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Bacteria isolated from bengal cat (*Felis catus* ×  
*Prionailurus bengalensis*) anal sac secretions produce  
volatile compounds associated with animal signaling

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## 25 Abstract

26 Anal sacs are an important odor producing organ found across the mammalian Order Carnivora.  
27 Secretions from the anal sac may be used as chemical signals by animals for behaviors ranging from defense  
28 to species recognition to signaling reproductive status. In addition, a recent study suggests that domestic  
29 cats utilize short-chain free fatty acids in anal sac secretions for individual recognition. The fermentation  
30 hypothesis is the idea that symbiotic microorganisms living in association with animals contribute to odor  
31 profiles used in chemical communication and that variation in these chemical signals reflects variation in  
32 the microbial community. Here we examine the fermentation hypothesis by characterizing volatile organic  
33 compounds (VOC) and bacteria isolated from anal sac secretions collected from a male bengal cat, a cross  
34 between a domestic cat and wild leopard cat (*Felis catus* × *Prionailurus bengalensis*).

35 Both left and right anal sacs of a male bengal cat were manually expressed (emptied) and collected.  
36 Half of the material was used to culture bacteria or to extract bacterial DNA and other half was used for  
37 VOC analysis. DNA was extracted from the anal sac secretions and used for a 16S rRNA gene sequence  
38 based characterization of the microbial community. Additionally, some of the material was plated out in  
39 order to isolate bacterial colonies. The same three taxa, *Bacteroides fragilis*, *Tessaracoccus*, and *Finegoldia*  
40 *magna* were abundant in the 16S rRNA gene sequence data and also isolated by culturing. Using Solid  
41 Phase Microextraction (SPME) gas chromatography-mass spectrometry (GC-MS), we tentatively identified  
42 52 compounds from bengal cat anal sac secretions and 67 compounds from cultures of the three bacterial  
43 isolates chosen for further analysis.. Among 67 compounds tentatively identified from bacteria isolates, 52  
44 were also found in the anal sac secretion.

45 We show that the bacterial community in the anal sac consists primarily of only a few abundant  
46 taxa and that isolates of these taxa produce numerous volatiles that are found in the combined anal sac  
47 volatile profile. Many of these volatiles are found in anal sac secretions from other carnivorans, and are  
48 also associated with known bacterial biosynthesis pathways. This supports the fermentation hypothesis and  
49 the idea that the anal sac is maintained at least in part to house bacteria that produce volatiles for the host.  
50

## 51 Introduction

52 Anal sacs are an odor producing organ common to many mammals, including members of the Order  
53 Carnivora (carnivorans) [1,2]. In carnivorans, anal sacs are two small structures found on each side of the  
54 anus [3], located between the internal and external sphincter muscles. The interior walls of each sac are  
55 lined with sebaceous and apocrine glands, and the anal sac secretes a foul smelling, oily substance that  
56 ranges in color from yellow to brown [4]. Scent secretions and marking behaviors play critical roles in  
57 animal communication. Anal sac secretions are used for defense by the hooded skunk (*Mephitis macroura*)  
58 [5] and the honey badger (*Mellivora capensis*) [6], territory marking by the spotted hyena (*Crocuta crocuta*)  
59 [7] and the wolf (*Canis lupus*) [8,9], individual identification by the ferret (*Mustela furo*) [10,11], the  
60 mongoose (*Herpestes auropunctatus*) [12], the giant panda (*Ailuropoda melanoleuca*) [13], striped hyena  
61 (*Hyaena hyaena*) [14] and the spotted hyena (*C. crocuta*) [13,15] and sex recognition by the brown bear  
62 (*Ursus arctos*) [16], the giant panda (*A. melanoleuca*) [17], and some mustelids (*Mustela* spp.) [11,18]. In  
63 domestic cats (*Felis catus*) and meerkats (*Suricata suricatta*) [19], anal sac secretions are used for territorial  
64 marking, and such secretions may have information about sex, reproductive state, and recognition of  
65 individuals [1,20]. Further, these chemical signals are species specific and chemical signals from scent  
66 glands in the Felidae were found to retain a phylogenetic signal [21].

