

**UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS DE JABOTICABAL**

**EFEITO DE IDADE E DIETAS COM DIFERENTES FONTES
DE PROTEÍNA E CARBOIDRATO SOBRE A MICROBIOTA
ASSOCIADA À MUCOSA GASTROINTESTINAL DE CÃES**

Ana Paula Judice Maria

Médica Veterinária

2017

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Orientador: Prof. Dr. Aulus Cavalieri Carciofi

**Tese de Doutorado apresentado à
Faculdade de Ciências Agrárias e
Veterinárias – Unesp, Campus de
Jaboticabal, como pré-requisito para a
obtenção do título de Doutora em Medicina
Veterinária, área Clínica Médica Veterinária**

2017

M332e Maria, Ana Paula Judice
Efeito da idade e dietas com diferentes fontes de proteína e carboidrato sobre a microbiota associada à mucosa gastrointestinal de cães / Ana Paula Judice Maria. -- Jaboticabal, 2017
v, 80 p. ; 29 cm

Tese (doutorado) - Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, 2017

Orientador: Aulus Cavalieri Carciofi

Banca examinadora: Marita Vedovelli Cardozo, Márcia de Oliveira Sampaio Gomes, Thaila Cristina Putarov, Lilian Rose Marques de Sá
Bibliografia

1. Cães. 2. Microbiota. 3. Gastrointestinal. I. Título. II. Jaboticabal-Faculdade de Ciências Agrárias e Veterinárias.

CDU 619:561.23:636.7

CERTIFICADO DE APROVAÇÃO

TÍTULO: EFEITO DA IDADE E DIETAS COM DIFERENTES FONTES DE PROTEÍNA
E CARBOIDRATO SOBRE A MICROBIOTA ASSOCIADA À MUCOSA
GASTROINTESTINAL DE CÃES

AUTORA: ANA PAULA JUDICE MARIA

ORIENTADOR: AULUS CAVALIERI CARCIOFI

Aprovada como parte das exigências para obtenção do Título de Doutora em MEDICINA
VETERINÁRIA, área: CLÍNICA MÉDICA VETERINÁRIA pela Comissão Examinadora:



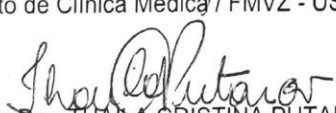
Prof. Dr. AULUS CAVALIERI CARCIOFI
Departamento de Clínica e Cirurgia Veterinária / FCAV / UNESP - Jaboticabal



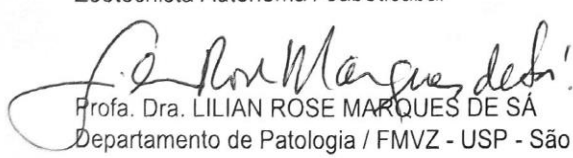
Pós-doutoranda MARITA VEDOVELLI CARDOZO
Departamento de Patologia Veterinária / FCAV / UNESP - Jaboticabal



Profa. Dra. MÂRCIA DE OLIVEIRA SAMPAIO GOMES
Departamento de Clínica Médica / FMVZ - USP - São Paulo, SP



Pesquisadora Dra. THAILA CRISTINA PUTAROV
Zootecnista Autônoma / Jaboticabal



Profa. Dra. LILIAN ROSE MARQUES DE SÁ
Departamento de Patologia / FMVZ - USP - São Paulo, SP

Jaboticabal, 31 de março de 2017.

DADOS CURRICULARES DO AUTOR

ANA PAULA JUDICE MARIA – Nascida em 23 de julho de 1983, em Ribeirão Preto – SP. Graduada em Medicina Veterinária pela Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho” (Unesp), Câmpus de Jaboticabal em fevereiro de 2010. Em Março de 2011 iniciou o curso de mestrado pelo programa de pós-graduação em Medicina Veterinária (Clínica Médica) da Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho” (FCAV/UNESP), concluindo-o em julho de 2013. Em agosto de 2013, iniciou o curso de doutorado pelo mesmo programa e instituição. Realizou doutorado sanduíche financiado pela Capes (Programa de Doutorado-sanduíche no Exterior - PDSE), no período de março de 2014 a março de 2015, junto ao Departamento de Ciência Animal (Department of Animal Science) da Universidade de Illinois em Urbana-Champaign (University of Illinois at Urbana-Champaign - UIUC), no Estados Unidos da America.

Dedico

Aos meu pais,
pelos exemplos de vida e fraternidade

AGRADECIMENTOS

Agradeço a Deus por estar presente e pela luz no meu caminho.

Agradeço a minha família por todo amor e carinho que recebo.

A minha mãe Rosa Maria Judice Maria, minha eterna amiga e companheira, pelo amor, compreensão e por sempre acreditar nos meus sonhos. Agradeço ao meu pai Fausto Maria, *in memoriam*, pelo exemplo de vida e por todos os ensinamentos deixados.

A minha irmã Carla Cristina Judice Maria e aos meus irmãos Fausto Maria Junior e Carlos Eduardo Judice Maria, pela dedicação, amizade e pelos inúmeros momentos de alegria que me proporcionam.

Ao meu orientador Aulus Cavalieri Carciofi, exemplo de dedicação à ciência, por orientar com sabedoria e paciência, e pela oportunidade de crescimento profissional e pessoal.

Ao Prof. Kelly Scott Swanson da Universidade de Illinois por contribuir com o projeto e pela orientação durante meu período de doutorado sanduiche.

A Dra. Ping Deng e a Profa Hannah D. Holscher por toda a contribuição com o projeto e dedicação e paciência ao meu ensinar.

A professora Lilian Rose Marques de Sá por sua orientação, disponibilidade e paciência, também por ser exemplo de dedicação a ciência e a educação universitária.

A professora Márcia de Oliveira Sampaio Gomes por compartilhar comigo sua experiência e principalmente pela sua amizade.

Aos membros da banca de qualificação e de defesa, Profa. Lilian Rose Marques de Sá, Profa. Márcia de Oliveira Sampaio Gomes Dra. Marita Vedovelli Cardozo, Dra. Thaila Cristina Putarov e Dra. Bruna Agy Loureiro pelas contribuições na melhoria deste trabalho.

Ao médico veterinário endoscopista, Franz N. Yoshitoshi, por sua fundamental participação neste estudo. Muito obrigada pela generosa disponibilidade e excelente trabalho exercido na execução deste trabalho.

Aos médicos veterinários anestesistas Ana Paula Gering e Raul Honda pela participação neste estudo. Muito obrigada pelo excelente trabalho e disponibilidade.

A Elaine, Claudinha e ao Diego pela amizade e carinho, e por toda a ajuda que recebi de vocês durante a minha pós graduação.

As minhas amigas Caroline, Renata, Mariana (Brucelinha), Nara, Bruninha, Carol Nagib, Raquelzinha, Mari Monte e Thailinha pela paciência, companheirismo e amizade sincera.0

Ao Renato pelo companheirismo, amizade, dedicação e paciência comigo.

Agradeço novamente aos animais que participaram desse estudo, pois também representam o amor de Deus; Graça, Virgilio, Conta, Leo, Leo Jr., Napoleão Jr., Juca, Peri, Luizinho, Ondina, Rímel, Marina, Poulain, Stephanie, Coca, Pepita, Sasha, Manu, Simba, Scooby, Scooty, Sivi, Pepe, Spike, Sheyk, Marley e Joaquim. Agradeço a todos os cães e gatos do Laboratório de Pesquisa em Nutrição de Cães e Gatos, por me proporcionarem ótimos momentos e amor.

A todo o grupo do Laboratório de Pesquisa em Nutrição de Cães e Gatos, pós-graduandos, estagiários e funcionários, sem ajuda de vocês esse trabalho não seria realizado.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela pelo auxílio financeiro do estudo (nº processo Fapesp 2011/23575-3). À Capes pela bolsa de estudo do Programa de Doutorado-sanduíche no Exterior (PDSE) pela bolsa de estudo do Programa de Demanda Social (DS).

À GUABI PET CARE, Campinas, SP, Brazil, pela estrutura e manutenção do Laboratório de Pesquisa em Nutrição e Doenças Nutricionais de Cães e Gatos “Prof. Dr. Flávio Prada”.

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Projeto intitulado “**Efeito de idade e dietas com diferentes fontes de proteína e carboidrato sobre a microbiota e a imunidade associadas à mucosa do trato gastrointestinal de cães**”, protocolo nº 546/16, sob a responsabilidade do Prof. Dr. Aulus Cavalieri Carciofi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL - SP, em reunião ordinária de 16 de fevereiro de 2016.

Vigência do Projeto	01/03/2016 a 30/12/2016
Espécie / Linhagem	Canina
Nº de animais	36
Peso / Idade	Média de 9kg / Cães Adultos média de 4 anos; cães idosos média de 10 anos.
Sexo	Machos e fêmeas
Origem	Canil experimental do Laboratório de Nutrição e Doenças Nutricionais Prof. Dr. Flávio Prada

Jaboticabal, 16 de fevereiro de 2016.



Profª Drª Lizandra Amoroso
 Coordenadora – CEUA

EFEITO DE IDADE E DIETAS COM DIFERENTES FONTES DE PROTEÍNA E CARBOIDRATO SOBRE A MICROBIOTA ASSOCIADA A MUCOSA DO TRATO GASTROINTESTINAL DE CÃES

RESUMO - O presente estudo avaliou e comparou a composição da microbiota associada à mucosa gastrointestinal de cães adultos e idosos, alimentados com rações contendo proteína de origem animal ou vegetal e fibras de diferentes fermentabilidades. O estudo também comparou a composição da microbiota associada a mucosa do duodeno, jejuno e cólon. O ensaio seguiu esquema fatorial 3 x 2, com três rações e duas idades. Foram utilizados 18 cães adultos ($2,6 \pm 0,9$ anos) e 18 cães idosos ($10,2 \pm 1,0$ anos). Foram produzidas três dietas: dieta NFF com fibra insolúvel não fermentável a base de cana-de-açúcar e farinha de vísceras de frango; dieta FF com polpa de beterraba, fibra fermentável e parcialmente solúvel, e farinha de vísceras; dieta SM com 30% de farelo de soja em substituição a farinha de vísceras de frango. Os animais foram submetidos a 30 dias de adaptação à dieta e nos dias 31 e 32 foram realizadas as endoscopias e colonoscopias para coleta de fragmentos do duodeno, jejuno e colon, para posterior análise da microbiota associada à mucosa gastrointestinal através do sequenciamento Illumina. Os dados foram avaliados por análise de variância e médias comparadas pelo teste de Tukey. Dados sem distribuição normal foram submetidos a transformação logarítmica na base 10, $\log(x + 1)$ ou raiz quadrada antes da análise estatística, e quando necessário submetidos ao teste não paramétrico de Kruskal-Wallis. A avaliação das dietas e do efeito da idade sobre a mucosa do cólon demonstraram que cães idosos e adultos possuem uma microbiota associada à mucosa do cólon diversa e com grupos bacterianos predominantes iguais. O envelhecimento mostrou alterações na microbiota da mucosa do cólon canino com menor abundância de algumas famílias e gêneros em cães idosos. Os macronutrientes também levaram a alterações na microbiota associada com a mucosa do cólon de cães. As fontes de fibra que possuem diferentes perfis de fermentação levaram a diferentes abundâncias de algumas bactérias. A dieta NFF levou a uma maior abundância de *[Prevotella]* e Lachnospiraceae gênero indefinido, já as dieta FF e SM acarretaram em maior abundância de Veillonellaceae e *Megamonas* na mucosa do cólon. Também a fonte de proteína de origem animal levou a uma maior abundância de grupos bacterianos relacionados com a degradação de proteínas, aminoácidos e derivados, tais como Peptococcaceae, *Peptococcus* e *Slackia*. A avaliação da microbiota associada à mucosa do jejuno, do duodeno e do cólon mostrou mudanças nos grupos de bactérias predominantes entre o intestino delgado (duodeno e jejuno) e o cólon. A caracterização da microbiota associada à mucosa gastrointestinal em cães saudáveis pelo sequenciamento Illumina é útil para a compreensão da diversidade das comunidades intestinais microbianas nos cães, e permitiu acessar o núcleo normal de alguns grupos bacterianos que podem estar alterados em doenças gastrointestinais. Portanto, o estudo demonstrou que a idade e os macronutrientes podem alterar a microbiota associada à mucosa do cólon, como também mostrou que o jejuno, duodeno e cólon abrigam o mesmo grupo de bactérias, mas em diferentes concentrações.

Palavras-chave: microbiota, mucosa, trato gastrointestinal, fibra, proteína.

EFFECT OF AGE AND DIETS WITH DIFFERENT SOURCES OF PROTEIN AND CARBOHYDRATE ON THE MICROBIOTA ASSOCIATED WITH THE GASTROINTESTINAL MUCOSA OF DOGS

ABSTRACT - The present study evaluated and compared the microbiota composition associated with gastrointestinal tract mucosa of adult and elderly dogs, fed with animal or vegetable protein and fiber of different fermentabilities. The study also compared the composition of the microbiota associated with the mucosa of the duodenum, jejunum and colon. The assay followed a 3 x 2 factorial scheme, with three rations and two ages, generating six experimental treatments. Three blocks of 12 dogs each were used, six adult dogs (2.6 ± 0.9 years) and six elderly (10.2 ± 1.0 years). The experimental diets were: diet with non-fermentable insoluble fiber based on sugarcane (NFF) and chicken offal meal; Diet with 30% of soybean meal (SM), replacing the flour of chicken viscera; Diet with fermentable and partially soluble fiber based on beet pulp (FF). Each block was structured as follows: day one to day 30 adaptation to the diet; day 31 and 32 endoscopies and colonoscopies with collection of fragments of the duodenum, jejunum and colon, for further analysis of microbiota associated to the gastrointestinal mucosa by Illumina sequencing. The data was evaluated by analysis of variance by the SAS MIXED procedure. Averages were compared by the Tukey test ($P < 0.05$). Data without normal staining were submitted to log 10 base transformation, $\log(x + 1)$ or square root before statistical analysis, and when necessary submitted to non-parametric Kruskal-Wallis test. The evaluation of diets and age effect on colon mucosa demonstrated that old and adults dogs harbored a diverse colon mucosa microbial profile, with the same predominant bacterial groups. Ageing showed changes in the canine colon mucosa microbiota with lower abundance of some family and genus groups of old dogs. Macronutrients related changes in the microbiota associated with the colon mucosa of dogs. The fiber sources with different fermentation profiles led to different abundances of some bacteria. The NFF diet led to higher abundance of *[Prevotella]* and Lachnospiraceae undefined genus and the FF and SM diet led to higher abundance of Veillonellaceae and *Megamonas*. In addition, the protein source of animal origin led to a higher abundance of bacteria groups related to protein degradation, amino acids and derivatives, such as Peptococcaceae, *Peptococcus* and *Slackia*. The evaluation of the microbiota associated with the mucosa of the jejunum, duodenum and colon showed many bacterial shifts between the small intestine (duodenum and jejunum) and the colon in the canine intestinal tract. The characterization of the microbiota associated with the gastrointestinal mucosa of healthy dogs by Illumina sequence present in this study is helpful to understanding the diversity of the microbial intestinal communities in the dogs and allowed the normal core of some bacterial groups that might be altered in gastrointestinal disease. Therefore, the study demonstrated that age and macronutrients may alter the microbiota associated with the colonic mucosa. The study also showed that the jejunum, duodenum and colon harbor the same group of bacteria, but in different concentrations.

Keywords: microbiota, mucosa, gastrointestinal tract, fiber, protein.

CAPÍTULO 1 – Considerações Gerais

1. INTRODUÇÃO

O microbioma do trato gastrointestinal é um ecossistema complexo e devido às diferenças anatômicas e fisiológicas, cada compartimento intestinal abriga um único ecossistema microbiano único. Cada cão também abriga um perfil microbiano muito singular e individual (SUCHODOLSKI et al., 2005). As bactérias presentes nesses micorbiomas realizam funções especializadas utilizando nutrientes advindos da dieta do hospedeiro e, em troca, fornecem metabólitos para a absorção do hospedeiro (SUCHODOLSKI, 2011a). Também existem diferenças na composição e no número total de bactérias nos diferentes compartimentos do trato trato gastrointestinal. A contagem bacteriana total e a riqueza de espécies aumentam ao longo do trato gastrointestinal e variam também entre o lúmen intestinal e a mucosa (MENTULA et al., 2005). As bactérias pertencentes aos filos Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, e Actinobacteria constituem mais de 99% de toda a microbiota intestinal de cães. Os grupos bacterianos remanescentes são representados pelos filos Spirochaetes, Tenericutes, Verrucomicrobia, Cyanobacteria, Chloroflexi e algumas linhagens bacterianas não classificadas (SUCHODOLSKI, 2011a; SWANSON et al., 2011).

A capacidade da microbiota intestinal em degradar a dieta e produzir ácidos graxos de cadeia curta é de suma importância para a saúde do trato gastrointestinal (TOPPING; CLIFTON, 2001). Esses ácidos graxos exercem papel fundamental na fisiologia do cólon, uma vez que, eles constituem a principal fonte de energia para os enterócitos e colonócitos, estimulam a proliferação celular do epitélio e do fluxo sanguíneo visceral, e intensificam a absorção de sódio e água, reduzindo a carga osmótica do carboidrato (SUNVOLD et al., 1995).

Pesquisas demonstraram os benefícios da alimentação com fibras alimentares e prebióticos para cães, incluindo um aumento na produção de butirato, acetato, propionato, lactato e ácido graxo de cadeia curta (VICKERS et al., 2001), aumento na produção fecal Bifidobactérias e diminuição nas concentrações de fenol fecal e indolo (SWANSON et al., 2002; FLICKINGER et al., 2003). Também é

reconhecido que as proteínas de origem animal aumentam as concentrações de produtos putrefativos e *Clostridium* spp nas fezes de cães (ZENTEK et al., 2003),

Além de sua reconhecida função em prover nutrientes ao organismo, o trato gastrointestinal é órgão imunológico muito ativo, que tem estrutura complexa e alberga diversos tipos celulares especializados que cumprem papel importante na proteção contra o ambiente externo (CUNNINGHAM-RUNDLES; LIN, 1998). Assim, a dieta deve fornecer adequadamente nutrientes para o trato gastrointestinal, aspecto importante para dar suporte a um bom desenvolvimento e funcionamento deste órgão. A fonte principal de nutrientes para a mucosa intestinal são os compostos absorvidos do lúmen. Estes compostos absorvidos do lúmen advêm dos ingredientes da dieta ou são produzidos e liberados pela microbiota intestinal (SUCHODOLSKI et al., 2011a). Gomes et al. (2011) demonstraram menor atividade fermentativa no intestino grosso de cães idosos, o que poderia estar correlacionado ao processo de imunosenescência já demonstrado em cães (DAY, 2010).

