1 **Original Research Paper**
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4 **Feline gut microbiota composition in association with Feline coronavirus infection: a**
5 **pilot study**
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23

24 **Abstract**

25 Feline coronaviruses (FCoV) colonize the intestinal tract, however, due to not fully understood

26 mutations, they can spread systemically and cause feline infectious peritonitis (FIP). Recent

27 studies on human medicine report that gut microbiota is involved in the development of

28 systemic disorders and could influence the immune response to viral diseases. The aim of this

29 study was to provide preliminary data on the fecal microbiota composition in healthy cats

30 compared to FCoV-infected cats, with and without FIP. Cats were equally grouped as healthy

31 FCoV-negative, healthy FCoV-positive or FIP affected (total n = 15). Fecal sample were

32 evaluated for the microbiota composition. A total of 3,231,916 sequences were analyzed. The

33 samples' alpha-diversity curves did not reach a proper plateau and, for the beta-diversity, the

34 samples seemed not to group perfectly by category, even if the healthy FCoV-positive group

35 showed a hybrid microbial composition between FCoV-negative and FIP groups. Although

36 there were no taxa significantly linked to the different conditions, some peculiar patterns were

37 recognized: Firmicutes was the most represented phylum in all the samples, followed by

38 Bacteroidetes and Actinobacteria. In FCoV-positive cats, the Firmicutes and Bacteroidetes

39 were respectively over- and under-represented, compared to the other groups. Among FIP cats,

40 three subjects shared a similar microbiome, one cat showed a different microbial profile and

41 the other one had the lowest number of diverse phyla. Despite the limited number of animals,

42 some differences in the fecal microbiome between the groups were observed, suggesting to

43 further investigate the possible correlation between gut microbiota and FCoV infection in cats.

44

45 **Keywords:** Feline infectious peritonitis; gut microbiota; feline coronavirus

Introduction

One topic of great interest in the last few years has been the evaluation of intestinal microbiota, which is the consortium of all living microorganisms that inhabit the gastrointestinal (GI) tract. This new insight into the complexity of the intestinal microbiota, by the use of next generation sequencing techniques, and its intimate relationship with the host has spurred researchers to better understand the importance of a balanced microbial ecosystem for the regulation of host health and immunity (Suchodolski, 2016). In the last few years molecular methods, mostly targeting the small subunit ribosomal RNA gene (16S rRNA), have been used for identification of bacterial microbiota. This gene is ubiquitously present in all bacteria and archaea and contains both conserved and variable sequence regions, enabling researchers to distinguish organisms at different phylogenetic levels. Such approach has demonstrated that the gastrointestinal tract of both human and animals harbors a highly complex microbial ecosystem, consisting of several hundreds of different bacterial phylotypes (Handl et al., 2011). The importance of microbiota for the host is highlighted by murine studies that clearly indicate a reciprocal regulation between microbiota and T cell development (Williams et al., 2006; Round and Mazmanian, 2009; Lopes et al., 2016). In human medicine, variations of the gut microbiota have been correlated not only with gastrointestinal diseases (Frank et al., 2007), but also with obesity (Ley et al., 2005), allergies, autoimmune (Vieira et al., 2014; Block et al., 2016) and infectious diseases (Goedert et al., 2016; Tincati et al., 2016).

Feline Infectious Peritonitis (FIP) is a systemic fatal disease affecting mostly young cats (Pedersen, 2014a) sustained by the feline coronavirus (FCoV), which commonly occurs in multicat environments. The FCoVs usually inhabit the intestinal tract (feline enteric coronavirus, FECV). During viral replication within the enterocyte, a mutated variant, also called FIP virus (FIPV) may be generated (Pedersen, 2014a). Whichever is the exact mutation, FIPV loses tropism for enterocytes and gains the capability to replicate inside macrophages

71   (Kipar et al., 2010). Moreover, FCoV induces macrophage-monocyte proliferation in haemo-

72   lymphatic tissues and thus allow a progressive viral adaptation to the replication within these

73   cells (Kipar and Meli, 2014). Although both viral biotypes (FECV and FIPV) can spread

74   systemically, only the FIPV may induce FIP, due to the higher rate of interactions between the

75   virus and monocytes. Moreover, the host immune response has a pivotal role in the development

76   of the disease. Indeed, the cell-mediated immunity seems to be protective. On the other hand,

77   it is known that the humoral response induces development of antibodies that enhance the *in*

78   *vitro* virus uptake in macrophages and this, in turn, contributes *in vivo* to the development of

79   the clinical signs seen during the effusive (wet) form of the disease. The non-effusive (dry)

80   form is considered as an intermediate status involving a partially effective cellular response

81   (Pedersen, 2014a).