67 The chemical composition of anal sac secretions has been analyzed in a number of animals in the  
68 Carnivora. Studies in the cheetah (*Acinonyx jubatus*), red fox (*Vulpes vulpes*), dog (*Canis familiaris*),  
69 coyote (*Canis latrans*), wolf (*C. lupus*), lion (*Panthera leo*), and mongoose (*H. auro punctatus*) have  
70 identified volatile short-chain free fatty acids, such as acetic acid, propanoic acid, and butanoic acid as  
71 being partially responsible for the odors [22–28]. The nature of these constituents led to the suggestion that  
72 they may be metabolites produced by bacteria in the sac from available substrates [22].

73 The fermentation hypothesis posits that bacteria metabolize secretions and produce volatile organic  
74 compounds, such as hydrocarbons, fatty acids, wax esters, and sulfur compounds [15,16,29] that are used  
75 in communication by the host [30,31]. Evidence in support of this hypothesis links bacterial action to  
76 specific, olfactory-mediated host behavior or to the production of certain odorants. For example, researchers  
77 have shown that trimethylamine, an odorant that plays a key role in mouse (*Mus musculus*) reproduction,  
78 requires commensal bacteria for its production [24]. Researchers have also inhibited odorant production in  
79 Indian mongooses (*H. auro punctatus*) and European hoopoes (*Upupa epops*) by treating the animals' scent  
80 glands with antibiotics [30,32].

81 In this study, we investigated the fermentation hypothesis by focusing in detail on bacterial isolates  
82 collected from a single animal, available at the time of our study. We studied a bengal cat, a hybrid between  
83 the Asian leopard cat (*P. bengalensis*) and the domestic cat (*F. catus*). We collected anal sac secretions in  
84 order to characterize their chemical profile and analyze bacterial community composition. Then we isolated  
85 and identified those bacteria that could be cultivated under anaerobic conditions from these samples.  
86 Volatiles produced by these isolates were identified and compared to those found in the anal sac secretions.  
87 This is the first study in felines to demonstrate that bacteria isolated from anal sacs produce key volatile  
88 compounds found in anal sac secretions.

89

## 90 **Materials and methods**

### 91 **Sample collection**

92 With the owner's consent, both left and right anal sacs were manually expressed in a male bengal  
93 cat (*F. catus* × *P. bengalensis*) by a veterinarian at the Berkeley Dog and Cat Hospital in Berkeley, CA.  
94 Samples of anal sac secretions were collected using Puritan cotton swabs and placed in 2 mL screw cap  
95 tubes. Three swabs (2 samples and one blank control) were used for 16S rRNA gene sequencing (placed  
96 into 100% ethanol), three swabs were used for GC/MS (plus two media controls and two jar blanks), and  
97 two swabs were used for culturing.

98

### 99 **DNA extraction and 16S rRNA gene sequencing and analysis**

100 Swabs of the anal sac secretions were placed in 100% ethanol prior to extraction. Genomic DNA  
101 was extracted using the MoBio PowerSoil DNA Isolation kit (MoBio, Carlsbad, CA, USA). Samples were  
102 transferred to bead tubes containing C1 solution and incubated at 65 °C for 10 min, followed by 3 min of  
103 bead beating. The remaining extraction protocol was performed as directed by the manufacturer.

104 DNA samples were sent to the Integrated Microbiome Resource, Centre for Comparative Genomics  
105 and Evolutionary Bioinformatics, Dalhousie University for sequencing. Bacterial diversity was  
106 characterized via amplification by a PCR enrichment of the 16S rRNA gene (V4-V5 region) using primers  
107 515F and 926R (Walters et al. 2016). After an initial denaturation step at 98 °C for 30 sec, we ran 30 cycles  
108 of the following PCR protocol: 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, followed by final extension  
109 at 72 °C for 4 m 30 s, and a final hold at 4 °C [33]. Prior to sequencing, the amount of input DNA per

