dogs, with five different voluntary food intake (VFI) tests plus one palatability test being performed. The overall results from those tests indicated that dogs consuming the HPHF diet ate less than those fed the other diets, without negatively affecting the palatability of the foods. Bosch et al. (2009) reported similar results, with a trend for lower VFI in 16 dogs fed a high-fermentable fiber diet (HFF: containing 8.5% sugar beet pulp) compared with dogs fed a low-fermentable fiber (LFF: containing 8.5% cellulose). From the existing evidence, dietary fibers may influence appetite regulation of dogs.

Lastly, the effects of feeding a HP diet to dogs has been observed in several studies. A recent study evaluated the body composition of 14 neutered dogs fed a diet containing 59.7 g protein/1000 kcal (P60) or a diet containing 94 g protein/1000 kcal (P94). After 26 weeks of feeding dogs to maintain BW, fat mass of dogs fed the P60 diet (2.3±0.57 kg) increased at a greater level than dogs fed the P94 diet (1.8±0.52 kg), but the body mass was not different (Kawauchi et al., 2017). Vasconcellos et al. (2009) conducted a weight loss study in obese cats, feeding a control (Co: 89.6 g protein/1000 kcal) or HP (118.9 g protein/1000 kcal) diet. Cats fed the HP diet increased energy intake during the weight loss phase and subsequently increased their energy

requirement in the maintenance phase compared with cats fed the Co diet (31.4±1.6% vs. 18.2±1.4%).

WEIGHT LOSS AND ITS OUTCOMES

Obese individuals not only encounter the morbid condition but they also suffer from concomitant diseases. Fortunately, the detrimental consequences of obesity could be reversible by weight loss. The foundation of successful weight loss is a combination of dietary energy restrictions using a proper-formulated diet and increase physical activity. Most diets that are designed for weight loss regimens have increased high-quality protein content and micronutrient to preserve lean muscle mass and avoid nutrient deficiency during a weight loss program. However, such diets have a low energy density due to substitution of high-calorie ingredients with functional dietary fiber (Blanchard et al., 2004; German et al., 2007; Floerchinger et al., 2015; Pallotto et al., 2017). One challenge during the weight loss process is to minimize hunger when food intake is restricted. Therefore, increased dietary fiber and protein may also aid in mitigating this effect (Weber et al., 2007; Bosch et al., 2009; Ben-Harchache et al., 2020). Several studies have shown the positive effects of weight loss. The reduction of leptin, insulin, glucose, and CRP concentrations were positively correlated while adiponectin concentration was negatively correlated with body fat mass and body weight loss (Jeusette et al., 2005; German et al., 2009; Starr et al., 2019). These findings suggest that weight loss improves metabolic responses.

GUT MICROBIOTA AND HOST HEALTH

The intestinal microorganisms or microbes consist of bacteria, fungi, protozoa, and viruses. The number of microbes (bacteria is the main population) is estimated to be 10^{14} to 10^{15} cells, which accounts for a 1:1 ratio of microbial cells to host cells (Sender et al., 2016). Interestingly, the microbial inhabitants create a complex ecosystem in the gut and directly influence host

physiology and metabolism through direct contact with the organisms and microbiota-derived metabolites (Savage, 1986). Microbiota-host interactions may have beneficial and detrimental effects. The commensal bacteria help with food digestion and absorption, compete with pathogenic bacteria for nutrients, produce antimicrobial substances, and prevent pathogen colonization, in the gastrointestinal tract (GIT), strengthen the intestinal epithelial cells and tight junctions, modulate gut-associated lymphoid tissue, and generate several metabolite compounds that benefit the host (Suchodolski, 2011). However, dysbiosis can be an important factor in many diseases, including inflammatory bowel disease, type 2 diabetes, and obesity (Weiss and Hennet, 2017).

Gut microbiome research has gained exponential interest in the past decade. Most studies to date have focused on bacterial taxonomy, but recent research has emphasized the functional gut microbiome on health and disease. The gut microbiome has been an important area of research in several clinical conditions such as gastrointestinal disorders (e.g., inflammatory bowel disease and colorectal cancer), type 2 diabetes, cardiovascular disease, and stress-related psychiatric disorders (Muñoz-Garach et al., 2016; Dinan and Cryan, 2017; Tang et al., 2017; Hills et al., 2019). Obesity-related diseases are also an area of interest and gut microbiota might be a new route for preventive or therapeutic intervention.

CANINE GUT HEALTH AND MICROBIOTA

Although knowledge of the canine gut microbiota has been expanded recently, a lot still needs to be elucidated. Molecular phylogenetic studies, using a comparative 16S rRNA gene analysis to typically analyze fecal samples, have revealed a complex microbial community in the canine gastrointestinal tract. The majority of gut microbes in the large intestine are anaerobic bacteria with the dominant bacterial phyla in dogs being Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Proteobacteria. Bacterial composition and diversity vary depending on several

factors, including animal characteristics (e.g., species, age, sex, breed), environmental factors (e.g., diet, medications, and lifestyle), health status, sample material and handling, and laboratory approaches (Deng and Swanson, 2015).

Obesity is an imbalance between energy intake and expenditure, resulting in excess body weight, accumulative fat, and persistent low-grade inflammation. It is a multifactorial disease that is now a global health concern and compromises social and economic costs. In recent years, omic technologies have become available and widely incorporated into research, providing an advanced understanding of the mechanisms involved in obesity development. The gut microbiome has been extensively studied and now thought to contribute to obesity. Bäckhed et al. (2004) reported greater body fat mass deposits in wild-type mice than in germ-free mice fed the same diet. Turnbaugh et al. (2006a) experimented with the activities of the unique microbial composition of obese subjects that could be transmissible from one to another. Germ-free mice were colonized with cecal microbiota harvested from obese or lean donors. The results showed a greater increase in total body fat in germ-free mice colonization with microbiota from obese versus lean donors. These findings suggested that microbiota participated in host metabolism and energy harvest regulation. Additionally, the abundance of different intestinal microbiota potentially affects body composition and might contribute to certain diseases such as obesity. Furthermore, Cani et al. (2007) reported that feeding a high-fat diet induced enterotoxemia and glucose intolerance, increased fat mass and body weight, and changed the abundance of *Bifidobacterium spp.* The findings revealed that dietary patterns can also change the microbial composition and consequences on the host. The characteristics of gut microbiota in obese individuals have been observed in humans and animals. These include a lower fecal bacterial diversity and richness, increased abundance of Actinobacteria and Firmicutes, decreased abundance of Bacteroidetes, and

increased Firmicutes: Bacteroidetes ratio in overweight or obese humans (Turnbaugh et al., 2006b; Davis, 2016). Similar results were observed in a study by Park et al. (2015). They also reported that gut microbial diversity was lower in obese dogs compared to the lean group, however, the predominant bacterial phyla in lean dogs was Firmicutes (85%), followed by Actinobacteria while obese dogs were primarily composed of Proteobacteria (76%) followed by Firmicutes. These features could be referred to as gut dysbiosis due to obesity. This condition alters gut barrier integrity, subsequently leading to a reduction in gut permeability, the breakdown of tight-junctions, and bacterial translocation which may be an early factor in the development of inflammation and insulin resistance (Saad et al., 2016). Reports from Swanson et al. (2011) and Coelho et al. (2018) revealed that the dog gut microbiome shared the similarity in genes and responses to stimuli (e.g., diets) compared to the human gut microbiome. Therefore, the findings in humans may be predictive of dog gut microbiome results and vice versa.

Obesity causes an imbalance in the gut microbiota, but can be restored in response to a weight-loss regimen. A weight-loss strategy may involve dietary intervention with restricted-energy intake and a HPHF diet. In a clinical controlled weight-loss study, consuming an energy-restricted HP (35% protein) diet supplemented with soluble fiber (mainly inulin) impacted the microbial composition by increasing bacterial gene richness in low gene counts individuals and abundance of *Faecalibacterium prausnitzii*, *Bacteroides dorei, Eubacterium eligens, Roseburia homini*, *and Ruminococcus* species and by decreasing *Eubacterium rectale* (Cotillard et al., 2013). A recent clinical weight-loss trial in client-owned obese dogs revealed changes in the microbial structure after weight loss. Alpha diversity (richness and evenness) and the relative abundance of Bacteroidetes and Fusobacteria increased whereas the abundance of Firmicutes and Firmicutes:Bacteroidetes ratio decreased (Bermudez Sanchez et al., 2020).

Estrogens [e.g., estradiol (mainly), estriol, estretrol, and estrone] are steroid hormones that are synthesized primarily from the gonads (i.e., ovary and testes). Their receptors [estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), G-protein-coupled estrogen receptor 1 (GPER1)] are expressed in various tissues, for instance, intestine, brain, bone, and adipose tissue (Eyster, 2016). As a result, estrogen not only influences reproductive organs but also nonreproductive systems (e.g., neuroendocrine, vascular, skeletal, immune, and digestive system) (Homma et al., 2005; Hamilton et al., 2017). Interestingly, extra-gonadal tissues, including the adrenal gland, brain, adipose tissue, skin, pancreas, and other sites yet to be identified also produce the estrogen hormone in a form of estradiol which is a potent form and has equal roles aid biological activities as gonadal-derived estrogen (Baracat et al., 2016). After metabolism, estrogens are excreted via urine, feces, or bile. Hypoestrogenic or hyperestrogenic conditions affect normal physiological functions. Several factors alter the homeostasis of the estrogen hormone, including the gut microbiota. Gut bacteria secrete the β-glucuronidase enzyme to metabolize estrogens to deconjugated forms, with deconjugated estrogens being reabsorbed into the bloodstream and recirculated via enterohepatic circulation to target tissues. Phytoestrogens, such as isoflavones, are present in various plants and seeds, particularly in soybeans. These compounds exert estrogenic activity and are also metabolized in the same manner as endogenous estrogens (Messina, 2014). Estrogen concentration is not only influenced by gut microbiota, but it also impacts the gut microbiome. Santos-Marcos et al. (2018) identified the differences in gut microbial communities between pre- and postmenopausal women with the Firmicutes:Bacteroidetes ratio being higher and the relative abundance of *Lachnospira* and *Roseburia* being lower in postmenopausal women than in premenopausal women. This suggested that the estrogen hormone plays a critical role in intestinal microbial diversity and richness.

Therefore, conditions that reduce the circulating estrogen level and alter the balance of gut microbiota composition (e.g., obesity and lack of estrogen hormone) potentially disrupt estrogen-gut microbiome homeostasis.

Gut microbiota profiles have been changed through the evolution of feeding behaviors and nutrition. Food is not only central to the daily lives of humans and animals, but is an important factor to shape the structure and functionality of microorganisms in the gut. Generally, most dietary nutrients are absorbed in the small intestine, however, some escape the digestion and absorption processes and pass to the hindgut. Diet-gut microbiota interactions take place mostly in the large intestine, where non-digestible food ingredients are digested and utilized by microbes. In return, gut microbes generate nutritional resources (e.g., SCFA, vitamins, and amino acids) that may be utilized by the host (Read and Holmes, 2017). Changes in dietary macronutrient profiles, including carbohydrates, protein, and fat, may result in significant shifts in the gut microbiota.

Dietary fibers or non-starch carbohydrates include plant cell wall polysaccharides, oligosaccharides (e.g., FOS and MOS), and resistant starch cannot be digested by enzymes possessed by non-ruminant animals, but can be broken down and utilized by microbiota. Therefore, they serve as food sources for gut microbes and may be a predictor of the structure of the gut microbial population. Bacteroidetes are generalist bacteria when it comes to glycan catabolism, while Firmicutes are specialists that are selective for specific glycans (Darrell et al., 2016). Gut bacteria use carbohydrate-active enzymes (CAZymes) to cleave glycosidic bonds of glycoconjugates, oligosaccharides, and polysaccharides to monosaccharides. CAZymes are categorized into five types, Glycoside Hydrolase (GH), GlycosylTransferase (GT), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE), and Auxiliary Activities (AA) (Carbohydrate-active enzymes database). Bacteroidetes encode an average of 137 GH and PL, while Firmicutes encode

an average of 40 GH and PL per genome (Kaoutari et al., 2013). Two *Bacteroides* species*, (B. thetaiotaomicron and B. ovatus),* which are members of the Bacteroidetes family are capable of utilizing all major glycan classes, except for cellulose present in plants (Martens et al., 2011). Other bacteria also participate in fermentation, for instance, *Bifidobacterium* species have the ability to hydrolyze inulin-type fructans and oligosaccharides (e.g., arabinoxylans, FOS, and galactooligosaccharides) (Parche et al., 2007). Some bacteria have a symbiotic relationship with another through the cross-feeding phenomenon. For example, Bifidobacteria hydrolyze degraded glycan products coming from Bacteroidetes fermentation. Other bacteria utilize fermentation products (e.g., lactate, succinate, propionate, and butyrate) produced from the degradation processes of other taxa (Louis and Flint, 2017; Turroni et al., 2018). The findings suggested that gut bacteria have their preferences for various kinds of fibers.

Dietary plant cell walls are valuable dietary fiber sources that are primarily composed of β-glucans, pectins, hemicelluloses, cellulose, and lignin. Pectin is a soluble, gel-forming fiber that is present in fruits, vegetables, and sugar beet pulp (Prandi et al., 2018). Bacteroidetes and Firmicutes are the two main bacterial phyla responsible for the degradation of pectin, with the *Eubacterium eligens, Faecalibacterium prausnitzii,* and *B. thetaiotaomicron* strains being some of the most active (Chung et al., 2017). Furthermore, Middelbos et al. (2010) reported that supplementation of 7.5% beet pulp (60% of total dietary fiber) in the diet fed to dogs increased Firmicutes (15% to 28%) abundance and decreased density of Fusobacteria (40% to 24), with no changes in Bacteroidetes.

Similar to other dietary fibers, β-glucans are resistant to enzymatic digestion in the small intestine and pass through to the large intestine where it is partially or completely fermented by colonic bacteria. The fermentation alters the community structure of the microbial population and

subsequently elicits specific functionality. For example, consumption of oat β-glucans increased the abundance of *Bifidobacterium, Lactobacillus,* and *Prevotella* species and lead to a reduction of cholesterol concentration (Zhou et al., 2015; Connolly et al., 2016). The gut microbiome impacts cholesterol and bile acids by various mechanisms. Some bacterial genera, including *Clostridium, Bifidobacterium, Lactobacillus, Bacteroides, and Enterococcus* can produce a catalytic enzyme, bile salt hydrolase (BSH) that deconjugates primary bile acids, making them less soluble and therefore less readily reabsorbed. Moreover, some bacterial strains such as *Eubacterium, Bacteroides, Bifidobacterium, Lactobacillus, and Peptostreptococcus* can directly convert cholesterol into coprostanol, which is excreted in feces (Kriaa et al., 2019). All of these mechanisms influence cholesterol homeostasis and subsequently reduce the serum cholesterol level.

Some dietary fiber types have been tested to determine their functions as prebiotics. FOS is one of the well-known prebiotics and is present in chicory and Jerusalem artichoke that have been used in human and companion animal food products. FOS has been associated with several health benefits. For instance, the reduction of hepatic glucose production was reported in response to a daily intake of 20 g FOS in diabetic humans (Luo et al., 2000). Additionally, consumption of FOS stimulated mineral absorption, mainly calcium, magnesium, sodium, zinc, and iron in adult dogs (Pinna et al., 2018). FOS encourages the growth of bifidobacteria and discourages the growth of potentially putrefactive microorganisms. Flickinger et al. (2003) reported the number of *Clostridium perfringens* was linearly decreased by FOS supplementation (1 to 3 g of scFOS/d) and the concentration of this bacteria species tended to lower when all FOS supplemented groups were compared with the control. The interaction of scFOS (1.5% inclusion) and protein concentration in the diet has been shown to affect fecal bacterial counts. A HP diet (CP: 30.4% DM) + scFOS

increased whereas a low-protein diet (CP: 22.9% DM) + scFOS decreased *Bifidobacterium spp.* (Pinna et al., 2018). However, Barry et al. (2009) reported that the inclusion of 0.4% of scFOS in the diet did not alter fecal bacterial populations in dogs when compared to a control.

The amount of protein consumed and its sources and processing, which determines protein digestibility, modulates the gut microbial composition and metabolic activity. A comparative review of ileal and fecal protein digestibility in non-ruminant species reported that the average apparent large intestinal nitrogen digestibility was 33.7% for dogs (Hendriks et al., 2012). This finding showed a significant disappearance of nitrogen in the large intestine due to the bacterial metabolism of non-digested nitrogenous compounds. The proteolytic activity present in the large intestine has been mainly attributed to the *Bacteroides, Clostridium, Propionibacterium, Streptococcus, Fusobacterium,* and *Lactobacillus* genera (Davila et al., 2013). Protease enzymes secreted from the bacteria, particularly *Bacteroides* species, might reduce maltase and sucrase enzyme activities in the brush border membrane of the enterocytes (Riepe et al., 1980). David et al. (2014) reported that the consumption of animal-based protein (30% of energy intake) increased the abundance of bile-tolerant anaerobes such as *Alistipes, Bilophila,* and *Bacteroides* but decreased the level of Firmicutes such as *Eubacterium rectale, Roseburia,* and *Ruminococcus* species. Additionally, Ford et al. (2020) reported that high protein intakes increase the abundance of *Lactobacillus, Lactococcus,* and *Streptococcus* in older women. High-dietary protein levels have been applied in feeding regimens and shown to decrease fat mass and preserve fat-free mass during weight loss, reduce the risk of sarcopenia in elderly people, and enhance lean tissue growth in livestock animals (Gilbert et al., 2018; Ford et al., 2020; Moon and Koh, 2020). Nevertheless, consumption of high and/or undigested proteins was reported to enhance the growth of gut pathogenic and protein-fermenting bacteria and therefore increase protein fermentation-derived

metabolites which are associated with increasing the risk of gastrointestinal diseases in humans and livestock animals (Gilbert et al., 2018). However, little is known about the effects of short-term or long-term consumption of high protein diets in dogs.

In line with high carbohydrate intake, the consumption of high dietary fat continues to increase in the modernized world. Both the type and amount of dietary fat modulate gut microbiota homeostasis. High dietary fat intake may increase the quantity of fat consumed, promoting the secretion of primary bile acids that can reach the colon and further be metabolized by gut microbiota. In particular, about 15% of conjugated primary bile acids are deconjugated and modified to secondary bile acids and are either reabsorbed or excreted via feces. Additionally, a secondary bile acid, deoxycholic acid, has an antimicrobial activity that can damage bacterial cell membranes and prohibit bacterial overgrowth (Ciaula et al., 2017). Caesar et al. (2015) compared diets containing either saturated (lard) or polyunsaturated fats (fish oil) fed to mice. They reported that *Bifidobacterium, Lactobacillus, Streptococcus,* and *Akkermasia muciniphila* were increased in fish-oil-fed mice, while *Bacteroides, Turicibactor,* and *Bilophila* were increased in lard-fed mice.

MICROBIOTA-DERIVED METABOLITES AND THEIR FUNCTIONS

Products of bacterial fiber fermentation, such as SCFA, especially acetate, propionate, and butyrate, have been well documented and have potential effects in maintaining gut health and regulating appetite control in the brain (Hu et. al., 2017). The vast majority of anaerobic bacteria are responsible for acetate production and this substance exerts the highest concentration among the SCFA. Many Firmicutes, Ruminococcaceae, Lachnospiraceae, and Clostridiaceae families are butyrate-producing bacteria, while bacteria in the Bacteroidetes family produce propionate through the succinate pathway (Reichardt et al., 2014). Butyrate has been well documented for its anti-

inflammatory and anti-carcinogenic effects in the colon and that it is a major fuel source for colonocytes (Hamer et al., 2008). Luceri et al. (2016) conducted a randomized controlled clinical trial in patients with diverticulitis, colorectal cancer, or IBD. The patients received a sodium butyrate (600 mmol/L) enema twice daily for 30 days. They reported improvements in endoscopic scores, mucosal atrophy, and upregulation of genes associated with mucosal repairs, however, the changes were not consistent among the patients. These variable results may have been due to the diversity of the disorders each individual had. Furthermore, butyrate and propionate have potential roles in appetite regulation. Chambers et al. (2015) investigated the efficacy of acute and long-term supplementation of 10 g/d inulin-propionate ester on appetite control. Propionate stimulated the secretion of PYY and GLP-1 hormones from colonocytes and reduced energy intake by 13.8%. After 24 wk, the participants had a reduction in body weight, intraabdominal adipose tissue distribution, and intrahepatocellular lipid content. Li et al. (2018) reported that acute oral administration of 5% (W/W) sodium butyrate successfully decreased food intake in mice.

Dietary proteins and peptides that are in excess of requirements and escape digestion undergo proteolysis and fermentation via gut microbes, resulting in the production of both beneficial and undesirable metabolites. These microbial metabolites include SCFA, BCFA, ammonia, polyamines, hydrogen sulfide, and phenolic and indolic compounds. BCFA originate from the fermentation of branch-chain amino acids (e.g., valine, leucine, and isoleucine) and comprise isobutyrate, isovalerate, and valerate. Isobutyrate has been known to be used as an energy source for colonocytes by Jaskiewicz et al. (1996). In vitro studies reported that valerate exerts an antiproliferative effect while isovalerate and isobutyrate had weak responses on cell differentiation and apoptosis on human colorectal adenocarcinoma cell lines (Heerdt et al., 1994; Siavoshian et al., 1997). To date, the benefits or detrimental effects of BCFA have not been well documented.

Fermentation of aromatic amino acids (e.g., phenylalanine, tyrosine, and tryptophan) yields phenolic and indolic compounds. Phenol and indole are produced by the degradation of tyrosine and tryptophan, respectively (Hughes et al., 2000). Studies have shown that indole might have beneficial effects on intestinal epithelium. Indole improved the inflammatory responses and increased the expression of genes involved in strengthening the mucosal barrier in human colon cancer cell lines (Bansal et al., 2010). Additionally, Shimada et al. (2013) reported that germ-free mice supplemented with indole increased mRNA expression of tight junction and adherens junction-associated molecules which implied the strength of barrier function. Contrary to indole, phenolic compounds are associated with increase paracellular permeability and reduce epithelial barrier function of colonocytes, which may promote carcinogenic activity (Hughes et al., 2008). In addition, the supplementation of 0.9% FOS was demonstrated to decrease fecal phenols and increase fecal SCFA concentrations in dogs (Propst et al., 2003). FOS has been demonstrated to counteract the production of protein-derived catabolites, instead, it enhances the production of saccharolytic fermentation and increases SCFA concentrations (Swanson et al., 2002; Flickinger et al, 2003).

Gut bacteria produce ammonia by deamination of amino acids and hydrolysis of urea (Wrong and Vince, 1984). Existing evidence indicates the deleterious effects of ammonia on the intestinal epithelium of the host. An in vitro study revealed that exposure to ammonia impaired gut barrier function (Hughes et al., 2008). Lin and Visek (1991) also reported that the perfusion of ammonium chloride or ammonium acetate into the large intestine of mice resulted in abnormalities of brush border and epithelial cells, with the aggregation of lymphocytes in the lamina propria.

Bile acids are synthesized from cholesterol in the liver in the form of primary bile acids (cholic acid, CA; and chenodeoxycholic acid, CDCA) and are subsequently conjugated to amino

acids, glycine or taurine. Later on, bile acids are subjected to biotransformations to secondary bile acids by gut microbes, particularly by the bacteria in the genera *Clostridium, Enterococcus, Bifidobacterium, Lactobacillus,* and *Bacteroides*. Two major transformations include hydrolysis of conjugated primary bile acids by BSH, then chemical modification by 7 α-dehydroxylation, and 7 α-dehydrogenation to generate secondary bile acids (Ridlon et al., 2016). Generally, there are about 70% primary bile acids [CA (35%) and CDCA (35%)], 28% secondary bile acids [deoxycholic acid (DCA, 25%), lithocholic acid (LCA, 1%), and ursodeoxycholic acid (UDCA, 2%)] in the biliary bile acid pool, which is similar to the bile acid profile in the small intestine. In contrast, the composition of bile acids in the colon comprises about 4% of unconjugated primary bile acids [CA (2%) and CDCA (2%)] and 65% of secondary bile acids [DCA (34%), LCA (29%), and UDCA (2%)] (Ridlon et al., 2005). A number of studies have demonstrated the associations of microbial disturbances in alterations of host bile acid profiles that occur in several disease states, including IBD, IBS, *Clostridium difficile* infection, colon cancer, cholesterol gallstones, asthma, and obesity. Unlike most of the inflammatory GI disorders that are related to the reduction of secondary bile acids and increment of primary conjugated bile acids, the composition of bile acids in obese conditions and certain GI diseases may be the opposite. Low concentrations of CA and CDCA were observed in obese individuals whereas high concentrations of DCA and LCA are examined in colon cancer and cholesterol gallstone disease patients (Low-Beer and Nutter, 1978; McGarr et al., 2005; Joyce and Gahan, 2017).

CONCLUSION

In summary, obesity is considered to be one of the major medical diseases in humans and companion animals, particularly in dogs and cats, with the incidence continuing to increase. Several factors contribute, including gonadectomy, however, little is known about the

pathophysiological changes, particularly gut microbial structure and microbial-derived metabolites that may be altered after spaying and could advocate the onset of the disease in dogs. Dietary interventions may be a practical solution for the prevention and management of obesity. Indeed, a weight-loss regimen using a specially formulated diet has been recognized as an effective strategy for the management of canine obesity, nevertheless, the information on metabolic changes, fecal microbial profiles, and fecal metabolites of dogs undergoing the process is limited and needs further investigation. The overall objective of this dissertation was to investigate the effects of HPHF diet consumption in adult female dogs undergoing weight gain and weight loss conditions. The four primary research aims are listed below.

The first aim was to evaluate the effects of dietary macronutrient profile on apparent total tract macronutrient digestibility and fecal microbiota populations, fermentative metabolite concentrations, and bile acid concentrations of adult female dogs after spay surgery. We hypothesized that HPHF diets would reduce nutrient and energy digestibilities and beneficially shift fecal microbiota, fermentative-end products, and bile acids after spay surgery as compared to a control diet.

The second aim was to investigate the effects of restricted feeding of a HPHF diet and weight loss on complete blood cell count, serum chemistry profile, serum hormone concentrations, and serum inflammatory marker concentrations, body composition, and voluntary physical activity of overweight dogs. We hypothesized that closely monitoring BW and adjusting intake of a HPHF diet would lead to steady weight loss and increase fat loss while maintaining lean mass, Additionally, we hypothesized that weight loss would increase voluntary physical activity, reduce blood lipids, and reduce inflammatory markers of overweight dogs.

The third aim was to evaluate the effects of restricted feeding of a HPHF diet and weight loss on fecal characteristics and fermentative metabolite concentrations, and fecal bile acid concentrations of overweight dogs. We hypothesized that weight loss and consumption of a HPHF diet would beneficially alter the fecal metabolite concentrations. The concentrations of fecal SCFA and certain fecal bile acids would be increased whereas fecal protein catabolites and total fecal bile acid would be decreased.

The fourth aim was to study the effects of restricted feeding of a HPHF diet and weight loss on fecal microbiota of overweight dogs. We hypothesized that weight loss and consumption of a HPHF diet would beneficially alter the fecal microbiota community (e.g., the relative abundances of *Bifidobacterium, Lactobacillus, Faecalibacterium, Romboutsia,* and *Fusobacterium* would increase while the relative abundances of *Catenibacterium, Streptococcus,* and *Megamonas* would decrease) and create a balanced gut microbial environment.

