PRELIMINARY NOTE

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Fecal microbiota profile of ovariohysterectomized cats submitted to estrogen replacement

Perfil da microbiota fecal de gatas ovariohisterectomizadas submetidas à reposição estrogênica

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ABSTRACT

This study aimed to observe the effects of 17 β-estradiol replacements on the fecal microbiota in spayed cats. Individual samples of fresh feces were collected and stored at -80° C. Sequencing of the V3/V4 regions of the 16S rRNA gene was used, and bioinformatic analysis was performed. Firmicutes/Bacteriodetes ratio was lower in the group receiving estrogen replacement compared to the SHAM group (P = 0,005). Jaccard index (P = 0.123) and Yue & Clayton index (P = 0.094) did not reveal alpha and beta diversity differences. The linear discriminant analysis effect size (LefSe) identified Firmicutes and *MegasPhaera* as the biomarkers for the SHAM group, and Burkholderiales, Betaproteobacteria, Sutterellaceae, *Suterella*, Proteobacteria, Proteobacteria unclassified and *Collinsella* for the group receiving estrogen replacement.

Keywords: Fecal microbiota. Neutering. Obesity. 17 **β**-estradiol.

RESUMO

O objetivo deste estudo foi observar os efeitos da reposição de 17 β-estradiol na microbiota fecal de gatas castradas. Amostras individuais de fezes frescas foram colhidas e armazenadas a -80°C. Foi realizado o sequenciamento das regiões V3/V4 do gene 16S rRNA e a análise bioinformática. A razão Firmicutes/Bacteriodetes foi menor no grupo que recebeu reposição estrogênica em comparação ao grupo SHAM (P = 0,005). O índice de Jaccard (P = 0,123) e o índice de Yue & Clayton (P = 0,094) não revelaram diferenças na alfa e beta diversidade. A análise discriminatória linear de tamanho do efeito (LefSe) identificou Firmicutes e Megasphaera como biomarcadores para o grupo SHAM, e Burkholderiales, Betaproteobacteria, Sutterellaceae, Suterella, Proteobacteria, Proteobacteria não classificada e Collinsella para o grupo que recebeu reposição estrogênica.

Palavras-chave: Microbiota fecal. Castração. Obesidade. 17 β-estradiol.

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Estrogen replacement in neutering cats reduces food intake and body weight (Backus, 2011). The manipulation of the fecal microbiota can also promote weight loss since undesirable changes influence the host's metabolism by one of three mechanisms: increased inflammation due to the release of lipopolysaccharides; energy extraction from the diet; and alteration of host satiety hormone production, resulting in hyperphagia (Belkaid & Hand, 2014; Fischer et al., 2017; Flint, 2011).

Characterization studies of the feline fecal microbiota have been carried out, as well as comparing the microbiota between lean, obese, and neutered cats (Deusch et al., 2015; Fischer et al., 2017; Ritchie et al., 2010). To verify the influence of estrogenic replacement on the fecal microbiota profile of ovariohysterectomized cats, 12 healthy, short-haired, and crossbred adult animals with an average body weight of 2.8 kg were used. The Institutional Ethical Committee for Animal Experimentation (Protocol Number 5609.2017.46) approved all procedures. The animals were divided into three randomized groups: the control group composed of four animals undergoing celiotomy (SHAM group), four animals undergoing ovariohysterectomy (OH group), and four animals undergoing ovariohysterectomy and receiving 17 β-estradiol valerate capsules (Magistral Pharmacia, Londrina, Brazil) orally by the handler ($OH+E_2$ group), administered daily for six months before meals, at a dosage of 4 μg in the first three months and 12 μg in the remaining three months. Vaginal smear slides were stained with Diff-Quick (Laborclin, Pinhais, Brazil) and examined at 40x magnification to provide interpretations regarding the hormonal phase and estrogenic stimulus. Species-specific estrogen measurements were performed by radioimmunoassay method using an ultrasensitive estradiol kit (Beckman Coulter, Inc., Brea, CA, USA). Throughout the experimental period, all animals received the same feed recommended for adult cats (Golden gatos; Premier pet, Dourado, Brazil). The nutritional composition provided by the manufacturer was 31% crude protein, 12% ether extract, 8% mineral matter, 3.5% fibrous matter, and 3912 kcal/kg metabolizable energy. The main ingredients used were chicken viscera flour, isolated swine protein, corn gluten bran, ground whole corn, broken rice, beet pulp, chicken fat, and fish oil. Body weight was recorded weekly, and statistical analysis for weight gain and Firmicutes/Bacteriodetes ratio was performed in SigmaPlot software (Systat Software, Inc., San Jose, CA, USA). Data were subjected to analysis of variance and considering repeated measures to compare the groups and periods when appropriate. A P value <0.05 was statistically significant.