110 sample was normalized using a SequalPrep Normalization Plate, following the standard protocol  
111 (ThermoFisher Scientific, Wilmington, DE, USA). The final library pool was quantified using the Qubit  
112 dsDNA HS assay (Invitrogen, Carlsbad, CA). We generated paired-end 300 bp sequencing reads of 16S  
113 PCR amplicons with multiple barcodes on the Illumina MiSeq, resulting in 450 bp amplicons of the V4  
114 region using v3 chemistry. The samples were diluted to the appropriate concentration, spiked with 5% PhiX  
115 control library, and sequenced using an Illumina MiSeq instrument with the manufacturer's standard 150  
116 nucleotides paired-end dual-index sequencing protocol.

117  
118 Raw, demultiplexed amplicon reads were processed using DADA2 v1.8, following the standard  
119 online tutorial [34]. The reads were trimmed down to 250 base pairs to remove low quality nucleotides. In  
120 addition, the quality of reads were ensured by trimming bases that did not satisfy a Q2 quality score. Reads  
121 containing Ns were discarded and we used two expected errors to filter the overall quality of the read (rather  
122 than averaging quality scores) [35]. Chimeric reads were also removed using DADA2 on a per sample  
123 basis. The remaining pairs of reads were merged into amplicon fragments and unique Amplicon Sequence  
124 Variants (ASVs) were identified. Reads that did not merge successfully were discarded. Upon completion  
125 of the DADA2 pipeline, all ASVs (n=52) that were found in the negative control swabs were removed from  
126 the analysis, only three of these ASVs were also found in the anal sac samples. ASVs were assigned  
127 taxonomy using the dada2 function "assignTaxonomy" and the Silva (NR v132) database [36–38]. One  
128 ASV that was assigned to "Eukaryotes" was removed. All ASVs with the same taxonomy (at the genus  
129 level) were grouped and then ranked by number of reads. No ASVs were assigned to mitochondria or  
130 chloroplast.

## 131 132 **Bacterial culturing and identification**

133 Anal sac secretions were vortexed with 1 mL Phosphate Buffer Saline (PBS). Two serial 1:10  
134 dilutions were performed and 100  $\mu$ L of each dilution was plated onto Columbia Blood Agar (CBA) and  
135 Brain Heart Infusion (BHI). Plates were incubated anaerobically in a BD GasPak EZ Container System  
136 with packets of CO<sub>2</sub> generator for 5 days at 37 °C. Morphologically distinct colonies were streaked for  
137 isolation on both CBA and BHI. The 16S rRNA gene was sequenced using Sanger sequencing using the  
138 27F and 1391R primers. Taxonomy was assigned by the result of BLAST queries to the nr database at  
139 NCBI (excluding unnamed/environmental sequences), a species name was given in cases where the identity  
140 was >98% to only a single species.

## 141 142 **Bacterial volatile analysis**

143 To extract volatiles from *Bacteroides fragilis* UCD-AAL1 and *Tessaracoccus sp.* UCD-MLA,  
144 cultures were grown in 5 mL BHI anaerobically for 24 hours at 37 °C. 100  $\mu$ L of the culture was transferred  
145 into each of three Restek (Bellefonte, PA) tubes filled with 5 mL of BHI. Two jar blanks (no media or  
146 bacteria) and two BHI media-only blanks were used as controls. The same procedure was followed for  
147 *Fingoldia magna* UCD-MLG, except that cultures were grown and incubated in BHI supplemented with  
148 5% defibrinated sheep blood (BBHI) anaerobically for 24 hours at 37°C.

149 Headspace extraction was performed with Solid Phase Microextraction (SPME) fibers (Part 57912-  
150 U, Sigma Aldrich) which had 50/30  $\mu$ m thickness and DVB/CAR/PDMA coating. Two SPMEs were  
151 inserted into the headspace of each Restek tube prior to anaerobic incubation at 37 °C for 24 hours. SPME  
152 fibers were introduced by piercing the fibers through the septa insert of the lids and making sure that the

153 fibers were injected but not touching the media containing the bacteria. An internal standard was introduced  
154 before sampling using 1  $\mu$ L of the standard solution (10 mL/L of decane-d<sub>22</sub> in ethanol) per jar.