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*CHAPTER 3: EFFECTS OF DIETARY MACRONUTRIENT PROFILE ON APPARENT TOTAL TRACT MACRONUTRIENT DIGESTIBILITY AND FECAL MICROBIOTA, FERMENTATIVE METABOLITES, AND BILE ACIDS OF FEMALE DOGS AFTER SPAY SURGERY

ABSTRACT

Obesity and estrogen reduction are known to impact the gut microbiota and gut microbial-derived metabolites in some species, but limited information is available in dogs. The aim of this study was to determine the effects of dietary macronutrient profile on apparent total tract macronutrient digestibility, fecal microbiota, and fecal metabolites of adult female dogs after spay surgery. Twenty-eight adult intact female beagles (age: 3.02 ± 0.71 yr, BW: 10.28 ± 0.77 kg; BCS: 4.98 ± 0.57) were used. After a 5-wk baseline phase (wk 0), 24 dogs were spayed and randomly allotted to one of three experimental diets (n=8/group): 1) control (CO) containing moderate protein and fiber (COSP), 2) high-protein, high-fiber (HPHF), or 3) high-protein, high-fiber plus omega-3 and medium-chain fatty acids (HPHFO). Four dogs were sham-operated and fed CO (COSH). All dogs were fed to maintain BW for 12 wk after spay, then allowed to consume twice that amount for 12 wk. Fecal samples were collected at wk 0, 12, and 24 for digestibility, microbiota, and metabolite analysis. All data were analyzed using repeated measures and linear Mixed Models procedure of SAS 9.4, with results reported as change from baseline.

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Apparent organic matter and energy digestibilities had greater decreases in HPHF and HPHFO than COSH and COSP. Increases in fecal acetate, total short-chain fatty acids, and secondary bile acids were greater and decreases in primary bile acids were greater in HPHF and HPHFO. Principal coordinates analysis of weighted UniFrac distances revealed that HPHF and HPHFO clustered together and separately from COSH and COSP at wk 12 and 24, with relative abundances of Faecalibacterium, Romboutsia, and Fusobacterium increasing to a greater extent and Catenibacterium, Bifidobacterium, Prevotella 9, Eubacterium, and Megamonas decreasing to a greater extent in HPHF or HPHFO. Our results suggest that HPHF diets alter nutrient and energy digestibilities, fecal metabolite concentrations, and fecal gut microbiota, but spay surgery had minor effects. Future research is needed to investigate how food intake, nutrient profile, and changes in hormone production influence gut microbiota and metabolites of dogs individually and how this knowledge may be used to manage spayed pets.

INTRODUCTION

Globalization and modernization have allowed people to afford and access food easily, greatly influencing lifestyle, eating behaviors, and life expectancy. Not all of these changes have been positive, however, as diseases characteristic of the modern age such as obesity, cardiovascular disease, and type 2 diabetes continue to increase. A similar situation exists in companion pets (e.g., dogs and cats). Nowadays, pet owners treat their pets as irreplaceable members of the family, but this relationship is a two-edged sword. A tight human-pet relationship may be beneficial in some respects, but owner attitudes toward food and misperceptions about proper feeding and exercise are risk factors for pet obesity (German, 2006; Courcier et al., 2010; Linder and Mueller, 2014; Endenburg et al., 2018; Muñoz-Prieto et al., 2018). Additionally, while

developments in veterinary medicine, such as surgical sterilization by ovariectomy, are useful in controlling pet overpopulation, they also contribute to pet obesity risk (Lefebvre et al., 2013).

Indeed, obesity is known to predispose humans and pets to several metabolic disorders, including hyperlipidemia, joint disease, and type 2 diabetes (German, 2006; Cabellero, 2019). More recently, the effect of obesity on the gastrointestinal microbiota (e.g., dysbiosis) and potential disease has also been reported in rodent models and humans (Santos-Marcos et al., 2019). Gastrointestinal bacteria are part of a complex ecosystem that influence host physiology and metabolism through direct contact and also indirectly by way of microbiota-derived metabolites (Suchodolski, 2011; Canfora et al., 2019). Disturbance of the gastrointestinal system might lead to detrimental outcomes such as inflammatory bowel disease, type 2 diabetes, obesity, and others (Weiss and Hennet, 2017). In addition to promoting obesity, altered production of estrogen may impact organ systems such as the gastrointestinal tract. Lack of estrogen hormone production by either biological aging or surgery has been shown to lead to changes in host physiology and alter the community structure of the gut microbial populations (Reichler, 2009; Zhao et al., 2019).

Modification of diet by changing ingredient composition and/or macronutrient profile may be an effective tool for the management of obesity and gut microbiota populations. Although numerous reports have described risk factors and consequences of obesity in humans and dogs, little is known about the pathophysiological changes following spay surgery that induce obesity or influence the phylogeny and activity of the gastrointestinal microbiota, which in turn may influence disease in dogs. Investigating these relationships may establish insight into the prevention and management of canine obesity. In a previous study, we reported the changes in body composition, metabolic status, and physical activity levels in female dogs fed HPHF diets following spay surgery (Phungviwatnikul et al., 2020). The objectives of this study were to

determine the effects of the same dietary macronutrient profile on apparent total tract macronutrient digestibility and fecal characteristics, microbiota populations, and microbial-derived metabolites of adult female dogs following spay surgery. We hypothesized that the HPHF (HPHF) diets would reduce nutrient and energy digestibilities and beneficially shift fecal microbiota (e.g., increased relative abundances of *Bifidobacterium, Lactobacillus, Fusobacterium, Turicibacter, Blautia, and Faecalibacterium*) and metabolites [e.g., increased fecal short-chain fatty acid (SCFA) concentrations and decreased fecal protein catabolite and total bile acid concentrations] in dogs following spay surgery as compared to dogs fed a control diet containing moderate concentrations of protein and fiber.

MATERIALS AND METHODS

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee prior to experimentation (IACUC #17008).

Experimental design:

Twenty-eight adult intact female beagles (age: 3.02 ± 0.71 yr, BW: 10.28 ± 0.77 kg; BCS: 4.98 ± 0.57) were used in a longitudinal spay study, which is fully described by Phungviwatnikul et al. (2020). Briefly, the experiment lasted 29 wk, with a 5-wk baseline phase and a 24-wk post-spay phase. Dogs had free access to fresh water at all times and were fed once daily. After the 5-wk baseline phase whereby all dogs were fed a control diet at a rate to maintain BW, 24 dogs were spayed using standard procedures. Four dogs were sham-operated and fed the control diet (CO) to serve as lean controls (COSH). Spayed dogs were randomly allotted to one of three dry diets (n=8/group): 1) CO containing a moderate amount of crude protein and a low amount of dietary

fiber; 2) high-protein, high-fiber diet (HPHF); and 3) high-protein, high-fiber diet containing additional omega-3 and medium-chain fatty acids (HPHFO). All three diets were formulated to meet all Association of American Feed Control Officials (AAFCO, 2016) nutrient recommendations for adult dogs at maintenance (**Table 3.1**). After the first 12 wk of the post-spay phase, dogs were fed an amount that exceeded needs (up to 200% the energy needed to maintain BW) for an additional 12 wk. Dogs were housed individually in pens (1.22 m wide × 1.85 m long) in a temperature-controlled room under a 12 h light: 12 h dark cycle in the Veterinary Medicine Basic Sciences Building at the University of Illinois. All dogs were allowed outside of their pens a couple of times a week for socialization with other dogs and humans in compatible groups. The pens were cleaned daily and dogs were bathed every 2 wk.

Fecal collection:

During the last wk of the baseline phase, at wk 12, and at wk 24, total feces were collected from the pen floor, weighed, and frozen at -20^{o}C until analysis. On the first day of the collection phase, one fresh fecal sample (within 15 min of defecation) was collected for measurement of pH, moisture content, microbiota populations, and fermentative metabolite concentrations. Fecal pH was measured immediately using an Accumet AP1001 Portable pH Meter Kit (Fisher Scientific, Waltham, MA) and then feces were aliquoted for other measures, including SCFA and protein fermentative products [ammonia; branched-chain fatty acids (BCFA); phenols; indoles]. Fecal aliquots (2 tubes/dog; ~2 g/tube) for analysis of phenols and indoles were frozen at -20°C immediately after collection. One aliquot (~5 g/dog) was collected and placed in 2 N hydrochloric acid for SCFA, BCFA, and ammonia analyses. An additional aliquot was collected for fresh fecal dry matter (DM) determination. Finally, 3-4 aliquots of fresh feces were collected in sterile

cryogenic vials (Nalgene, Rochester, NY), frozen on dry ice, and stored at -80°C for microbiota and bile acid analyses.

Fecal scores:

All fecal samples during the collection phase were scored according to the following scale: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; 5 = watery, liquid that can be poured.

Fecal chemical analyses:

Fecal samples (aside from fresh fecal aliquots) were dried at 55°C in a forced-air oven and ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. Samples were analyzed according to procedures of the Association of Official Analytical Chemists (AOAC) for dry matter (DM; 105°C), organic matter, and ash (AOAC, 2006; methods 934.01 and 942.05). Crude protein was calculated from Leco (FP2000 and TruMac) total nitrogen values according to AOAC (2006; method 992.15). Total lipid content (acid-hydrolyzed fat) was determined according to the methods of the American Association of Cereal Chemists (1983) and Budde (1952). Gross energy was measured using an oxygen bomb calorimeter (model 1261, Parr Instruments, Moline, IL). Digestibility of energy and macronutrients were determined using the following equation: (nutrient intake (g/d) – fecal output (g/d))/(nutrient intake (g/d)) ×100.

Fecal fermentative metabolite concentrations:

Fecal SCFA and BCFA concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H_3PO_4 on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively. Fecal ammonia concentrations were determined according to the method of Chaney and Marbach (1962). Fecal phenol and indole concentrations were determined using gas chromatography according to the methods described by Flickinger et al. (2003).

Fecal bile acids:

The protocol for quantifying bile acids was adapted and modified from the methods previously described by Batta et al. (2002). Briefly, an aliquot of 10-15 mg lyophilized stool was added to 200 µL of 1-butanol containing internal standards (cholic acid-d_4 and lithocholic acid-d_4) followed by adding 20 µl of hydrochloric acid. Samples were incubated for 4 h at 65°C. Following incubation, samples were completely evaporated at 65°C under nitrogen gas, 200 µl trimethylsilylation derivatization agent was added and samples were incubated for 30 min. The samples were then evaporated under nitrogen gas and resuspended in 200 µl hexane, vortexed, and centrifuged at 4°C for 10 min at 3,000 × g. The supernatant was then analyzed by gas chromatography and mass spectrometry according to methods described by Blake et al. (2019). Cholic acid (CA), chenodeoxycholic acid CDCA), lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) were measured.

Fecal microbiota populations:

Total DNA from fecal samples was extracted using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Carlsbad, CA) with bead beating using a vortex adaptor. The concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA). 16S rRNA gene amplicons of the V4 region were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with a Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) that target a 252 bp-fragment of that region were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. The quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 2% agarose E-gel (Life Technologies, Carlsbad, CA) and extracted using a Qiagen gel purification kit (Qiagen, Carlsbad, CA). Cleaned size-selected pooled products were run on an Agilent 2100 Bioanalyzer to confirm the appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the W. M. Keck Center for Biotechnology at the University of Illinois.

Bioinformatics and statistical analyses for assessing fecal microbial communities:

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.14) and QIIME 2.2019.4 (Bolyen et al., 2020) was used to process the resulting sequence data. Briefly, high-quality (quality value ≥ 20) sequence data derived from the sequencing process were

demultiplexed. Data were then denoised and assembled into amplicon sequence variants (ASV) using DADA2 (Callahan et al., 2016). The SILVA 132 database (Quast et al., 2013) was used to assign taxonomy. An even sampling depth (15,427 sequences per sample) was used for assessing alpha- and beta-diversity measures. Beta-diversity was assessed using weighted and unweighted UniFrac distance (Lozupone and Knight, 2005) measures and presented using principal coordinates analysis (PCoA) plots.

Statistical analyses:

All data were analyzed using the Mixed Models procedure of SAS (version 9.4; SAS Institute, Cary, NC). To compare the effects of spay surgery, the data from dogs fed the control diet (COSH: intact control) and COSP (spayed control) were compared against one another. Dietary effects were tested by comparing data from spayed dogs (COSP, HPHF, and HPHFO). Food intake, fecal characteristics, apparent total tract macronutrient digestibility, fecal metabolites, and fecal microbiota were evaluated based on the change from baseline data at wk 12 (restricted feeding) and wk 24 (ad libitum feeding). These response criteria data were analyzed using repeated-measures analysis and evaluated differences due to treatment, time, and treatment*time interaction. One dog from the COSP group was excluded from analyses because it was an outlier in terms of food intake, physical activity, and other metabolic measures. All results are presented as least squares means ± standard error of the mean (SEM). A $P<0.05$ was considered significant and a $P<0.10$ was considered a trend.

RESULTS

Food intake, fecal characteristics, and apparent total tract macronutrient and energy digestibility:

Baseline food intake, fecal characteristics, and apparent total tract macronutrient and energy digestibilities are presented in **Supplementary Table 3.1**. When comparing dogs in the spayed groups, the increases in as-is and DM food intake (g/d), energy intake (kcal/d), and fecal output (g/d) of animals in all groups (HPHF, HPHFO, and COSP) were greater ($P<0.001$) at wk 24 than wk 12 **(Table 3.2)**. Change in fecal DM percentage was lower ($P<0.001$) and change in fecal volume (g/d; DMB) was higher ($P<0.001$) in HPHF and HPHFO dogs than COSP dogs. Apparent total tract fat, OM, and energy digestibilities were decreased to a greater ($P<0.05$) extent in dogs fed HPHF or HPHFO than those fed COSP. Fecal pH, fecal scores, and protein and total dietary fiber digestibilities were not altered by treatment or time (**Table 3.2**). There were no differences in food intake, fecal characteristics, or apparent total tract macronutrient and energy digestibilities between COSH and COSP dogs **(Table 3.3)**.

Fecal fermentative metabolites:

Baseline fecal fermentative metabolite concentrations are presented in **Supplementary Table 3.1**. When comparing dogs in the spayed groups, HPHF and HPHFO dogs had a higher ($P=0.0001$) increase in fecal acetate concentration than COSP dogs **(Table 3.2)**. HPHF and HPHFO dogs also had a greater ($P<0.01$) reduction in fecal valerate concentrations than COSP dogs. Fecal isovalerate, valerate, and total fecal BCFA concentrations were higher ($P<0.05$) and fecal propionate concentrations were lower ($P<0.05$) at wk 12 than wk 24. Fecal butyrate concentrations were not affected by treatment or time. The only difference between COSH and

COSP dogs was that fecal valerate concentrations were increased at a higher ($P<0.05$) level in COSP dogs than COSH dogs **(Table 3.3)**.

Fecal bile acids:

Baseline fecal bile acid concentrations are presented in **Supplementary Table 3.2**. When comparing dogs in the spayed groups, decreases in fecal CA and total primary bile acid concentrations were greater ($P<0.05$) in HPHFO dogs than COSP dogs **(Table 3.4)**. Increase in fecal LCA concentrations tended to be greater ($P=0.093$) in HPHFO dogs than in COSP dogs. Fecal secondary bile acid percentage tended to be greater ($P=0.084$), while fecal primary bile acid percentage tended to be lower ($P=0.084$) in HPHFO dogs than COSP dogs. Fecal UDCA concentrations were decreased at a greater ($P<0.05$) level at wk 24 than wk 12, but were not affected by diet. Fecal total bile acid, total secondary bile acid, CDCA, and DCA concentrations were not different due to treatment or time.

When comparing dogs fed the control diet (COSH and COSP), fecal CDCA and total bile acid concentrations were increased at a greater ($P<0.05$) level in COSH dogs than COSP dogs, while fecal LCA and total secondary bile acid concentrations tended to be greater ($P=0.098$ and $P=0.085$, respectively) in COSH dogs than COSP dogs **(Table 3.5)**. Also, fecal total bile acid concentrations tended to be increased at a higher ($P=0.084$) amount at wk 24 than wk 12. Fecal CA, DCA, UDCA, and total primary bile acid concentrations or fecal primary and secondary bile acid percentages were not altered by treatment or time.

Fecal microbiota:

When comparing all four treatments, alpha diversity metrics were impacted by treatment and time. Amplicon sequence variants (ASV) were affected ($P<0.05$) by treatment and time, and Shannon diversity index tended to be affected ($P=0.06$) by treatment and time. Observed OTU were not different at wk 12 (COSH: 101±7.5; COSP: 115±7.9; HPHF: 118.63±7.0; HPHFO: 115.75±3.5), but were lower ($P<0.05$) in COSH dogs (99.5±7.7) than in HPHFO dogs (114.63±2.1) at wk 24. Observed OTU for COSP (111.71±6.6) and HPHF (116.25±5.6) were not different at wk 24. The Shannon index was higher ($P<0.05$) in HPHFO dogs than COSH dogs at wk 12 and 24, and it tended to be higher in HPHF dogs than in COSH dogs at wk 12 ($P=0.089$) and 24 ($P=0.062$) **(Figure 3.1A)**. Beta diversity, which is represented by PCoA of weighted UniFrac distances revealed that HPHF and HPHFO dogs clustered together and separately from COSH and COSP dogs at wk 12 and 24 ($P<0.05$) **(Figure 3.1B)**. Unweighted UniFrac distances were not different among groups **(Figure 3.1C)**. When comparing only COSH and COSP dogs, there were no significant differences in alpha diversity or beta diversity indices (data not shown).

Baseline fecal microbiota relative abundances are presented in **Supplementary Table 3.3**. The relative abundance increase of *Allobaculum* tended to be greater ($P=0.055$) in COSP dogs than COSH dogs **(Table 3.7)**. All other differences in COSP and COSH dogs were due to time. The relative abundances of the Actinobacteria phyla and *Bifidobacterium* and *Ruminococcus torques group* genera were decreased at a greater ($P<0.05$) level, whereas the relative abundance of the *Eubacterium* genera tended to be decreased at a greater ($P=0.082$) level at wk 24 than wk 12. The relative abundance of the *Streptococcus* genera was increased at a greater ($P<0.01$) level at wk 24 than wk 12. The relative abundance of the *Megamonas* genera tended to be decreased at a greater ($P=0.072$) level at wk 12 than wk 24.

When comparing spayed dogs only, alpha diversity metrics were not different (data not shown). Beta diversity, however was altered by diets. The PCoA of weighted UniFrac distances revealed that HPHF and HPHFO dogs clustered together and separately from COSP dogs at wk 12 and wk 24 **(Figure 3.1D)**. In spayed dogs, the increase in the relative abundance of Actinobacteria was greater (P<0.01) in COSP dogs than HPHF and HPHFO dogs **(Table 3.6; Figure 3.2A)**. In contrast, the increase in the relative abundance of Fusobacteria was greater (P<0.01) in HPHF and HPHFO dogs than COSP dogs **(Table 3.6; Figure 3.2D)**. The other bacterial phyla were not affected by diets in spayed dogs.

At the genus level, the relative abundance increases of fecal *Faecalibacterium, Romboutsia,* and *Fusobacterium* were greater (P<0.05), while the decrease of fecal *Catenibacterium* was greater (P<0.05) in HPHF and HPHFO dogs than COSP dogs **(Table 3.6; Figure 3.3)**. The relative abundance decreases of *Bifidobacterium* (P=0.058), *Prevotella 9* (P=0.079)*, Eubacterium* (P=0.062)*,* and *Megamonas* (P=0.071) tended to be greater in HPHF and HPHFO dogs than COSP dogs **(Table 3.6; Figure 3.3)**. The other bacterial phyla and genera were not altered by diet.

DISCUSSION

The gastrointestinal microbial inhabitants create a balanced and complex ecosystem in the gut and directly influence host physiology and metabolism through direct contact and microbiota-derived metabolites. The commensal bacteria aid in the digestion of food and nutrient absorption, compete with pathogenic bacteria for nutrients, produce antimicrobial substances, and prevent pathogen colonization in the gastrointestinal tract, strengthen the intestinal epithelial cells and tight junctions, modulate gut-associated lymphoid tissue, and generate several metabolite compounds

that benefit the host (Suchodolski, 2011). Although it influences host metabolism in many ways, obesity may also affect gastrointestinal function and lead to an imbalanced gut microbiota (German, 2006; Gomes et al., 2018). Obesity has been shown to reduce fecal bacterial diversity and richness, increase abundance of Actinobacteria and Firmicutes, decrease abundance of Bacteroidetes, and increase the Firmicutes:Bacteroidetes ratio in humans (Turnbaugh et al., 2009). Similar results have been reported in dogs (Handl et al., 2013; Park et al., 2015), with lower gut microbial diversity and greater abundance of Actinobacteria reported in obese compared to lean animals. This gut dysbiosis may disrupt gut barrier integrity, including the breakdown of tight-junctions and bacterial translocation, which activates inflammatory responses and may initiate insulin resistance (Saad et al., 2016). Dysbiosis may also impact the production of metabolites in the colon, contributing to changes in gastrointestinal and host health (Pilla and Suchodolski, 2020).

Because high-fiber, high-protein diets are effective in obesity management (Liu et al., 2003; Weigle et al., 2005; Soenen et al., 2012; Bermudez Sanchez et al., 2020), their influence on fecal characteristics, microbiota, and metabolites are important and were the focus of the current study. Barley-based β-glucans, beet pulp, short-chain fructooligosaccharides (scFOS), cellulose, and other fibers are known to reduce caloric density and nutrient digestibility and increase stool output (Fahey et al., 1990; Donadelli and Aldrich, 2019), responses that were observed in this study. Many of these fibers are also well recognized for their health benefits as it pertains to SCFA production and digesta viscosity, which can aid in managing obesity and metabolic disorders. Some fibers such as β-glucans, increase digesta viscosity. Greater viscosity impairs lipid emulsification by bile salts in the small intestine, which disturbs lipid digestion and absorption (Nakashima et al., 2018). Greater viscosity may also influence lipase, amylase, and trypsin activities, leading to lower nutrient digestibility (Dikeman and Fahey, 2006).

By definition, dietary fibers are not digested by host enzymes. They may be broken down and utilized by microbiota in the large intestine; however, SCFA are derived primarily from the fermentation of dietary fibers although some are produced during protein fermentation. The fermentability of a dietary fiber depends on its physicochemical properties. SCFA provide energy to colonocytes, improve gut barrier function, reduce luminal pH that aids in pathogen resistance, and improve lipid and carbohydrate metabolism (Canfora et al., 2017; Alexander et al., 2018; Minamoto et al., 2019). As expected, fecal total SCFA and acetate concentrations were greater in dogs consuming high-fiber diets in the current study, with restricted fed and ad libitum phases not differing from one another.

Dietary proteins and peptides that escape digestion may also undergo fermentation via microbiota, resulting in the production of both potentially beneficial and undesirable metabolites. Protein catabolites include BCFA, ammonia, polyamines, hydrogen sulfide, phenolic and indolic compounds, and SCFA. BCFA originate from the fermentation of branched-chain amino acids (e.g., valine and leucine) and comprise isobutyrate and isovalerate. Although valerate is technically a SCFA, it is derived from the other branched-chain amino acid (i.e., isoleucine) (Aguirre et al., 2016). Furthermore, ammonia is the end-product of bacterial deamination of amino acids (Wrong and Vince, 1984). Existing evidence suggests deleterious effects of ammonia on the intestinal epithelium, but the benefits or detrimental effects of BCFA have not been well elucidated in vivo (Lin and Visek, 1991; Hughes et al., 2008). In the current study, dogs fed high-fiber diets had lower fecal valerate concentrations, but ammonia concentrations were not different from the controls. While high-fiber diets typically reduce protein catabolite concentrations, the fermentability of fiber affects this response (Kerr et al., 2013; Panasevich et al., 2013; Zhou et al., 2014). Moreover, because the diets tested herein contained >40% protein, some protein was likely

present for fermentation in the large intestine. In vitro studies have reported that valerate exerts an antiproliferative effect on cell differentiation and apoptosis on human colorectal adenocarcinoma cell lines (Heerdt et al., 1994; Siavoshian et al., 1997). A recent study conducted by McDonald et al. (2018) suggested that valerate had the potential to inhibit the growth of *Clostridioides difficile*, an opportunistic pathogen, in culture media and *Clostridioides difficile*-infected mice. More research is necessary to assess the impacts of BCFA and ammonia on the gastrointestinal health of dogs, but appears to be minor in this study.

Bile acid conversion is another important metabolic feature of the gut microbiota. Primary bile acids (CA; CDCA) are synthesized from cholesterol in the liver and are subsequently conjugated to the amino acids glycine or taurine in dogs. If bile acids are not reabsorbed by the small intestine, they are subject to biotransformation to secondary bile acids by microbiota in the large intestine. Although the information on fecal bile acid profiles in dogs is limited, recent studies have reported bile acid profiles of healthy dogs and dogs with gastrointestinal diseases. Guard et al. (2019) reported that fecal bile acids in healthy dogs are primarily comprised of secondary bile acids [83.2%; DCA (61.5%), LCA (21.2%), and UDCA (0.5%)], with only 10.6% being primary bile acids [CA (5.5%); CDCA (5.1%)]. Dogs with gastrointestinal disorders (e.g., chronic inflammatory enteropathy and exocrine pancreatic insufficiency) tend to have lower bile acid conversion, resulting in greater primary bile acid and lower secondary bile acid concentrations (Blake et al., 2019; Li et al., 2021).

In regard to diet, fecal bile acids may be affected by several ingredients and/or nutrients, including fat, prebiotics, and fiber. For instance, in overweight dogs, fecal total bile acid, DCA and LCA concentrations tended to be greater in dogs supplemented with inulin (Alexander et al., 2018). In healthy dogs fed a grain-based vs. grain-free diet, fecal total bile acid concentrations

were similar, but the proportion of primary and secondary bile acids were modified. Dogs fed the grain-free diet, which contained greater oligosaccharide and soluble fiber concentrations, had greater fecal primary and lower secondary bile acid concentrations (Pezzali et al., 2020). In the current study, dogs fed the high-fiber, high-protein diets had lower fecal CA and primary bile acids, but similar total and secondary bile acids suggesting greater conversion. Interestingly, spayed vs. sham-operated dogs eating the control diet also differed in their fecal bile acid profile. Spayed dogs had lower fecal CA, LCA, total secondary, and total bile acid concentrations. In a human study, a similar finding obtained from Phylogenetic Investigation of Communities by Reconstruction of Unobserved States analysis (PICRUSt) showed that premenopausal women had higher bile acid secretion than postmenopausal women (Santos-Marcos et al., 2018). Such findings suggest an influence of estrogen on the regulation and excretion of bile acids, but more research is needed to identify what direct or indirect effects may be at play.

Gut bacterial composition and diversity can vary greatly, depending on animal characteristics (e.g., species, age, sex, breed), environmental factors (e.g., medications, and lifestyle), health status, sample material and handling, and laboratory approaches (Deng and Swanson, 2015). In this study, dietary treatments, gonadectomy, and changes in food intake and body weight over time may have affected gut microbiota populations. Although the canine gut microbiome has been characterized in recent years, little has been elucidated in the obese dog population. According to a previous report by Phungviwatnikul et al. (2020), BW gain, BCS and metabolic changes (e.g., increased fat mass and leptin concentrations) were observed in dogs after being allowed to eat ad libitum for 12 wk. In the current study, *Bifidobacterium*, *Allobaculum*, *Catenibacterium*, *Megamonas*, *Romboutsia*, *Ruminococcus gnavus* group, and *Streptococcus* were changed over time, suggesting that increased diet intake (and available substrate in the colon or

altered transit time) and/or weight gain as described by Phungviwatnikul et al. (2020) modified these bacterial taxa. There is some evidence in both humans and dogs that Actinobacteria are enriched in obese individuals (Turnbaugh et al., 2009; Handl et al., 2013; Forster et al., 2018), a response that was not observed with weight gain in the current study. In obese vs. lean mice, lower bacterial richness has been observed (Acharya et al., 2019). Similar differences have been reported in obese vs. lean dogs (Handl et al., 2013; Park et al., 2015; Bermudez Sanchez et al., 2020). Higher relative abundances of *Megamonas* and *Streptococcus* have been reported in obese children and higher relative abundance of *Catenibacterium* has been reported in obese adult humans with metabolic syndrome (Kröger et al., 2020; Gallardo-Becerra et al., 2020; Pisanu et al., 2020). Additionally, the relative abundance of *Megamonas* decreased and relative abundance of *Streptococcus* tended to decrease, whereas the relative abundance of *Catenibacterium* increased in overweight and obese individuals after 3 months of caloric restriction (Pisanu et al., 2020). Although the shifts in fecal *Streptococcus* in the current study agreed with these studies, the other taxa did not, suggesting other factors are involved.

