The Effect of Obesity and Weight Loss on the Feline Fecal Microbiota, Inflammation and Intestinal Health Markers

by

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ABSTRACT

The effect of obesity and weight loss on the feline fecal microbiota, inflammation and intestinal health markers

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University of Guelph, 2018

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Dr. Adronie Verbrugghe
Dr. J. Scott Weese

Obesity in cats is a universal epidemic, with disastrous health repercussions. Obesity in humans and mice is associated with a low-grade inflammatory state, and an “obese microbiome”. However, similar study of cats is limited.

Studies with client-owned cats include challenges that may influence results, such as optimal sample collection. Therefore, the impact of 4-day storage at ambient temperature on feline fecal microbial abundance and diversity was evaluated, with no observed significant changes. These findings suggest that short-term storage of fecal samples at ambient temperature has no detrimental impact.

Exploring obesity-related effects on microbiota and assessing the obesity-associated inflammatory response can lead to the development of alternative strategies to address feline adiposity and related health consequences. The effects of feline obesity and weight loss on the fecal microbiota, as well as inflammation and intestinal health markers, were investigated in obese cats before and after a standardized weight loss program, and compared to lean cats. There was little apparent impact of weight loss on the fecal microbiota in obese cats, as no differences
in relative abundance of taxa and biodiversity indices between lean and obese cats before weight loss. However, a group of enriched operational taxonomic units was identified in obese cats before weight loss compared to lean cats, with nearly half belonging to Firmicutes. Some members of which may be involved in energy-harvesting efficiency. In addition, one of the enriched members belonged to the Prevotellaceae family, which includes folate producers. An increase in folate levels is considered a negative intestinal health marker in cats. Higher serum folate concentrations were also observed in cats before compared to after weight loss, and higher serum cobalamin concentrations were measured in lean cats compared to both obese groups. Inflammatory markers did not differ between obese and lean cats following a diet adaptation period, yet some pro-inflammatory markers (e.g. Flt3 and interleukin β-1) were increased after weight loss, which suggests an inflammatory process associated with weight loss.

In conclusion, obesity in cats is suggested to be affiliated with energy harvesting and compromised intestinal health. An inflammatory response may be triggered by a traditional weight loss plan.
ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude to my advisor, Dr. Adronie Verbrugghe, for taking me on as a DVSc student at a very short notice, and for her extensive support and guidance in every aspect of graduate studies, while being considerate of my complex personal-life matters. Dr. Verbrugghe provided me with tools and taught me skills through involvement in clinics, research and teaching, and assisted me in staying on track during my program.

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I want to extend my gratitude also to all the cats and owners who participated in my study. This study would have not happened without these people’s determination and persistence.

Finally, a huge thank you to my beloved grandmother and mother, for their immense support through my program, cheering for me every step of the way, and to my beloved son, for his unconditional love, giving me strength in the roughest points of the program. Grandma, this thesis is dedicated to your memory.
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GLOSSARY

AAHA - American Animal Hospital Association

AMOVA - Analysis of molecular variance

APP – Acute phase protein

BCS – Body condition score

BF – Body fat

BMI – Body mass index

BP – Base pairs

BW – Body weight

CCL5 - C-C motif chemokine ligand 5

CONV-D - Conventionalization with cecal content performed on germ-free mice

CONV-R - Conventionally-raised mice

CPN60 - Chaperonin-60

CF – Crude fat

CP – Crude protein

CRP - C-reactive protein

CV – Coefficient of variation

DGGE - Denaturing gradient gel electrophoresis

DXA - Dual-energy X-ray absorptiometry

ER – Energy requirements

FISH - Fluorescence in situ hybridization

GF – Germ-free

GI – Gastrointestinal
GM-CSF - Granulocyte macrophage-colony stimulating factor

HOMOVA - Homogeneity of molecular variance

IFN-γ - Interferon-γ

IL-8 – Interleukin-8

IL-18 – Interleukin-18

IL-4 – Interleukin-4

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

IL-10 – Interleukin-10

IL-13 – Interleukin-13

IL-12 – Interleukin-12

IL-2 – Interleukin-2

IQR – Interquartile range

KC - keratinocyte-derived chemokine

LDA - Linear discriminatory analysis

LEfSe - Linear discriminatory analysis effective size

LL – Lower limit

LPL - Lipoprotein lipase

LPS – lipopolysaccharides

MCP-1 - Monocyte chemoattractant protein-1

MCS – Muscle condition score

NAFLD - Non-alcoholic fatty liver disease

NFE – Nitrogen-free extract
NRC – National Research Council
OAWL – Obese after weight loss
OBWL – Obese before weight loss
OTU – Operational taxonomic unit
PCR - Polymerase chain reaction
PCoA - Principal coordinate analyses
PDGF-BB - Platelet-derived growth factor, isoform B
qPCR - Quantitative PCR
RAGE - receptor for advanced glycation end products
RDP - Ribosomal database project
RDW – Red cell distribution width
SAA - Serum amyloid A
SCF - Stem cell factor
SCFA - Short-chain fatty acid
SDF-1 - Stromal-cell derived factor
SE – Standard error
SIBO - small intestine bacterial overgrowth
TGGE - Temperature gradient gel electrophoresis
TNF-α – Tumor necrosis factor-α
UL – Upper limit
WSAVA - World Small Animal Veterinary Association
ZO-1 - Zonula occludens-1
DECLARATION OF WORK PERFORMED

I declare that with the exception of specifics mentioned below, all work reported in this thesis was performed by me.

Restraining of cats and blood draws were performed (in addition to myself) by volunteers (undergraduates and fellow graduate students from Dr. Verbrugghe’s laboratory) and by technicians from the Small Animal Clinic, Ontario Veterinary College, University of Guelph, Guelph, Ontario.

Complete blood count, serum biochemical profile (full and partial), were analyzed by technicians at the Animal Health Laboratory, University of Guelph, Guelph, Ontario.

Analyses of cobalamin, folate and S100A12 were performed at the Gastrointestinal Laboratory, Texas A&M University, College Station, Texas.

Enzyme linked immunoassays (for acute phase proteins) were performed by a technician from Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario.

Fecal samples collection for Chapter 2 was performed by Charlotte Chau and Amanda Santarossa.
Statistical analyses for Chapter 4 were performed by Gabrielle Monteith, Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario.
CHAPTER 1: LITERATURE REVIEW

1.0 INTRODUCTION

Obesity in companion animals is a worldwide problem, especially in western industrialized countries. The most common definition of obesity is the accumulation of excessive amounts of adipose tissue in the body [1]. The primary reason for fat storage is a positive energy balance, resulting from either excess food/energy intake or inadequate energy utilization, although many other factors can influence this balance.

Currently, in the US, the prevalence of weight-excess or obesity in adult humans is 69% [2]. In companion animals, 54% of adult dogs and 59% of adult cats are classified as overweight or obese by their veterinarian [3]. Moreover, many pet owners are unaware of, or even worse, underestimate their pet’s body condition and weight status, something that is a major cause for concern, since awareness is the first step in battling this epidemic [4-7].

Obesity is a profound health problem in humans and animals. The degree of severity can be determined using a variety of methods such as body condition score (BCS) [8, 9], body mass index (BMI) [10-12], or morphometric measurements in dogs and cats [12, 13]. Obesity is associated with increased risk for diseases such as type II diabetes mellitus, orthopedic diseases, respiratory and urinary tract diseases in humans [14]. Similar findings have been reported for companion animals [15-20].
The search for potential mechanisms underlying the pathogenesis of obesity and related diseases revealed in humans and mice that there is a low-grade inflammatory response accompanying obesity, which is described by the term “metainflammation”, and is mediated by two primary pathways [21, 22]. One pathway is via white adipose tissue, which acts as an endocrine organ to secrete inflammatory mediators [23, 24]. The other pathway is related to obesity-induced changes in gut microbiota. There is increased evidence suggesting an “obese microbiome” (i.e. genome, genes and their functions) in humans, rodents and dogs, which is influenced by various factors, such as genetics, age and dietary factors [25, 26]. Still, it is unclear whether this “obese microbiome” is a cause or a consequence of obesity. In the past decade the interaction between obesity and the gut microbiota (i.e. the microbial community, contains bacteria, fungi, viruses, and protozoa, but mostly refers to the bacterial population) [25, 26] has been studied, indicating increased Firmicutes-to-Bacteriodetes ratio in humans [27] with a reduction in *Methanobrevibacter smithii* [28]. Microbial diversity was also shown to be reduced [26]. Overall, the effects of the GI bacteria on obesity, and their interaction are controversial, complex, and not fully understood. It is suggested that nutrient excess and obesity induce GI microbiota-derived pro-inflammatory signaling in the host, leading to exacerbation of the obesity-associated inflammation.

While previous studies have described the intestinal microbiota in healthy cats [29, 30], evaluation of the relationship between the feline microbiota and obesity is lacking. This review further explores the feline normal and adiposity-related microbiota, the effect of obesity on intestinal health and the associated inflammatory response.
1.1 DEFINITION, RISK FACTORS, PREVALENCE AND COMORBIDITIES OF OBESITY IN COMPANION ANIMALS

Obesity or being overweight are terms with a variety of definitions, such as the accumulation of excessive amounts of adipose tissue in the body [1]; or the excess body fat (BF) sufficient to result in impairment of health or body function [31]. More specifically, there is a differentiation between an “overweight” status and an “obese” status. Being overweight is a condition in which an animal is 15%, or 10 to 20% over its optimal weight [32, 33]; obese companion animals are more than 20% above their optimal weight [33]. From a body composition standpoint, some studies suggest that overweight animals have 25-35% BF, whereas animals with BF > 35% are considered obese [8, 9] (Table 1.1). These definitions share a common ground, which is the accumulation of excess BF.
Table 1.1. Body condition score compared to body fat (%) assessment in cats (feline and canine figures were adapted from the American Animal Hospital Association (AAHA) nutritional assessment guidelines) [34]

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<th>BF% [35]</th>
<th>Description</th>
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<tbody>
<tr>
<td>1/5</td>
<td>1/9</td>
<td></td>
<td></td>
<td>&lt; 5%</td>
<td></td>
</tr>
<tr>
<td>1.5/5</td>
<td>2/9</td>
<td></td>
<td></td>
<td>5 – 9%</td>
<td></td>
</tr>
<tr>
<td>2/5</td>
<td>3/9</td>
<td></td>
<td></td>
<td>10 – 14%</td>
<td></td>
</tr>
<tr>
<td>2.5/5</td>
<td>4/9</td>
<td></td>
<td></td>
<td>15 – 19%</td>
<td></td>
</tr>
<tr>
<td>3/5</td>
<td>5/9</td>
<td></td>
<td></td>
<td>20 – 24%</td>
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</tr>
<tr>
<td>3.5/5</td>
<td>6/9</td>
<td></td>
<td></td>
<td>25 – 29%</td>
<td></td>
</tr>
<tr>
<td>4/5</td>
<td>7/9</td>
<td></td>
<td></td>
<td>30 – 34%</td>
<td></td>
</tr>
<tr>
<td>4.5/5</td>
<td>8/9</td>
<td></td>
<td></td>
<td>35 – 39%</td>
<td></td>
</tr>
<tr>
<td>5/5</td>
<td>9/9</td>
<td></td>
<td></td>
<td>≥ 40%</td>
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**Abbreviations:** BCS, body condition score; BF, body fat

Neutering, dietary factors (e.g. ad libitum feeding, high-fat diets, snacks/treats/human foods given in addition to the daily energy requirements), behavioural factors, certain pharmaceuticals (e.g. glucocorticosteroids), genetics and diseases (e.g. hypothyroidism, hyperadrenocorticism) were reported to be risk factors for obesity in pets [1]. More specifically in cats, risk factors to be considered include inactivity, neutering, male sex, cross-bred, in-door living with a total of one or
two cats [5, 36], increased leg length, and owner underestimation of the cat’s body condition score [4-6]. The impact of other risk factors such as commercial dry food and feeding regimen is still debated [5-7]. More research is required for a better assessment of these, and other potential, risk factors.

On the one hand, a cross-sectional study from 2005 found that 35% of cats assessed by private US clinics were overweight or obese [7]. On the other hand, data from Banfield clinics in the US indicated that in 2007 only 10% of cats were considered as overweight or obese, whereas 5 years later, in 2011, there was an increase of 90% in prevalence, leading to 19% overweight or obese cats [37]. That being said, in 2016 an annual veterinary survey conducted by the Association for Pet Obesity Prevention reported a much higher prevalence of feline overweightness and obesity (59%) [3], which is similar to the higher end of prevalence-range found in surveys conducted in Europe, New Zealand and Japan (11.5-63%) [6, 38-40]. In many prevalence studies the assessment of overweightness or obesity was conducted using a body condition scoring (BCS) scale of 1-5 (score of 4 being overweight and 5 being obese) [38, 39], 1-9 (score of 6,7 being overweight and 8,9 being obese) [3, 40] or a combination of both [6, 7] (Table 1.1). The authors’ definition of overweight versus obese is slightly different or includes “obese” under the umbrella of overweightness, especially when using a combination of BCS assessment methods. This may promote reduced accentuation of the obese prevalence, and overrepresentation of overweight patients.

Feline obesity is a concomitant factor to many diseases, and its detrimental consequences include increased risk for diabetes mellitus [16], insulin resistance [17], and urinary disorders [18].
Additional negative health consequences were extrapolated from dogs, and include pancreatitis [19], orthopedic problems [41], cardiorespiratory diseases [20, 42] and neoplasia [15, 43]. Moreover, increased caloric intake decreases longevity and quality of life [44-47] and is probably associated with various other disease risks that are currently not understood.

Unfortunately, data linking caloric intake or obesity and longevity is not available in cats. Kealy et al. (2002) were the first to estimate longevity in larger mammals compared to rodents. In this study, 24 pairs of Labrador retrievers were followed throughout their entire life. In each pair, one dog had initially ad libitum access to food (until the age of 3 years), while the other dog was restricted in energy. Dogs fed ad libitum had a higher fat deposition, were more likely to develop chronic diseases like osteoarthritis earlier in life, and had a shorter life span. Dogs that were restricted in energy lived on average two years longer [44]. In a study of cause-specific mortality in 900,000 humans, body mass index (BMI) was identified as a strong predictor of mortality. It appears that as BMI increases, median life span is reduced [10]. Nonetheless, higher weight and BMI are associated with increased survival and lower death in both people and dogs with heart failure. This association is referred to as the “obesity paradox” [48-50]. The mechanism behind it is unclear. In cats with heart failure, it seems that both high and low weights are associated with lower survival, although more research is required to examine this relationship [51].

Quality of life was assessed in overweight and obese cats (BCS > 3/5). In a study performed by Christmann et al. (2015), a cat-owner questionnaire assessed the cat’s happiness, vitality, begging behavior, appetite, stool volume and flatulence. Overall, owners noticed significant improvement in their cat’s quality of life with increased weight loss, which manifested especially as an increase
in the cats’ energy level and happiness. No negative behavioural effects were observed during the weight loss programme [47]. A more recent study included behavioural assessment of cats during weight loss, and concluded more affectionate display with food intake restriction, as perceived by the owners, independent of the weight loss diet consumed [52].

Therefore, due to the risk for obesity-associated diseases and the reduction of quality of life and longevity, obesity should be regarded as an animal welfare issue.

1.2 METHODS FOR OBESITY ASSESSMENT IN CATS

According to the World Small Animal Veterinary Association (WSAVA) and the American Animal Hospital Association (AAHA) nutritional assessment guidelines [35, 53], body composition assessment is an essential part of nutritional assessment in every cat at every visit to the veterinarian. This is especially crucial for obesity management, since it assists in determining ideal body weight (BW) and energy requirements, as well as communicating the veterinarian’s concerns related to unhealthy body composition to the owner [35]. Body composition can be defined by dividing the body into two different compartments – fat mass and fat free mass (includes bone, lean soft tissue mass and water) [54]. Unfortunately, only a few methods are broadly validated, and most of them are impractical and expensive for use in veterinary practice. Discussing the different available tools, aids in reconciling differences in the literature and assists in choosing the best strategy to assess body composition in clinical settings.
1.2.1 Dual-Energy X-ray Absorptiometry (DXA)

Lean soft tissue mass, bone and fat mass can be evaluated by the dual-energy X-ray absorptiometry (DXA) (Figure 1.1), while using x-rays at two levels of energy to differentiate and quantify tissues [55]. This method was validated in cats against computerized tomography (CT) [56], and is considered as the gold standard for body composition assessment [57]. Nonetheless, due to its cost, the need of animal sedation and expertise in its operation, it is not commonly used in veterinary practice [58].

Figure 1.1. An image of a dual-energy X-ray absorptiometry machine

1.2.2 Body Weight

The measurement of BW on its own in cats, cannot imply on excess of BF [59]. However, as an easy and non-invasive test, individual BW should be repeatedly measured as part of routine physical examination [35, 53], preferably on the same scale [60]. Repeated BW measures are very useful in clinics, and combined with additional life stage and medical information, these give an
indication for required obesity-management intervention and follow up of weight loss success during treatment of obesity.

1.2.3 Body and Muscle Condition Scores

Body condition score and muscle condition score (MCS) assessments are practical and repeatable tools in clinic to subjectively and semi-quantitatively assess the fat and lean soft tissue mass, through visualization and manual palpation [9, 34, 35, 58]. The WSAVA and AAHA nutritional assessment guidelines recommend that their evaluation will be performed as part of every physical examination of dogs and cats [34, 35, 53]. The most common BCS systems used in veterinary practice are the 5-point and the 9-point scales [9]. Fat mass measured by the 9-point scale where 1 is considered emaciated and 9 is considered obese (Table 1.1), as well as the 4-point MCS system, ranging from normal muscling to severe muscle loss, correlate well to DXA measurements, show considerable repeatability among scorers [9, 61, 62] and therefore serve as the most reliable tool for body composition assessment in veterinary practice. That being said, a BCS of 9 indicates BF $\geq 40\%$; hence, in order to better assess BF in these cases, the use of additional tools is required [57, 61].

1.2.4 Morphometric Measurements

Morphometric measurements are non-invasive metric measurements of body parts with a measurement tape, which are converted, most often using mathematical equations, to a measurement of body composition. It is suggested that morphometric measurements are a better indicator of body composition than BCS [13, 63], but further affirmation and validation is required. Such measurements in cats include BMI, girth and body fat index [11, 13, 64-66].
**Body Mass Index (BMI)**

Body mass index is another assessor of body composition in companion animals. In cats, several methods to assess BMI exist. All methods are performed on the cats while standing, with a measuring tape. The two equations reported in literature for use in cats are different than the one used in humans [which is the weight divided by the squared height (kg/m^2)] [67]. Both equations were not validated in cats against DXA or any other body composition assessment method as of yet:

1. BMI (%) = 100 x BW (kg) / [body length (m) x body height (m)],
where length is the distance from the point of the shoulder to the tuber ischium, and height is the distance from the tip of the shoulder (though the olecranon) to the proximal edge of the central metacarpal pad, was developed by Nelson *et al.* (1990) [17].

2. BMI (%) = 100 x ((Ribcage (m) / 0.7067) - LIM (m)) / 0.9156 - LIM (m).
The ribcage measurement is the circumference measured at the 9th rib, and the “limb index measurement” (LIM) is the distance between the patella and the calcaneus of the left leg, was developed by Hawthorne and Butterwick (2000) [63].

**Girth circumference (Girth)**

Girth circumference in cats is measured with a measuring tape behind the last rib [11, 64]. This measurement is helpful to distinguish overweight/obese cats from their lean counterparts, as well
as to monitor weight loss. Since girth is measured in one place only, good repeatability of results may be of a concern.

**Body Fat Index (BFI)**

The body fat index (BFI) comprises of six metric measurements that are performed in the cat while standing – head circumference, thoracic circumference, front leg circumference and length, hind leg length and body length (Figure 1.2). The measurements are then entered into an online software that uses a specific algorithm [65]. The output of that algorithm is BFI, an estimation of BF mass. Research showed that this method has a higher correlation to DXA than the BCS method, and precision increases with increased BF [68].