155 For the anal sac samples, the swabs containing the anal sac fluid were placed in septa screw cap  
156 jars that each contained a SPME. After a 24 hour incubation period, the SPMEs were removed. Then we  
157 performed a liquid extraction of volatiles by adding 20 mL of methanol to the jars and incubating for 24  
158 hours.

159

## 160 **GC-MS analysis**

161 Chromatography occurred on a 7890 GC (Agilent Technologies Inc., Santa Clara, CA) with a ZB-  
162 WAX 30 m  $\times$  250  $\mu$ m capillary column, coated with a 0.25  $\mu$ m film stationary phase (Part 7HG-G007-11,  
163 100% polyethylene glycol from Phenomenex, Torrance, CA) equivalent to DB-Wax or Carbowax. Helium  
164 was used as the carrier gas at 1 ml/min in constant flow mode. The inlet was set to 260  $^{\circ}$ C and SPMEs were  
165 splitlessly desorbed during the run. The oven temperature was programmed to increase from 40  $^{\circ}$ C (held  
166 for 5 min) to 110  $^{\circ}$ C at a rate of 5  $^{\circ}$ C min<sup>-1</sup>, and raise to 250  $^{\circ}$ C (held for 10 min) at a rate of 40  $^{\circ}$ C min<sup>-1</sup>.  
167 A transfer line set at 250  $^{\circ}$ C led to a 5977A mass spectrometer (Agilent Technologies Inc., Santa Clara,  
168 CA) with a solvent delay of 5 min. The MS swept from 50 to 500 m/z. The mass spectrometer was operated  
169 in the selected scan mode. The MS source was set to 230  $^{\circ}$ C and the MS quad set to 150  $^{\circ}$ C. A standard  
170 mix of C<sub>8</sub>-C<sub>20</sub> alkanes was analyzed to calculate the Kovats Retention Indices and to monitor control of the  
171 instruments.

172 Methanolic extract of cat anal secretion was analyzed by GC-MS as tert-butyldimethylsilyl  
173 (TBDMS) derivatives (Knapp, 1979). 2 mL out of 20 mL was used in the analysis. Samples were placed in  
174 glass conical vials and dried, reacted with a mixture of 50  $\mu$ L N-methyl-N-tert-  
175 butyldimethylsilyltrifluoroacetamide (MTBSTFA; Sigma-Aldrich Co. LLC., St Louis, MO, U.S.A.) and 50  
176  $\mu$ L acetonitrile at 60 $^{\circ}$ C for an hour.

177

## 178 **GC/MS data analysis workflow**

179 MassHunter Profinder B.08.00 (Agilent Technologies Inc.) was used to deconvolute, integrate and  
180 align the data. Peaks with amplitudes of less than 1000 counts were ignored. Compounds must have been  
181 present in at least 60% of replicates from one treatment to be included in statistical analyses. Statistical  
182 analysis was performed using GeneSpring MPP (Agilent Technologies, Inc.), where  $p \leq 0.05$  was used  
183 throughout to test for statistical significance using a T-test with Bonferroni correction. Tentative compound  
184 identification was based on the combined comparing mass spectra to the NIST 2014 Library and by a  
185 comparison of the calculated matching of standard alkane retention indices (LRI) values, when available.

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187

## 188 **Results and discussion**

### 189 **16S rRNA gene sequencing and bacteria identification**

190 The 16S rRNA gene PCR sequencing and analysis of the feline anal sac showed that 98% of the  
191 reads that were placed into ASVs were assigned to six genera (Table 1). These ASVs generally represent  
192 genera that contain anaerobic members known to be associated with mammals. *Tessaracoccus* species are  
193 have been isolated in sediment and have also been found in the gut of mammals including rhinoceros and  
194 humans [39–43]. *Bacteroides* is a genus of bacteria also often associated with mammals [44,45].