Permanently removing the production of estrogens by gonadectomy is known to increase food consumption and BW and alter metabolism in dogs and cats (Belsito et al., 2009; Phungviwatnikul et al., 2020), but effects on the gut microbiota are unknown. Estrogens are steroid hormones synthesized primarily from the gonads (i.e., ovaries and testes) and involved in several biological activities (Hirschberg, 2012). Estrogens may be excreted in the urine, but are also excreted via bile and undergo enterohepatic circulation (Cross et al., 2018; Fuentes and Silveyra, 2019). Biliary estrogens are in the form of sulfate and glucuronide conjugates, which may be metabolized by microbial sulfatase and β-glucuronidase enzymes. This deconjugation allows estrogens to be reabsorbed into the bloodstream and recirculated via enterohepatic circulation to

target tissues (Baker, 2017). Due to enterohepatic circulation, a bi-directional relationship exists between estrogens and the gut microbiota. Estrogen concentrations are not only influenced by gut microbiota, but they may also impact the gut microbiome. Santos-Marcos et al. (2018) identified differences in gut microbial communities between pre- and postmenopausal women matched for body mass index, with the relative abundances of Firmicutes, *Lachnospira*, and *Roseburia,* and the Firmicutes:Bacteroidetes ratio being higher and the relative abundances of Actinobacteria and *Prevotella* being lower in postmenopausal women without significant differences observed in alpha diversity. The results from a murine study revealed alterations of gut microbiota via gonadectomy and feeding regimens. Gonadectomized female mice had lower Proteobacteria under normal feeding conditions whereas in conditions of overfeeding, Actinobacteria was lower but Firmicutes:Bacteroidetes ratio was higher than that of intact females (Santos-Macos et al., 2020).

Based on the existing literature, reduced estrogen production not only affects host metabolism, but is believed to disrupt estrogen-gut microbiome homeostasis. In the current study, the diversity and composition of gut microbiota of COSH and COSP dogs before and after spay was not affected other than *Allobaculum* relative abundance being greater in spayed vs. sham-operated dogs at wk 12 and 24. A similar outcome was observed by Acharya et al. (2019). Those researchers reported the effects of 17β-estradiol implantation in ovariectomized lean and obese mice fed a high-fat diet, with *Allobaculum* being more abundant in the non-implanted mice than 17β-estradiol implanted mice regardless of body weight. This finding might suggest that *Allobaculum* spp. are negatively associated with estrogen production. The lack of microbiota changes due to spay in the current study are somewhat surprising and may be due to inappropriate sampling times (circulating estrogen hormone concentrations in dogs are rapidly declined after gonadectomy) (Ibrahim and Zaid, 2017), low sample size of COSH dogs, or other factors. To be

certain, more research is necessary in larger populations and possibly using different sampling times.

Even though spay surgery and body composition did not appear to have a large impact, dietary intervention was shown to have an effect on the gut microbiota in this study. Recent studies testing extruded, raw, fresh, and human-grade diets have shown how different microbiome communities may be even in healthy dogs (Algya et al., 2018; Do et al., 2021). Although dietary fibers are often the focus, additional nutritional factors including dietary proteins and fatty acids, as well as changes in bile acid or hormone concentrations may affect the microbiota composition and activity. In this study, several bacterial taxa were impacted by the feeding of a HPHF diet, including SCFA-producing bacteria (e.g., *Faecalibacterium; Prevotella; Ruminococcus*) and bacteria associated with protein fermentation (e.g., *Fusobacterium*) and bile salt deconjugation (e.g., *Bifidobacterium* and *Lactobacillus*).

Bifidobacterium species have the ability to hydrolyze and utilize inulin-type fructans and oligosaccharides (e.g., arabinoxylans, FOS, and galactooligosaccharides), therefore consumption of prebiotics generally increase the abundance of this bacterial genera (Parche et al., 2007). Inulin supplementation also increases the abundances of several other bacterial genera such as *Lactobacillus* and *Faecalibacterium,* and decrease abundance of *Bacteroides* (Bastard et al., 2020). Additionally, Kovatcheva-Datchary et al. (2015) and Sandberg et al. (2019) reported that consumption of barley kernel bread increases the abundance of *Prevotella* and is associated with high valerate concentrations (Tap et al., 2015). Unexpectedly, the changes in the relative abundances of *Bifidobacterium* and *Prevotella 9* were higher or tended to be higher, respectively, in COSP dogs than in HPHF or HPHFO dogs. Although the control diet did not contain any functional dietary fibers (e.g., scFOS), it did contain a significant amount of β-glucans from the

barley inclusion (23% as-is), which may have contributed to the alterations of these bacteria. Furthermore, an increase in the relative abundance of *Prevotella* is often associated with high-carbohydrate and high-fiber diets, whereas increased relative abundance of *Bacteroides* often occurs in response to high-protein diets (Wu et al., 2011; Kovatcheva-Datchary et al., 2015; Moreno-Pérez et al., 2018). Hence, Prevotella and Bacteroides often respond inversely to dietary intervention (Wu et al., 2011; Korean et al., 2013). This relationship may also explain the relationship of both taxa in this study, with the relative abundance of *Prevotella 9* in COSP dogs fed the control diet (45% DM, NFE) being higher compared to dogs fed HPHF (17% DM, NFE). Additionally, higher relative abundance of *Bacteroides* was also observed in dogs fed HPHF diets (42% to 43% DM, CP) compared to dogs fed COSP (22% DM, CP) although the difference did not reach statistical significance. In this experiment, the relative abundance of *Faecalibacterium* was increased to a greater extent in HPHF and HPHFO dogs than COSP dogs. Although they did not reach statistical significance, butyrate concentrations were numerically higher in HPHF and HPHFO dogs compared to COSP dogs, which agrees with the increase in *Faecalibacterium* - a known butyrate producer (Louis et al., 2004).

Although fiber utilization is often the focus of diet intervention studies, several bacterial genera elicit proteolytic activity, including *Bacteroides, Clostridium, Propionibacterium, Streptococcus, Fusobacterium,* and *Lactobacillus*. Therefore, high protein intake and/or reduced protein digestibility due to increased consumption of fiber may enhance the growth of these bacteria if it results in more protein reaching the large intestine (Davila et al., 2013; Adam et al., 2014; Hervik and Svihus, 2019). In the current study, the relative abundances of Fusobacteria and *Fusobacterium* were increased greater in dogs fed HPHF and HPHFO. In humans, Fusobacteria abundance and excessive protein intake is associated with the development of colorectal cancer

and inflammatory bowel diseases (Hou et al., 2011; Kelly et al., 2018), however, high abundances of Fusobacteria and *Fusobacterium* are normally present among healthy adult domesticated dog breeds and correlated with improved gastrointestinal tract functionality (Alessandri et al., 2019).

Both the type and amount of dietary fat may modulate gut microbiota homeostasis. Omega-3 poly-unsaturated fatty acids have anti-obesity effects and have been shown to increase the abundances of *Bifidobacterium, Lactobacillus, Streptococcus, Roseburia,* and *Akkermasia muciniphila* in mice and humans (Caesar et al., 2015; Torres-Fuentes et al., 2015; Watson et al., 2018). In the current study, the relative abundances of those bacteria were not increased in HPHFO dogs as hypothesized. The lack of response is probably due to the amount of fish oil present in the diets tested, which was likely too low to impact the gut microbiota.

Bile acids may modulate the gastrointestinal microbiota and vice versa. Gut microbiota such as *Clostridium, Bifidobacterium, Lactobacillus, Bacteroides,* and *Enterococcus* produce bile salt hydrolase enzymes, which hydrolyze conjugated primary bile acids to form deconjugated acids that are less soluble and therefore less readily reabsorbed by the intestinal epithelium. Another biotransformation of bile acids is chemical modification by 7 α-dehydroxylation (by *Eubacterium* and *Clostridium XIVa* cluster) and 7 α-dehydrogenation enzymes to generate secondary bile acids, which may be reabsorbed and returned to the liver to some extent (Ridlon et al., 2016; Kriaa et al., 2019). In dogs, *C. hiranonis* has been shown to be the primary bacterial group responsible for BA conversion (Pilla et al., 2020; Li et al., 2021).

Secondary bile acids such as DCA have antimicrobial activity that can damage bacterial cell membranes and prohibit bacterial overgrowth (Ciaula et al., 2017). Furthermore, there is evidence that unconjugated bile acids, in particular, DCA, are toxic and have inhibitory potential on *Lactobacillus* and *Bifidobacterium* species at a level 10 times stronger than CA (Kurdi et al.,

2006). These reasons could partially explain the unexpected reduction of *Bifidobacterium* and *Lactobacillus* species in HPHFO dogs because although DCA concentrations were not significantly altered by treatments, it was numerically higher in HPHFO dogs compared to HPHF or COSP dogs. Although *Bifidobacterium* and *Lactobacillus* are often the focus of microbiome studies, it should be stated that the reduction in their relative abundances did not lead to any negative effects on gastrointestinal functionality of dogs in the current study. This is similar to other studies that have reported similar findings (Sandri et al., 2017; Algya et al., 2018; Do et al., 2021). Some bacterial groups such as *Eubacterium, Bacteroides, Bifidobacterium, Lactobacillus,* and *Peptostreptococcus* are able to convert cholesterol into coprostanol, which is excreted in the feces. Additionally, microbial desulfation by *Peptococcus, Clostridium,* and *Fusobacterium* can prevent bile acid excretion (Kriaa et al., 2019). All of these mechanisms not only influence gut microbial structure, but may also shift cholesterol homeostasis and subsequently reduce serum cholesterol concentrations as observed in our previous study testing the HPHF and HPHFO diets (Phungviwatnikul et al., 2020).

Overall, the results of this experiment confirm that gonadectomy and overeating are major risk factors contributing to weight gain and obesity in dogs. Consumption of HPHF diets may help, however, as dogs consuming those diets were shown to have reduced apparent total tract digestibilities of fat, organic matter, and energy compared to dogs fed the control diet containing a moderate amount of protein and fiber. Although our results demonstrate that gonadectomy and overeating may also alter the gastrointestinal microbiota and metabolites, dietary intervention led to more robust changes. Gut dysbiosis has been reported with obesity in dogs and other species, but was not observed in the current study with overeating and weight gain. Consumption of HPHF diets increased fecal total short-chain fatty acid and acetate concentrations and reduced fecal

valerate, cholic acid, and total primary bile acid concentrations compared to controls. Consumption of HPHF diets also shifted the gastrointestinal microbiota, showing separate clustering from control-fed dogs, reduced relative abundances of fecal Actinobacteria, *Bifidobacterium*, *Prevotella*, *Catenibacterium*, and *Megamonas*, and increased relative abundances of fecal *Faecalibacterium*, *Ruminococcus*, and *Fusobacterium*. Reduced estrogen production has been shown to impact gut microbiota in previous studies, but few changes were noted in the current study. Further research with greater sample sizes, a longer length of time, and/or different diets will further elucidate the effects of estrogen reduction and weight gain on the gut microbiota of dogs.

TABLES AND FIGURES

Table 3.1. Ingredient and chemical composition of experimental diets

Item	Treatment[1]		
	CO	HPHF	HPHFO
Ingredient	*% as-is*		
Poultry meal (low ash)	11.36	34.16	34.16
Soy protein concentrate	8.00	22.00	22.00
Barley	23.00	11.00	11.00
Beet pulp	1.00	10.00	10.00
Brewer's rice	38.60	8.00	8.00
Chicken fat	10.50	5.30	4.00
Liquid digest	3.00	3.00	3.00
Powder palatant	2.00	2.00	2.00
Cellulose	1.00	2.00	2.00
Short-chain fructooligosaccharides[2]	-	1.00	1.00
Coconut oil[3]	-	-	0.80
Fish oil	-	-	0.50
Sodium chloride	0.50	0.50	0.50
Potassium chloride	0.45	0.45	0.45
Mineral premix[4]	0.18	0.18	0.18
Vitamin premix[5]	0.18	0.18	0.18
Choline chloride	0.13	0.13	0.13
Natural antioxidant[6]	0.10	0.10	0.10
Analyzed composition			
Dry matter (DM), %	90.50	92.16	91.97

Table 3.1 (cont.). Ingredient and chemical composition of experimental diets

		%, DM	
Ash	4.74	8.29	8.48
Crude protein	22.31	41.94	42.91
Acid-hydrolyzed fat	15.59	12.00	12.50
Crude fiber	2.60	5.08	4.48
Total dietary fiber	12.10	20.90	21.00
Insoluble fiber	6.80	13.60	14.00
Soluble fiber	5.30	7.30	7.00
Nitrogen-free extract[7]	45.26	16.87	15.11
Gross energy[7], kcal/g	4.61	4.66	4.64
Metabolizable energy (ME)[7], kcal/g	3.69	3.08	3.09
Macronutrients on energy basis (% of ME)			
Protein	21.16	47.68	48.55
Fat	35.91	33.13	34.35
Carbohydrate	42.93	19.18	17.10

[1]Treatment: CO = control diet containing a moderate amount of crude protein and dietary fiber, HPHF = high-protein, high-fiber diet, HPHFO = high-protein, high-fiber diet containing additional omega-3 and medium-chain fatty acids.

[2]Short-chain fructooligosaccharides: Fortifeed scFOS prebiotic fiber, Ingredion Inc., Westchester, IL USA.

[3]Coconut oil: Gold label virgin coconut oil, Healthy Traditions, Inc., Blum, TX, USA.

[4]Provided per kg diet: Mn (as MnSO4), 66.00 mg; Fe (as FeSO4), 120 mg; Cu (as CuSO4), 18.00 mg; Co (as CoSO4), 1.20 mg; Zn (as ZnSO4), 240 mg; iodine (as KI), 180 mg; Se (as Na2SeO3), 0.24 mg.

[5]Provided per kg diet: vitamin A, 5.28 mg; vitamin D3, 0.04 mg; vitamin E, 120.00 mg; vitamin K, 0.88 mg; thiamin, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B12, 0.11 mg.

[6]Natural antioxidant: Naturox liquid antioxidant, blend of vegetable oil, natural mixed tocopherols, lecithin and rosemary extract.

Table 3.1 (cont.). Ingredient and chemical composition of experimental diets

[7]Nitrogen-free extract = 100 - (ash + crude protein + acid hydrolyzed fat + total dietary fiber); metabolizable energy = 8.5 kcal ME/g fat + 3.5 kcal ME/g protein + 3.5 kcal ME/g nitrogen-free extract; gross energy was measured by bomb calorimetry.

Table 3.2. Change from baseline food intake, fecal characteristics, apparent total tract macronutrient and energy digestibilities, and fecal metabolites of adult spayed female dogs fed different diets under restricted or ad libitum feeding regimens after spay surgery

Item[2]	Dietary treatment group[1]						SEM	P-values		
	COSP		HPHF		HPHFO			Treatment	Time	Treatment × Time
	wk12	wk24	wk12	wk24	wk12	wk24				
Consumption										
Food intake (as-is), g/d	17.28	66.49	11.75	106.17	15.01	94.11	15.637	0.7324	0.0001	0.5104
Food intake (DM), g/d	15.65	60.19	14.49	101.51	17.00	89.74	14.224	0.5935	0.0001	0.4909
Energy intake, kcal/d	73.04	247.55	20.97	357.60	28.84	324.66	60.430	0.9414	0.0001	0.5090
Fecal characteristics										
Fecal pH	0.09	0.02	0.29	0.22	0.23	0.15	0.127	0.4903	0.3842	0.9996
Fecal score[3]	-0.04	-0.23	-0.19	-0.25	-0.19	-0.13	0.318	0.9350	0.3664	0.4052
Fecal dry matter (DM), %	-1.08	-0.75	-4.88	-4.87	-5.01	-6.41	0.673	0.0002	0.6069	0.5441
Fecal output (DM), g/day	-0.49	7.23	17.72	36.61	17.51	28.93	2.895	<0.0001	0.0006	0.3476
Macronutrient and energy digestibility										
Protein digestibility, %	1.72	-0.02	1.03	0.16	1.30	1.41	1.457	0.8388	0.1100	0.3388
Fat digestibility, %	0.73	0.06	-1.63	-2.23	-1.35	-1.46	0.532	<0.0001	0.0495	0.5390
Total dietary fiber digestibility, %	4.18	-0.40	-0.56	-3.22	-0.94	0.87	4.254	0.7187	0.3361	0.3661
Organic matter digestibility, %	1.10	-0.13	-5.80	-7.28	-6.26	-5.94	0.946	<0.0001	0.1883	0.3989
Energy digestibility, %	1.11	-0.05	-5.04	-5.89	-4.92	-4.63	0.948	<0.0001	0.3143	0.5410
Fecal metabolites										
Acetate, µmol/g	-25.01	-18.71	248.51	281.56	251.32	313.72	46.104	0.0001	0.2846	0.7657

Table 3.2 (cont.). Change from baseline food intake, fecal characteristics, apparent total tract macronutrient and energy digestibilities, and fecal metabolites of adult spayed female dogs fed different diets under restricted or ad libitum feeding regimens after spay surgery

Propionate, µmol/g	-7.53	23.35	-96.42	-50.21	-50.37	-17.94	41.285	0.2954	0.0453	0.9210
Butyrate, µmol/g	3.34	-1.15	39.85	34.55	29.41	18.56	13.699	0.1794	0.3086	0.9110
Total SCFA[4], µmol/g	-28.43	4.26	191.94	265.90	230.37	314.33	87.413	0.0302	0.1370	0.8699
Isobutyrate, µmol/g	1.57	-0.29	2.10	1.80	1.66	0.62	0.810	0.5316	0.0453	0.4690
Isovalerate, µmol/g	0.77	-1.04	1.03	0.54	1.04	-0.08	1.176	0.7687	0.0284	0.5485
Valerate, µmol/g	1.50	0.39	-0.46	-0.47	-0.97	-0.80	0.546	0.0087	0.2187	0.1134
Total BCFA[4], µmol/g	3.74	-1.05	2.67	1.87	1.73	-0.27	1.751	0.8060	0.0103	0.2109
Ammonia, µmol/g	49.50	0.79	22.87	65.90	119.54	126.88	37.443	0.1707	0.98229	0.3417

[1]Dietary treatment group: COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO.

[2]Mean values of restricted feeding phase (change from wk 0 to wk 12) and ad libitum phase (change from wk 0 to wk 24).

[3]Fecal scores: 1 = hard, dry pellets; small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed and moist stool retains shape; 4 = soft, unformed stool, assumes shape of container; 5 = watery, liquid that can be poured.

[4]Total SCFA: total short-chain fatty acids = acetate + propionate + butyrate; total BCFA: total branched-chain fatty acids = isobutyrate + isovalerate + valerate.

Table 3.3. Change from baseline food intake, fecal characteristics, apparent total tract macronutrient and energy digestibilities, and fecal metabolites of adult female dogs fed a control diet under restricted or ad libitum feeding regimens after spay surgery

Item[2]	Dietary treatment group[1]				SEM	P-values		
	COSH		COSP			Treatment	Time	Treatment × Time
	wk12	wk24	wk12	wk24				
Consumption								
Food intake (as-is), g/d	-16.50	-17.53	17.30	66.51	22.694	0.1089	0.3625	0.3434
Food intake (DM), g/d	-14.93	-15.86	15.66	60.20	20.538	0.1089	0.3625	0.3434
Energy intake, kcal/d	-59.93	-62.36	73.66	248.17	86.023	0.1086	0.3853	0.3725
Fecal characteristics								
Fecal pH	0.21	0.26	0.10	0.03	0.159	0.3672	0.9122	0.5366
Fecal score[3]	0.00	0.38	-0.03	-0.31	0.252	0.7231	0.7498	0.2083
Fecal dry matter (DM), %	0.12	1.44	-1.05	-0.73	0.856	0.1882	0.3527	0.5700
Fecal output (DM), g/day	-2.24	-2.69	-0.49	7.23	3.119	0.2395	0.2743	0.2238
Macronutrient and energy digestibility								
Protein digestibility, %	1.18	1.19	1.86	0.12	1.394	0.9286	0.4162	0.4098
Fat digestibility, %	0.11	0.01	0.75	0.08	0.469	0.4874	0.2293	0.3688
Total dietary fiber digestibility, %	0.71	2.37	4.51	-0.07	4.363	0.9183	0.6874	0.3972
Organic matter digestibility, %	0.46	0.63	1.16	-0.07	0.907	0.9983	0.4463	0.3184
Energy digestibility, %	0.40	0.54	1.17	0.01	0.933	0.9312	0.4922	0.3810

Table 3.3 (cont.). Change from baseline food intake, fecal characteristics, apparent total tract macronutrient and energy digestibilities, and fecal metabolites of adult female dogs fed a control diet under restricted or ad libitum feeding regimens after spay surgery

Fecal metabolites								
Acetate, µmol/g	16.15	-6.49	-26.23	-19.93	27.592	0.5126	0.7927	0.6430
Propionate, µmol/g	-9.84	-10.33	-9.87	21.00	35.883	0.7755	0.5008	0.4873
Butyrate, µmol/g	-5.47	-33.90	2.09	-2.40	14.479	0.6411	0.1933	0.5658
Total SCFA[4], µmol/g	0.85	-50.72	-34.01	-1.33	51.022	0.9256	0.8540	0.4199
Isobutyrate, µmol/g	1.09	1.16	1.50	-0.36	1.149	0.6947	0.3443	0.3075
Isovalerate, µmol/g	1.42	1.30	0.59	-1.22	1.231	0.2822	0.2089	0.2697
Valerate, µmol/g	-0.74	-1.76	1.50	0.39	0.722	0.0271	0.1194	0.9436
Total BCFA[4], µmol/g	1.77	0.70	3.71	-1.08	1.928	0.9792	0.0966	0.2698
Ammonia, µmol/g	63.06	93.55	49.50	0.79	38.728	0.3791	0.7393	0.1698

[1]Dietary treatment group: COSH: sham-operated dogs fed CO; COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO.
[2]Mean values of restricted feeding phase (change from wk 0 to wk 12) and ad libitum phase (change from wk 0 to wk 24).

[3]Fecal scores: 1 = hard, dry pellets; small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed and moist stool retains shape; 4 = soft, unformed stool, assumes shape of container; 5 = watery, liquid that can be poured.

[4]Total SCFA: total short-chain fatty acids = acetate + propionate + butyrate; total BCFA: total branched-chain fatty acids = isobutyrate + isovalerate + valerate.

Table 3.4. Change from baseline fecal bile acid concentrations and percentages of adult spayed female dogs fed different diets under restricted or ad libitum feeding regimens after spay surgery

Item[2]	Dietary treatment group[1]						SEM	P-values		
	COSP		HPHF		HPHFO			Treatment	Time	Treatment × Time
	wk12	wk24	wk12	wk24	wk12	wk24				
Cholic acid	-0.44	1.26	-0.60	-0.81	-1.90	-2.31	0.635	0.0114	0.5514	0.0768
Chenodeoxycholic acid	-0.24	0.03	-0.34	-0.39	-0.42	-0.46	0.105	0.1000	0.3149	0.0741
Lithocholic acid	-0.27	-0.25	-0.09	-0.12	0.16	0.03	0.108	0.0933	0.4124	0.5725
Deoxycholic acid	-1.31	-0.20	-0.77	-0.84	1.32	0.65	0.716	0.1530	0.8210	0.4125
Ursodeoxycholic acid	-0.04	-0.08	-0.06	-0.10	-0.05	-0.06	0.055	0.8075	0.0291	0.5526
Total primary bile acid	-0.68	1.29	-0.94	-1.20	-2.31	-2.77	0.700	0.0107	0.5438	0.0772
Total secondary bile acid	-1.62	-0.54	-0.92	-1.05	1.44	0.62	0.811	0.1371	0.9401	0.4299
Total bile acid	-2.30	0.75	-1.86	-2.25	-0.88	-2.15	0.925	0.6353	0.5760	0.1076
Secondary bile acid[3], %	8.75	-5.54	13.61	16.19	25.90	29.86	7.728	0.0837	0.5159	0.1417
Primary bile acid[3], %	-8.75	5.54	-13.61	-16.19	-25.90	-29.86	7.728	0.0837	0.5159	0.1417

[1]Dietary treatment group: COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO.

[2]Mean values of bile acids (μg/mg) during restricted feeding phase (change from wk 0 to wk 12) and ad libitum phase (change from wk 0 to wk 24).

[3]Primary bile acid (sum of cholic acid and chenodeoxycholic acid) and secondary bile acid (sum of lithocholic acid, deoxycholic acid, and ursodeoxycholic acid) are expressed as a percent of total bile acid measured.

Table 3.5. Change from baseline fecal bile acid concentrations and percentages of adult female dogs fed a control diet under restricted or ad libitum feeding regimens after spay surgery

Item[2]	Dietary treatment group[1]				SEM	P-values		
	COSH		COSP			Treatment	Time	Treatment × Time
	wk12	wk24	wk12	wk24				
Cholic acid	1.14	2.99	-0.43	1.26	0.877	0.1408	0.1027	0.9788
Chenodeoxycholic acid	0.13	0.45	-0.24	0.03	0.140	0.0419	0.1883	0.9123
Lithocholic acid	0.15	0.13	-0.27	-0.25	0.158	0.0980	0.9364	0.7704
Deoxycholic acid	1.07	2.10	-1.31	-0.20	0.932	0.1011	0.0820	0.9507
Ursodeoxycholic acid	0.00	-0.01	-0.05	-0.09	0.044	0.2602	0.2868	0.3439
Total primary bile acid	1.27	3.44	-0.68	1.29	0.987	0.1483	0.1444	0.9403
Total secondary bile acid	1.22	2.22	-1.62	-0.54	1.055	0.0848	0.1032	0.9521
Total bile acid	2.48	5.66	-2.30	0.75	1.240	0.0107	0.0843	0.9705
Secondary bile acid[3], %	-4.17	-17.39	6.96	-7.32	17.824	0.5044	0.2130	0.9600
Primary bile acid[3], %	4.17	17.39	-6.96	7.32	17.824	0.6964	0.1112	0.7205

[1]Dietary treatment group: COSH: sham-operated dogs fed CO; COSP: spayed dogs fed CO.

[2]Mean values of bile acids (μg/mg) during restricted feeding phase (change from wk 0 to wk 12) and ad libitum phase (change from wk 0 to wk 24).

[3]Primary bile acid (sum of cholic acid and chenodeoxycholic acid) and secondary bile acid (sum of lithocholic acid, deoxycholic acid, and ursodeoxycholic acid) are expressed as a percent of total bile acid measured.