Individual samples of fresh feces were collected at the end of the experiment and stored at -80°C. Cell lysis and DNA extraction were performed using the magnetic breads technique with a proprietary protocol (Neoprospecta Microbiome Technologies, Florianópolis, Brazil). The bacterial identification was performed using high-performance sequencing of the V3 / V4 regions of the 16S rRNA gene. The DNA sequences of the microorganisms were analyzed using a proprietary pipeline. Bioinformatic analysis was performed with Mothur software (http://www.mothur.org/wiki/Download_mothur) and loaded on the Neobiome platform for viewing. The taxonomic classification was obtained in the design of the ribosomal database (Cole et al., 2014). The strings were assigned to the phylotypes at the genus level for analysis. The relative abundances were calculated, and the main phyla and genera (abundance> 1%) found in each group at various times was presented in a column chart. Alpha and beta diversity analyses were performed. The Chao index and diversity by Simpson index estimated richness. The difference between community members and structure found in each sample was measured by the Jackard index, which considers the wealth of the community, and by the Yue & Clayton index, which considers the diversity (richness and uniformity of the phylotypes present in each sample) (Yue & Clayton, 2005). The similarity between the community members and the structure present in each sample was represented by the two-dimensional analysis of the primary coordinates (PCoA). The comparison of community members (Jacquard index) and structure (Yue & Clayton index) between the groups was performed using the Parsimony test and the analysis of molecular variance (AMOVA), considering $P < 0.05$ as statistically significant. The LefSe identifies

the microorganisms most likely to explain the observed effects on diversity.

The OH and OH+E_2 groups had more weight gain than the SHAM group. Despite this, there was weight loss (P = 0.03) and the appearance of superficial cells without estrous signs using 12 µg of 17 β-estradiol, compared with the OH and SHAM groups. The global averages at the phylum level in decreasing order of abundance were: Firmicutes (71%), Actinobacteria (16%), Bacteroidetes (10%), Bacteria unclassified (2%), and Proteobacteria (1%). Interestingly, the Firmicutes/Bacteriodetes ratio was significantly lower in the group receiving estrogen replacement (61/15; 75/12; 55/12; 40/37) compared to the SHAM group (88/1; 88/1; 93/2; 76/1) (P = 0.005).

The relative abundance at the genus level, with the distribution of the 25 main bacterial genera isolated from animal feces, is shown in Figure 1. There was no difference regarding alpha diversity between groups. The values (mean ± SD) of Chao, Simpson, and Shannon indices in the OH+E_2, OH, and SHAM groups were 58.58 ± 13.38, 10.69 ± 3.51, 2.74 ± 0.38; 48.73 ± 9.06, 9.73 ± 2.80, 2.68 ± 0.28; 45.30 ± 5.62, 8.53 ± 3.14, 2.46 ± 0.32, respectively. Beta diversity using the Jaccard and Yue & Clayton indices are shown in the PCoA graphs in Figure 2. AMOVA test did not reveal any difference between the groups for the Jaccard index (P = 0.123) and the structure of communities for the Yue & Clayton index (P = 0.094). The cladogram (Figure 3) shows the grouping of similar samples (clusters) and the distance between them. The LefSe identified Firmicutes and *MegasPhaera* as the biomarkers for the SHAM group. Burkholderiales, Betaproteobacteria, Sutterellaceae, *Suterella*, Proteobacteria, Proteobacteria unclassified, and *Collinsella* were the biomarkers for the OH+E_2 group (Figure 4).

The five bacterial phyla identified are comparable to previous studies in cats regarding the predominance of the phylum Firmicutes (Deusch et al., 2015; Fischer et al., 2017; Ritchie et al., 2010). However, unlike those same authors, this study found the phylum Actinobacteria as the second most abundant, instead of phylum Bacteroidetes. This may be due to the type of diet and are consistent with Bermingham et al. observations (Bermingham et al., 2013), with cats provided dry food containing moderate concentrations of proteins and carbohydrates have a greater abundance of Actinobacteria and less abundance of Fusobacteria and Proteobacteria in comparison with cats fed wet food, containing high concentrations of proteins and low concentrations of carbohydrates. The lowest Firmicutes/Bacteroidetes ratio observed in the OH+E_2 group is desirable for manifesting a lean phenotype. However, despite the lower weight gain compared with OH and OH+E_2 groups, the SHAM group showed a higher Firmicutes/Bacteriodetes ratio, which is at odds with what is commonly reported for mice and humans, although this relationship remains controversial (Turnbaugh et al., 2009).