195 *Anaerococcus*, *Peptoniphilus* and *Finegoldia* are all Gram Positive Anaerobic Cocci (GPAC) formerly part  
 196 of the *Peptostreptophilus* genus and are found in mammalian guts and urinary tracts [46–48].  
 197 *Peptostreptococcus* is another mammalian-associated GPAC, with around 15 species in the genus. At least  
 198 one member of the group has been found as an obligate anaerobic bacteria in cats and dogs [49,50].

199 We cultured 25 bacterial isolates from this anal sac and found only *Tessaracoccus*, *Escherichia*,  
 200 *Bacteroides*, *Finegoldia*, and *Clostridium* isolates under the conditions used (TABLE 2). Of the cultured  
 201 bacteria, three isolates were members of genera found in high abundance in the anal sac: *Tessaracoccus*,  
 202 *Bacteroides fragilis*, and *Finegoldia magna*. We therefore chose to focus our experimental efforts on these  
 203 genera.

204

205 **Table 1. 16S rRNA gene based survey of the bengal cat anal sacs.**

Right Anal Sac		Left Anal Sac		Genus
# of reads	%	# of reads	%	
15250	83%	17840	83%	<i>Tessaracoccus</i>
1532	8%	1986	9%	<i>Anaerococcus</i>
709	4%	746	3%	<i>Finegoldia</i>
325	2%	390	2%	<i>Bacteroides</i>
182	1%	114	1%	<i>Peptoniphilus</i>
117	1%	111	1%	<i>Peptostreptococcus</i>
18115	98%	21187	98%	n/a

206 Table shows the number of reads mapping to ASVs, summed by taxonomy at the genus level (e.g.  
 207 everything with the same genus was collapsed into a single column). Not shown are any reads that were  
 208 placed into ASVs for which the genera of the taxonomic assignments summed to less than 1% of the total  
 209 number of sequencing reads.

210

211

212 **Table 2. Identified isolates in this study**

Strain ID	Genus	Species
UCD-MLI	<i>Bacteroides</i>	<i>fragilis</i>
UCD-AAL1	<i>Bacteroides</i>	<i>fragilis</i>
UCD-MBD1A	<i>Clostridium</i>	<i>perfringens</i>
UCD-MBD2A	<i>Clostridium</i>	<i>perfringens</i>
UCD-MD1A	<i>Clostridium</i>	<i>perfringens</i>
UCD-MD1C	<i>Clostridium</i>	<i>perfringens</i>
UCD-MD1E	<i>Clostridium</i>	<i>perfringens</i>
UCD-MD2A	<i>Clostridium</i>	<i>perfringens</i>

UCD-MLB	<i>Escherichia</i>	<i>coli</i>
<b>UCD-MLG</b>	<b><i>Finegoldia</i></b>	<b><i>magna</i></b>
<b>UCD-MLA</b>	<b><i>Tessaracoccus</i></b>	<b>sp.</b>
UCD-MLH	<i>Tessaracoccus</i>	sp.
UCD-ACL3	<i>Tessaracoccus</i>	sp.
UCD-AD1A	<i>Tessaracoccus</i>	sp.
UCD-AD2B1	<i>Tessaracoccus</i>	sp.
UCD-AD2B2	<i>Tessaracoccus</i>	sp.
UCD-AD2B3	<i>Tessaracoccus</i>	sp.
UCD-AD3C	<i>Tessaracoccus</i>	sp.
UCD-MLE	<i>Tessaracoccus</i>	sp.
UCD-MLF	<i>Tessaracoccus</i>	sp.
UCD-MBD1B	<i>Tessaracoccus</i>	sp.
UCD-MBD1C	<i>Tessaracoccus</i>	sp.
UCD-MBD1D	<i>Tessaracoccus</i>	sp.
UCD-MBD1E	<i>Tessaracoccus</i>	sp.
UCD-MBD3A	<i>Tessaracoccus</i>	sp.
UCD-MD3B	<i>Tessaracoccus</i>	sp.