Table 3.6. Change from baseline relative abundances (%) of fecal microbiota of adult female dogs fed different diets under restricted or ad libitum feeding regimens after spay surgery

Phylum	Genus	Dietary treatment group[1,2]						SEM	P-values		
		COSP		HPHF		HPHFO			Treatment	Time	Treatment × Time
		wk12	wk24	wk12	wk24	wk12	wk24				
Actinobacteria		5.00	0.00	-2.38	-2.75	-3.13	-4.25	7.267	0.0063	0.2203	0.3386
	Bifidobacterium	5.71	0.63	-1.43	-2.30	-3.01	-3.61	3.321	0.0576	0.0143	0.2353
	Collinsella	-0.66	-0.51	-1.13	-0.56	-0.25	-0.69	0.667	0.7814	0.7255	0.2697
Bacteroidetes		0.57	3.57	6.50	0.25	7.00	5.00	4.976	0.6348	0.4144	0.2303
	Bacteroides	-0.82	-0.90	7.60	2.69	6.76	5.29	4.349	0.1544	0.2193	0.5032
	Prevotella 9	0.79	3.24	-1.85	-2.14	-0.23	-0.94	2.000	0.0791	0.4876	0.1610
Firmicutes		-6.57	-3.00	-15.87	-7.13	-12.63	-13.13	7.962	0.4554	0.2039	0.4490
	Allobaculum	9.66	1.25	1.37	-3.58	1.03	-0.40	5.065	0.2974	0.0201	0.3706
	Blautia	-3.33	-2.91	-1.13	-0.09	-1.21	-2.38	2.463	0.4914	0.8677	0.2773
	Catenibacterium	-3.89	-0.36	-9.82	-8.77	-5.30	-4.74	3.252	0.0374	0.0121	0.3240
	Clostridium sensu stricto 1	-0.37	-0.73	1.14	1.28	0.95	1.04	0.977	0.1077	0.9327	0.9151
	Enterococcus	0.00	0.02	0.00	0.28	-0.01	0.25	0.106	0.5594	0.0196	0.4468
	Eubacterium	0.06	0.02	0.05	0.00	-0.02	-0.03	0.025	0.0626	0.0163	0.4224
	Faecalibacterium	-0.01	0.79	3.95	4.92	5.97	4.01	1.615	0.0060	0.9423	0.2812
	Lachnoclostridium	-0.20	-0.21	-0.10	0.16	0.23	0.04	0.298	0.4033	0.6913	0.0559
	Lachnospiraceae NK4A136 group	0.03	-0.01	0.04	0.18	0.24	0.21	0.126	0.1436	0.5108	0.0857

Table 3.6 (cont.). Change from baseline relative abundances (%) of fecal microbiota of adult female dogs fed different diets under restricted or ad libitum feeding regimens after spay surgery

	Lactobacillus	3.69	0.70	3.78	3.31	-5.19	-7.69	4.550	0.0294	0.4002	0.8934
	Megamonas	-4.46	-1.54	-14.08	-12.92	-13.86	-12.08	5.659	0.0713	0.0030	0.4757
	Peptoclostridium	-1.63	-1.99	-1.20	0.87	0.45	0.89	2.222	0.5531	0.2708	0.3569
	Romboutsia	-0.13	-0.23	0.36	-0.20	2.19	0.89	0.618	0.0080	0.0132	0.3962
	Roseburia	-0.04	-0.02	-0.05	-0.05	-0.05	-0.06	0.065	0.3642	0.8397	0.3176
	Ruminococcus gnavus group	-3.21	-4.11	-2.50	-2.36	-1.59	-1.85	2.305	0.7847	0.4940	0.6834
	Ruminococcus torques group	0.43^{cd}	0.00^{d}	1.91^{bc}	2.06^{bc}	3.68^{a}	2.00^{ab}	0.609	<0.0001	0.0105	0.0407
	Streptococcus	-0.53	8.73	0.91	8.45	-1.68	8.15	3.573	0.5253	<0.0001	0.5964
Fusobacteria		-6.57	-2.14	10.38	10.00	9.00	11.50	3.879	0.0033	0.9773	0.5834
	Fusobacterium	0.10	-2.16	10.23	9.96	8.82	11.29	3.858	0.0034	0.9907	0.6023
Proteobacteria		0.71	1.86	1.75	-0.25	0.13	1.25	1.725	0.9049	0.9214	0.2696

[1]Dietary treatment group: COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO

[2]Mean values during restricted feeding phase (change from wk 0 to wk 12) and ad libitum phase (change from wk 0 to wk 24).

[a,b,c] Mean values within the same row with unlike superscript letters represent significant treatment*feeding regimen interactions ($P < 0.05$).

Table 3.7. Change from baseline relative abundances (%) of fecal microbiota of adult female dogs fed a control diet under restricted or ad libitum feeding regimens after spay surgery

Phylum	Genus	Dietary treatment group[1,2]				SEM	P-values		
		COSH		COSP			Treatment	Time	Treatment × Time
		wk12	wk24	wk12	wk24				
Actinobacteria		4.25	-1.50	5.00	0.00	2.118	0.3931	0.0038	0.3706
	Bifidobacterium	3.73	-1.85	5.71	0.63	1.780	0.2678	0.0306	0.9506
	Collinsella	0.30	-0.03	-0.66	-0.51	0.768	0.5416	0.8583	0.6483
Bacteroidetes		-3.50	0.50	0.57	3.57	3.462	0.5045	0.1934	0.8453
	Bacteroides	0.17	2.97	-0.82	-0.90	2.651	0.5515	0.3608	0.3332
	Prevotella 9	-2.95	-2.18	0.79	3.24	1.876	0.1346	0.3342	0.6082
Firmicutes		-0.50	-7.50	-6.57	-3.00	4.657	0.912	0.738	0.3151
	Allobaculum	-0.67	-7.95	9.66	1.25	2.986	0.0550	0.0476	0.8735
	Blautia	1.95	0.78	-3.33	-2.91	1.774	0.1228	0.7630	0.5323
	Catenibacterium	-4.14	-6.67	-3.89	-0.36	3.227	0.5108	0.8515	0.2676
	Clostridium sensu stricto 1	-0.20	-0.10	-0.37	-0.73	0.566	0.7233	0.5175	0.3650
	Enterococcus	0.00	0.00	0.00	0.02	0.009	0.4790	0.4790	0.4790
	Eubacterium	0.01	-0.03	0.06	0.02	0.020	0.1198	0.0816	0.9745
	Faecalibacterium	0.64	0.43	-0.01	0.79	0.412	0.8183	0.4443	0.2081
	Lachnoclostridium	0.13	0.03	-0.20	-0.21	0.171	0.2957	0.3649	0.4923
	Lachnospiraceae NK4A136 group	-0.08	-0.13	0.03	-0.01	0.065	0.2944	0.2764	0.9309
	Lactobacillus	2.98	1.40	3.69	0.70	3.504	0.9992	0.5978	0.8694
	Megamonas	-0.83	2.06	-4.46	-1.54	4.179	0.6279	0.0717	0.7784

Table 3.7 (cont.). Change from baseline relative abundances (%) of fecal microbiota of adult female dogs fed a control diet under restricted or ad libitum feeding regimens after spay surgery

	Peptoclostridium	1.66	1.64	-1.63	-1.99	1.383	0.1264	0.8547	0.8732
	Romboutsia	-0.08	-0.01	-0.13	-0.23	0.184	0.6248	0.9307	0.6311
	Roseburia	0.01	0.04	-0.04	-0.02	0.046	0.3896	0.3953	0.9322
	Ruminococcus gnavus group	-0.83	-0.93	-3.21	-4.11	2.258	0.4267	0.5885	0.6642
	Ruminococcus torques group	0.42	0.10	0.43	0.00	0.119	0.8092	0.0396	0.7268
	Streptococcus	0.13	5.60	-0.53	8.73	554.505	0.9207	0.0099	0.4564
Fusobacteria		1.50	6.25	0.14	-2.14	2.350	0.1957	0.6383	0.1984
	Fusobacterium	1.22	6.42	0.10	-2.16	2.336	0.1954	0.5758	0.1731
Proteobacteria		-1.25	2.00	0.71	1.86	1.369	0.6647	0.1896	0.5133

[1]Dietary treatment group: COSH: sham-operated dogs fed CO;
COSP: spayed dogs fed CO.

[2]Mean values during restricted feeding phase (change from wk 0 to wk 12) and ad libitum phase (change from wk 0 to wk 24).

Figure 3.1

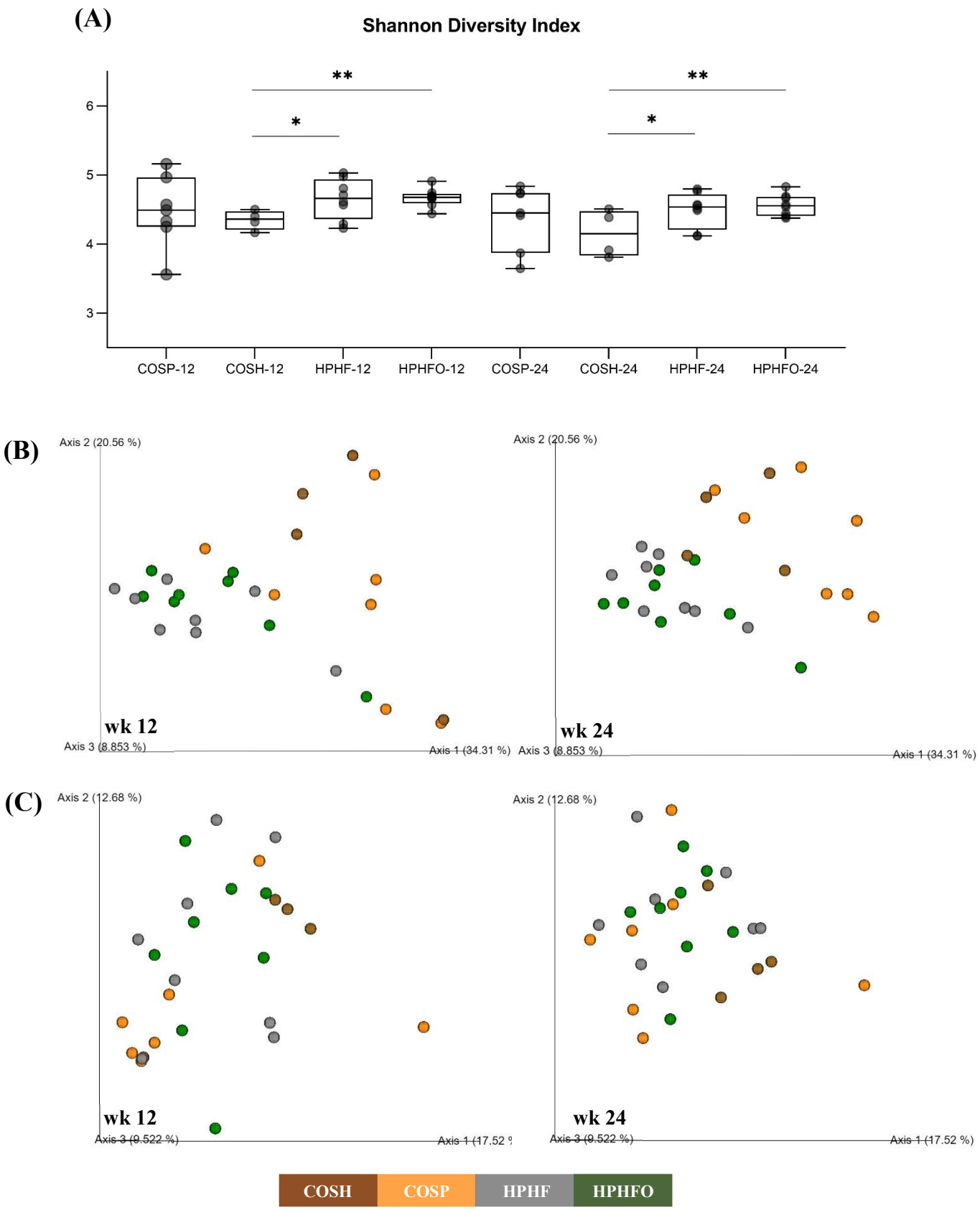
(A)
Shannon Diversity Index
**
*
**
*
6
5
4
3
COSP-12
COSH-12
HPHF-12
HPHFO-12
COSP-24
COSH-24
HPHF-24
HPHFO-24
(B)
Axis 2 (20.56 %)
wk 12
Axis 3 (8.853 %)
Axis 1 (34.31 %)
Axis 2 (20.56 %)
wk 24
Axis 3 (8.853 %)
Axis 1 (34.31 %)
(C)
Axis 2 (12.68 %)
wk 12
Axis 3 (9.522 %)
Axis 1 (17.52 %)
Axis 2 (12.68 %)
wk 24
Axis 3 (9.522 %)
Axis 1 (17.52 %)
COSH
COSP
HPHF
HPHFO

Figure 3.1 (cont.)

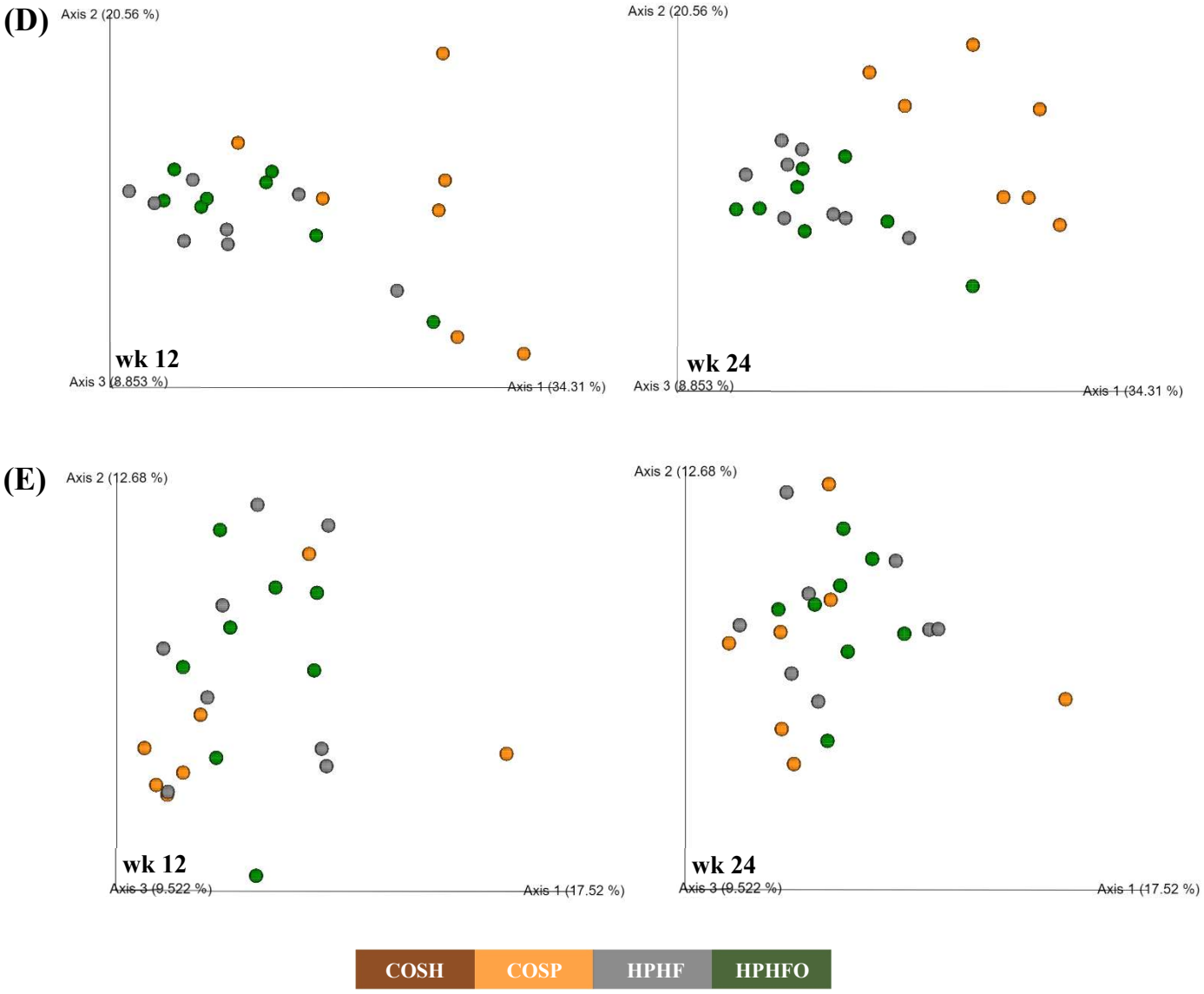

Figure 3.1. Fecal microbial communities of sham-operated dogs fed a control diet (COSH), of spayed dogs fed a control diet (COSP), spayed dogs fed a high-protein, high-fiber diet (HPHF), and spayed dogs fed a high-protein, high-fiber diet with additional omega-3 and medium-chain fatty acids (HPHFO). (A) Shannon diversity index suggested that species richness was lower ($P<0.05$) in COSH dogs than HPHFO dogs at wk 12 and 24, and tended to be lower ($P<0.10$) in COSH dogs than HPHF dogs at wk 12 and 24. COSP-12: COSP dogs at wk 12; COSP-24: COSP dogs at wk 24; HPHF-12: HPHF dogs at wk 12; HPHF-24: dogs at wk 24; HPHFO-12: HPHFO dogs at wk 12; HPHFO-24: HPHFO dogs at wk 24. **Mean values differ ($P<0.05$). *Mean values tend to differ ($P<0.10$). (B) Principal coordinates analysis (PCoA) plots of weighted UniFrac distances of fecal microbial communities of all dogs revealed that dogs fed HPHF or HPHFO clustered together and separately from dogs fed the control diet (COSH or COSP) at wk 12 and wk24 ($P<0.05$). (C) PCoA plots of unweighted UniFrac distances of fecal microbial communities of all dogs were not different among treatment groups ($P>0.05$). (D) PCoA plots of weighted UniFrac distances of fecal microbial communities of spayed dogs revealed that dogs fed HPHF or HPHFO clustered together and separately from dogs fed the control diet (COSP) at wk 12 and wk 24 ($P<0.05$). (E) PCoA plots of unweighted UniFrac distances of fecal microbial communities of spayed dogs ($P>0.05$).

Figure 3.2

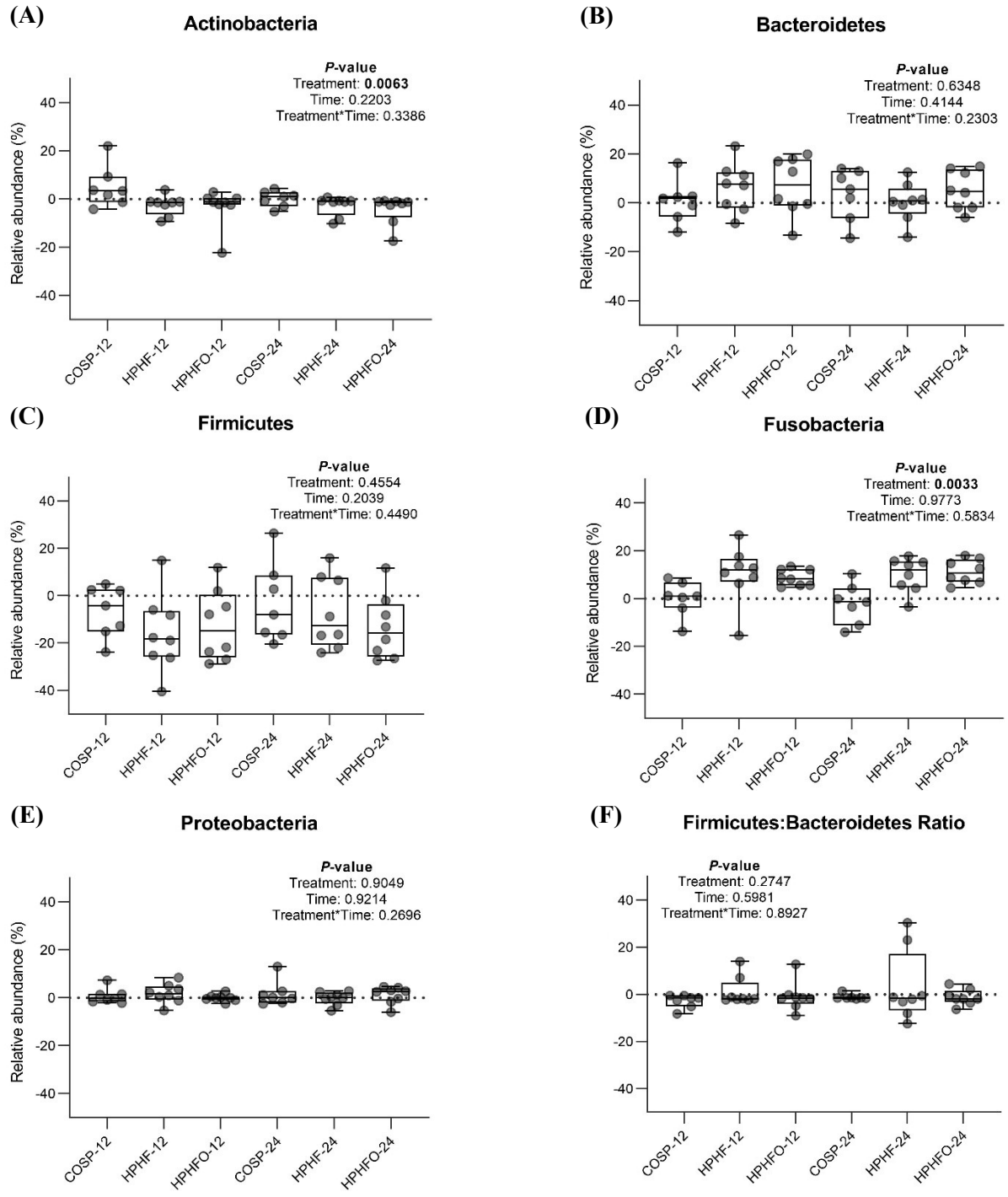
(A)
Actinobacteria
P-value
Treatment: 0.0063
Time: 0.2203
Treatment*Time: 0.3386
(B)
Bacteroidetes
P-value
Treatment: 0.6348
Time: 0.4144
Treatment*Time: 0.2303
(C)
Firmicutes
P-value
Treatment: 0.4554
Time: 0.2039
Treatment*Time: 0.4490
(D)
Fusobacteria
P-value
Treatment: 0.0033
Time: 0.9773
Treatment*Time: 0.5834
(E)
Proteobacteria
P-value
Treatment: 0.9049
Time: 0.9214
Treatment*Time: 0.2696
(F)
Firmicutes:Bacteroidetes Ratio
P-value
Treatment: 0.2747
Time: 0.5981
Treatment*Time: 0.8927
Relative abundance (%)
40
20
0
-20
-40
COSP-12
HPHF-12
HPHFO-12
COSP-24
HPHF-24
HPHFO-24

Figure 3.2. Change from baseline relative abundance (%) of fecal (A) Actinobacteria, (B) Bacteroidetes, (C) Firmicutes, (D) Fusobacteria, (E) Proteobacteria phyla of spayed dogs fed the control diet (COSP), spayed dogs fed a high-protein, high-fiber diet (HPHF), and spayed dogs fed a high-protein, high-fiber diet with additional omega-3 and medium-chain fatty acids (HPHFO). COSP-12: COSP dogs at wk 12; COSP-24: COSP dogs at wk 24; HPHF-12: HPHF dogs at wk 12; HPHF-24: dogs at wk 24; HPHFO-12: HPHFO dogs at wk 12; HPHFO-24: HPHFO dogs at wk 24. Data are represented as the change from baseline (wk 0) least squares means ± SEM.

Figure 3.3

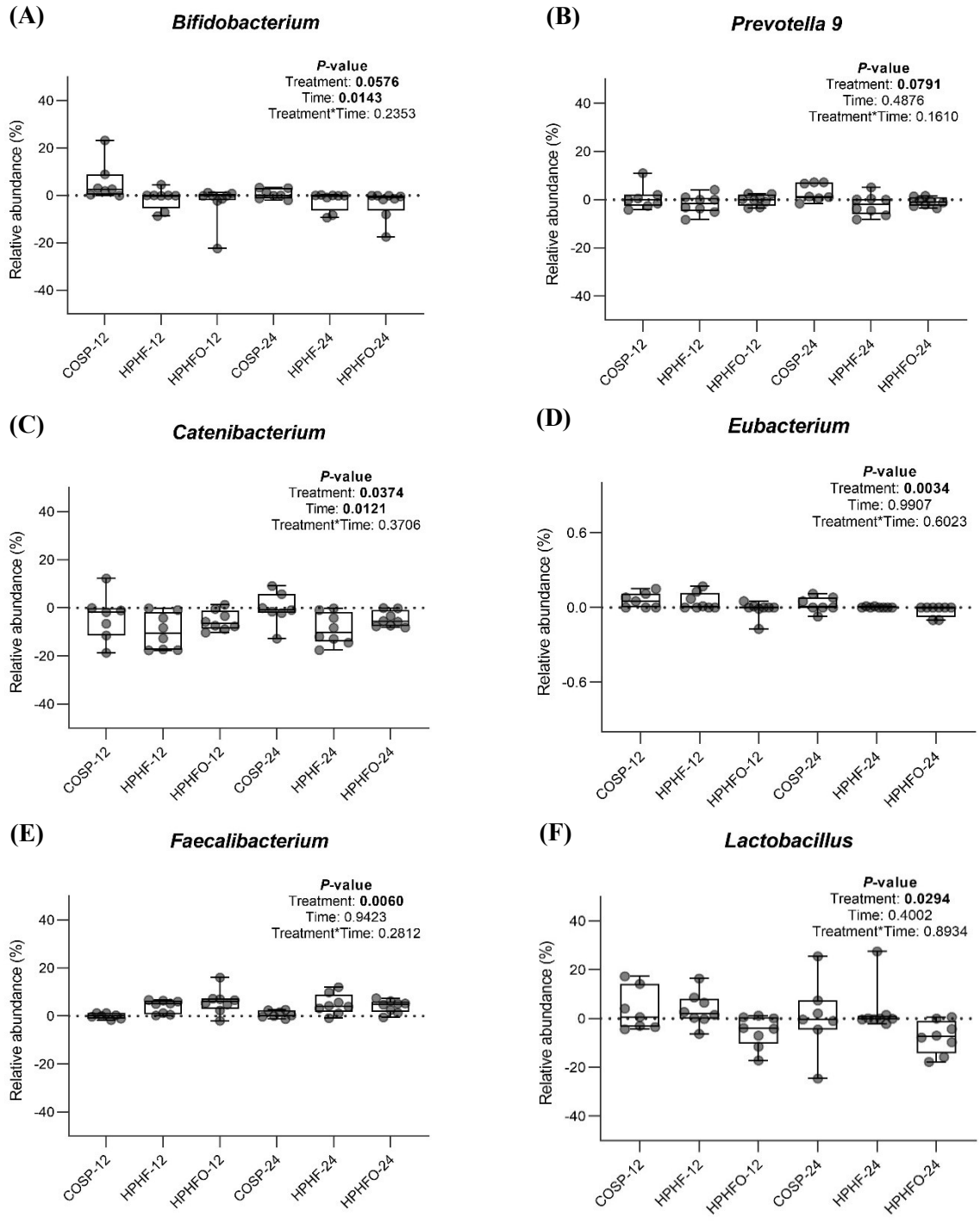
(A)
Bifidobacterium
P-value
Treatment: 0.0576
Time: 0.0143
Treatment*Time: 0.2353
(B)
Prevotella 9
P-value
Treatment: 0.0791
Time: 0.4876
Treatment*Time: 0.1610
(C)
Catenibacterium
P-value
Treatment: 0.0374
Time: 0.0121
Treatment*Time: 0.3706
(D)
Eubacterium
P-value
Treatment: 0.0034
Time: 0.9907
Treatment*Time: 0.6023
(E)
Faecalibacterium
P-value
Treatment: 0.0060
Time: 0.9423
Treatment*Time: 0.2812
(F)
Lactobacillus
P-value
Treatment: 0.0294
Time: 0.4002
Treatment*Time: 0.8934
Relative abundance (%)
COSP-12
HPHF-12
HPHFO-12
COSP-24
HPHF-24
HPHFO-24

Figure 3.3 (cont.)

