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Influência da obesidade, restrição energética e castração na microbiota intestinal de cães e gatos

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INFLUÊNCIA DA OBESIDADE, RESTRIÇÃO ENERGÉTICA E CASTRAÇÃO NA MICROBIOTA INTESTINAL DE CÃES E GATOS[1]

Autora: Manuela Marques Fischer
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RESUMO

Os métodos de tratamento para a obesidade em cães e gatos focam na restrição calórica, seja restringindo a ingestão de alimento ou alimentando o animal com dietas hipocalóricas. Entretanto, esses métodos frequentemente falham, sendo necessárias estratégias alternativas para promover a perda de peso. O objetivo desse estudo foi investigar as diferenças na microbiota fecal entre animais magros e obesos e determinar se a castração e/ou a perda de peso estão associadas com mudanças na população microbiana. No primeiro experimento, a composição da microbiota fecal foi avaliada nos gatos magros inteiros, magros castrados e obesos castrados, antes e depois da perda de peso. Os gatos obesos foram submetidos a seis semanas de restrição energética e apresentaram redução na massa gorda após a perda de peso ($P<0,001$), embora o peso corporal não tenha mudado ($P>0,05$). Firmicutes, seguido de Bacteroidetes foram os filos predominantes em todos os grupos. O grupo dos magros castrados tiveram o perfil de bactérias que era esperado para os obesos, com maior abundância de Firmicutes e menor de Bacteroidetes ($P<0,05$). Não foram observadas diferenças entre os grupos magros inteiros e obesos castrados. A população microbiana dos gatos obesos mostrou poucas alterações com a perda de peso. No segundo experimento, o teste foi realizado quando os cães estavam magros, após consumo *ad libitum* para promover o ganho de peso e após a perda de peso. As seguintes concentrações séricas foram analisadas: glicose, colesterol, triglicerídeos, albumina, creatinina, fosfatase alcalina (FA), alanina aminotransferase (ALT), proteínas totais (PT), insulina e leptina. As amostras de fezes foram analisadas para determinar a abundância de Bacteroidetes e Firmicutes. As concentrações de triglicerídeos, colesterol, albumina, FA, ALT e PT foram maiores ($P<0,05$) nos cães obesos quando comparados aos magros. Bacteroidetes foi mais abundante ($P<0,001$) nos magros e Firmicutes não diferiu entre os grupos ($P>0,05$). Após a perda de peso, os níveis de colesterol e PT e a abundância de Bacteroidetes permaneceram inalteradas estatisticamente. Conclui-se então que, nos modelos testados, há diferenças na microbiota fecal entre os grupos dos estudos realizados. Entretanto, no estudo com os gatos a obesidade pareceu não influenciar o crescimento das diferentes populações de microorganismos.

Palavras-chave: microorganismos, perda de peso, saúde gastrointestinal

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INFLUENCE OF OBESITY, ENERGY RESTRICTION AND NEUTERING ON THE GUT MICROBIOTA OF DOGS AND CATS[2]

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Advisor: Prof. Dr. Alexandre de Mello Kessler

ABSTRACT

Treatment methods for obesity in dogs and cats focus on calorie restriction, either by restricting the calorie intake of the animal, or by feeding energy diluted diets. However, these methods often fail, requiring additional strategies to promote weight loss. The objective of this study was to investigate differences in the gut microbiota between lean and obese animals and determine whether neutering and/or weight loss are associated with changes in the microbial populations. In the first experiment, the composition of the faecal microbiota was evaluated in lean intact, lean neutered and obese neutered cats, before and after weight loss. The obese cats were submitted to six weeks of energy restriction and showed less fat body mass after weight loss ($p<0.001$), although the body weight has not changed ($P>0.05$). Firmicutes followed by Bacteroidetes were the predominant bacterial phyla in all groups. The lean neutered cats had a bacterial profile of what one would expect from the obese cats, with greater abundance ($P<0.05$) of Firmicutes and lower abundance ($P<0.05$) of Bacteroidetes. There were no significant differences between lean intact and obese neutered. The microbe populations of obese cats showed very few changes with weight loss. In the second experiment, testing was performed when the dogs were lean, after ad libitum feeding to promote weight gain and after weight loss. Serum concentrations were analyzed: glucose, cholesterol, triglycerides, albumin, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), total proteins (TP), insulin and leptin. Faecal samples were analyzed to determine the abundances of Bacteroidetes and Firmicutes. Triglycerides, cholesterol, albumin, PA, ALT and TP were greater ($P<0.05$) in obese dogs when compared to the lean. The abundance of Bacteroidetes was greater ($P<0.001$) in the lean group and the phylum Firmicutes showed no differences among the groups ($P>0.05$). After weight loss, the levels of cholesterol and TP and the abundance of Bacteroidetes remained unchanged statistically. In conclusion, differences in the faecal microbiota were observed among the groups of both studies. However, in the study with cats, obesity seems not to influence the growth of diverse populations of microrganisms.

Key words: microorganisms, weight loss, gastrointestinal health

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RELAÇÃO DE ABREVIATURAS E SÍMBOLOS

ANOVA	analysis of variance
ALP	alkaline phosphatase
ALT	alanine aminotransferase
BCS	body condition score
BW	body weight
D_2O	deuterium oxide
DMD	dry maintenance diet
DNA	ácido desoxirribonucleico, deoxyribonucleic acid
FISH	fluorescent in situ hybridisation
FOS	fosfoligossacarídeos
g	gram
h	hour
kJ	kilojoule
ME	metabolizable energy
mg	miligrama, miligram
mL	mililitro, milliliter
NRC	National Research Council
PCR	polymerase chain reaction
qPCR	real time quantitative polymerase chain reaction
rRNA	ribossomal ribonucleic acid
SAS	Statistical Analisis System
SEM	standard error of a mean
TP	total proteins
vs.	versus
y	year
WG	weight gain
WL	weight loss
µ	micro

CAPÍTULO I

1. INTRODUÇÃO

A obesidade é uma das desordens mais comuns de cães e gatos e sua prevalência é estimada em 25 a 40% em diversos países. A doença ocorre devido a um desbalanço entre o consumo e o gasto energético e está associada a um risco maior no desenvolvimento de dislipidemias, resistência insulínica e outras doenças endócrinas e metabólicas. O seu tratamento em pequenos animais é um dos principais desafios da prática clínica. Fatores como castração, confinamento e alimentação com dietas hipercalóricas e palatáveis contribuem para o desbalanço energético. Os métodos de tratamento para a obesidade focam na restrição calórica, seja restringindo a ingestão de alimento ou alimentando o animal com dietas hipocalóricas. Entretanto, a falta de comprometimento e de cumprimento das recomendações pelo proprietário resulta em falhas nos programas de perda de peso. Assim, surge a necessidade de estratégias alternativas para promover a perda de peso.

A obesidade tem sido associada a diferenças na relação entre populações de Firmicutes e Bacteroidetes, dois grandes filos de bactérias da microbiota intestinal. Em humanos, de acordo com Ley et al. (2006), a proporção relativa de Bacteroidetes é menor em pessoas obesas quando comparadas às pessoas magras. Recentemente essa diferença vem sendo pesquisada em cães e gatos, porém poucos estudos têm caracterizado a microbiota filogenética do intestino de caninos e felinos. As informações obtidas nessa pesquisa irão melhorar a compreensão do papel dos microorganismos intestinais no desenvolvimento da obesidade e na progressão da doença. Se as diferenças na distribuição das duas populações de microorganismos existirem entre cães e gatos magros e obesos, novos métodos de tratamento para alterar a distribuição da população (seja por meios dietéticos ou por compostos bioativos) poderão fornecer aos proprietários outra estratégia para promover o peso corporal saudável em seus animais obesos e retardar a progressão da doença.

Assim, este estudo teve como objetivo fornecer informações sobre a distribuição de certas populações da microbiota intestinal em relação à obesidade e restrição de energia em cães e gatos e à castração em gatos.

2. REVISÃO BIBLIOGRÁFICA

2. 1 Obesidade em cães e gatos

A obesidade é uma doença em que o tecido adiposo se acumula de forma a afetar a saúde do paciente (German, 2006). O tecido adiposo serve como um reservatório de ácidos graxos que são usados como fonte de energia durante o estado de jejum pós-prandial. Como usa muito pouca energia, pouco contribui para o gasto energético basal (Laflamme, 2012). Entretanto, é um ativo produtor de hormônios, citocinas e outras substâncias de sinalização de células, chamadas de adipocinas (Trayhurn & Wood, 2005). Muitas adipocinas são mediadoras inflamatórias, que contribuem para a inflamação crônica da obesidade. Algumas das citocinas inflamatórias em maiores quantidades liberadas a partir do tecido adiposo em indivíduos obesos incluem o Factor de Necrose Tumoral-α, IL-1β e IL-6, Proteína C-reactiva e outras (Trayhurn & Wood, 2005). A inflamação na obesidade se estende para além do tecido adiposo. O fígado é afetado, seja através de infiltração gordurosa diretamente, seja através de mediadores inflamatórios derivados do tecido adiposo (Shoelson et al., 2006). O baixo grau de inflamação persistente da obesidade está associado a doenças crônicas como a osteoartrite e diabetes mellitus (Shoelson et al., 2006) e com o aumento do estresse oxidativo, que também contribui para doenças relacionadas à obesidade (Urakawa et al., 2003).

Como nos humanos, a epidemia da obesidade em animais de companhia está crescendo e em países industrializados é a doença nutricional mais frequente. Ela é resultante de um desbalanço entre o consumo e o gasto de energia e diagnosticada em cães e gatos com peso 20% superior ao ideal (Toll et al., 2010). Pesquisas realizadas na Europa demonstraram uma prevalência de obesidade canina em torno de 39% na França (Colliard et al., 2006), 50% no Reino Unido (Holmes et al., 2007) e 34% na Áustria (Handl et al., 2009). Sabe-se que a obesidade é um fator de risco para muitas doenças em cães, como as ortopédicas (Kealy et al., 2000), respiratórias (Bach et al., 2007), distúrbios hormonais (Martin et al., 2006) e doença do trato urinário (Lund et al., 2006). Além disso, mesmo cães moderadamente obesos têm uma expectativa de vida reduzida e desenvolvem doenças como a osteodistrofia mais cedo na vida (Kealy et al., 2002).

Em gatos, há alguns anos, uma clínica veterinária na Escócia observou que 39% dos 118 gatos atendidos estavam com sobrepeso ou obesidade (Courcier et al., 2010), com base em uma escala de condição corporal de nove pontos (Laflamme, 1997). Outras pesquisas estimaram 28% (Freeman et al., 2006) e 52% (Russel et al., 2000) de gatos com sobrepeso ou obesidade. Os problemas decorrentes parecem ser ainda mais graves nos gatos: diabetes mellitus, doenças ortopédicas, dermatológicas, doenças orais, lipidose hepática, infecções do trato urinário e alto risco anestésico (LUND *et al*., 2005; GERMAN, 2006). Alguns fatores como alimentação à vontade de dietas altamente palatáveis e gordurosas (Scarlett et al., 1994), castração e confinamento (Lund et al., 2005) contribuem para o aumento na prevalência de sobrepeso nos gatos.

2.1.1 Fatores de risco

Raça

É um fator de risco para cães. Segundo Zoran et al. (2010), diversas raças de cães são mais propensas a desenvolver obesidade, como Labrador, Retrievers, Beagle ou Boxer. Cair Terrier, Cavalier King Charles Spaniel, Scottish Terrier and Cocker Spaniel também são raças predispostas (Lund et al., 2006). Em contrapartida, algumas raças de galgos e cães pastores parecem ser mais resistentes à obesidade (Diez & Nguyen, 2006).