Diante do exposto, a utilização de diferentes fontes de proteína e de fibras podem modular a microbiota associada à mucosa do colon de cães, podendo interferir diferente em cães adultos e idosos. assim como a caracterização da microbiota intestinal de cães idosos. Como segunda hipótese do estudo, a microbiota associada a mucosa do trato gastrointestinal difere entre os segmentos do mesmo. Portanto, o objetivo desse estudo foi caracterizar o efeito de idade e dietas com proteínas de origem animal ou vegetal e diferentes tipos de fibra sobre a microbiota associada a mucosa do colon, como também caracterizar a microbiota associada a mucosa do duodeno, jejuno e colon de cães.

2. REVISÃO DE LITERATURA

2.1 Microbiota intestinal

O microbioma do trato gastrointestinal é composto por bactérias, arqueobactérias, fungos, protozoários e vírus e pode ser definido como o agregado de todos esses microrganismos que habitam o trato gastrointestinal e o sistema de interações que esses organismos têm entre si e com as células hospedeiras

(BLAKE: SUCHODOLSKI, 2016). A microbiota do intestino é um ecossistema complexo e diverso, mantendo uma variedade de papéis que contribuem para a saúde em geral. A relação simbiótica que existe entre a microbiota e o trato gastrointestinal do hospedeiro é crítica para o bom funcionamento dos processos nutricionais, de desenvolvimento, imunológicos e fisiológicos em animais e, assim, contribui para a saúde do hospedeiro (CUNNINGHAM-RUNDLES; LIN; 1998; HOOPER et al, 2001; OUWEHAND et al., 2005).

Uma das funções atribuída a microbiota intestinal é a contribuição para a nutrição e metabolismo do hospedeiro. Substratos não digeridos que chegam ao lúmen do cólon são fermentados pelas bactérias residentes e como resultado da atividade bioquímica da microbiota, ocorre a formação dos ácidos graxos de cadeia curta (acetato, propionato e butirato). O ácidos graxos de cadeia curta, constituem a principal fonte de energia para os enterócitos e colonócitos, estimulam a proliferação celular do epitélio (SUNVOLD et al., 1995).

Enquanto muitas bactérias intestinais utilizam carboidratos, algumas fermentam proteínas quando abundante na dieta e/ou no cólon (MACFARLANE; MACFARLANE, 2012). A fermentação microbiana da proteína e aminoácidos não digeridos e absorvidos pelo intestino gera ácidos graxos de cadeia ramificada, compostos putrefativos, como fénel, indol, amônia e algumas aminas biogênicas. (CUMMINGS; MACFARLANE, 1991; SMITH; MACFARLANE, 1997). Os catabólitos da proteína não só resultam em mau odor das fezes, mas podem também ser tóxicos em altas concentrações (KUZMUK et al., 2005). Já o consumo de carboidratos fermentáveis, como amido resistente, polissacarídeos não-amiláceos como os mananoligossacarídeos, frutoligossacarídeos, estaquiase, rafinose e açúcares não absorvidos que alcançam o cólon, tem como benefício a produção de ácidos graxos de cadeia curta (acetato, propionato, e butirato) e lactato (TOPPING; CLIFTON, 2001).

Swanson et al. (2011) usaram o sequenciamento e relataram a filogenia e a capacidade funcional do microbioma gastrointestinal de cães saudáveis. Em relação a capacidade funcional do microbioma intestinal, os autores reportaram como as mais prevalentes categorias funcionais: carboidratos (12 a 13% das sequências), metabolismo proteico (8 a 9%), metabolismo do DNA (7%), parede celular e cápsula

(7 a 8%), aminoácidos e derivados (7%), virulência (6 a 7%), e cofatores, vitaminas, grupos prostéticos, e pigmentos (6%).

A microbiota intestinal tem um papel importante na estrutura epitelial, como demonstrado em modelos de animais (AL-ASMAKH; ZADJALI, 2015). Os camundongos *germe-free* possuem alterações na morfologia intestinal, motilidade, fisiologia e função quando comparados com camundongos sem patógenos específicos e de tipo selvagem. Camundongos *germe-free* mostram uma diminuição na área de superfície do intestino delgado, vilosidades e criptas ileais mais curtas, lâmina própria mais fina, tempo de trânsito mais longo, concentrações de ácidos graxos intestinais menores e osmolaridade reduzida (AL-ASMAKH; ZADJALI, 2015).

Outro papel importante da microbiota residente é parte integrante da barreira intestinal, que protege o hospedeiro de patógenos. Este mecanismo de proteção é inclui competição por nutrientes e locais de adesão à mucosa, o que cria um ambiente fisiologicamente restritivo para patógenos invasores (NEISH et al., 2009). Os ácidos graxos de cadeia curta oriundos da fermentação bacteriana inibem o crescimento bactérias patogênicas, por meio da redução do pH luminal e fecal. (HOOPER et al, 2001; NRC, 2006; NEISH, 2009).

A microbiota também ajuda no desenvolvimento e adequado funcionamento do sistema imunológico. Bactérias comensais transformam carboidratos complexos em ácidos graxos de cadeia curta, que são benéficos e fornecem energia para as células endoteliais, aumentam as células T reguladoras anti-inflamatórias e modulam a motilidade intestinal (BLAKE; SUCHODOLSKI, 2016). No nascimento, o sistema imunológico é imaturo e se desenvolve após colonização da microbiota intestinal. Esta microbiota por sua vez, estimula o aumento do número de placas de Peyer, liberação de imunoglobulinas (especialmente a imunoglobulina A secretora), reconhecimento das células dendríticas e coordena sinais pró-inflamatórios e anti-inflamatórios (NEISH, 2009).

2.2 Microbiota do trato gastrointestinal

O trato gastrointestinal dos mamíferos abriga grande diversidade de microorganismos, com uma densidade aproximada de 10^4 a 10^5 no estômago e 10^9

a 10^{11} unidades formadoras de colônias/ml de digesta no cólon (BENNO et al., 1992; MENTULA et al., 2005). A densidade e diversidade bacteriana aumenta consideravelmente do estômago para o cólon (SUCHODOLSKI et al., 2005; SUCHODOLSKI et al., 2008). As alterações fisiológicas que ocorrem ao longo do trato gastrointestinal, incluindo a natureza ácida do estômago, os sais biliares e enzimas presentes no intestino delgado, influenciam diretamente em quais espécies de microbiota habitam cada região (HOODA et al., 2013). No estômago de cães saudáveis, as bactérias pertencem principalmente ao filo Proteobacteria (> 90% das sequências) e apenas algumas pertencem ao filo Firmicutes (0.3%) (GARCIA-MAZCORRO et al., 2012).

A comunidade microbiana do duodeno em cães saudáveis consisti em seis filios: Firmicutes (46.4% das sequências do gene rRNA 16S obtidas), Proteobacteria (26.6%), Bacteroidetes (11.2%), Spirochaetes (10.3%) Fusobacteria (3.6%) e Actinobacteria (1%) (XENOULIS et al., 2008). Outro estudo avaliou a microbiota do jejuno em cães sadios, e identificou o filo Proteobacteria como o mais abundante (46%), seguido por Firmicutes (15%), Actinobacteria (11.2%), Spirochaetes (14.2%), Bacteroidetes (6.2%) e Fusobacteria (5.4%) (SUCHODOLSKI et al., 2009). Já o conteúdo do íleo em cães sadios predomina os filios Fusobacteria, Firmicutes e Bacteroidetes (SUCHODOLSKI; CAMACHO; STEINER, 2008).

Os filios predominates presentes no cólon de cães incluem Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, e Actinobacteria (SUCHODOLSKI; CAMACHO; STEINER, 2008; HANDL et al., 2011; SWANSON et al., 2011). Entretanto, amostras de cólon de cães sadios apresentaram co-dominância dos filios: Fusobacteria, Bacteroidetes e Firmicutes, aproximadamente 30% de cada filo (SUCHODOLSKI et al. 2008). Embora a população microbiana normal do trato gastrointestinal de cada indivíduo seja única, as principais mudanças populacionais podem ser indicativas de estado de saúde ou estado de doença, e grandes mudanças podem ocorrer durante as doenças do trato gastrointestinal (CHABAN et al., 2012; SUCHODOLSKI et al., 2012a).

2.3 Dieta e microbiota intestinal

A microbiota intestinal pode ser influenciada por vários fatores, incluindo dieta, fase de vida, ambiente, estado de doença, ou medicações (CHEN et al., 2014). Experimentos recentes utilizaram sequenciamento para avaliar o efeito de fibra dietética e de carboidratos não digeríveis. Mudanças em grupos de bactérias não convencionais, como aumento da concentração de *Blautia*, *Lachnospira*, *Veillonella*, *Megasphaera*, e *Faecalibacterium*, e diminuição de *Clostridium*, *C. hiranonis*, e *Fusobacterium*, foram observadas com o aumento da fibra dietética ou de prebióticos em estudos recentes com cães e gatos (BELOSHAPKA et al., 2013; HOODA et al., 2013; PANASEVICH et al. 2015).

Estudos anteriores observaram a influência da dieta na microbiota intestinal de cães utilizando 454 pirosequenciamento. Beloshapka et al. (2013) avaliaram os efeitos de dietas de carne crua com ou sem inulina ou extratos de parede celular de levedura na população intestinal de bactérias em cães adultos saudáveis. Seis cães Beagles saudáveis (idade média = 5,5 anos; peso corporal médio = 8,5 kg) foram distribuídos aleatoriamente para 1 dos 6 tratamentos dietéticos: (1) dieta carne controle; (2) carne + 1,4% inulina na matéria seca (MS); (3) carne + 1,4% parede celular de levedura na MS; (4) controle frango; (5) frango + 1,4% inulina na MS; and (6) frango + 1,4% parede celular de levedura MS. Esses pesquisadores concluíram que foram obtidas um total de 358.693 sequências, com uma avaliação de 4.000 sequências selecionadas aleatoriamente para fornecer estimativas de diversidade. Esses pesquisadores também concluíram que os filos bacterianos predominantes presentes em todos os cães incluem Fusobacteria (~43% das sequências), Firmicutes (~37% das sequências), Bacteroidetes (10-15% das sequências), Proteobacteria (5% das sequências), e Actinobacteria (2-3% das sequências). Os autores também concluíram que os gêneros predominantes foram *Fusobacterium* (16-36% das sequências), *Cetobacterium* (8-33% das sequências), *Clostridium* (12-21% das sequências), e *Bacteroides* (6-18% das sequências). No entanto, apesar dos avanços continuarem a serem feitos a fim de desenvolver ainda mais o conhecimento atual de populações microbianas do intestino canino, diferenças nos métodos de extração de DNA, diferenças nos primers de amplificação e plataformas de sequenciamento usadas (por exemplo, 454-pirosequenciamento versus 16S

rRNA clone bibliotecas), dificultam a avaliação das variações de resultados entre os diferentes estudos.

2.4 O envelhecimento e a microbiota intestinal

Estudos sobre as alterações fisiológicas decorrentes do envelhecimento em cães e gatos tornaram-se mais frequentes, talvez como resultado do aumento na expectativa de vida destes animais. Embora muitos cães permaneçam ativos na sua maturidade, a maioria se torna menos ativo e pode mostrar sinais do envelhecimento a partir dos seis ou sete anos (LAFLAMME, 2005).

Algumas das alterações do trato digestório de cães idosos consiste no aumento da ocorrência de doença periodontal, dificuldade em preensão e mastigação, frequência aumentada de diarreia, vômito e regurgitação (KIRK, 2000). Cães mais velhos apresentam menor área de superfície das vilosidades do duodeno, menor altura das vilosidades do jejuno e profundidade maior das criptas do cólon (LARSEN; FARCAS, 2014). Kuzmuk et al. (2005) estudaram os efeitos de dieta e idade e observaram respostas fisiológicas e morfológicas diferentes entre cães jovens (1 a 2 anos) e idosos (11 a 12 anos) quando alimentados com dieta baseada em produtos de origem vegetal ou animal. A altura das vilosidades do jejuno era maior em cães jovens que consumiram a dieta baseada em produtos vegetais, tanto em comparação com os cães jovens que consumiram dieta baseada em produto animal como em relação aos cães idosos que receberam as duas dietas.

A microbiota intestinal cumpre papel importante no processo de digestão e metabolismo do hospedeiro, provendo ainda mecanismo de defesa natural contra os patógenos invasores (NRC, 2006). O envelhecimento pode também afetar as populações bacterianas intestinais e as concentração dos seus produtos. Embora a microbiota de cães adultos tenha sido estudada, pouco é conhecido sobre as mudanças que acontecem com o avançar da idade (SIMPSON; MARTINEAU; JONES, 2002; GOMES et al., 2011). Um estudo comparou as populações de bactérias em cães Beagle de diferentes idades (menos de 12 meses versus mais de 11 anos de idade) utilizando o método de cultivo, os pesquisadores encontraram menor número de *Bacteroides*, *Peptostreptococci*, *Bifidubacterium*, *Lactobacillus* e

Staphylococcus no cólon, enquanto *Clostridium perfringens* e *Streptococci* aumentaram em cães idosos (BENNO et al., 1992).

Outro estudo utilizando método de cultivo em placa encontrou aumento de *Clostridium*, *Lactobacillus* e *Bacteroides* em cães idosos em comparação com cães adultos jovens (KEARNS; HAYEK; SUNVOLD, 1999). Um estudo de impressão molecular baseado em eletroforese em gel de gradiente desnaturante (DGGE) comparou jovens adultos (2,5 anos) e velhos (11 anos) e mostrou apenas menor número de *Bacteroides* em cães idosos na microbiota fecal canina (SIMPSON; MARTINEAU; JONES, 2002). Gomes et al. (2011) não encontraram evidências de um efeito de idade sobre contagens fecais microbianas com números semelhantes de aeróbios totais, anaeróbios totais, *E. coli*, *Clostridium*, *Lactobacillus* e *Bifidobacterium* pelo método cultivado. Porém, as alterações relacionadas à idade em produtos de fermentação nesse estudo sugerem uma alteração na atividade metabólica bacteriana ou na taxa de absorção intestinal desses compostos nos animais idosos.

As causas de alteração na composição da microbiota intestinal de cães com a idade avançada ainda são incertas. Hopkins, Sharp e MacFarlane (2001) sugeriram que algumas cepas bacterianas pudessem tirar proveito de novos nichos ecológicos, induzindo assim troca na composição da microbiota intestinal. Foi proposto que a adesão reduzida à mucosa possa ser fator envolvido na menor colonização por algumas espécies de bifidobactérias em indivíduos velhos (SAUNIER; DORÉ, 2002).

2.5 Métodos de alto rendimento para avaliação microbiana

O conhecimento sobre a microbiota do trato gastrointestinal foi obtido principalmente utilizando técnicas baseadas na cultura, porém apenas uma fração dos organismos presentes no trato gastrointestinal pode ser cultivada (KERR; BELOSHAPKA; SWANSON, 2013). Atualmente, os métodos de cultivo são mais utilizados para estudos clínicos, quando se visa um patógeno específico (por exemplo, *Salmonella*), teste de susceptibilidade antimicrobiana, estudos epidemiológicos, é uma método utilizado para caracterizar as propriedades metabólicas dos isolados e seus fatores de virulência (SUCHODOLSKI, 2011b).

Ao longo da última década, as técnicas moleculares promoveram melhor compreensão da composição, dinâmica e funcionalidade do ecossistema microbiota-hospedeiro em cães (RITCHIE; STEINER; SUCHODOLSKI, 2008; SUCHODOLSKI, 2011b; SWANSON et al., 2011), aumentando o progresso na área. Métodos baseados no DNA para a análise da microbiota surgiram e podem ser ferramentas úteis para identificar e quantificar de maneira eficaz as populações microbianas. As ferramentas moleculares baseadas no gene 16S rRNA microbiano disponíveis atualmente, incluem a PCR quantitativa (qPCR); hibridização *in situ* fluorescente (FISH); técnicas baseadas em gel, tais como análise de polimorfismo de comprimento de fragmento de restrição (RFLP), electroforese em gel de gradiente desnaturante (DGGE) e electroforese em gel com gradiente de temperatura (TGGE); e técnicas de sequenciamento tais como o pirosequenciamento 454 (Roche Applied Science, Indianapolis, IN, USA), sequenciamento Illumina (Illumina Inc., San Diego, CA, USA), e sequenciamento Sanger. As técnicas de sequenciamento também são usadas para a avaliação funcional do microbioma. A tabela 1, apresenta um resumo das técnicas atualmente disponíveis para a avaliação da microbiota e do microbioma (KERR; BELOSHAPKA; SWANSON, 2013).

Pesquisas focadas no uso de fibra dietética e carboidratos não digeríveis mediram alguns grupos microbianos ligados à saúde (como, *Bifidobacterium spp.*, *Lactobacillus spp.*) ou potenciais patógenos (como, *Clostridium perfringens* e *Escherichia coli*). Sabe-se agora, que o intestino canino alberga centenas de espécies microbianas, os gêneros *Bifidobacterium* e *Lactobacillus* representam apenas aproximadamente 1% das sequências no intestino e que *C. perfringens* e *E. coli* são bactérias comensais presentes em animais saudáveis (SUCHODOLSKI, 2011a). Dada esta recente evolução, a relevância biológica dos estudos baseados na cultura e no qPCR realizados devem ser interpretados com cautela. As novas tecnologias melhoram nossa visão e compreensão da microbiota do trato gastrointestinal.

Tabela 1. Métodos moleculares utilizados para análise do microbioma.