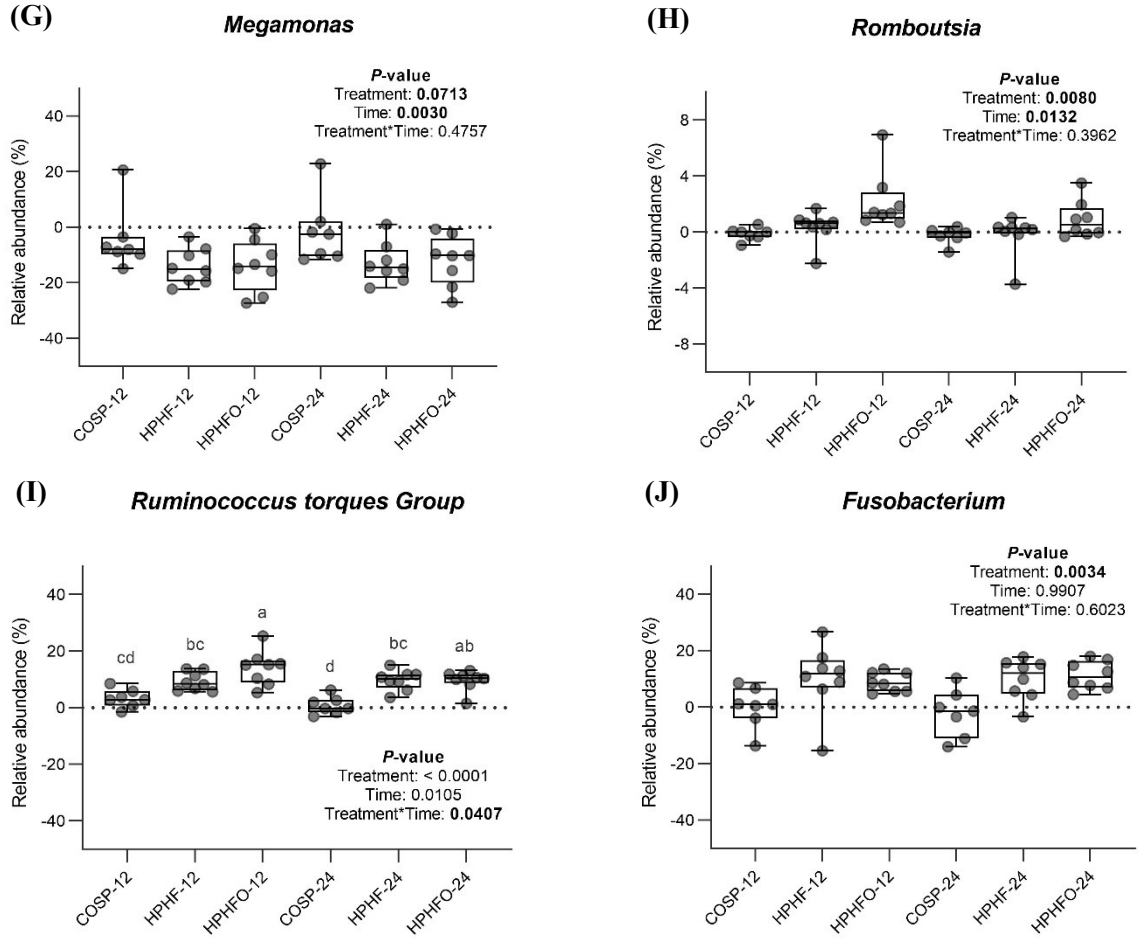

Figure 3.3. Change from baseline relative abundance (%) of fecal (A) *Bifidobacterium*, (B) *Prevotella 9*, (C) *Catenbacterium*, (D) *Eubacterium*, (E) *Faecalibacterium*, (F) *Lactobacillus*, (G) *Megamonas*, (H) *Romboutsia*, (I) *Ruminococcus Torques* group, and (J) *Fusobacterium* genera of spayed dogs fed a CO diet (COSP); spayed dogs fed a high-protein, high-fiber diet (HPHF), and spayed dogs fed a high-protein, high-fiber diet with additional omega-3 and medium-chain fatty acids (HPHFO). COSP-12: COSP dogs at wk 12; COSP-24: COSP dogs at wk 24; HPHF-12: HPHF dogs at wk 12; HPHF-24: dogs at wk 24; HPHFO-12: HPHFO dogs at wk 12; HPHFO-24: HPHFO dogs at wk 24. Data are represented as the change from baseline (wk 0) least squares means ± SEM. $^{a-c}$Different letters differ ($P<0.05$).

Supplementary Table 3.1. Baseline food intake, fecal characteristics, apparent total tract macronutrient and energy digestibilities, and fecal metabolites of adult female dogs fed different diets

Item	Dietary treatment group[1]					P-values
	COSH	COSP	HPHF	HPHFO	SEM	Treatment
Consumption						
Food intake (as-is), g/d	225.0	202.5	220.5	217.2	15.21	0.535
Food intake (DM), g/d	203.6	183.3	199.6	196.6	13.76	0.535
Energy intake, kcal/d	858.8	773.6	843.3	831.0	64.23	0.575
Fecal characteristics						
Fecal pH	5.28	5.35	5.26	5.48	0.153	0.4493
Fecal score[2]	2.88	2.92	2.88	3.06	0.194	0.7795
Fecal dry matter (DM), %	26.00	25.86	25.14	25.05	0.748	0.7617
Fecal output (DM), g/day	19.74	17.62	18.96	18.77	1.619	0.8668
Macronutrient and energy digestibility						
Protein digestibility, %	87.74	87.56	88.59	88.76	1.413	0.7701
Fat digestibility, %	96.88	96.50	96.68	96.73	0.422	0.8333
Total dietary fiber digestibility, %	61.51	60.55	62.86	62.43	4.331	0.9676
Organic matter digestibility, %	91.68	91.54	91.88	91.84	0.923	0.9883
Energy digestibility, %	91.46	91.29	91.71	91.72	0.954	0.9728
Fecal metabolites						
Acetate, µmol/g	405.7	467.4	462.4	424.1	32.05	0.611
Propionate, µmol/g	273.9	236.7	300.5	238.3	3789.6	1.000

Supplementary Table 3.1 (cont.). Baseline food intake, fecal characteristics, apparent total tract macronutrient and energy digestibilities, and fecal metabolites of adult female dogs fed different diets

Butyrate, µmol/g	58.01	53.67	46.77	47.88	25.604	0.6988
Total SCFA[4], µmol/g	737.6	757.8	809.7	710.2	62.66	0.594
Isobutyrate, µmol/g	3.38	3.39	3.00	3.82	0.645	0.8136
Isovalerate, µmol/g	4.41	4.85	4.20	4.81	0.898	0.9057
Valerate, µmol/g	2.96	1.57	1.53	1.67	0.424	0.1859
Total BCFA[4], µmol/g	10.75	9.87	8.73	10.29	1.538	0.8230
Ammonia, µmol/g	111.4	139.2	142.1	113.9	19.526	0.5880

[1]Dietary treatment group: COSH: sham-operated dogs fed CO; COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO.

[2]Fecal scores: 1 = hard, dry pellets; small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed and moist stool retains shape; 4 = soft, unformed stool, assumes shape of container; 5 = watery, liquid that can be poured.

[3]Total SCFA: total short-chain fatty acids = acetate + propionate + butyrate; total BCFA: total branched-chain fatty acids = isobutyrate + isovalerate + valerate.

Supplementary Table 3.2. Baseline fecal bile acid concentrations and percentages of adult female dogs fed different diets

Item	Dietary treatment group[1]					P-values
	COSP	COSP	HPHF	HPHFO	SEM	Treatment
Cholic acid	0.94	0.78	0.92	2.46	0.645	0.2020
Chenodeoxycholic acid	0.26	0.49	0.40	0.49	0.111	0.5531
Lithocholic acid	0.43	0.65	0.65	0.44	0.096	0.2126
Deoxycholic acid	3.59	3.46	5.20	3.71	0.653	0.1970
Ursodeoxycholic acid	0.06	0.14	0.11	0.10	0.048	0.4375
Total primary bile acid	1.19	1.27	1.32	2.96	0.734	0.2579
Total secondary bile acid	4.07	4.25	5.96	4.25	0.721	0.2134
Total bile acid	5.27	5.52	7.28	7.21	1.120	0.4877
Secondary bile acid[2], %	81.66	74.00	81.73	66.74	7.535	0.4409
Primary bile acid[2], %	18.34	26.00	18.27	33.26	7.535	0.4409

[1]Dietary treatment group: COSH: sham-operated dogs fed CO; COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO.

[2]Primary bile acid (sum of cholic acid and chenodeoxycholic acid) and secondary bile acid (sum of lithocholic acid, deoxycholic acid, and ursodeoxycholic acid) are expressed as a percent of total bile acid measured.

Supplementary Table 3.3. Baseline relative abundances (%) of fecal microbiota of adult female dogs fed different diets

Phylum	Genus	Dietary treatment group[1]					P-values
		COSH	COSP	HPHF	HPHFO	SEM	Treatment
Actinobacteria		3.75	3.59	3.88	6.25	2.105	0.8876
	Bifidobacterium	2.00	1.30	2.50	4.64	2.335	0.7687
	Collinsella	1.73	2.05	1.45	1.30	0.491	0.6690
Bacteroidetes		17.75	11.66	16.50	9.63	2.384	0.1254
	Bacteroides	6.25	6.58	9.50	5.88	2.902	0.4961
	Prevotella 9	7.90[a]	3.42[b]	4.09[ab]	1.88[b]	1.661	0.0048
Firmicutes		66.25	71.62	64.62	70.75	5.038	0.4004
	Allobaculum	8.50	1.54	4.88	3.75	3.584	0.6780
	Blautia	3.88	7.50	4.95	6.43	1.312	0.3209
	Catenibacterium	11.75	9.27	10.00	5.75	3.467	0.4943
	Clostridium sensu stricto 1	0.23	1.17	0.06	0.46	0.632	0.8375
	Enterococcus	0.00	0.00	0.00	0.01	0.008	0.5717
	Eubacterium	0.03	0.01	0.00	0.04	0.020	0.4124
	Faecalibacterium	1.00	1.43	2.00	0.88	0.507	0.3780
	Lachnoclostridium	0.25	0.45	0.31	0.35	0.188	0.9782
	Lachnospiraceae NK4A136 group	0.25	0.17	0.20	0.11	0.092	0.5575
	Lactobacillus	4.10	5.85	2.28	10.20	3.069	0.1716
	Megamonas	15.00	10.86	15.00	14.75	3.117	0.7369
	Peptoclostridium	3.25	5.75	6.13	5.75	1.097	0.3506
	Romboutsia	0.50	0.26	0.88	0.13	0.402	0.6658
	Roseburia	0.01	0.06	0.06	0.06	0.041	0.7521
	Ruminococcus gnavus group	2.75	5.66	3.26	2.58	1.541	0.5343
	Ruminococcus torque group	0.00	0.43	0.25	0.25	0.177	0.9726

Supplementary Table 3.3 (cont.). Baseline relative abundances (%) of fecal microbiota of adult female dogs fed different diets

	Streptococcus	0.23	0.70	0.25	3.14	1.519	0.1411
Fusobacteria		6.25	9.69	10.13	9.00	3.081	0.8252
	Fusobacterium	6.25	9.69	10.13	9.00	3.081	0.8252
Proteobacteria		5.75	3.26	4.63	4.38	1.165	0.2442

[1]Dietary treatment group: COSH: sham-operated dogs fed CO; COSP: spayed dogs fed CO; HPHF: spayed dogs fed HPHF; HPHFO: spayed dogs fed HPHFO

[a,b] Mean values within the same row with unlike superscript letters represent significant treatment effects ($P < 0.05$).

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*CHAPTER 4: WEIGHT LOSS AND HIGH-PROTEIN, HIGH-FIBER DIET CONSUMPTION IMPACT BLOOD METABOLITE PROFILES, BODY COMPOSITION, VOLUNTARY PHYSICAL ACTIVITY, FECAL MICROBIOTA, AND FECAL METABOLITES OF ADULT DOGS

ABSTRACT

Canine obesity is associated with reduced lifespan, metabolic dysfunction, and dysbiosis, but dietary intervention may aid in its management. This study aimed to determine the effects of restricted feeding of a high-protein, high-fiber (HPHF) diet and weight loss on body composition, physical activity, blood metabolites, and fecal microbiota and metabolites of overweight dogs. Twelve dogs (age: 5.5±1.1 yr; BW: 14.8±2.0 kg, BCS: 7.9±0.8) were fed a high-protein (42.0% dry matter), high-fiber (26.8% dry matter) diet during a 4-wk baseline phase to maintain BW. After baseline (wk 0), dogs were initially fed at 80% of that needed to maintain BW and then adjusted to target 1.5% weekly weight loss for 24 wk. Body composition using dual-energy x-ray absorptiometry and blood samples (wk 0, 6, 12, 18, 24), voluntary physical activity (wk 0, 7, 15, 23), and fresh fecal samples for microbiota and metabolite analysis (wk 0, 4, 8, 12, 16, 20, 24) were measured over time. Microbiota data were analyzed using QIIME 2. All data were analyzed statistically over time using SAS 9.4.

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After 24 wk, dogs lost 31.2% of initial BW and had 1.43±0.73% weight loss per wk. BCS decreased (P<0.0001) by 2.7 units, lean mass percentage increased (P<0.0001) by 11.3%, and fat mass and percentage decreased (P<0.0001) by 3.1 kg and 11.7%, respectively, with weight loss. Many serum metabolites and hormones were altered, with triglycerides, leptin, insulin, C-reactive protein, and interleukin-6 decreasing (P<0.05) with weight loss. Relative abundances of fecal *Bifidobacterium*, Coriobacteriaceae UCG-002, undefined Muribaculaceae, *Allobaculum, Eubacterium, Lachnospira, Negativivibacillus, Ruminococcus gauvreauii group,* uncultured Erysipelotrichaceae, and *Parasutterella* increased (P<0.05), whereas Prevotellaceae Ga6A1 group, *Catenibacterium, Erysipelatoclostridium, Fusobacterium, Holdemanella, Lachnoclostridium, Lactobacillus, Megamonas, Peptoclostridium, Ruminococcus gnavus group,* and *Streptococcus* decreased (P<0.01) with weight loss. Fecal ammonia and secondary bile acids decreased, while fecal valerate increased with weight loss. Several significant correlations were observed between gut microbial taxa and key biological parameters. Our results suggest that restricted feeding of a HPHF diet and weight loss promotes fat mass loss, minimizes lean mass loss, reduces inflammatory marker and triglyceride concentrations, and modulates fecal microbiota phylogeny and activity in overweight dogs. Even though microbial changes were observed with weight loss, a state of dysbiosis was not observed in overweight dogs.

INTRODUCTION

Obesity is recognized as one of the adverse effects coming from the global nutrition transition that has occurred in developed countries over the past few decades, whereby the incidence of high-calorie food consumption and sedentary lifestyle has increased (Popkin et al., 2012). Presently, obesity is a global epidemic disease, and its prevalence continues to increase. A

similar trend has been observed in companion pets (e.g., dogs and cats). The human-companion animal bond has been researched extensively and shown to improve human health outcomes (e.g., reduction in depression, anxiety, and mortality) (Friedman and Krause-Parello, 2018; Miyake et al., 2020). However, this bond and a changing living environment also promote pet obesity as a result of owner misinterpretation of ideal body condition scores (BCS), inappropriate feeding, insufficient exercise, and gonadectomy. Additional factors that increase pet obesity risk include genetics, age, sex, and health conditions (German, 2006; Laflamme, 2006; Courcier et al., 2010; Linder and Mueller, 2014; Rowe et al., 2017; Simpson et al., 2019).

Unquestionably, obesity is not only a multifactorial disease, but it also increases risk of type 2 diabetes, orthopedic disease, cardiorespiratory disorders, intestinal dysbiosis, gastrointestinal disorders, and cancers (German, 2006; Weiss and Hennet, 2017; Upadhyay et al., 2018; Emerenziani et al., 2019). Because obesity causes alteration of adipokine production, its consequences affect insulin secretion and glucose and lipid homeostasis, contributing to the development of obesity-related metabolic dysfunction and dysbiosis (Kahn et al., 2006; Ndumele et al., 2006; Saad et al., 2016). Hence, obesity, insulin resistance, and gut microbiota are linked and thought to be potential pathophysiological causes of one another. Intestinal bacteria increase dietary energy extraction and produce metabolites and cytokines that affect host metabolism and consequent weight gain. Likewise, obesity diminishes gut microbial diversity and alters its composition, alters tight junction protein production, increases intestinal permeability. Increased intestinal permeability leads to lipopolysaccharide translocation into the systemic system and induces inflammation and insulin resistance (Lee et al., 2020).

Fortunately, the risk of obesity-related morbidities can be reduced by restricted feeding and consequent weight reduction. Generally, successful weight loss comes from a combination of

increased physical activity and the restricted feeding of an appropriately formulated diet. High-protein and/or high-fiber diets are typically considered to be the best option when it comes to weight loss programs for dogs and cats (Blanchard et al., 2004; German et al., 2007; Floerchinger et al., 2015; André et al., 2017; Kieler et al., 2017; Pallotto et al., 2017; Salas-Mani et al., 2018; Bermudez Sanchez et al., 2020).

Several studies have shown the positive effects of weight loss in dogs, with benefits including improved metabolism, metabolic outcomes, and mobility, decreased disease risk, improved quality of life, and increased life expectancy (German, 2016). Additionally, weight loss reduces serum leptin, insulin, glucose, interleukin-6 (IL-6), and C-reactive protein (CRP) concentrations and increases serum adiponectin concentration and physical activity level (Jeusette et al., 2005; German et al., 2009; Warren et al., 2011; Bastien et al., 2015; Floerchinger et al., 2015; Starr et al., 2019). The relationships between weight loss and gut microbiota have been noted in a few canine studies (Kieler et al., 2017; Salas-mani et al., 2018; Burmudez Sanchez et al., 2020). However, none of those experiments integrated and investigated longitudinal changes in serum metabolites, gut microbiota, and fecal metabolites during weight loss. Additionally, identifying strong correlations among those variables may be key to understanding energy balance and identifying potential remedies of obesity.

The objective of this study was to determine the effects of weight loss on body composition, voluntary physical activity, blood metabolite profiles, serum markers of inflammation, fecal microbiota populations, and fecal metabolites of overweight dogs fed a high-protein, high-fiber (HPHF) diet. We hypothesized that closely monitoring BW and adjusting intake of a HPHF diet would lead to steady weight loss and increased fat loss while maintaining lean mass. Additionally, weight loss was hypothesized to increase voluntary physical activity, reduce blood lipids, and

decrease serum inflammatory markers. Based on the results of our previous study testing a similar diet in dogs (Phungviwatnikul et al., 2021), weight loss and consumption of a HPHF diet was expected to beneficially alter the fecal microbiota community (e.g., increased *Bifidobacterium, Lactobacillus, Faecalibacterium, Romboutsia, Fusobacterium* and decreased *Catenibacterium, Streptococcus,* and *Megamonas*) and metabolite concentrations [e.g., increased short-chain fatty acid (SCFA) and total bile acids concentrations and decreased protein catabolites] in overweight dogs.

MATERIALS AND METHODS

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee prior to experimentation (IACUC #18268).

Experimental design:

Twelve overweight adult spayed female beagles (age: 5.5 ± 1.1 yr; BW: 14.8 ± 2.0 kg; BCS: 7.9 ± 0.75) were used in a longitudinal weight loss study. The experiment consisted of 28 wk, with a 4-wk baseline phase, followed by a 24-wk weight loss phase. All dogs were considered healthy except for being overweight. Complete blood count and serum biochemistry panel were within the reference range. Dogs had not received any medications that could affect blood parameters and gut microbiota for at least 4 wk before and during the experiment. The maintenance energy requirements (MER) of all dogs were obtained during a 4-wk baseline phase, where animals were fed the experimental diet.

The diet was formulated to meet all Association of American Feed Control Officials (AAFCO, 2018) nutrient recommendations for adult dogs at maintenance **(Table 4.1)**. Several

dietary fiber and prebiotic sources and functional ingredients were used in the diet. Inclusion of barley, beet pulp, cellulose, psyllium hush, scFOS, and brown flax seed have been shown to provide beneficial effects on the host, with this mixture providing a balance of insoluble and soluble dietary fibers in the diet. Additionally, the inclusion rate of scFOS and high concentrations of barley and beet pulp in diets were reported in previous literature and were well-tolerated by dogs (Respondek et al., 2007; de Godoy, 2011; Donadelli and Aldrich, 2019). Furthermore, several functional ingredients were included in the diet. L-carnitine aids in long-chain fatty acid transport and energy production, especially during weight loss, and has been shown to improve energy expenditure of dogs (Varney et al., 2020). The concentration of L-carnitine inclusion followed AAFCO (2018) recommendations. Fish oil was included in the diet for its anti-inflammatory and blood lipid-lowering properties in dogs (Adler et al., 2018; de Godoy et al., 2018). Green tea extract was included in the diet because it has been shown to improve insulin sensitivity and lipid profile of obese dogs (Serisier et al., 2008). Chromium, added in the form of chromium picolinate, is an essential trace mineral that is required for the cellular uptake of glucose (Schachter et al., 2001). Lastly, vitamin C and vitamin E are important in metabolic function and have antioxidant properties (El-Warrak et al., 2012; Gordon et al., 2020). The concentrations of fish oil, green tea extract, chromium picolinate, vitamin C, and vitamin E were based on the published literature.

After the baseline phase (wk 0), dogs were fed at a rate to lose approximately 1.5% BW per wk (range from 1-2% for dogs) as recommended by the American Animal Hospital Association – weight management guidelines for dogs and cats (Brooks et al., 2014). To achieve weight loss, dogs initially received 80% of the food required to maintain BW during the baseline phase and then energy intake was adjusted weekly based on the level of weight loss. Dogs were weighed and BCS (9-point scale; Laflamme, 1997) were evaluated weekly, with all being performed in the

morning before feeding. Once dogs met their target weight, food intake (FI) was adjusted to maintain BW.

Dogs were housed individually in pens (1.22 m wide × 1.85 m long) in a temperature-controlled room under a 12 h light:12 h dark cycle in the Veterinary Medicine Basic Sciences Building at the University of Illinois. Dogs had free access to fresh water and were fed twice daily (9:00 am and 5:00 pm) throughout the study. Food offerings and refusals were measured daily to calculate intake. Dogs were allowed outside of their pens a couple days a week for socialization with other dogs in compatible groups and humans, except on collection days. Pens were cleaned daily and dogs were bathed every 2 wk.

Chemical analysis of diets:

The experimental diet was subsampled and ground through a 2-mm screen using a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ). The sample was analyzed according to procedures of the Association of Official Analytical Chemists (AOAC) for dry matter (DM; 105°C) and ash [organic matter (OM) was calculated from ash] (AOAC, 2006; methods 934.01, 942.05). Crude protein content was calculated from Leco total N values (TruMac N, Leco Corporation, St. Joseph, MI; AOAC, 2006). Total lipid content (acid-hydrolyzed fat) of the sample was determined according to the methods of the American Association of Cereal Chemists (AACC, 1983) and Budde (1952). Gross energy of the diet was measured using an oxygen bomb calorimeter (model 1261, Parr Instruments, Moline, IL). Total dietary fiber (TDF) content was determined according to Prosky et al. (1985).

Body composition:

Body composition was evaluated by dual-energy x-ray absorptiometry [DEXA: Hologic X-ray Bone Densitometer QDR 4500 Elite Acclaim Series] at the University of Illinois Veterinary Teaching Hospital at baseline (wk 0) and after 6, 12, 18, and 24 wk of the weight loss. To perform DEXA scans, dogs were sedated by an intramuscular injection of Dexdomitor (0.02 mg/kg) and Torbugesic (0.2 mg/kg), and positioned in sternal recumbency. The four legs, trunk, and head of each dog were scanned individually, and measurements of fat, lean, and bone mineral content were taken in each body region. Body fat percentage was calculated for each part and the entire body. After the measurement, an intramuscular injection of the reversal agent for dexmedetomidine, atipamezole (0.2 mg/kg BW), was given.

Voluntary physical activity:

At baseline (wk 0), wk 7, wk 15, and wk 23, accelerometers (Actical devices: Mini Mitter, Bend, OR) were used to measure voluntary physical activity. During activity monitoring periods, Actical devices were attached to collars worn around the neck for six consecutive d. Mean activity was presented in activity counts per epoch (epoch length = 0.25 min), with light h (0700-1900) and dark h (1900-0700) also being measured.

Complete blood count, serum chemistry profile, blood hormones, and inflammatory markers:

Fasted (at least 10 h) blood samples were collected via jugular or cephalic puncture at baseline (wk 0) and after 6, 12, 18, and 24 wk of weight loss. Samples were immediately transferred to appropriate vacutainer tubes. Sterilized glass serum tubes (#366430 BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) were used for serum chemistry profile, hormones (leptin,

insulin), and inflammatory marker (CRP, IL-6) analyses. Plastic whole blood tubes with K_2EDTA additive (#365974 BD Microtainer, Becton Dickinson, Franklin Lakes, NJ) were used for complete blood count. Tubes were centrifuged at 2,000 × g for 15 min at 4ºC for serum collection. Serum chemistry profile and complete blood count were analyzed using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN) at the University of Illinois Veterinary Medicine Diagnostics Laboratory.

The concentrations of serum leptin, insulin, CRP, and IL-6 were measured using commercial ELISA kits (leptin: #EZCL-31K, MilliporeSigma, Burlington, MA; insulin: #10-1203-01, Mercodia, Winston Salem, NC; CRP: #ab157698, Abcam, Cambridge, MA; IL-6: #ab193686, Abcam, Cambridge, MA).

Fecal sample collection:

Fresh fecal samples (within 15 min of defecation) were collected at baseline (wk 0) and after 4, 8, 12, 16, 20, and 24 wk of weight loss for measurement of fecal scores, pH, moisture content, microbiota populations, and fermentative metabolite concentrations. Fecal pH was measured immediately using an Accumet AP1001 Portable pH Meter Kit (Fisher Scientific, Waltham, MA) and then feces were aliquoted for other measures, including SCFA and protein fermentative products [ammonia; branched-chain fatty acids (BCFA)]. One fecal aliquot (~5 g/dog) was collected and placed in 2 N hydrochloric acid in a 1:1 (weight: weight) ratio and stored at -20ºC for SCFA, BCFA, and ammonia analyses. An additional aliquot was collected for DM determination. Finally, 4 aliquots of fresh feces were collected in sterile cryogenic vials (Nalgene, Rochester, NY), frozen on dry ice, and stored at -80ºC for microbiota and bile acid analyses.

Fecal scores:

All fecal samples during the collection phase were scored according to the following scale 1 to 7 (Greco, 2015): 1 = very hard and dry, often expelled as individual pellets, no residue left on ground when picked up; 2 = firm but not hard, segmented in appearance, little or no residue on ground when picked up; 3 = log-shape, moist surface, leaves residue on ground but hold form when picked up; 4 = very moist, soggy, log-shaped, leaves residue and loses form when picked up; 5 = very moist but has a distinct shape, piles rather than distinct logs, leaves residue and loses form when picked up; 6 = has texture but no defined shape, present as piles or spots, leaves residue when picked up; 7 = watery, no texture, flat puddles.

Fecal chemical analyses:

Fecal samples were analyzed according to procedures of the Association of Official Analytical Chemists (AOAC, 1975) for DM using a 105ºC oven. Fecal SCFA and BCFA concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H_3PO_4 on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively. Fecal ammonia concentrations were determined according to the method of Chaney and Marbach (1962).

The protocol for quantifying bile acids was adapted and modified from the methods previously described by Batta et al. (2002). Briefly, an aliquot of 10-15 mg lyophilized stool was added to 200 µL of 1-butanol containing internal standards (cholic acid-d_4 and lithocholic acid-d_4) followed by adding 20 µl of hydrochloric acid. Samples were incubated for 4 h at 65ºC. Following

incubation, samples were completely evaporated at 65°C under nitrogen gas, 200 µl trimethylsilylation derivatization agent was added and samples were incubated for 30 min. The samples were then evaporated under nitrogen gas and resuspended in 200 µl hexane, vortexed, and centrifuged at 4°C for 10 min at 3,000 × g. The supernatant was then analyzed by gas chromatography and mass spectrometry according to methods described by Blake et al. (2019). Cholic acid (CA), chenodeoxycholic acid CDCA), lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) were measured.

Fecal microbiota populations:

Total DNA from fecal samples was extracted using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Carlsbad, CA) with bead beating using a vortex adaptor. The concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA). 16S rRNA gene amplicons of the V4 region were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with a Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) that target a 252 bp-fragment of that region were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. The quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 2% agarose E-gel (Life Technologies, Carlsbad, CA) and extracted using a Qiagen gel purification kit (Qiagen, Carlsbad, CA). Cleaned size-selected pooled products were run on an Agilent 2100 Bioanalyzer

to confirm the appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the W. M. Keck Center for Biotechnology at the University of Illinois.

Fecal microbiota bioinformatics and statistical analyses:

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.14) and QIIME 2.2019.4 (Bolyen et al., 2019) was used to process the resulting sequence data. Briefly, high-quality (quality value ≥ 20) sequence data derived from the sequencing process were demultiplexed. Data were then denoised and assembled into amplicon sequence variants (ASV) using DADA2 (Callahan et al., 2016). The SILVA 132 database (Quast et al., 2013) was used to assign taxonomy. An even sampling depth (23,385 sequences per sample) was used to assess alpha- and beta-diversity measures. Beta-diversity was assessed using weighted and unweighted UniFrac distance (Lozupone and Knight, 2005) measures and presented using principal coordinates analysis (PCoA) plots.