82   The *in vivo* diagnosis of FIP may be challenging, especially in dry forms, but several tests may

83   help supporting a clinical diagnosis of wet FIP. Nowadays, the definitive diagnosis is usually

84   achieved post-mortem, by demonstrating the presence of intralesional FCoV through

85   immunohistochemistry (Pedersen, 2014b; Tasker, 2018). *In vivo*, the suspicion of FIP is based

86   on signalment and clinical history, as well as on laboratory data (Pedersen, 2014b; Stranieri et

87   al., 2017; Tasker, 2018). During wet forms of the disease, the effusion analysis is usually

88   diagnostic (Giordano et al., 2015; Stranieri et al., 2017; Tasker, 2018).

89   The feline gut microbiota shares many similarities with its human counterpart and, as previously

90   described for people and animals, the development of infectious diseases, especially when

91   associated with a strong immune system involvement, may be enhanced by an unbalanced

92   composition of the gut microbiota (Suchodolski et al., 2015; Tizard and Jones, 2018). Thus, the

93   aim of this study was to compare the fecal microbiota composition, evaluated through next

94   generation sequencing (NGS), of FCoV negative clinically healthy cats with that of FCoV

95   positive clinically healthy cats and of cats with FIP, in order to highlight possible differences

Materials and methods

Animals/Caseload

Twenty-one cats, submitted for clinical examinations to both the Veterinary Teaching Hospital of our University and to private practitioners, were recruited.

The inclusion criteria, applied after the screening analyses, in order to reduce possible confounding factors, were: (1) Private owned cats living indoor. (2) No administration of antibiotics in the previous sixty days. (3) Negative testing for feline immunodeficiency virus (FIV) antibodies and feline leukemia virus (FeLV) antigen. (4) Age ≤2.5 years. (5) Availability of fresh feces (6) Absence of clinical signs (for inclusion in the groups of clinically healthy FCoV positive or negative cats). (7) Presence of clinical signs and clinicopathological abnormalities consistent with FIP (Stranieri et a., 2018), followed by a post-mortem confirmation of diagnosis (for the inclusion in FIP group). (8) All the cats were fed only with similar diets based on dry and canned commercial food.

Sample collection

At admission, 2 mL of whole blood was collected from each cat by venipuncture of the jugular vein: 1 mL was transferred into an EDTA tube and 1 mL into a plain tube (Venoject, Terumo Italia). When effusion was present, it was sampled by ultrasound-guided drainage and stored in EDTA tubes. According to the standard operating procedures of our laboratory whole blood and effusion were analyzed within 12-18 h from the sampling. From each animal, a fresh fecal sample (at least 15 g) was collected and immediately stored, frozen at -20 °C.

The FIP cats, due to the severe course of the disease, were humanly euthanized, after owner's consent. A complete necropsy, followed by routine histology and immunohistochemistry and RT-PCR for Feline coronavirus, was performed. Immunohistochemistry and RT-PCR were

121   performed on tissue biopsies from liver, spleen, mesenteric lymph node, intestine, kidney and

122   lung and, in cats with neurological signs also on brain and cerebellum, in order to confirm the

123   clinical suspicion of FIP. In one case, since the owner declined necropsy, the diagnosis of FIP

124   was confirmed by immunocytochemistry on the effusion sample.

125   All the above procedures, were performed within routine diagnostic workouts and therefore,

126   according to the decisions of the Ethical Committee of the university of Milan, residual aliquots

127   of samples or tissues collected under informed consent of the owners can be used for research

128   purposes without any additional formal request of authorization to the Ethical Committee. (EC

129   decision 29 Oct 2012, renewed with the protocol n° 02-2016).