213 All identified isolates from the anal sacs used in this study, bolded taxa are those for which we generated  
214 volatile data. Identifications were made from BLAST results of Sanger sequencing of the 16S rRNA gene.  
215

216

217 *Tessaracoccus* is a genus in the Propionibacteriaceae family of the phylum Actinobacteria. The  
218 characterized species in this genus are facultatively anaerobic and Gram positive bacteria. Representatives  
219 from this genus have been isolated from a wide variety of environments, including rhinoceros feces [42],  
220 the gut of humans [41], metalworking fluid [51], marine sediment [39], and activated sludge biomass [52].  
221 For our work on *Tessaracoccus*, we focused our efforts on a single isolate, *Tessaracoccus* sp. UCD-MLA.  
222 This full-length 16S rRNA gene sequence was 97% identical to multiple members of the genus and we  
223 could not assign a species ID with confidence [53].

224 *Bacteroides fragilis* is the type species of the genus *Bacteroides* in the Bacteroidaceae family  
225 (phylum Bacteroidetes) and is an obligate anaerobe [54]. It has previously been found in numerous places,  
226 including the oral cavity of cats [55], as well as the gut microbiome of dogs [56]. The 16S rRNA gene of  
227 our chosen isolate, *Bacteroides fragilis* UCD-AAL1, was 99% identical to representatives of this species at  
228 NCBI.

229 The only species in the genus of *Finegoldia* (Peptoniphilaceae family within the Firmicutes  
230 phylum), *Finegoldia magna*, is an obligately anaerobic Gram positive coccus that is part of the normal flora  
231 of the human gastrointestinal and genitourinary tracts [47] and has been previously found in cats [57] as

232 well as dogs [58]. The 16S rRNA gene from our single isolate, *Finegoldia magna* UCD-MLG, was 99%  
233 identical to representatives of this species at NCBI.

234  
235

## 236 **Anal sac secretion constituents and Bacteria isolates headspace SPME**

237 We tentatively identified 127 compounds from the domestic cat anal gland secretion. Out of 127  
238 tentatively identified compounds, 89 compounds were found in liquid extraction of anal secretion after  
239 TBDMS derivatization (S1 Table), and 52 compounds were measured by SPME-GC-MS in the anal sac  
240 secretion. These compounds were tentatively identified on the basis of the precise interpretation of its  
241 accurate mass spectra, MS fragmentation, and Kovats index information. These VOC metabolites were  
242 identified in the following compound chemical classes: heterocyclic compounds (12 %), alcohols (16 %),  
243 fatty acids (17 %), ketones (11 %), aromatic carbons (13 %), amines (9 %), aldehydes (7 %), esters (6 %).