Métodos	Descrição	Características	Principais vantagens	Principais desvantagens
qPCR	Amplifica e quantifica uma molécula de DNA alvo	Tintura ou sonda utilizada para ligar o DNA de cadeia dupla, o que faz com que a intensidade das emissões fluorescentes aumente	Baixo custo; Alta sensibilidade permitindo a detecção de seqüências em baixas concentrações	Escopo limitado
FISH	Detecção sensível de seqüências de ácidos nucleicos específicas em células metafásicas ou interfásicas	Procedimento manual de amostras biológicas; Intensidades de fluorescência medidas usando FLEX (um sistema de microscópio de fluorescência quantitativo)	Permite a localização e estudo da organização espacial das células à medida que ocorrem no seu habitat natural	Dispendioso; Não é facilmente escalável para exames de doenças
RFLP	Técnica de fingerprinting de alto rendimento usada para explorar mudanças na estrutura e composição de comunidades microbianas.	Amostra de DNA digerida por enzimas de restrição para caracterizar microbiota de regiões específicas. Os fragmentos foram então separados de acordo com o comprimento por electroforese em gel.	Fornecer uma visão ampla dos sistemas microbianos	Primers não específicos
DGGE	Os fragmentos de rRNA 16S amplificados por PCR foram separados em gel de poliacrilamida contendo gradiente desnaturante (por exemplo, ureia, formamida).	método à base de gel de fingerprinting	Fornecer uma visão ampla dos sistemas microbianos	Apenas semi-quantitativo e insensível
TGGE	Fragmentos 16S de rRNA amplificados por PCR separados em gel de poliacrilamida contendo gradiente de temperaturas	método à base de gel de fingerprinting	Gera diferenças qualitativas na ecologia microbiana	Apenas semi-quantitativo e insensível
Sequenciamento 454	Pirosequenciamento por emissão de luz	400 a 600 leituras de base	Cobertura 16S é boa	O custo limita a cobertura <i>shotgun</i>
Sequenciamento Illumina	Fluorescente; sequenciamento passo a passo	100 a 150 leituras de base	Cobertura muito alta devido à alta saída do instrumento e custo muito baixo	Aumento dos custos e do tempo de bioinformática
Sequenciamento Sanger	Fluorescente; terminador dideoxynucleotídeo	750 leituras de base ou superior	Comprimento e precisão de leitura elevada	Comparado com o sequenciamento de última geração, é caro e tem baixa taxa de rendimento

Fonte: KERR; BELOSHAPKA; SWANSON, 2013

3. OBJETIVOS GERAIS

O presente estudo caracterizou os efeitos do consumo de dietas extrusadas com fontes de fibra de diferentes grau de fermentação e com fontes de proteínas de origem animal ou vegetal na avaliação da microbiota associada a mucosa do cólon de cães adultos e idosos. Além disso, como segundo objetivo, o estudo caracterizou a microbiota associada a mucosa ao longo do trato gastrointestinal de cães.

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CAPITULO 2 – AGE AND DIET EFFECTS ON COLON MUCOSA MICROBIOTA OF DOGS¹

Short title: Colon Microbiota of dogs

Funding: This work was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), process number 2011/23575-3. The scholarship of the first author was supported by Capes - Programa de Doutorado-sanduíche no Exterior (PDSE) and Programa de Demanda Social (DS).

¹ Escrito de acordo com as normas da revista *PLOS ONE*

AGE AND DIET EFFECTS ON COLON MUCOSA MICROBIOTA OF DOGS

Ana Paula J. Maria^{1¶}, Ping Deng^{2¶}, Hannah D. Holscher^{2¶}, Márcia de O. S. Gomes^{3¶}, Franz N. Yoshitoshi^{4¶}, Kelly S. Swanson^{2¶*}, Aulus C. Carciofi^{1¶*}

¹ Department of Veterinary Clinic and Cirurgic, São Paulo State University, School of Agricultural and Veterinarian Sciences, Jaboticabal, SP, Brasil;

² Department of Animal Science, University of Illinois at Urbana Champaign, Urbana, IL, United States of America;

³ Department of Internal Medicine, University of São Paulo, College of Veterinary Medicine and Animal Science, São Paulo, SP, Brasil;

⁴ Endoscopet - Endoscopy and Surgery, São Paulo, SP, Brasil.

Presented partially as an oral communication at the 19th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN), Toulouse, France, September, 17st to 19th, 2015.

*Corresponding author

E-mail: aulus.carciofi@gmail.com (ACC)

E-mail: ksswanso@illinois.edu (KSS)

¶These authors contributed equally to this work.

Abstract

The aim of this study was to determine the effects of feeding different fermentability of fibers and protein sources of animal or vegetable origins on colon mucosa microbiota communities of old and adult dogs using Miseq Illumina sequencing. Eighteen young adult (2.6 ± 0.9 yr) and 18 geriatric (10.2 ± 1.1 yr) Beagle dogs were assigned to three isonutritive extruded kibble dietary groups containing: a non-fermentable insoluble fiber (NFF; 34% poultry meal; 8% sugarcane fiber), a fermentable fiber (FF; 35% poultry meal; 10% beet pulp) and a soybean meal (SM; 30% soybean meal; no additional fiber source). Dogs were fed the experimental diets for 30 days, followed by intestinal mucosa sample collection on day 31 and 32. DNA was extracted and the V4 region of the 16S rRNA gene was amplified and subjected to Miseq Illumina sequencing. Predominant colon mucosa bacterial phyla included Firmicutes (44.1%) and Bacteroidetes (39.2%). *Prevotella* was lower in colon mucosa of adult dogs fed SM diet compared to older dogs fed the same diet and compared to adult dogs fed NFF and FF diets. Also, Mogibacteriaceae and Mogibacteriaceae undefined genus were higher in colon mucosa of adult dogs fed NFF diet compared with old dogs fed the same diet and compared to adult dogs fed FF and SM diets. Alcaligenaceae and *Suterella* were higher in colon mucosa of old dogs compared to adult dogs fed the FF diet and compared to old dogs fed NFF and SM diet. *Slackia*, Peptococcaceae and *Peptococcus* were higher ($P < 0.05$) in colon mucosa of dogs fed poultry-based diets (NFF and FF) but *Veillonellaceae*, *Megamonas* and *Catenibacterium* were lower ($P < 0.05$) compared with soybean-based (SM) diet. Lachnospiraceae undefined genus were higher ($P < 0.05$) in colon mucosa of dogs fed NFF diet compared with dogs fed FF and SM diets. The abundance of *Slackia*, Coriobacteriaceae, *Bacteroides*, Bacteroidaceae, Paraprevotellaceae undefined genus, Lachnospiraceae and *Plesiomonas*, were lower ($P < 0.05$) in colon mucosa of geriatric dogs compared with adult

dogs. Therefore the different sources of protein and fiber related changes in the predominant colon mucosa microbiota of dogs, as well as the ageing.

Introduction

The intestinal microbiota can be defined as the dynamic collection of microorganisms within the gastrointestinal (GI) tract and the system of interactions these organisms have with each other and with the host cells [1-2]. The intestinal microbiota plays an important role in the digestion and metabolism of the host, providing a natural defense, mechanism against invading pathogens, aiding in development of a healthy epithelium and immune system [3-5]. These microbiota communities can be influenced by infection, exposure to antibiotics, dietary shifts [6-7] and ageing [8] and other factors.

Nowadays companion animals are living longer and healthier lives because of advances in both veterinary medicine and nutrition. [9]. Age-related physiologic differences among of various life stages and the outcomes of modifying the diet at these times are still largely untested [9]. To date, many studies have characterized the effect of dietary intervention, such as dietary fiber, animal-derived protein, carbohydrates, and symbioses on GI microbiota in dogs and cats [10-14]. Although the adult dog microbiota has been studied, little is known about the changes that occur with advancing of age [15-17]. Most of the available information about the attached gut mucosa microbiota in dogs has been obtained in studies of idiopathic inflammatory bowel disease [18-19] fecal samples or luminal aspirations, which still unclear are the data about the bacterial populations that might be intimately associated with the intestinal mucosa [20]. There is a little information of the microbiota associated with the gastrointestinal mucosa in ageing dogs and the impact of dietary

intervention on this condition [20]. Further identification and characterization of canine microbiota will be useful in evaluating the effects of age on intestinal health [9].

In a previous study, old dogs fed an extruded diet supplemented with beet pulp as a fermentable fiber source had reduced nutrient digestibility compared with adult animals. The diet formulated with soybean meal as a protein and fiber source was highly digestible and promotes the intestinal formation of SCFA and spermidine and high IgA intestinal secretion for both adult and old dogs. Old dogs exhibited increased fecal concentrations of spermine, putrescine, and cadaverine without other changes in fermentation products compared with adult animals [21]. The aims of the present study were investigate and characterize the microbiota associated with the colon mucosa of adult and old dogs fed kibble diets formulated with fibers of different fermentability and animal or vegetable sources of protein on the mucosal bacteria.

Material and methods

Animals and diets

The protocol of the present study was previously approved by the Ethics Committee in the Use of Animals (Protocol no. 004542/12) of the School of Agricultural and Veterinarian Sciences, São Paulo State University and is in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation.

Thirty-six healthy beagles were used. The dogs were separated into two age groups; one group had 18 old dogs [10.2 ± 1.0 yr of age and 11.8 ± 2.1 kg body weight (BW)] and the other group had 18 adult dogs (2.6 ± 0.9 yr of age and 11.05 ± 1.2 kg BW). Dogs were twin-housed in kennels (1.5x 3.5 m) with a solarium at the Laboratory of Research on Nutrition and Nutritional Diseases of Dogs and Cats, São Paulo State University (Jaboticabal, Brazil). Three isonutritive kibble diets with different sources of fiber and protein (Table 1) were used:

Table 1. Formula and analyzed composition of the experimental diets with different sources of fiber and protein

Item	Diets ¹		
	NFF	FF	SM
Ingredients (%)			
Maize grain	29.36	25.36	24.06
Poultry by-product meal	33.6	35.6	11.2
Broken rice	20.0	20.0	20.0
Soybean meal	-	-	30.0
Beet pulp	-	10.4	-
Sugarcane fiber ²	8.3	-	-
Poultry fat	5.4	5.3	8.6
Liquid palatant	2.0	2.0	2.0
Potassium chloride	0.4	0.4	0.4
Sodium chloride	0.4	0.4	0.4
Vitamin-mineral premix ³	0.3	0.3	0.3
Choline chloride	0.1	0.1	0.1
Mold inhibitor ⁴	0.1	0.1	0.1
Antioxidant ⁵	0.04	0.04	0.04
Calcium carbonate	-	-	0.7
Dicalcium phosphate	-	-	2.1
Analysed chemical composition (% DM basis) ⁶			
Dry matter	95.2	93.5	94.6
Mineral matter	10.5	9.5	8.7
Crude protein	28.6	30.5	28.3
Fat	12.3	11.4	11.2
Total Dietary Fiber	12.2	13.7	11.3
Insoluble Fiber	12.2	12.5	10.5
Soluble Fiber	0.0	1.2	0.8
Starch	33.6	33.6	35.3
Gross energy (kcal/kg)	4672	4763	4721
Density after extrusion (g/L)	380	410	380
Starch gelatinization degree (%)	86.7	87.6	88.5

¹ NFF = diet with sugarcane fiber and poultry by-product meal; FF = diet with beet pulp and poultry by-product meal; SM = diet with 30% of soybean meal.

² Vit2be Fiber, Dilumix, Leme, SP, Brazil.

³ Provided per kg of diet: iron, 100 mg; copper, 10 mg; magnesium, 10 mg; zinc, 150 mg; iodine, 2 mg; selenium, 0.3 mg; vitamin A, 18,000 IU; vitamin D3, 1,200 IU; vitamin E, 200 IU; thiamin, 6 mg; riboflavin, 10 mg; pantothenic acid, 40 mg; niacin, 60 mg; vitamin B6, 6 mg; folic acid, 0.30 mg; vitamin B12, 0.1 mg.

⁴ Mold Zap: Ammonium dipropionate, acetic acid, sorbic acid and benzoic acid – Alltech do Brasil Agroindustrial Ltda, Curitiba, 81170-610 PR, Brazil.

⁵ Banox: butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and calcium carbonate – Alltech do Brasil Agroindustrial Ltda, Curitiba, 81170-610 PR, Brazil.

⁶ Analysed in duplicate with coefficient of variation below 5%.

(1) a diet with insoluble and non-fermentable fiber (NFF) was based on sugarcane fiber and poultry by-product meal; (2) A diet with soluble and fermentable fiber (FF) was based on beet pulp and poultry by-product meal; (3) a diet with 30% of soybean meal (SM), which was partially replaced the protein source and the source of fiber, which includes fermentable oligosaccharides. Diets were formulated according to the recommendations for dog's maintenance (22), and were manufactured in the extruder facility of the School of Agricultural and Veterinarian Sciences, São Paulo State University. Diets were extruded under identical processing conditions in a single-screw extruder (Mab 400S, Extrucenar, Monte Alto, Brazil). Dogs were offered two meals daily and were fed to maintain constant BW throughout the study. Food amount provided was initially determined by calculating the daily maintenance energy requirement of kennel dogs ($ME, \text{kcal} = 130 \times \text{kg BW}^{0.75}$) (NRC, 2006). Dogs were weighed weekly and if necessary, the amount of food was adjusted. Water was provided *ad libitum*.

Experimental procedure

The experiment was designed as a 3 x 2 factorial arrangement with three diets and two ages, totaling six treatments. Dogs were assigned into three blocks of 12 animals with six adult and six old animals, two replicates per food and age in each block, totaling six animals per treatment. Each block lasted 32 days, in which days 1 to 30 consisted in the adaptation to the diet. Intestinal mucosa samples were collected on days 31 and 32.

Prior to the colonoscopic biopsy procedure, dogs were fasted for 12 hr and then sedated with 1.0 mg chlorpromazine/kg (Sanofi-Aventis pharmaceuticals Ltda, Suzano, Brazil) intramuscularly. After sedation, anesthesia induction was performed with propofol (Cristália chemical pharmaceutical products ltd, São Paulo, Brazil) at a dose of 5.0 mg/kg, given slowly through an intravenous drip. After the animals lost the laryngotracheal reflex, endotracheal intubation was performed with a tracheal tube of appropriate size for each animal. The anesthesia was maintained with isoflurane (Virbac Animal Health of Brazil, Jurubatuba, Brazil) in sufficient concentration to keep the animal on the anesthetic plane. For the biopsy procedure by lower gastrointestinal endoscopy, the animals were placed in lateral recumbency. A flexible endoscope (Karl Storz, 60914NKS, Karl Storz GmbH & Co. KG, Germany) was used along with a biopsy forceps alligator mouth type of 2.2 mm (Karl Storz, 60252LX, Karl Storz GmbH & Co. KG, Tuttlingen, Germany) to collect the mucosa samples. During the procedure all animals received intravenous fluid therapy and after the procedure they were individually assisted for recovery from anesthesia. Two fragments were collected from the mucosa of colon of each dog. Samples were frozen in liquid nitrogen and then stored at -80°C until DNA extraction for microbial analysis.

DNA extraction and sequencing

Genomic DNA was extracted from intestinal mucosa samples using the PowerLyzer™ PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA,

USA) according to the manufacturer's instructions. Concentration of extracted DNA was quantified using a Qubit[®] 2.0 Fluorometer (Life Technologies, Invitrogen, Grand Island, NY, USA). Polymerase chain reaction (PCR) amplicons from the V4 region of the 16S rRNA gene were prepared for sequencing following a similar procedure as as described previously [23] and primers as described previously [24]. The samples were amplified in a thermocycler DNA Engine[®] Thermal Cycler (Bio-Rad Laboratories, Inc., Foster City, CA, USA), using the PCR protocol: initial denaturing at 95°C for 5min, 35 cycles of denaturing at 95°C for 20s, annealing at 54 °C for 30s, extension at 72 °C for 1min, and final extension for 10min for sample. Amplicons from PCR were purified utilizing Agencourt[®] AMPure XP beads (Beckman-Coulter Inc., Indianapolis, IN, USA). PCR products were pooled and concentrated using the MinElute[®] Gel Extraction Kit (QIAGEN Group, Valencia, CA, USA), following the manufacturer's instructions. The quality of DNA was assessed by electrophoresis using precast agarose gels (E-Gel[®] EX Gel 1%, Invitrogen, Grand Island, NY, USA). Further the DNA concentrations were measured using Qubit[®] 2.0 Fluorometer (Life Technologies, Invitrogen, Grand Island, NY, USA). Amplicons were combined in equimolar ratios to create a DNA pool that was used for sequencing. DNA quality was assessed before sequencing using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The Illumina sequencing was performed at the W. M. Keck Center for Biotechnology at the University of Illinois utilizing a MiSeq 2x300 nt v3 technology (Illumina Inc., San Diego, CA, USA).

Bioinformatics and statistics

The data analysis was performed with QIIME 1.8.0 [25], following quality filtering [26]. Briefly, high quality (quality value > 25) sequence data sequences were demultiplexed and quality filtered using `split_libraries_fastq.py` default parameters. Resulting sequences were clustered into Operational taxonomic units (OTU) using closed-reference OTU picking

against the Greengenes 13_8 reference OTU database (97% similarity threshold). An even sampling depth of 6448 sequences per sample was used for assessing alpha- and beta-diversity measures, samples with less than 6448 sequences were excluded from subsequent analyses.

Sequence percentages at each taxonomic level were analyzed using the Mixed Models procedure of Statistical Analysis System 9.2 (SAS Institute Inc., Cary, NC, USA), testing the main effects of age, diet and their interaction from colon samples. Random effects of block and animal were included in all models. All variables were first tested for hypotheses of normality and variance homogeneity of the treatments. When the F-test was significant, means were separated for treatments using LSMEAN with Tukey's adjustment to control for multiple comparison. When the data were not normally distributed, these data were transformed using the logarithmic with base 10, $\log(x+1)$ or square root prior to statistical analysis. If normality could not be achieved through transformations, the non-parametric Kruskal-Wallis test was performed. A probability of $P < 0.05$ was considered significant and values of $P < 0.1$ were considered a trend.

Results

All dogs accept the three diets and remained clinically healthy without adverse effects (e.g., vomiting and diarrhea) throughout the study. Bacterial DNA was extracted from 36 colon mucosa specimens. Due to the low abundance of DNA concentrations, 5 samples of the mucosa colon (lost samples: 2 adult dog fed NFF; 1 old dog fed NFF; 1 adult dog fed FF; 1 adult dog fed SM) were lost. Therefore, a total of 31 intestinal mucosa samples were subjected to MiSeq Illumina sequencing. A total of 18,894,896 sequences were obtained from the current data set, of those a total of 7,629,557 sequences were remained after quality filtering. The sequencing identified a total of 10 bacterial phyla, 57 families and 97 genera in

the mucosa of all dogs. According to phylogenetic diversity whole tree and rarefaction curves (S1 and S2 Fig), microbial diversity and species were similar among age ($P = 0.244$) and diet ($P > 0.05$).