Statistical analyses:

FI, CI, BW, BCS, body composition, serum chemistry, complete blood cell count, blood hormones, inflammatory markers, fecal characteristics, fecal metabolites, and fecal microbiota data were analyzed using the linear Mixed Models procedure of SAS (version 9.4; SAS Institute, Cary, NC). Data were analyzed using repeated measures analysis, with differences due to time being the focus. All results are presented as least squares means ± standard error of the mean (SEM). A $P<0.05$ was considered significant and a $P<0.10$ was considered a trend. The R programming language (RStudio version 1.1.463) was used to calculate the Spearman correlation

coefficient between 1) physiological parameters (BW, BCS, FI, CI, serum metabolites and hormones, and body composition) and the gut microbiota (wk 0, 6, 12, 18, 24); 2) between fecal metabolite and bile acid parameters and the gut microbiota (wk 0, 4, 8, 12, 16, 20, 24); and 3) between serum hormones (leptin and insulin) and fat mass (wk 0, 6, 12, 18, 24), a $P<0.05$ was considered significant, and the result was displayed using a heat map and a linear plot.

RESULTS

Food intake, caloric intake, BW, and BCS:

At baseline, average MER was 89.09 ± 19.5 × BW $kg^{0.75}$, average BW was 14.8 ± 2.0 kg, and average BCS was 7.9 ± 0.75. BCS decreased by 2.7 units ($P<0.0001$) over 24 wk of restricted feeding and weight loss. Nine out of 12 dogs achieved an ideal BCS (BCS 5/9) by wk 24, with 3 remaining overweight dogs having a BCS of 5.5 to 6. During weight loss, the average MER (wk 1 to wk 24) of all dogs was reduced to 70.51 ± 7.66 × BW $kg^{0.75}$, with MER being ~60-62 × BW $kg^{0.75}$ from wk 14 to 24 when a consistent level of weight loss was reached. After the 24 wk weight loss phase, dogs lost 31.2% of initial BW ($P<0.0001$), with 1.43 ± 0.73% weight loss per wk. Dogs consumed an average of 457.5 ± 61.4 kcal/d throughout the entire weight loss phase (wk 1 to wk 24), but settled on an approximate caloric intake of 360-400 kcal/d from wk 14 to 24 when a consistent level of weight loss was reached **(Figure 4.1)**. Energy intake was reduced by 30.96% by wk 24 compared to baseline ($P<0.0001$). Once dogs reached their target BW and had BCS 5, which started at wk 14 in some dogs, they received an amount of food to maintain BW once again.

Body composition:

After 24 wk of restricted feeding and weight loss, lean mass, fat mass, and fat

percentage were reduced (P<0.0001) by 1.27 kg, 3.12 kg, and 11.72%, respectively, whereas lean mass percentage was increased (P<0.0001) by 11.33% **(Table 4.2)**. Additionally, bone mineral content was decreased (P<0.01) by 0.01 kg. The alterations of lean mass and fat mass per kg BW loss were consistent throughout the experiment, with an average reduction of 0.26 kg in lean mass and 0.76 kg in fat mass.

Voluntary physical activity:

Average daily physical activity and activity during the light cycle were affected by time **(Table 4.3)**. Total activity was highest at wk 23, with a level that was higher (P<0.05) than that measured at wk 15, but not different than baseline or wk 7. Light cycle activity was highest at wk 23, with a level that was higher (P<0.05) than levels measured at wk 7 and wk 15, but not baseline. Activity during the dark cycle was highest at wk 23, which tended to be higher (P<0.10) than that measured at wk 7 and wk 15, but not baseline.

Serum metabolites, complete blood count, and blood hormones and inflammatory markers:

Serum creatinine, blood urea nitrogen (BUN), total bilirubin, and chloride concentrations were increased (P<0.01), serum triglycerides, creatine phosphokinase, calcium, alkaline phosphatase, and corticosteroid isoenzyme of alkaline phosphatase were reduced (P<0.01), and serum cholesterol tended to be decreased (P<0.10) with restricted feeding and weight loss **(Table 4.4)**. Other serum metabolites (total protein; albumin; globulin; albumin:globulin ratio; gamma-glutamyltransferase; phosphorus; sodium; potassium; sodium:potassium ratio) were affected by time, but were not consistently altered with restricted feeding and weight loss. Total white blood cells, neutrophils, lymphocytes, mean cell volume, and mean corpuscular hemoglobin

concentrations were decreased (P<0.01) with restricted feeding and weight loss **(Table 4.5)**. All blood hormone and inflammatory cytokine concentrations decreased (P<0.05) with restricted feeding and weight loss **(Table 4.6)**. After 24 wk, serum leptin (P<0.0001), insulin (P<0.0001), CRP (P<0.05), and IL-6 (P<0.05) concentrations were decreased by 68.5%, 61.7%, 57.4%, and 37.5%, respectively.

Fecal characteristics, fermentative metabolites, and fecal bile acids:

Fecal DM percentage increased (P<0.05), while fecal scores decreased (firmer stool; P<0.001) with restricted feeding and weight loss **(Table 4.7)**. Fecal acetate concentrations tended to decrease (P=0.051) and fecal ammonia concentrations decreased (P<0.05), while fecal valerate concentrations increased (P<0.01) with restricted feeding and weight loss. Fecal pH and other fecal metabolites were not altered over time. Fecal DCA concentrations decreased (P<0.05), fecal secondary bile acid concentrations tended to decrease (P=0.058), and fecal UDCA concentrations increased (P<0.01) with restricted feeding and weight loss **(Table 4.8)**. The other bile acid concentrations and percentages were not altered.

Fecal microbiota:

Alpha diversity indices, including the Shannon diversity index **(Figure 4.2A)**, were not affected by restricted feeding and weight loss. Beta diversity, which is represented by PCoA plots of unweighted **(Figure 4.2B)** and weighted **(Figure 4.2C)** UniFrac distances, revealed that fecal microbial populations shifted away from that measured at baseline (wk 0) with restricted feeding and weight loss (P<0.05).

Four bacterial phyla and over 30 bacterial genera were altered with restricted feeding and weight loss **(Supplementary Table 4.1**; **Figure 4.3)**. Relative abundances of fecal Proteobacteria,

Bifidobacterium, Coriobacteriaceae UCG-002, undefined Muribaculaceae*, Allobaculum, Eubacterium, Negativibacillus, Ruminococcus gauvreauii* group*,* uncultured Erysipelotrichaceae, and *Parasutterella* were consistently increased (P<0.05) with restricted feeding and weight loss. Relative abundances of fecal Prevotellaceae Ga6A1 group*, Catenibacterium, Erysipelatoclostridium, Holdemanella, Lachnoclostridium, Lactobacillus, Megamonas, Peptoclostridium, Ruminococcus gnavus* group*,* and *Streptococcus* were consistently decreased (P<0.01) with restricted feeding and weight loss. Relative abundances of fecal Actinobacteria, Fusobacteria, Campilobacterota, *Adlercreutzia, Slackia, Prevotella, Anaerofilum, Blautia, Clostridium sensu stricto 1, Enterococcus, Faecalibaculum, Faecalitalea, Lachnospira, Helicobacter, Fusobacterium* were different (P<0.05) and the relative abundances of fecal *Parabacteroides*, *Allisonella, Candidatus Stoquefichus, Epulopiscium,* uncultured Ruminococcaceae, and *Sutterella* tended to be different (P < 0.10) with restricted feeding and weight loss, but with an inconsistent pattern.

Correlations between key biological parameters and gut microbiota:

Several significant correlations were observed between gut microbial taxa and key biological parameters including BW, BCS, FI, CI, fecal bile acids (CA, CDCA, LCA, DCA, UDCA, primary bile acids, secondary bile acids, total bile acids), and fecal metabolites (total SCFA, acetate, propionate, butyrate, total BCFA, isobutyrate, isovalerate, valerate, ammonia) (**Supplementary Figure 4.1)**. Some of the correlations were also reported in the previous literature. In brief, BW was positively (P<0.05) correlated with the relative abundance of *Enterococcus* (r = 0.4443), but negatively (P<0.05) correlated with the relative abundance of *Romboutsia* (r = -0.2322), and *Sutterella* (r = -0.2549). BCS was positively (P<0.05) correlated

with the relative abundance of *Enterococcus* (r = 0.3346), but negatively (P<0.05) correlated with the relative abundance of *Lachnospira* (r = -0.2926). FI and CI were positively (P<0.05) correlated with the relative abundance of *Enterococcus* (FI: r = 0.5544; CI: r = 0.5544) and *Streptococcus* (FI: r = 0.3086; CI: r = 0.3087), but negatively (P<0.05) correlated with the relative abundance of *Bifidobacterium* (FI: r = -0.2384; CI: r = -0.2384) and *Allobaculum* (FI: r = -0.4275; CI: r = -0.4275).

Serum cholesterol concentrations were positively (P<0.05) correlated with the relative abundance of *Parasutterella* (r = 0.6303), but negatively (P<0.05) correlated with the relative abundance of *Blautia* (r = -0.4125) and *Romboutsia* (r = -0.2258). Serum IL-6 concentrations were positively (P<0.05) correlated with the relative abundance of *Catenibacterium* (r = 0.3388) and *Sutterella* (r = 0.3999). A negative (P<0.05) correlation existed between serum CRP concentrations and the relative abundance of *Faecalibacterium* (r = -0.0058).

Fecal total BCFA concentrations were positively (P<0.05) correlated with the relative abundance of Proteobacteria (r = 0.4326) and *Parasutterella* (r = 0.6580), but negatively (P<0.05) correlated with the relative abundance of Firmicutes (r = -0.3156) and *Blautia* (r = -0.4812). Fecal butyrate concentrations were positively (P<0.05) correlated with the relative abundance of *Allobaculum* (r = 0.3972) whereas fecal acetate concentrations were negatively (P<0.05) correlated with the relative abundance of *Bifidobacterium* (r = -0.3367). A positive (P<0.05) correlation existed between fecal valerate concentrations and the relative abundance of *Prevotella* (r = 0.3296). The relative abundance of *Bifidobacterium* was positively correlated with fecal total bile acid (r = 0.2188) and secondary bile acid (r = 0.2420) concentrations, whereas the relative abundance of *Lactobacillus* was negatively (P<0.05) correlated with fecal DCA (r = -0.2490) and secondary bile acid (r = -0.2358) concentrations.

DISCUSSION

Obesity is a chronic progressive disease and its prevalence has been increased in number over the past few decades. The marked increase in the prevalence of obesity cases in both humans and pets reflects the adaptation of dietary patterns and lifestyles to the modern world, the failure of healthcare institutions in the management of obesity, and a result of the human-pet relationship (AAHA 2003; Courcier et al., 2010; Rowe et al., 2017; Blüher, 2019; Lumbis and Scally, 2020). Companionship with pets fulfills the animal's requisite needs and provides health benefits to humans, however, it also is an important risk factor of pet obesity (Bland et al., 2010). Humans and pets (e.g., dogs and cats) have various similarities in regard to contributing factors of obesity and obesity-related comorbidities (e.g., type 2 diabetes, cardiorespiratory disorders, and joint diseases), which is likely because of misperceptions of ideal BW and feeding patterns, shared environmental elements, and lifestyles (German, 2006; German, 2015; Caballero, 2019). Not only is obesity a predisposition to secondary diseases, but it is also an economic burden on pet owners (Bomberg et al., 2017). Based on the records of the Nationwide Mutual Insurance Company (Columbus, OH), a US-based pet insurance company, approximately 20 percent of the claims filed were related to conditions or diseases associated with obesity, resulting in more than $90 million in veterinary expenses (Pet Product News, 2021). Therefore, obesity prevention and weight management are critically important for the health and companionship of pets. In humans, various weight-loss strategies have been used, including lifestyle modifications (e.g., altered diet, behavior, exercise), application of pharmacological agents, and surgery (Kushner, 2018). Likewise, the foundation to weight reduction in pets is increasing physical activity and implementing proper dietary modification.

In humans, several dietary patterns such as the Mediterranean diet, very-low-carbohydrate ketogenic diet, low-fat diet, high-protein diet, or high-fiber diet have been proposed to manage obesity and comorbidities (Shai et al., 2008; Bueno et al., 2013; Cuenca-Sánchez et al., 2015; Campos-Nonato et al., 2017; Gardner et al., 2018; Zhang et al., 2018). Most pet diets designed for weight loss have a low energy density due to substitution of high-calorie ingredients with functional dietary fibers (Blanchard et al., 2004; German et al., 2007; Floerchinger et al., 2015; André et al., 2017; Kieler et al., 2017; Pallotto et al., 2017; Salas-Mani et al., 2018; Bermudez Sanchez et al., 2020). Such diets also have increased concentrations of high-quality proteins and micronutrients to preserve lean muscle mass and avoid nutrient deficiency during reduced intake. Increased dietary fiber and protein may also aid in mitigating hunger during weight loss (Weber et al., 2007; Bosch et al., 2009; Ben-Harchache et al., 2020). Restricted feeding of HPHF diets has successfully been used in pets (Jeusette et al., 2005; German et al., 2009; Warren et al., 2011; Bastien et al., 2015; Floerchinger et al., 2015; Salas-Mani et al., 2018; Starr et al., 2019; Bermudez Sanchez et al., 2020). However, the effects of these diets on metabolic responses, physical activity, fecal microbiota, and fecal metabolites have not been studied at the same time. These data and relationships may provide a greater understanding of the underlying mechanisms and identify new approaches to alleviate obesity and its comorbidities in dogs.

In the current study, restricted feeding and weight loss led to reduced BW, BCS, fat mass, and blood triglycerides, cholesterol, leptin, insulin, CRP, and IL-6 as expected and similar to that reported in dogs previously (German et al., 2009; Rafaj et al., 2017). Even though alpha diversity was not impacted, restricted feeding and weight loss in the current study also shifted fecal microbiota populations, with four bacterial phyla and more than 30 bacterial genera being different over time. Changes in gut microbial diversity, composition, and functionality have been linked to

many disease states over the past decade, including neurological disorders, allergic diseases, gastrointestinal diseases, cardiovascular diseases, diabetes, and obesity (Mangiola et al., 2016; Patterson et al., 2016; Meng et al., 2018; Blake et al., 2019; Angelucci et al., 2019; Barcik et al., 2020; Kazemian et al., 2020; Li et al., 2021). Most pertinent here, the gut microbiota's potential contribution to weight gain, obesity, and metabolic dysfunction has been of interest, with reduced microbial diversity and an increased Firmicutes:Bacteroidetes ratio being reported in obese vs. lean individuals (Ley et al., 2005; Turnbaugh et al., 2009; Handl et al., 2013; Park et al., 2015). Those responses are not always observed, however, including the current study and other recent publications (Schwiertz et al., 2010; Frost et al., 2019; Magne et al., 2020; Phungviwatnikul et al., 2021).

Although most microbiota-obesity knowledge comes from humans and rodent models, a few recent dog studies have focused on this topic. Bermudez Sanchez et al. (2020) reported that weight loss increased alpha diversity (richness and evenness) and the relative abundances of Bacteroidetes and Fusobacteria, but decreased the relative abundance of Firmicutes and the Firmicutes:Bacteroidetes ratio in dogs. In another study, however, the biodiversity and relative abundance of bacterial taxa at the phylum level were not different due to weight loss in dogs (Salas-Mani et al., 2018). In the current study, alpha diversity was not impacted by restricted feeding and weight loss, but many bacterial genera shifted. The increased relative abundance of *Allobaculum* and decreased relative abundances of *Lactobacillus, Megamonas,* and *Catenibacterium* were in agreement with previous canine weight loss studies (Salas-Mani et al., 2018; Burmudez Sanchez et al., 2020)

In most studies, including the current one, it is difficult or impossible to distinguish fecal microbiota changes due to weight loss and consequent metabolic changes from those due to

restricted feeding or dietary change because reduced feeding and weight loss occur concurrently. Restricted feeding alone will reduce the substrate load reaching the large intestine, potentially affecting fecal microbial populations. Dietary fiber has a strong impact on the gut microbiota and is one of the most important dietary modifications in weight loss diets. A wide range of dietary fibers exist, with each having unique physicochemical characteristics, effects on host physiology and metabolism, and impacts on the gut microbiota populations. Some soluble fibers such as β-glucans and psyllium husk increase gut luminal viscosity and gut microbial fermentability, altering gastrointestinal transit time and enhancing microbiota-derived metabolite production (Gill et al., 2021). SCFA (i.e., acetate, propionate, and butyrate) are the primary metabolites of fermentation coming from dietary fibers and other non-digestible carbohydrates. These organic acids reduce luminal pH and serve as fuel for colonocytes, promoting gastrointestinal health, have anti-inflammatory and anti-carcinogenic effects, and assist in appetite regulation (van der Hee and Wells, 2021). The amount and type of SCFA produced depends on several factors, including bacterial taxonomic groups present, dietary ingredient type and amounts, and gut transit time (Wong et al., 2006). In the current study, fecal SCFA concentrations were not altered by restricted feeding. This lack of change may be related to the sample analyzed (e.g., feces), as the majority of SCFA are absorbed by colonocytes leaving little for excretion in feces.

Dietary fibers not only serve as food sources for gut microbes, but also may be a predictor of the structure of the gut microbial population. Several human and animal (rodents and dogs) studies have reported that the consumption of barley β-glucans increase relative abundances of fecal *Prevotella* and *Lactobacillus* (Kovatcheva-Datchary et al., 2015; Garcia-Mazcorro et al., 2018; Sanberg et al., 2019; Phungviwatnikul et al., 2021), and is positively associated with fecal valerate concentrations (Tap et al., 2015). Furthermore, dietary barley malt melanoidins increase

the relative abundances of *Parasutterella, Bifidobacterium*, and *Lactobacillus* (Aljahdali et al., 2020). Psyllium husk supplementation increased the abundance of *Lachnospira*, which was associated with increased fecal water content (Jalanka et al., 2019). In addition, Mayengbam et al. (2019) observed a negative correlation between *Lachnospira* relative abundance and BW. Others have shown that the gut microbial population are affected by prebiotic inulin-type fructans (e.g., FOS, oligofructose, and inulin) (Kelly, 2008), with increased relative abundance of *Bifidobacterium* being the most consistent finding (Barry et al., 2010; Vandeputte et al., 2017; Healey et al., 2018; Bastard et al., 2020). *Bifidobacterium* populations are thought to be important in maintaining gut health, as it has been linked with IBD remission in humans and dogs (Papa et al., 2012; White et al., 2017). Moreoever, previous studies have shown that *Bacteroides* are more prevalent in individuals consuming high protein and fat concentrations, while *Prevotella* is more common in individuals consuming high fiber and carbohydrate concentrations (Wu et al., 2011; Kovatcheva-Datchary et al., 2015; Moreno-Pérez et al., 2018). Finally, several bacterial strains of the *Clostridium, Eubacterium, Ruminococcus,* and *Bacteroides* genera ave known to have cellulose-degrading properties (Hamaker and Tuncil, 2014).

High-protein and amino acid-rich diets aid in minimizing lean mass loss and stimulating muscle protein synthesis (Wolfe, 2002). In the current study, although dogs were restricted fed, they consumed approximately 2 times the daily protein requirement (Brooks et al., 2014), had constant rates of fat and lean mass loss, and the proportions of fat mass and lean mass loss were 71% and 29%, which were similar to the findings reported by Pasiakos et al. (2013). These results suggest that this protein intake was appropriate for weight loss. High intake of protein will lead to greater amino acid catabolism, leading to higher ammonia and urea production and higher concentrations in the bloodstream and intestinal lumen. Urea and non-digestible peptides will

reach the large intestine and may be catabolized by gut microbes (Shen et al., 2015). The utilization of endogenous and exogenous nitrogenous substances by gut microbiota such as bacteria of the genera *Clostridium, Peptostreptococcus, Fusobacterium, Bacteroides, Veillonella, Bifidobacterium, Lactobacillus, Eubacterium,* and *Peptococcus* affect the biosynthesis of microbial protein, fermentation products of amino acids (e.g., ammonia, SCFA, and BCFA), and amino acid homeostasis of the host (Suzuki et al., 1979; Dai et al., 2011). In the current study, serum ammonia concentrations decreased over time, being positively associated the relative abundance of *Lactobacillus* and negatively associated with the relative abundance of *Bifidobacterium.* An enrichment of gut *Bifidobacterium* has been reported with a high-protein, caloric-restriction treatment in a previous study (Dong et al., 2020). An potential factor may have been the impact of SCFA on intestinal lumen pH. Higher acid load triggers mucosal secretion of bicarbonate, creating an alkaline reaction that is favorable to ammonia absorption. This event may also explain the increase in circulating BUN in the current (Wrong and Vince, 1984).

The impacts of different types of diets and nutrients on gut microbiota have been extensively studied, but the relationships of dietary restriction and gut microbiota have not yet been fully described, particularly in dogs. Metabolic effects observed during caloric restriction resemble those found during fasting and re-feeding cycles, wherein the body switches between energy sources (glucose to fatty acids) by increasing fatty acid catabolism from adipose tissue and stimulating downstream β-oxidation, and also increasing skeletal muscle protein breakdown to provide amino acids for gluconeogenesis (Cahill, 2006). This condition influences gut microbial activity and structure and vice versa. The decreased relative abundances of fecal *Megamonas, Sutterella,* and *Streptococcus* were consistent with findings in obese individuals after enrolling in a caloric-restricted weight loss program (Pisanu et al., 2020). Although calorie-restricted feeding

had strong impacts on the gut microbiota, fiber supplementation (10 g inulin + 10 g resistant maltodextrin per day) may ameliorate those effects and increase the abundance of *Bifidobacterium* and *Parabacteroides* (Benítez-Páez et al., 2021), which were in line with the findings in the current study.

The effects of restricted feeding is not limited to the gut microbiota, but also impacts the metabolism of bile acids and other metabolites. The host and gut microbiota work together to diversify the chemical composition of bile acids. The host synthesizes primary bile acids (i.e., CA and CDCA) from cholesterol in the liver, whereas the gut microbiota promotes deconjugation and biotransformation of primary bile acids to secondary bile acids (i.e., DCA, LCA, and UDCA) (Ciaula et al., 2017). von Schwartzenberg et al. (2021) reported that caloric restriction decreases total fecal bile acid, DCA, and LCA concentrations as a result of reduced bile secretion (due to decreased fat intake) and changes in the composition of bile acid-metabolizing taxa. The gut bacteria in the genera *Clostridium, Bacteroides, Enterococcus, Bifidobacterium,* and *Lactobacillus* produce bile salt hydrolases, which deconjugate bile acids. Other bacterial taxa, such as *Eubacterium* and *Clostridium* species, convert primary bile acids to secondary bile acids via 7α-dehydroxylation (Ridlon et al., 2016; Kriaa et al., 2019). Additionally, Ju et al. (2019) reported that *Parasutterella* lower fecal CA and DCA concentrations in *Parasutterella*-colonized mice. Similar findings were noted in the current study, with total fecal secondary bile acid and DCA concentrations being reduced and relative abundance of *Parasutterella* being increased following decreased food consumption. The results of the current study conflicted with others when it came to the relative abundance of *Eubacterium* and *Clostridium sensu stricto 1*, however, with these bacterial taxa being increased in the current study, but decreased in those reported by Pilla et al. (2020) and Li et al. (2021). Additionally, bile acids, particularly conjugated DCA and CDCA,

exhibit bacteriostatic and bactericidal activities against lactobacilli strains (Wang et al., 2021). This fact may partially explain the reduction in *Lactobacillus* in the current study. UDCA not only has several therapeutic effects (e.g., anticholestatic, antiproliferative, antioxidant, and anti-inflammation), but also counteracts DCA and LCD (Winston and Theriot, 2020). The inverse concentration of fecal UDCA and DCA was also noted in the current study. As a result of caloric deprivation, intestinal motility and bile pigments elimination are diminished. The buildup of bilirubin in the intestine results in an enhanced enterohepatic circulation, which increases plasma reflux (Kotal et al., 1996). Furthermore, bilirubin accumulation is toxic to certain gut bacteria such as *Streptococcus* species (Chen and Yuan, 2020), which may partly explain the shifts of that bacterial taxa in the current study.

Correlation analyses identified a high number of microbe-physiological outcome associations that require further study. A total of 32 gut bacterial taxa were associated with multiple clinical parameters. In the current study, *Enterococcus* showed direct correlations with BW, BCS, FI, CI, and fat mass, whereas *Lachnospira* was inversely related to BCS. These findings are in line with that of human surgically-induced weight loss patients (Sanminguel et al., 2017). Additionally, Kong et al. (2019) reported negative correlations between *Allobaculum* and *Bifidobacterium* and obesity in mice, which supports the negative correlations between those bacteria and CI in the current study. Martínez-Cuesta et al. (2021) reported that *Romboutsia* was diminished in obese individuals, which agreed with the negative correlation observed between that bacterial taxa and BW in the current study. Moreover, fecal *Catenibacterium* has been reported to be more abundant in the obese and associated with metabolic syndrome and inflammation in a previous study conducted in children (Gallard-Becerra et al., 2020), which was in line with the positive correlation between this bacterial group and serum IL-6 in the current study. Furthermore, in the current study,

the relative abundance of fecal *Faecalibacterium* was negatively correlated with serum CRP concentrations, supporting its anti-inflammatory nature that has been reported previously (Verhoog et. al., 2019). Chen et al. (2021) reported that *Sutterella* had a positive correlation with cyclooxygenase-2, which may induce the expression and secretion of IL-6 (Hinson et al., 1996), supporting the positive correlation between *Sutterella* and IL-6 in the current study.

Several correlations between the gut bacterial taxa and fermentation products were noted, with some relationships likely reflecting the cross-feeding that occurs in the large intestine. *Bifidobacterium* degrades undigestible carbohydrates and yields acetate, lactate, succinate, and BCFA which may be utilized by butyrate-producing bacteria such as *Allobaculum* to produce butyrate (Teixeira et al., 2018; Fu et al., 2019). These relationships between *Bifidobacterium*, *Allobaculum,* acetate, and butyrate were observed in the current study. Additionally, a positive relationship between *Prevotella* and fecal valerate was consistent with the results reported by Tap et al. (2015). Negative correlations existed between *Lactobacillus,* fecal secondary bile acids, and DCA, which may reflect the antimicrobial property of DCA on *Lactobacillus* (Wang et al., 2021). On the contrary, *Bifidobacterium* was positively correlated with fecal total bile acid and secondary bile acids, which was similar to the findings reported by Wan et al. (2020) and supporting its role in bile acid conversion (Ridlon et al., 2005).

While some correlations agreed with the literature, several correlations were inconsistent with previous reports. Hou et al. (2017), for instance, reported that *Sutterella* was enriched in obese children, but the opposite was observed in the current study. Similarly, while a negative relationship was observed between *Parasutterella* and serum cholesterol concentrations, positive relationships existed between *Blautia,* BW, and serum cholesterol, and a positive relationsip between *Romboutsia* and serum cholesterol were reported in obese adult humans (Zeng et al.,

2019), the opposite was observed in this study. The reasons for these discrepancies may be due to host species differences, the dietary interventions of each study, design or length of study, or other unknown variables.

Overall, the results in this experiment suggest that obesity is a complex disease that not only affects a multitude of body systems, but also impacts the structure and activity of gut microbiota. Weight loss and restricted feeding with a high-protein, high-fiber diet induce physiological adaptation and modify the gut microbial composition, subsequently improving the biological parameters of overweight dogs. The effects of weight loss and restricted feeding are inseparable in this study, however, so that must be acknowledged. Caloric restriction-induced BW and fat mass loss decreased circulating triglyceride, leptin, insulin, CRP, and IL-6 concentrations, diminished fecal ammonia and secondary bile acids, increased the relative abundances of fecal *Bifidobacterium*, *Allobaculum, Eubacterium, Lachnospira, Ruminococcus gauvreauii group,* and *Parasutterella,* and decreased the relative abundances of fecal *Catenibacterium, Lactobacillus, Megamonas, Ruminococcus gnavus group,* and *Streptococcus.* Several strong correlations between physiological variables and gut microbiota were observed and demonstrate that certain bacterial taxa may exacerbate the progression of obesity and comorbidities and vice versa. Bacteria with strong correlations with host metabolism should be studied further, as they may serve as biomarkers in the future. Further research may also identify the impacts of weight reduction independently from restricted feeding, in particular on gut microbiota, and further clarify the independent effects between dietary regimen and weight loss.