**Figure 1.2.** Required measurements for the assessment of body fat index % (image source: © Hill’s Pet Nutrition, Inc; Hill’s Healthy Weight Protocol [http://www.hwp.hillsvet.ca/](http://www.hwp.hillsvet.ca/))
1.3 OBESITY AND THE INFLAMMATORY RESPONSE

The excess of BF leading to obesity, as assessed by the methods described above, is accompanied by the presence of a low-grade inflammatory response [22]. The inflammatory response is derived by two primary pathways, the white adipose tissue and the GI tract microbiota, including effects on GI permeability. The involvement of white adipose tissue is discussed below, the role of gut microbiota will be further discussed under section 1.7.2.

1.3.1 White Adipose Tissue

Most research has focussed on adipose tissue as a cause of obesity-associated inflammation. White adipose tissue was once considered a passive tissue. However, over the past few years, it has been proven that this tissue is anything but passive, and acts as an active endocrine organ [69].

*Hypertrophy and Hyperplasia of Adipocytes*

White adipose tissue functions mainly to store energy and as a thermal insulator. It consists of 35-70% adipocytes, and the remaining tissue includes preadipocytes, mesenchymal stem cells, endothelial and smooth muscle cells, as well as macrophages and other immune cells. Obesity is associated with excess BF, and develops mainly through hypertrophy of adipocytes in adulthood. Until adulthood is achieved, both hyperplasia and hypertrophy of adipocytes occur [70] (Figure 1.3). Preadipocytes differentiate to mature adipocytes, allowing hyperplastic (increase in cell number) expansion of the adipose tissue in life when needed (before adulthood), and then adipocytes become hypertrophic (increase in cell size) when energy intake exceeds requirements [70].
Adipokine Secretion by White Adipose Tissue and Inflammatory Mediators Secreted by Other Tissues Involved in the Obesity-Associated Inflammation

The hypertrophic expansion of white adipose tissue during weight gain goes along with inflammation. The hypertrophic adipocytes secrete monocyte chemoattractant protein-1 (MCP-1), a chemokine, to attract macrophages which infiltrate the adipose tissue through the blood [71]. In mice, it is suggested that obese white adipose tissue mainly contains pro-inflammatory M1 macrophages, unlike white adipose tissue in lean subjects that contain mainly anti-inflammatory M2 macrophages [72]. Both adipocytes and macrophages in white adipose tissue, as well as other cells (e.g. monocytes) release inflammatory mediators, called adipokines (mainly cytokines and chemokines), which are a wide range of hormones and protein factors [71, 73]; however, the ratio of the secretion between cells may vary with animal species [71].

In humans and rodents there are more than 100 recognized adipokines, including cytokines, chemokines, acute phase proteins (APPs) and hormones [24]. Adipokines have a modulatory role in a variety of biological systems, including haemovascular functions, inflammatory and immune-
related effects, as well as impacts on cell proliferation [74]. More specifically, obesity triggers the activation of the inflammasome pathway, which results in up-regulation of many inflammatory genes, as well as increased plasma secretion of interleukin (IL)-1β and IL-18 that act as important mediators in the inflammatory response. Their plasma concentrations in humans were shown to decrease with weight loss [75]. Tumor necrosis factor-α (TNF-α), a cytokine expressed mainly by macrophages, is the first inflammatory cytokine reported in human obesity to relate to insulin resistance and glucose metabolism [76]. Tumor necrosis factor-α activates signaling pathways (e.g. mitogen-activated protein kinases), which results in the secretion of IL-1β, IL-6, and its concentrations are affiliated with IL-8 secretion [71, 77]. The infiltrating macrophages and T cells secrete also Fas ligand, a cytokine that with increased concentrations in advanced obesity, enhances the expression of its receptor, Fas (a part of the TNF receptor family) [78]. Fas ligand and Fas play a major role in apoptosis of cells expressing Fas receptor; however, Fas activation in obese white adipose tissue can also induce a pathway for pro-inflammatory cytokine/chemokine secretion, such as IL-6 and MCP-1, as was shown in mice and to an extent in humans [78-81]. Interleukin-6 is secreted by white adipose tissue, the liver and the muscle, considered as an immune-regulator, involved in haematopoiesis [82] and is suggested to be involved (depending on its level) in glucose homeostasis [71]. Both IL-6 and TNF-α have a role in propagating APPs secretion from the liver [69, 83]. Granulocyte macrophage-colony stimulating factor (GM-CSF) is a pro-inflammatory cytokine, an immune-modulator of myeloid cells such as macrophages, neutrophils and dendritic cells [84]; however, there are incongruities in the literature regarding the activity and importance of GM-CSF in obesity-associated inflammation in mice. There is some evidence to show that GM-CSF-deficient mice are obese [85], although, a recent study contradicted these previous results [86]. Interferon-γ (IFN-γ) and IL-12 also assists in regulation
of obesity-associated inflammation in mice. While IFN-\(\gamma\) is expressed by T cells and natural killer cells [87], the IL-12 cytokine family is mostly expressed by antigen-presenting cells [88]. The IL-12 cytokines are heterodimeric proteins, composed of different alpha and beta chains (e.g. p35 and p40 respectively), and by dimerization of specific chains, four combinations of IL-12 are created [88]. Interferon-\(\gamma\) works through modulation of macrophage activity, their cytokine secretion in white adipose tissue, promotion of adiposity, and contribution to glucose homeostasis [87, 89]. Interleukin-12 has similar activity, but in addition to the association between plasma concentrations of members of the IL-12 cytokine family and obesity, it appears that mRNA expression of the IL-12 gene differs between tissues, increase in some tissues, while decreases in others, as further explained. In obese mice, some chains, such as p40 and p28 mRNA expression, are elevated with obesity in white adipose tissue and skeletal muscle tissue, but their expression is diminished in heart tissue [88]. This implies on IL12 involvement in a pathway leading to atherosclerosis, while allowing exacerbated activity of TNF-\(\alpha\) and IL-6, to reach the same end result [88, 90]. Platelet-derived growth factor, isoform B (PDGF-BB) is a cytokine with a pro-fibrotic activity, mainly in the liver, but also in additional tissues [91], and circulating concentrations are expected to increase with obesity-associated inflammation. However, the association between PDGF-BB and obesity is not clear, as research results in mice are currently contradictory, and PDGF-BB/obesity-related research in humans is very limited [92, 93]. Interleukin-2 (IL-2) is another cytokine with an important role in cytokine synthesis and the proliferation-modulation of T cells. That being said, a recent human study did not show a significant increase in IL-2 serum concentrations in humans with general obesity. The same study showed a significant increase in serum concentrations of IFN-\(\gamma\) and TNF-\(\alpha\) in these individuals [94]. Stem cell factor (SCF) has pro-inflammatory properties, especially with regards to migration
of haematopoietic progenitor cells to the chronically-inflamed adipose tissue. Red (blood) cell distribution-width (RDW) is a marker for human metabolic syndrome. Stem cell factor is negatively associated with RDW, hence, SCF is suggested to negatively associate with obesity [95]. Similar to SCF, Flt-3L is a haematopoietic pro-inflammatory cytokine important for the development of dendritic cells, and for propagating the chronic inflammatory response, such as the case in obesity [96]. Stromal-cell derived factor (SDF)-1 is a chemokine that targets liver cells, such as sinusoidal endothelial cells and stellate cells. Stromal-cell derived factor-1 participates in chronic inflammation and promotes fibrosis in steatohepatitis in mice [97]. Increased serum secretion of IL-8, also a chemokine, is associated with augmented neutrophil chemotaxis and infiltration to the adipose tissue. Neutrophils produce adipokines as well, and have a role in propagating insulin resistance [71]. In mouse models, keratinocyte-derived chemokine (KC) is another prominent chemokine, analogous to IL-8 in humans, and adipose tissue expression, as well as plasma concentrations of KC, increase in mouse obesity [98]. An additional chemokine, RANTES, was only recently shown to take part in the obesity-associated inflammation and in the development of obesity. RANTES mainly adjusts the infiltration of immune cells into the adipose tissue, but in RANTES-knock-out mice fed a high-fat diet, also a significantly reduced weight gain occurred [99].

One of the roles of inflammatory cytokines such as IL-1β, TNF-α, IL-1, IL-6 and IL-8, is propagating hepatic production and secretion of APPs, e.g. serum amyloid A (SAA) and C-reactive protein (CRP) [100]. Serum amyloid A, a chemottractant, has a major role in down-regulation of the inflammatory response [101]. An increase in serum SAA concentrations was observed in obese humans, with a reduction in concentrations after weight loss [69, 83, 102, 103]. C-reactive protein
is a very potent inflammatory mediator, highly associated with cardiac health [104], and participates in the obesity-associated inflammation. In humans, elevated serum CRP concentrations were found in overweight and obese individuals to correlate with BMI, independently of confounders such as age, race and smoking status [105].

Unlike the adipokines mentioned above, IL-4, IL-10 and IL-13 have some anti-inflammatory properties [106]. Interleukin-4 and IL-13 are produced in adipose tissue of lean individuals/subjects by adipocytes, and promote activation of M2 macrophages, leading to CD4+ activation. In hypertrophic obese white adipose tissue, the ratio of CD8+/CD4+ increases, leading to increased pro-inflammatory cytokine secretion, as discussed above [71]. Interleukin-10 is secreted mainly by monocytes and macrophages, and is involved in glucose homeostasis [71].

Also adipose tissue secretion of the hormones, leptin and adiponectin, has been studied widely in humans and mice [69, 107]. Leptin is a protein, encoded by the ob gene. Leptin serum concentrations increase with increased BF [108]. The ob gene transcription is mediated by a variety of inflammatory mediators and metabolites, such as glucocorticoids, insulin, pro-inflammatory cytokines (e.g. TNF-α, IL-6) and lipopolysaccharides (LPS), but is also influenced by phases of the estrus cycle or hormonal status [108-110]. Leptin is primarily responsible for the regulation of energy depots and boosts energy expenditure [111]. When leptin concentrations in serum increase after a meal, it suppresses appetite [112]. Genetic mutations in the leptin gene or its receptor occur rarely in humans and mice and cause extreme obesity [113, 114]. Adiponectin is a protein exclusively produced by mature adipocytes in white adipose tissue. In humans, adiponectin promotes insulin sensitivity, inhibits atherosclerosis, and is thought to be anti-inflammatory [115,
More specifically, work with two cloned receptors in mice demonstrated enhanced lipid β-oxidation in muscle and liver tissue, reduced hepatic gluconeogenesis, as well as increased muscle glucose uptake [74].

Some members of the S100 calcium-binding proteins, also known as calgranulins, were found to be novel biomarkers for obesity-related inflammation, as well as mediators. All calgranulins are pro-inflammatory, although different calgranulins possess different qualities. S100A12/calgranulin C is one of these proteins, and can be expressed in different cells in inflamed tissues, such as myeloid cells, epithelial cells, smooth muscle cells, etc. That being said, the mechanism behind increased serum concentrations of S100A12 is not fully clear, although it is suspected to result from an increase in circulating myeloid cells, due to the high association found between increased serum S100A12 concentrations to increase mRNA of S100A12 in mononuclear cells in the peripheral blood [117]. S100A12 is more resistant to oxidation compared to other calgranulins, and therefore, is able to maintain its biologic function in increased oxidative states, such as obesity [118]. S100A12 can independently activate cytokines/chemokines, such as IL-6 and MCP-1, and can be an important triggering-component for an acute inflammation [118].

Calgranulins bind to multi-ligand receptor for advanced glycation end products (RAGE), and S100A12 is suggested to have the strongest ability to activate RAGE [119]. More specifically, the S/S variant in the RAGE receptor in obese individuals stimulates the augmented binding of S100A12 with cytokines such as TNF-α and IL-6 in mononuclear cells in peripheral blood [120].

An additional study in RAGE-deficient mice that were fed with a high-fat diet, indicated prevention of weight gain due to the lack of RAGE [121]. Therefore, these data provide an
additional pathway for the development of obesity and an obesity-associated inflammation, although similar information is not available in cats.

The Obesity-Associated Inflammatory Response in Cats – What is Known So Far?

In comparison to humans and rodents [69, 107], relatively limited information is available on obesity-related gene expression and secretion of adipokines in cats [23, 122]. Research mainly focussed on leptin and adiponectin. Feline obesity is related to increased energy intake or reduced energy expenditure, and an increase in BF involves in most cases hyperleptinemia [123] and decreased sensitivity to leptin [123]. The feline adiponectin gene is very similar to the human and canine gene, and is transcribed to different molecular forms. The adiponectin protein though, is much more similar to the canine protein rather than the human protein [124]. In cats, concentrations of high molecular weight adiponectin are usually reduced with obesity, and increase with weight loss [74, 124]. However, similar to dogs, research in cats is inconsistent in cats on whether adiposity is the principal factor affecting the concentration of circulating adiponectin, or whether it is also affected by nutrient intake, independent of change in BW [74, 125]. Serum and plasma leptin and adiponectin concentrations are suggested to correlate positively and negatively with obesity status and insulin resistance in cats, respectively [75, 122, 123, 126-128]. However, it is unclear whether leptin and/or adiponectin contribute to the pathogenesis of feline insulin resistance/sensitivity [125, 129, 130].

Consistent and conclusive data regarding the feline obesity-dependent inflammatory response are lacking. A recent feline study by Van de Velde et al. (2013) compared chronically obese and lean cats, and evaluated adipokine mRNA expression and morphological features of adipose tissue. It
was found that the intensity of the feline inflammatory response propagated by obesity depended on adipose tissue location, it was more profound in subcutaneous adipose tissue compared to visceral adipose tissue, which could affect the onset, severity and treatment of obesity-related comorbidities, such as insulin resistance. Feline obesity was overall characterized by increased adipocyte cell size, changes in adipokine gene expressions [increased mRNA expression of leptin, IL-6, CCL-5, and MCP-1], in addition to the presence of T-lymphocytes, which aligns with human obesity [122]. Based on these findings, a similar increase in serum adipokines concentrations was expected; however, a local adipose tissue inflammation doesn’t necessarily cause a systemic inflammation, as was observed in two additional studies by Hoenig et al. (2007 and 2013). The study by Hoenig et al. (2007) investigated the effects of two isocaloric diets, with different macronutrient levels (protein, carbohydrates), on leptin and adiponectin, fat distribution, and glucose metabolism in both obese and lean cats. The study by Hoenig et al. (2013) examined the effects of weight gain in lean cats on fat distribution, inflammatory biomarkers and insulin sensitivity. The results from both Hoenig studies demonstrated equal distribution of fat tissue between the subcutaneous and the visceral regions, decreased insulin sensitivity with increased adiposity, but aside from a serum increase in leptin and decrease in adiponectin in obese cats compared to lean cats, no significant changes occurred in the concentrations of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) in the plasma [126, 131]. These results are in agreement with another feline study, investigating the effects of weight loss on plasma concentrations of APPs (haptoglobin and SAA) and adiponectin. No significant differences were observed in plasma APP concentrations before and after weight loss, though a significant increase in adiponectin occurred after weight loss [132].
Therefore, the feline obesity-related inflammatory response is not fully understood, and in order to understand its similarity/dissimilarity to other species, and the relationship to comorbidities such as insulin resistance, more research is warranted.

1.4 NORMAL FELINE GASTROINTESTINAL MICROBIOTA

The cat is a carnivorous animal and possesses a relatively simple GI tract in comparison to herbivorous and omnivorous monogastric species [133], and depends less on the GI microbiota for energy harvesting. Cats are accustomed to a high animal-derived protein diet, with relatively low digestible carbohydrate content [134], hence the residing microbiota is expected to be composed of more proteolytic bacteria. Thus far, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria were found to be the most prevalent bacterial phyla in the feline GI tract and fecal samples [135, 136]. However, inconsistent and contradictory evidence in the bacterial composition, as well as bacterial intra-species variability, can be found. These controversies are subject to a variety of factors, such as animal (e.g. age, sex), dietary, environmental or assessment methodologies (e.g. sampling, storage, extraction) [137, 138]. Recent studies investigating the composition of the normal feline fecal microbiota using molecular tools are represented in Table 1.2.

Bacterial composition variability exists between animals. For example, the feline microbiome was found to be more diverse than the canine microbiome, mainly at the level of bacterial species or strain [139], and inter-species variance is higher than the intra-individual or inter-individual variance within species [140, 141]. Furthermore, bacterial gut diversity increases due to dietary
patterns, from carnivory to omnivory and herbivory [141]. That being mentioned, canine and feline microbiota are similar to human and mice at the predominant phyla level, where a major difference lies in the relatively high abundance of *Fusobacteria* in the intestinal and fecal microbiota of dogs and cats, which reaches 10% or more of the sequences [137].
<table>
<thead>
<tr>
<th>Sample size</th>
<th>Samples</th>
<th>Storage Temperature</th>
<th>Method of Assessment</th>
<th>Predominant Microbiota Composition of a Healthy Feline GI Tract</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 cats</td>
<td>Fecal</td>
<td>-20°C</td>
<td>Fluorescence in situ hybridization (FISH)</td>
<td><em>Bifidobacterium</em> spp., <em>Bacteroides</em> spp., <em>Lactobacillus-Enterococcus</em> subgp., and <em>C. histolyticum</em></td>
<td>Inness <em>et al.</em>, 2007 [29]</td>
</tr>
<tr>
<td>4 cats</td>
<td>1 Stomach, 2 Duodenum, 2 Jejunum, 3 Ileum 4 Colon, 1 Rectum</td>
<td>-80°C</td>
<td>16S rRNA gene analysis (clone libraries)</td>
<td><em>Firmicutes</em> (67%), <em>Proteobacteria</em> (14%), <em>Bacterioidetes</em> (10%), <em>Fusobacteria</em> (5%), <em>Actinobacteria</em> (4%)</td>
<td>Ritchie <em>et al.</em>, 2008 [135]</td>
</tr>
<tr>
<td>5 indoor cats; 4 outdoor cats</td>
<td>Fecal</td>
<td>-20°C</td>
<td>cpn60 gene analysis (clone libraries)</td>
<td><em>Actinobacteria</em> – <em>Bifidobacterium</em> spp. 13 – 8% (indoor vs. outdoor) <em>Firmicutes</em> – 41 – 72 % (indoor vs. outdoor) <em>Proteobacteria</em> - ~1% (indoor only) <em>Bacterioidetes</em> – 16 – 2% (indoor vs. outdoor)</td>
<td>Desai <em>et al.</em>, 2009 [136]</td>
</tr>
<tr>
<td>12 cats</td>
<td>Fecal</td>
<td>N/A</td>
<td>16S rRNA gene 454-pyrosequencing</td>
<td><em>Firmicutes</em> – mainly <em>Clostridiales</em>, <em>Lactobacillales</em>, and <em>Erysipelotrichales</em>; <em>Actinobacteria</em> - mainly <em>Coriobacteriales</em></td>
<td>Garcia-Mazcorro <em>et al.</em>, 2011 [142]</td>
</tr>
<tr>
<td>12 cats</td>
<td>Fecal</td>
<td>-80°C</td>
<td>Massive parallel 16S rRNA gene pyrosequencing</td>
<td><em>Firmicutes</em> (92-99%) – mainly <em>Clostridia</em>; <em>Actinobacteria</em> (7-8%) – mainly <em>Coriobacteriales</em>; <em>Bacterioidetes</em> (&lt; 1%) – mainly <em>Prevotella</em> and <em>Bacteroides</em>; <em>Fusobacteria</em> (&lt;0.1%) – mainly <em>Fusobacterium</em> sp.</td>
<td>Handl <em>et al.</em>, 2011 [139]</td>
</tr>
<tr>
<td>4 cats</td>
<td>Fecal</td>
<td>-80°C</td>
<td>Shotgun 454-pyrosequencing</td>
<td><em>Firmicutes</em> (36.3%), <em>Bacteroidetes/Chlorobi</em> (36.1%), <em>Proteobacteria</em> (12.4%), <em>Actinobacteria</em> (7.7%)</td>
<td>Barry <em>et al.</em>, 2012 [140]</td>
</tr>
</tbody>
</table>
1.5 MICROBIOTA ASSESSMENT

1.5.1 Challenges in Assessment of Microbiota

Results of fecal microbiota-related analyses can be impacted by a variety of factors. Such factors, as fecal sampling techniques, storage conditions [143, 144], DNA extraction [145] or sequencing techniques (see section 1.5.2), have been investigated to some extent in humans, with inconclusive results in regards to their effects. In companion animals, data regarding these factors is very limited. A recent study examined, among other things, the effect of two non-invasive collection methods, litter box sample versus rectal swab, on the feline fecal microbiota. Significant differences were observed between methods, when rectal swabs contained more bacteria compared to litter box samples [146]. One of the least controlled challenges encountered in field studies, especially in cats, is storage conditions of fecal samples. A substantial amount of time may pass between defecation to sample collection, not to mention, until submission for laboratory analyses. A study examining the effect of short term refrigeration (< 2 weeks) on fecal microbiota of dogs and cats found very mild changes in microbiota membership and composition [147]. However, some owners would prefer to store their cat’s stool samples, following collection, outside of the refrigerator, for hygiene purposes. Therefore, the impact of ambient temperature on the feline fecal microbiota requires further investigation.