Ten phyla were identified in colon mucosa samples. Firmicutes (44.1%) was the predominant bacterial phylum present in colon mucosa samples from all dogs, followed by Bacteroidetes (39.2%), Fusobacteria (6.5%), Proteobacteria (5.6%), Actinobacteria (4.3%) and Deferribateres (0.2%). The remaining phyla, Cyanobacteria, Synergistetes, Tenericutes and Verrucomicrobia, were present as less of 0.1% of all bacterial sequences. No effects ($P > 0.05$) of age, diet, or age x diet interaction, were observed at the phylum level (Table 2; S1 Table). Firmicutes was largely comprised of the order Clostridiales (37.2%), Lactobacillales (5.9%), and Erysipelotrichales (0.3%). Among the Bacteroidetes phyla, Bacteroidales was the predominant order (39%).

Predominant colon mucosa bacterial families include *Veillonellaceae* (21.8%), *Paraprevotellaceae* (21.1%), *Prevotellaceae* (11.3%), *Bacteroidaceae* (6.6%), *Fusobacteriaceae* (6.5%), *Lachnospiraceae* (6.1%) and *Lactobacillaceae* (5.6%) and the most abundant genera were [*Prevotella*] (15.3%), *Megamonas* (15%), *Prevotella* (11.3%), *Bacteroides* (6.6%), *Fusobacterium* (6.5%) and *Lactobacillus* (5.6%), results also presented in Table 2 and S1 Table.

1 **Table 2. Predominant bacterial phyla, family and genera (expressed as a percentage of total sequences) in colonic intestinal**
 2 **mucosa of dogs from two age groups fed diets with different sources of fiber and protein.**

Item Phylum	Family	Genus		Age*Diet			Age		Diets ¹			P - Value						
				NFF	FF	SM	Older	Adult	NFF	FF	SM	Age*Diet	Age	Diet				
Actinobacteria ²	Coriobacteriaceae	<i>Slackia</i>	Older	4.20	4.30	3.40	3.95	4.75	4.28	4.71	4.08	0.7278	0.2199	0.4530				
			Adult	4.26	5.11	4.79												
			Older	1.38	1.18	1.26	1.26 ^b	2.11 ^a	1.47	1.77	1.81	0.4782	0.0422	0.7648				
			Adult	1.46	2.40	2.37												
			Older	0.17	0.12	0.08	0.12 ^b	0.29 ^a	0.25 ^a	0.26 ^a	0.11 ^b	0.1564	0.0022	0.0362				
			Adult	0.34	0.41	0.14												
Bacteroidetes	Bacteroidaceae	<i>Bacteroides</i>	Older	46.49	34.94	43.10	41.55	37.14	44.92	35.99	37.12	0.0962	0.1499	0.0570				
			Adult	43.48	37.69	30.38												
			Older	5.38	5.41	4.13	4.99 ^b	8.50 ^a	7.44	6.92	5.89	0.9156	0.0057	0.5533				
			Adult	9.59	8.34	7.67												
			[Paraprevotellaceae]	g_	Older	28.26	15.56	25.43	23.13	19.40	27.13	16.93	19.74	0.2260	0.3370	0.1087		
					Adult	26.17	18.94	13.27										
	[Prevotella] ²	g_	[Prevotella] ²	Older	4.49	4.40	3.39	4.14 ^b	7.93 ^a	7.29	5.72	5.10	0.6639	0.0140	0.4713			
				Adult	10.31	6.94	6.77											
				Older	23.78 ^{Aa}	11.17 ^{Aa}	22.02 ^{Aa}	18.99	11.43	19.81	11.56	14.26	0.0475	0.0376	0.1452			
				Adult	15.84 ^{Aa}	11.95 ^{Aa}	6.51 ^{Bb}											
				Deferribacteres ²	Deferribacteraceae ⁵	<i>Mucispirillum</i> ²	Older	0.15	0.05	0.10	0.09	0.29	0.15	0.08	0.35	0.7505	0.7936	0.1879
							Adult	0.13	0.11	0.63								
Firmicutes	Lachnospiraceae	g_	Older	41.32	47.62	45.20	44.69	42.93	39.97	46.52	44.95	0.9454	0.5623	0.2133				
			Adult	38.49	45.37	44.82												
			Older	6.48	5.69	4.11	5.43 ^b	7.1 ^a	7.34	5.94	5.51	0.4182	0.0466	0.1926				
			Adult	8.22	6.05	7.05												
			Older	3.49	2.32	2.13	2.67	3.35	4.20 ^a	2.63 ^b	2.20 ^b	0.4721	0.1603	0.0063				
			Adult	5.02	2.91	2.21												
	Peptococcaceae	<i>Peptococcus</i>	Older	0.38	0.45	0.17	0.34	0.40	0.44 ^a	0.47 ^a	0.19 ^b	0.8999	0.5498	0.0428				
			Adult	0.51	0.47	0.21												
	Veillonellaceae	<i>Megamonas</i>	Older	20.33	21.23	28.55	23.26	19.2	16.28 ^b	21.39 ^{ab}	26.02 ^a	0.4266	0.1681	0.0372				
			Adult	11.73	21.98	23.38												
			Older	12.96	13.88	23.17	16.59	12.25	9.43 ^b	13.97 ^{ab}	19.86 ^a	0.4382	0.1357	0.0208				
			Adult	5.56	14.52	16.33												
	[Mogibacteriaceae]	g_	Older	0.18 ^{Ba}	0.10 ^{Aa}	0.05 ^{Aa}	0.11	0.23	0.38	0.10	0.04	0.0192	0.0656	0.0030				
			Adult	0.57 ^{Aa}	0.10 ^{Ab}	0.02 ^{Ab}												
	Erysipelotrichaceae ¹	<i>Catenibacterium</i>	Older	0.20	0.37	0.38	0.32	0.35	0.24	0.34	0.43	0.0606	0.8952	0.4963				
			Adult	0.28	0.30	0.48												
			Older	0.07	0.11	0.19	0.12	0.06	0.04 ^b	0.09 ^{ab}	0.15 ^a	0.8841	0.0585	0.0213				

		Adult	0.01	0.07	0.11								
Fusobacteria ²		Older	4.24	8.14	4.03	5.48	7.61	5.38	6.79	7.46	0.1038	0.1168	0.8673
		Adult	6.52	4.96	11.36								
Proteobacteria ²		Older	3.56	4.71	4.11	4.14	7.24	5.26	5.78	6.03	0.9447	0.1877	0.6477
	Alcaligenaceae	Adult	6.98	6.74	8.01								
	<i>Sutterella</i>	Older	0.53 ^{Ab}	1.42 ^{Aa}	0.65 ^{Ab}	0.87	0.77	0.60 ^b	1.07 ^a	0.80 ^{ab}	0.0049	0.4778	0.0195
		Adult	0.67 ^{Aa}	0.71 ^{Ba}	0.94 ^{Aa}								
	Comamonadaceae ²	Older	0.62	0.32	0.43	0.45	0.57	0.58	0.32	0.64	0.5984	0.9589	0.6001
		Adult	0.50	0.31	0.88								
	<i>Comamonas</i> ²	Older	0.07	0.01	0.05	0.04	0.03	0.05 ^a	0.01 ^b	0.04 ^a	0.4079	0.1619	0.0307
		Adult	0.03	0.02	0.04								
	Enterobacteriaceae ²	Older	1.06	1.67	1.58	1.40	2.49	0.95	2.14	2.74	0.6462	0.7068	0.5030
		Adult	0.69	2.59	4.02								
	<i>Plesiomonas</i> ²	Older	0.07	0.03	0.00	0.01 ^b	0.93 ^a	0.13	0.28	1.01	0.5166	0.0166	0.5537
		Adult	0.09	0.48	2.13								

3 ¹NFF: diet with sugarcane fiber and poultry by-product meal; FF: diet with beet pulp and poultry by-product meal; SM: diet with 30% soybean meal to partially replace

4 the poultry by-product meal and add fiber.

5 ²-Log₁₀

6 ^{a, b} Means in the same row and not sharing a common superscript lowercase letters differ ($P < 0.05$).

7 ^{A, B, C} Means in the same column and not sharing common superscript capital letters differ ($P < 0.05$).

Effect of age and diet interaction was observed in a few families and genus (Table 2; S1 Table). The adult dogs fed NFF diet had higher ($P < 0.05$) mucosa colon abundance of Mogibacteriaceae and Mogibacteriaceae undefined genus (members of Clostridiales and Firmicutes) when compared with the older dogs fed the same diet and with the adult dogs fed FF and SM diets. The mucosa colon abundance of [*Prevotella*] (member of Bacteroidales and Bacteroidetes) were lower ($P < 0.05$) for adult dogs fed SM diet than for older dogs fed the same diet and when compared with the adults dogs fed NFF and FF diets. The mucosa colon abundance of Alcaligenaceae and *Sutarella* (members of Bulkholdeliars and Proteobacteria) were higher ($P < 0.05$) for the older dogs fed FF than for the adult dogs fed the same diet and when compared with the older dogs fed NFF and SM diets.

Diet shifted the relative abundances of several predominant family and genera in the colon mucosa (Table 2; S1 Table), independent of age. The colon mucosa abundance of Peptococcaceae, *Peptococcus* (members of Clostridiales and Firmicutes) and *Slackia* (member of Coriobacteriales and Actinobacteria) were higher ($P < 0.05$) for dogs fed NFF and FF diets compared to the SM-fed dogs. The abundance Lachnospiraceae undefined genus (member of Clostridiales and Firmicutes) were higher ($P < 0.05$) for animals fed NFF diet than for the dogs fed FF and SM diets, while the colon mucosa abundance of *Comamonas* (member of Bulkholdeliars and Proteobacteria) was lower ($P < 0.05$) in dogs fed FF diet than NFF and SM-fed dogs. Furthermore, dogs fed NFF diet had lower colon mucosa abundance of Veillonellaceae, *Megamonas* (members of Clostridiales and Firmicutes) and *Catenibacterium* (member of Erysipelotrichaceae and Firmicutes) ($P < 0.05$) when compared with SM-fed dogs.

There were a number of significant shifts between adult and senior dogs at the family and genus level in the colon mucosa (Table 2; S1 Table), independent of diet. Most notably,

Coriobacteriaceae, *Slackia* (members of Coriobacteriales and Actinobacteria), Bacteriodaceae, *Bacterioides*, Paraprevotellaceae undefined genus (members of Bacteroidales and Bacteroidetes), Lachnospiraceae (member of Clostridiales and Firmicutes) and *Plesiomonas* (member of Enterobacteriales and Proteobacteria) were lower ($P < 0.05$) on mucosa colon of senior dogs compared to the adult dogs.

Discussion

Data on the genome sequencing demonstrated that aging and long term dietary interventions induced changes in the human gastrointestinal microbiome [27-30]. It has been well established that the number and activity of the gastrointestinal microbiota can be manipulated by diet, but most of the studies in dogs have focused on analysis of fecal samples [11, 12, 14, 31]. Bacteria from biopsies in the intestine are quantitatively different from the stool under colonoscopy and quantitatively and qualitatively different from the fresh stool in humans subjects [32]. Other factors have been discussed that might determine the composition of the intestinal microbiota such as age [15, 16, 17], and breed [17]. Therefore, this study was conducted to gain more knowledge about how gastrointestinal mucosa microbial populations of adult and old dogs are impacted by different fermentability of fibers and protein sources of animal or vegetable origins. This is the first study that looked for how colon mucosa microbiome is affected by dietary components in adult and old dogs through Illumina sequencing.

The major bacterial phylum present in colon mucosa of dogs were Firmicutes and Bacteroidetes, these find were similar to previous reports in the canine feces [11, 33, 34, 35]. Although Firmicutes and Bacteroidetes are predominant phyla present in the canine feces, a few other studies have found a greater presence of Fusobacteria [12, 14] on the feces. These differences may be a result of differences in the type of sample (mucosa versus feces), or

differences in microbial compositions of individuals sampled or owing to differences in extraction methods, primers used or by the variable regions of the 16S rRNA amplified.

Prevotella is saccharolytic, possess enzymes that degrade complex indigestible carbohydrates, both xylan and cellulose through carbohydrate-active enzymes such as xylanase, carboxymethylcellulase, and endoglucanase [36, 37]. In the present study, all the 3 diets had similar amounts of fiber, but they had different sources of fiber and probably the dietary fiber type led to a higher *Prevotella* abundance in the dogs that were fed NFF and FF diet. The NFF diet was formulated with sugarcane fiber, an insoluble and low fermentable fiber source [38, 39] which is composed of approximately 45.8% cellulose, 28.1% hemicellulose, and 9.3% lignin [40]. The FF diet was formulated with beet pulp fiber, beet pulp is a non-viscous moderately fermentable source of fiber [NRC, 2006], which is composed of approximately of 25% cellulose and 31% hemicellulose and 16% soluble polysaccharides [41]. One study using diferents sources of fiber found Prevotellaceae family consisted of more than 25% of the total fecal microbiota in pigs fed the chicory forage diet, which is about 3 to 22 times higher than in pigs fed the other diets (sugar beet pulp and wheat bran) [42]. Moreover, a study showed that children from a rural African village of Burkina, where the diet is high in fiber content, showed a significant enrichment in Bacteroidetes and *Prevotella* [43] In addition, an association between *Prevotella* dominated microbiota and fiber intake has been showed in human [44]. These finds indicates that bacteria belonging to *Prevotella* are important to some fiber source degradation.

Alcaligenaceae and *Suterella* belongs to the Proteobacteria phylum, these both bacteria are assacharolytic that can utilize urea, a range of aminoacids and can produce nitric oxide [45]. It is know that the protein fermentation of animal origin in the colon induces increase of fecal ammonia and BCFA formation than vegetable protein sources [46, 47]. The

FF diet was formulated with beet pulp, a fermentable and no viscous fiber [NRC, 2006], and with poultry-by product meal as protein source. In the first part of this study, the beet pulp had decreased protein digestibility in old dogs [21], thereby the increased protein or amino acid content that reached the colon been available as a substrate to these group of bacteria. Barry and colleagues evaluated the effects of dietary fiber on the feline gastrointestinal metagenome and found increased Proteobacteria on fecal samples with the pectin diet supplementation compared to cellulose or fructooligosaccharides supplementation [48]. A study compared extruded diet versus a diet composed by raw human grade beef meat, representing about the 70% of the diet, found an increased abundance of Proteobacteria on fecal samples of dogs fed the raw diet [49]. These bacteria group may be affect by the diet macronutrients. Moreover, *Suterella* have been identified in fecal samples and in intestinal biopsy samples from individuals with Crohn's disease and ulcerative colitis [50] and in dogs with acute hemorrhagic diarrhea [51].

Lachnospiraceae (clostridial cluster XIVa) is a strictly anaerobic family of clostridia that are major constituents of mammalian gastrointestinal tract microbiomes and have been associated with the maintenance of gut health [52]. Genome comparisons of the carbohydrate-active enzymes, transporters, and metabolic pathways of the Lachnospiraceae in comparison with the Clostridiaceae, reveal these groups to be more highly specialized for the degradation of complex plant material, like cellulose and hemicellulose components [52]. In the present study, the higher abundance of Lachnospiraceae undefined genus were found in dogs fed NFF diet, that was formulated with sugarcane fiber, an insoluble and low fermentable fiber source [38, 39], which is composed of approximately 45.8% cellulose, 28.1% hemicellulose, and 9.3% lignin [40]. Moreover, this group of bacteria are able to produce short-chain fat acids, including acetate, butyrate, and propionate substance that are important for both microbial and

host epithelial cell growth [5, 53, 54]. Depletions of specific *Clostridium* clusters, like Lachnospiraceae, have been associated with inflammatory bowel disease in humans [55] and in dogs with idiopathic inflammatory bowel disease [56]. These finds warrant further investigations on the potentially protective role of these bacterial groups in gastrointestinal disease of dogs.

Veillonellaceae and *Megamonas* are member of the Clostridium IX [57], these bacterial groups are assacharolytic and utilize end products of sugar metabolisms of other gastrointestinal bacteria [58; 59], such as lactate or succinate, to produce propionate. Propionate is a beneficial product of the gastrointestinal microbiota as it has anti-inflammatory potential, is utilized by adipose tissue and the liver, plays a role in the satiety sensation, influences glucose and energy homeostasis, and improves insulin sensitivity [60, 61]. In the first part of this study, dogs fed FF and SM diets had increase total short-chain fatty acids and propionate concentrations in feces, because beet pulp and soybean are more fermentable in the colon of dogs, an increase in microbial activity in relation to sugar cane would be expected. *Megamonas* is a predominant genus of the family Veillonellacee and is reported to increase in the feces of dogs fed diet supplemented with inulin [12].

Catenibacterium is a member of Erysipelotrichi class and was more abundant in dogs fed SM diet, but their biological function is unclear. The Erysipelotrichi class has been associated with colon mucus barrier impenetrability in mice [62]. In dogs, the decreased abundance of Erysipelotrichi class and Erysipelotrichaceae family has been associated with acute hemorrhagic diarrhea [51].

In the present study, some bacterial family and genus populations were altered by protein source. The bacterial *Peptococcus* are able to decarboxylation and deamination of amino acids having ammonia as final product [2]. These bacteria were higher in the colon

mucosa of dogs fed NFF and FF diets, both diets containing poultry by-product meal as a protein source. The fecal ammonia concentration was also higher to dogs fed NFF and FF diets [21]. Probably, the protein source, whether due to amino acid balance or digestibility, may alter the microbial balance within the intestinal tract of dogs [12].

Slackia is an asaccharolytic member of Coriobacteriaceae and Actinobacteria [63] and a higher abundance was in dogs fed NFF and FF diets. The *Slackia* genus was correlated with the with fecal score in cats naturally occurring chronic diarrhea, suggesting that increased numbers of these organisms may be important to gut health [64]. In dogs, decreased proportion of the family Coriobacteriaceae was observed in dogs with inflammatory bowel disease and other faecal dysbiosis in comparison to healthy subjects [56].

Comamonas belong to the Betaproteobacteria, are asaccharolytic rod that can utilize urea, a range of amino-acids, and can produce nitric oxide [65]. The authors did not find enough information about this group of bacteria in dogs. No available information about association of other species with specific gastrointestinal sites, diseases or functions of the gastrointestinal microbiota.

In human, the age-related changes in the gut microbiota composition include a decline in microbiota diversity, a decrease in saccharolytic bacteria and an increase in proteolytic bacteria [66] decreased abundance of dominant species and increased abundance of subdominant species [67]. Comprehensive and detailed reviews in this field were performed [27, 28]. The studies were mostly in line in reporting an age-related reduction of diversity in the gut ecosystem, as well as an increased colonization by opportunistic species and pathobionts, such as streptococci, staphylococci, enterobacteria and enterococci. Accompanied by rearrangements in the saccharolytic and proteolytic population, with a reduction in species known for producing short-chain fatty acids, in particular butyrate [67].