TABLES AND FIGURES

Table 4.1. Ingredient and analyzed chemical composition of the high-protein, high-fiber experimental diet

Ingredient	% as-is	Analyzed composition	%, DM
Poultry meal	35.00	Dry matter (DM), %	92.47
Soy protein concentrate	22.00	Organic matter	89.65
Barley	14.00	Ash	10.32
Beet pulp	10.00	Crude protein	42.04
Brewer's rice	4.075	Acid hydrolyzed fat	12.14
Chicken fat	3.00	Crude fiber	3.90
Liquid palatant	3.00	Total dietary fiber	26.81
Cellulose	2.00	- Insoluble fiber	15.00
Fish oil	2.00	- Soluble fiber	11.81
Psyllium husk	1.00	Nitrogen-free extract[1]	8.69
Short-chain fructooligosacharides[2]	1.00	Metabolizable energy (ME)[1], kcal/g	2.81
Brown flax seed	0.50	Gross energy, kcal/g[1]	4.49
Powder palatant	0.50	Macronutrients on energy basis (% ME)	
Sodium chloride	0.50	- Protein	52.41
Potassium chloride	0.45	- Fat	36.76
Vitamin premix[3]	0.18	- Carbohydrate	10.83
Mineral premix[4]	0.18		
L-carnitine, 50%	0.15		
Choline chloride	0.13		
Green tea extract	0.10		
Natural antioxidant[5]	0.10		
Vitamin C	0.06		
Vitamin E	0.05		
Chromium methionine[6]	0.025		

[1]Nitrogen-free extract = 100 - (ash + crude protein + acid hydrolyzed fat + total dietary fiber); metabolizable energy = 8.5 kcal ME/g fat + 3.5 kcal ME/g protein + 3.5 kcal ME/g nitrogen-free extract; gross energy was measured by bomb calorimetry.

[2]Short-chain fructooligosaccharides: Fortifeed scFOS prebiotic fiber, Ingredion Inc., Westchester, IL USA.

[3]Provided per kg diet: vitaminA, 5.28 mg; vitaminD3, 0.04 mg; vitamin E, 120.00 mg; vitamin K, 0.88 mg; thiamin, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B12, 0.11 mg.

Table 4.1 (cont.). Ingredient and analyzed chemical composition of the high-protein, high-fiber experimental diet

[45]Provided per kg diet: Mn (as MnSO4), 66.00 mg; Fe (as FeSO4), 120 mg; Cu (as CuSO4), 18.00 mg; Co (as CoSO4), 1.20 mg; Zn (as ZnSO4), 240 mg; iodine (as KI), 180 mg; Se (as Na2SeO3), 0.24 mg.

[5]Natural antioxidant: Naturox liquid antioxidant, blend of vegetable oil, natural mixed tocopherols, lecithin and rosemary extract.

[6]Chromium methionine: Microplex®, a national feed ingredient for animals that contains organic chromium, Zinpro Corporation, Eden Prairie, MN USA.

Table 4.2. Body composition of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Variables	wk 0	wk 6	wk 12	wk 18	wk 24	SEM	P-values
Body composition							
Total body mass, kg	14.28[a]	12.19[b]	11.86[c]	10.54[d]	9.85[e]	0.451	**<0.0001**
Total lean muscle mass, kg	7.67[a]	7.42[ab]	7.06[b]	6.57[c]	6.40[c]	0.231	**<0.0001**
Total fat mass, kg	6.36[a]	5.54[b]	4.56[c]	3.74[d]	3.24[e]	0.264	**<0.0001**
Total bone mineral content, kg	0.24[a]	0.24[b]	0.24[c]	0.23[d]	0.23[e]	0.007	**0.0013**
Fat mass percentage, %	44.42[a]	41.83[b]	38.20[c]	35.22[d]	32.70[e]	1.026	**<0.0001**
Lean mass percentage, %	53.88[e]	56.37[d]	59.79[c]	62.60[b]	65.19[a]	0.989	**<0.0001**
Lean mass loss per kg BW	-	0.81	0.77	0.72	0.73	0.033	0.1307
Fat mass loss per kg BW	-	0.25	0.24	0.29	0.27	0.035	0.4479

[a-e] Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

Table 4.3. Voluntary physical activity of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Variables	wk 0	wk 7	wk 15	wk 23	SEM	P-values
Voluntary physical activity (counts/epoch)						
Daily activity	29.13^{ab}	25.58^{ab}	22.92^{b}	32.21^{a}	1.950	**0.0091**
12-hour of light activity	44.77^{ab}	37.28^{b}	34.46^{b}	50.72^{a}	3.144	**0.0027**
12-hour of dark activity	13.49	13.89	11.39	13.70	1.329	0.2842
Light-to-darkness ratio of activity counts	3.59^{xy}	2.94^{y}	3.02^{y}	4.10^{x}	0.345	**0.0514**

[1] Mean activity was represented as activity counts per epoch (epoch duration, 15 seconds).

^{a,b} Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

^{x,y} Mean values within the same row with unlike superscript letters tend to differ significantly ($P < 0.10$).

Table 4.4. Serum metabolites of overweight adult female dogs fed a high-protein, high-fiber diet undergo weight loss

Variables	wk 0	wk 6	wk 12	wk 18	wk 24	Reference[1]	SEM	P-values
Serum chemistry panel								
Creatinine, mg/dL	0.51^{b}	0.52^{b}	0.55^{ab}	0.57^{a}	0.57^{a}	0.5-1.5	0.025	**0.0022**
BUN[2], mg/dL	11.25^{c}	11.33^{c}	12.17^{bc}	13.25^{ab}	14.33^{a}	6-30	0.778	**<0.0001**
Total protein, g/dL	5.89^{a}	5.94^{a}	5.93^{a}	5.62^{b}	5.86^{a}	5.1-7.0	0.076	**<0.0001**
Albumin, g/dL	3.30^{x}	3.18^{z}	3.29^{xy}	3.21^{xyz}	3.19^{yz}	2.5-3.8	0.083	**0.0577**
Globulin, U/L	2.59^{b}	2.76^{a}	2.64^{b}	2.41^{c}	2.67^{ab}	2.7-4.4	0.056	**<0.0001**
Albumin:globulin Ratio	1.28^{ab}	1.17^{c}	1.24^{bc}	1.35^{a}	1.21^{bc}	0.6-1.1	0.048	**<0.0001**
Total ALP[2], U/L	46.08^{a}	34.83^{b}	32.58^{bc}	27.92^{d}	28.08^{cd}	7-92	7.579	**<0.0001**
CALP[2], U/L	22.33^{a}	15.83^{b}	15.00^{b}	13.83^{b}	12.25^{b}	0-40	7.407	**<0.0001**
ALT[2], U/L	28.50	31.00	26.25	25.00	28.83	8-65	4.219	0.4584
GGT[2], U/L	2.08^{c}	2.67^{bc}	3.33^{ab}	3.67^{a}	2.58^{bc}	0-7	0.316	**<0.0001**
Total bilirubin, mg/dL	0.14^{c}	0.18^{b}	0.19^{ab}	0.22^{ab}	0.23^{a}	0.1-0.3	0.013	**<0.0001**
CPK[2], U/L	138.42^{a}	138.33^{ab}	146.08^{a}	136.92^{ab}	119.00^{b}	26-310	22.861	**0.0086**
Total cholesterol, mg/dL	189.25^{xy}	190.17^{xy}	191.08^{x}	178.83^{xy}	177.50^{y}	129-297	15.839	**0.0787**
Triglycerides, mg/dL	59.58^{a}	54.75^{ab}	50.75^{ab}	48.50^{b}	46.25^{b}	32-154	4.187	**0.0011**
Calcium, mg/dL	10.09^{a}	10.10^{a}	10.09^{a}	9.75^{b}	9.76^{b}	1.6-11.4	0.067	**<0.0001**
Phosphorus, mg/dL	3.36^{x}	3.38^{x}	2.99^{xy}	2.93^{y}	3.07^{xy}	2.7-5.2	0.184	**0.0590**
Sodium, mmol/L	146.42^{a}	145.08^{b}	145.25^{b}	145.83^{ab}	145.83^{ab}	141-152	0.274	**0.0018**
Potassium, mmol/L	4.50^{ab}	4.36^{bc}	4.48^{abc}	4.34^{c}	4.53^{a}	3.9-5.5	0.045	**0.0014**
Sodium:potassium Ratio	32.50^{bc}	33.42^{ab}	32.50^{bc}	33.83^{a}	32.17^{c}	28-36	0.357	**0.0014**
Chloride, mmol/L	109.83^{b}	110.58^{ab}	110.75^{ab}	111.33^{a}	111.67^{a}	107-118	0.583	**0.0030**

Table 4.4 (cont.). Serum metabolites of overweight adult female dogs fed a high-protein, high-fiber diet undergo weight loss

Glucose, mg/dL	87.92	89.42	90.67	86.58	87.17	68-126	2.747	0.1034

[1] University of Illinois Veterinary Diagnostic Laboratory Reference Ranges

[2] BUN: blood urea nitrogen; Total ALP: total alkaline phosphatase; CALP: corticosteroid isoenzyme of ALP; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; CPK: creatine phosphokinase.

[a-d] Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

[x-z] Mean values within the same row with unlike superscript letters tend to differ significantly ($P < 0.1$).

Table 4.5. Complete blood cell counts of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Variables	wk 0	wk 6	wk 12	wk 18	wk 24	Reference[1]	SEM	P-values
Complete blood cell counts								
Total white blood cells, 10^6/µL	8.01^{a}	7.12^{a}	6.96^{a}	5.61^{b}	5.24^{b}	6-17	0.586	**<0.0001**
Neutrophils, 10^3/µL	5.97^{a}	5.23^{ab}	4.98^{ab}	4.11^{bc}	3.62^{c}	3-11.5	0.475	**<0.0001**
Lymphocytes, 10^3/µL	1.45^{a}	1.34^{ab}	1.16^{ab}	1.03^{b}	1.14^{ab}	1-4.8	0.146	**0.0229**
Monocytes, 10^3/µL	0.42	0.34	0.32	0.30	0.32	0.2-1.4	0.045	0.2830
Eosinophils, 10^3/µL	0.16	0.20	0.09	0.13	0.15	0.1-1.0	0.033	0.1728
Red blood cells, 10^6/µL	6.37	6.45	6.69	6.42	5.98	5.5-8.5	0.289	0.2761
Hemoglobin, g/dL	14.73	14.66	15.18	14.61	13.98	12-18	0.657	0.5506
Hematocrit, %	43.78^{xy}	44.22^{xy}	45.67^{x}	43.65^{y}	44.13^{xy}	35-52	1.797	**0.0705**
MCV[2], fl	68.99^{a}	68.90^{ab}	68.64^{abc}	68.27^{bc}	68.07^{c}	60-77	0.829	**0.0009**
MCH[2], pg	23.13^{a}	22.78^{b}	22.71^{b}	22.47^{b}	22.73^{b}	20-25	0.237	**0.0001**
MCHC[2], g/dL	33.53^{a}	33.03^{c}	33.11^{bc}	33.33^{abc}	33.42^{ab}	32-36	0.322	**0.0002**
Platelet, 10^3/µL	284.92	282.58	257.08	271.83	285.58	200-700	12.260	0.1489

[1] University of Illinois Veterinary Diagnostic Laboratory Reference Ranges

[2] MCV: mean cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

[a,b,c] Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

[x,y] Mean values within the same row with unlike superscript letters tend to differ significantly ($P < 0.10$).

Table 4.6. Blood hormones and inflammatory markers of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Variables	wk 0	wk 6	wk 12	wk 18	wk 24	SEM	P-values
Hormones							
Leptin, ng/mL	12.60[a]	9.47[b]	6.67[c]	4.68[cd]	3.97[d]	1.456	**<0.0001**
Insulin, mU/L	19.38[a]	15.58[ab]	12.15[bc]	10.98[c]	7.43[c]	1.919	**<0.0001**
Inflammatory markers							
C-reactive protein, ng/mL	3721[a]	1937[ab]	2312[ab]	1743[ab]	1587[b]	1243.6	**0.0485**
Interleukin-6, ng/mL	0.32[a]	0.26[ab]	0.25[ab]	0.24[ab]	0.20[b]	0.052	**0.0366**

[a-d] Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

Table 4.7. Fecal characteristics and fecal metabolites of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Variables	wk 0	wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	SEM	P-values
Fecal characteristics									
Fecal pH	5.48	5.46	5.50	5.36	5.50	5.57	5.43	0.055	0.1775
Fecal score[2]	2.83[a]	2.83[a]	2.33[b]	2.25[b]	2.17[b]	2.17[b]	2.00[b]	0.121	**< 0.0001**
Fecal dry matter (DM), %	23.42[b]	24.13[ab]	24.90[ab]	26.25[a]	25.43[ab]	25.76[ab]	24.65[ab]	0.711	**0.0497**
Fecal metabolites	---------- µmol/g DM ----------								
Acetate	630.80[x]	589.65[xy]	511.27[y]	527.88[xy]	570.10[xy]	542.65[xy]	582.87[xy]	29.113	**0.0507**
Propionate	221.12	228.47	196.08	191.03	200.61	192.43	223.01	16.253	0.1634
Butyrate	74.48	76.93	80.42	82.25	91.95	90.05	87.87	5.937	0.1528
Total SCFA[1]	926.39	895.05	787.77	801.16	862.67	825.13	893.76	43.554	0.1523
Isobutyrate	6.27	6.82	6.12	6.52	6.46	6.44	7.06	0.699	0.8473
Isovalerate	7.54	9.08	8.07	7.95	8.20	7.75	7.76	0.984	0.7316
Valerate	0.85[c]	1.05[bc]	1.04[bc]	1.36[abc]	1.71[ab]	1.94[a]	1.53[abc]	0.214	**0.0002**
Total BCFA[1]	14.66	16.95	15.23	15.82	16.38	16.13	16.35	1.764	0.8367
Total VFA[1]	941.05	912.00	803.00	816.98	879.04	841.25	910.11	44.108	0.1600
Ammonia	147.69[a]	124.13[ab]	115.68[ab]	124.82[ab]	126.35[ab]	111.98[b]	103.39[b]	10.526	**0.0108**

[1]Total SCFA: total short-chain fatty acids = acetate + propionate + butyrate; total BCFA: total branched-chain fatty acids = isobutyrate + isovalerate + valerate; total VFA: total volatile fatty acids = acetate + propionate + butyrate + isobutyrate + isovalerate + valerate.

[2]Fecal score: 1 = very hard and dry, often expelled as individual pellets; 2 = firm but not hard, segmented in appearance; 3 = log-shape, moist surface; 4 = very moist, soggy, log-shaped; 5 = very moist but has a distinct shape, piles rather than distinct logs; 6 = has texture but no defined shape, present as piles or spots; 7 = watery, no texture, flat puddles (Greco, 2015).

[a,b,c] Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

[x,y] Mean values within the same row with unlike superscript letters tend to differ significantly ($P < 0.10$).

Table 4.8. Fecal bile acids of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Variables	wk 0	wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	SEM	P-values
Bile acids, µg/mg									
Cholic acid	0.08	0.09	0.06	0.05	0.06	0.06	0.07	0.021	0.9371
Chenodeoxycholic acid	0.22	0.25	0.19	0.14	0.15	0.18	0.19	0.048	0.6941
Lithocholic acid	1.32	1.46	1.33	1.40	1.32	1.28	1.24	0.109	0.7023
Deoxycholic acid	4.14^{a}	4.27^{ab}	3.78^{ab}	4.03^{ab}	3.51^{ab}	3.03^{ab}	2.77^{b}	0.402	**0.0141**
Ursodeoxycholic acid	0.05^{b}	0.07^{ab}	0.08^{ab}	0.12^{a}	0.10^{a}	0.10^{a}	0.08^{ab}	0.021	**0.0032**
Total primary bile acid	0.30	0.34	0.25	0.19	0.22	0.24	0.26	0.067	0.8386
Total secondary bile acid	5.51^{xy}	5.79^{x}	5.19^{xy}	5.54^{xy}	4.93^{xy}	4.41^{xy}	4.09^{y}	0.498	**0.0578**
Total bile acids	5.81	6.13	5.44	5.74	5.15	4.64	4.35	0.529	0.1146
Secondary bile acid[1], %	95.00	94.81	95.34	96.44	95.38	94.52	93.93	0.886	0.3602
Primary bile acid[1], %	5.00	5.19	4.66	3.56	4.62	5.48	6.07	0.886	0.4074

[1]Primary bile acid (sum of cholic acid and chenodeoxycholic acid) and secondary bile acid (sum of lithocholic acid, deoxycholic acid, and ursodeoxycholic acid) are expressed as a percent of total bile acid measured.

[a,b] Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

[x,y] Mean values within the same row with unlike superscript letters tend to differ significantly ($P < 0.10$).

Figure 4.1

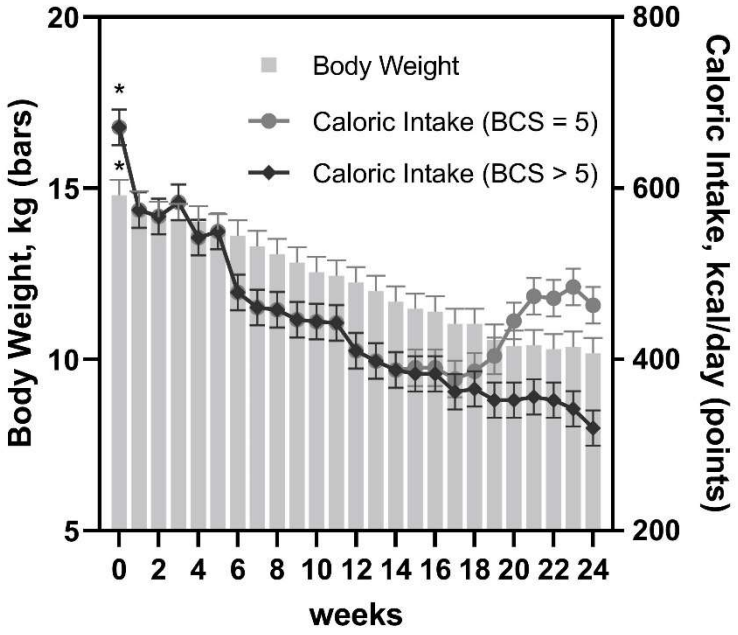


Figure 4.1. Caloric intake (kcal/d) and body weight (kg) data of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss. Data are presented as least square means ± SEM.

Figure 4.2

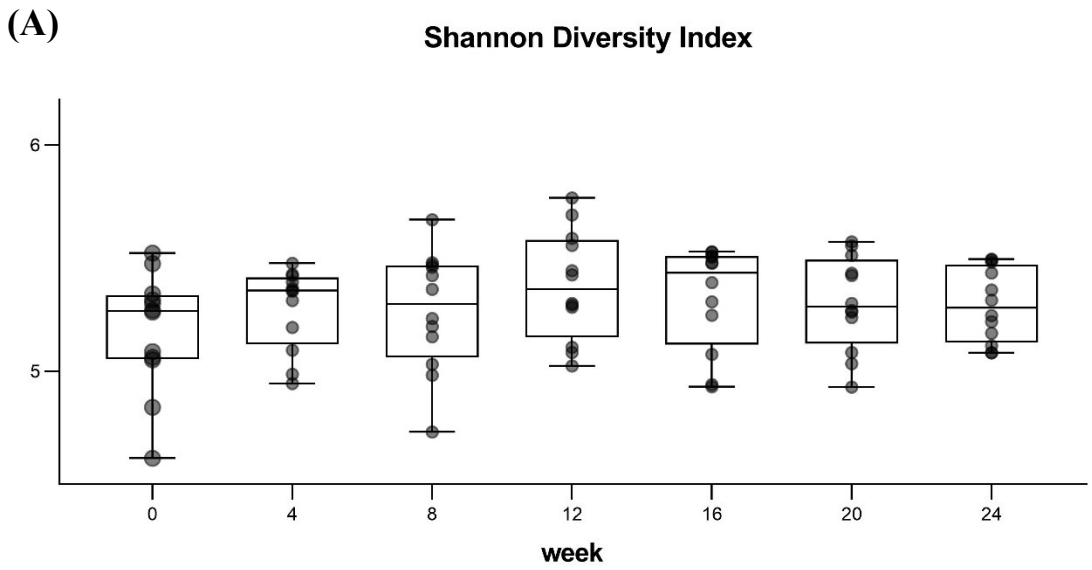


Figure 4.2 (cont.)

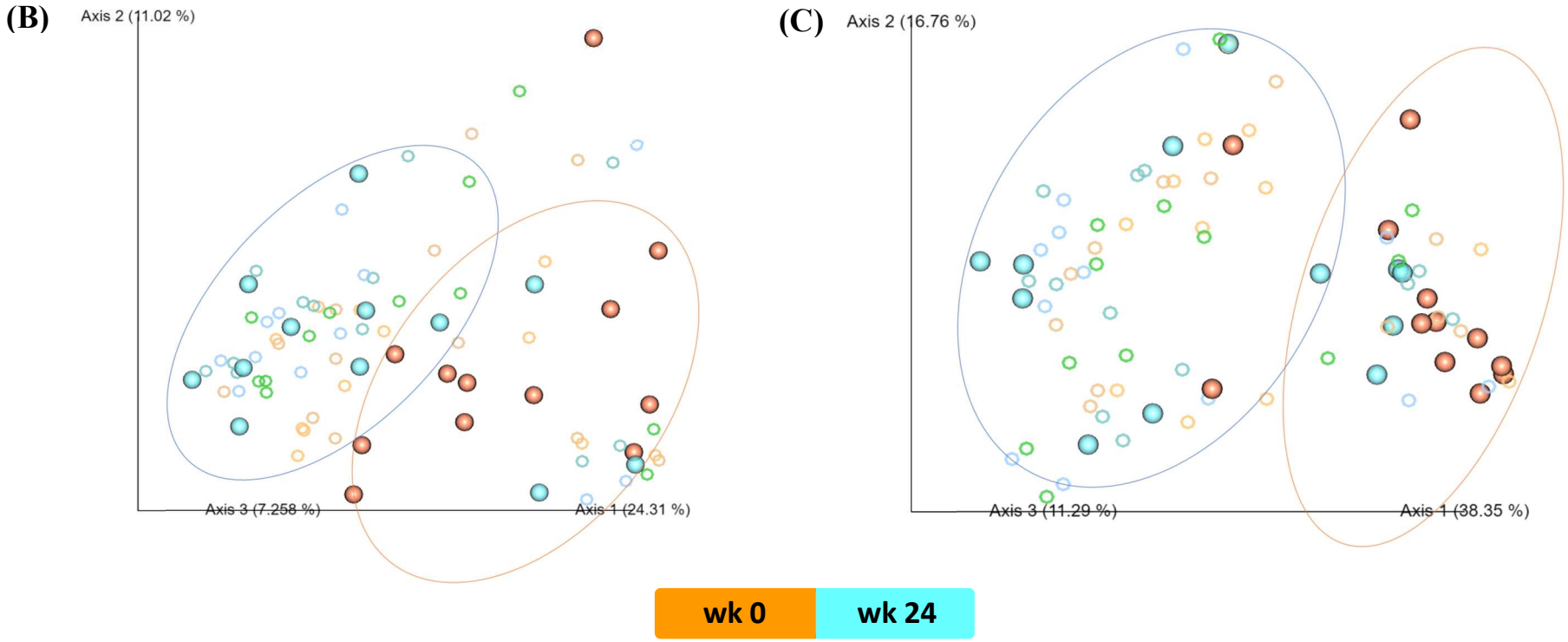


Figure 4.2. Fecal microbial communities of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss. (A) Shannon diversity index suggested that species richness was not different ($P>0.05$) due to restricted feeding and weight loss. (B) Principal coordinates analysis (PCoA) plots of unweighted UniFrac distances of fecal microbial communities revealed that dogs at wk 0 clustered together and separately from dogs at other time points ($P<0.05$). (C) PCoA plots of weighted UniFrac distances of fecal microbial communities revealed that dogs at wk 0 clustered together and separately from dogs at other time points ($P<0.05$).