Idade

A incidência de obesidade aumenta com a idade (Robertson, 2003). Segundo Armstrong & Lund (1996), a idade média para o diagnóstico varia entre os 5 e os 8 anos. Até aos 4 anos, o nível de obesidade registrado em cães é inferior a 20% em comparação com mais de 50% na faixa etária dos 7 a 8 anos, e cerca de 70% acima dos 9 anos. Martin et al. (2006) também observaram que o excesso de peso aumentou significativamente com a idade.

Castração

Segundo Fettman et al. (1997), a castração em gatos predispõe à obesidade reduzindo o gasto de energia e aumentando o apetite e, consequentemente, o consumo de alimento. Diversos outros estudos sugerem a castração como fator de risco para o desenvolvimento da obesidade felina (Root, 1995; Scarlett et al., 1994; Nguyen et al., 2004; Lund et al., 2005; Colliard et al., 2009). Em cães, a obesidade é mais frequente em fêmeas do que em machos (Krook et al. 1960). Martin et al. (2006) em sua pesquisa observaram que os cães castrados eram mais obesos do que os inteiros. Jeusette et al. (2004) relataram um aumento significativo no consumo de alimento após a castração de Beagles fêmeas, com consequente ganho de peso.

Alterações endócrinas

A obesidade é altamente associada a várias disfunções endócrinas que são caracterizadas por desequilíbrio e/ou resistência hormonal. A redução de peso geralmente normaliza estas alterações endócrinas, implicando a obesidade como uma causa direta (Kil & Swanson, 2010). Martin et al. (2006) fizeram uma pesquisa com 31 cães naturalmente obesos e apenas 6 deles não apresentaram nenhum distúrbio hormonal e 18 apresentaram distúrbio na função da tireóide. O hipotireoidismo é uma das doenças endócrinas mais comuns em cães e a obesidade é a característica principal da doença, ocorrendo em 41-48% dos cães com hipotireoidismo (Panciera, 1994; Dixon et al., 1999).

Além disso, fatores como estilo de vida do proprietário, status socio-econômico, idade, tipo de habitação (casa ou apartamento) e nível de atividade física estão associados com a obesidade dos animais (Colliard, 2006; Courcier et al. 2010; Kienzle, 1998). Tipo de dieta, petiscos e comidas de mesa também são fatores relacionados à obesidade em cães e gatos (Colliard, 2006; Courcier et al. 2010; Robertson, 1999).

2. 2 Microbiota intestinal de humanos e ratos

Nos intestinos humanos e animais habitam cerca de dez a 100 trilhões $(10)^{14}$ de microorganismos, a grande maioria bactérias (Ley et al. 2005, Ley et al. 2006). Nos seres humanos e nos ratos, os filos mais abundantes são os Firmicutes (60-80%) e os Bacteroidetes (20-40%) e a maioria dos representantes desses dois filos não crescem fora do seu hospedeiro (Ley et al. 2005, Ley et al. 2006). A composição da microbiota intestinal depende da idade, sexo, geografia, etnia, família e dieta, e pode ser modulada por prebióticos, probióticos e antibióticos. Alterações microbianas no intestino humano foram propostas como uma possível causa de obesidade (Ley et al. 2006). Certos filos e classes de bactérias estão associadas à melhor transferência de calorias da dieta para o hospedeiro (Turnbaugh et al., 2008). O filo das Bacteroidetes parece possuir menos genes para enzimas envolvidas no metabolismo lipídico e de carboidratos do que o filo dos Firmicutes (Kallus & Brandt, 2012). No entanto, dentro do filo Bacteroidetes, Bacteroides thetaiotaomicron melhorou a absorção de nutrientes pelo hospedeiro (Hooper et al., 2001). Estudos em camundongos mostraram maior abundância de Firmicutes nos animais obesos alimentados com dietas ocidentais, concomitante com uma diminuição na abundância de Bacteroidetes (Ley et al. 2005, Turnbaugh et al., 2008).

A distribuição da “microbiota obesa” tem sido observada em humanos obesos e a proporção relativa de Bacteroidetes é menor em pessoas obesas quando comparadas às pessoas magras (Ley et al., 2006). Turnbaugh et al. (2009) verificaram menor proporção de Bacteroidetes e maior proporção de Actinobacteria em indivíduos obesos do que em magros, não havendo diferença significativa na proporção de Firmicutes. Também foi encontrado um nível significativamente mais elevado de espécies de Lactobacillus (do filo Firmicutes) em pacientes obesos quando comparado aos controles magros (Armougom et al., 2009).

O sequenciamento do RNA 16S ribossomal demonstrou que ratos obesos com deficiência de leptina possuíam maior proporção de Firmicutes em relação a de Bacteroidetes do que os ratos magros, independentemente das diferenças de consumo alimentar (Ley et al., 2005). Turnbaugh et al. (2006) demonstraram que a colonização de ratos *germ-free* com a microbiota de ratos obesos resultou em maior ganho de gordura do que o observado em ratos que receberam a microbiota de ratos magros. Em outra pesquisa desenvolvida por Turnbaugh et al. (2009), foram utilizados camundongos colonizados com fezes humanas, que consumiram dieta ocidental vs. dieta com baixo teor de gordura durante duas semanas. A microbiota foi então transplantada para ratos *germ-free*. Os que receberam a microbiota dos animais que consumiram a dieta ocidental ganharam significativamente mais adiposidade do que os que receberam a microbiota dos animais que consumiram a dieta com baixo teor de gordura. No entanto, também tem sido sugerido que a dieta, e não a obesidade, está correlacionada com as alterações da microbiota intestinal (de La Serre, 2010), e que os mecanismos, tais como a indução de sobrealimentação por microorganismos do intestino podem ter um papel no desenvolvimento da obesidade (de Lartigue et al., 2011; Raybould, 2012).

2. 3 Microbiota intestinal de cães e gatos

Para Deng & Swanson (2015), os filos predominantes nos intestinos caninos e felinos são: Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria e Actinobacteria. As proporções variam entre as espécies hospedeiras e em estudos relatados na literatura essa variabilidade pode tanto estar associada ao animal, seja por raça, dieta ou idade, quanto ao ambiente ou às metodologias laboratoriais.

Em cães, utilizando 16S rRNA gene amplicon-based Sanger sequencing, quatro filos principais foram identificados no cólon de seis animais saudáveis: Firmicutes (47.7%); Proteobacteria (23.3%); Fusobacteria (16.6%) e Bacteroidetes (12.4%) (Suchodolski et al., 2008). Em outra pesquisa, onde as mesmas amostras de fezes foram avaliadas, porém empregando duas metodologias diferentes, os autores encontraram resultados diferentes. Quando utilizado 16S rRNA gene amplicon-based 454-pyrosequencing, os autores encontraram Fusobacteria (23 – 40% das leituras), Firmicutes (14 – 28% das leituras), Bacteroidetes (31 – 34% das leituras), Actinobacteria (0.8 – 1·4% das leituras) e Proteobacteria (5 – 7% das leituras) como filos dominantes nas fezes caninas (Middelbos et al., 2010). E quando testado o shotgun 454-pyrosequencing, a abundância dos filos foi diferente: Bacteroidetes (37 – 38% das sequências), Firmicutes (31 – 35% das sequências), Proteobacteria (13 – 15% das sequências), Fusobacteria (7 – 9% das sequências) e Actinobacteria (1% das sequências) (Swanson et al., 2011). Garcia-Mazcorro et al. (2012) também observaram discrepâncias de resultados na abundância fecal da microbiota de cães saudáveis utilizando análises de FISH e 454-pyrosequencing.

Em um estudo publicado recentemente, Handl et al. (2013) avaliaram a composição da microbiota fecal de 22 cães obesos e 21 magros, todos de proprietários e também de nove cães Beagle pertencentes ao canil de pesquisas da Universidade, dos quais cinco foram alimentados ad libitum e quatro serviram como controles magros. O filo predominante (>90%) em todos os animais foi o Firmicutes. Outros filos identificados em menores quantidades nos animais de proprietários foram Actinobacteria, Fusobactéria, Proteobacteria e Bacteroidetes e nos do canil de pesquisa foram Actinobacteria, Proteobacteria e Bacteroidetes. Nos cães obesos, o filo Actinobacteria foi significativamente mais abundante quando comparado aos cães magros. Essa pesquisa demonstrou que a microbiota intestinal canina é altamente diversificada e mostra uma variação interindividual considerável.

Em um estudo com gatos, Ritchie et al. (2010) identificaram 133 sequências de genes 16S rRNA da microbiota fecal de 15 animais de estimação e observaram que a maioria das sequências pertencia ao filo Firmicutes, seguido por Proteobacteria e Bacteroidetes. Nessa pesquisa não foram utilizados gatos obesos, portanto não foi avaliada a influência da obesidade. Outras pesquisas também encontraram maior abundância do filo Firmicutes no cólon e nas fezes de gatos adultos (Ritchie et al., 2008; Desai et al., 2009) e filhotes (Hooda et al., 2013). Garcia-Mazcorro et al. (2011) relataram o Firmicutes como sendo o filo mais abundante (92-95%), seguido de Actinobacteria (4-7%) e com menos de 1% os filos Proteobacteria, Bacteroidetes e Fusobacteria. Barry at el. (2012) também relataram o filo

Firmicutes como sendo o predominante (36-50%), porém seguido do filo Bacteroidetes (24-36%) e Proteobateria (11-12%). Já em outras pesquisas, foi observada a predominância de Bacteroidetes/Chlorobi (68%), seguido por Firmicutes (13%) e Proteobacteria (6%) (Handl et al., 2011; Tun et al., 2012).

2. 4 Efeito da dieta no trato gastrointestinal de cães e gatos

Embora o interesse no papel da microbiota intestinal em animais de companhia tenha crescido (Wynn, 2009; Kil e Swanson, 2010), na grande maioria das vezes o foco é direcionado para estudar os impactos dos macronutrientes da dieta sobre a microbiota intestinal.

Foram observadas alterações na população microbiana do trato gastrointestinal de gatos quando testadas diferentes concentrações de carboidratos (Bermingham et al., 2011.), proteínas (Lubbs et al., 2009; Vester et al., 2009; Hooda et al., 2013) e fontes de fibra (Barry et al., 2010; Middelbos et al., 2010) na dieta. Embora controverso, uma atenção recente na patofisiologia da obesidade tem focado na hipótese de que as mudanças na composição da microbiota intestinal aumenta a extração da energia da dieta, o que está associado com o aumento do peso corporal e adiposidade (Kallus & Brandt, 2012). Bermingham et al. (2011), investigaram a população microbiana fecal de gatos alimentados com dieta seca ou úmida e observaram que uma alteração a curto prazo na dieta levou a grandes alterações na população bacteriana das fezes. Os gatos alimentados com dieta seca, que contém mais carboidrato (33% de proteína bruta e 46% de carboidrato na MS), tiveram maior abundânia de Actinobacteria e menor abundância de Fusobacteria e Proteobacteria comparados aos alimentados com dieta úmida (42% de proteína bruta e 5% de carboidrato na MS).