1.5.2 Microbiota Assessment Methods

Until the past two decades, traditional culture-based techniques predominantly provided insight into the bacterial composition of the GI tract of humans and animals, by means of phenotypic characterization. These techniques were relatively approachable to researchers (e.g. inexpensive,
highly available); however, the end product was not thoroughly representative of the microbiota phylotype composition or prevalence because only a small proportion of GI bacteria can be effectively grown under normal culture conditions [148]. Although Goodman et al. (2011) recently showed an improvement in cultivation ability to 70% of the human fecal microbiota [149], these methods are cumbersome and not widely available.

Fingerprinting methods such as Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) separate amplicons based on their difference in base pair composition. The amplicons, when exposed to increased temperature or denaturing agent migrate throughout the gel at various speeds, which cause the formation of a specific banding pattern for the sample, a fingerprint. Fingerprinting methods have been used for qualitative assessment (e.g. for microbiota comparison between the canine GI segments [150]); however, these methods have their short comings, particularly an inability to clearly define changes [151]. While these methods may identify differences, their ability to explain what accounts for those differences and assess specific taxa is limited.

Analyses using molecular methods rely mainly on the bacterial 16S rRNA gene and other conserved genes (e.g. Chaperonin-60, CPN60) [136]. The 16S rRNA gene is organized in units called operons [152]. It contains highly preserved and stable regions, as well as regions with augmented variability, which permit in-depth phylogenetic characterization and classification of the microbiota [149, 152]. Polymerase chain reaction (PCR) is performed using a heat-stable DNA polymerase that amplifies the 16S rRNA gene region, to potentially create millions of amplicons. These amplicons on their own cannot be used for absolute quantification of the GI microbiota,
since different bacteria (even within the same species) possess a different number of copies of this gene [153]; however, amplicons number can imply on bacterial ecological tactics, or in other words, imply on bacterial response to accessible resources, such as increased amplification [154].

Quantitative PCR (qPCR) is an adaptation of PCR, meant to quantitatively assess the abundance of the GI microbiota [142]. When a fluorescent molecule, such as fluorescently-labeled sequence-specific probes or primers, is incorporated to the PCR process, an increase in fluorescent signal indicates an increase in the DNA quantity [155]. However, actual cell number cannot be assessed from qPCR, due to the increase in operon numbers throughout growth for example, which highlights a disadvantage of this method [156].

In contrast to qPCR, fluorescence in situ hybridization (FISH) is a method that aims to accurately quantify bacterial cells by using fluorescently-labeled oligonucleotides, and identifying the 16S rRNA. With the appropriate oligonucleotides, it can distinguish microbiota at all taxonomic levels, although it is very challenging [157]. This method can also provide additional information on microbiota location in respect to the gastrointestinal wall, and on its morphology [158]. Although the technique provides very beneficial information in humans and companion animals, and promotes the understanding of the relationship between microbiota and gastrointestinal diseases in dogs [158] and cats [29, 159, 160], it is considered labor intense and expensive, and therefore is used in small-scale studies, with a small sample size [158-160].

Sequencing techniques enable 16S rRNA amplicon identification by assessing the base pairs order. The Sanger technique was one of the first methods used in human and companion animal research,
using construction of gene clone libraries [161]. In a feline study conducted by Ritchie et al. (2008), Sanger sequencing was used to report the most predominant fecal bacterial phyla in healthy cats [135]. Another study assessed the feline fecal microbiota, targeting the *cpn60* gene, and used Sanger sequencing to show a difference in the composition compared to Ritchie et al. (2008) [136]. However, this method is laborious and has limited throughput, as amplicons must be cloned into bacteria, with individual bacterial colonies sequenced.

High-throughput sequencing techniques are more preferable and efficient [162]. High-throughput methods such as 454-pyrosequencing or the Illumina sequencing (next generation sequencing) are able to sequence thousands to millions of base pairs in a short amount of time, allowing for in-depth study of the microbiota and relative quantification of amplicons [162]. Handl et al. (2011) demonstrated a difference from previous data in regards to the biodiversity of the healthy feline GI microbiota using pyrosequencing [139]. Illumina sequencing techniques have been applied in kittens recently, to characterize the fecal microbiota following dietary changes, such as feeding with high-protein low-carbohydrates diet vs. moderate-protein moderate-carbohydrate diet [163]. High-throughput sequencing techniques are being used only in the last decade, and due to its cost, only small number of samples were analysed in most studies. Therefore, the information obtained so far from studies conducted in all species should be considered with caution. In addition, these techniques have their disadvantages as well, since the use of universal bacterial primers may underestimate specific bacteria. Guanine and cytosine rich bacteria (e.g. *Bifidobacterium*) were found to be under-represented in 16S rRNA gene sequencing studies, where the use of a combination between universal bacterial primers and specific ones resulted in more detailed characterization of the residing bacteria [30].
Therefore, in order to better assess GI microbial diversity, use of molecular tools became prominent [136, 139, 163, 164]. As mentioned, molecular tools also have their limitations. Microbial diversity can be influenced by many factors, such as the DNA extraction method performed, or by the use of different PCR primers, which may lead to the underestimation of groups with low abundance, or overestimation of some bacterial groups [165, 166]. Hence, interpretation of studies involving molecular methods, especially in regards to bacterial quantification, should be made with caution, and use of combination of methods or application of correctional steps (the use of primer combinations) would better reflect true bacterial composition [167].

**1.6 OBESITY AND THE GASTROINTESTINAL MICROBIOTA**

Intestinal dysbiosis is defined as the microbial diversity aberration from what is considered normal [168, 169]. Obesity was defined earlier in this review as a low grade inflammatory state [21, 22]. Intestinal dysbiosis is highly associated with inflammatory states in humans, mice and companion animals [160, 170-173], and consequently its relation to obesity has been investigated. However, information is especially limited in cats [28, 174, 175].

To date it is unclear whether the microbiota changes that occur in obese subjects are a cause or consequence of obesity. Some mechanisms that may work simultaneously have been proposed. Such mechanisms include amplified energy harvest by the GI microbiota [176], increased nutrient utilization which promotes short-chain fatty acid (SCFA) formation [177], secondary bile acids
formation [178] and adipogenesis [179], down-regulation or over-expression of obesity-related genes [180], or elevated LPS formation [181]. Also, the gut microbiota interacts with the host’s mitochondria by signaling, using the endocrine, humoral and immune systems [182]. When mitochondrial function is diminished, such as the case in Crohn’s disease, prevalence of specific bacteria may be reduced (e.g. butyrate producers) or increased (e.g. $\text{H}_2\text{S}$ producers) [182]. Overall, the interaction between gut microbiota and host mitochondria is utilized by both pathogenic and commensal bacteria. These mechanisms independently or in combination with obesity-related increase in gut permeability [183, 184], result in elevated bacterial translocation, endotoxin permeation, and therefore, local and systemic inflammation [185] (Figure 1.4).

**Figure 1.4.** Contribution of obesity and intestinal microbiota to the obesity-associated inflammatory response [69, 139-151, 159-161, 167]
1.6.1 The Association between Microbiota and Obesity

Microbiota Promotes Obesity Development through Nutrient Harvest and Metabolic Pathways

Dietary nutrient composition was shown to modulate intestinal microbial biodiversity and fat deposition. Increased energy harvest from nutrient excess or the ability to utilize indigestible carbohydrates (e.g. resistant starch, variety of soluble and insoluble fibre) result in the production of SCFA and monosaccharides that can be absorbed through the colonocytes and trigger hepatic lipid formation [186], gluconeogenesis, and serve as substrates for mitochondrial oxidation [187]. Fecal samples of obese humans were 20% higher in SCFA compared to their lean counterparts, indicating increased energy harvesting abilities in obese subjects, thus providing additional energy to the host [188]. Some anaerobic bacteria (5-10%), belonging to Bacteroides, Eubacterium and Clostridium genera, have the capacity to degrade primary bile acids to secondary bile acids, which can interact with the host mitochondria and contribute to energy metabolism [178]. Hence, fat deposition is partially induced by the GI microbiota, implying the presence of “obese microbiome” [186] (Figure 1.4).

This statement was assessed in germ-free (GF) mice that were compared to conventionally-raised (CONV-R) mice, before and after colonization with unfractionated microbiota yielded from the cecum of adult CONV-R donors (i.e. conventionalization). Differences in BF % were determined by using DXA. Prior to conventionalization, CONV-R mice were found to have 42% higher total BF, as well as significantly higher epididymal fat, than the GF mice, although consuming 29% less of the same rodent diet. Once 10-day or 14-day conventionalization with cecal content were performed on GF mice (then called CONV-D mice), their total BF and epididymal fat increased by nearly 60%, and their food consumption decreased by 27% compared to when they were germ-
free. This study also investigated the mechanism behind the increase in fat accumulation, and showed that in CONV-D mice, there is prominent microbial digestion of dietary polysaccharides, resulting in monosaccharide formation and their subsequent transfer into the liver for lipogenesis. The results of this study indicated that there was no change in the whole DNA content of epididymal fat pads between GF and CONV-D mice. Furthermore, qPCR demonstrated no significant change in the expression of genes associated with adipogenesis or lipogenesis. In conjunction with histochemical data, it was concluded that the increase in fat in the epididymal fat pad is a result of adipocyte hypertrophy [186]. Another study involving conventionalization, showed that fecal microbial transplantation, in GF mice increased the density of capillaries in the villus epithelium of the small intestines, thereby increasing absorption surface area and nutrient harvest by the host [189].

The GI microbiota affect metabolic pathways that enhance adiposity. That is the case with a strain of *B. thetaiotaomicron* (saccharolytic bacterial species), which was reported to induce host monosaccharide transporters in mice [190, 191]. Microbial components can also suppress the expression of host genes such as fasting-induced adiposity factor (Fiaf), also known as angiopoietin-like protein 4 (ANGTPL4), a suppression that causes an increase in activity of lipoprotein lipase (LPL) in adipocytes, and enhances storage of liver-derived triglycerol in the fat cells of the host [190]. Lipoprotein lipase is produced by the fat tissues, and the intestines [192, 193], and has a major role in the regulation of fatty acid translocation from organs with triglyceride-rich lipoproteins (e.g. the fat tissues, muscle). Fasting-induced adiposity factor was shown to inhibit *in vitro* [194] and *in vivo* [186] LPL activity in mice. Also in mice, administration of *Lactobacillus rhamnosus* CNCMI-4317 caused an increase in Fiaf expression, while modifying
oxidative phosphorylation by the mitochondria [195]. More specifically, an increase in intestinal Fiaf in mice protected them from the obesogenic dietary effects of high-fat and digestible carbohydrates, and kept them lean [180].

Information in humans, as well as in companion animals, is still scarce. A recent canine study investigated the effects of obesity and weight loss on fecal microbiota and SCFA concentrations. The study suggested that gut microbiota associated with increased SCFA production may negatively impact the rate of weight loss in dogs. However, more research is required to understand the association between GI/fecal microbiota and obesity/weight loss in companion animals, specifically in cats [196].

**Microbiota-induced Inflammatory Response Enhances Obesity**

Previous studies in mice have shown that free fatty acids can bind to toll-like receptor 4 (TLR4), which activates cells within the innate immune system, causing pro-inflammatory cytokine secretion, and an increase in BF [197]. It appears though, that TLR4 needs CD14 to regulate cytokine secretion. Lipopolysaccharides are components of the gram-negative bacteria external membrane. LPS induces an innate inflammatory response in the host and leads to weight gain in mice via CD14 signalling. However, a knockout of CD14 protected mice from weight gain after LPS production/administration in a low dose, unlike a situation like sepsis. Overall, it appears that LPS in a sufficient yet small amount can cause metabolic endotoxemia (2-3 times increase in plasma LPS concentrations) and mimic, in a slightly different pathway the high-fat diet-induced inflammatory response and obesity [198]. In a study by Osto et al. (2009), administration of LPS to healthy cats for 10 days (10-1000 ng/kg/h) caused subacute endotoxemia and a corresponding
inflammatory response throughout the administration period. The inflammatory response was manifested by increased fever, elevated neutrophils in the liver, and in the first 5 days, reduced insulin sensitivity and increased glucose. However, after 5 days, body temperature and glucose levels normalized, suggesting desensitization to LPS, when administered in low levels and for a set period. Serum triglycerides increased initially, but then decreased rapidly. Still, the triglyceride content in the liver was significantly increased after 10 days, which is an indicator for the dyslipidemia that occurs with subacute endotoxemia [199]. Therefore, the inflammatory response modulated through LPS is perceived as an integral part of the obesity equation and its comorbidities, especially insulin resistance (Figure 1.4).

In humans and mice there are reports suggesting an “obese microbiome” - a higher Firmicutes-to-Bacteroidetes ratio [27, 200], with overall decreased microbial diversity and richness [26, 201]. Nonetheless, there are inconsistencies, and some researchers do not report significant changes in microbial composition with obesity [188]. In companion animals, not only is information regarding mechanisms of interaction between obesity and microbiota limited, but also information regarding the GI bacterial biodiversity and composition is lacking. Two studies in companion animals, one in dogs and one in cats, demonstrated some differences between lean and obese animals [146, 202]. Yet, in both studies no diet acclimatization was performed [146, 202], and diet has a major influence on microbial composition and biodiversity [175, 203]. A recent third study, by Pallotto et al (2018), investigated the effects of a 16-week weight loss plan on the microbiota of eight obese, neutered, male cats, housed in kennels. While there were some changes in the fecal microbiota structure, and to some extent, composition (changes in prevalence of several genera in Firmicutes, Proteobacteria and Bacteroidetes), this study included a small sample of cats, did not
examine the effects of obesity, but merely weight loss, and was conducted in a very controlled environment. Therefore, results from these studies in companion animals should be interpreted with caution [204].

1.6.2 The Effects of Obesity and GI Microbiota on Intestinal Permeability

It is suggested from rodent models and human studies that the gut barrier permeability (i.e. the properties of the gut membrane that allow different molecules to penetrate from the lumen) is increased with obesity, and is related to the gut microbiota [75, 185]. Obesity is suggested to influence gut barrier permeability through three potential interacting mechanisms – dietary nutrient profile and pattern, deviation from normal microbial composition (i.e. microbial dysbiosis) leading to LPS production, and nutritional deficiencies [205] (Figure 1.4).

Obesity-associated Mechanisms Increase Intestinal Permeability

In mice, 4 weeks of a high-fat diet caused weight gain, increased levels of LPS-producing bacteria, as well as plasma LPS concentrations, reaching eventually the threshold for metabolic endotoxemia and induction of metabolic disease (obesity and insulin resistance) through CD14 [198]. In a follow up study by the same research group, the effects of antibiotic treatment (ampicillin and neomycin) on the GI microbiota of ob/ob mice or mice that were fed a high-fat diet for 4 weeks were examined. Feeding a high-fat diet significantly reduced the mRNA expression of zonula occludens-1 (ZO-1) with a trend for reduction of mRNA expression of occludin (tight junction proteins) in adipose tissue. Thus, a high-fat diet endangers intestinal tight junction integrity and increases intestinal permeability. Once the mice were treated with antibiotics, dysbiosis that occurred due to the high-fat diet was resolved and tight junction protein
expression was restored, which underlines the potentially negative effect of diet on the microbiota, and implies on the significant role of the GI microbiota in intestinal permeability and metabolic endotoxemia [206].

Beyond fat, additional nutrients such as fructose may also have a negative influence on intestinal permeability. In a study by Bergheim et al. (2008), mice had 8 weeks ad libitum access to regular water or water sweetened with different sweeteners – glucose, fructose, sucrose or artificial sweetener. Mice exposed to glucose had the highest caloric intake or weight gain. However, mice exposed to fructose had the highest increase in portal blood LPS concentrations, TNF-α mRNA expression and lipid peroxidation compared to all other groups. Therefore, it is suggested that the type of carbohydrate consumed may also influence GI microbial composition, and concomitantly, endotoxemia and intestinal permeability [207]. That being said, it seems that in both mice and humans, high energy consumption as well as high-fat diet promote endotoxemia. A high-fat diet is more efficient in transporting LPS from the gut to blood circulation when compared to a high-carbohydrate diet [208, 209].

In most cases the positive energy balance leading to obesity is caused by excess nutrient intake, mainly fat [210]. Most overweight and obese companion animals are overfed with a complete and balanced diet, but some are fed more than 10% of their daily energy intake as unbalanced foods (e.g. treats, table scraps), or are generally being fed with an unbalanced high-fat homemade diet, which may eventually cause nutritional deficiencies [211]. Some minerals and vitamins have a significant role in the function of the GI intestinal barrier, such as magnesium [212], zinc [213], calcium [214], vitamin D [215] and A [216]. For example, zinc activates hepatocyte nuclear factor-
4α, which is crucial to the differentiation and regulation of the tight junction proteins. Zinc deficiency causes inactivation of this factor, epithelial barrier dysfunction and altered permeability [217].

Assessment Methods for Intestinal Permeability

Assessing intestinal permeability, especially in cases of focal/multi-focal sites with increased permeability, is difficult. Direct tests to evaluate intestinal integrity/permeability include tight junction protein expression, or distribution, or transepithelial potential [218, 219]. However, these methods are quite invasive, and require harvesting of intestinal tissue.

In companion animals, especially in dogs, initial indicators of intestinal disease, which can also serve as indirect intestinal health markers, include serum concentrations of cobalamin and folate. In small intestinal disease, folate absorption may be poor, and cause low serum folate concentrations. When serum folate concentrations are high, it may be an indicator of increased synthesis by bacteria, and consequently absorption in the small intestine. High serum concentrations of folate in dogs usually occur in small intestine bacterial overgrowth (SIBO). Low serum concentrations of cobalamin suggest malabsorption in the distal small intestine, mostly due to SIBO in dogs [220]. Since SIBO is a form of bacterial dysbiosis, and bacterial dysbiosis may cause increased intestinal permeability, abnormal serum concentrations of both cobalamin and folate can suggest jeopardized intestinal health. That being said, serum folate concentrations can be relatively easily altered, depending on dietary content, and overall, both vitamin concentrations can also be depleted due to concomitant diseases (e.g. exocrine pancreatic insufficiency) or be within normal ranges in SIBO [221]. Gastrointestinal bacteria may produce toxins, aside from
LPS, such as ammonia and $D$-lactate. In cats, it was found that especially $D$-lactate and depleted concentrations of serum cobalamin may suggest bacterial dysbiosis, and serve as intestinal health indicators [222]. Serum concentrations of folate in cats are commonly high within normal reference range, and elevated concentrations are rare, and not necessarily indicative of SIBO and hence intestinal permeability [220].