Data on changes of dominant microbiota associated with ageing are also lacking for dogs. The present study indicated that the Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria, Actinobacteria and Deferribateres, the predominant bacterial members found on the colon mucosa microbiota were not change by the ageing in dogs and that Coriobacteriaceae, *Slackia*, Bacteroidaceae, *Bacteroides*, Paraprevotellaceae undefined genus, Lachnospiraceae and *Plesiomonas* were decreased in old dogs. These results are difficult to compare to other studies with old dogs since they used different methodologies to evaluate the microbiota and different regions of colon mucosa or feces.

Few studies explored the effect of age on the colon microbiota of dogs [15, 16, 17, 68]. One study compared research Beagle dogs of different ages (less than 12 months vs. more than 11 years of age) and using cultivated method found lower levels of *Bacteroides*, *Peptostreptococci*, *Bifidobacteria*, *Lactobacilli* and *Staphylococci* in the colon, whereas *Clostridium perfringens* and *Streptococci* were higher in elderly dogs [15]. Other study using cultivated methods found increased *Clostridium spp*, *Lactobacillus spp* and *Bacteroides spp* in senior dogs compared with young adult dogs [68]. A molecular fingerprinting study based on denaturing gradient gel electrophoresis (DGGE) compared younger adult (2.5 years old) and old (11 years old) and showed only a lower numbers of *Bacteroides* in old dog on canine fecal microbiota [Simpson et al. 2002]. Gomes and colleagues found no evidence of an age (4 weeks years old versus 10 years old) effect on microbial fecal counts with similar numbers of total aerobes, total anaerobes, *E. coli*, *Clostridium spp*, *Lactobacillus spp* and *Bifidobacterium spp* by cultivated method [17]

In conclusion, this study demonstrated that old and adults dogs harbored a diverse colon mucosa microbial profile, with the same predominant bacterial groups. Also indicated macronutrients related changes in the predominant colon mucosa microbiota of dogs. The

fiber sources with different fermentation profiles led to different abundances of some bacteria. The NFF diet led to higher abundance of *[Prevotella]* and Lachnospiraceae undefined genus and the FF and SM diet led to higher abundance of Veillonellaceae and *Megamonas*. Also the protein source of animal origin led to an higher abundance of bacteria groups related to protein degradation, amino acids and derivatives, such as Peptococcaceae, *Peptococcus* and *Slackia*. Ageing showed changes in the canine colon mucosa microbiota with lower abundance of some bacterial groups on colon mucosa of old dogs. Future studies addressing understanding age-related changes in the colon mucosa of dogs, the associated microbial metabolic activity, together with the effects of different dietary components on the microbiota, is essential to defining optimal diet for elderly dogs to enhance their quality of life.

Acknowledgments

The authors acknowledge the technical support of GUABI PET CARE, Campinas, SP, Brazil to the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”.

Author Contributions

Conceived and designed the experiments: APJM, ACC. Performed the experiments: APJM, FN, PD. Analyzed the data: APJM, PD, HDH, KSS, ACC. Wrote the paper: APJM, PD, HDH, MOSG, ACC, KSS.

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Supporting Information

S1 Fig. Alpha diversity results. Phylogenetic diversity metric based on PD_whole_tree method on the colon. No significant difference was observed between the two ages ($P=0.244$).

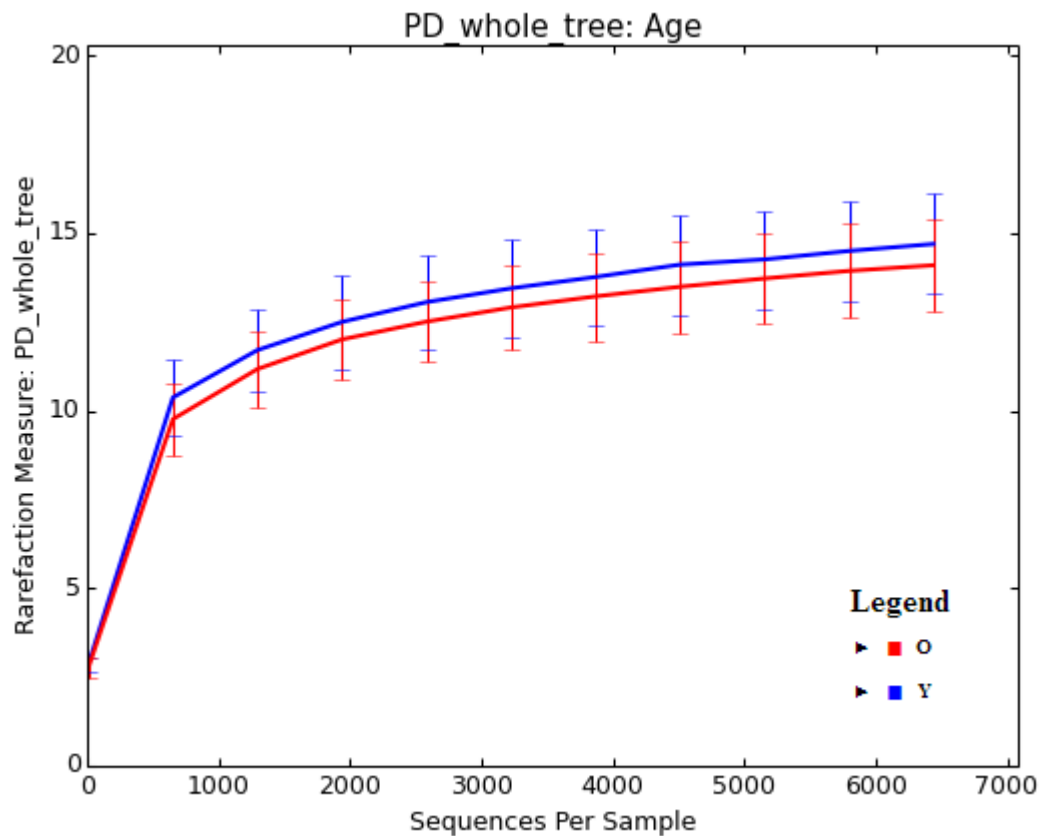


Figure 1. Rarefaction analysis of 16 S rRNA gene sequences obtained from canine colon mucosa samples. Lines represent the average of each group (blue = young adult dogs; red = old dogs), while the error bars represent the standard deviations. The analysis was performed on a randomly selected subset of 6448 sequences per sample.

S2 Fig. Alpha diversity results. Phylogenetic diversity metric based on PD_whole_tree method on the colon. No significant difference was observed between the three diets ($P>0.05$).

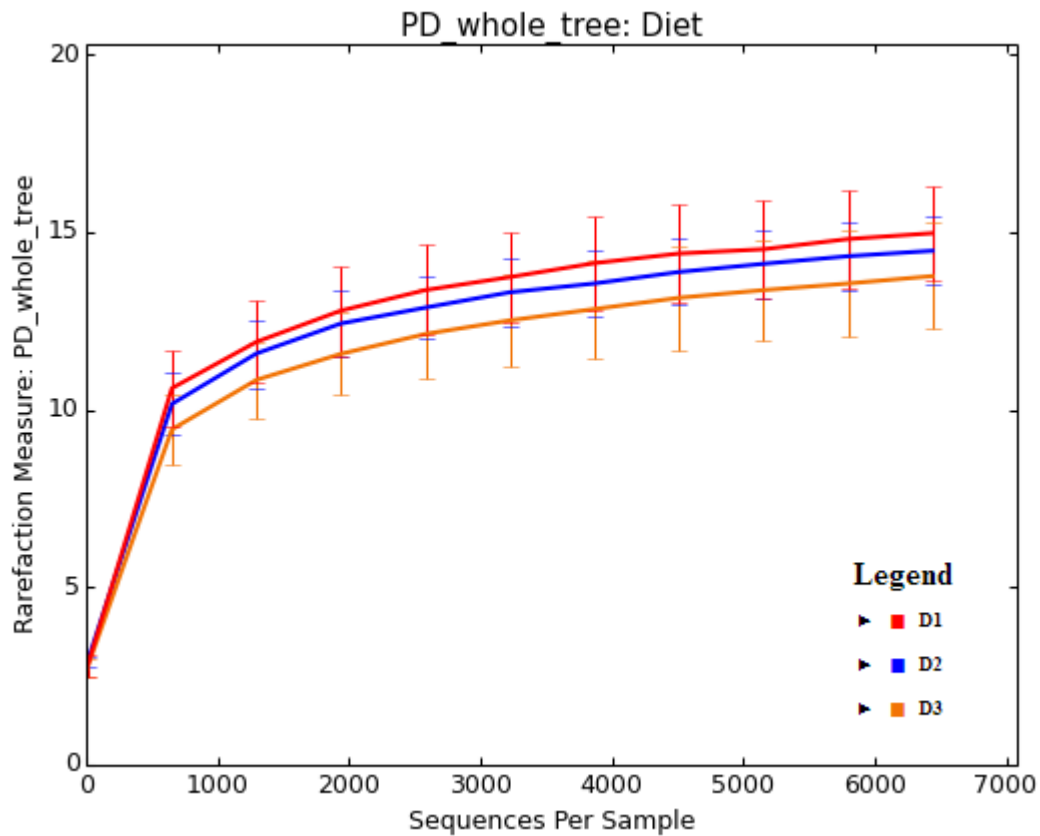


Figure 2. Rarefaction analysis of 16 S rRNA gene sequences obtained from canine colon mucosa samples. Lines represent the average of each group (orange= NFF diet; blue = FF diet; red = SM diet), while the error bars represent the standard deviations. The analysis was performed on a randomly selected subset of 6448 sequences per sample.

Aerococcaceae ¹		Adult	0.07±0.09	0.19±0.08	0.01±0.08								
		Older	0.01±0.01	0.01±0.01	0.03±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.7507	0.6159	0.6387
Facklamia ¹		Adult	0.02±0.01	0.01±0.01	0.02±0.01								
		Older	0.01±0.01	0.01±0.01	0.03±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.9481	0.6159	0.6387
f__Carnobacteriaceae ¹		Adult	0.02±0.01	0.01±0.01	0.02±0.01								
		Older	0.01±0.01	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.8361	0.9117	0.5978
Lactobacillaceae ¹		Adult	0.03±0.01	0.00±0.01	0.00±0.01								
		Older	5.80±2.27	7.81±2.06	4.57±2.06	6.04±1.27	4.92±1.38	4.85±1.69	7.07±1.53	4.51±1.53	0.5664	0.4223	0.6325
Lactobacillus ²		Adult	3.78±2.53	6.31±2.27	4.54±2.27								
		Older	5.80±2.27	7.81±2.06	4.57±2.06	6.04±1.27	4.92±1.38	4.85±1.69	7.07±1.53	4.51±1.53	0.7762	0.4223	0.6325
Streptococcaceae ¹	Streptococcus ¹	Adult	3.78±2.53	6.31±2.27	4.54±2.27								
		Older	0.57±0.22	0.15±0.21	0.34±0.20	0.35±0.12	0.35±0.14	0.54±0.17	0.22±0.15	0.30±0.15	0.9627	0.1882	0.5863
Turicibacteraceae	Turicibacter	Adult	0.49±0.25	0.30±0.22	0.23±0.23								
		Older	0.57±0.21	1.18±0.19	0.34±0.19	0.70±0.12	0.38±0.13	0.46±0.17	0.79±0.15	0.37±0.15	0.0885	0.0860	0.1328
o__Clostridiales f__ ¹	g__ ¹	Adult	0.36±0.23	0.36±0.21	0.44±0.21								
		Older	0.63±0.46	0.83±0.42	1.36±0.42	0.95±0.24	0.95±0.27	0.72±0.33	0.78±0.30	1.35±0.30	0.4345	0.6897	0.3486
Clostridiaceae		Adult	0.82±0.52	0.71±0.46	1.34±0.46								
		Older	3.41±1.08	6.11±0.98	3.32±0.98	4.31±0.58	5.21±0.64	4.38±0.8	5.95±0.72	3.95±0.72	0.4957	0.3062	0.1413
	g__	Adult	5.48±1.21	5.67±1.08	4.61±1.08								
		Older	2.78±0.92	5.30±0.84	2.16±0.84	3.43±0.50	4.29±0.55	3.51±0.69	5.07±0.62	2.99±0.62	0.3704	0.2594	0.0682
	Clostridium ¹	Adult	4.32±1.03	4.72±0.92	3.91±0.92								
		Older	0.62±0.37	0.82±0.34	1.16±0.34	0.88±0.20	0.93±0.22	0.87±0.27	0.89±0.25	0.96±0.25	0.5156	0.2838	0.9203
Lachnospiraceae		Adult	1.16±0.41	0.96±0.37	0.71±0.37								
		Older	6.48±1.14	5.69±1.05	4.11±1.05	5.43±0.80 ^B	7.1±0.84 ^A	7.34±0.95	5.94±0.89	5.51±0.89	0.4182	0.0466	0.1926
	g__	Adult	8.22±1.24	6.05±1.14	7.05±1.14								
		Older	3.49±0.60	2.32±0.55	2.13±0.55	2.67±0.35	3.35±0.38	4.20±0.46 ^A	2.63±0.42 ^B	2.20±0.42 ^B	0.4721	0.1603	0.0063
	Blautia	Adult	5.02±0.67	2.91±0.60	2.21±0.60								
		Older	0.95±0.18	1.28±0.17	0.75±0.17	1.00±0.10	0.99±0.11	0.98±0.13	1.21±0.12	0.80±0.12	0.7149	0.9477	0.0699
	Dorea ¹	Adult	1.00±0.20	1.11±0.18	0.85±0.18								
		Older	1.51±0.57	1.21±0.51	0.90±0.51	1.19±0.33	2.10±0.36	1.74±0.45	1.36±0.40	1.83±0.40	0.4347	0.1034	0.6815
	[Ruminococcus] ¹	Adult	1.93±0.63	1.45±0.57	2.88±0.56								
		Older	0.52±0.31	0.86±0.29	0.30±0.28	0.55±0.18	0.61±0.20	0.40±0.25	0.66±0.22	0.68±0.22	0.0606	0.6320	0.5414
Peptococcaceae	Peptococcus	Adult	0.25±0.35	0.42±0.31	1.13±0.31								
		Older	0.38±0.12	0.45±0.11	0.17±0.11	0.34±0.07	0.40±0.07	0.44±0.09 ^A	0.47±0.08 ^A	0.19±0.08 ^B	0.8999	0.5498	0.0428
Peptostreptococcaceae ¹		Adult	0.51±0.14	0.47±0.12	0.21±0.12								
		Older	0.02±0.15	0.41±0.14	0.03±0.14	0.16±0.09	0.15±0.10	0.15±0.13	0.28±0.11	0.03±0.11	0.6447	0.9604	0.6092
	g__ ¹	Adult	0.31±0.17	0.13±0.15	0.03±0.15								
		Older	0.01±0.13	0.41±0.12	0.03±0.12	0.15±0.07	0.08±0.08	0.04±0.10	0.27±0.09	0.03±0.09	0.8599	0.4824	0.7850
Ruminococcaceae ¹		Adult	0.08±0.14	0.13±0.13	0.03±0.13								
		Older	2.64±0.96	3.18±0.88	1.83±0.88	2.59±0.53	3.35±0.59	3.82±0.73	2.99±0.66	2.11±0.66	0.5870	0.4911	0.1208
	g__ ¹	Adult	5.20±1.08	2.068±0.98	2.37±0.96								
		Older	2.34±0.76	2.83±0.69	1.66±0.69	2.31±0.41	2.91±0.46	3.21±0.57	2.70±0.51	1.91±0.51	0.5984	0.4310	0.1334
	Faecalibacterium ¹	Adult	4.23±0.85	2.48±0.76	2.15±0.76								
		Older	0.23±0.23	0.33±0.21	0.15±0.21	0.25±0.13	0.40±0.14	0.53±0.17	0.27±0.16	0.17±0.16	0.3403	0.6551	0.2007
Veillonellaceae		Adult	0.89±0.25	0.17±0.23	0.18±0.23								
		Older	20.33±3.56	21.23±3.25	28.55±3.25	23.26±1.93	19.2±2.13	16.28±2.65 ^B	21.39±2.40 ^{AB}	26.02±2.40 ^A	0.4266	0.1681	0.0372
	Megamonas	Adult	11.73±3.98	21.98±3.56	23.38±3.56								
		Older	12.96±3.52	13.88±3.21	23.17±3.21	16.59±1.90	12.25±2.10	9.43±2.61 ^B	13.97±2.36 ^{AB}	19.86±2.36 ^A	0.4382	0.1357	0.0208