Hooda et al. (2013), utilizaram 16S rRNA pyrosequencing-454 para avaliar os efeitos da proporção de proteína:carboidrato da dieta, sobre a microbiota fecal de gatos filhotes com 8 e 16 semanas de idade. Eles observaram maior abundância de Actinobacteria e menor de Fusobacteria nos animais alimentados com a dieta moderada em proteína e carboidrato em comparação com os alimentados com a dieta alta em proteína e pobre em carboidratos. Além das diferenças a nível de filo, muitas mudanças a nível de gênero e espécie foram observadas. Middelbos et al. (2010), investigaram os efeitos da inclusão de 7.5% de polpa de beterraba na população microbiana fecal de cães. Utilizando 16S rRNA gene amplicon-based 454-pyrosequencing, eles observaram que a inclusão da fibra diminuiu a abundância de Fusobacteria e aumentou a de Firmicutes, quando comparado à 0% de inclusão da polpa de beterraba. Barry et al. (2010) avaliaram os efeitos de dietas para gatos contendo 4% de fruto-oligossacarídeos (FOS), 4% de pectina e 4% de celulose. A dieta com FOS aumentou a abundância de Actinobacteria e a dieta com pectina aumentou a de Firmicutes e de bactérias totais.

Manter uma população microbiana equilibrada e estável é fundamental para manter a saúde gastrointestinal de cães e gatos, ainda que esses animais não dependam de fermentação microbiana para aquisição de energia (Deng & Swanson, 2015).

3 OBJETIVOS E HIPÓTESES

O objetivo dessa pesquisa foi investigar a distribuição das populações de bactérias presentes no intestino de cães e gatos, associando fatores como obesidade, restrição de energia e castração.

As hipóteses testadas foram:

1. A obesidade altera a predominância dos principais filos de bactérias presentes no intestino de cães e gatos.

2. A restrição de energia e a perda de peso alteram a microbiota intestinal de cães e gatos.

3. Cães que se tornam obesos têm sua microbiota intestinal modificada e, ao perder peso, a microbiota se reestabelece.

4. A castração pode alterar a microbiota intestinal de gatos magros e obesos.

CAPÍTULO II

Running head: Gut microbiota in lean and obese cats.

Effects of obesity, energy restriction and neutering on the gut microbiota of cats

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Key words: gut microbiota, feline, obesity, neutered, and energy restriction.

Summary

Surveys report that 25% to 57% of cats are overweight or obese. There are many postulated reasons, but of endogenous causes neutering is the most evinced. Current treatment methods often fail; thus new strategies to promote weight loss are needed. Recently, obesity in some species has been associated with gut microbial populations that affect energy extraction, body weight and adiposity. Although obesity is common in cats, the relationship with intestinal microbiota has not been reported. The aims of this study were to determine if alterations in intestinal microbiota are associated with obesity, energy restriction and neutering in cats. Using 16S rRNA gene primers, we evaluated the composition of faecal microbiota in 8 lean intact, 8 lean neutered, and 8 obese neutered cats before and after 6 weeks of energy restriction. The obese cats had less fat mass after energy restriction ($P<0.001$), although body weight did not significantly change ($P>0.05$). Firmicutes, followed by Bacteroidetes were the predominant bacterial phyla in all groups. Lean neutered cats had a bacterial profile similar to obese rodents and humans, with a greater abundance ($P<0.05$) of Firmicutes and lower abundance ($P<0.05$) of Bacteroidetes compared to the other 3 groups. There were no significant differences between lean intact and obese neutered cats. Microbe populations of obese cats had few changes with weight loss. The short duration of energy restriction may have affected the findings of this study. Additional work is needed to understand how feline faecal microbiota are affected by neutering, obesity and weight loss.

Introduction

Obesity is one of the most common feline nutritional disorders in the United States. Surveys report incidence rates of between 25% and 57% of cats are considered to be overweight or obese (Colliard et al., 2009; Scarlett et al., 1994). Obesity can be defined as an excess of body fat sufficient to impair health or body function and is generally recognized as 20-25% above ideal body weight in humans. Obese cats face an increased risk of musculoskeletal problems, diabetes mellitus, and hepatic lipidosis. (Lund et al. 2005; Zoran, 2010).

The fundamental underlying cause in all cases of obesity is an imbalance between energy intake and energy expenditure that results in persistent energy surplus. Exogenous factors include activity level, external influences on food intake, diet composition and palatability, and environment and lifestyle. Endogenous factors that contribute to obesity include age, sex, reproductive status, hormonal abnormalities, and genetic predisposition. Of all endogenous factors, neutering is the most evinced. Multiple studies have shown that intact adult cats generally weigh less than neutered animals of the same breed and size (Duch et al., 1978; Fettman et al., 1997; Flynn et al., 1996; Houpt et al., 1979; Root 1995). Several recent studies have demonstrated that ovariohysterectomy and castration in cats leads to an increase in food intake and weight gain (Belsito et al., 2009; Flynn et al., 1996; Kanchuk et al., 2003; Martin et al., 2001; Nguyen et al., 2004; Wei et al., 2014). Treatment methods focus on calorie restriction; however, lack of owner compliance often results in relapses in weight management programs. Thus, the development of additional strategies to promote weight loss in cats is needed.

Recently, obesity development has been associated with the presence or absence of certain gut microbial populations (Firmicutes and Bacteriodetes) that affect energy extraction and therefore body weight and adiposity in other species. In humans, a distribution of "obese microbiota" has been observed (Ley et al., 2006). It is reported that obese individuals have greater populations of Firmicutes, and more Bacteriodetes compared to their lean counterparts (Ley et al., 2005). Colonization of germ-free mice with the "obese microbiota" resulted in greater fat gain compared with germ-free mice receiving microbiota from lean mice (Turnbaugh et al., 2006). However, it has also been suggested that diet, not obesity, is correlated with gut microbiota changes (de La Serre, 2010), and that mechanisms such as induction of overeating by gut microbes may have a role in obesity development (de Lartigue et al., 2011; Raybould, 2012). Although controversial, recent interest in the pathophysiology of obesity has focused on the hypothesis that compositional changes in gut microbiota enhance dietary energy extraction, which is associated with increased body weight and adiposity (Kallus and Brandt, 2012).

Control of gut populations using diet or antibiotics may be a viable strategy to promote a healthy body weight in cats and reduce the onset of other secondary diseases. However, while several studies have characterized the phylogenetic microbiota of the feline gut (Ritchie at al., 2010; Hooda et al., 2013, Minamoto et al. 2012), few have examined the effect of neutering, obesity and weight loss (Kieler et al. 2012). Therefore the aims of this study were to compare the composition of faecal microbiota in lean neutered and intact cats, as well as obese neutered cats (before and after weight loss), to determine if neutering or energy restriction causes a shift in faecal microbial populations.

Materials and methods

Animals and diets

Approval of experimental protocols was granted by the Institutional Animal Care and Use Committee of the University of California, Davis and complied with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

Twenty-four adult (median age 6 y), specific pathogen free, domestic shorthair cats owned by the University of California were used in this study. Ten cats were neutered males, 4 were intact males, 4 were intact females and 6 were spayed females. A 9-point body condition score (BCS) system was used (Laflamme, 2007), where a score of 5 was considered ideal, a score >5 and <7 considered overweight, and a score >7 considered obese. The obese cats were group housed in a light (14-h-light/10-h-dark cycle) and temperature (18–24°C) controlled facility at the University of California, Davis, in an enriched environment (perches, rotating toys, and scratching poles) and were brushed and socialized once a day. They were individually housed only to be fed twice a day and for feces and blood collections. Fresh water was available at all times, except prior to body composition determination. All cats had consumed the same extruded dry-type diet packed specifically for our facility for at least 8 weeks prior to entering and throughout the study. All cats were fed the same batch of diet for the duration of the study. The nutrient composition of the diet is 38% protein, 17% fat, 32% carbohydrate, 4% crude fiber, and 9% ash (on a dry matter basis) (calculated metabolizable energy = 16.19 kJ/g on a dry matter basis). The diet met the nutritional recommendations for all life stages in cats (AAFCO, 2012).

Study design

Prior to the start of the study, each cat underwent a physical examination and blood collection for a serum chemistry panel and complete blood count. The experiment was divided into 3 phases. In the first phase, all the obese neutered cats (n=8) consumed the diet ad libitum for 10 days, during which time their body weights (BW) and food intakes stablized. During the 2nd phase, the cats were fed 60-70% of their previously measured energy intake for a period of 6 weeks. Body composition was determined, fecal and blood samples were taken prior to the start and at the end of phase 2. Body weight was measured weekly and body condition score was determined every other week by the same person. In the third phase, 8 lean intact and 8 lean neutered cats were group housed and consumed the same diet for at least 8 weeks. Food intake was not measured in these two groups, but cats were weighed weekly and remained weight stable during the period of the study. On the night before of blood and fecal collection for body composition determination and microbe analysis, cats were body condition scored and moved into individual cages. Following collection of final blood and fecal samples all cats were returned to group housing in the feline facility. A total of 32 fecal samples were collected.

Parameters evaluated

Body composition determination: Estimation of body fat mass (FM) and lean mass was accomplished using the deuterium oxide (D_2O) isotopic dilution method of Backus et al. (2000), with modifications (Wei et al., 2011). Deuterium oxide was purchased from Fisher Scientific (Pittsburgh, PA, USA). A basal blood sample (3 cc),

without D_2O enrichment was obtained by jugular venipuncture. Cats were fasted (12 h) prior to collection of this sample, and water was withheld from cats 2 h prior to collection. Deuterium oxide (0.4 g D_2O/kg body weight) was administered to the cats subcutaneously and allowed to equilibrate for 3 h, after which a D_2O enriched blood sample (3 cc) was collected. Condensed serum water samples were analyzed on an ATI Mattson Infinity Series Fourier transform infrared spectrometer equipped with a class 2A laser (Madison, WI, USA).

Fecal DNA extraction, pyrosequencing, and bacterial composition analysis: Fresh fecal samples for each cat were collected once daily over three consecutive days into sterile cryotubes, stored at -80°C, combined and homogenized. A subsample was collected for extraction, isolation, and purification of nucleic acids. Analyses by quantitative PCR were performed (Hartman et al., 2009). The abundances of five predominant microbial phyla in the feline gut were analyzed - *Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria* and *Actinobacteria*. Bacterial DNA was extracted by a bead-beating method using a commercial DNA extraction kit (Mobio Powersoil Kit) following the manufacturer's instructions. The bead-beating step was performed on a homogenizer for 60 seconds at a speed of 4 m/s. Amplification of the 16S rRNA genes was carried out using universal bacterial primers (530F-1100R) to amplify DNA in a single-step, 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (QIAGEN) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads. Samples were sequenced using Roche 454 FLX titanium

instruments and reagents according to the manufacturer's guidelines. The Q25 sequence data were processed using a proprietary analysis pipeline (www.mrdnalab.com) (Capone et al., 2011; Swanson et al., 2011). Briefly, sequences were depleted of barcodes and primers, then sequences less than 150 bp were removed, as were sequences with ambiguous base calls and homopolymer runs exceeding 6 bp. Operational taxonomic units (OTUs) were generated by clustering at 3% divergence (97% similarity) from de-noised sequences, and chimeras were removed. Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006) and compiled into each taxonomic level as the percentage of sequences within each sample that map to the designated taxonomic classification.

Statistical analysis

The experimental data was analyzed in a randomized block design by ANOVA. Data were blocked by sex and/or age, and the effects were evaluated by F-test. Means of the four groups tested were compared by Student-Newman-Keuls test. Data of species frequency was submitted to log transformation for statistical significance testing, because they were not normally distributed. Multivariate analysis was performed on family and species data, using principal components modules of SAS JMP Software. A p value less than 0.05 was considered significant.