Many molecules have been tested, especially labeled sugars [223], to indirectly assess intestinal permeability. The most commonly labeled sugars used in mice and humans are lactulose and mannitol, which are fermented in the colon [224]. Mannitol crosses the lumen through the transcellular route due to its low molecular weight, whereas lactulose crosses the membrane through the paracellular route. Increased ratio of lactulose-to-mannitol may imply altered intestinal permeability in both the small intestine and colon [225]. Studies in obese mice, investigating the lactulose-to-mannitol ratio demonstrate increased intestinal permeability; however, results from human studies are inconsistent and may suggest that the examined ratio is not a good marker for permeability assessment in obese patients [226]. In dogs, a dual sugar absorption test was performed, using the ratio between two labeled sugars, lactulose and rhamnose, that were administered orally to dogs, and their urinary excretion/blood concentrations were determined. The ratio of lactulose to rhamnose in dogs was found to be a reliable indicator of intestinal damage [227, 228]. Cats are suggested to have increased intestinal permeability when compared to dogs under normal circumstances based on indirect assessment of intestinal permeability using labeled sugars (as previously described in dogs); therefore, abnormal intestinal permeability is harder to assess, and may not always reflect an intestinal disease or inflammation [229, 230].
Additional non-invasive biomarkers that can assist in detecting damage to the barrier integrity include blood, urinary and fecal markers. Claudin-3 is a tight junction protein that appears in the urine when permeability increases, and its increase in the urine is correlated to its histochemically observed loss from the intestinal barrier [231]. In dogs, altered colonic expression (detected post mortem via using labeled tight junction-specific antibodies) of caludin-2, zonulin, occludin, and additional tight junction proteins, has been found in cases of GI disease, such as canine idiopathic lymphocytic-plasmacytic colitis [232]. Calprotectin, lactoferrin and elastase are neutrophil derived proteins, and their presence in the stool in high concentrations may reflect on intestinal inflammation [233]. Fecal and serum assessment of calprotectin have been validated in dogs [234], but not in cats.

Intestinal permeability was shown to increase in humans with systemic inflammation caused by LPS administration. Following LPS administration, a strong connection was established between the serum IL-10 concentrations and polyethylene glycol, an intestinal permeability marker, which indicated an affiliation between triggering of the innate immune system, and the degree of intestinal permeability [235]. This study and others suggest that obesity, and the activation of the inflammatory response, are also involved in increased risk to gut barrier health. Nonetheless, more research is warranted, especially in companion animals.

1.7 CONCLUSION

Several factors impact the development of the obesity-associated inflammatory response, including the intestinal microbiota, through diverse mechanisms. These mechanisms have been
investigated in humans and in mice to some extent, but the existence of such a systemic low-grade inflammatory response in cats, as well as the contribution of intestinal microbiota, have not been well established, and require further research.

1.8 OBJECTIVES AND HYPOTHESES

Currently, characterization of normal feline microbiota is available in the literature; however, description of the obese-cat microbiota and research regarding the association between both feline obesity and weight loss, and intestinal microbiota, are quite scarce. Also, literature on the presence of an obesity-associated inflammation in obese cats, and the data on its extent and implications, remain inconclusive and limited. Hence, more established evidence for the presence of the obesity-related inflammatory response in cats is required, including an understanding of the inflammatory markers taking part, and their potential influence on intestinal microbiota and intestinal health.

Therefore the research objectives of this thesis included:

1. Validation of the use of feline fecal samples kept at ambient temperature for 4 days, for microbiota analysis, using next generation sequencing (Illumina Miseq) (Chapter 2).
2. Characterization of the differences in fecal microbiota between obese and lean client-owned cats following a period of dietary adaptation, and investigation of the differences in fecal microbiota in obese cats before and after a standardized weight loss plan using next generation sequencing (Illumina Miseq) (Chapter 3).
3. Examination of the association between both feline obesity and weight loss and a variety of serum inflammatory and intestinal health markers, including serum cytokines (e.g. TNF-α and IL-6), chemokines (e.g. MCP-1 and SDF), APPs (e.g. serum amyloid A), cobalamin, folate (Chapter 4).

It was hypothesized that:

1. Short-term storage of fecal samples at ambient temperature would not affect the microbial biodiversity (Chapter 2).

2. Differences would be detected in feline fecal microbial population-members and structure between lean and obese cats, which would revert with weight loss in obese cats (Chapter 3).

3. An obesity-associated low-grade inflammatory response would be entrenched, and associated with impaired gastrointestinal health, while a corrective process would occur with weight loss (Chapter 4).
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THE EFFECT OF STORAGE AT AMBIENT TEMPERATURE ON THE FELINE FECAL MICROBIOTA

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CHAPTER 2: THE EFFECT OF STORAGE AT AMBIENT TEMPERATURE ON THE FELINE FECAL MICROBIOTA

Abstract

Background: Feline fecal microbiota analyses can potentially be impacted by a variety of factors such as sample preparation, sequencing method and bioinformatics analyses. Another potential influence is changes in the microbiota from storage of samples prior to processing. This study examined the effect of ambient temperature exposure on the feline fecal microbiota composition. Fecal samples were collected from 12 healthy cats, within 15 minutes after defecation. Samples were aliquoted and the first aliquot was frozen at -80°C within one hour of defecation. Remaining aliquots were maintained at ambient temperature (20 to 23°C) and frozen at -80°C at 6, 12, 24, 36, 48, 72 and 96h after collection. DNA was extracted from all aliquots, and polymerase chain reaction (PCR) was performed. The PCR products were sequenced with next-generation sequencing (Illumina MiSeq).

Results: No significant differences were observed in alpha and beta biodiversity indexes, as well as relative abundance of different taxa over time (P>0.05 for all tests between time points). Principal coordinate analyses demonstrated that samples cluster mainly by cat, with no significant differences between time points (AMOVA, P>0.05; HOMOVA, P>0.05). Linear discriminant analysis effect size method was performed and failed to detect any enriched taxa, between time points. Random forest algorithm analysis indicated homogeneity across time points.

Conclusions: Although existing evidence from human fecal storage studies is contradictory, a recent study in companion animals agreed with the current study, demonstrating that maintenance
of feline fecal samples at ambient temperature for up to 4 days has no effect on the bacterial membership and structure.

2.0 INTRODUCTION

The human gastrointestinal (GI) microbial community (the ‘microbiota’) is extremely complex in composition, and very high in concentration, reaching $10^{12}$-$10^{14}$ cell/g of intestinal content.

The microbiota is dominated by bacteria, but also consists of Archaea, viruses, fungi and parasites. The bacterial concentration progresses from the stomach to the colon, with the highest concentration found in the colon [1-4]. Similar numbers are found in dogs and cats, although composition and bacterial species dominance are different from humans [5, 6]. Increased evidence exists in humans and companion animals for the health implications and clinical importance of the commensal or symbiotic relationship between the intestinal bacteria and their host. The intestinal microbiota plays a crucial role in the development of the host immune system, protection against pathogens, toxins and mutagens and utilization of excess nutrients or nutrients that are unavailable to the host [7]. The microbiota produces short chain fatty acids (SCFAs) that serve as energy sources to the colonocytes, produces vitamins, and aids in mineral absorption and intestinal integrity, along with a myriad of other effects, many of which remain to be properly defined [5, 8, 9].

Any deviation from the ‘normal’ microbiota is referred to as intestinal dysbiosis [5, 10]. In humans, animal models and companion animals, dysbiosis can be associated with a range of disease states, particularly inflammation [11-14]. Alterations of the normal gut microbiota
balance [15, 16], due to inherent, environmental or immunological factors can be involved in the pathogenesis of intestinal inflammatory diseases [11, 17-19], or with other organ-related diseases, such as diabetes mellitus, obesity or asthma [2, 20-22].

In order to characterize the human or animal microbiota, a variety of methods can be used. In the past, the use of bacterial cultivation techniques dominated, but results are limited because of the inability to grow a large percentage of the microbiota using standard culture methods [23, 24]. Recently developed high-throughput techniques, such as Illumina MiSeq, are capable of quick massive parallel sequencing, providing more into-depth understanding of human and companion animal microbiome, and are considered as the preferred analytical method nowadays [25]. Aside to the effects of DNA extraction methods [26] or molecular tools, fecal sampling technique and sample storage conditions can potentially influence phylogenetic identification.

The effects of different sampling and storage methods on the fecal bacterial population of healthy and diseased human subjects were examined [27]. Fecal samples were aliquoted within 10 minutes of defecation and stored at different temperatures: -80°C, at -20°C for a week, at +4°C for 24 hours and at ambient temperature for 24 hours. No significant differences were found in the number of operational taxonomic units (OTUs), diversity or richness between the different storage temperatures [27]. This study agreed with the results of previous studies [28], but is in contradiction with others, that reported a mild gradual difference in the fecal bacterial composition when samples were stored at ambient temperature for 24 hours compared to the composition assessed around defecation [29, 30]. Studies evaluating the impact of storage conditions on fecal microbiota in companion animals are scarce. One study in dogs and cats
identified limited change in the microbiota from short term (<2 week) refrigeration [31]. However, the impact of room temperature storage was not assessed. This is an important aspect to understand for field studies, particularly of species such as cats. Fecal collections for microbiota-related studies in client-owned cats are often challenging, as significant time may pass between defecation, sample collection, and submission for analyses. During this time the sample may be left at room temperature (e.g. overnight in the litter box) before proper storage.

The objective of this study was to determine the effect of ambient temperature exposure on the feline fecal microbiota.

2.1 MATERIALS AND METHODS

Sample collection

Fresh fecal samples were collected from 12 healthy cats located at a cat boarding facility in Guelph, Ontario from June to July 2016. The subjects were determined to be healthy based on information provided to the facility manager by cat owners prior to boarding. Cats were observed closely by facility personnel and fecal samples were collected within 15 minutes of defecation. Samples were maintained unrefrigerated until arrival at the laboratory. Upon arrival, samples were weighed (Denver MAXX, Denver Instrument, Bohemia, New York, USA), manually homogenized and aliquoted into 200 mg samples. One aliquot was frozen at -80°C within one hour of defecation (time point (T) 0). The remaining aliquots were kept at ambient temperature (20 to 23°C) in a biosafety cabinet (Class II, Type A2 Biosafety Cabinet, Thermo Fischer Scientific, Waltham, Massachusetts, USA). Aliquots were frozen at -80°C at the following time
points: 6, 12, 24, 36, 48, 72, 96h. Downstream processing was then performed on samples that had all undergone the same freeze-thaw cycle and were processed as a batch.

**DNA extraction**

For DNA extraction, a commercial stool extraction kit (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek Inc., Doraville, Georgia, USA) was used according to the manufacturer’s instructions. The DNA was collected in 1.5 ml micro-centrifuge tubes, and stored at -80°C until further analysis.

**Polymerase Chain Reaction (PCR)**

The quantity of extracted DNA was assessed using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Nano Drop Technologies Inc. (Thermo Fisher Scientific), Waltham, Massachusetts, USA), with readings ranging from 33 to 661 ng/ml. To prepare the 16S rRNA gene amplicons for the Illumina MiSeq system (Illumina, San Diego, California, USA), all DNA samples were diluted (if needed) to a range of 30 to 100 ng/ml. The V4 region of the 16S rRNA gene was amplified using the forward primer S-D-Bact-0564-a-S-15 (5’-AYTGGGYDCAAAGNG-3’), reverse primer S-D-Bact-0785-b-A-18 (5’-TACNVGGGTATCTAATCC-3’), KAPA HiFi ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA), and PCR grade water. Two PCR cycles were conducted in a thermal cycler (Mastercycler Pro, Eppendorf Canada Ltd, Mississauga, Ontario, Canada). The purified PCR products were evaluated with 1.5% agarose gel for gel electrophoresis and DNA was measured using spectrophotometry.
DNA sequencing

Using an Illumina MiSeq system, the samples were amplified by bridge amplification and sequenced with terminator nucleotides [32]. At least 100,000 reads/sample with sequences of approximately 500 bp in length from 2x250 paired end reads were obtained [32].

Bioinformatics analyses and statistics

Mothur v1.36.1 was used for bioinformatics analyses, as well as some of the statistical analyses [33, 34]. Additional statistical analyses were performed using JMP 13.0 (SAS Campus Drive, Cary, North Carolina, USA). Paired end reads were assembled and filtered to remove sequences greater than 250 base pairs (bp) in length. Sequences with any ambiguous base calls or runs of homopolymers greater than 8 bp were removed.

Sequences were aligned to the Silva16S rRNA reference database [35], and those that did not align with the correct region were removed. In addition, chimeras were identified using uchime [36] and removed. Sequences were classified using the RDP classifier (v14) [37], and those taxonomic assignments were used to create OTUs using a closed (database-dependent) OTU picking approach. Archeae were removed. Subsampling was performed based on the smallest number of sequences from a sample, to standardize sequence number used for analysis [38].

Alpha-diversity indexes (Shannon diversity [39], Simpson diversity [40] and Chao1 [41]) were calculated to assess evenness, diversity and richness, respectively, and compared between time points using a nonparametric multiple comparisons test (Wilcoxon Each Pair). Relative abundances were calculated for the different taxonomic levels, at each time point. Differences in
relative abundance of taxa accounting for ≥ 1% of sequences within phyla and ≥ 0.1% within genera were evaluated using nonparametric multiple comparison test (Wilcoxon Each Pair), with p-values adjusted using the Benjamini-Hochberg correction to control for the false discovery rate [42]. Beta-diversity was assessed, using the classical Jaccard index [43] and Yue & Clayton index of dissimilarity [44] to examine community membership and population structure, respectively. For visualisation of differences in membership and structure between cats and time points, dendrograms were created, and significance of clustering according to time point was determined using parsimony and unifrac.unweighted [45]. Beta-diversity was visualized using principal coordinate analyses (PCoA) and further assessed using analysis of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA). Linear discriminatory analysis (LDA) effective size (LEfSe) [46] was conducted as well for the identification of genomic features between time points. Random forest algorithm analysis was used to assess the ability to predict group classification, by cat or by time point [47].

2.2 RESULTS

Ninety-three samples were processed from the 12 cats. Three aliquots (two at T96, and one at T72) were not obtained due to small fecal samples size from two cats. A total of 9,118,609 sequences passed all filters, with a median of 93,909 sequences per sample, and a range of 50,315 to 207,127 sequences per sample. A random subsample of 50,315 sequences per sample was used to normalize samples for analysis.
There were no significant differences in evenness, diversity and richness between the different time points (all $P > 0.05$) (Figure 2.1). Median bacterial relative abundance accounting for $\geq 1\%$ of phyla and $\geq 0.1\%$ of genera, are presented in Figure 2.2 and Figure 2.3 respectively. No significant differences in relative abundances were noted at any taxonomic level (all adjusted $P > 0.05$). There was a numerical decrease in *Megasphaera* (Figure 2.3); however, this was not statistically significant (unadjusted $P=0.06$, adjusted $P=0.81$).

Figure 2.1: Comparison of bacterial population evenness (A), diversity (B) and richness (C) in 12 healthy cats between time points 0, 6, 12, 24, 36, 48, 72 and 96 hours.

Figure 2.2: Comparison of median relative abundances in of predominant phyla originating from fecal samples of 12 healthy cats, between time points 0, 6, 12, 24, 36, 48, 72 and 96 hours.
Figure 2.3: Comparison of median relative abundances in of predominant genera originating from fecal samples of 12 healthy cats, between time points 0, 6, 12, 24, 36, 48, 72 and 96 hours.

No differences in community membership (Classical Jaccard index – unifrac $P = 0.74$; parsimony $P > 0.05$ for all comparisons) or population structure (Yue & Clayton – unifrac $P = 0.83$; parsimony $P > 0.05$ for all comparisons) were observed. Clustering by cat, but not time point, was apparent on the dendrograms for both community membership and structure (Figure 2.4). Principal coordinate analyses indicated that fecal microbiota mainly clustered by cat, with no significant differences in community membership (Figure 2.5A and B) or structure between time points (AMOVA $P > 0.05$ and HOMOVA $P > 0.05$ for all comparisons).
**Figure 2.4:** Dendrogram of the Classical Jaccard index representing the community membership of the fecal microbiota in 12 healthy cats, compared between time points 0, 6, 12, 24, 36, 48, 72 and 96 hours. Each cat is represented with a different colour.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis failed to identify enriched taxa between time points. Random forest algorithm analysis was performed both to evaluate the ability to differentiate between time points and between cats. When random forest was analysed according to time point, 92% error rate was established, indicating homogeneity across time points, and a very poor ability to separate samples into their appropriate groups. However, when “cat” was analysed as the group, the error rate was only 15%, indicating a much stronger ability to assign samples to the appropriate cat.
**Figure 2.5:** Two dimensional principal coordinate analysis of population membership of the fecal microbiota of 12 healthy cats, assessed at time points 0, 6, 12, 24, 36, 48, 72 and 96 hours. (A) By time point; (B) By cat

### 2.3 DISCUSSION

The results from the study imply that there are no significant changes in feline fecal microbiota evenness, diversity and richness, as well as community membership and structure over 96 hours, while fecal samples are kept at ambient temperature.

Microbiota assessment in feline fecal samples can provide important insight. However, multiple variables can impact the results. It is important to understand potential external influences or biases that might affect the ability to properly define the microbiota and detect true biological differences. The potential impact of sample storage is one potential concern, particularly in studies that involve collection of samples from the community and inherent delays until processing. Understanding the potential influences of sample storage is important for proper design and interpretation of studies.
A variety of ecological indices can be used to assess microbial biodiversity. Despite the use of a range of methods, including assessment of relative abundances and alpha and beta diversity indices, no significant impact of storage on the fecal microbiota was identified. In addition to statistical analyses, clear numerical, non-statistically significant, differences were evident. While care must be taken when considering any non-significant results, these results should not be completely dismissed as statistical differences can be clouded by the degree of inter-sample variation and power limitations. The most readily apparent numerical difference was a decrease in the relative abundance over time of *Megasphaera*, a genus of relatively fastidious anaerobes [48]. Therefore, the trend for decreased *Megasphaera* abundance over time could correspond to air exposure during storage, something that might have been accentuated by manual homogenization during preparation of fecal samples. However, since the methods used in this study do not depend on viable microorganisms, it is unclear whether poor aerotolerance can accurately explain these results. *Megasphaera* was shown to play a dominant role in bacterial composition of the feline GI tract [49]. It is associated with ruminal fermentation of lactate into short-chain fatty acids (SCFA), and is especially related to butyrate formation [50]. Due to its fermentation capacity, the bacterium was shown to have beneficial effects on the GI health of some monogastric mammals [51, 52]. However, further research is required for the understanding of the underlined role of *Megasphaera* in the cat, an obligate carnivore [49], especially when increased protein content may promote a steep decrease in *Megasphaera* abundance, as well as jeopardise the GI microbiota health [53].

While the extent of studies investigating the impact of storage on feline fecal microbiota has been limited, the results of this study are similar to other studies that have shown limited or no
impact of short-term storage. One study investigated the effect of storage at 4°C on fecal samples from seven dogs and ten cats, after 0, 3, 7 and 14 days [31]. Only a couple of significant changes were observed in the feline fecal microbiota, such as a decrease in the relative abundance of Erysipelotrichaceae *incertae sedis* after 7 days, or enrichment with *Psychrobacter* and *Arthrobacter* (Proteobacteria and Actinobacteria respectively) after 14 days [31]. In comparison to this refrigeration study, it would be expected that changes would occur prior to 7 days of storage at ambient temperature, which was not observed in the current study. A human study involving fecal samples from four healthy children, kept at room temperature for 12, 24, 48 and 72 hours, reported a minor change in community structure over time [29]. A small but significant increase in diversity was identified after 12 hours along with a decrease in strains of *Ruminococcus* and *Faecalibacterium* [29]. Corresponding changes were not noted in the present study, with possible explanations being differences in sample preparation as well as inter-species variation in fecal microbiota composition. In contrast to the study by Roesch *et al.* (2009), non-sterile containers were used for sample preservation in the current study and fecal samples were homogenised, as performed similarly by Weese *et al.* (2014) [29, 31]. Homogenization of stool assists in standardization of aliquots [4, 31, 54]. However, some researchers opt not to homogenise samples as it also causes increased oxygen exposure, which can influence the microbiota membership and structure over time [27]. Additional methodological parameters that can contribute to differences in sample membership and population structure are freeze-thaw cycles [55], differences in extraction methods [56] as well as sequencing methods. Regardless, while there have been variable results from studies in different species using different storage conditions, changes noted over the short timeframe of this study have been mild or absent, consistent with these data.
In general, changes in membership or population structure would correlate to either degradation of microbial DNA or bacterial growth. Dietary ingredients may include microbial metabolic inhibitors. Most feline diets are composed of both animal products and plants ingredients. Plant ingredients contain chlorophyll metabolites such as pheophorbide \( \alpha \) and pyropheophorbide \( \alpha \) [57], which inhibit bacterial efflux pumps [58]. The inhibitory effect of these metabolites could be one potential explanation for the lack of bacterial growth in the current study. Nonetheless, the dietary history of the cats was unavailable and further tests were not conducted to confirm this assumption.