		Adult	5.56±3.93	14.52±3.52	16.33±3.52									
	Phascolarctobacterium	Older	5.47±0.73	5.79±0.67	4.09±0.67	5.09±0.40	5.80±0.44	5.39±0.55	6.07±0.49	4.88±0.49	0.4382	0.2381	0.2448	
		Adult	5.21±0.82	6.34±0.73	5.75±0.73									
	Veillonella ³	Older	1.89±0.60	1.57±0.55	1.30±0.55	1.58±0.32	1.12±0.35	1.46±0.43	1.30±0.39	1.28±0.39	0.5966	0.3103	0.9787	
		Adult	0.98±0.67	1.03±0.60	1.31±0.60									
	[Mogibacteriaceae]	Older	0.18±0.07 ^b	0.10±0.06	0.05±0.07	0.11±0.04	0.23±0.04	0.38±0.05 ^A	0.10±0.05 ^B	0.04±0.05 ^B	0.0192	0.0656	0.0030	
		Adult	0.57±0.08 ^{Aa}	0.10±0.07 ^B	0.02±0.07 ^B									
	Erysipelotrichaceae ¹	Older	0.20±0.14	0.37±0.13	0.38±0.13	0.32±0.09	0.35±0.10	0.24±0.12	0.34±0.11	0.43±0.11	0.0606	0.8952	0.4963	
		Adult	0.28±0.16	0.30±0.14	0.48±0.15									
	Allobaculum	Older	0.09±0.04	0.20±0.04	0.06±0.03	0.12±0.02	0.14±0.02	0.10±0.03	0.17±0.03	0.11±0.03	0.0788	0.4985	0.2341	
		Adult	0.12±0.04	0.13±0.04	0.17±0.04									
	Catenibacterium	Older	0.07±0.05	0.11±0.04	0.19±0.04	0.12±0.03	0.06±0.03	0.04±0.04 ^B	0.09±0.03 ^{AB}	0.15±0.03 ^A	0.8841	0.0585	0.0213	
		Adult	0.01±0.05	0.07±0.05	0.11±0.05									
	[Eubacterium] ¹	Older	0.04±0.09	0.06±0.08	0.12±0.08	0.07±0.06	0.14±0.06	0.08±0.07	0.08±0.06	0.16±0.06	0.7280	0.3420	0.3270	
		Adult	0.14±0.10	0.10±0.09	0.19±0.09									
Fusobacteria ¹		Older	4.24±2.09	8.14±1.91	4.03±1.91	5.48±1.24	7.61±1.37	5.38±1.70	6.79±1.54	7.46±1.54	0.1038	0.1168	0.8673	
		Adult	6.52±2.34	4.96±2.09	11.36±2.09									
	Fusobacteriaceae ²	Older	4.24±2.09	8.14±1.91	4.03±1.91	5.48±1.24	7.61±1.37	5.38±1.70	6.79±1.54	7.46±1.54	0.0839	0.1168	0.8673	
		Adult	6.52±2.34	4.96±2.09	11.36±2.09									
	Fusobacterium ¹	Older	4.24±2.09	8.14±1.91	4.03±1.91	5.48±1.24	7.61±1.37	5.38±1.70	6.79±1.54	7.46±1.54	0.1035	0.1168	0.8673	
		Adult	6.52±2.34	4.96±2.09	11.36±2.09									
Proteobacteria ¹		Older	3.56±2.13	4.71±1.94	4.11±1.94	4.14±1.12	7.24±1.24	5.26±1.54	5.78±1.39	6.03±1.39	0.9447	0.1877	0.6477	
		Adult	6.98±2.38	6.74±2.13	8.01±2.13									
	Alcaligenaceae	Sutterella	Older	0.53±0.15 ^B	1.42±0.14 ^{Aa}	0.65±0.14 ^B	0.87±0.08	0.77±0.09	0.60±0.11 ^B	1.07±0.10 ^A	0.80±0.10 ^{AB}	0.0049	0.4778	0.0195
		Adult	0.67±0.17	0.71±0.15 ^b	0.94±0.15									
	Comamonadaceae ¹	Older	0.62±0.42	0.32±0.38	0.43±0.38	0.45±0.22	0.57±0.25	0.58±0.31	0.32±0.28	0.64±0.28	0.5984	0.9589	0.6001	
		Adult	0.50±0.47	0.31±0.42	0.88±0.42									
	g__ ¹	Older	0.36±0.25	0.18±0.22	0.25±0.22	0.26±0.13	0.32±0.14	0.33±0.18	0.17±0.16	0.37±0.16	0.4388	0.6083	0.8218	
		Adult	0.28±0.27	0.16±0.25	0.51±0.25									
	Comamonas ¹	Older	0.07±0.03	0.01±0.02	0.05±0.02	0.04±0.01	0.03±0.02	0.05±0.02 ^A	0.01±0.02 ^B	0.04±0.02 ^A	0.4079	0.1619	0.0307	
		Adult	0.03±0.03	0.02±0.03	0.04±0.03									
	Limnohabitans ¹	Older	0.19±0.14	0.12±0.13	0.12±0.13	0.14±0.08	0.20±0.09	0.19±0.11	0.11±0.10	0.22±0.10	0.7912	0.8376	0.7743	
		Adult	0.19±0.16	0.08±0.14	0.33±0.15									
	Neisseriaceae ¹	Older	0.00±0.04	0.00±0.03	0.00±0.03	0.00±0.02	0.04±0.02	0.00±0.03	0.05±0.03	0.00±0.03	>0.05	0.3689	0.7318	
		Adult	0.00±0.04	0.10±0.04	0.00±0.04									
	g__ ⁴	Older	0.00±0.01	0.00±0.01	0.00±0.01	0.00±0.01	0.01±0.01	0.00±0.01	0.02±0.01	0.00±0.01	>0.05	0.1955	0.7607	
		Adult	0.00±0.01	0.03±0.01	0.00±0.01									
	Conchiformibius ⁴	Older	0.00±0.03	0.00±0.02	0.00±0.02	0.00±0.01	0.02±0.02	0.00±0.02	0.03±0.02	0.00±0.02	>0.05	0.3948	0.9954	
		Adult	0.00±0.03	0.07±0.03	0.00±0.03									
	Rhodocyclaceae ¹	Older	0.13±0.11	0.06±0.10	0.17±0.10	0.12±0.06	0.14±0.07	0.12±0.08	0.06±0.07	0.21±0.07	0.4830	0.7672	0.5600	
		Adult	0.12±0.13	0.05±0.11	0.25±0.11									
	g__Dechloromonas ¹	Older	0.13±0.11	0.05±0.10	0.17±0.10	0.12±0.06	0.13±0.06	0.12±0.08	0.05±0.07	0.20±0.07	0.5718	0.7297	0.5707	
		Adult	0.11±0.12	0.04±0.11	0.24±0.11									
	Campylobacteraceae ³	Campylobacter ⁴	Older	0.05±0.99	0.04±0.91	0.01±0.90	0.08±0.55	1.17±0.60	1.51±0.75	0.13±0.68	0.24±0.68	>0.05	0.2954	0.4725
		Adult	3.20±1.11	0.13±0.99	0.41±0.99									
	Helicobacteraceae ¹	Helicobacter ¹	Older	0.22±0.47	0.46±0.43	0.84±0.43	0.51±0.26	0.89±0.29	0.48±0.36	0.99±0.33	0.63±0.33	0.6128	0.3377	0.1869
		Adult	0.76±0.53	1.59±0.47	0.34±0.47									
	Succinivibrionaceae ¹	Older	0.60±0.24	0.59±0.22	0.30±0.22	0.49±0.16	0.75±0.17	0.56±0.20	0.66±0.18	0.64±0.18	0.1957	0.2512	0.8594	

Enterobacteriaceae ¹	g ₋₋₋ ¹	Adult	0.48±0.26	0.71±0.24	1.02±0.24									
		Older	0.08±0.06	0.11±0.06	0.11±0.06	0.10±0.04	0.10±0.04	0.05±0.05	0.14±0.04	0.09±0.04	0.0634	0.8241	0.3641	
	Anaerobiospirillum ²	Adult	0.02±0.07	0.19±0.06	0.07±0.06									
		Older	0.52±0.24	0.49±0.22	0.19±0.22	0.39±0.16	0.65±0.17	0.51±0.20	0.51±0.18	0.55±0.18	0.1572	0.1620	0.9599	
		Adult	0.45±0.27	0.52±0.24	0.95±0.24									
		Older	1.06±1.36	1.67±1.25	1.58±1.25	1.40±0.86	2.49±0.91	0.95±1.07	2.14±0.99	2.74±0.99	0.6462	0.7068	0.5030	
Pasteurellaceae ¹	g ₋₋₋ ¹	Adult	0.69±1.49	2.59±1.36	4.02±1.36									
		Older	1.02±0.72	1.58±0.66	1.52±0.66	1.36±0.39	1.47±0.43	0.83±0.54	1.77±0.48	1.64±0.48	0.8983	0.9179	0.4998	
	Escherichia ¹	Adult	0.59±0.81	1.99±0.72	1.77±0.72									
		Older	0.03±0.02	0.03±0.02	0.05±0.02	0.04±0.01	0.04±0.01	0.03±0.01	0.04±0.01	0.05±0.01	0.6960	0.8681	0.6432	
	Plesiomonas ¹	Adult	0.01±0.02	0.05±0.02	0.06±0.02									
		Older	0.07±0.88	0.03±0.81	0.00±0.81	0.01±0.54 ^B	0.93±0.58 ^A	0.13±0.69	0.28±0.63	1.01±0.63	0.5166	0.0166	0.5537	
Moraxellaceae ¹	g ₋₋₋ ⁴	Adult	0.09±0.98	0.48±0.88	2.13±0.88									
		Older	0.01±0.20	0.01±0.19	0.01±0.19	0.01±0.11	0.28±0.12	0.24±0.15	0.17±0.14	0.02±0.14	0.3138	0.1568	0.9224	
		Adult	0.50±0.23	0.34±0.20	0.02±0.20									
		Older	0.01±0.17	0.01±0.16	0.01±0.16	0.01±0.09	0.23±0.10	0.19±0.13	0.16±0.12	0.02±0.12	>0.05	0.3948	0.9954	
	Acinetobacter ¹	Adult	0.38±0.19	0.32±0.17	0.02±0.17									
		Older	0.09±0.04	0.03±0.04	0.05±0.04	0.05±0.02	0.05±0.03	0.06±0.03	0.05±0.03	0.04±0.03	0.7280	0.8841	0.5882	
Pseudomonadaceae ¹	Pseudomonas ¹	Adult	0.02±0.05	0.08±0.04	0.04±0.04									
		Older	0.05±0.03	0.03±0.02	0.03±0.03	0.04±0.01	0.03±0.02	0.04±0.02	0.03±0.02	0.03±0.02	0.6834	0.5544	0.7046	
		Adult	0.02±0.03	0.03±0.03	0.04±0.03									
		Older	0.09±0.09	0.07±0.08	0.06±0.09	0.07±0.05	0.13±0.06	0.07±0.07	0.09±0.06	0.14±0.06	0.9316	0.9254	0.8354	
		Adult	0.05±0.10	0.10±0.09	0.22±0.09									

¹-Log10

²- Log(x+1)

³- SQRT

⁴- No parametric analyses

^{a, b} Means in the same row and not sharing a common superscript lowercase letters differ ($P < 0.05$).

^{A, B, C} Means in the same column and not sharing common superscript capital letters differ ($P < 0.05$).

¹ IF: diet with sugarcane fiber and poultry by-product meal; FF: diet with beet pulp and poultry by-product meal; SM: diet with 30% soybean meal to partially replace the poultry by-product meal and add fiber.

CAPITULO 3 – MICROBIOTA DIVERSITY ALONG GUT MUCOSA OF DOGS¹

Short title: Gut Microbiota of dogs

Funding: This work was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), process number 2011/23575-3. The scholarship of the first author was supported by Capes - Programa de Doutorado-sanduíche no Exterior (PDSE) and Programa de Demanda Social (DS).

¹ Escrito de acordo com as normas da revista *PLOS ONE*

MICROBIOTA DIVERSITY ALONG GUT MUCOSA OF DOGS¹

Ana Paula J. Maria^{1¶}, Ping Deng^{2¶}, Hannah D. Holscher^{2¶}, Thaila C. Putarov^{1¶}, Franz N. Yoshitoshi^{3¶}, Kelly S. Swanson^{2¶*}, Aulus C. Carciofi^{1¶*}

¹ Department of Veterinary Clinic and Cirurgic, São Paulo State University, School of Agricultural and Veterinarian Sciences, Jaboticabal, SP, Brasil;

² Department of Animal Science, University of Illinois at Urbana Champaign, Urbana, IL, United States of America;

³ Endoscopet - Endoscopy and Surgery, São Paulo, SP, Brasil.

Presented partially as an oral communication at the 19th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN), Toulouse, France, September, 17st to 19th, 2015.

*Corresponding author

E-mail: aulus.carciofi@gmail.com (ACC)

E-mail: ksswanso@illinois.edu (KSS)

¶These authors contributed equally to this work.

Abstract

The aim of this study was to describe the diversity of the microbiota associated to the duodenum, jejunum and colon mucosa in dogs using Miseq Illumina sequencing. Thirty-six Beagles dogs were assigned to kibble dietary during 30 days. Intestinal mucosa sample collection from the duodenum, jejunum, and colon were performed by endoscopy and colonoscopy on day 31 and 32. DNA was extracted and the V4 region of the 16S rRNA gene was amplified and subjected to Miseq Illumina sequencing. Predominant intestinal mucosa bacterial phyla included Firmicutes (45.3%), followed by Bacteroidetes (34.3%), Proteobacteria (10.2%), Actinobacteria (5.7%), Fusobacteria (4.2%) and Deferribacteres (0.2%) taking together all the 3 segments. Actinobacteria and Proteobacteria were in higher abundance in the jejunum and duodenum mucosa compared with the colon ($P < 0.001$) and Bacteroidetes and Deferribacteres were in higher abundance in the colon mucosa ($P < 0.01$). *Veillonellaceae* was the predominant family in duodenum, jejunum and colon. Among duodenum and jejunum mucosa, *Prevotellaceae* and *Lactobacillaceae* were also predominant families. Furthermore, in the colon mucosa the predominant colon bacterial families were [*Paraprevotellaceae*], *Prevotellaceae* and *Fusobacteriaceae*. The molecular approach as described in this study facilitated the identification of several shifts along the intestinal tract mucosa of healthy dogs. Along the canine intestinal tract a number of significant shifts were found in family and genera levels, showing the differences among small and large intestine.

Introduction

The intestinal microbiota can be defined as the dynamic collection of microorganisms within the gastrointestinal (GI) tract and the system of interactions of these organisms have with each other and with the host cells [1]. The resident microbiota plays an important role in

the digestion and metabolism of the host; it has a physiologic effect on gastrointestinal motility, acts in the development of the intestinal epithelium and the immune system [2], and also provides a natural defense mechanism against invading pathogens [2, 3, 4]. Further, the microbiota supplies nutrients such as vitamins, lactate, and short-chain fatty acids to host tissues [5]. However, the microbiota composition can be influenced by diet [6, 7, 9, 10], antibiotics used [11], GI disease [13], and ageing [14]. These complex interactions among the microbiota, immune system, and host genetics that influence the balance between health and disease [1].

The intestinal microbiota is a dynamic system and its composition varies within an individual, like different locations of the GI tract, luminal vs. mucosa-adherent, or different time points; [15, 16] and among individuals [17]. Most of the available information about the attached gut mucosa microbiota in dogs has been obtained in studies of idiopathic inflammatory bowel disease [18, 19] using molecular approaches. One study used cultured dependent methods in healthy dogs [20] to characterize the microbiota on the intestinal mucosa in dogs. Others studies characterized the microbiota of the gastrointestinal tract have already been performed using 16S rRNA gene sequences in dogs and cats [15, 16] but these studies used luminal intestinal content or solid intestinal content for the identification of the microbial population along the intestinal tract. Identification and characterization of canine intestinal mucosa microbiota will be useful by the molecular approach in the intestinal tract of healthy dogs and studies in this area are warranted. The aim of the present study was to investigate the predominant microbiota associated along the gastrointestinal mucosa of healthy dogs using the Illumina sequence.

Material and methods

Animals and diets

All the animal procedures were approved by the Ethics Committee in the Use of Animals of the School of Agricultural and Veterinarian Sciences, São Paulo State University according to the Brazilian animal protection law (Protocol n° 546/16).

Thirty six healthy beagles were divided into two age groups; 18 older dogs [10.2 ± 1.0 yr of age and 11.8 ± 2.1 kg body weight (BW)] and 18 adult dogs (2.6 ± 0.9 yr of age and 11.05 ± 1.2 kg BW). Three isonutritive kibble diets with different sources of fiber and protein were tested: a diet with insoluble and non-fermentable fiber (NFF) was based on sugarcane fiber and poultry by-product meal; a diet with soluble and fermentable fiber (FF) was based on beet pulp and poultry by-product meal; a diet with 30% of soybean meal (SM), which was both the source of fiber and partially replaced the protein source. Diets were formulated according to the recommendations for dog's maintenance [21], and were manufactured in the extruder facility of the School of Agricultural and Veterinarian Sciences, São Paulo State University.

Diets were extruded under identical processing conditions in a single-screw extruder (Mab 400S, Extrucenter, Monte Alto, Brazil). Dogs were offered two meals daily and were fed to maintain constant BW during the study. Food amount provided was initially determined by calculating the daily maintenance energy requirement of kennel dogs (ME, kcal = $130 \times \text{kg BW}^{0.75}$) [3]. Dogs were weighed weekly and if necessary the food amount was adjusted. Water was provided *ad libitum*. Dogs were twin-housed in kennels (1.5x 3.5 m) with a solarium at the Laboratory of Research on Nutrition and Nutritional Diseases of Dogs and Cats, São Paulo State University (Jaboticabal, Brazil).

Experimental procedure

The experiment was designed as a 3 x 2 factorial arrangement with three diets and two ages, totaling six treatments. Dogs were separated into three blocks of 12 animals, each

containing 6 adults and 6 old animals, with two replicates per food and age in each block, totaling six animals per treatment. Each block lasted 32 days, in which days 1 to 30 was adaptation phase. Intestinal mucosa samples were collected on days 31 and 32. Prior to the endoscopic and colonoscopic biopsy procedure, dogs were fasted for 12 hr and then sedated with 1.0 mg chlorpromazine/kg (Sanofi-Aventis pharmaceuticals Ltda, Suzano, Brazil) intramuscularly. After sedation, anesthesia induction was performed with propofol (Cristália chemical pharmaceutical products ltd, São Paulo, Brazil) at a dose of 5.0 mg/kg, given slowly through an intravenous drip. After the animals lost the laryngotracheal reflex, endotracheal intubation was performed with a tracheal tube of appropriate size for each animal. The anesthesia was maintained with isoflurane (Virbac Animal Health of Brazil, Jurubatuba, Brazil) in sufficient concentration to keep the animal on the anesthetic plane. For the biopsy procedure by upper and lower gastrointestinal endoscopy, the animals were placed in lateral recumbency. A flexible endoscope (Karl Storz, 60914NKS, Karl Storz GmbH & Co. KG, Germany) was used along with a biopsy forceps alligator mouth type of 2.2 mm (Karl Storz, 60252LX, Karl Storz GmbH & Co. KG, Germany) to collect the mucosa samples. During the procedure all animals received intravenous fluid therapy and after the procedure they were individually assisted for recovery from anesthesia and health condition. Two fragments were collected from the mucosa of duodenum, jejunum and colon of each dog. Samples were frozen in liquid nitrogen and then stored at -80°C until DNA extraction for microbial analysis. Fragments from ileum were not collected due to the difficult access to this region by endoscopic and colonoscopy procedure.