Results

The animals had no adverse clinical changes throughout the experiment. There were no effects of sex or age on any of the variables. The lean cats had a greater ($P<0.05$) percentage of lean body mass than obese cats (Table 1). Although body weight did not significantly change ($P>0.05$), obese cats had a reduction in the percentage of fat body mass after weight loss ($P<0.001$). The majority of sequences from the 32 fecal samples that were analyzed were classified as Firmicutes (65,8%) followed by Bacteroidetes (25,2%), Proteobacteria (3,52%), Actinobacteria (2,20%) and Fusobacteria (0,3%) (data not shown). The number of Firmicutes was greater ($P<0.05$) in the lean neutered cats when compared to other 2 groups. In contrast, Bacteroidetes was lower in this group ($P<0.05$) (Table 2). There were no significant differences between lean intact and obese neutered, or cats.

Among the Firmicutes, Lachnospiraceae, Peptostreptococcaceae, Veillonellaceae and Ruminococcaceae were the predominant families in cat faeces (Table 3). Peptostreptococcaceae was the most abundant family in lean neutered cats and greater ($P<0.05$) when compared with the other groups. Among the Bacteroidetes, Prevotellaceae was the most abundant bacterial family present in all groups; however it was lower ($P<0.05$) in lean neutered cats compared with the other 2 groups. The principal component analysis of the predominant families are shown in Figure 1.

The microbe populations of obese cats showed very few changes in bacterial species with weight loss (Table 4). *Blautia producta, Catenibacterium mitsuokai, Clostridium bartletti, Clostridium hiranonis, Clostridium methylpentosum, Megasphaera elsdenii, Oscillospira spp, Prevotella copri, Ruminococcus flavefaciens, Ruminococcus gnavus* and *Sarcina ventriculi* were predominant species in cat faeces. Faecal *Clostridium hiranonis* was higher ($P<0.05$) in lean neutered cats compared to the

other groups. In contrast, faecal *Prevotella copri, Faecalibacterium prausnitzii* and *Clostridium bolteae* was lower in this group (P<0.05). Among the groups of cats, the major differences between species were observed between the lean neutered and obese neutered cats. Of the 12 species with a significant difference (P <0.05), 8 were different between these groups. The principal component analysis of the predominant species are shown in Figure 2.

Discussion

Although the body weight had not changed, the obese cats had less fat body mass after weight loss, suggesting that 6 weeks of energy restriction was sufficient for a change in body composition. However, weight loss was not accompanied by a change in microbial populations. Using universal bacterial 16S rRNA gene primers we identified 5 bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria (Table 2), which is comparable to previous studies analyzing faecal microbiota in cats (Ritchie et al., 2008; Ritchie et al., 2010; Hooda et al., 2013). The majority of sequences from the 32 fecal samples were classified as Firmicutes, followed by Bacteroidetes. Firmicutes are known to be the predominant bacterial group in the intestinal tract of animals and our results were consistent with previous findings in cats (Ritchie et al., 2008, Ritchie at al., 2010, Hooda et al., 2013) and dogs (Middelbos et al., 2010, Handl et al., 2013) using 16S rRNA gene primers. We did not observe differences in microbial populations of lean and obese cats, as previously reported in mice (Turnbaugh et al., 2008) or humans (Ley et al., 2006; Turnbaugh et al., 2009). Our results are in agreement with Handl et al. (2013), who did not observe major shifts in

the faecal microbial composition between lean and obese dogs.

To our knowledge, this is the first study that has compared gut microbial diversity in intact vs. neutered, lean vs. obese and obese vs. obese cats after calorie restriction. Currently there are no studies in other species determining the effect of neutering on faecal microbiota. In the present study, we observed that lean neutered cats had a greater number of Firmicutes and a lower number of Bacteroidetes compared to the other 2 groups. This bacterial profile is what one would have predicted from obese, not lean cats. Studies in humans and mice have shown that alterations in gastrointestinal microbiota are associated with the development of fat mass (Ley et al., 2005). Weight loss in the obese cats in this study had no effect on the microbiome. This may have been due, in part, to the short period of energy restriction in this study.

Several studies suggest that changes in dietary composition can alter the microbiota in cats. In one study that fed healthy cats wet and dry diets for 5 weeks, differences were observed in faecal bacterial populations, suggesting that 5-weeks following a dietary change is sufficient to alter gastrointestinal microbiota (Bermingham et al., 2011). Other researchers also observed alterations in microbial populations with changes in dietary protein (Lubbs et al., 2009; Vester et al., 2009) or fiber sources (Barry et al., 2010). In our study, the same diet was used during the weight loss phase for the obese cats, demonstrating that reducing energy intake by 30-40% for 6 weeks was not enough to induce changes in the faecal microbiota.

In conclusion, the present study reports some changes in the faecal microbial population in domestic cats; however, these changes require further investigation. Neutering, but not energy restriction was associated with modifications in the feline

intestinal microbiota. Further research is needed to discern whether obesity or neutering is associated with alterations in the microbiome.

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Table 1. Body composition of lean intact, lean neutered and obese cats (before and after weight loss)

	Lean Intact[1]	Lean Neutered[1]	Obese Neutered[1]	Obese after weight loss[1]	SEM	*P*
Body weight (kg)	4.27[b]	4.88[b]	6.57[a]	6.18[a]	0.599	0.0001
Lean body mass (%)	76.1[a]	71.6[ab]	59.8[c]	67.1[b]	5.87	0.0001
Fat body mass (%)	23.9[c]	28.4[bc]	40.2[a]	32.9[b]	5.87	0.0001

[a,b,c]Mean values with unlike superscript letters within a row were significantly different (P,0.05).
[1]8 cats per group

Table 2. Predominant bacterial phyla (expressed as a percentage) in the faeces of lean (intact and neutered) and obese (neutered) cats

	Lean Intact	Lean Neutered	Obese Neutered	Obese after weight loss	SEM	*P*
Firmicutes	63.3[b]	81.9[a]	57.4[b]	60.5[b]	16.9	0.0305
Bacteroidetes	27.58[a]	11.29[b]	33.24[a]	28.72[a]	14.09	0.0231
Proteobacteria	4.09	3.03	3.20	3.74	3.31	0.9129
Actinobacteria	1.6	1.76	3.02	2.42	1.59	0.2809
Fusobacteria	0.38	0.07	0.37	0.39	0.50	0.5296
Unclassified	3.0	1.85	2.80	4.26	2.25	0.2263

[a,b]Mean values with unlike superscript letters within a row were significantly different (P,0·05).

Table 3. Bacterial families (expressed as a percentage of sequences) in the faeces of lean and obese cats

	Lean Intact	Lean Neutered	Obese Neutered	Obese after weight loss	SEM	*P*
Firmicutes						
Clostridiaceae	3.72	5.49	5.66	1.26	7.699	0.6416
Enterococcaceae	0.03	0.22	0.07	0.23	0.444	0.7374
Erysipelotrichaceae	3.55	5.65	3.66	3.82	3.686	0.6313
Incertae Sedis XIII	0.05	0.03	0.39	0.20	0.365	0.1880
Lachnospiraceae	19.44	19.49	12.95	16.53	6.596	0.1777
Peptostreptococcaceae	15.67[b]	32.23[a]	9.67[b]	14.24[b]	12.662	0.0077
Ruminococcaceae	5.31	7.82	7.09	9.69	6.674	0.6279
Streptococcaceae	0.34	1.97	0.98	0.12	3.120	0.6428
Veillonellaceae	13.50	7.27	14.09	11.69	7.127	0.2360
Bacteroidetes						
Bacteroidaceae	2.19	0.85	2.92	2.98	2.375	0.2663
Bacteroidales*	1.58	0.67	2.00	0.98	1.099	0.0930
Porphyromonadaceae	0.14	0.15	0.30	0.21	0.256	0.5961
Prevotellaceae	23.45[a]	9.44[b]	27.72[a]	24.33[a]	13.63	0.0578
Proteobacteria						
Alcaligenaceae	1.93	0.43	1.85	1.95	1.97	0.3500
Succinivibrionaceae	2.04	2.43	1.22	1.53	2.05	0.6520
Actinobacteria						
Bifidobacteriaceae	0.62	0.30	0.83	0.29	0.682	0.3218
Coriobacteriaceae	0.93	1.46	2.18	2.11	1.442	0.2790
Fusobacteria						
Fusobacteriaceae	0.38	0.07	0.37	0.37	0.508	0.5474

[a,b]Mean values with unlike superscript letters within a row were significantly different (P,0·05).
*Unknown family within the order Bacteroidales

Table 4. Bacterial species (expressed as a percentage of sequences) in the faeces of lean and obese cats

	Lean Intact	Lean Neutered	Obese Neutere	Obese after	SEM	*P*
Firmicutes						
Acidaminococcus Fermentans	0.28^{ab}	0.05^{b}	0.80^{a}	0.12^{b}	0.383	0.0085
Bacillus humi	0.55	0.55	0.21	1.71	1.099	0.1925
Blautia Producta	7.79	8.69	4.16	4.75	3.061	0.1129
Blautia Ruminococcus	1.89	2.75	1.50	1.72	1.006	0.1263
Catenibacterium Mitsuokai	2.20	3.01	2.19	2.85	3.498	0.8195
Clostridium Bartletti	1.61	6.18	1.58	2.28	4.151	0.4806
Clostridium Bolteae	0.35^{a}	0.08^{b}	0.32^{a}	0.36^{a}	0.289	0.0229
Clostridium Disporicum	0.03	0.39	0.03	0.01	0.553	0.7645
Clostridium Ghonii	0.01	0.04	0.08	1.64	2.276	0.4420
Clostridium Hiranonis	10.87^{b}	22.32^{a}	5.72^{b}	7.89^{b}	8.582	0.0195
Clostridium Methylpentosum	2.14^{ab}	2.04^{b}	2.82^{ab}	4.10^{a}	1.566	0.0499
Clostridium Perfringens	0.36	1.11	1.02	0.65	0.971	0.3731
Clostridium Symbiosum	1.07	0.88	1.89	2.23	1.058	0.1005
Clostridium Thermocellum	0	0	0.15	0.01	0.216	0.4851
Clostridium Xylanolyticum	0.31	0.25	0.37	0.65	0.279	0.0824
Coprococcus Eutactus	0	0	0.06	1.95	1.783	0.0738
Dorea Formicigenerans	0.65	0.70	0.31	0.43	0.417	0.2573
Enterococcus Cecorum	0.04	0.24	0.04	0.24	0.463	0.9144
Eubacterium Biforme	0.68	0.46	0.60	0.47	0.439	0.4490
Faecalibacterium Prausnitzii	1.90^{a}	1.12^{b}	2.13^{a}	2.52^{a}	0.883	0.0104
Lactobacillus Intestinalis	0.09	1.66	0.04	0.03	1.639	0.2549
Megamonas Hypermegale	0.19	0.04	0.20	0.38	0.350	0.1983
Megasphaera Elsdenii	11.96	6.92	11.75	10.56	7.171	0.1219
Mogibacterium Pumilum	0.03^{b}	0^{b}	0.41^{a}	0.18^{ab}	0.332	0.0030
Oscillospira spp	2.39	1.72	3.17	2.92	2.299	0.2897
Phascolarctobacterium spp	0.49^{a}	0.17^{b}	0.88^{a}	0.44^{ab}	0.464	0.0075
Roseburia Eubacterium	3.23	1.90	1.81	1.59	1.936	0.8304
Roseburia Faecis	1.43	1.10	0.48	0.60	0.810	0.3116
Roseburia Intestinalis	0.41	0.45	0.17	0.26	0.299	0.1823
Ruminococcus Flavefaciens	0.64	4.99	1.44	2.71	7.226	0.1528
Ruminococcus Gnavus	2.87	3.65	1.80	2.04	1.514	0.1381
Ruminococcus Torques	0.36^{ab}	0.83^{a}	0.12^{b}	0.27^{ab}	0.385	0.0077
Sarcina Ventriculi	3.36^{b}	3.95^{ab}	4.54^{a}	0.65^{b}	7.491	0.0376
Staphylococcus Carnosus	0.18^{ab}	0.15^{ab}	0.04^{b}	0.59^{a}	0.355	0.0457

Streptococcus Bovis	0.35	2.13	0.93	0.13	3.295	0.9192
Syntrophomonas Curvata	1.23	0.85	0.42	0.45	0.784	0.1764
Bacteroidetes						
Bacteroides acidifaciens	0.84	0.35	0.90	0.34	0.727	0.3593
Bacteroides coprocola	0.65	0.17	0.41	0.49	0.439	0.1010
Bacteroides stercoris	0.39	0.13	1.12	0.87	1.486	0.2171
Bacteroides Vulgatus	0.21	0.30	0.31	0.43	0.596	0.9804
Prevotella Copri	27.10[a]	11.04[b]	32.06[a]	27.04[a]	15.14	0.0046
Proteobacteria						
Succinivibrio spp	1.94	2.34	1.17	1.34	1.983	0.5305
Sutterella spp	1.94	0.42	1.90	1.94	1.989	0.1543
Actinobacteria						
Bifidobacterium	0.46	0.26	0.58	0.23	0.524	0.4159
Bulleidia spp	0.67	0.30	1.22	0.52	0.634	0.1085
Collinsella Stercoris	0.27	0.53	0.55	0.36	0.414	0.7299
Collinsella Tanakaei	0.36[b]	0.40[b]	0.88[ab]	1.27[a]	0.816	0.0218
Fusobacteria						
Fusobacterium Varium	0.37	0.08	0.38	0.33	0.525	0.3347

[a,b]Mean values with unlike superscript letters within a row were significantly different (P,0·05).