The first fecal sample in the current study was stored only after an hour from its collection, due to the location of the boarding facility and preparation time. Also, fecal samples were stored only for four days. It is possible that some changes in microbial structure and composition have occurred during the first hour, however, no continuous significant effects were observed. Four days should be sufficient time in field studies to transport samples to better storage conditions, such as refrigeration or freezing. The sample size was relatively small, but it was based on a recent storage study in companion animals [31], as well as on human storage studies, that used a similar smaller sample size.

Nonetheless, it is possible that with immediate refrigeration or freezing, and a larger sample size numerical trends in microbiota composition would become significant. Since very little research exists on the effects of storage on fecal samples in general, and even more so in cats, more
research is warranted prior to solid conclusions in regards to short-term fecal storage recommendations.

2.4 CONCLUSIONS

This study demonstrate that several-day-storage of healthy cats’ feces at ambient temperature, has no effect on microbial biodiversity. Although sample freezing at -80°C is recommended for long term storage, the current study suggests that short term storage, up to 4 days, at ambient temperatures can be appropriate, especially when field studies are performed.
2.5 REFERENCES


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CHAPTER 3: BACTERIAL FECAL MICROBIOTA IS ONLY MINIMALLY AFFECTED BY A STANDARDIZED WEIGHT LOSS PLAN IN OBESE CATS

ABSTRACT

Background: Obesity in pets is a prominent worldwide problem with detrimental health consequences. Research in humans and mice suggests that obesity influences the abundance and diversity of gastrointestinal (GI) microbiota, and that an “obese microbiome” has a considerable influence on energy metabolism and fat storage in the host. Microbiota membership and composition have been previously assessed in healthy cats; however, research on the effects of obesity and weight loss on the cat’s fecal microbiota is very limited. Therefore, this study’s objective was to evaluate differences in fecal microbiota abundance and biodiversity between lean and obese cats, and in obese cats before and after a standardized weight loss.

Fourteen lean and 17 obese healthy client-owned cats were fed a veterinary therapeutic weight loss food (Hill’s Prescription Diets Metabolic Feline dry) at maintenance energy requirement for 4 weeks. At the end of this adaptation period (end of week 4) lean cats finished the study, whereas obese cats continued to a 10-week weight loss period on the same food, fed at individually-tailored weight loss energy requirements. Body weight, body condition and muscle condition score were recorded biweekly throughout the study. At the end of each period, a fecal sample and food-consumption records were obtained from the owners. Fecal samples were stored at -80°C until analyses were performed. DNA was extracted from all fecal samples, and polymerase chain reaction (PCR) was performed. Sequencing of PCR products was conducted using next-generation sequencing (Illumina MiSeq).
Results: No significant differences were seen in the relative abundance of taxa and in biodiversity indices between cats in either group ($P > 0.05$ for all tests). Nevertheless, some significantly enriched taxa, mainly belonging to Firmicutes, were visible in linear discriminant analysis effect size test in obese cats before weight loss compared to lean cats.

Conclusions: The association between feline obesity and the fecal bacterial microbiota was demonstrated in enriched taxa in obese cats compared to lean cats, which may be related to enhanced efficiency of energy-harvesting. However, in obese cats, the fecal microbiota abundance and biodiversity were only minimally affected during the early phase of a standardized weight loss plan.

3.0 INTRODUCTION

The obesity epidemic is a prominent problem in humans and companion animals. There are a few definitions for obesity, but they are all in agreement that the term reflects an excess of body fat that jeopardizes the animal’s health [1-4]. In Europe, the United States and New Zealand, estimations from the last decade indicate that 11.5% – 63% of cats are overweight or obese [5-8]. Obesity is mainly caused by an imbalance between energy intake and energy expenditure [9]. Additional factors contribute to obesity, such as genetics, age, sex, desexing, physical inactivity, and endocrine diseases [7, 8, 10-12]. However, more recent findings indicate that gastrointestinal (GI) microbiota (i.e. gastrointestinal microbial community) plays an important role in the development of obesity. The microbiota can be addressed as a “metabolic organ”, acting to support the host with metabolic functions that the host is not capable of performing, such as fermentation of plant polysaccharides [13-15]. The microbiota can also affect metabolic
pathways in the host, for example promote metabolic pathways that enhance adipose tissue storage [13, 16]. Moreover, the microbiota is also involved in low-grade inflammation, which occurs with obesity. It is suggested that lipopolysaccharide (LPS), a structural component in external membrane of gram-negative bacteria, triggers inflammation in the host through the innate immune response [17].

Increased relative abundance of Firmicutes versus Bacteroidetes, and hence a higher Firmicutes-to-Bacteroidetes ratio were observed in obese humans and mice compared to their lean counterparts [18, 19]. However, some studies did not observe these differences [15]. Obesity was also associated with reduced microbial richness and diversity in humans and mice [20, 21]. Nonetheless, little research exists in companion animals to demonstrate these findings. Research in dogs demonstrated increased relative abundance of the phylum Actinobacteria and genus *Roseburia* in lean versus obese dogs [22]; however, in this study the subjects differed in their origin (lean research dogs and obese pet dogs) and the dogs were not fed the same food [22]. In cats, research on the relationship between obesity and fecal microbiota is even scarcer, inclusive of merely two studies. One published feline study examined the differences in microbiota between lean and overweight/obese cats used two fecal collection methods – rectal swabs versus litter box samples. In both collection methods, differences in microbiota between lean and obese cats were found. However, in the study they used shelter cats, with no medical history known, and the food they consumed was different (no diet adaptation period). Hence, their results may have been affected by the diet or medical condition, and therefore, are inconclusive [23]. Still, similarly to the canine study mentioned above [22], dietary standardization was not performed and the effect of weight loss was not assessed [23].
Another feline study examined the effect of weight loss on microbiota in eight kennel cats. However, the sample size used was quite small, results were not compared to matching lean kennel cats, and the study environment was very controlled, hence, less reflective of the true effects in population [24].

Therefore, the aim of the current study was to characterize the fecal microbiota differences between lean and obese client-owned cats following a period of dietary acclimatization, as well as to investigate the effects of a standardized weight loss plan on the bacterial fecal microbiota of obese cats. It was hypothesized that the feline fecal microbial composition and structure will differ between lean and obese cats and that these microbial differences will revert with weight loss in obese cats, to resemble the microbiota of lean cats.

3.1 MATERIALS AND METHODS

Experimental Design

Fourteen lean (body condition score (BCS) 4-5/9, 10 males and 4 females) and 17 obese (BCS ≥ 8/9, 11 males and 6 females) [25] cats were enrolled in the study. All cats were client-owned animals from the Guelph, Ontario region, lived indoors, were neutered and between 2-9 years of age. All cats were determined to be healthy, apart from obesity, based on physical exam, medical and dietary information provided by cat owners, as well as complete blood count (CBC) and serum biochemistry panel. There was no history of antimicrobial and anti-inflammatory medications administered in the 90 day period prior to study enrolment. The study took place between May 2015 and December 2016. A figure of the study’s experimental design is available in Appendix 3a.
The study was conducted at the Ontario Veterinary College, University of Guelph. The experimental design was approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #2496).

**Adaptation Period**

At the time of enrolment, body weight (BW), BCS and muscle condition score (MCS) [25, 26] were documented. Next, all cats underwent a one-week transition period to a veterinary therapeutic food (Hill’s Prescription Diets Metabolic Feline dry, Topeka, Kensus, USA) intended for weight loss and adult maintenance (Table 3.1), followed by a 4-week adaptation period during which all cats received the study food for 100% of their daily ration. Individual maintenance energy requirements (ER) for both lean cats (LEAN) and obese cats before weight loss (OBWL) were calculated in accordance with the National Research Council (NRC) (LEAN - 100 Kcal/kg^{0.67}; OBWL - 130 Kcal/kg^{0.4}) based on ideal body weight [27]. Food intake was recorded daily by the owners. Three weeks following study enrolment, body weight was assessed and the amount of food offered was adjusted to maintain a stable body weight. At the end of the adaptation period (week 5) BW, BCS, and MCS were recorded. Body condition score was evaluated using a 9-point scale, validated for cats by Laflamme (1997) [25]. Muscle condition score was assessed using a 4-point system, described and validated for cats by Michel et al. [28]. Also, body mass index (BMI) and girth were assessed in all cats at this time. Body mass index was calculated according to Nelson et al. (1990) [29] and girth was measured right behind the last rib [30]. All measurements were conducted by the same investigator (MT) throughout the study to reduce variability. Fecal samples were obtained from the owners within 24 hours after
defecation and frozen at -80°C until further analysis. The lean cats completed the study at week 5, while the obese cats continued with a 10-week weight loss plan.

**Table 3.1.** Proximate and total dietary fibre analyses of the veterinary therapeutic food\(^a\) intended for weight loss and adult maintenance

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>Content(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>g/100g</td>
<td>5.5</td>
</tr>
<tr>
<td>Crude fat (by acid hydrolysis) (CF) (DM)</td>
<td>g/100g</td>
<td>13.0</td>
</tr>
<tr>
<td>Crude protein (CP) (DM)</td>
<td>g/100g</td>
<td>38.6</td>
</tr>
<tr>
<td>Nitrogen-free extract (NFE) (DM)(^c)</td>
<td>g/100g</td>
<td>24.1</td>
</tr>
<tr>
<td>Crude fibre (DM)</td>
<td>g/100g</td>
<td>6.3</td>
</tr>
<tr>
<td>Total dietary fibre (DM)</td>
<td>g/100g</td>
<td>18.5</td>
</tr>
<tr>
<td>Crude ash (DM)</td>
<td>g/100g</td>
<td>5.8</td>
</tr>
<tr>
<td>Energy density (DM)(^d)</td>
<td>kcal/100g</td>
<td>330</td>
</tr>
</tbody>
</table>

*Table legend:* DM, dry matter

The food was fed to lean cats (LEAN, n=14) for adult maintenance for 5 weeks and to obese cats for adult maintenance for 5 weeks (OBWL, n=17), followed by a 10-week weight loss period (OAWL, n=16).

\(^a\) Hill’s Prescription Diets Metabolic Feline (dry), which contained Chicken by-product meal, brewers rice, corn, gluten meal, powdered cellulose, dried tomato, pomace, flaxseed, dried beet pulp, chicken liver flavor, coconut oil, pork fat, lactic acid, potassium chloride, calcium sulfate, L-lysine, choline chloride, carrots, DL methionine, vitamins (Vitamin E supplement, L-ascorbyl-2-polyphosphate (source of vitamin C), niacin supplement, thiamine mononitrate, calcium pantothenate, pyridoxine hydrochloride, Vitamin A supplement, riboflavin supplement, biotin, Vitamin B12 supplement, folic acid, Vitamin D3 supplement), taurine, L-carnitine, minerals (manganese sulfate, ferrous sulfate, zinc oxide, copper sulfate, calcium iodate, sodium selenite), mixed tocopherols for freshness, natural flavors, \(\beta\)-carotene.

\(^b\) Nutrient content refers to an average of two consecutive laboratory analyses from the same bag, which were performed by Maxxam Analytics International Corporation, Mississauga, Ontario, Canada;

\(^c\) Calculated using the equation: NFE (g/100g) = 100 – (CP + CF + TDF + ash) [27];

\(^d\) Calculated using the equation: Energy density (kcal/100g) = (CF \times 8.5) + (CP \times 3.5) + (NFE \times 3.5) [27]
**Weight Loss Period**

At week 5 the individual ER for weight loss were calculated for the obese cats in accordance with the NRC \(0.6 \times 130\ \text{Kcal/kg}^{0.4}\), based on ideal body weight [27]. The weight loss period continued for 10 weeks. During this period, food intake was recorded daily by the owners and BW, BCS, and MCS were assessed bi-weekly to monitor for effective and safe weight loss (with a target BW loss of 0.5-2\% of the initial BW [27, 31]), as well as to monitor maintenance of lean body mass. If weekly weight loss rate was below 0.5\% or exceeded 2\%, individual ER adjustments were made – lowering or increasing the ER initially by 5\%, respectively [12]. After 10 weeks, assessment of BMI and girth were repeated. At that time, fecal samples collected by the owners for the obese cats after weight loss (OAWL) were also obtained and stored as previously described.

**Sample Preparation and DNA Extraction**

All fecal samples were analysed in parallel. Whole fecal samples were thawed overnight in their original container in a refrigerator (+4°C), manually homogenized in a biosafety cabinet (Class II, Type A2 Biosafety Cabinet, Thermo Fischer Scientific, Waltham, Massachusetts, USA), and aliquoted into 200 mg samples.

DNA extraction was conducted using a commercial stool extraction kit (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek Inc., Doraville, Georgia, USA) in accordance with the manufacturer’s instructions. DNA products following extraction were stored at -80°C until further analysis.
Polymerase Chain Reaction (PCR)

A spectrophotometer (NanoDrop 1000 Spectrophotometer, Nano Drop Technologies Inc. (Thermo Fisher Scientific), Waltham, Massachusetts, USA) was used to assess the quantity of extracted DNA. All DNA samples were diluted (if needed) to a range of 30 to 100 ng/ml. The V4 region of the 16S rRNA gene was amplified using polymerase chain reaction (PCR) with the forward primer: S-D-Bact-0564-a-S-15 (5’-AYTGGGYDAAAGNG-3’), reverse: S-D-Bact-0785-b-A-18 (5’-TACNVGGGTATCTAATCC-3’), KAPA HiFi ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA), and PCR grade water. In order to prepare the PCR products for Illumina MiSeq (Illumina, San Diego, California, USA) sequencing, the purified PCR products were amplified with Illumina adapters through PCR (Mastercycler Pro, Eppendorf Canada Ltd, Mississauga, Ontario, Canada), and then purified again. Prior to Illumina sequencing, the finalized PCR products were evaluated using gel electrophoresis and DNA was measured using spectrophotometry.

DNA sequencing

Bridge amplification was performed with the Illumina MiSeq system (Illumina, San Diego, USA), using terminator nucleotides that were incorporated to the amplified PCR products with the removal of the terminator group [32].

Bioinformatics and statistical analyses

Mothur v1.36.1 was used for sequence processing [33, 34]. Further statistical analyses were performed using JMP 13.0 (SAS Campus Drive, Cary, North Carolina, USA). Assembly and filtration of paired end reads were done to remove sequences greater than 250 bp in length and
those with any ambiguous base calls or runs of homopolymers greater than 8 bp. Alignment of sequences to the Silva16S rRNA reference database [35] was implemented, with the removal of sequences that did not align with the correct region. Uchime was conducted to identify chimeras [36], which were then removed. Ribosomal Database Project (RDP) classifier (v14) was used for taxonomic assignment of sequences [37]. Archaea were removed. A closed operational taxonomic unit (OTU) picking approach was then performed. In order to standardize sequence number used for analysis, subsampling was completed based on the smallest number of sequences from a sample [38].

Normality of data distribution was assessed using the Shapiro-Wilk test. Evenness, diversity and richness were calculated using Shannon diversity [39], Simpson diversity [40] and Chao1 [41] respectively (alpha-diversity indices), and compared between the groups (LEAN, OBWL and OAWL) using a nonparametric multiple comparisons test (Wilcoxon Each Pair). Relative abundances were calculated for the different taxonomic levels, for each group. Differences in relative abundance of phyla accounting for ≥ 1% and of the rest of the taxa accounting for ≥ 0.1% of sequences were evaluated using nonparametric multiple comparison test (Wilcoxon Each Pair for comparisons between LEAN to OBWL and LEAN to OAWL; Wilcoxon Signed-Rank for the comparison between OBWL to OAWL), with p-values adjusted using the Benjamini-Hochberg correction (R. Core Team, 2013, R Foundation for Statistical Computing, Vienna, Austria; \(^1\) SAS Campus Drive, Cary, North Carolina, USA) to control for false discovery [42]. Relative abundances are presented as median with range (minimum to maximum). The classical Jaccard index [43] and Yue & Clayton index of dissimilarity [44] (beta-diversity indexes) were performed to examine community membership and population structure,
respectively. To reflect the differences in membership and structure between the groups, dendrograms were generated, and significance of clustering according to group was determined using parsimony and unweighted unifrac tests [45]. Beta-diversity indices were also visualized using principal coordinate analyses (PCoA), with further comparison of groups by analysis of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA). In order to identify difference in taxa between groups, linear discriminatory analysis (LDA) effective size (LefSe) [46] was conducted. Random forest algorithm analysis was calculated to assess the ability to predict group classification [47].

Statistical analyses for body weight and body composition measurements were performed using SAS v9.3 (SAS Campus Drive, Cary, North Carolina, USA). Normality of the data was assessed using the Shapiro-Wilk test. Differences in BW, BCS, BMI and girth measurements between LEAN to OBWL and OAWL groups were examined using student T-test/paired T-test or the corresponding Wilcoxon-Mann-Whitney/Wilcoxon Signed-Rank, depending on whether the samples were paired. Significance was set at $P < 0.05$ for all comparisons. Normally distributed data are expressed as mean ± standard error (SE). Body condition score was not normally distributed and is presented as median with range (minimum to maximum).

3.2 RESULTS

Fourteen lean and 17 obese cats were enrolled in the study. One obese cat did not complete the study due to reduced owner cooperation with food restriction, which resulted in weight maintenance rather than weight loss. This cat was included in the OBWL group, but not in the
OAWL group. Only a stool sample and food intake data were obtained for one cat because of its fractious nature, leaving 13 cats for assessment of BW and body composition in the LEAN group. All cats tolerated the food well, no cat refused to eat the food, and none showed signs of illness or maldigestion (mean food consumption ± SE within groups – LEAN: 215.2 ± 9.9 kcal, OBWL: 217.8 ± 5.9 kcal, OAWL: 134.6 ± 2.3 kcal).

**Body weight and body composition**

Body weight remained stable in all the cats during the 4-week adaptation period, however mean BW was significantly lower in LEAN (4.49 ± 0.22 kg) versus OBWL (6.95 ± 0.32 kg) ($P < 0.0001$). The 10-week weight loss plan was successful as body weight was significantly lower in OAWL (6.30 ± 0.28 kg) compared to OBWL (6.95 ± 0.32 kg) ($P < 0.0001$). On average cats lost 687.50 ± 0.02 g over the 10-week period, which represents an average weight loss of 0.94 ± 0.02 % per week. Also a significant decrease in BCS between OBWL (9 (8 to 9)) and OAWL (8 (6 to 9)) was observed ($P = 0.0012$). Body mass index and girth in LEAN (41.58 ± 1.30 and 38.38 ± 1.05 respectively) were significantly lower compared to OBWL (60.45 ± 2.92 and 52.11 ± 1.18 respectively) ($P < 0.0001$), and were also significantly lower in OAWL (55.62 ± 2.76 and 48.25 ± 1.39 respectively) compared to OBWL, $P < 0.0001$. Altogether, BW, BCS, BMI and girth in OAWL were still significantly higher than in LEAN cats after the 10-week weight loss plan ($P < 0.0001$ for BW, BCS and girth, and $P < 0.0002$ for BMI).
**Fecal microbiota analysis**

Fecal samples analyses resulted in a total of 9,193,399 sequences that passed all filters, with a median of 105,984 sequences per sample (range 22,314-557,519). A random subsample of 22,314 sequences per sample was used for sample normalization.