130

131   *Screening analyses*

132   The purpose of clinicopathological tests was to confirm the absence of subclinical changes in

133   healthy cats and to evaluate the clinical status of those affected by FIP. A complete blood cell

134   count was performed on whole blood in EDTA using the Sysmex XT-2000iV hematology laser

135   analyzer (Sysmex Corporation), along with a blood smear evaluation. Serum samples were

136   obtained by centrifugation (3750 g x 5 min) of blood collected in plain tubes and used to run a

137   biochemical routine panel (including total proteins, albumin, bilirubin, creatinine, alanine

138   aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea) with an automated

139   spectrophotometer (RX Daytona, Randox Laboratories), using reagents provided by the

140   manufacturer. Tests for the detection of FIV antibodies and FeLV antigens were performed on

141   serum with a lateral flow ELISA kit (SNAP FIV/FeLV Combo Test, IDEXX Veterinary

142   Diagnostic).

143   Additional laboratory tests were performed for cats suspects of FIP to further support the

144   clinical diagnosis of the disease (Pedersen, 2014b; Tasker, 2018). Specifically, serum protein

145   electrophoresis was performed on agarose gel using the automated analyzer Hydrasis (Sebia

146   Italia) and the specific manufacturer's reagents (Hydragel 7/15 β1–β2, Sebia Italia), as

147   previously reported (Stranieri et al., 2017). Based on the total protein concentration, measured

148   with the biuret method, and on the percentages of the electrophoretic fractions, the

149   concentration (g/L) of each electrophoretic fraction was calculated. Moreover, feline α-1-acid-

150   glycoprotein (AGP) was measured in serum using a radial immunodiffusion (SRID) kit

151   (Tridelta Development Ltd), following the manufacturer's instructions (Duthie et al., 1997;

152   Paltrinieri et al., 2007; Hazuchova et al., 2017). When present, effusion was analyzed by

153   measuring total protein concentration with a refractometer, and cell counts using the Sysmex

154   XT-2000iV hematology laser analyzer mentioned above. A cytologic evaluation was also

155   performed. Particular attention was given to ΔTNCC (the ratio between DIFF and BASO counts

156   of the Sysmex XT-2000iV) due to its high diagnostic accuracy for FIP-related effusion

157   (Giordano et al., 2015; Stranieri et al., 2017). In two cases, due to the severe health conditions,

158   it was not possible to perform an adequate blood sample for the screening analyses. However,

159   a complete necropsy followed by tissue biopsies was performed in order to confirm the

160   suspicion for FIP.

161   For molecular testing, 10 mg of each frozen fecal specimen was diluted in 400 µL of PBS. The

162   mixture was vortexed, incubated at 40 °C for 5 min and then centrifuged ($5500\,g \times 4$ min). The

163   supernatant was transferred into a new sterile Eppendorf tube, incubated at 95 °C for 5 min and

164   then centrifuged ($11000\,g$ x 1 min). RNA was extracted from the supernatant using a kit for

165   viral RNA extraction (NucleoSpin® RNA Virus, Macherey-Nagel), following manifacturer's

166   instructions. Amplification of a 177 bp fragment of the conserved 3' untranslated region (3'

167   UTR) using a nested RT-PCR was performed as previously described (Herrewegh et al., 1995).

168   FCoV RNA was used as positive control and RNase-free water as negative control. RT-PCR

169   amplicons were visualized under an ultraviolet transilluminator following electrophoresis

170   through a 2% agarose, ethidium bromide stained gel. The same protocol was used for tissue

171   samples obtained during necropsy. Based on the presence or absence of amplicons, samples

172   were considered as positive or negative for FCoV, respectively.

173   For immunohistochemistry, the biopsy samples were fixed in 10% isosmotic formalin. The

174   samples were processed as already described in other studies (Zini et al., 2018). The primary

175   antibody, manually added by an operator, was a mice monoclonal antibody against FCoV (clone

176   FIPV3-70 Serotec). The immunocytochemistry was performed only on one sample (cat 5F)

177   using a similar procedure, except for the antigen unmasking step.