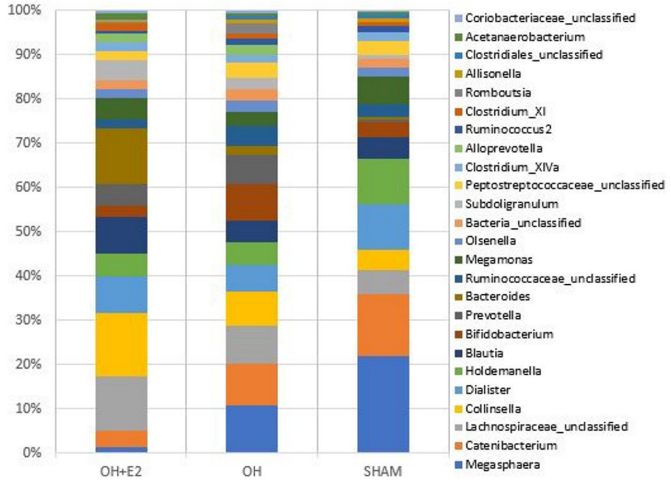

Figure 1 – Relative abundance of the main genera found in fecal samples of three groups of cats. OH+E_2 = cats undergoing ovariohysterectomy and then supplemented with estrogen; OH = cats undergoing ovariohysterectomy; SHAM = cats experiencing celiotomy.

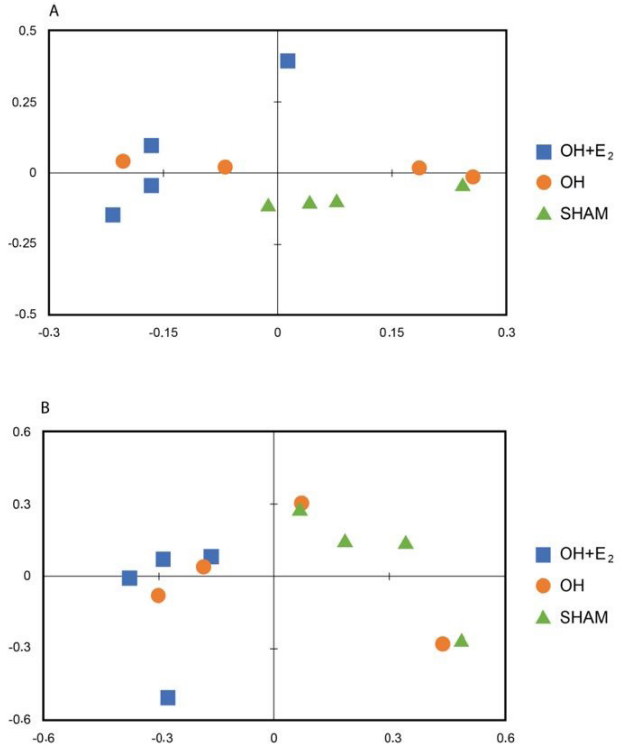

Figure 2 – Principal component analysis chart (PCoA) representing the fecal microbial communities of cats in the OH+E_2, OH, and SHAM groups in terms of composition (Jaccard), in (A), and structure (Yue & Clayton), in (B) OH+E_2 = cats undergoing ovariohysterectomy and then supplemented with estrogen; OH = cats undergoing ovariohysterectomy; SHAM = cats experiencing celiotomy.