**Relative abundance**

Median bacterial relative abundance was examined across all taxa between groups. After performing Benjamini-Hochberg adjustment, significant differences between groups were no longer identified for any taxa (Figure 3.1 & 3.2, Table 3.2, $P > 0.05$).

**Figure 3.1:** Comparison of median relative abundances of predominant phyla originating from fecal samples of healthy lean cats (LEAN, $n=14$) and obese cats (OBWL, $n=17$) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and obese cats after a 10-week weight loss period on the same food (OAWL, $n=16$).
Figure legend: LEAN, lean cats; OBWL, obese cats before weight loss; OAWL, obese cats after weight loss

Figure 3.2: Comparison of median relative abundances of predominant genera originating from fecal samples of healthy lean cats (LEAN, n=14) and obese cats (OBWL, n=17) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and obese cats after a 10-week weight loss period on the same food (OAWL, n=16).
### Table 3.2. Summary of medians for relative abundances from fecal microbiota of healthy lean and obese cats

<table>
<thead>
<tr>
<th></th>
<th>Phyla (9/9)*</th>
<th>Class (10/25)*</th>
<th>Order (15/56)*</th>
<th>Family (20/99)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LEAN</td>
<td>OBWL</td>
<td>OAWL</td>
<td></td>
</tr>
<tr>
<td><strong>Phyla (9/9)</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td>81.0 (63.7-91.0)</td>
<td>80.2 (5.3-93.4)</td>
<td>76.0 (46.3-92.1)</td>
<td></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>5.4 (1.9-23.2)</td>
<td>8.2 (2.9-19.0)</td>
<td>7.4 (1.2-43.0)</td>
<td></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>2.9 (0.8-32.8)</td>
<td>2.5 (0.4-30.1)</td>
<td>4.0 (0.5-13.6)</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>1.9 (0.4-5.1)</td>
<td>2.1 (0.5-4.3)</td>
<td>2.0 (0.6-5.3)</td>
<td></td>
</tr>
<tr>
<td>Bacteria_unclassified</td>
<td>1.3 (0.5-10.4)</td>
<td>1.6 (0.3-3.7)</td>
<td>0.7 (0.0-3.4)</td>
<td></td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.4 (0.1-7.3)</td>
<td>0.5 (0.1-2.5)</td>
<td>0.4 (0.0-7.8)</td>
<td></td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>0.3 (0.0-1.5)</td>
<td>0.3 (0.0-8.7)</td>
<td>0.2 (0.0-3.5)</td>
<td></td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>0.1 (0.0-3.7)</td>
<td>0.1 (0.0-3.7)</td>
<td>0.1 (0.0-2.13)</td>
<td></td>
</tr>
<tr>
<td><strong>Fibrobacteres</strong></td>
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<td>0.0 (0.0-1.1)</td>
<td>0.0 (0.0-0.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Class (10/25)</strong>*</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Clostridia</td>
<td>35.8 (13.1-59.3)</td>
<td>41.1 (20.1-70.9)</td>
<td>38.6 (17.8-68.0)</td>
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<tr>
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<td>7.7 (0.4-15.0)</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>Gammaproteobacteria</td>
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</tr>
<tr>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Erysipelotrichales</td>
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<td>7.7 (0.4-15.0)</td>
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</tr>
<tr>
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</tr>
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<td>1.3 (0.2-11.9)</td>
<td></td>
</tr>
<tr>
<td>Coriobacteriales</td>
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</tr>
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<td>1.7 (0.0-6.4)</td>
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</tr>
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</tr>
<tr>
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</tr>
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<td>0.5 (0.0-5.3)</td>
<td></td>
</tr>
<tr>
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<td>0.3 (0.0-7.5)</td>
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<tr>
<td>Campylobacterales</td>
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<tr>
<td>Xanthomonadales</td>
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<td>0.4 (0.0-7.1)</td>
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<tr>
<td><strong>Family (20/99)</strong>*</td>
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<td></td>
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<tr>
<td>Peptostreptococcaceae</td>
<td>13.1 (4.1-32.5)</td>
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<td>12.8 (0.3-50.7)</td>
<td></td>
</tr>
<tr>
<td>Veillonellaceae</td>
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<td>14.3 (0.4-39.1)</td>
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<tr>
<td>Erysipelotrichaceae</td>
<td>16.8 (0.7-33.3)</td>
<td>10.6 (0.3-25.3)</td>
<td>7.7 (0.4-15.0)</td>
<td></td>
</tr>
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<td>Lachnospiraceae</td>
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<td>9.7 (6.2-23.2)</td>
<td>8.4 (3.8-17.0)</td>
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<td>3.4 (1.2-9.7)</td>
<td>5.9 (0.7-11.6)</td>
<td></td>
</tr>
<tr>
<td>Bifidobacteraceae</td>
<td>0.9 (0.4-29.0)</td>
<td>0.7 (0.1-24.9)</td>
<td>1.3 (0.2-11.9)</td>
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<tr>
<td>Clostridiales_unclassified</td>
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<td>1.3 (0.2-4.4)</td>
<td>1.5 (0.5-11.1)</td>
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<tr>
<td>Clostridiales_1</td>
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<td>0.6 (0.1-16.6)</td>
<td>1.4 (0.1-18.6)</td>
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</tr>
<tr>
<td>Coriobacteraceae</td>
<td>1.1 (0.3-4.8)</td>
<td>1.3 (0.2-5.1)</td>
<td>1.4 (0.2-7.4)</td>
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<tr>
<td>Enterococcaceae</td>
<td>1.9 (0.2-12.0)</td>
<td>2.3 (0.3-11.6)</td>
<td>1.5 (0.1-9.4)</td>
<td></td>
</tr>
</tbody>
</table>
The relative abundances across taxa presented here represent the fecal microbiota of healthy lean cats (LEAN, n=14) and obese cats (OBWL, n=17) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and obese cats after a 10-week weight loss period on the same food (OAWL, n=16). Cut-off for phyla and genera in the study were 1% and 0.1% respectively. However, only a portion of the most abundant members for all taxa (besides to phyla) are presented in the table. No significant differences were found between groups, using Wilcoxon Each Pair and Wilcoxon Signed-Rank, depending on the groups’ comparison, followed by the Benjamini-Hochberg adjustment ($P > 0.05$ for all comparisons). Cut-off for phyla and genera in the study were 1% and 0.1% respectively.

The numbers after each taxa represent the number of members in the specific taxa presented in the table out of the overall number of members in that taxa retrieved by the analyses.

<table>
<thead>
<tr>
<th>Genera (20/199)*</th>
<th>Succinivibrionaceae</th>
<th>Planococccaeae</th>
<th>Lactobacillaceae</th>
<th>Enterobacteriaceae</th>
<th>Pettcpocccaeae_1</th>
<th>Firmicutes_unclassified</th>
<th>Bacteroidaceae</th>
<th>Acidaminococcaceae</th>
<th>Verrucomicrobiaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 (0.0-9.3)</td>
<td>1.4 (0.3-8.0)</td>
<td>0.8 (0.0-6.3)</td>
<td>0.5 (0.1-4.6)</td>
<td>1.3 (0.0-3.5)</td>
<td>0.4 (0.1-4.2)</td>
<td>0.9 (0.2-2.7)</td>
<td>0.7 (0.1-2.3)</td>
<td>0.2 (0.1-1.6)</td>
</tr>
<tr>
<td></td>
<td>0.9 (0.0-6.8)</td>
<td>2.2 (0.6-8.3)</td>
<td>0.5 (0.1-11.2)</td>
<td>0.7 (0.1-10.6)</td>
<td>0.4 (0.0-5.3)</td>
<td>0.4 (0.1-1.8)</td>
<td>0.7 (0.2-3.1)</td>
<td>0.3 (0.0-2.5)</td>
<td>0.3 (0.0-7.5)</td>
</tr>
<tr>
<td></td>
<td>0.4 (0.0-32.0)</td>
<td>1.2 (0.1-8.3)</td>
<td>0.8 (0.1-4.6)</td>
<td>0.7 (0.0-4.5)</td>
<td>0.7 (0.0-4.1)</td>
<td>0.5 (0.0-5.3)</td>
<td>1.0 (0.1-2.8)</td>
<td>0.2 (0.0-3.8)</td>
<td>0.3 (0.1-0.9)</td>
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</tr>
</tbody>
</table>

Table legend: LEAN, lean cats; OBWL, obese cats before weight loss; OAWL, obese cats after weight loss.
Alpha and beta diversity indices

There were no significant differences in alpha diversity between the groups (all $P > 0.05$) (Figure 3.3). There were also no differences in community membership (Classical Jaccard index – unifrac $P = 0.17$; parsimony $P > 0.05$ for all comparisons) or population structure (Yue & Clayton – unifrac $P = 0.93$; parsimony $P > 0.05$ for all comparisons).

Figure 3.3: Comparison of bacterial population evenness (Shannoneven), diversity (Invsimpson) and richness (Chao1) in (A) healthy lean cats (LEAN, n=14) and (B) obese cats (OBWL, n=17) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and (C) obese cats after a 10-week weight loss period on the same food (OAWL, n=16).

Figure legend: LEAN, lean cats; OBWL, obese cats before weight loss; OAWL, obese cats after weight loss

No significant clustering per group was evidenced, and there was a strong intra-cat relationship (Figure 3.4). Principal coordinate analyses also demonstrated no apparent clustering based on community membership (Figure 3.5) or structure between groups (AMOVA $P > 0.05$ and
HOMOVA $P > 0.05$ for all comparisons). Random forest algorithm analysis indicated 72% error rate for assigning a cat to a group, and therefore, homogeneity across groups.

**Figure 3.4:** Dendrogram of the Classical Jaccard index representing the community membership of the fecal microbiota in healthy lean cats (L, n=14) and obese cats (OBWL, n=17) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and obese cats after a 10-week weight loss period on the same food (OAWL, n=16). Samples were coded with cat number, followed by time point (T5 = week 5, end of adaptation period, T15 = week 15, end of weight loss period). Each group is represented with a different colour (see legend).

*Figure legend:* LEAN, lean cats; OBWL, obese cats before weight loss; OAWL, obese cats after weight loss
Figure 3.5: Three dimensional principal coordinate analysis of population membership of the fecal microbiota of healthy lean cats (LEAN, n=14) and obese cats (OBWL, n=17) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and obese cats after a 10-week weight loss period on the same food (OAWL, n=16). Each group is represented with a different colour (see legend).

Figure legend: LEAN, lean cats; OBWL, obese cats before weight loss; OAWL, obese cats after weight loss

Linear discriminatory analysis effective size (LEfSe)

LEfSe analysis failed to identify enriched taxa between the OBWL to OAWL groups. However, a comparison of the LEAN to the OBWL group revealed 13 OTUs differentially enriched in the OBWL group (Table 3.3), six of which are members of the phylum Firmicutes, two belong to the phylum Proteobacteria and two to Actinobacteria. One OTU of the phylum Tenericutes was
found differentially enriched in the LEAN group. When comparing the LEAN to the OAWL group, the only difference was enrichment of *Pseudomonas* (phylum Proteobacteria).

**Table 3.3.** Significantly enriched operational taxonomic units (OTUs) from fecal microbiota of healthy lean and obese cats

<table>
<thead>
<tr>
<th>Comparison</th>
<th>LEAN</th>
<th>OBWL</th>
<th>OAWL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAN vs. OBWL</td>
<td>Tenericutes</td>
<td>Anaeroplasma</td>
<td>Firmicutes</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LEAN vs. OAWL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table legend:* OTUs, operational taxonomic units; LEAN, lean cats; OBWL, obese cats before weight loss; OAWL, obese cats after weight loss

These OTUs are from fecal microbiota of healthy lean cats (LEAN, n=14) and obese cats (OBWL, n=17) following a 4-week adaptation period with a veterinary therapeutic food intended for weight loss and adult maintenance, and obese cats after a 10-week weight loss period on the same food (OAWL, n=16). The presented OTUs have a linear discriminant analysis score > 2, and are organized in a descending manner.
3.3 DISCUSSION

Based on human and rodent studies, it was expected that microbial differences would occur between lean and obese cats and that these microbial differences would revert with weight loss in obese cats. However, bacterial fecal microbiota was only minimally affected in the current study.

In genetically obese mice, a 50% decrease in the relative abundance of Bacteroidetes compared to lean mice on the same high-polysaccharide diet was identified, along with a corresponding increase in Firmicutes and reduced diversity [18]. Similar changes were subsequently identified in adult obese humans [19]. In addition, weight loss, using either fat or carbohydrate-restricted diets, was also found to restore the original ratio between Firmicutes to Bacteroidetes, and this restoration was correlated to the % of weight loss [19]. More recent findings indicate that diet composition, and not caloric intake, had the greatest effect on the fecal microbiota of mice, when comparing lean mice to obese mice after weight loss, i.e. two groups of mice with similar weight and body composition [48]. These conclusions further corroborated other studies that showed that the diet effect (especially fat content) and initial body composition in weight reduction of obese mice and humans were the main contributors to the significant changes in the fecal microbiota [49-51]. Yet, a similar, broad effect was not noted in the current feline study as the relative abundance of taxa and biodiversity indices were not different between lean and obese cats, and in obese cats before and after weight loss. This could relate to various factors, such as differences between cats and other species, environmental influence, the pathophysiology of obesity in cats, the degree and duration of the initial obese status, the nature of the weight loss intervention, degree of cooperation of owners and the duration of the study period. Although in the current study, the weight loss period lasted only 10 weeks and the final fecal sample was
taken when weight loss was still occurring, the food used was the same for all groups/periods, excluding diet as a confounder.

Despite the lack of broad taxonomic changes through comparison of relative abundances, differences in the microbiotas were identified when comparing lean to obese before weight loss. Six of the thirteen OTUs that were significantly enriched in obese cats before weight loss belong to Firmicutes, a finding which is consistent with the over-representation of certain Firmicutes members in obese subjects in other species. [18]. This obesity-associated increase in Firmicutes in humans was thought to be related to increased ability of energy harvest/storage [18]. Additional research in genetic-related obesity, as well as in mice fed a high-fat diet, was not able to correlate microbiota composition and membership changes with energy-harvest markers (SCFA and energy content in feces). Therefore, it was suggested that the changes observed indicated a potential microbial adaptation to diet or obesity over time, and hence, a more complicated, and not completely understood, interaction between the microbiota and energy harvesting [52].

In the current study, Planococcaceae incertae sedis, a taxa that belongs to the Planococcaceae family, was found to be enriched in obese cats before weight loss compared to lean cats. Two unclassified members from this family were also found to be enriched in pigs that had a higher residual feed intake, meaning, they were more efficient in energy harvesting [53]. In obese twins, a lower proportion of Bacteroidetes versus Actinobacteria was found, although the abundance of Firmicutes was not different. The same study pin-pointed obesity-related genes from the microbiome, and found that 75% of the enriched obesity-related genes in obese twins originated
from Actinobacteria, whereas the residual 25% originated from Firmicutes. Many of these genes’ functions are related to carbohydrate, amino-acid or lipid metabolism [51].

Firmicutes, as well as Actinobacteria, were enriched in obese cats before weight loss compared to lean cats. In humans, obesity-specific Prevotellaceae, which belongs to the phylum Bacteroidetes, as well as some families that belong to the phylum Proteobacteria were enriched in morbidly obese individuals compared to individuals who went through gastric bypass or had normal weight. Enrichment of Prevotellaceae and Proteobacteria was also demonstrated in obese cats before weight loss compared to lean cats enrolled in the current study. The Prevotellaceae family has members that facilitate protein and carbohydrate fermentation, as well as acetate, H₂ and folate producers – hence, may have implications on energy metabolism and intestinal health [54, 55]. At last, in contrast to the current study, Verrucomicrobia was more abundant in normal-weight individuals or in individuals who had gastric bypass surgery, rather than in obese people [55]. Nonetheless, further understanding of the interaction between obesity and microbial features in the different species requires metagenomics research to further explore the obesity-related metabolic pathways and functional potential of the microbiome.

The presence of enriched taxa in obese cats prior to weight loss, compared to lean cats, as well as absence of differences in enriched taxa between obese cats before and after weight loss, may strengthen the findings that body composition and % weight loss, while excluding diet as a confounder, are the main drivers of changes in fecal microbiota [48-51]. Although BW and BCS significantly decreased with energy restriction, the weight loss observed may not have been sufficient to cause significant changes in the biodiversity indices, relative
abundance or LEfSe analysis in obese cat over the course of the weight loss plan. Still, fewer differences in enriched taxa were observed when comparing obese cats after weight loss to the lean cats. This might mean that with weight loss, the microbial population membership and composition are changing slowly, yet 10-week weight loss period is not enough to completely revert the obesity-related differences in enriched taxa to a lean body condition.

Challenges occurred with fecal collection during the current study. The owners were advised to maintain the fecal samples at 4°C until transfer to the clinic, yet, some owners kept them at ambient temperature. Still, it is important to note that a recent study investigated the effects of storage at ambient temperature on the feline fecal microbiota and confirmed the validity of using feline fecal samples that were kept at ambient temperature for up to 4 days for microbiota-related analyses [56].

In the current study, aside to the relatively short weight loss period that was not sufficient for obese cats to reach their ideal body weight, the sample size was relatively small for both the obese and the lean cats, and can be considered a limitation. However, since no similar study was previously conducted, the samples size was aligned with other feline studies, investigating the effects of GI inflammatory conditions on the microbial population [57, 58]. Nevertheless, it is possible that with a larger sample size, and a longer period of caloric restriction would promote greater weight loss, more distinct and consistent changes in the fecal microbiota would be reported.
3.4 CONCLUSIONS

Enriched taxa in LEfSe analysis were observed especially when comparing obese cats before weight loss to their lean counterparts. Nearly half of the enriched taxa in the obese group belonged to Firmicutes, which concurs with previously reported studies in humans and mice. This may be related to better energy-harvesting abilities in the host; however, a metagenomic approach is warranted to explore the functional potential of the feline obese fecal microbiome. The current study also demonstrated minimal effects of a 10-week standardized and successful weight loss plan on microbial biodiversity, while excluding diet as a confounder. This may imply that sudden short term energy restriction is not enough to revert microbiome changes. Nevertheless, more research is warranted allowing a longer weight loss period and a larger sample size.
3.5 REFERENCES


CHAPTER 4: THE IMPACT OF A STANDARDIZED WEIGHT LOSS PLAN ON INFLAMMATORY AND INTESTINAL HEALTH MARKERS IN OBESE CATS

ABSTRACT

Background: Feline obesity is a worldwide epidemic, with deleterious health outcomes. Research in humans and animals suggests that obesity causes a low-grade inflammatory response and negatively impacts intestinal health. Similar research in cats is limited. Therefore, the current study’s objective was to explore the systemic inflammatory response and serum markers of intestinal health in feline obesity before and after a standardized weight loss plan. During a 4-week adaptation period, 13 lean and 17 obese healthy client-owned cats were fed a veterinary therapeutic weight loss food (Hill’s Prescription Diets Metabolic Feline dry) at maintenance energy requirement (ER). After 4 weeks, lean cats completed the study and obese cats continued with a weight loss plan for 10 weeks during which obese cats were fed the same food, for individual weight loss ER. Body weight and body condition score were assessed biweekly. At the end of each period, food-logs recorded by owners were obtained and serum was analysed for intestinal health markers (i.e. cobalamin, folate) and serum inflammatory markers (i.e. S100A12, acute phase proteins (serum amyloid A, C-reactive protein) and 19 cytokines/chemokines).