178

179   *Microbiota Analyses*

180   Microbiota analyses were outsourced to an external laboratory. Investigation of microbial

181   communities (Metabarcoding analysis) in each frozen fecal sample was performed by amplicon

182   sequencing of a hypervariable genomic region (V3-V4 region 16SrRNA gene amplification)

183   using an NGS approach on Illumina Platform. Total DNA was extracted from each fecal sample

184   using a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN S.r.l.). The quality of the

185   genomic DNA was verified using a 2200 TapeStation DNA Screen Tape device (Agilent) and

186   an ND-1000 spectrophotometer (NanoDrop) and its concentration ascertained using a Qubit

187   assay (Life Technologies). The DNA was normalized to 5 ng/µL and then 2.5 µL was used for

188   PCR amplification. Indexed NGS libraries were evaluated with the D1000 screen Tape (Agilent

189   Tape Station 2200) and then quantified with ABI9700 qPCR instrument using the KAPA

190   Library Quantification Kit in triplicates, according to the manufacture's protocol (Kapa

191   Biosystems). Five µL of the pooled library at a final concentration of 4 nM were used for

192   sequencing using Illumina Miseq with a 250 Paired end-read sequencing module.

193

194   *Statistical Methods*

195   Pre-processing steps were performed and the raw reads quality of the extracted sequences was

196   checked (using FastQC v0.11.2). Reads were trimmed with Phred scale quality threshold of 19

197   (representing the probability of an incorrect base call) allowing the reads to be truncated after

198   base quality dropped below 18 (Phred scale). Reads IDs were edited

199   (multiple_split_libraries_fastq.py, Qiime script) in order to be compatible for the following

200   QIIME pipeline scripts. The 97% clustered Qiime formatted Greengenes v.13.8 reference

201   database was used.Before the statistical analysis, alpha rarefaction and beta diversity were

202   evaluated. Alpha rarefaction was an evaluation of within-sample diversity by species richness.

203   A graphical alpha-rarefaction plot was created using Qiime pipeline. Beta diversity is an

204   estimation of between-sample diversity by microbial profile. A graphical representation was

205   calculated using Bray Curtis Qiime beta-diversity pipeline.

206   Finally, statistical analyses were performed with "R" statistical software (software R v3.2;

207   packages edgeR v3.10.5, Robinson M.D., 2010 and Phyloseq v1.14.0, McMurdie P.J., 2013).

208   Samples were assigned to groups relying on samples' metadata and the Taxonomy table (genus

209   level) was normalized. For each group of samples, a statistical analysis was performed using

210   the edgeR ExactTest function, to generate a list of statistically relevant taxonomies that are

211   differentially present among the conditions. Exact test specifications for differential expression

212   between two groups of taxonomy tables was performed. It implements the exact test proposed

213   by Robinson and Smyth (2008) for a difference in mean between two groups of random

214   variables following a negative binomial distribution.

215   Finally, comparison of different phyla, classes and orders relative abundance among groups

216   was performed using Analyse-it for Microsoft Excel. Specifically, a Kruskall-Wallis test was

217   performed, followed, when statistically relevant results were found, by a Wilcoxon Mann-

218   Whitney test, for the comparison between two groups. Statistical significance was set at $P < 0.05$

219

220　**Results**

221　*Screening analyses*

222　Based on the results of the screening analyses including FCoV PCR on feces and IHC on

223　tissues, after the application of inclusion criteria, 15 cats were selected and classified as follows:

224　five healthy negative for FCoV PCR on feces (H group), five healthy positive for FCoV PCR

225　on feces (COR group) and five positive for FCoV PCR on feces and affected by FIP (FIP group)

226　confirmed by IHC. Of the 15 animals enrolled, eight were female and seven were male. Breeds

227　were quite variable, even though the domestic shorthair was the most represented (six cats),

228　followed by two ragdolls, an exotic, a holy birman, a norwegian forest, a bobtail, a maine coon,

229　a scottish fold and a sphynx. Haematology, biochemistry and the clinical examination, were

230　unremarkable for all the clinically healthy cats, regardless the FCoV positivity, whereas cats of

231　the FIP group showed changes consistent with the disease (Table 1).

232

233　*Microbiota analysis*

234　A total of 3,231,916 sequences, with an average of 215,461 sequences/sample (median 219,276,

235　range 195,516.5 – 235,660.5) were of adequate quality and were subsequently analyzed. The

236　alpha-diversity rarefaction curves did not reach a proper plateau for almost all the samples (Fig.