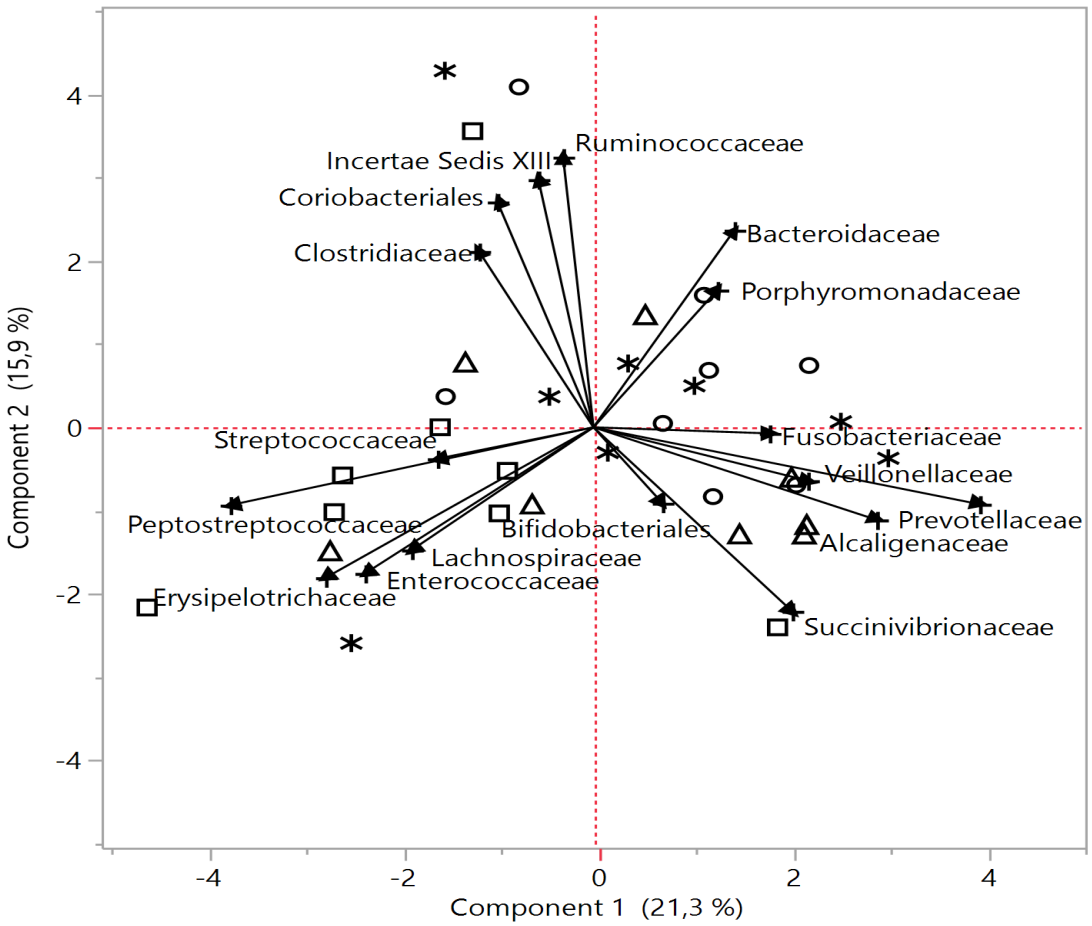

Figure 1. Principal component analysis scatter plot of 17 families of bacteria observed in four different groups of cats (Δ Lean intact; □ Lean neutered; ○ Obese neutered; * Obese after weight loss). The vectors shown are the eigenvectors of the correlation matrix. The arrows indicate the direction and crescent magnitude of the frequency of each family. The total variance explained by the components 1 and 2 is 37,2%.

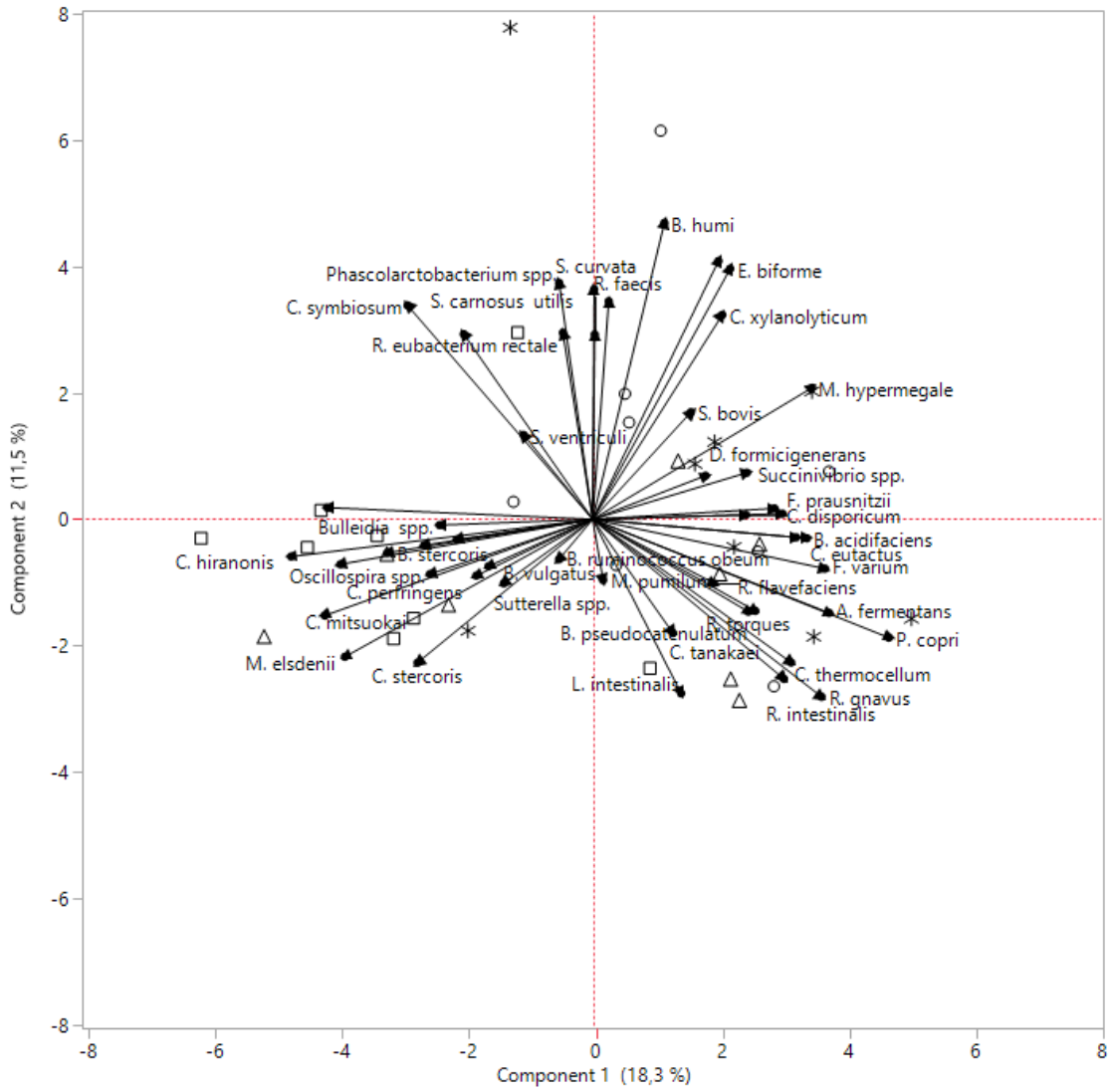

Figure 2. Principal component analysis scatter plot of 48 species of bacteria observed in four different groups of cats (Δ Lean intact; □ Lean neutered; ○ Obese neutered; * Obese after weight loss). The vectors shown are the eigenvectors of the correlation matrix. The arrows indicate the direction and crescent magnitude of the frequency of each species. The total variance explained by the components 1 and 2 is 29,8%.

CAPÍTULO III

RUNNING HEAD: FAECAL MICROBIOTA IN DOGS

Short-term obesity alters blood metabolites and faecal microbiota of adult Beagle dogs

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Summary

The knowledge of the gut microbiota composition in obesity in dogs could provide a basis for future studies to investigate the effects of dietary, antibiotic, or probiotic manipulations on canine gastrointestinal microbiota. The aim of this research was to investigate whether weight gain and weight loss alters the blood parameters and the abundance of Firmicutes and Bacteroidetes in the faecal microbiota of adult Beagle dogs. Testing was performed when the dogs were lean, after *ad libitum* feeding to promote weight gain and after weight loss. Serum concentrations were analyzed: glucose, cholesterol, triglycerides, albumin, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), total proteins (TP), insulin and leptin. Faecal samples were analyzed using quantitative real-time PCR to determine the abundances of Bacteroidetes and Firmicutes. Triglycerides, cholesterol, albumin, PA, ALT and TP were greater ($P<0.05$) in obese dogs when compared to the lean. The abundance of Bacteroidetes was greater ($P<0.001$) in the lean group and the phylum Firmicutes showed no differences among the groups ($P>0.05$). After weight loss, the levels of cholesterol and TP and the abundance of Bacteroidetes remained unchanged. This study showed that obesity, even during a short time, promote changes that weight loss did not reverse in its entirety.