Results: Body weight and body condition were higher in obese than in lean cats, and reduced following weight loss. Serum cobalamin concentrations were higher in lean compared to obese cats before and after weight loss. Serum folate concentrations were higher in obese cats, before weight loss compared to after. Increased serum concentrations of serum amyloid A, interleukin-1β, flt-3L, stromal-cell derived factor and stem-cell factor were observed in cats after weight loss.
compared to obese cats before weight loss, while higher concentrations of inflammatory markers were not observed in obese cats before weight loss compared to lean cats. Interleukin-2 was higher in lean compared to obese cats before weight loss.

**Conclusions**: Short-term weight loss initiates improvement in intestinal health. However, at the same time, solid evidence for a systemic inflammatory response associated with obesity was not observed in cats. On the contrary, cats might be more sensitive to develop a mild inflammatory response as a result of energy restriction and weight loss.

### 4.0 INTRODUCTION

Feline obesity, defined as an excess of body fat that is associated with increased risk for negative health implications [1-4], is a universal epidemic. Prevalence estimates in Europe, the United States and New Zealand, indicate that 11.5% – 63% of cats are overweight or obese [5-8]. Obesity in rodents, humans, and to an extent, companion animals, is reported to induce a low-grade inflammatory response, termed “metainflammation” (i.e. metabolically triggered inflammation), generating an innate immune system response [9-12]. Obesity instigates secretion of pro-inflammatory adipokines in adipose tissue, whilst weight loss usually leads to a reduction in their secretion, and an improvement in metabolic state, as was shown in humans [13, 14].

Obesity-associated pro-inflammatory mediators that were investigated in pets include cytokines (i.e. interleukin (IL)-1, IL-6, IL-18 tumor necrosis factor (TNF)-α) and positive acute phase proteins (i.e. haptoglobin, serum amyloid A (SAA), and C-reactive protein (CRP)), [11, 15-17]. S100A12 is considered a novel marker/mediator in the obesity-associated inflammation in
humans [18]. The relationship between S100A12 and obesity in cats is still undefined. This metainflammation is one mechanism thought to trigger a variety of health consequences in cats, such as diabetes mellitus and insulin resistance, which impact life span and quality of life [19-22]. In genetically obese mice, this inflammatory response was also shown to be related to increased intestinal permeability and reduce absorptive function, predisposing to toxin infiltration and endotoxemia [12]. Obesity may impact intestinal permeability through deviation from normal microbial structure (i.e. bacterial dysbiosis), which could cause an increase in lipopolysaccharide (LPS)-producing bacteria and LPS production [23]. The direct assessment of intestinal permeability via tight junction protein expression, for example, requires harvesting of intestinal tissue [12]. However, measuring serum cobalamin and folate can be used for non-invasive assessment of intestinal health in humans and dogs [24, 25]. In cats, gastrointestinal bacterial dysbiosis is suggested when reduced serum concentrations of cobalamin are observed [24].

The association between metainflammation and intestinal permeability is not yet clear in cats. Moreover, findings regarding the effects of obesity and weight loss on pro-inflammatory mediators are inconsistent [16, 17, 26]. On one hand, adipokine mRNA expression in adipose tissue was found to be associated with obesity in chronically obese cats [10]. On the other hand, although an increase in serum adipokine concentrations was presumed, 60% weight gain in lean cats did not alter systemic concentrations of pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα [17]. It is important to note that only a few cytokines were assessed in feline-obesity related studies, using enzyme linked immunoassays (ELISAs). Serum concentrations of acute phase proteins, including SAA and haptoglobin, were also not different between obese cats
before and after weight loss, however, this study did not assess differences between lean and obese cats [16]. Altogether, the degree of obesity-related inflammatory response and its corresponding health repercussions in cats are not clear.

Hence, the current study aimed to investigate the effect of feline adiposity and weight loss on pro-inflammatory markers including the acute phase proteins SAA and CRP, 19 cytokines/chemokines and S100A12, as well as effects on initial diagnostics of intestinal health, folate and cobalamine. It was hypothesized that an obesity-associated low-grade inflammatory response would be observed, and be linked to compromised gastrointestinal health, while weight loss would aid in correcting this metainflammation.

4.1 MATERIAL AND METHODS

Experimental design

Data was collected from thirteen lean (body condition score (BCS) 4-5/9 [27], 9 males and 4 females) and 17 obese (BCS ≥ 8/9 [27], 11 males and 6 females) cats enrolled in the study described in Chapter 3. As mentioned previously, all subjects were considered healthy based on a medical-history and dietary questionnaire recorded by owners, physical exam, as well as no abnormalities on a complete cell blood count and serum biochemistry panel. All cats were desexed, indoor, client-owned, between 2-9 years of age and from the Guelph, Ontario region. The study took place between May 2015 and December 2016. A figure of the study’s experimental design, which includes the combined study design described in Chapter 3 and in the current chapter, is available in Appendix 3a.
The study was conducted at the Ontario Veterinary College, University of Guelph. The experimental design was approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #2496).

**Adaptation Period**

At the start of the study, each cat’s BW, BCS, muscle condition score (MCS), body mass index (BMI) and girth) were assessed [20, 27-32]. Assessments of BCS, MCS, girth and BMI are described in Chapter 3. Morphometry for determination of body fat index (BFI) was conducted only in obese cats [30, 31]. All measurements were performed by the same investigator (MT) throughout the study to reduce variability. Next, all cats were started on a veterinary therapeutic food (Hill’s Prescription Diets Metabolic Feline dry Hill’s Pet Nutrition. Topeka, Kansas, USA) intended for weight loss and weight maintenance (Please see Table 3.1, Chapter 3). A 1-week transition period from the cats’ original diet to the study food was followed by a 4-weeks period of diet adaptation, during which cats received the study food for 100% of their daily ration. Energy requirements for maintenance of each individual cat in both the lean (LEAN) and obese groups (obese before weight loss (OBWL)) were determined in accordance with the National Research Council (NRC) (LEAN - 100 Kcal/kg\(^{0.67}\); OBWL - 130 Kcal/kg\(^{0.4}\)), based on ideal body weight [33]. Body weight and BCS assessment were conducted 3 weeks after the start of the study. At the end of the adaptation period, BW, BCS, BMI, girth, and for obese cats only - BFI, were assessed again and a food-log in which owners entered daily food intake data, was obtained. A blood sample was drawn from the jugular or cephalic vein for analysis of serum concentrations of inflammatory markers, i.e. SAA, CRP, S100A12 and 19
cytokines/chemokines, as well as intestinal health markers, i.e. folate, cobalamine. Study participation of lean cats was concluded after this adaptation period, while obese cats were followed through a 10-week weight loss period.

**Weight Loss Period**

As described in Chapter 3, obese cats continued with an individually customized weight loss plan with the same food for 10 weeks, once the adaptation period concluded. Each cat’s daily ER for weight loss was calculated based on the NRC recommendations (0.6 × 130 Kcal/kg\(^{0.4}\)) [33]. Daily food intake was recorded by the cat owners, and BW and BCS were evaluated bi-weekly to monitor for safe weight loss, with a target weekly BW loss between 0.5-2% of the weight assessed at the end of the adaptation period [33, 34]. Body weight and body composition assessment was repeated after 10 weeks of weight loss. At that time, food logs were obtained from the owners and blood samples were collected for the obese cats after weight loss (OAWL).

**Laboratory Analyses**

Blood collected for analyses of serum concentrations of inflammatory and intestinal health markers was centrifuged (Sorvell Legend RT Centrifuge, Fisher Scientific, Ottawa, Canada) for 2 h following refrigeration at 4°C at 3000 rpm for 10 mins. The serum retrieved was aliquoted and frozen at -80°C. Frozen serum samples were batched from both the lean and obese groups, and were analyzed together. Assays for feline SAA (Cat Serum Amyloid A (SAA), Immunology Consultants Laboratory, Inc., Portland, USA) and CRP (Feline C-Reactive Protein (CRP), Immunology Consultants Laboratory, Inc) were two-site ELISA assays and were performed in accordance to manufacturer instructions. The results were read using a plate reader (Epoch
Microplate Spectrophotometer. BioTek Instruments, Inc., Vermont, USA) and the mean of the duplicates was used for further analysis. Serum concentrations of cobalamin, folate and S100A12 were analyzed at the Gastrointestinal Laboratory, Texas A&M University (College Station, Texas). Serum cobalamin and folate were measured using a chemiluminescent enzyme immunoassay (Immulite 2000 Immunoassay System. Siemens Healthcare GMBH, Erlangen, Germany), in which an automated alkaline denaturation procedure was involved. Serum S100A12 was assessed as described in Bridges et al. (2014) [35]. Serum concentrations of 19 inflammatory cytokines and chemokines (Table 4.1) were evaluated with a commercially available feline-specific multiplex cytokine/chemokine magnetic bead panel (MILLIPLEX MAP Feline Cytokine/Chemokine Magnetic Bead Panel (Cat# FCYTMAG-20K-PMX)). Cytokines and chemokines were analyzed using a 96-well plate assay. All serum and quality controls samples were run in duplicates. The plates were read using an appropriate plate reader and software (Bio-Rad Bio-Plex 200 System. Bio-Rad, Hercules, California, USA; Bio-Plex Manager Software Version 6.1.1.). The quality control samples, bead counts, and standard curves were evaluated based on manufacturer recommendations. Coefficient of variation (CVs) for the results were assessed for each set of duplicates. If CV per duplicate was $< 20\%$, the results were deemed acceptable and the mean of the duplicates was used for further analysis.[36] However, for CV’s $\geq 20\%$, individual results were assessed [37]. If the individual results of the duplicates were not in agreement, results from that sample were omitted.

**Statistical Analyses**

Statistical analyses were performed using SAS v9.3 (SAS Institute Inc., Cary, North Carolina, USA). The experimental model was not symmetric, since the parameters examined in the lean
group were only assessed once, following the adaptation period, whereas the same parameters were assessed over two time points in the obese group (after adaptation/before weight loss, after weight loss). Therefore, following a Shapiro-Wilk test for normality, a student’s T-test or a Wilcoxon-Mann-Whitney test were performed when parameters were compared between the LEAN group to the OBWL or OAWL. Paired T-test or Wilcoxon Signed-Rank test were performed when comparing parameters between OBWL to OAWL groups. For all comparisons, significance was set at $P < 0.05$. Normally distributed data are expressed as mean ± standard error (SE) or as mean of the back transformed values (lower limit (LL)-upper limit (UL)). Data that did not have a normal distribution are presented as median ± interquartile range (IQR).

4.2 RESULTS

Thirteen lean and 17 obese cats were enrolled in the study. One obese cat did not complete the study since compliance with the weight loss plan was challenging for the owner, and the cat did not lose body weight. This cat was included in the OBWL group, but excluded from the OAWL group. For the cytokine/chemokine analyses in the obese groups, only 16 of 17 samples from the OBWL groups, and 14 of 16 samples from the OAWL group were included for statistical analyses, due to high CV per duplicate for three samples, and a possible technical error during the multiplex analyses.

All cats tolerated the food well and had no signs of relevant comorbidities. Cats did not refuse to eat the food (mean energy consumption ± SE within groups – LEAN: 217.6 ± 9.9 kcal, OBWL: 217.8 ± 5.9 kcal, OAWL: 134.6 ± 2.3 kcal).
**Body Weight and Body Composition**

Body weight remained stable in all the cats during the adaptation period. Results for changes in BW, BCS, BMI, girth between groups as well as data regarding average weight loss and rate can be found in Chapter 3. Body fat index was higher in the OAWL cats (35 ± 0.25) compared to BFI in OBWL cats (48 ± 0.14), \( P < 0.0001 \).

**Inflammatory Markers**

Interleukin-2 was higher in the LEAN group compared to the OBWL group (\( P = 0.0407 \)). No other differences in serum cytokine/chemokine and S100A12 concentrations were observed between the LEAN cats and OBWL cats. Stem cell factor was lower in the LEAN group compared to the OAWL group (\( P = 0.0457 \)), but other than that, there were no differences in serum cytokine/chemokine and S100A12 concentrations between the LEAN and OAWL cats. All other differences were observed between the OBWL group and the OAWL group; i.e. serum KC concentrations were higher in OBWL cats (\( P = 0.0334 \)), while serum concentrations of IL-1β, Flt-3L, SAA, SCF, SDF were higher in the OAWL group (\( P = 0.0297, P = 0.0498, P = 0.0004, P = 0.0022, P = 0.0019 \), respectively). All remaining serum cytokines/chemokines and S100A12 concentrations were not different between OBWL and OAWL. Also no differences were observed among any of the groups for serum concentrations of the positive acute phase proteins, SAA and CRP (\( P > 0.05 \)) ([Table 4.1](#)).
Table 4.1. Serum inflammatory and intestinal health markers in lean (LEAN) and obese cats (OBWL) following a 4-week adaptation period and in obese cats after a 10-week weight loss period (OAWL) with the same food.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>LEAN Mean ± SE / (LL-UL)</th>
<th>OBWL Mean ± SE / (LL-UL)</th>
<th>OAWL Mean ± SE / (LL-UL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flt-3L (pg/mL)</td>
<td>109.2 ± 7.5</td>
<td>86.9 ± 8.6 †</td>
<td>108.7 ± 10.6 †</td>
</tr>
<tr>
<td>IFN-γ (pg/mL)</td>
<td>65.1 (34.3-123.2)</td>
<td>48.7 (27.4-86.6)</td>
<td>54.0 (29.3-99.6)</td>
</tr>
<tr>
<td>IL-13 (pg/mL)</td>
<td>16.7 (8.9-31.5)</td>
<td>12.2 (6.9-21.6)</td>
<td>15.1 (8.3-27.6)</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>115.4 (49.2-270.7)</td>
<td>102.4 (47.5-220.9)</td>
<td>99.7 (47.7-222.1)</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>62.3 (34.1-113.9)</td>
<td>38.9 (22.6-67.1)</td>
<td>44.1 (24.9-78.2)</td>
</tr>
<tr>
<td>SCF (pg/mL)</td>
<td>154.6 (119.2-200.4) ‡</td>
<td>159.9 (126.5-202.0) †</td>
<td>240.7 (188.7-307.0) ‡‡</td>
</tr>
<tr>
<td>SDF (pg/mL)</td>
<td>1114.5 ± 521.1</td>
<td>1058.8 ± 161.8</td>
<td>1713.7 ± 287.0</td>
</tr>
<tr>
<td>IL-18 (pg/mL)</td>
<td>46.8 (25.2-86.8)</td>
<td>26.4 (15.1-46.0)</td>
<td>36.4 (20.3-65.2)</td>
</tr>
<tr>
<td>SAA (µg/mL)</td>
<td>0.01 (4.1-3.4-3.3)</td>
<td>4-3 (1.4-3.1-11.6) †</td>
<td>0.02 (8.6-3-69.8) ‡†</td>
</tr>
<tr>
<td>GM-CSF (pg/mL)</td>
<td>4.2 ± 9.5</td>
<td>4.2 ± 0.0</td>
<td>4.2 ± 0.0</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>9.9 ± 1.4</td>
<td>9.9 ± 0.3 †</td>
<td>9.9 ± 0.0 †</td>
</tr>
<tr>
<td>IL-2 (pg/mL)</td>
<td>8.3 ± 12.9 †</td>
<td>2.3 ± 0.0</td>
<td>2.8 ± 0.0</td>
</tr>
<tr>
<td>PDGF-BB (pg/mL)</td>
<td>1564.3 ± 1174.2</td>
<td>1615.3 ± 984.8</td>
<td>872.0 ± 2819</td>
</tr>
<tr>
<td>IL-12 pg/mL</td>
<td>245.0 ± 183.8</td>
<td>256.9 ± 152.6</td>
<td>315.0 ± 134.85</td>
</tr>
<tr>
<td>Fas (13) (pg/mL)</td>
<td>2.8 ± 1.0</td>
<td>2.8 ± 0.0</td>
<td>2.8 ± 0.0</td>
</tr>
<tr>
<td>IL-8 (pg/mL)</td>
<td>32.1 ± 10.0</td>
<td>26.7 ± 11.3</td>
<td>34.9 ± 17.7</td>
</tr>
<tr>
<td>KC (pg/mL)</td>
<td>2.4 ± 3.5</td>
<td>4.7 ± 13.7 †</td>
<td>3.0 ± 5.6 †</td>
</tr>
<tr>
<td>RANTES (pg/mL)</td>
<td>35.7 ± 33.3</td>
<td>45.4 ± 21.5</td>
<td>54.0 ± 30.1</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>666.9 ± 1464.7</td>
<td>115.9 ± 754.2</td>
<td>115.9 ± 0.0</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>4.9 ± 3.0</td>
<td>4.9 ± 0.0</td>
<td>4.9 ± 0.0</td>
</tr>
<tr>
<td>S100A12 (ng/mL)</td>
<td>20.5 ± 17.6</td>
<td>27.1 ± 20.4</td>
<td>25.9 ± 20.3</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>168.7 ± 60.9</td>
<td>211.0 ± 143.6</td>
<td>199.6 ± 105.2</td>
</tr>
<tr>
<td><strong>Intestinal Health Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate (ng/mL)</td>
<td>18.8 (16.2-21.8)</td>
<td>20.1 (17.7-22.9) †</td>
<td>17.4 (15.3-19.9) †</td>
</tr>
<tr>
<td>Cobalamin (pg/mL)</td>
<td>913 ± 44 ‡‡</td>
<td>882.0 ± 38.0 ‡</td>
<td>879.0 ± 76.7 ‡</td>
</tr>
</tbody>
</table>

* † ‡ ‡‡ †† Refers to statistical significance levels.
Abbreviations: OBWL, obese before weight loss; OAWL, obese after weight loss; SE, standard error; LL-UL, lower limit – upper limit; IQR, interquartile range; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon γ; IL, interleukin; KC, keratinocyte chemoattractant; MCP-1, monocyte chemoattractant protein 1; PDGF-BB, platelet-derived growth factor BB; RANTES (i.e. CCL5), C-C motif chemokine ligand 5; SCF, stem cell factor (SCF); SDF-1, stromal-cell derived factor; TNFα, tumor necrosis factor α

Table legend: *Sample size for the 19 cytokines and chemokines: LEAN, n = 13; OBWL, n = 16; OAWL, n = 14.
Sample size for S100A12, CRP, SAA, cobalamin and folate: LEAN, n = 13; OBWL, n = 17; OAWL, n = 16;
* Significant difference between LEAN to OBWL (P < 0.05)
† Significant difference between OBWL to OAWL (P < 0.05)
‡ Significant difference between LEAN to OAWL (P < 0.05)

Intestinal Health Markers

Serum cobalamin concentrations were higher in LEAN cats compared to OBWL cats (P = 0.0099) and were also higher in the LEAN group compared to the OAWL group (P = 0.0215).

No differences were observed in serum concentrations of cobalamin between OBWL and OAWL cats. Serum folate concentrations were significantly higher in the OBWL group compared to OAWL group (P = 0.0030). No other differences were observed for serum folate concentrations between groups (Table 4.1).

4.3 DISCUSSION

In other species, such as rodents and humans, the association between obesity [9, 13] and weight loss [14] and the inflammatory response is well established. However, previous research in cats suggested that obesity and weight loss do not necessarily impact systemic concentrations of the APPs, SAA and haptoglobin; and the pro-inflammatory cytokines, IL-1, TNFα and IL-6 [16, 17].
The current study also failed to reveal a systemic inflammatory response in obese cats as higher serum concentrations of APPs, cytokines, and chemokines were not observed in OBWL compared to lean cats. Despite no increase of systemic inflammatory mediators with obesity, their concentration and expression could still be increased in adipose tissue. Indeed, Vandeveld et al. (2013) observed location-dependent alterations of adipokine gene expression in adipose tissue of obese versus lean cats in favour of pro-inflammatory cytokines and chemokines [10, 17]. Unexpectedly, serum IL-2 concentrations were higher in lean cats compared to OBWL, although IL-2 is considered a pro-inflammatory immunomodulator. A study of systemic cytokines in obese prepubertal children found similar higher serum concentrations of IL-2 in lean children compared to obese children [38]. In contrast to studies in humans and mice [39-41], serum S100A12 concentrations did not differ between groups in the current study, which deserves further investigation regarding its importance in the pathogenesis of obesity, or the suggested metainflammation.