244 A total of 67 compounds were tentatively identified from the SPME analysis of the three bacterial  
245 isolates (19 compounds from *B. fragilis* UCD-AAL1, 44 compounds from *Tessaracoccus* sp UCD-MLA,  
246 and 23 compounds from *F. magna* UCD-MLG) (Table 2). Among these 67 compounds, 52 compounds  
247 were also found in the anal sac secretion. 11 compounds (octan-1-ol, 1-(H)-indole, nonanoic acid,  
248 pentadecanoic acid, toluene, trans-2-pentenoic acid, non-2-enal, tetradecanal, n-hexadecanoic acid,  
249 octadecanoic acid, and (Z)-docos-13-enoic acid) found in the anal sac secretion have also been reported in  
250 other mammalian anal sac secretions [10,13,14,17,19,20,26,27]. Octan-1-ol and 1-(H)-indole were found  
251 in the anal sac secretion and in all three bacterial isolates. Octan-1-ol is a compound that produces a pungent  
252 odor and was previously reported in *C. lupus* anal sac secretion [26]. 1-(H)-indole is known to be widely  
253 distributed in the natural environment and can be produced by a variety of bacteria that have a strong fecal  
254 odor. 1-(H)-indole has also been found in various mammalian anal secretions, such as *V. vulpes* [59], *F.*  
255 *catus* [20], *A. melanoleuca* [13], *C. lupus* [26], and *M. furo* [10]. Toluene is an aromatic compound found  
256 naturally in petroleum and coal, and is a major component of gasoline [60]. Raymer *et. al.* reported toluene  
257 found in *C. lupus* [26]. Although anaerobic bacterial toluene degradation is a well known pathway [61],  
258 toluene biosynthesis is less common in bacteria. It is possible that our tentatively identified result could be  
259 a different compound but is likely to contain the alkyl benzene structure. Non-2-enal, and tetradecanal are  
260 chain aldehyde compounds. Non-2-enal was previously found in *C. lupus* [26] and tetradecanal was found  
261 in both *S. suricatta* [19,27], and *A. melanoleuca* [17] anal sac secretions. It is likely these aldehydes are  
262 formed by bacterial oxidation of a ubiquitous fatty acid such as oleic acid [62]. Out of 52 compounds, six  
263 fatty acids were tentatively identified from our anal sac secretion; nonanoic acid, pentadecanoic acid, trans-  
264 2-pentenoic acid, n-hexadecanoic acid, octadecanoic acid, and (Z)-docos-13-enoic acid. Fatty acids are one  
265 of the most common compound groups known to be present in various animal anal sac secretions  
266 [13,14,16,17,19,27] and all of the fatty acids found in our anal sac secretion have been previously reported  
267 in other mammalian anal sac secretions. Nonanoic acid is a nine carbon fatty acid known to have unpleasant  
268 rancid odor, and previously found in *S. suricatta* anal sac secretions [19]. Pentadecanoic acid was found in  
269 *P. leo* [27], *S. suricatta* [19], and *A. melanoleuca* [13,17] anal sac secretions. Trans-2-pentenoic acid was  
270 found in *H. hyaena* [14] anal sac secretions. N-hexadecanoic acid was found in *P. leo* [27], *S. suricatta*  
271 [19], *A. melanoleuca* [17] anal sac secretions. (Z)-docos-13-enoic acid was found in *A. melanoleuca*  
272 [13,17] anal sac secretions. Fatty acids are a very common group biosynthesized by variety of organisms.  
273 The biogenesis typically starts with acetyl CoA, which is extended with malonate units to ultimately  
274 assemble fatty acids containing an even number of carbons [62]. All six fatty acids found in our anal sac  
275 secretion were also found in the *Tessaracoccus* sp UCD-MLA culture. It is possible that the highly abundant



276 (in our sample) *Tessaracoccus* is largely contributing in the production of fatty acid compounds in this anal  
 277 sac.

278 41 compounds found in our bengal cat anal sac secretion have not been previously described in an  
 279 anal sac secretion. Cyclohexanone and dimethyl trisulfide were found from the anal sac secretion and also  
 280 from all three bacteria isolates. Cyclohexanone is known to be generated by the cyclohexanol degradation  
 281 metabolomic pathway, which is widely used by bacteria [63]. Including dimethyl trisulfide, volatile sulfur  
 282 compounds (hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyl disulfide) such methionine derived  
 283 volatiles are often generated by bacteria [62] and have also been reported to be produced by the *Bacteroides*  
 284 family [64]. Hydrocarbons, aliphatic alcohols, and ketones are volatiles most likely formed by modification  
 285 of products of the fatty acid biosynthetic pathway [62]. Alkanes and methyl ketones are often known to be  
 286 produced from decarboxylation in bacteria [62]. Methyl ketone group compounds (2-methyl  
 287 cyclopentanone, 1-(1,3-thiazol-2-yl)ethanone, 1-phenylethan-1-one) were tentatively identified from the  
 288 cat anal sac secretion and at least one of the bacteria isolates. Another final modification process of the fatty  
 289 acid metabolomic pathway is the reduction of carboxy groups to aldehydes and into aliphatic alcohols.  
 290 Several aliphatic alcohol (decan-1-ol, butan-1-ol, 2-methylhexadecan-1-ol, 2-hexyldecan-1-ol) were  
 291 tentatively identified from the anal sac secretion and from bacteria isolates. Butan-1-ol and its  
 292 bishomologues up to C16 have been found in different combinations in several bacteria [62,65,66].  
 293 Aromatic compounds are common natural products in plants but also known to be produced by bacteria  
 294 [62]. Especially among the aromatic alcohols, such phenols, 2-penylethanol is one of the most aromatic  
 295 compound produced by diverse bacteria. In our present study, aromatic alcohols, such as phenyl methanol,  
 296 and 3,5-di-(tert)-buthylphenol were tentatively identified from both anal sac and bacterial isolates. These  
 297 aromatic alcohols can be assumed to be generated by the shikimate pathway which is present only in  
 298 microorganisms and plants, never in animals [67].