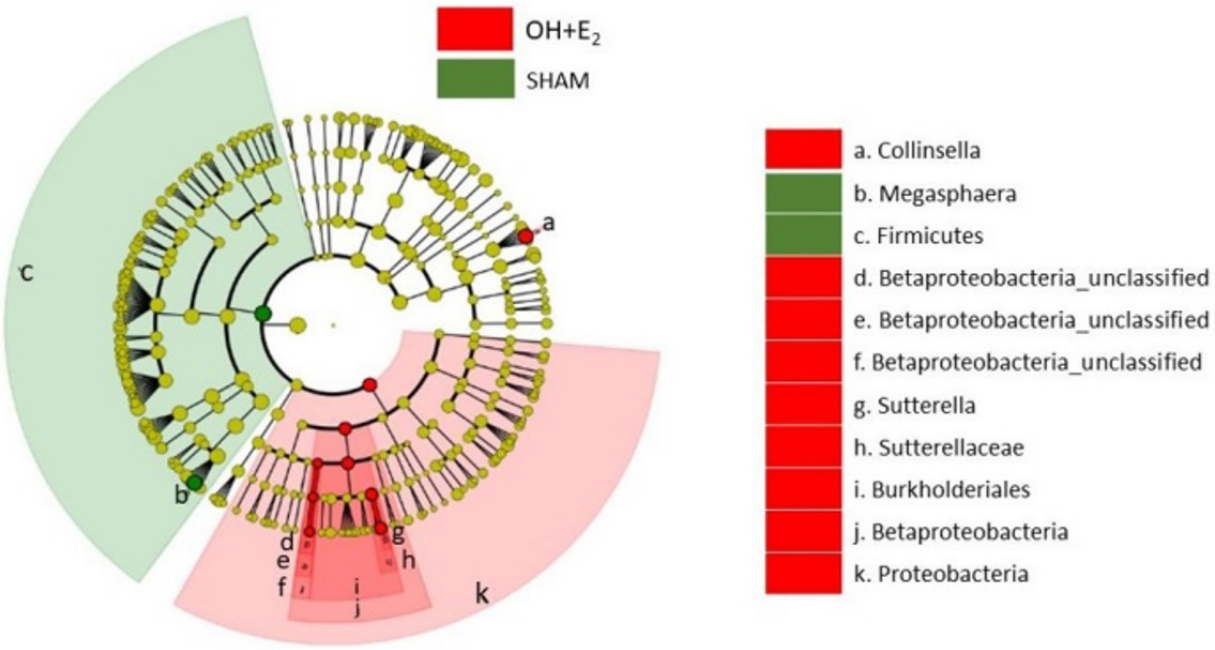

Figure 3 – Cladogram represents the distance between fecal samples from the OH+E_2 and SHAM groups. OH+E_2 = cats undergoing ovariohysterectomy and then supplemented with estrogen; SHAM = cats experiencing celiotomy.

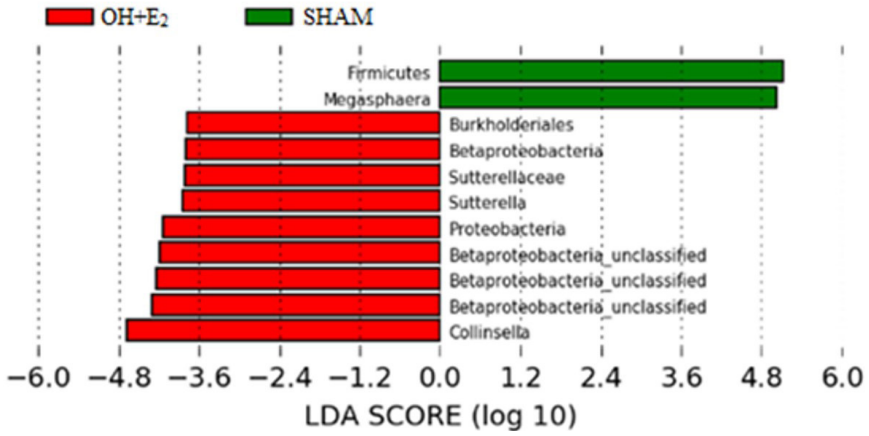

Figure 4 – Linear discriminant analysis effect size (LefSe) found in fecal samples from the OH+E_2 and SHAM groups. OH+E_2 = cats undergoing ovariohysterectomy and then supplemented with estrogen; SHAM = cats experiencing celiotomy.

Although not statistically significant, the long-term effects of estrogen replacement on the differences in the structure and composition of communities attributed to biomarkers are still noteworthy (P = 0.094). However, the physiological significance of these bacterial signatures requires further investigation. There were some limitations to the present study. The sample size was small, and only one sample was taken at the end of the experiment. However, these results help expand the research involving the interrelation of hormonal strategies, microbiota, and obesity promoting feline health.

Conflict of Interest

The authors declared no conflict of interest.

Ethics Statement

All procedures involving animals were approved and followed the ethical standards for animal experimentation and were aproved by the Ethic Committee on the Use of Animals of the State University of Londrina (CEUA/UEL Protocol Number 5609.2017.46).

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