Introduction

As in humans, the epidemic of obesity in pets is growing and in industrialized countries is the most common nutritional disease. Obesity is the result of an imbalance between consumption and energy expenditure and diagnosed in dogs and cats weighing 20% more than the ideal (Toll et al., 2010). Surveys have shown a prevalence of canine obesity around 39% in France (Colliard et al., 2006), 50% in the UK (Holmes et al., 2007), 34% in Austria (Handl et al. 2009), 34% in USA (Lund et al., 2006) and 44% in China (Mao et al., 2013). Obesity in dog is associated with several health conditions, including hormonal disturbances (Martin et al., 2006), hyperlipidemia (Jeusette et al., 2005), cruciate ligament rupture (Adams et al., 2011), respiratory distress (Bach et al., 2007), orthopedic disease (Kealy et al., 2000) and urinary tract disease (Lund et al., 2006). Moreover, even moderately obese dogs have a reduced life expectancy and develop diseases such as osteoarthritis earlier in life (Kealy et al., 2002). According to Zoran et al. (2010), various breeds of dogs are more prone to develop obesity, such as Labrador Retrievers, Beagles and Boxers. Although highly studied, there is a little research relating the effects of obesity in the intestinal microbiota of dogs. Recently, obesity development has been associated with the presence or absence of certain gut microbial populations that affect dietary energy extraction and thus influence body weight and adiposity. It was found that obese individuals had greater frequency of phyla Firmicutes than Bacteroidetes when compared to their lean counterparts (Ley et al., 2005). To our knowledge, there is only one study that has compared the gut bacterial community in obese and lean dogs. Handl et al. (2013) evaluated the composition of the faecal microbiota in 22 lean and 21 obese pet dogs and they did not observe clear differences in microbial composition in these dogs.

Obesity also is associated with modifications in blood parameters, as cholesterol, triglycerides, insulin and leptin concentrations. Increased serum triglyceride and/or cholesterol concentrations have been observed in obese dogs (Bailhache et al., 2003; Jeusette et al., 2005a, Park et al., 2014). Significant decreases in both serum triglyceride and cholesterol concentrations were observed in obese dogs submitted to weight loss (Diez et al., 2004; Jeusette et al., 2005a). Jeusette et al. (2005b) also observed increases in leptin and insulin concentrations in obese dogs, with significant decreases of these levels during weight loss.

It was hypothesised that weight gain would result in detrimental changes in metabolic parameters and in the gut microbiota, but weight loss would reverse these effects. So, the aim of this study was to investigate whether weight gain and weight loss alter the blood parameters and the frequency of Firmicutes and Bacteroidetes in the faecal microbiota of adult Beagle dogs.

Materials and methods

Animals and diets

The Ethics Committee for Animal Welfare of the Federal University of Rio Grande do Sul granted approval of experimental protocols.

Ten adult (average age 3 y) intact Beagle dogs, owned by the Federal University of Rio Grande do Sul were used for this study. Five dogs were male and five were female. A 9-point body condition score (BCS) system was used (Laflamme, 2007), where a score of 4 and 5 were considered ideal, a score >5 and <7 was considered overweight,

and a score >7 was considered obese. At the beginning of the study, all dogs were considered with ideal BCS. The dogs were group housed by sex in a light (14-h-light/10-h-dark cycle) and temperature (18–24°C) controlled facility at the Federal University of Rio Grande do Sul, Porto Alegre, and were socialized twice a day. They were individually housed only to be fed twice a day and for feces and blood collections. Fresh water was available at all times, except 2 h prior to blood collections. All dogs had consumed a dry maintenance diet, a commercial, nutritionally complete and balanced extruded dry-type diet (Purina Proplan®) for 6 weeks prior to entering the study. Throughout the study, all dogs were fed the same batch of the diet. The same diet was also used during the weight loss phase. The nutrient composition of the diet is 26% protein, 15% fat, 37% carbohydrate, 3% crude fiber, and 7% ash (on a dry matter basis) (calculated metabolizable energy = 16.31 kJ/g on a dry matter basis). The diet meets the nutritional recommendations for adult dogs (AAFCO, 2008).

Study design

Prior to the start of the study, the health of each dog was assessed by physical examination and blood collection for serum chemistry and complete blood count measurements. The experiment was divided into 3 phases. In the first phase [lean (L)], the dogs consumed the diet for 6 weeks; at which time their body weights (BW) and food intakes were stable. During the 2nd phase [weight gain (WG)], the dogs were fed *ad libitum* over a period of 14 weeks, to increase their weight, when they reached the minimum BCS of 7. In the third phase [weight loss (WL)], the dogs were fed 60-70% of their previously measured energy intake to achieve weight loss until they reached a BCS

of 5 (13 weeks). Fresh feces and blood samples were taken at the end of all phases. Throughout the experiment, body weight was measured weekly and body condition score was determined every other week.

Parameters evaluated

Serum chemistry: Serum was separated by centrifugation from whole blood samples. These serum samples were then analyzed for glucose, cholesterol, triglycerides, albumin, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), total proteins (TP), insulin and leptin concentrations. Measurements were conducted as described in the directions of commercially available kits.

Fecal collection and quantitative assessment of phyla Firmicutes and Bacteroidetes by real time PCR: Fresh fecal samples for each dog were collected once daily over two consecutive days into sterile cryotubes, stored at -80° C, and then combined for homogeneity. A subsample was collected for DNA extraction. The abundances of Firmicutes and Bacteroidetes predominant microbial phyla in the canine gut were analyzed.

DNA Extraction and Polymerase Chain Reaction (PCR)

For the DNA extraction, 100 mg of the fecal sample was weighed and submitted to a Power Fecal® DNA Isolation Kit (MO BIO, CA), following the manufacturer's protocol. The total DNA extracted from all feces samples was carried out through a polymerase chain reaction (PCR). The PCR to bacterioidetes and firmicutes phylum was

carried out using the primers listed in the Table 1 in a reaction volume of 20 μL containing 1U Taq DNA polymerase and reaction buffer 1X (Invitrogen ®), 1.0 mM $MgCl_2$, 200 μM of dNTPs, 1 μL of genomic DNA, 0.4 μM of each primer (forward and reverse), completed with deionized water (MilliQ, Millipore ®). The PCR was performed in a thermocycler AMPLITHERM® model TX 96 and the thermal cycle conditions as follows: 94°C for 2 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 1 minute and 72°C for 15 seconds. PCR products were analyzed by electrophoresis gel with 1.5% agarose, stained with ethidium bromide solution and viewed under ultraviolet light.

Quantification of excreted ETEC

The quantitative PCR (qPCR) technique was used to determine the number of excreted Bacterioidetes and Firmicutes phylum cells μl^{-1} in the fecal samples. The oligonucleotides sequences used in this study are in Table 1. The primers sequence was determined by GenScript bioinformatics tool – PCR in Real Time (https://www.genscript.com/ssl-bin/app/primer). The positives controls for each reaction (firmicutes and bacterioidetes) were synthesized based on the fragment amplified by each respective primers and it's were described in Table 1.

All the qPCR reactions were realized in triplicates. Quantitative PCR reactions in real time were carried out in a final volume of 20 μL, containing 10 μL of 2X Fast-Plus Eva Green Master Mix (Biotium ®), 0.4 μM of each primer (forward and reverse), 1 μL of DNA, completed with deionized water (MilliQ, Millipore ®). The reactions were performed in the Eco®TM Real Time PCR System (Illumina ®). The DNA was amplified

by qPCR in real time using the thermal cycle conditions as follows: 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The melting curve was carried out immediately after the amplification with a linear temperature transition rate of 0.1°C.s^{-1} from 55 to 95°C with continuous determination of fluorescence acquisition.

To evaluate the performance of qPCR reaction efficiency, linear range of accurate quantization and sensitivity of each target gene serial dilutions of genomic DNA (DNAbact and DNAfirm) with known concentrations (standard curve) to amplification were made under the same conditions mentioned above. A dilution series to cover a wide range of concentrations included seven points, to ensure that the reaction was performed with the same efficiency at different concentrations. After that, based on preliminary assay, it was established four dilutions to run in each plate reaction assay. The graph generated by the genomic DNA concentration and respective CT values were tested and the R^2 of the regression equation calculated. The standard curve was built and the slope ratio value, which indicates the test sensitivity, led to the calculation of the efficiency. The standard curve was the log of the initial sample quantity against the number of qPCR cycles, and was generated by the Eco®TM Real Time software.

Statistical analysis

The experimental data was analyzed in a factorial design by ANOVA. Factors were sex (male and female) and experimental groups (lean, weight gain and weight loss), and the effects were evaluated by F-test. Means of the 3 groups tested were compared by Student-Newman-Keuls test.

Results

The animals showed no clinical changes throughout the experiment. There were effects of sex in BCS (P=0.016), cholesterol (P=0.016), triglycerides (P=0.008) and ALT (P=0.049). The females showed greater values when compared to the males, except for ALT. The average of the BCS of female was 6.2 vs. 5.5 of males, the average of cholesterol was 192.7 mg/dL vs. 139.2 mg/dL and the average of triglycerides was 53.8 mg/dL vs. 41.9 mg/dL (Table 2). The dogs had a significant increase (P<0.001) in body weight and body condition score during the weight gain phase. However, after energy restriction, the body weight returned to baseline. Glucose, creatinine, leptin and insulin were not statistically different (P>0.05) among the groups. Triglycerides, albumin, PA and ALT were greater (P<0.05) in the group of weight gain. Cholesterol and TP were greater (P<0.05) in the groups of weight gain and weight loss, as showed in Table 2.

Regarding to the faecal microbiota, the phylum Firmicutes showed no differences among the groups (P>0.05). The abundance of Bacteroidetes was greater (P<0.001) in the lean group (Table 3), with 282.9 copies/μl x 10^8 detected. After weight gain, this number decreased to 45.73 copies/μl x 10^8 and after weight loss the number of copies did not change statistically.

Discussion

Hyperlipidemia is being increasingly recognized as clinically important in dogs (Xenoulis and Steiner, 2010). In this study, significant increases in serum triglycerides

and total cholesterol occurred in obese dogs, in agreement with other studies (Bailhache et al., 2003; Jeusette et al., 2005a; Peña et al., 2008; Stone et al., 2009; Park et al., 2104, Okada et al., 2015). However, the averages of triglyceride and cholesterol level did not exceed 500mg/dL and 750mg/dL, respectively. It has been recommended that hypertriglyceridemia that exceeds 500 mg/dL and cholesterol levels over 750 mg/dL should be treated in order to avoid possible complications (Whitney, 1992; Ford, 1996). After weight loss, triglycerides concentrations returned to baseline, as in other researches (Diez et al., 2004; Jeusette et al., 2005a), while cholesterol levels did not differ statistically. This could be due to a short time of weight loss. The numbers suggest a decrease in cholesterol levels; possibly it would return to baseline levels if the weight loss period lasts longer.

Glucose, leptin and insulin were not statistically different ($P>0.05$). No effect of obesity or weight loss on glucose concentrations has been previously reported in dogs (Borne et al., 1996; Diez et al., 2004), except for two recent studies (Adolphe et al., 2014; Okada et al., 2015). Both researches induced weight gain in lean dogs and observed higher concentrations of glucose in obese animals. In this study, it was expected that leptin and insulin were greater in obese dogs, but this was not observed. Jeusette et al. (2005b) observed increases in leptin and insulin concentrations in obese dogs, with significant decreases of these levels during weight loss. As in humans and rodents, plasma leptin concentrations in dogs and cats increase with increasing body fat mass and adipocyte size (Adolphe et al., 2014; Park et al., 2014; Park et al., 2015) and leads to decreased after weight loss (Jeusette at al., 2005a; Jeusette et al., 2005b; Yamka et al., 2006).

In the present study, ALP and ALT were greater in obese dogs and returned to baseline after weight loss. A recent study also found lower ALT and ALP levels in obese dogs after weight loss (Peña et al., 2014). Okada et al. (2015) overfed dogs for 4 weeks and they found significant elevations in ALP after acute weight gain. In contrast, Diez et al. (2004), observed no effect of weight loss on plasma ALP and ALT. Although a recent research has shown that metabolic and cardiovascular alterations occur within only 12 weeks of obesity in an obese dog (Adolphe et al., 2014), some of the results observed in the current study may be due to a short time period of weight gain and weight loss. The variability of the dogs must also be taken into account.