237　1). This means that the sequencing depth was enough to identify only the most abundant

238　bacteria for each sample, excluding the rarest ones. The evaluation of beta-diversity showed

239　that the samples were not grouped perfectly by category. However, the COR group seemed to

240　have a hybrid microbial composition, between the microbial composition of H and FIP groups

241　(Fig. 2). According to the statistical analysis there were no taxa significantly linked to the

242　different conditions (zero differential taxa found for FIP vs H, FIP vs COR, COR vs H).

243　However, it was possible in some cases, to identify specific pattern between groups or for single

244　animals as detailed below.

Eleven different phyla were identified, with the majority represented by six of them (Fig. 3). *Firmicutes* was the main represented phylum, followed by *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. Nevertheless, COR group showed a major abundance of *Firmicutes* and minor of *Bacteroidetes*, compared to the other groups. This was also confirmed by the *Bacteroidetes:Firmicutes* ratio (0.9 in H group, 0.5 in FIP group and 0.1 in COR group; P =0.13. Although the phylum relative abundance was not significantly different among the three groups, *Bacteroides* and *Firmicutes* were close to the significance level (P =0.13 and 0.08 respectively).

In the FIP group, three cats shared a similar microbiota composition, while cat 1F showed a completely different composition and cat 2F showed a lower number of diverse phyla (Fig. 4). The same pattern could be observed in relative class abundance (Table 2). COR group had a major proportion of *Erysipelothrichi* and *Clostridia* and lower *Bacteroidia* with respect of the other two groups (P =0.05, 0.10 and 0.13, respectively). Again, cat 1F showed a completely different pattern of microbiota composition and cat 2F had a lower number of different classes (Fig. 5). The same trend was observed also in relative order abundance. For family and genus, the great amount of data made impossible to find any evident pattern. *Lactobacillus* presence was quite variable in healthy cats, whereas in the FIP group it was present only the cat with dry form (4F).

Discussion

Until recently, traditional bacterial culture was commonly used to identify gut microbial population, but it is now recognized that the vast majority of intestinal microorganisms cannot be cultured using standard plating techniques (Suchodolski, 2016). The study of microbiota composition is a new field of interest in veterinary medicine. Microbiota evaluation is tricky both in the execution and in the interpretation of results, moreover the use of next generation

270 sequencing technique is quite expensive. For this reason, in literature several studies involved

271 a low number of animal (Schmid et al., 2018; Płoneczka-Janeczko et al., 2017; Desai et al.,

272 2009)

273 To our knowledge, this is the first study about the gut microbiota composition in cats with feline

274 coronaviruses.

275 Alpha rarefaction and beta diversity analyses are the most common and historically relevant

276 statistics for metagenome studies aimed to determine easily and visually the presence of groups

277 between samples. The alpha rarefaction aims to demonstrate that enough reads were sequenced

278 for each sample for the main taxa identification. The beta diversity graphically represents the

279 distance between microbiological communities from each sample. No differences in the fecal

280 microbiota were observed among the three groups. However, despite the low number of

281 animals, in several cases, P values were close to statistical significance. The analysis of a higher

282 number of animals is needed to confirm the impact of systemic infection on the intestinal

283 microbiota, as previously reported in people (Goedert et al., 2016).

284 Moreover, the peculiar enteric tropism FCoV could explain these results. Whether is the

285 microbiota influenced by FCoV presence, or if certain animals with peculiar microbiota

286 composition are predisposed to FCoV infection is unclear. In literature, only two studies

287 investigated the correlation between gut microbiota and coronaviral diseases, specifically in

288 swine transmissible gastro-enteritis virus (TGEV) infection (Koh et al., 2015; Liu et al., 2015).

289 However, results were not comparable with our findings because of the different pathogenic

290 role of FCoV compared to TGEV, as FECV infection is usually asymptomatic, and because of

291 the different diet and bacterial phylotypes observed in swine compared to cats.