DNA extraction and sequencing

Genomic DNA was extracted from intestinal mucosa samples using the PowerLyzer™PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA,

USA) according to the manufacturer's instructions. Concentration of extracted DNA was quantified using a Qubit[®] 2.0 Fluorometer (Life Technologies, Invitrogen, Grand Island, NY, USA). Polymerase chain reaction (PCR) amplicons from the V4 region of the 16S rRNA gene were prepared for sequencing following a similar procedure as describe before [22] and primers according to a publish study [23]. The samples were amplified in a thermocycler DNA Engine[®] Thermal Cycler (Bio-Rad Laboratories, Inc., Foster City, CA, USA), using the PCR protocol: initial denaturing at 95°C for 5min, 35 cycles of denaturing at 95°C for 20s, annealing at 54 °C for 30s, extension at 72 °C for 1min, and final extension for 10min for sample. Amplicons from PCR were purified utilizing Agencourt[®] AMPure XP beads (Beckman-Coulter Inc., Indianapolis, IN, USA). PCR products were pooled and concentrated using the MinElute[®] Gel Extraction Kit (QIAGEN Group, Valencia, CA, USA), following the manufacturer's instructions. The quality of DNA was assessed by electrophoresis using precast agarose gels (E-Gel[®] EX Gel 1%, Invitrogen, Grand Island, NY, USA). Further the DNA concentrations were measured using Qubit[®] 2.0 Fluorometer (Life Technologies, Invitrogen, Grand Island, NY, USA). Amplicons were combined in equimolar ratios to create a DNA pool that was used for sequencing. DNA quality was assessed before sequencing using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The Illumina sequencing was performed at the W. M. Keck Center for Biotechnology at the University of Illinois utilizing a MiSeq 2x300 nt v3 technology (Illumina Inc., San Diego, CA, USA).

Bioinformatics and statistics

The data analysis was performed with QIIME 1.8.0 [24], following quality filtering [25]. Briefly, high quality (quality value > 25) sequence data sequences were demultiplexed and quality filtered using `split_libraries_fastq.py` default parameters. Resulting sequences were clustered into Operational taxonomic units (OTU) using closed-reference OTU picking

against the Greengenes 13_8 reference OTU database (97% similarity threshold). An even sampling depth of 6448 sequences per sample was used for assessing alpha- and beta-diversity measures, samples with less than 6448 sequences were excluded from subsequent analyses. Weighted and unweighted UniFrac distances between all samples were visualized using Principle Coordinates Analysis (PCoA) at an even sampling depth of 6448 sequences per sample.

Sequence percentages at each taxonomic level were analyzed using the Mixed Models procedure of Statistical Analysis System 9.2 (SAS Institute Inc., Cary, NC, USA), testing the main effect of segment from all samples (duodenum, jejunum and colon). Random effects of block and animal were included in all models. All variables were first tested for hypotheses of normality and variance homogeneity of the treatments. When the F-test was significant, means were separated for treatments using LSMEAN with Tukey's adjustment to control for multiple comparison. When the data was not normally distributed, it was transformed using the logarithmic with base 10, $\log(x+1)$ or square root prior to statistical analysis. If normality could not be achieved through transformations, the non-parametric Kruskal-Wallis test was performed. A probability of $P < 0.05$ was considered significant.

Results

A total of 21 duodenum mucosa samples (3 adult dog fed NFF; 4 old dog fed NFF; 3 adult dog fed FF; 5 old dog fed FF; 5 adult dog fed SM; 2 old dog fed SM), 14 jejunum mucosa samples (2 adult dog fed NFF; 2 old dog fed NFF; 3 adult dog fed FF; 2 old dog fed FF; 2 adult dog fed SM; 3 old dog fed SM) and 31 colon mucosa samples (4 adult dog fed NFF; 5 old dog fed NFF; 5 adult dog fed FF; 6 old dog fed FF; 5 adult dog fed SM; 6 old dog fed SM) were subjected to MiSeq Illumina sequencing. Due to the low abundance of DNA concentrations, 14 duodenum, 22 jejunum and 5 colon mucosa samples were lost. Therefore,

a total of 66 intestinal mucosa samples were subjected to MiSeq Illumina sequencing. A total of 18,894,896 sequences were obtained from the current data set, of those a total of 7,629,557 sequences were remained after quality filtering. The sequencing identified a total of 10 bacterial phyla, 57 families and 97 genera in the mucosa. According to phylogenetic diversity whole tree and rarefaction curves (S1 Fig), microbial diversity and species richness were similar among intestinal segments ($P > 0.05$).

Principal coordinates analysis (PCoA) of weighted UniFrac distances among samples based on their 97% OTU composition and abundances (S2 Fig) indicated that gut microbial communities were more similar within segments than across all segments. Intestinal mucosa microbial communities across the segments were similar within duodenum and jejunum and different between colon and duodenum (S2 Fig). Otherwise, unweighted UniFrac PCoA in Fig 2B, no clear separation was seen between segments when the abundance of bacterial species was not accounted for.

Predominant bacterial phyla present in the 3 segments (duodenum, jejunum and colon) of dog intestinal mucosa included Firmicutes (45.3%), followed by Bacteroidetes (34.3%), Proteobacteria (10.2%), Actinobacteria (5.7%), Fusobacteria (4.2%) and Deferribacteres (0.2%). The relative abundance of Bacteroidetes and Fusobacteria were higher in the colon mucosa when compared with the duodenum and jejunum mucosa ($P < 0.05$). In contrast, the abundance of Proteobacteria, Actinobacteria and Deferribacteres was lower in the colon mucosa compared to the duodenum and jejunum mucosa ($P < 0.05$; Table 1).

Predominant duodenum and jejunum bacterial families included Veillonellaceae (23.06% and 22.25%, respectively), Prevotellaceae (17.12% and 18.7%) and Lactobacillaceae (11.75% and 11.06%), and predominant colon bacterial families included Veillonellaceae

Table 1. Predominant bacterial phyla, family and genera (expressed as a percentage of total sequences) in intestinal mucosa of dogs.

Item	Phylum	Family	Genus	Duodenum	Jejunum	Colon	SEM	P Value
Actinobacteria		Bifidobacteriaceae	Bifidobacterium	7.07 ^a	6.82 ^a	4.32 ^b	0.44	<.0001
			Coriobacteriaceae ²	0.86 ^b	0.93 ^b	1.66 ^a	0.23	0.0010
			Collinsella ²	0.69 ^b	0.79 ^b	1.46 ^a	0.23	0.0017
Bacteroidetes		Bacteroidaceae ¹	Bacteroides ¹	28.98 ^b	32.08 ^b	39.22 ^a	1.87	<.0001
			Prevotellaceae	2.75 ^b	3.23 ^b	6.56 ^a	0.61	<.0001
			Prevotella	17.12 ^a	18.70 ^a	11.32 ^b	1.89	<.0001
			[Paraprevotellaceae] ¹	8.12 ^b	8.95 ^b	21.06 ^a	4.51	<.0001
			g__ ⁴	1.86 ^b	1.82 ^b	5.77 ^a	0.66	<.0001
			[Prevotella] ¹	6.26 ^b	7.12 ^b	15.28 ^a	1.70	<.0001
Deferribacteres ⁴	Deferribacteraceae ⁴	Cloacibacterium ¹	0.87 ^a	0.72 ^a	0.22 ^b	0.19	<.0001	
		Mucispirillum ⁴	0.76 ^a	0.61 ^a	0.22 ^b	0.17	0.0003	
Firmicutes ⁴		Gemellaceae ⁴	Gemella ⁴	0.23 ^a	0.20 ^a	0.18 ^b	0.08	0.0199
			g__ ⁴	47.00	45.08	44.14	1.79	0.4624
			Carnobacteriaceae ⁴	0.64 ^a	0.13 ^b	0.11 ^b	0.18	0.0012
			Lactobacillaceae	0.45 ^a	0.10 ^b	0.06 ^b	0.14	0.0024
			Clostridiaceae ¹	0.12 ^a	0.06 ^b	0.01 ^b	0.02	0.0003
			Lactobacillus ²	11.75 ^a	11.06 ^a	5.57 ^b	0.98	<.0001
			g__ ¹	2.02 ^b	1.79 ^b	4.74 ^a	0.41	<.0001
			Clostridium ⁴	1.66 ^b	1.42 ^b	3.84 ^a	0.39	<.0001
			Lachnospiraceae ¹	0.37 ^b	0.36 ^b	0.90 ^a	0.12	<.0001
			g__ ²	3.24 ^b	4.07 ^b	6.13 ^a	0.49	<.0001
			Dorea ⁴	1.41 ^b	1.83 ^b	2.9 ^a	0.26	<.0001
			Peptostreptococcaceae ¹	0.42 ^b	0.56 ^b	1.60 ^a	0.23	<.0001
			g__ ⁴	0.09	0.03	0.15	0.06	0.1571
			Veillonellaceae	0.03 ^b	0.01 ^b	0.12 ^a	0.05	0.0001
			Phascolarctobacterium	23.06	22.25	21.75	1.64	0.8201
			Veillonella	6.86 ^a	5.99 ^{ab}	5.42 ^b	0.42	0.0294
			Erysipelotrichaceae ³	3.64 ^a	3.52 ^a	1.36 ^b	0.27	<.0001
			Catenibacterium	0.20	0.30	0.34	0.05	0.0568
			[Eubacterium] ⁴	0.03 ^b	0.08 ^{ab}	0.10 ^a	0.02	0.0057
			Fusobacteria ¹	Fusobacteriaceae ¹	Fusobacterium ¹	0.01 ^b	0.04 ^a	0.10 ^a
Proteobacteria ¹		Comamonadaceae ¹	Comamonas ¹	2.10 ^b	2.36 ^b	6.51 ^a	0.78	<.0001
			g__ ¹	14.42 ^a	13.6 ^a	5.52 ^b	2.60	0.0022
			Limnohabitans ¹	2.16 ^a	3.56 ^a	0.47 ^b	1.09	<.0001
			Neisseriaceae ⁴	1.17 ^a	1.82 ^a	0.27 ^b	0.55	0.0001
			g__ ⁴	0.70 ^{ab}	1.25 ^a	0.16 ^b	0.37	0.0001
			Conchiformibius ⁴	1.10 ^a	0.15 ^b	0.02 ^c	0.39	<.0001
			Rhodocyclaceae ¹	0.33 ^a	0.06 ^b	0.01 ^c	0.12	0.0001
			Dechloromonas ¹	0.77 ^a	0.09 ^b	0.01 ^b	0.26	<.0001
			Campylobacteraceae ⁴	0.88 ^a	1.04 ^a	0.12 ^b	0.30	<.0001
			Enterobacteriaceae ¹	0.84 ^a	0.99 ^a	0.12 ^b	0.28	<.0001
			Campylobacter ⁴	0.01 ^b	0.02 ^b	0.51 ^a	0.36	0.0002
			g__ ¹	5.55 ^a	5.34 ^{ab}	1.95 ^b	1.10	0.0016
			Pasteurellaceae ⁴	5.27 ^a	5.14 ^a	1.45 ^b	1.00	0.0007
			g__Escherichia ¹	0.14 ^a	0.11 ^{ab}	0.04 ^b	0.02	0.0006
Plesiomonas ⁴	0.02 ^b	0.00 ^b	0.44 ^a	0.30	0.0149			
Moraxellaceae ¹	1.94 ^a	0.43 ^b	0.13 ^c	0.70	<.0001			
Pseudomonadaceae ¹	g__ ⁴	1.86 ^a	0.41 ^b	0.11 ^c	0.67	<.0001		
Pseudomonas ¹	0.47 ^a	0.27 ^{ab}	0.05 ^b	0.14	0.0033			
			0.62 ^a	0.89 ^a	0.10 ^b	0.19	<.0001	

¹ Log10

² Log(x+1)

³ SQRT

⁴ No parametric analyses.

^{a, b} Means in the same row and not sharing a common superscript lowercase letters differ ($P < 0.05$).

(21.75%), [Paraprevotellaceae] (21.06%), Prevotellaceae (11.32%) and Fusobacteriaceae (6.51%).

A number of significant shifts were observed among different intestinal segments at the family and genus level (Table 3). The colon mucosa had higher abundance of Coriobacteriaceae, *Collinsella*, Bacteroidaceae, *Bacteroides*, [Paraprevotellaceae], [Prevotella], Clostridiaceae, Clostridiaceae undefined genus, *Clostridium*, Lachnospiraceae Lachnospiraceae undefined genus, *Dorea*, Peptostreptococcaceae undefined genus, Fusobacteriaceae, *Fusobacterium*, Campylobacteraceae, *Campylobacter*, and Enterobacteriaceae, *Plesiomonas* when compared with duodenum and jejunum mucosa ($P < 0.05$). Furthermore, Eubacterium and Catenibacterium (Erysipelotrichales and Firmicutes) were higher ($P < 0.05$) in colon mucosa when compared with the duodenum mucosa.

The abundance of Bifidobacteriaceae, *Bifidobacterium*, Prevotellaceae, *Prevotella* Weeksellaceae, *Cloacibacterium*, Deferribacteraceae, *Muscipirillum*, Lactobacillaceae, *Lactobacillus*, *Veillonella*, Comamonadaceae, Comamonadaceae undefined genus, *Dechloromonas*, Enterobacteriaceae undefined genus and *Pseudomonas* remained relatively higher ($P < 0.05$) abundances in duodenum and jejunum mucosa compared to the colon mucosa.

Furthermore, Gemellaceae undefined genus, *Conchiformibius*, Neisseriaceae undefined genus, Pasteurellaceae undefined genus and *Phascolarctobacterium*

(Veillonellaceae family) were higher in the duodenum mucosa when compared to colon mucosa, while the abundance of Neisseriaceae undefined genus and Pasteurellaceae undefined genus showed a significant gradual decrease ($P < .0001$) along the intestinal tract from the duodenum to colon.

Discussion

A few number of studies have evaluated mucosal samples or intestinal content of different gastrointestinal tract segments in dogs [16, 20, 26]. In the present study, microbiota associated with the gastrointestinal mucosa were characterized using Illumina sequencing analysis. Phylogenetic similarities were found between the different compartments obtained from each dog. Unweighted UniFrac analysis, which accounts for the presence or absence of taxa but not their abundances, no group separation occurred. Taken together, these findings indicate that composition and relative abundances are more similar within segments than across segments and suggest that the intestinal segments have the same community but the abundance of this change across the GI tract. The intestinal microbiota also varies from one collection site in the GI tract to another (i.e., duodenum vs. colon vs. feces). In previous studies, a general increase was observed in diversity and total number of bacteria moving from the duodenum to the colon, as well as aerobic or facultative anaerobic bacteria predominate in the small intestine while anaerobes thrive in the large intestine [15, 16]. Bacterial culture analysis of dog intestinal content also revealed significant differences in the ratio of aerobic to anaerobic bacteria between the jejunum and feces. While the jejunum harbored a relative similar ratio of aerobes to anaerobes, anaerobic bacteria dominated in feces [26].

The Firmicutes phylum dominated the mucosal of duodenum, jejunum and colon (44% to 47% of the sequences). This finding is consistent with previous study that used molecular

techniques to analyze the microbiota in the proximal portion of the small intestine and the colon in healthy dogs [16] human [27] and cats [15]. Among the Firmicutes, Clostridiales was the most abundant bacterial order in the colon (32.77%) and a major constituent of the microbial community in the duodenum mucosa (28.41%). Suchodolski and colleagues (2008) using 16S rRNA gene clone libraries found Clostridiales was the most abundant bacterial order in the duodenum and jejunum (~40%) and a major constituent of the microbial community in the ileum and the colon of dogs [16]. Another study in cats also found Clostridiales like the most abundant bacterial class in the feline intestinal relative tract [15].

Veillonellaceae member of Firmicutes phylum was the most abundant family in three segments with no difference in abundance between segments. Veillonellaceae are assacharolytic and utilize end products of sugar metabolisms of other gastrointestinal bacteria (such as lactate or succinate) to produce propionate [28]. In human, studies have shown an increased abundance of *Veillonella* in fecal samples of irritable bowel patients [29, 30]. However, this group of bacteria was decreased in duodenum mucosa of dogs with idiopathic inflammatory bowel disease [16]. Moreover, Lactobacillaceae and *Lactobacillus* were present in most abundance in duodenum (11.75%) and jejunum (11.06%) mucosa compared to colon (5.57%) mucosa. These authors also observed high abundance of Lactobacillaceae in the duodenum, jejunum, and colon of dogs, while *Lactobacillus* was found as a minor fraction (1.4%) of all identified clones in the ileum. In addition, Lactobacillales were found in high abundance from the jejunum and colon of cats [15].

Lachnospiraceae family is a butyrate producer and were present in small intestine and colon with higher abundance in the colon segment. Butyrate can be used as an energy source by the gut epithelial cells, and it has anticarcinogenic and anti-inflammatory properties [31]. The decrease in the relative abundance of the butyrate-producing Lachnospiraceae in the

gastrointestinal microbiota is associated with compromised health status of subjects suffering from colorectal cancer [32], ulcerative colitis [33] in human. In duodenal biopsies from dogs with inflammatory bowel disease, 454-pyrosequencing also revealed decreased proportions of Lachnospiraceae in the duodenum when compared with healthy dogs [19].

The phylum *Bacteroidetes* (28.98%) was the second abundant bacterial phylum in the duodenal, jejunum and colon mucosa with higher abundance in the colon mucosa. Regarding the colon mucosa, our study found that Firmicutes (44.14%) was the most abundant phylum; Bacteroidetes (39.22%) was the second abundant bacterial phylum, followed by the Fusobacteria (6.51%) phylum. One study found co-dominance phyla of the Fusobacteria, Bacteroidetes and Firmicutes phylum (around 30% each) in the colon content of healthy dogs using 16S rRNA gene clone sequencing [16]. Differences in the methods used (i.e. 16S rRNA gene analysis vs high-throughput DNA molecular sequencing), diets, or environmental differences may explain the different results between the studies [10].

Among the phylum Bacteroidetes, Prevotellaceae members were found in higher abundance in the duodenum and jejunum mucosa, while [Paraprevotellaceae] members were found in higher abundance in the colon as well as Bacteroidaceae members. These bacterial groups are saccharolytic and share the common feature that they produce succinic acid, acetic acid, and in some cases propionic acid, as the major end-products [34]. In duodenal biopsies from dogs with idiopathic inflammatory bowel disease lower proportions of Prevotellaceae and Bacteroidaceae when compared with healthy dogs [19].

Proteobacteria were also present in higher abundance in the duodenum (14.42%) and jejunum (13.6%) and lower abundance in the colon (5.52%) mucosa. This find is in accordance with a previous study [16]. Proteobacteria were found in higher abundance on

feces of dogs with acute hemorrhagic diarrhea [19] and cats with inflammatory bowel disease presented higher abundance of Enterobacteriaceae in duodenal biopsies [13].