As expected, the abundance of Bacteroidetes decreased after weight gain. However, the abundance of Firmicutes did not change after weight gain or weight loss. In humans and mice, it has been suggested that the presence of two phyla (called Firmicutes and Bacteriodetes) may play a role in increasing the body weight and fat mass of their host, thereby contributing to host obesity (Ley et al., 2006). It has been shown in obese humans and in obese mice that they possess higher gut populations of Firmicutes than Bacteriodetes, compared to their lean counterparts, regardless of how much food was consumed (Ley et al., 2005). According to Deng & Swanson (2015), similarly to the human gut, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria are the predominant microbial phyla in the canine and feline gut. However, their proportions vary among host species and individual researches. Variability may be due to animal (e.g. breed, diet and age), living environment or laboratory methodologies.

In dogs, only a few studies have been conducted. In one study, four major phyla

were identified in the colon of six healthy dogs using 16S rRNA gene amplicon-based Sanger sequencing: Firmicutes (47.7%); Proteobacteria (23.3%); Fusobacteria (16.6%); Bacteroidetes (12.4%) (Suchodolski et al., 2008). Using the same fecal samples of healthy dogs and two different methodologies, authors found different results. In one study, using 16S rRNA gene amplicon-based 454-pyrosequencing, the authors found Fusobacteria (23 – 40% of the reads), Firmicutes (14 – 28% of the reads), Bacteroidetes (31 – 34% of the reads), Actinobacteria (0.8 – 14% of the reads) and Proteobacteria (5 – 7% of the reads) to be the co-dominant phyla in canine faeces (Middelbos et al., 2010). In the other study, it was used shotgun 454-pyrosequencing and Bacteroidetes (37 – 38% of the sequences), Firmicutes (31 – 35% of the sequences), Proteobacteria (13 – 15% of the sequences), Fusobacteria (7 – 9% of the sequences) and Actinobacteria (1% of the sequences) were the dominant bacterial phyla in canine faeces (Swanson et al., 2011). In the present study, it was used quantitative real-time PCR and, for that reason, is difficult to compare the results. Garcia-Mazcorro et al. (2012) also found in healthy dogs discrepancies in faecal microbial abundance data obtained using FISH and 454-pyrosequencing for analysis.

Handl et al. (2013) evaluated the composition of the faecal microbiota in 22 lean and 21 obese pet dogs using both qPCR and pyrosequencing techniques did not observe clear differences in microbial populations of the dogs. In the present study, it was found lower abundance of Bacteroidetes in obese dogs, and weight loss did not reverse the effect of obesity. Kieler et al. (2012) also showed that overweight and obese cats had a significantly different gut microbiota when compared to lean cats. However, they found lower abundance of both Firmicutes and Bacteroidetes in the obese ones. With little data available, is possible to observe that similarities between human and animal gut

microbiota exist, although there is discrepancies of results regarding to the abundance of phyla and species of bacteria.

In conclusion, the obese status, even during a short time, causes metabolic responses in healthy dogs. The weight loss, per se, can revert or reduce the concentrations of some blood metabolites. The weight gain caused a decrease in the abundance of Bacteroidetes in the faecal microbiota and this effect was not changed by weight loss.

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Table 1. Sequence of nucleotide taxon-specific primer pairs used in this study.

Assay	**Primer name**	**Sequence 5' – 3'**	**Length (pb)**	**Reference**
Bacterioidetes	DNAbact	GGAACATGTGGTTTAATTCGA TGATACGCGAGGAACCTTACC CGGGCTTAAATTGCAGTGGAA TGATGTGGAAACATGTCAGTG AGCAATCACCGCTGTGAAGGT GCTGCATGGTTGTCGTCAGCT	126	This study
	Bacterioides_F	GGARCATGTGGTTTAATTCGA TGAT	25	Guo et al., 2008
	Bacterioides_R	AGCTGACGACARCCATGCAG	20	
Firmicutes	DNAfirm	GGAGYATGTGGTTTAATTCGA AGCAACGCGAAGAACCTTACC AGGTCTTGACATCCCTCTGAC ACTCCTAGAGATAGGGGGTCC CCTTCGGGGGCGAGGTGACAG GTGSTGCATGGYTGTCGTCAG CT	128	This study
	Firmicutes_F	GGAGYATGTGGTTTAATTCGA AGCA	25	Guo et al., 2008
	Firmicutes_R	AGCTGACGACARCCATGCAS	20	

DNAbact: Positive control of bacterioidetes phyla; DNAfirm: Positive control of firmicutes phyla; F: forward primer; R: reverse primer

Table 2. Body weight and body condition score (BCS); glucose, cholesterol, triglycerides, albumin, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), total proteins, insulin and leptin concentrations of dogs prior to and following weight gain and after weight loss.

					P Value		
	L	WG	WL	SEM	Sex	Treatment	Sex X Treatment
Characteristics							
Body weight (kg)	11.8[b]	15[a]	12.1[b]	1.22	0.073	0.001	0.452
BCS	4.9[b]	7.4[a]	5.25[b]	0.74	0.016	0.001	0.437
Serum chemistry							
Glucose (mg/dL)	98.7	99.9	89.5	9.38	0.861	0.081	0.163
Cholesterol (mg/dL)	114.8[b]	215.4[a]	167.6[a]	56.8	0.016	0.005	0.829
Triglyceride (mg/dL)	37.3[b]	61.3[a]	45[b]	11.3	0.008	0.001	0.326
Albumin (g/L)	22.14[c]	35.34[a]	31.17[b]	4.32	0.307	0.001	0.903
Creatinine (mg/dL)	0.63	0.74	0.67	0.14	0.559	0.601	0.986
ALP (U/L)	60.9[b]	130.1[a]	59.5[b]	58.2	0.440	0.059	0.344
ALT (U/L)	35.56[b]	65.85[a]	40.24[b]	12.2	0.049	0.001	0.269
Total Proteins (g/L)	45.48[b]	70.58[a]	68.21[a]	7.9	0.126	0.001	0.451
Insulin (uU/mL)	14.43	17.72	16.59	5.44	0.509	0.772	0.921
Leptin (ng/mL)	5.38	6.1	4.77	1.53	0.859	0.449	0.599

[a,b,c]Mean values with unlike superscript letters within a row were significantly different ($P<0{\cdot}05$).

Table 3. Details of Beagle dogs used in the study, showing the number of copies of faecal phyla Bacteroidetes and Firmicutes during the three phases of the trial.

Dog ID	Sex	Bacteroidetes (copies/μl x 10^8)[1]			Firmicutes (copies/μl x 10^8)[2]		
		L	WG	WL	L	WG	WL
1	M	382.5	385.5	0.0103	4.904	0.3795	0.196
2	F	436.5	0.0003	5.815	1.945	1.655	0.468
3	M	74.55	0.2455	3.86	3.505	0.777	0.0789
4	F	77.9	0.666	3.485	1.6	0.2841	0.4946
5	M	304	68.65	0.0351	0.6745	12.628	5.4210
6	F	339	0.397	0.0222	1.1597	1.7909	0.0233
7	F	401.5	0.0018	7.95	0.0703	0.397	0.0941
8	M	496.5	0.0013	7.11	3.3987	3.1266	1.0894
9	F	71	0.885	4.4	0.5578	0.2433	0.0041
10	M	245	0.914	8.915	0.0731	0.265	0.0040
Average		282.9[a]	45.73[b]	4.16[b]	1.79	2.15	0.79

[a,b]Mean values with unlike superscript letters within a row were significantly different (P<0·05).

[1]P values of sex: 0.3431; sex X treatment: 0.6919

[2]P values of sex: 0.0761; sex X treatment: 0.8069

CONSIDERAÇÕES FINAIS

A obesidade é uma doença preocupante entre clínicos veterinários, pois sua incidência em cães e gatos está crescendo rapidamente. As consequências são diversas e podem ser graves. Pouco se sabe sobre a associação entre obesidade e microbiota intestinal nesses animais e os estudos encontrados na literatura são controversos. Essa pesquisa teve como objetivo fornecer informações sobre a distribuição de certas populações da microbiota intestinal em relação à obesidade e restrição de energia em cães e gatos e, ainda, investigar se a castração poderia ser um fator de alteração na microbiota de gatos.

Em humanos e ratos, as bactérias do filo *Bacteroidetes* são predominantes nos indivíduos magros e as do filo *Firmicutes* predominantes nos obesos. No estudo com gatos, os animais magros castrados tiveram o perfil de microbiota fecal esperado para os obesos, ou seja, houve predominância de *Firmicutes*. Porém, isso não pode ser atribuído exclusivamente à castração, afinal dois outros grupos também eram de animais castrados (obesos). Não foram encontrados na literatura outros estudos comparando animais castrados e inteiros. Portanto, novas pesquisas são necessárias para elucidar esta questão. Já na pesquisa com os cães, a obesidade mostrou efeitos mais evidentes, com alterações em parâmetros sanguíneos e também na microbiota fecal. A perda de peso foi capaz de reverter alguns dos efeitos da obesidade temporária, porém não de todos.

Conhecer a composição da microbiota fecal desses animais pode ser o primeiro passo para uma nova alternativa de tratamento da obesidade. Os estudos futuros devem focar na investigação dos efeitos das manipulações dietéticas, prebióticas ou probióticas sobre a microbiota intestinal e do potencial para utilização no tratamento nutricional ou farmacêutico da obesidade e outras doenças.

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APÊNDICE 1. Normas para a publicação de artigos na revista British Journal of Nutrition

DETAILED MANUSCRIPT PREPARATION INSTRUCTIONS

Language

Papers submitted for publication must be written in English and should be as concise as possible. We recommend that authors have their manuscript checked by someone whose first language is English before submission, to ensure that submissions are judged at peer review exclusively on academic merit.

We list a number of third-party services specialising in language editing and / or translation, and suggest that authors contact as appropriate. Use of any of these services is voluntary, and at the author's own expense.

Spelling should generally be that of the Concise Oxford Dictionary (1995), 9th ed. Oxford: Clarendon Press. Authors are advised to consult a current issue in order to make themselves familiar with BJN as to typographical and other conventions, layout of tables etc. Sufficient information should be given to permit repetition of the published work by any competent reader of BJN.

Published examples of BJN article types can be found below:

Research Article
Review Article
Horizons Article
Letter to the Editor

Authorship

The Journal conforms to the International Committee of Medical Journal Editors (ICMJE) definition of authorship, as described by P.C. Calder (Br J Nutr (2009) 101, 775).

The contribution of individuals who were involved in the study but do not meet these criteria should be described in the Acknowledgments section.

Ethical standards

The required standards for reporting studies involving humans and experimental animals are detailed in an Editorial by G.C. Burdge (Br J Nutr (2014) 112).

Experiments involving human subjects

The notice of contributors is drawn to the guidelines in the World Medical Association (2000) Declaration of Helsinki: ethical principles for medical research involving human subjects, with notes of clarification of 2002 and 2004 (http://www.wma.net/en/30publications/10policies/b3/), the Guidelines on the Practice of Ethics Committees Involved in Medical Research Involving Human Subjects (3rd ed., 1996; London: The Royal College of Physicians) and the Guidelines for the ethical conduct of medical research involving children, revised in 2000 by the Royal College of Paediatrics and Child Health: Ethics Advisory Committee (Arch Dis Child (2000) 82, 177–182). Articles reporting randomised trials must conform to the standards set by the Consolidated Standards of Reporting Trials (CONSORT) consortium.