Figure 4.3

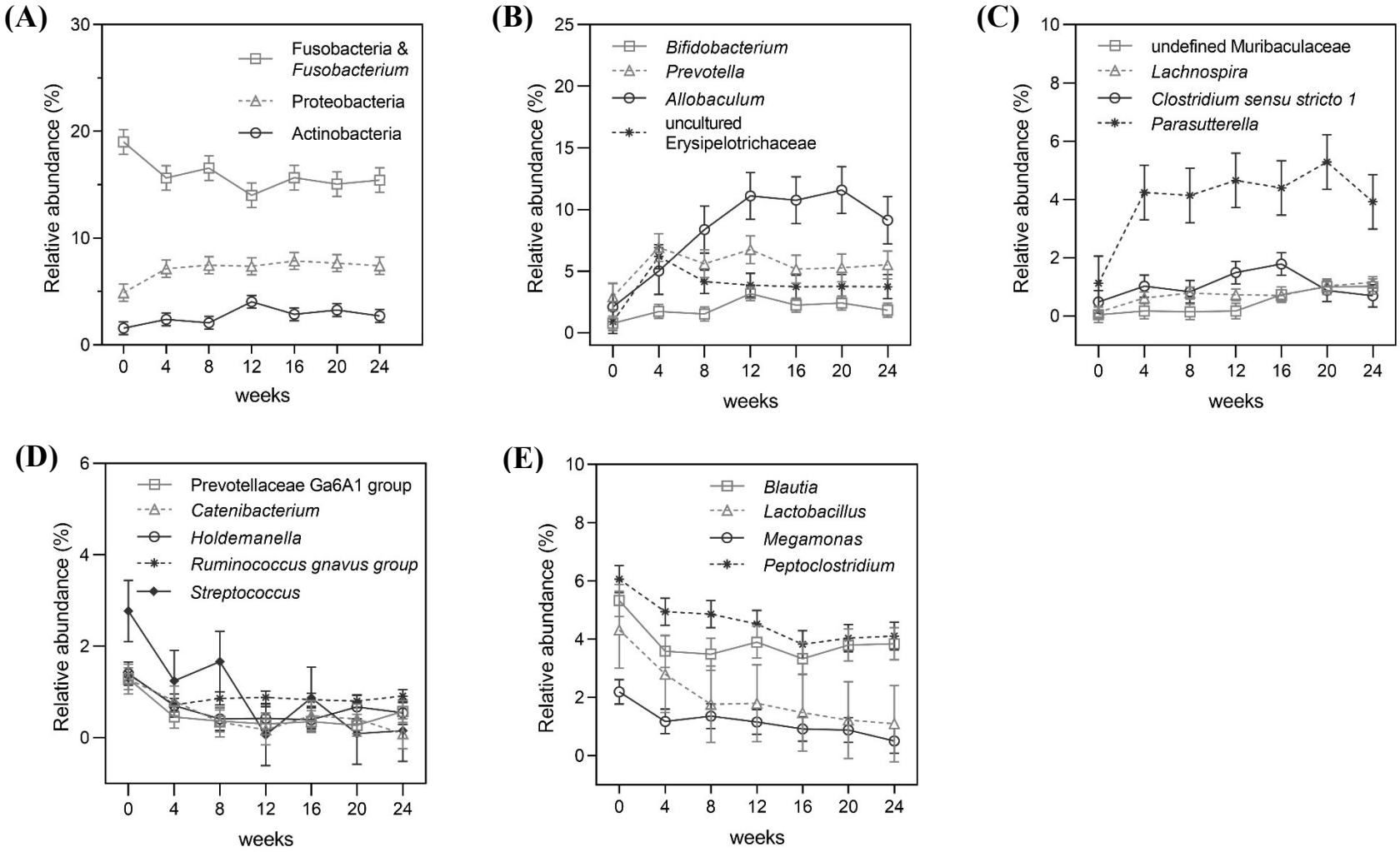

(A)
Relative abundance (%)
weeks
Fusobacteria & Fusobacterium
Proteobacteria
Actinobacteria
(B)
Relative abundance (%)
weeks
Bifidobacterium
Prevotella
Allobaculum
uncultured Erysipelotrichaceae
(C)
Relative abundance (%)
weeks
undefined Muribaculaceae
Lachnospira
Clostridium sensu stricto 1
Parasutterella
(D)
Relative abundance (%)
weeks
Prevotellaceae Ga6A1 group
Catenibacterium
Holdemanella
Ruminococcus gnavus group
Streptococcus
(E)
Relative abundance (%)
weeks
Blautia
Lactobacillus
Megamonas
Peptoclostridium

Figure 4.3. Relative abundances of fecal microbiota of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss that were increased ($P<0.05$) or decreased ($P<0.05$) over time. (A) Relative abundances of fecal Fusobacteria, *Fusobacterium*, Proteobacteria, and Actinobacteria. (B) Increased relative abundances of fecal *Bifidobacterium, Prevotella, Allobaculum,* and uncultured Erysipelotrichaceae. (C) Increased relative abundance of fecal undefined Muribaculaceae, *Lachnospira, Clostridium sensu stricto 1,* and *Parasutterella.* (D) Decreased relative abundance of fecal Prevotellaceae Ga6A1 group, *Catenibacterium, Holdemanella, Ruminococcus gnavus group,* and *Streptococcus.* (E) Decreased relative abundance of fecal *Blautia, Lactobacillus, Megamonas,* and *Peptoclostridium.*

Supplementary Table 4.1. Relative abundances (%) of fecal microbiota of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Phyla	Genera	wk 0	wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	SEM	P-values
Actinobacteria		1.55^{b}	2.40^{ab}	2.07^{b}	4.04^{a}	2.87^{ab}	3.27^{ab}	2.72^{ab}	0.614	**0.0052**
	Adlercreutzia	0.000^{b}	0.020^{ab}	0.049^{ab}	0.053^{a}	0.044^{ab}	0.045^{ab}	0.051^{ab}	0.0145	**0.0195**
	Bifidobacterium	0.77^{b}	1.72^{a}	1.52^{a}	3.16^{a}	2.24^{a}	2.41^{a}	1.82^{a}	0.568	**0.0067**
	Collinsella	0.62	0.48	0.34	0.44	0.25	0.41	0.48	0.108	0.4506
	Coriobacteriaceae UCG-002	0.00^{b}	0.03^{b}	0.05^{a}	0.28^{a}	0.22^{a}	0.28^{a}	0.23^{a}	0.073	**< 0.0001**
	Parvibacter	0.01	0.02	0.02	0.02	0.03	0.03	0.03	0.009	0.2256
	Slackia	0.15^{a}	0.14^{ab}	0.09^{ab}	0.09^{ab}	0.07^{b}	0.08^{ab}	0.10^{ab}	0.032	**0.0378**
Bacteroidetes		19.02	21.37	21.84	21.44	21.91	21.88	23.70	1.677	0.2533
	Alloprevotella	1.57	1.65	1.71	1.31	1.30	1.07	1.60	0.358	0.5385
	Bacteroides	13.19	12.14	14.00	12.88	14.31	14.23	14.94	1.052	0.3213
	Parabacteroides	0.02^{y}	0.04^{xy}	0.03^{xy}	0.05^{xy}	0.06^{xy}	0.05^{xy}	0.08^{x}	0.030	**0.0666**
	Prevotellaceae Ga6A1 group	1.29^{a}	0.45^{b}	0.36^{b}	0.30^{b}	0.35^{b}	0.27^{b}	0.57^{b}	0.242	**0.0004**
	Prevotella	2.92^{b}	6.92^{a}	5.60^{ab}	6.74^{a}	5.16^{ab}	5.27^{ab}	5.50^{ab}	1.136	**0.0024**
	Undefined Muribaculaceae	0.04^{c}	0.17^{bc}	0.14^{bc}	0.17^{bc}	0.73^{ab}	1.00^{a}	1.01^{a}	0.270	**< 0.0001**
Firmicutes		55.52	53.38	52.05	53.11	51.65	52.09	50.71	2.158	0.5710
	Allisonella	0.012^{y}	0.044^{xy}	0.019^{xy}	0.037^{x}	0.029^{xy}	0.032^{xy}	0.027^{xy}	0.0155	**0.0982**
	Allobaculum	2.08^{c}	5.01^{bc}	8.37^{ab}	11.09^{a}	10.76^{ab}	11.58^{a}	9.13^{ab}	1.907	**< 0.0001**
	Anaerofilum	0.126^{b}	0.085^{b}	0.157^{ab}	0.191^{ab}	0.259^{a}	0.164^{ab}	0.206^{a}	0.0560	**0.0013**
	Anaeroplasma	0.06	0.03	0.05	0.04	0.08	0.04	0.07	0.024	0.9547
	Blautia	5.33^{a}	3.59^{b}	3.49^{b}	3.90^{ab}	3.33^{b}	3.80^{ab}	3.84^{ab}	0.552	**0.0163**

Supplementary Table 4.1 (cont.). Relative abundances (%) of fecal microbiota of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

Butyricicoccus	0.16	0.17	0.17	0.16	0.19	0.21	0.19	0.029	0.6210
Candidatus Stoquefichus	0.043^{xy}	0.008^{y}	0.045^{xy}	0.046^{xy}	0.038^{xy}	0.045^{xy}	0.242^{x}	0.0602	**0.0665**
Catenibacterium	1.28^{a}	0.80^{ab}	0.34^{b}	0.17^{b}	0.48^{b}	0.41^{b}	0.08^{b}	0.327	**0.0017**
Clostridia UCG-014	0.08	0.07	0.04	0.03	0.02	0.02	0.04	0.025	0.1064
Clostridium sensu stricto 1	0.48^{b}	1.02^{ab}	0.83^{ab}	1.49^{ab}	1.78^{a}	0.87^{ab}	0.69^{ab}	0.388	**0.0173**
Dubosiella	0.53	1.36	0.80	0.97	0.70	0.76	0.48	0.272	0.1694
Enterococcus	0.74^{a}	0.29^{ab}	0.05^{ab}	0.02^{ab}	0.00^{b}	0.01^{b}	0.03^{ab}	0.168	**0.0028**
Erysipelatoclostridium	0.78^{a}	0.17^{b}	0.19^{b}	0.18^{b}	0.14^{b}	0.21^{b}	0.22^{b}	0.139	**< 0.0001**
Erysipelotrichaceae UCG-003	0.58	0.19	0.48	0.41	0.35	0.61	0.33	0.283	0.5392
Epulopiscium	0.01	0.10	0.09	0.10	0.31	0.16	0.05	0.111	**0.0816**
Eubacterium	0.011^{b}	0.057^{ab}	0.064^{ab}	0.090^{ab}	0.098^{a}	0.135^{a}	0.116^{a}	0.0310	**0.0030**
Eubacterium brachy group	0.16	0.29	0.27	0.35	0.27	0.30	0.29	0.079	0.4322
Faecalibaculum	0.03^{b}	0.54^{ab}	0.34^{ab}	0.33^{ab}	0.47^{ab}	0.77^{a}	0.51^{ab}	0.149	**0.0070**
Faecalitalea	0.125^{a}	0.005^{b}	0.078^{ab}	0.060^{ab}	0.085^{ab}	0.016^{ab}	0.018^{ab}	0.0575	**0.0363**
Faecalibacterium	7.98	7.69	7.77	6.92	7.61	6.64	7.46	0.742	0.7101
Fournierella	0.06	0.03	0.03	0.00	0.00	0.02	0.01	0.018	0.2608
Holdemanella	1.40^{a}	0.70^{ab}	0.41^{b}	0.42^{b}	0.39^{b}	0.67^{b}	0.54^{b}	0.255	**0.0005**
Intestinimonas	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.005	0.2190
Lachnoclostridium	0.71^{a}	0.35^{b}	0.36^{b}	0.38^{b}	0.30^{b}	0.32^{b}	0.42^{b}	0.106	**0.0012**
Lachnospira	0.12^{b}	0.62^{ab}	0.78^{a}	0.73^{a}	0.70^{ab}	1.03^{a}	1.15^{a}	0.201	**< 0.0001**

Supplementary Table 4.1 (cont.). Relative abundances (%) of fecal microbiota of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

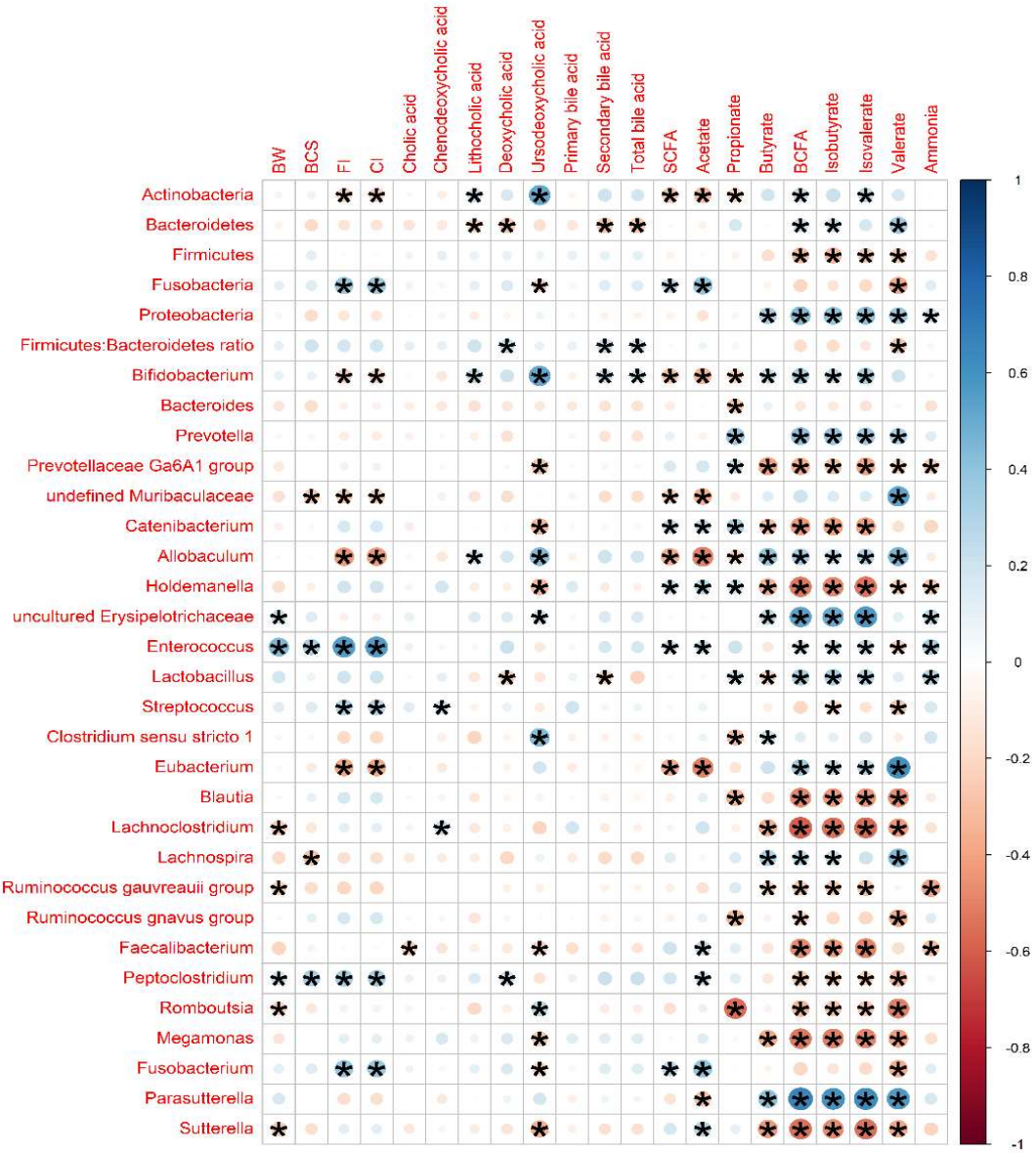
	Lachnospiraceae NK4A136 group	0.28	0.28	0.26	0.24	0.21	0.25	0.26	0.048	0.7854
	Lactobacillus	4.32^{a}	2.80^{ab}	1.76^{ab}	1.80^{ab}	1.48^{b}	1.22^{b}	1.10^{b}	1.319	**0.0074**
	Megamonas	2.19^{a}	1.18^{ab}	1.36^{ab}	1.16^{b}	0.92^{b}	0.88^{b}	0.51^{b}	0.425	**0.0015**
	Negativibacillus	0.03^{b}	0.12^{ab}	0.13^{ab}	0.18^{a}	0.16^{a}	0.23^{a}	0.16^{a}	0.037	**0.0002**
	Peptoclostridium	6.06^{a}	4.94^{ab}	4.86^{ab}	4.52^{b}	3.83^{b}	4.04^{b}	4.11^{b}	0.474	**0.0003**
	Peptococcus	0.46	0.49	0.42	0.43	0.32	0.36	0.37	0.109	0.5387
	Phascolarctobacterium	1.31	1.46	1.31	1.16	1.20	1.30	1.53	0.156	0.5209
	Romboutsia	1.78	1.26	1.26	1.76	1.68	1.71	1.61	0.358	0.5902
	Ruminococcus gauvreauii group	0.12^{b}	0.20^{ab}	0.22^{ab}	0.23^{ab}	0.19^{ab}	0.24^{a}	0.26^{a}	0.045	**0.0243**
	Ruminococcus gnavus group	1.39^{a}	0.72^{b}	0.86^{b}	0.88^{b}	0.83^{b}	0.80^{b}	0.91^{b}	0.141	**0.0002**
	Ruminococcus torques group	2.12	2.23	2.39	2.26	2.16	2.29	2.64	0.274	0.5835
	Sellimonas	0.34	0.38	0.36	0.32	0.36	0.40	0.38	0.042	0.7104
	Streptococcus	2.77^{a}	1.24^{ab}	1.66^{a}	0.06^{b}	0.87^{b}	0.09^{b}	0.15^{b}	0.670	**0.0007**
	Terrisporobacter	0.05	0.03	0.04	0.21	0.09	0.10	0.08	0.054	0.1496
	Turicibacter	1.98	1.36	1.24	1.37	1.14	1.31	1.59	0.305	0.3495
	UCG-005	0.03	0.04	0.03	0.03	0.03	0.02	0.03	0.010	0.7443
	Uncultured Erysipelotrichaceae	0.89^{b}	6.18^{a}	4.14^{a}	3.87^{a}	3.74^{a}	3.76^{a}	3.73^{a}	0.969	**0.0001**
	Uncultured Lachnospiraceae	0.55	0.30	0.38	0.39	0.33	0.36	0.30	0.080	0.1622
	Uncultured Ruminococcaceae	0.033^{y}	0.040^{xy}	0.045^{xy}	0.049^{xy}	0.059^{x}	0.047^{xy}	0.049^{xy}	0.0056	**0.0569**
Campilobacterota		0.013^{a}	0.070^{a}	0.018^{a}	0.006^{b}	0.057^{a}	0.027^{a}	0.023^{a}	0.0189	**0.0120**

Supplementary Table 4.1 (cont.). Relative abundances (%) of fecal microbiota of overweight adult female dogs fed a high-protein, high-fiber diet during weight loss

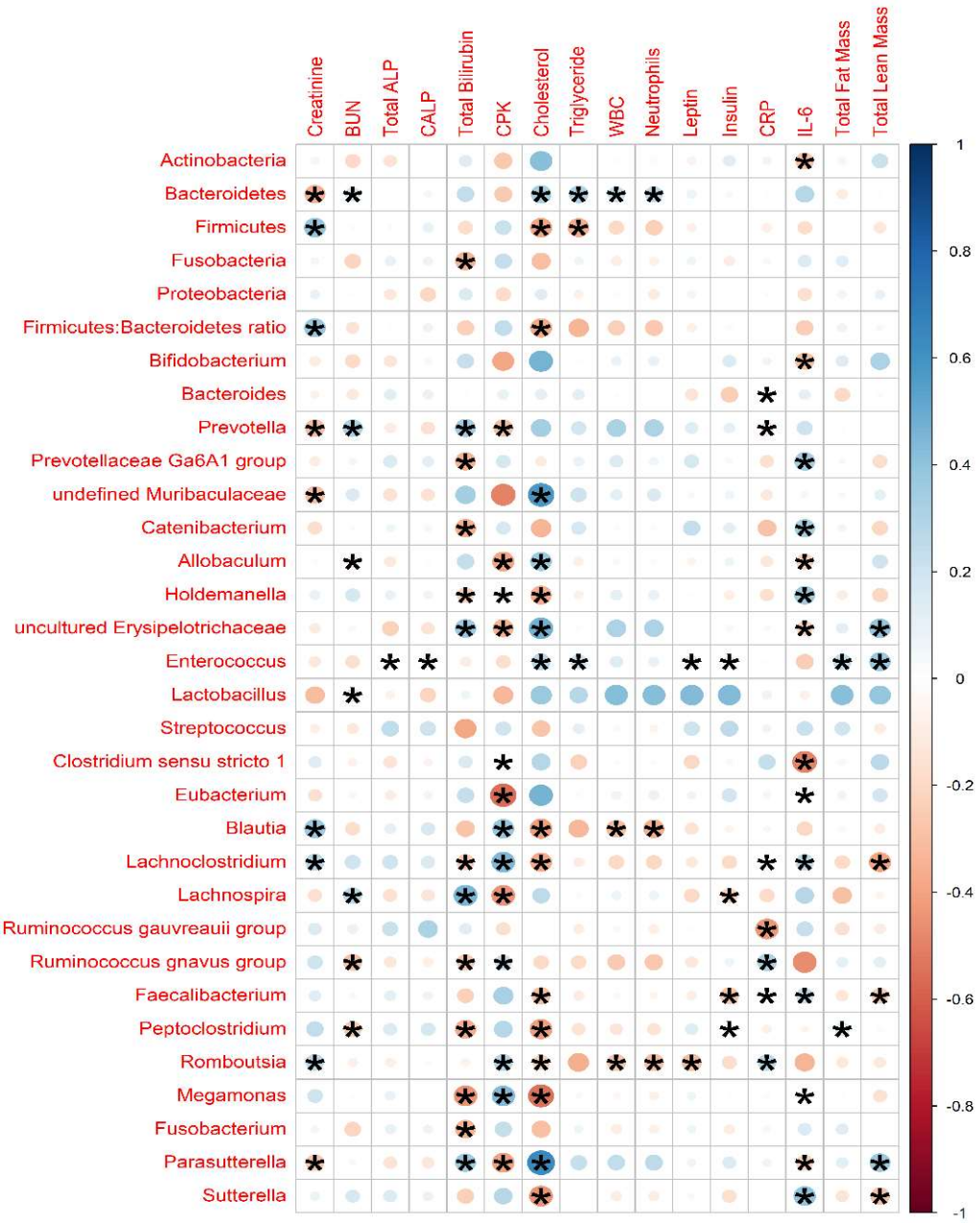
	Helicobacter	0.013^{ab}	0.070^{a}	0.019^{ab}	0.006^{b}	0.056^{ab}	0.026^{ab}	0.023^{ab}	0.0190	**0.0120**
Fusobacteria		19.00^{a}	15.63^{ab}	16.55^{ab}	14.02^{b}	15.65^{ab}	15.05^{ab}	15.44^{ab}	1.156	**0.0266**
	Fusobacterium	19.00^{a}	15.63^{ab}	16.55^{ab}	14.02^{b}	15.65^{ab}	15.05^{ab}	15.44^{ab}	1.156	**0.0267**
Proteobacteria		4.89^{b}	7.13^{ab}	7.47^{a}	7.37^{a}	7.86^{a}	7.67^{a}	7.41^{a}	0.810	**0.0041**
	Anaerobiospirillum	0.57	0.56	0.86	0.66	1.12	0.73	1.04	0.445	0.7611
	Parasutterella	1.12^{b}	4.24^{a}	4.14^{a}	4.65^{a}	4.39^{a}	5.28^{a}	3.92^{a}	0.936	**0.0004**
	Sutterella	3.11^{x}	2.23^{xy}	2.43^{xy}	2.02^{xy}	2.32^{xy}	1.63^{y}	2.41^{xy}	0.466	**0.0840**
	Succinivibrio	0.01	0.07	0.03	0.04	0.03	0.02	0.03	0.023	0.3134
	Firmicutes:Bacteroidetes ratio	3.40	2.97	2.50	2.79	2.47	2.67	2.41	0.359	0.2064

^{a,b,c}Mean values within the same row with unlike superscript letters differ significantly ($P < 0.05$).

^{x,y}Mean values within the same row with unlike superscript letters tend to differ significantly ($P < 0.10$).



Supplementary Figure 4.1. Heatmap of correlations between key biological parameters [BW, BCS, FI, CI, fecal bile acids (CA, CDCA, LCA, DCA, UDCA, primary bile acid, secondary bile acid, total bile acid), and fecal metabolites (SCFA, acetate, propionate, butyrate, BCFA, isobutyrate, isovalerate, valerate, ammonia)] and fecal bacterial taxa. The correlation (*R*) values are displayed in blue and red colors, representing positive and negative correlations, respectively. *P<0.05 was considered significant.



Supplementary Figure 4.2. Heatmap of correlations between key biological parameters [serum metabolites (creatinine, BUN, total ALP, CALP, total bilirubin, CPK, cholesterol, triglycerides), WBC, neutrophils, serum hormones (leptin, insulin), serum inflammatory markers (CRP, IL-6), total fat mass, and total lean mass] and fecal bacterial taxa. The correlation (*R*) values are displayed in blue and red colors, representing positive and negative correlations, respectively. *P<0.05 was considered significant.

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CHAPTER 5: SUMMARY

Obesity prevalence has risen dramatically in the past few decades. Canine obesity has been classified as a disease that has predisposing factors and consequences similar to that of humans. In line with humans, the preventative and therapeutic options of obesity include dietary intervention, physical activity and behavioral modifications, pharmacotherapy, and surgery. Among all, dietary intervention is a suitable, economical, and noninvasive strategy for pets. Additionally, macronutrient-modified diets have been shown to improve metabolic and gastrointestinal health and remodel gut microbiota in humans and rodents. Yet, only a few studies have tested the effects of the diets on gastrointestinal health markers and gut microbiota of dogs undergoing weight gain and/or weight loss conditions. Therefore, a full investigation on the alterations that result from dietary change, including changes to physiological outcomes, fecal microbiota, and fecal microbial-derived metabolites in dogs post-surgery or undergoing weight loss may provide a better understanding of how each factor plays a role in the management and treatment of canine obesity.

Two canine experiments were conducted to evaluate the effects of dietary macronutrient modifications on metabolic responses and gastrointestinal health in adult dogs undergoing weight gain and weight loss conditions. In the first experiment, three dry extruded diets containing different dietary macronutrient profiles were tested to investigate their effects on apparent total tract macronutrient digestibility, fecal bile acid concentrations, fecal fermentative-end product concentrations, and fecal microbiota of adult female dogs after spay surgery. We hypothesized that high-protein, high-fiber (HPHF) diets would reduce nutrient and energy digestibility and beneficially shift fecal microbiota, fermentative-end products, and bile acids after spay surgery as compared to a control diet. Apparent organic matter and energy digestibilities had greater

decreases in HPHF and HPHFO than COSH and COSP. Increases in fecal acetate, total short-chain fatty acids, and secondary bile acids were greater and decreases in primary bile acids were greater in HPHF and HPHFO. PCoA of weighted UniFrac distances revealed that HPHF and HPHFO clustered together and separately from COSH and COSP at wk 12 and 24, with relative abundances of *Faecalibacterium, Romboutsia*, and *Fusobacterium* increasing to a greater extent and *Catenibacterium, Bifidobacterium, Prevotella 9, Eubacterium, and Megamonas* decreasing to a greater extent in HPHF or HPHFO. The results suggested that dietary modification had major influences on the gut microbiota and metabolites while gonadectomy and overconsumption regimen had fewer effects. Consumption of HPHF diets altered the gut microbiome and their metabolites of obese dogs by reducing fat, organic matter, and energy digestibilities compared to dogs fed the control diet that contained lower amounts of protein and fiber. Although reduction of estrogen hormone had shown drastic changes in previous human and rodent studies, few changes were noted in the current study.

In the second experiment, the effects of restricted feeding of a HPHF diet and weight loss on body composition, complete blood cell count, serum chemistry profile, serum hormone concentrations, serum inflammatory marker concentrations, voluntary physical activity, fecal microbiota, fecal bile acid concentrations, and fecal fermentative metabolite concentrations of overweight dogs were tested. We hypothesized that restricted feeding of a HPHF diet and weight loss would increase voluntary physical activity, reduce blood lipids and inflammatory markers, and alter blood hormone concentrations. Additionally, we also hypothesized that the dietary regimen would beneficially alter the fecal metabolite concentrations by increasing the concentration of fecal SCFA and certain fecal bile acids whereas decreasing fecal protein catabolites and total fecal bile acid. Lastly, we hypothesized that the dietary regimen would

beneficially alter the fecal microbiota community and create a balanced gut microbial environment.

After 24 wk, dogs lost 31.2% of initial BW and had 1.43±0.73% weight loss per wk. BCS decreased by 2.7 units, lean mass percentage increased by 11.3%, and fat mass and percentage decreased by 3.1 kg and 11.7%, respectively, with weight loss. Total activity level was highest at the end of the weight-loss period. Many serum metabolites and hormones were altered, with triglycerides, leptin, insulin, CRP, and IL-6 decreasing with weight loss. Relative abundances of fecal *Bifidobacterium*, Coriobacteriaceae UCG-002*,* undefined Muribaculaceae*, Allobaculum, Eubacterium, Lachnospira, Negativivibacillus, Ruminococcus gauvreauii group,* uncultured Erysipelotrichaceae*,* and *Parasutterella* increased, whereas Prevotelaceae Ga6A1 group*, Catenibacterium, Erysipelatoclostridium, Fusobacterium, Holdemanella, Lachnoclostridium, Lactobacillus, Megamonas, Peptoclostridium, Ruminococcus gnavus group,* and *Streptococcus* decreased with weight loss. Fecal ammonia and secondary bile acids decreased, while fecal valerate increased with weight loss. The results suggested that restricted feeding of a HPHF diet and weight loss promoted fat mass loss, minimized lean mass loss, reduce inflammatory markers and triglyceride concentration, and moderated fecal microbiota phylogeny and activity in overweight dogs.

Overall, this dissertation demonstrated that a HPHF diet can be incorporated effectively into both weight maintenance and weight loss programs. The diet appeared to result in positive outcomes on canine health by altering metabolic responses and modulating fecal microbiota and metabolites of adult female dogs undergoing weight gain and/or weight loss. However, some limitations and gaps existed in both experiments. In the first experiment, the inclusion of such a high level of barley in the control diet (23% as-is) was likely responsible for the positive health

effects on dogs and gut microbiota. This likely attenuated many of the negative effects of obesity that was anticipated in the COSP dogs, and as a result, contributed to smaller significant effects among the diets than expected. Therefore, testing a control diet having a lower or equal inclusion rate of barley compared to that of the HPHF diet could be useful in future studies. Additionally, the concentrations of some ingredients in the HPHFO diets (e.g., fish oil; coconut oil) were likely too low to provide strong effects and may explain the lack of functional benefits observed in the HPHFO diet in that study. In previous canine studies, the inclusion of 2% fish oil was recommended to help reduce plasma cholesterol concentrations. Studies testing the inclusion of at least 2% fish oil on metabolic and gut microbiota responses may be of interest in the future. Lastly, the effects of estrogen reduction on gut microbes seemed to be minor in the current study, which have been due to the small sample size and/or inappropriate sampling times. Future research with greater sample sizes, different sampling times (possibly within one wk and 2 to 4 wk after spay surgery), and/or different diets may provide further insight into the effects of estrogen reduction and weight gain on the gut microbiota of dogs.

In the second experiment, only one diet was tested so its impacts on canine health may be limited and without providing integral explanations for the results observed. Future research with greater treatment numbers and/or different dietary types and dietary regimens may reveal better solutions for the management of canine obesity. Additionally, more research is necessary to determine the differences observed due to reduced food intake and those due to reduced body weight. In the current study, these variables coincided so each component could not be interpreted individually.