292 In contrast to what was previously reported for cats (Weese et al., 2015), our results showed a

293 low abundance of *Proteobacteria*. In humans, increase in *Proteobacteria* relative abundance is

294 associated with "dysbiosis" and gastrointestinal symptoms (Kaakoush et al., 2012; Suchodolski

295   et al., 2012; Shin et al., 2015). In our study, none of the cats had gastrointestinal disorders and

296   this could explain the *Proteobacteria* lower abundance, together with the individual variability.

297   In humans, low *Bacteroidetes* to *Firmicutes* ratio is another marker of dysbiosis (Shin et al.,

298   2015). Interestingly in our study, the COR group showed a lower *Bacteroidetes* to *Firmicutes*

299   ratio compared to the others. This could be related to the FCoV enteric tropism (stronger than

300   in FIP cats), even if none of the animals showed gastrointestinal signs. This findings in COR

301   group, could be a hint for an alteration in microbiota stability, even in absence of overt clinical

302   signs.

303   In FIP group, two cats showed a completely different microbiota composition compared to the

304   others. Specifically, cat 2F had a lower number of diverse phyla. Despite the possible individual

305   variability, such a lack in gut microbiota diversity has previously been reported in people treated

306   with antibiotics (Modi et al.; 2014). In our caseload, as this was an exclusion criterion, this low

307   abundance in cat 2F may be related to other variables (e.g. diet).

308   *Lactobacillus* seems to have a probiotic activity, however, elevated concentration had been

309   reported in systemic disorders, such as type II diabetes in people, thus making unclear its

310   protective role (Sato et al., 2014). In the FIP group, *Lactobacillus* was only found in cat 4F,

311   affected by a dry form. The meaning of this finding is unclear. Besides, few studies have

312   investigated this genus in cats, so it could be interesting to evaluate its presence in a larger

313   number of animals, both healthy and diseased.

314

315   **Conclusions**

316   The gut microbiota composition observed in FCoV infected cats provide preliminary insight

317   into an area that could be relevant for both feline health and deeper understanding of the

318   microbiota and immune system interplay. Due to the application of strict inclusion criteria to

319   reduce all the factors that could influence microbiota composition, the present study focused on

320 a small sized sample and this can certainly be a limitation, although in literature several studies

321 on the same topic have be conducted on a similar or even smaller sample size. Therefore, our

322 findings should be confirmed on a larger caseload, possibly including also FCoV seronegative

323 and non shedder cats, in order to definitively exclude any possible viral influence on gut

324 microbiota. Nevertheless, these results could give new insight on causes and clinical

325 significance of the microbiota changes associated with FCoV infection and possibly on FIP

326 pathogenesis.

327

334

335 **Conflict of interest statement**

336 None of the authors has any financial or personal interest that could influence or bias the

337 content of the paper.

338

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Figure legends

478

479   Fig. 1. Alpha rarefaction for the entire caseload. Each sample is represented in a different

480   colour. F= FIP affected cats; H= healthy FCoV negative cats; C= healthy FCoV positive cats.

481

482   Fig. 2. Beta diversity. Principal coordinatesanalysis of weighted distances. Proportions of

483   variance explained by each principal coordinate axis is denoted in the corresponding axis label.

484   In red cats belonging to healthy coronavirus positive group (C), in blue cats affected by FIP (F)

485   and in orange healthy cats, negative for coronavirus (H). Each dot represents a gut microbial

486   community from a single cat. Dots that are close to each other represent microbial communities

487   similar in sequence composition. PC1 axis represents the highest variance; PC2 the second

488   highest variance; PC3 represents the third highest variance;

489

490   Fig. 3. Relative abundance of the predominant phyla in the fecal microbiota in the three groups

491   (y-axis). Group of FIP affected cats (F, n =5), healthy coronavirus negative (H, n =5) and

492   positive (C, n =5) on x-axis. Each colour correspond to different phylum.

493

494   Fig. 4. Relative abundance of the predominant phyla for each subject in the fecal microbiota.

495   FIP affected cats (F, n =5), healthy coronavirus negative (H, n =5) and positive (C, n =5). Each

496   colour correspond to different phylum.

497

498   Fig. 5. Relative abundance of the predominant classes for each subject in the fecal microbiota.

499   FIP affected cats (F, n =5), healthy coronavirus negative (H, n =5) and positive (C, n =5). Each

500   colour correspond to different class.

501

502