Required disclosures: A paper describing any experimental work on human subjects must include the following statement in the Experimental Methods section: “This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients

were approved by the [insert name of the ethics committee; a specific ethics number may be inserted if you wish]. Written [or Verbal] informed consent was obtained from all subjects/patients. [Where verbal consent was obtained this must be followed by a statement such as: Verbal consent was witnessed and formally recorded].” For clinical trials, the trial registry name, registration identification number, and the URL for the registry should be included.

PLEASE NOTE: From 1 October 2014, as a condition for publication, all randomised controlled trials that involve human subjects submitted to BJN for review must be registered in a public trials registry. A clinical trial is defined by the ICMJE (in accordance with the definition of the World Health Organisation) as any research project that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes. Registration information must be provided at the time of submission, including the trial registry name, registration identification number, and the URL for the registry.

Experiments involving the use of other vertebrate animals

Papers that report studies involving vertebrate animals must conform to the ‘ARRIVE Guidelines for Reporting Animal Research’ detailed in Kilkenny et al. (J Pharmacol Pharmacother (2010) 1, 94-99) and summarised at www.nc3rs.org.uk. Authors must ensure that their manuscript conforms to the checklist that is available from the nc3Rs website. The attention of authors is drawn particularly to the ARRIVE guidelines point 3b (‘Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study’s relevance to human biology’, point 9c (‘Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment’) and point 17a (‘Give details of all important adverse events in each experimental group’). The Editors will not accept papers reporting work carried out involving procedures that cause or are considered likely to cause distress or suffering which would confound the outcomes of the experiments, or experiments that have not been reviewed and approved by an animal experimentation ethics committee or regulatory organisation.

Required disclosures: Where a paper reports studies involving vertebrate animals, authors must state in the Experimental Methods section the institutional and national guidelines for the care and use of animals that were followed and that all experimental procedures involving animals were approved by the [insert name of the ethics committee or other approving body; wherever possible authors should also insert a specific ethics/approval number].

Manuscript Format

The requirements of BJN are in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals produced by the ICMJE.

Typescripts should be prepared with 1.5 line spacing and wide margins (2 cm), the preferred font being Times New Roman size 12. At the ends of lines, words should not be hyphenated unless hyphens are to be printed. Line numbering and page numbering are required.

Manuscripts should be organised as follows:

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Papers should be accompanied by a cover letter including a brief summary of the work and a short explanation of how it advances nutritional science. The text for the cover letter should be entered in the appropriate box as part of the online submission process.

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The title page should include:

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Each paper must open with an unstructured abstract of not more than 250 words. The abstract should be a single paragraph of continuous text without subheadings outlining the aims of the work, the experimental approach taken, the principal results (including effect size and the results of statistical analysis) and the conclusions and their relevance to nutritional science.

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It is not necessary to introduce a paper with a full account of the relevant literature, but the introduction should indicate briefly the nature of the question asked and the reasons for asking it. It should be no longer than two manuscript pages.

Experimental methods

The methods section must include a subsection that describes the methods used for statistical analysis (see the section on statistical analysis in the Appendix) and the sample size must be justified by the results of appropriate calculations and related to the study outcomes.

For studies involving humans subjects or experimental animals, the Methods section must include a subsection that reports the appropriate ethical approvals for the study (see Ethical Standards above).

All analytical procedures must be accompanied by a statement of within and between assay precision.

Diets: The nutrient composition of diets used in studies published in BJN must be described in detail, preferably in a table(s). Experimentally relevant differences in composition between diets are essential. For instance, studies of fat nutrition should always include fatty acid compositions of all diets.

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These should be given as concisely as possible, using figures or tables as appropriate. Data must not be duplicated in tables and figures.

Discussion

While it is generally desirable that the presentation of the results and the discussion of their significance should be presented separately, there may be occasions when combining these sections may be beneficial. Authors may also find that additional or alternative sections such as ‘conclusions’ may be useful. The discussion should be no longer than five manuscript pages.

Acknowledgments

Here you may acknowledge individuals or organizations that provided advice and/or support (non-financial). Formal financial support and funding should be listed in the following section.

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Please provide a very brief description of the contribution of each author to the research. Their roles in formulating the research question(s), designing the study, carrying it out, analysing the data and writing the article should be made plain.

References

References should be numbered consecutively in the order in which they first appear in the text using superscript Arabic numerals in parentheses, e.g. 'The conceptual difficulty of this approach has recently been highlighted[1,2]'. If a reference is cited more than once, the same number should be used each time. References cited only in tables and figure legends should be numbered in sequence from the last number used in the text and in the order of mention of the individual tables and figures in the text.

Names and initials of authors of unpublished work should be given in the text as 'unpublished results' and not included in the References. References that have been published online only but not yet in an issue should include the online publication date and the Digital Object Identifier (doi) reference, as per the example below.

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Journal articles

Rebello SA, Koh H, Chen C et al. (2014) Amount, type, and sources of carbohydrates in relation to ischemic heart disease mortality in a Chinese population: a prospective cohort study. Am J Clin Nutr 100, 53-64.

Villar J, Ismail LC, Victora CG et al. (2014) International standards for newborn weight, length, and head circumference by gestational age and sex: the Newborn Cross-Sectional Study of the INTERGROWTH-21st Project. Lancet 384, 857-868.

Alonso VR & Guarner F (2013) Linking the gut microbiota to human health. Br J Nutr 109, Suppl. 2, S21–S26.

Bauserman M, Lokangaka A, Gado J et al. A cluster-randomized trial determining the efficacy of caterpillar cereal as a locally available and

sustainable complementary food to prevent stunting and anaemia. Public Health Nutr. Published online: 29 January 2015. doi: 10.1017/S1368980014003334.

Books and monographs

Bradbury J (2002) Dietary intervention in edentulous patients. PhD Thesis, University of Newcastle.

Ailhaud G & Hauner H (2004) Development of white adipose tissue. In Handbook of Obesity. Etiology and Pathophysiology, 2nd ed., pp. 481–514 [GA Bray and C Bouchard, editors]. New York: Marcel Dekker.

Bruinsma J (editor) (2003) World Agriculture towards 2015/2030: An FAO Perspective. London: Earthscan Publications.

World Health Organization (2003) Diet, Nutrition and the Prevention of Chronic Diseases. Joint WHO/FAO Expert Consultation. WHO Technical Report Series no. 916. Geneva: WHO.

Keiding L (1997) Astma, Allergi og Anden Overfølsomhed i Danmark – Og Udviklingen 1987–199I (Asthma, Allergy and Other Hypersensitivities in Denmark, 1987–1991). Copenhagen, Denmark: Dansk Institut for Klinisk Epidemiologi.

Sources from the internet

Nationmaster (2005) HIV AIDS – Adult prevalence rate. http://www.nationmaster.com/graph-T/hea_hiv_aid_adu_pre_rat (accessed June 2013).

Figures

Figures should be supplied as separate electronic files. Figure legends should be grouped in a section at the end of the manuscript text. Each figure should be clearly marked with its number and separate panels within figures should be clearly marked (a), (b), (c) etc. so that they are easily identifiable when the article and figure files are merged for review. Each figure, with its legend, should be comprehensible without reference to the text and should include definitions of abbreviations. The nature of the information displayed in the figures (e.g. mean (SEM)) and the statistical test used must be stated.

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Footnotes are given in the following order: (1) abbreviations, (2) superscript letters, (3) symbols. Abbreviations are given in the format: RS, resistant starch. Abbreviations in tables must be defined in footnotes in the order that they appear in the table (reading from left to right across the table, then down each column). Symbols for footnotes should be used in the sequence: *†‡§||¶, then ** etc. (omit * or †, or both, from the sequence if they are used to indicate levels of significance).

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Additional data (e.g. data sets, large tables) relevant to the paper can be submitted for publication online only, where they are made available via a link from the paper. The paper should stand alone without these data. Supplementary Material must be cited in a relevant place in the text of the paper.

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Original Articles

Original articles represent the most common form of articles published in the journal. Typically they describe the results of experiments carried out in order to test a novel hypothesis. Original articles should contain the following sections: Summary, Introduction, Materials and Methods, Results, Discussion, References.

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Short communications are brief articles that present particularly novel or exciting results, introduce new theories or ideas, or offer new methodological approaches. This format provides an opportunity for authors to (a) provide important results in concise form or (b) introduce significant new concepts or methods that are supported by a limited empirical data set. The papers should be highly original and represent ideas that will challenge current paradigms or approaches. They should stimulate thought, serving as precursors to new research programs or working groups. In these manuscripts the headings required for original articles may be omitted, but the structure of the paper should more or less be the same. The length of the short communication should not exceed 3500 words plus 1-2 tables or figures.

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The title should not exceed 35 words. Please provide a short title of 60 characters or less for the running head. List all the authors and their affiliations, and indicate the corresponding author by a footnote named “correspondence” where name, the complete postal address, telephone and fax numbers as well as e-mail address are given.

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The summary should not exceed 300 words, while giving the major objectives, methods, results, conclusions and practical applications of the research.

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Include up to 6 keywords. Keywords will be used for indexing purposes, as will the title; therefore please select words that are not included in the title.

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Include any acknowledgement before the reference list.

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VITA

Manuela Marques Fischer, filha de Maria Isabel Rodrigues Marques e Décio Lartigau Fischer, nasceu em 22 de março de 1982 em Porto Alegre. Cursou a maior parte do ensino fundamental e médio no Colégio São José, em Pelotas.

Ingressou no Curso de Medicina Veterinária da Universidade Federal do Rio Grande do Sul (UFRGS) no segundo semestre de 2002 e obteve o título da graduação em dezembro de 2008. Fez diversos estágios no Hospital de Clínicas Veterinárias da UFRGS, no Setor de Suínos e na Coordenadoria de Inspeção Industrial e Sanitária dos Produtos de Origem Animal – CISPOA. No último ano da graduação estagiou no Laboratório de Ensino Zootécnico – LEZO. Cursou o estágio curricular no Laboratório de Pesquisa em Nutrição e Doenças Nutricionais de Cães e Gatos da UNESP-Jaboticabal.

Em março de 2009, ingressou no Programa de Pós-Graduação em Zootecnia da UFRGS, área de concentração "Produção Animal", linha de pesquisa em "Nutrição e Alimentação de Não-Ruminantes". Obteve o título de Mestre em Zootecnia em fevereiro de 2011, com a defesa da dissertação intitulada "Efeitos de diferentes fontes de fibra na digestibilidade de nutrientes, nas respostas metabólicas pós-prandiais e na saúde intestinal de gatos" com orientação do Professor Alexandre de Mello Kessler e co-orientação do Professor Aulus Cavalieri Carciofi.

Em março de 2010, implementou o Setor de Nutrição Clínica de Cães e Gatos do HCV da UFRGS, onde desde então é coordenadora.

Em abril de 2011, ingressou no Doutorado no Programa de Pós-Graduação em Zootecnia da UFRGS, área de concentração "Produção Animal", linha de pesquisa em "Nutrição e Alimentação de Não-Ruminantes" com orientação do Professor Alexandre de Mello Kessler. De agosto de 2012 a janeiro de 2013, foi bolsista do programa de Doutorado Sanduíche no grupo de pesquisa liderado pela Prof[a] Andrea Fascetti na Universidade da California, em Davis (UCDavis).

Durante a vida acadêmica participou de projetos de pesquisa em nutrição de aves, suínos, cães e gatos, apresentou trabalhos em congressos nacionais, ministrou aulas para graduação, pós-graduação, publicou artigos e resumos. Atualmente trabalha como responsável técnica de empresas do ramo da nutrição pet e é coordenadora e professora do primeiro curso de pós-graduação *lato sensu* em nutrição de cães e gatos do país.