The abundance of Actinobacteria phylum was higher in duodenum (7.07%) and jejunum (6.82%) compared with the colon (4.32%) mucosa. Among the Actinobacteria phylum, the *Bifidobacterium* were observed in lower concentrations in the colon (2.41%) compared to duodenum and jejunum mucosa. One study found no *Bifidobacterium* were observed in all gastrointestinal contents of dogs [16]. Another study with intestinal tissue and contents samples of dogs based on bacterial culture, a lower abundance of *Bifidobacterium* (2×10^2 cfu) has also been reported in the proximal colon of dogs [35] and from the all intestinal tract [20]. *Bifidobacterium* members are anaerobic or microaerophilic bacteria, therefore these bacteria can be found in more abundance in small intestine. *Bifidobacterium* are considered beneficial microorganisms, part of the normal human intestinal microbial community, and have decreased abundance in irritable bowel syndrome in human [36].

Moreover, Coriobacteriaceae was observed in higher concentration in colon compared to duodenum and jejunum mucosa. This group of bacteria are nonmotile obligate anaerobes that can ferment a wide range of different carbohydrates including complex sugars, because it is obligate anaerobes, this can be found more in the colon than in the small intestine. Dogs with acute hemorrhagic diarrhea and inflammatory bowel disease had lower abundance of Coriobacteriaceae when compared with healthy dogs [19; 37].

In the present study, the Fusobacteria is anaerobic and increased from duodenum to colon, however the abundance of this bacteria were minor. Mentula and colleagues (2005) found higher concentration in feces (10^9 CFU/g) compared with the jejunum chime (10^4 CFU/g) [26]. In healthy human [20] and horses [38] lower abundance were observed in

studies evaluating the intestinal microbial community. Recent studies have shown the increased abundance of *Fusobacterium* associated with ulcerative colitis in humans [33].

A limitation of this study is that the animals were submitted to different diets and had different ages. The both factors can influence the microbiota community. However, the first part of this study (data not publish) compared the 3 diets and 2 ages in colon mucosa and found no difference in the major microbiota shifts with the consumption of different diets and no effect of age. Therefore, the samples number of each segment was satisfactory to perform the study.

In conclusion, many bacterial shifts were observed between the small intestine (duodenum and jejunum) and the colon in the canine intestinal tract. The Illumina sequence of the microbiota associated with the gastrointestinal mucosa in healthy dogs present in this study is helpful to understanding the diversity of the microbial intestinal communities in the dogs and allowed the normal core of some bacterial groups that might be altered in gastrointestinal disease.

Acknowledgments

The authors acknowledge the technical support of GUABI PET CARE, Campinas, SP, Brazil to the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”.

Author Contributions

Conceived and designed the experiments: APJM, ACC. Performed the experiments: APJM, TCP, FN, PD. Analyzed the data: APJM, PD, HDH, KSS, ACC. Wrote the paper: APJM, PD, TCP, HDH, KSS, ACC.

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Supporting Information

S1 Fig. Alpha diversity results. Phylogenetic diversity metric based on PD_whole_tree method from the intestinal segments. No significant difference was observed between the three segments ($P>0.05$).

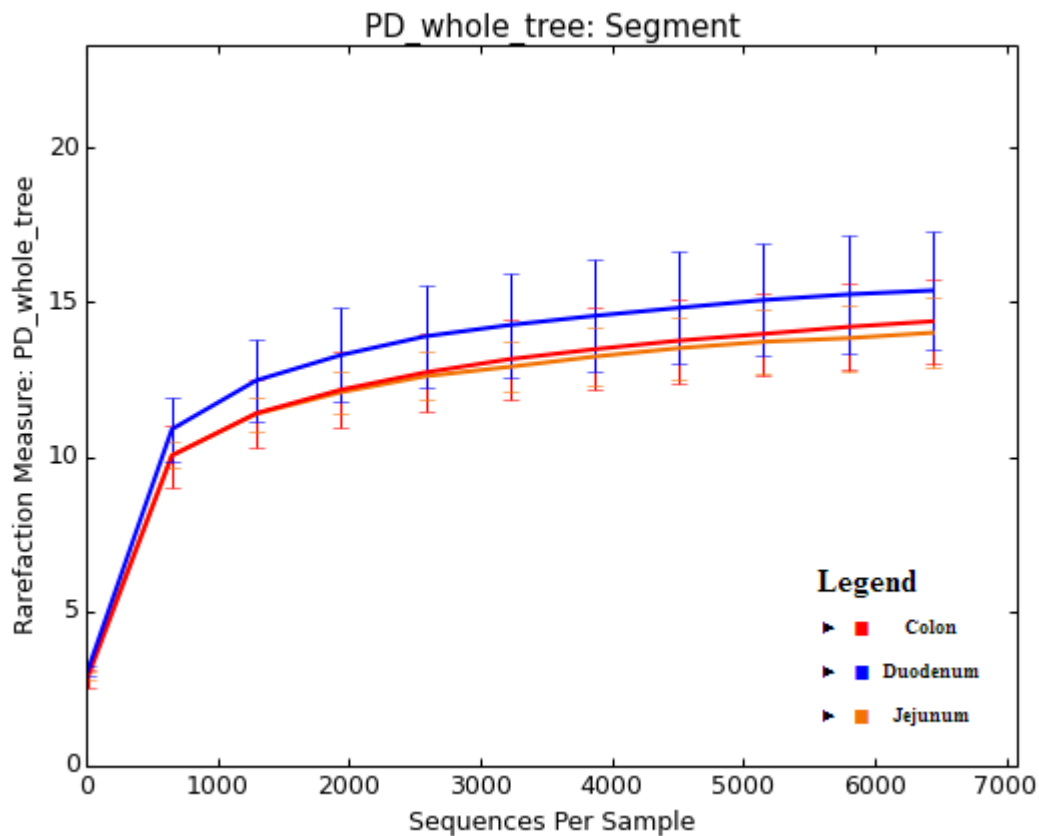
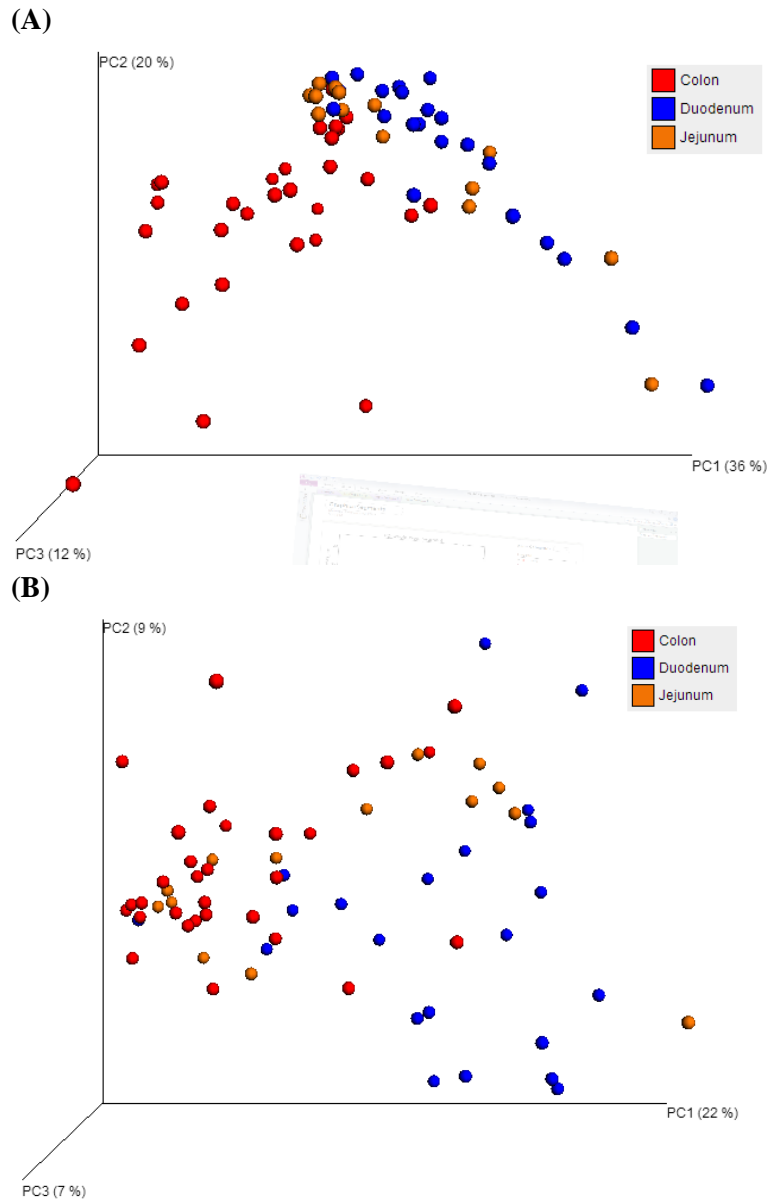


Figure 3. Rarefaction analysis of 16 S rRNA gene sequences obtained from canine gut mucosa samples. Lines represent the average of each group (orange= jejunum; blue = duodenum; red = colon), while the error bars represent the standard deviations. The analysis was performed on a randomly selected subset of 6448 sequences per sample.

S2 Fig. PCoA of weighted (A) and unweighted (B) UniFrac distances between samples of different intestinal segments from dogs.



(A): PCoA plot based on weighted UniFrac distances metric of the attached gut mucosa microbiota ($n = 36$ /group) based on their 97% OTU composition and relative abundances of duodenum (blue points), jejunum (orange points) and colon (red points). Points that are closer in space are more similar in their 97% OTU composition and relative abundances. B: PCoA plot based on unweighted UniFrac distances metric of the attached gut mucosa microbiota ($n = 36$ /group) based on their 97% OTU composition. Points that are closer in space are more similar in their 97% OTU composition.

S3. Table. Predominant bacterial phyla, family and genera (expressed as a percentage of total sequences) in intestinal mucosa of dogs.

Item			Duodenum	Jejunum	Colon	SEM	P Value			
Phylum	Family	Genus								
Actinobacteria	Actinomycetaceae ¹	Actinomyces ¹	7.07 ^a	6.82 ^a	4.32 ^b	0.44	<.0001			
	Corynebacteriaceae ¹	Corynebacterium ¹	0.19	0.09	0.07	0.06	0.2296			
	Bifidobacteriaceae	Bifidobacterium	5.77 ^a	5.76 ^a	2.41 ^b	0.45	<.0001			
	Coriobacteriaceae ²	Collinsella ²	0.69 ^b	0.79 ^b	1.46 ^a	0.23	0.0017			
		Slackia ¹	0.16	0.14	0.20	0.05	0.6959			
Bacteroidetes	Bacteroidaceae ¹	Bacteroides ¹	28.98 ^b	32.08 ^b	39.22 ^a	1.87	<.0001			
	Prevotellaceae	Prevotella	2.75 ^b	3.23 ^b	6.56 ^a	0.61	<.0001			
	S24-7 ⁴	g__ ⁴	17.12 ^a	18.70 ^a	11.32 ^b	1.89	<.0001			
	[Paraprevotellaceae] ¹	g__ ⁴	0.19	0.02	0.04	0.04	0.1222			
		[Prevotella] ¹	8.12 ^b	8.95 ^b	21.06 ^a	4.51	<.0001			
	[Weeksellaceae] ¹	g__ ⁴	1.86 ^b	1.82 ^b	5.77 ^a	0.66	<.0001			
		g__ ⁴	6.26 ^b	7.12 ^b	15.28 ^a	1.70	<.0001			
	Deferribacteres ⁴	Deferribacteraceae ⁴	Cloacibacterium ¹	0.87 ^a	0.72 ^a	0.22 ^b	0.19	<.0001		
			Mucispirillum ⁴	0.76 ^a	0.61 ^a	0.22 ^b	0.17	0.0003		
	Firmicutes ⁴	Gemellaceae ⁴	g__ ⁴	0.23 ^a	0.20 ^a	0.18 ^b	0.08	0.0199		
Gemella ⁴			47.00	45.08	44.14	1.79	0.4624			
Aerococcaceae ¹		Facklamia ¹	g__ ⁴	0.64 ^a	0.13 ^b	0.11 ^b	0.18	0.0012		
			g__ ⁴	0.45 ^a	0.10 ^b	0.06 ^b	0.14	0.0024		
Carnobacteriaceae ⁴		Lactobacillus ²	Gemella ⁴	0.19	0.03	0.04	0.05	0.0725		
			Lactobacillus ²	0.11	0.06	0.01	0.02	0.3281		
Streptococcaceae ¹		Streptococcus ¹	Carnobacteriaceae ⁴	0.12 ^a	0.06 ^b	0.01 ^b	0.02	0.0003		
			Streptococcus ¹	11.75 ^a	11.06 ^a	5.57 ^b	0.98	<.0001		
Turicibacteraceae		Turicibacter	Lactobacillaceae	0.91	0.35	0.34	0.26	0.3749		
			Lactobacillaceae	0.79	0.82	0.56	0.10	0.0963		
Clostridiales f_ ⁴		g__ ⁴	Streptococcaceae ¹	0.72	0.83	0.96	0.16	0.4891		
			Turicibacteraceae	2.02 ^b	1.79 ^b	4.74 ^a	0.41	<.0001		
Clostridiaceae ¹		g__ ¹	Clostridiales f_ ⁴	1.66 ^b	1.42 ^b	3.84 ^a	0.39	<.0001		
			Clostridiaceae ¹	0.37 ^b	0.36 ^b	0.90 ^a	0.12	<.0001		
Lachnospiraceae ¹		g__ ²	Clostridium ⁴	3.24 ^b	4.07 ^b	6.13 ^a	0.49	<.0001		
			Blautia	1.41 ^b	1.83 ^b	2.9 ^a	0.26	<.0001		
			Dorea ⁴	0.82	0.97	0.99	0.10	0.3282		
			[Ruminococcus] ⁴	0.42 ^b	0.56 ^b	1.60 ^a	0.23	<.0001		
			Peptococcaceae ²	0.62	0.63	0.59	0.12	0.2975		
			Peptostreptococcaceae ¹	0.48	0.38	0.36	0.06	0.3685		
Ruminococcaceae ⁴		g__ ⁴	Peptostreptococcaceae ¹	0.09	0.03	0.15	0.06	0.1571		
			g__ ⁴	0.03 ^b	0.01 ^b	0.12 ^a	0.05	0.0001		
Veillonellaceae		g__ ⁴	Ruminococcaceae ⁴	2.67	2.82	2.88	0.27	0.8494		
			Faecalibacterium ⁴	2.34	2.50	2.54	0.29	0.9015		
Fusobacteria ¹		Fusobacteriaceae ¹	Allobaculum ³	0.24	0.29	0.30	0.08	0.1831		
			Catenibacterium	23.06	22.25	21.75	1.64	0.8201		
			[Eubacterium] ⁴	14.95	12.52	12.73	1.19	0.7264		
			Fusobacterium ¹	6.86 ^a	5.99 ^{ab}	5.42 ^b	0.42	0.0294		
			Alcaligenaceae ¹	3.64 ^a	3.52 ^a	1.36 ^b	0.27	<.0001		
			Comamonadaceae ¹	g__ ¹	[Mogibacteriaceae] ¹	0.09	0.07	0.15	0.04	0.3929
					Erysipelotrichaceae ³	0.20	0.30	0.34	0.05	0.0568
			Proteobacteria ¹	Alcaligenaceae ¹	Allobaculum ³	0.16	0.17	0.13	0.03	0.6912
					Catenibacterium	0.03 ^b	0.08 ^{ab}	0.10 ^a	0.02	0.0057
					[Eubacterium] ⁴	0.01 ^b	0.04 ^a	0.10 ^a	0.03	0.0004
Fusobacterium ¹		2.10 ^b			2.36 ^b	6.51 ^a	0.78	<.0001		
Comamonadaceae ¹		g__ ¹	Alcaligenaceae ¹	14.42 ^a	13.6 ^a	5.52 ^b	2.60	0.0022		
			g__ ¹	0.71	0.85	0.84	0.09	0.5882		
	Comamonas ¹		0.65	0.80	0.83	0.09	0.3011			
	Limnochabitans ¹		2.16 ^a	3.56 ^a	0.47 ^b	1.09	<.0001			
Comamonadaceae ¹	g__ ¹	Comamonas ¹	1.17 ^a	1.82 ^a	0.27 ^b	0.55	0.0001			
		Limnochabitans ¹	0.18	0.42	0.03	0.14	0.2878			
Comamonadaceae ¹	g__ ¹	Limnochabitans ¹	0.70 ^{ab}	1.25 ^a	0.16 ^b	0.37	0.0001			

Neisseriaceae ⁴		1.10 ^a	0.15 ^b	0.02 ^c	0.39	<.0001
	g ₄	0.33 ^a	0.06 ^b	0.01 ^c	0.12	0.0001
	Conchiformibius ⁴	0.77 ^a	0.09 ^b	0.01 ^b	0.26	<.0001
Rhodocyclaceae ¹		0.88 ^a	1.04 ^a	0.12 ^b	0.30	<.0001
	Dechloromonas ¹	0.84 ^a	0.99 ^a	0.12 ^b	0.28	<.0001
Campylobacteraceae ⁴	Campylobacter ⁴	0.01 ^b	0.02 ^b	0.51 ^a	0.36	0.0002
Helicobacteraceae ⁴	Helicobacter ⁴	0.22	0.31	0.70	0.16	0.3462
Succinivibrionaceae ⁴		0.52	0.64	0.61	0.10	0.5304
	g ₄	0.04	0.11	0.10	0.03	0.0788
	Anaerobiospirillum ⁴	0.48	0.53	0.51	0.10	0.7037
Enterobacteriaceae ¹		5.55 ^a	5.34 ^{ab}	1.95 ^b	1.10	0.0016
	g ₁	5.27 ^a	5.14 ^a	1.45 ^b	1.00	0.0007
	g_Escherichia ¹	0.14 ^a	0.11 ^{ab}	0.04 ^b	0.02	0.0006
	Plesiomonas ⁴	0.02 ^b	0.00 ^b	0.44 ^a	0.30	0.0149
Pasteurellaceae ⁴		1.94 ^a	0.43 ^b	0.13 ^c	0.70	<.0001
	g ₄	1.86 ^a	0.41 ^b	0.11 ^c	0.67	<.0001
Moraxellaceae ¹		0.47 ^a	0.27 ^{ab}	0.05 ^b	0.14	0.0033
	Acinetobacter ¹	0.17	0.13	0.03	0.04	0.2256
Pseudomonadaceae ¹	Pseudomonas ¹	0.62 ^a	0.89 ^a	0.10 ^b	0.19	<.0001

¹ Log10

² Log(x+1)

³ SQRT

⁴ No parametric analyses

^{a, b} Means in the same row and not sharing a common superscript lowercase letters differ ($P < 0.05$).

¹ IF: diet with sugarcane fiber and poultry by-product meal; FF: diet with beet pulp and poultry by-product meal; SM: diet with 30% soybean meal to partially replace the poultry by-product meal and add fiber.