The effect of weight loss on serum APPs, cytokines and chemokines concentrations was more profound than the effect of obesity. Serum concentrations of KC were decreased, while serum concentrations of SCF, IL-1β, Flt-3L, SDF and SAA were increased in obese cats after weight loss. Keratinocyte chemoattractant, a cytokine secreted by adipose tissue, is highly involved in establishing the inflammatory response in adipose tissue in humans, by promoting macrophage accumulation and activation [42]. Therefore, higher serum KC concentrations in obese cats before weight loss compared to after weight loss, as seen in the current study, are expected. SCF, although pro-inflammatory, participates in the migration of haematopoietic progenitor cells to the site of chronic inflammation and was found to negatively correlate with red cell
distribution-width, an inflammatory marker which indicates the presence of metabolic syndrome in humans. Hence, it was suggested that SCF negatively correlates to obesity [43]. This may explain the higher serum SCF concentrations in the OAWL group compared to the OBWL group. Serum SCF concentrations were also higher in OAWL group compared to the lean group, but no differences were observed between lean and OBWL cats.

This may suggest that energy restriction and weight loss in cats are a greater stimulus for inflammation than obesity. Drastic energy restriction in obese cats may instigate hepatic lipidosis [44, 45], a disease that resembles to non-alcoholic fatty liver disease (NAFLD) in humans. In humans, NAFLD is often accompanied by metabolic changes, as well as inflammatory changes [45]. Also drastic weight loss following bariatric surgery in humans, can trigger liver damage and NAFLD [46], causing an increase in cytokines, such as IL-6, IL-8, TNF-α, IL-1β, Flt-3L, SDF, and APPs (e.g. SAA, CRP) [46-49]. In the current study, although weight loss did not affect serum concentrations of IL-6, IL-8, and TNF-α, serum concentrations of IL-1β, Flt-3L, SDF and SAA (in addition to the previously mentioned SCF) were higher in obese cats after weight loss, compared to before weight loss. Interleukin-1β is one of the main cytokines secreted in liver disease in humans, and is known to instigate the secretion of APPs [50], for example SAA. Serum amyloid A has an important role as a chemoattractant, as well as in down-regulating the inflammatory process [51]. While Tvarijonaviciute et al. (2012) failed to reveal any effect of weight loss on serum SAA concentrations in cats [16], an increase in serum SAA concentrations was observed in obese humans, with a reduction following weight loss [13, 52]. This is in contrast with the current study, as serum SAA concentrations were elevated after weight loss. Elevated concentrations of SAA were also suggested in hepatic lipidosis in cows...
[53], however, this has not been previously reported in cats. The chemokine, SDF, targets liver sinusoidal endothelial cells and hepatic stellate cells [54] and plays a role in the inflammatory response in steatohepatitis and fibrosis in mice [55]. Flt-3L, a haematopoietic pro-inflammatory cytokine, with similar structure as SCF, is pertinent for the development of dendritic cells and for the stimulation of the inflammatory response [56]. Interestingly, an in vitro study in mice showed that administration of IL-2 had a protective effect against this Flt-3L effect [57]. The current study, examined these pro-inflammatory markers for the first time in cats in relation to obesity and energy restriction. To the author’s knowledge, no research is currently available with regard to hepatic lipidosis.

It is notable that the authors aimed to avoid drastic energy restriction in order to prevent hepatic lipidosis from occurring. During the weight loss plan cats were fed 60% of their maintenance ER, as currently recommended for weight loss in veterinary practice [33, 58]. This means a 40% energy restriction of maintenance ER. This is lower than the degree of energy restriction reported in literature to induce feline hepatic lipidosis, which was identified to be between 50% and 75% energy restriction of maintenance ER [45, 59, 60]. Individual daily energy intake was also adjusted based on the rate of weight loss. A safe weekly weight loss rate between 0.5-2% of the BW assessed at the end of the adaptation period was the goal in the study, as currently recommended for weight loss plans in veterinary practice [33, 34]. Moreover, the resulted average weekly weight loss % was within this range. As no clinical signs of malaise and inappetance were observed in the cats or reported by the owners, no additional diagnostic tests (e.g. serum biochemistry, abdominal ultrasound, liver histology and/or cytology) were performed at the end of each period to verify liver health. The increase in pro-inflammatory cytokines and SAA
observed may suggest an inflammatory process in the cats following a short period of energy restriction and weight loss, and may imply a sensitivity to develop hepatic lipidosis.

In Chapter 3 it was demonstrated that obese cats before weight loss had specific enriched microbiota taxa compared to the lean group. These taxa belonged mainly to Firmicutes, but also included a representor of the Prevotellaceae family, which contains members with increased folate production abilities. Interesting findings were also observed in the current study when examining the effect of feline obesity and weight loss on intestinal health markers. Serum cobalamin concentrations were lower in both obese cat groups compared to lean cats. Also, serum folate concentrations were lower in obese cat after weight loss compared to before weight loss. Both were within the laboratory’s reference range in all cats, although for folate, concentrations were on the high end of the range for obese cats after weight loss. Serum Cobalamin and folate are indicators for gastrointestinal disease in humans and dogs [24, 25]. Reduced serum cobalamin concentrations are suggestive of increased intestinal permeability in humans [61]. In addition, obesity in humans is associated with a risk for cobalamin deficiency [62]. Similar conclusive evidence does not exist in cats; although, it is suggested that due to the positive therapeutic outcome of intestinal disease when cobalamin concentrations are replenished, a similar association with intestinal health can be made for both cats and dogs [25]. In general, both can be affiliated with healthy intestinal absorptive functionality [23-25]. However, unlike direct assessment tests for intestinal permeability, such as the assessment of tight junction proteins expression, serum cobalamin and folate concentrations can be altered due to numerous factors (e.g. level in food) [25]. Nonetheless, as long as such factors can be excluded, assessment of cobalamin and folate can be used as a “first-step approach”, due to non-
invasive and ease of measuring, availability, and low-cost. A recent feline study did not demonstrate an association between BW and serum cobalamin concentrations [63]. Despite this study, and considering an association between serum cobalamin and folate and intestinal health and absorption, the current findings, whilst still being within normal reference range, may suggest better intestinal properties in lean compared to obese cats and improvement of intestinal health with weight loss.

The small sample size, as well as the relatively short weight loss period, through which cats did not reach their targeted ideal BW yet, pose a limitation for this study. Also, the study design assessed only one time point in lean cats compared to two time points in obese cats. A bigger sample size, and a longer weight loss period, with additional check points for all groups throughout, for all inflammatory and intestinal health markers, would have been beneficial to better understand the relevance and importance of obesity-associated inflammatory response in cats, as well as its health implications. Also, aiming for different weight loss rates maybe be beneficial to assess the impact of the rate of weight loss on the mentioned inflammatory and intestinal health markers.

4.4 CONCLUSIONS

The current study failed to provide evidence for a systemic obesity-associated inflammatory response in cats. However, energy restriction and weight loss seem to trigger a systemic inflammation in obese cats during weight loss. This might be linked to feline hepatic lipidosis, which warrants further investigation. Moreover, assessment of serum cobalamin and folate,
although all concentrations were within normal reference ranges, potentially suggest improved intestinal health in lean compared to obese cats, which could be achieved with weight loss in obese cats.
4.5 REFERENCES


CHAPTER 5: DISCUSSION AND FUTURE RESEARCH

5.0 DISCUSSION

Human and rodent studies indicate that obesity is associated with a low-grade metabolic inflammatory response (i.e. “metainflammation”) [1], and is associated with the secretion of pro-inflammatory adipokines from the adipose tissue and activated macrophages [2]. In some studies, obesity was also shown to be associated with changes in fecal microbial population membership and structure [3, 4]. Weight loss in humans was shown to revert adipokine secretion, as well as alter microbiota membership and composition to better match lean individuals [4, 5].

The aim of this research project was to investigate the association between feline obesity and weight loss and the fecal microbiota, inflammation and intestinal health markers. However, an initial challenge was appropriate storage of the fecal samples collected by the owners, since previous work in companion animals only examined the effects of refrigeration, and not ambient temperature, on the fecal microbiota.

The results of Chapter 2 enabled the use of feline fecal samples kept at ambient temperature. The results of Chapter 3 and 4 have substantiated the need for additional research into the systemic or local role of obesity as a low-grade inflammatory state in cats, the potential inflammatory effects of weight loss and their causes, as well as the effects of feline obesity and weight loss on the fecal microbiota and intestinal health. The conclusions from this research project were noted at the end of each chapter.
In summary, in Chapter 3, no broad differences were observed after both the adaptation and weight loss periods between lean and both obese groups, or when comparing biodiversity indices and microbial relative abundance between the obese groups. Yet, some significant differences were noted by linear discriminatory analysis effective size (LEfSe) analysis. Interestingly, members of the phylum Firmicutes were over-represented in obese cats before weight loss compared to lean cats. While caution must be taken to avoid drawing excessive conclusions from phylum-level changes, a high ratio of Firmicutes to Bacteroidetes has been observed in previous obese human and mice studies, with reversion of this ratio following weight loss [3, 4].

Therefore, while broad changes in the microbiota were not evident, there still may be differences present in lean and obese cats that could be associated with weight status. Also, it was previously shown that a significant percentage of obesity-associated genes originate from different taxa of the phylum Firmicutes [6]. Of additional interest was enrichment in Planococcaceae *incertae sedis* (family Planococcaceae), Prevotellaceae and Proteobacteria members in obese cats before weight loss. Enrichment in Planococcaceae *incertae sedis* was associated with increased energy harvesting in pigs [7], whilst enrichment in the two latter taxa members was demonstrated in morbidly obese humans compared to individuals who went through bariatric surgery or had a normal weight [8]. Interestingly, the Prevotellaceae family has members that enable H2, acetate and folate production [9], suggesting an association between the enrichment in members of this family in obese cats and the significantly higher serum folate levels noted in the same group of cats (although still within normal reference range), as described in Chapter 4.

In contrast to studies in humans and mice [4, 10], there were no significant differences between obese cats before and after weight loss. It is possible that this was because of the relatively short
timeframe, as weight loss continued for merely 10 weeks, and obese cats were still far from their ideal body weight and body condition score (BCS). More specifically, they still had an obese BCS, body mass index and body fat index %. One should also consider that the initial degree of obesity and how long the cat has been obese may impact microbial changes. It could be that chronic obesity, to varying levels, compared to acute weight gain, will have a different effect on the microbiota. Potentially, the microbiota of chronically obese cats may have been more adapted to the obese state, have reached some sort of a steady state, and hence, were less affected by the 10-week weight loss period. A post study power analysis on some of the inflammatory markers that did not differ significantly was conducted, and showed that a larger sample size (around 80 cats) was required to achieve significance. Therefore, longer durations of study or larger sample sizes may be required to evaluate the impact of weight loss on the microbiota, as changes in the microbiota may be gradual and a 10-week weight loss period may not be sufficient.

Chapter 4 describes the associations of obesity and weight loss with changes in inflammatory and intestinal health markers. Obesity had no observed effect on the inflammatory state in obese compared to lean cats. Also previous feline studies showed mainly differences in inflammatory markers at the adipose tissue levels [11], rather than systemic differences [12, 13]. Harvesting adipose tissue samples is more invasive, and hence, was not performed in the current study.

Despite the absence of difference between lean and obese cats, there was an increase in keratinocyte chemoattractant (KC) in the obese cats before weight loss, compared to obese cats after weight loss. This finding may be relevant as in humans this cytokine is secreted by the
adipose tissue, and is very involved in prompting and substantiating the inflammatory response in the adipose tissue [14]. Serum KC concentrations were not significantly higher than the concentrations in the lean group though, which may imply to a difference in the meaning of its activity in cats, or simply may be related to the relatively small sample size of cats.

An interesting result of this study was the increase in serum concentrations of several pro-inflammatory cytokines (such as interleukin β-1 and somatic cell-derived factor) and an acute phase protein (serum amyloid A) in the obese cats after weight loss, compared to before weight loss. These findings were not expected, based on similar human and mice studies. These results suggest that an underlying inflammatory response associated with food restriction and weight loss could be present. Hepatic-derived inflammation is known to occur in humans with non-alcoholic fatty liver disease, and can be associated with drastic weight loss that happens under circumstances such as bariatric surgery; however, to our knowledge, the inflammatory state in cats with hepatic lipidosis has not been described so far [15]. Still, cats did not show overt clinical signs of hepatic lipidosis. As an extrapolation of these results, it is suggested that a broader population of cats may exhibit subclinical inflammation during weight loss. The relevance of this is unclear but bears investigation.

Serum cobalamin and folate are considered as indicators of intestinal health, and potentially intestinal absorption, as long as additional influencers of their serum concentration (e.g. dietary content) are excluded [16]. Serum cobalamin concentrations were significantly higher in lean cats compared to both obese groups. In humans, reduced serum cobalamin concentrations are associated with reduced intestinal integrity [17], and although this association is not fully proven
in companion animals, a similar connection has been suggested [16]. In a similar fashion, when small intestinal bacterial overgrowth (SIBO) occurs, presence of folate-producing bacteria can increase, which will elevate serum folate concentrations. In cats, SIBO is quite rare; however, we did see higher serum concentrations of folate (although still within reference ranges) in obese cats before weight loss, compared to the same cats after weight loss, suggesting that a degree of small intestinal dysbiosis might have been present in obese cats, and that this was ameliorated with weight loss. Nonetheless, since fecal samples were used to assess the microbiota, determination of the exact anatomical area (e.g. regions of small intestine vs. large intestine etc.) of the dysbiosis was not possible. Despite the difficulty assessing proximal intestinal tract changes through evaluation of feces, one of the enriched taxa in the obese cats before weight loss was Prevotellaceae (phylum Bacteroidetes). Members from this taxa have been shown to be good predictors for amount of estimated folate-synthesis genes in humans [9], something that might account for the higher serum folate concentrations in obese cats before weight loss. Combined with the finding of lower serum cobalamin concentrations, it may suggest a more jeopardized intestinal health in obese cats before weight loss.

In conclusion, the results obtained from the initial research project (Chapter 2) facilitated the use of fecal samples maintained at ambient temperature from the main research project. The results acquired from the main project (Chapter 3 and 4), indicate that obesity and a short weight loss period in cats have implications on metabolic outcomes and therefore on intestinal health, but for more conclusive results, further microbiota, metagenomics and metabolomics research is warranted, with a longer weight loss period, preferably until ideal body weight is achieved, and a larger sample size of cats.
5.1 CLINICAL IMPLICATIONS AND LIMITATIONS

The findings of this research project may have clinical implications. It should be kept in mind that the observed mild changes in microbiota in obese cats before weight loss can impact metabolic processes in the host, depending on the gene potential of the bacteria. “Obese microbiome” is suggested to exist, based on the findings of enriched taxa in obese cats before weight loss compared to lean cats, and based on evidence for these taxa functionality from previous studies. The microbiota have different metabolic pathways that manifest to better utilize available nutrients and/or to produce metabolites depending on availability of nutrients, metabolic processes in the host, etc [18-20]. It could be that in cats microbiota changes are more at metagenome and metabolome level, rather than in relative abundance and biodiversity indices. Therefore, once further research is conducted to assess the metagenomics and metabolomics of enriched taxa in the obese cats (e.g. obesity-associated genes and metabolic pathways respectively), it could be that a metagenomics/microbiota-manipulation approach should be taken to assist in mitigation/prevention of metabolic complications of obesity and weight loss, as well as to promote a safer weight loss in obese cats.

In addition to these clinical implications, once additional research is performed on a larger sample size and until weight loss is concluded, more information will be unveiled regarding the metabolic repercussions of obesity, the connection to intestinal health, and regarding a weight loss-associated inflammation. The results should have us reconsider the existence of a systemic “metainflammation” in obese cats, and to reflect on whether the existence of such inflammation
is more local at the adipose tissue level, and therefore, adipokine secretion occurs in a more paracrine way. This will potentially have implications on supplementary treatment routes (e.g. administration of medications). The implications of the average weight loss rate achieved in the current study and the diet used, should be considered. It could be that the energy restriction and rate of weight loss were too intense, that it promoted the development of hepatic lipidosis. In the current study, veterinary recommendations for weight loss were used [21], and perhaps future studies should use different recommendations to avoid the observed outcome.

Also, although the diet used was meant for weight loss, and was clinically proven to be effective at achieving weight loss, it could be that some of the nutrients’ levels were deficient due to the food intake restriction that was applied in the study (despite the evidential safety of the weight loss performed [22, 23]). Some nutrients deficiencies (e.g. choline) may prompt hepatic lipidosis [22]. It could be that the obese cats were deficient in these nutrients, which initiated the development of the weight loss-associated inflammation.

Overall, the information obtained from this part of the study can be utilized to diminish/prevent metabolic consequences of obesity, modify weight loss rate or other constituents in the weight loss plan (such as nutrient profile of diet or supplementation of nutraceuticals), and prevent complications of weight loss, for the most beneficial outcome for the cat.

The main limitations of this research project were the relatively small sample size, the study design (lean cats had only one sampling time point), and the relatively short weight loss period, since cats did not reach their ideal body weight at the end of the study. Additional limitations
include the lack of supplementary health monitoring tests to assess for hepatic damage of energy restriction (such as abdominal ultrasound, complete biochemistry inclusive of full lipid profile, as well as serum analysis of metabolites like carnitine and choline) or for a tissue-associated inflammation (adipose tissue samples), before and after weight loss. Inability to sample proximal intestinal sites also limits conclusions that can be made about the microbiota in those regions as only fecal samples were assessed.

5.2 FUTURE RESEARCH

In the future, studies to be performed should include studies using samples already collected during the described projects, as well as new research projects. It was previously mentioned that different microbial metabolic pathways and metabolites produced, and not necessarily microbial composition, are the main microbial-associated changes that occur in obesity/weight loss. Therefore, studies that can be done using samples collected during the current projects include metagenomics and metabolomics assessment (following an evaluation of the appropriate storage time after defecation for these analyses) using frozen DNA and fecal samples from participants in Chapter 2 and 3, as well as the assessment of whole blood adipokine mRNA expression and the serum metabolomic profiles of all cats participating in Chapter 3 and 4. The latter will assist in substantiating or excluding a reasoning behind the observed weight loss-associated inflammation. Finally, similar research projects to the one performed should be conducted with a larger sample size of cats (e.g. 80 obese cats), and a weight loss plan that continues until ideal body weight is achieved. Since systemic inflammation was not observed in the current study, adipose tissue inflammation should be investigated, as well as liver tissue inflammation for cats.
after weight loss, especially if a similar weight loss plan (similar restriction and rate achieved) is performed. Also, considering results of a previous study that suggested a different profile of inflammatory marker mRNA expression, depending on location of adipose tissue, it is suggested that tissue samples from different adipose deposits (e.g. visceral versus subcutaneous) should be analyzed for numerous inflammatory markers and compared to findings in serum. Additional intestinal health markers should be examined and incorporated into the study, depending on feasibility and invasiveness (e.g. different methods for lipopolysaccharides assessment, tight junction proteins assessment, microbiota-associated metabolites, primary and secondary bile acids). The results obtained from all research aspects will assist in bettering our knowledge regarding the obesity/weight loss associated inflammatory processes, which will be applied to modify current approach, and provide alternative or complementary weight loss strategies, with suggested implications to other species.
5.3 REFERENCES


APPENDICES

Appendix 3a: The effect of obesity and weight loss on the feline fecal microbiota, inflammatory and intestinal health markers - study design [CHAPTER 3 - 24-30; CHAPTER 4 - 30, 31]

Abbreviations: BCS, body condition score; MCS, muscle condition score; BMI, body mass index; Girth, girth circumference; BFI, body fat index