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301 **Table 3. Tentatively identified compounds (compound name and formula listed) from cultures and**  
 302 **anal sac.**

Compound name	Formula	Reference	Anal sac	Bacterial isolates		
				<i>Bf</i>	<i>Tess</i>	<i>Fm</i>
cyclohexanone	C <sub>6</sub> H <sub>10</sub> O		+	+	+	+
dimethyl trisulfide	C <sub>2</sub> H <sub>6</sub> S <sub>3</sub>		+	+	+	+
octan-1-ol	C <sub>8</sub> H <sub>18</sub> O	Wolf [8]	+	+	+	+
decan-1-ol	C <sub>10</sub> H <sub>22</sub> O		+	+	+	+

1-(H)-indole	C <sub>8</sub> H <sub>7</sub> N	Red fox [27] ; Domestic cat [20] ; Giant panda [13]; Wolf [8] ; Ferret [10]	+	+	+	+
dimethyl disulfide	C <sub>2</sub> H <sub>6</sub> S <sub>2</sub>		+		+	+
m-xylene	C <sub>8</sub> H <sub>10</sub>		+		+	+
2,5-dimethylpyrazine	C <sub>6</sub> H <sub>8</sub> N <sub>2</sub>		+		+	+
phenylmethanol	C <sub>7</sub> H <sub>8</sub> O		+		+	+
nonanoic acid	C <sub>9</sub> H <sub>18</sub> O <sub>2</sub>	Meerkats [19]	+	+	+	
pentadecanoic acid	C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>	Lion [27] ; Meerkats [19] ; Giant panda [13,17]	+		+	+
trichloromethane	CHCl <sub>3</sub>			+	+	+
toluene	C <sub>7</sub> H <sub>8</sub>	Wolf [8]	+		+	
2-aminocynoacetamide	C <sub>3</sub> H <sub>5</sub> N <sub>3</sub> O		+		+	
butan-1-ol	C <sub>4</sub> H <sub>10</sub> O		+		+	
trans-2-pentenoic acid	C <sub>5</sub> H <sub>8</sub> O <sub>2</sub>	Striped hyena [14]	+		+	
1,3-diazine	C <sub>4</sub> H <sub>4</sub> N <sub>2</sub>		+		+	
azetidine	C <sub>3</sub> H <sub>7</sub> N		+	+		
2-Methyl-2-propanyl acrylate	C <sub>7</sub> H <sub>12</sub> O <sub>2</sub>		+	+		
ethenylbenzene	C <sub>8</sub> H <sub>8</sub>		+		+	

2-methylpyrazine	C5H6N2		+		+	
2-methylcyclopentanone	C6H10O		+			+
methyl 3-amino-2-methylpropanoate	C5H11NO2		+		+	
2-ethylpyrazine	C6H8N2		+		+	
1-isopropoxy-1-propene	C6H12O		+			+
2-ethyl-5-methylpyrazine	C7H8N2		+		+	
2-ethylhexyl formate	C9H18O2		+		+	
2-Isobutyl-3,6-dimethylpyrazine	C10H16N2		+		+	
cycloocta-1,5-diene	C8H12		+			+
N-methyl-1-(methylthio)-2-nitroethenamine	C4H8N2O2S		+		+	
1-(1,3-thiazol-2-yl)ethanone	C5H5NOS		+		+	
1-phenylethan-1-one	C8H8O		+			+
2-hexylaziridine	C8H17N		+	+		
methyl (Z)-N-hydroxybenzenecarboximidate	C8H9NO2		+	+		
non-2-enal	C9H17NS	Wolf [8]	+		+	
2-decen-1-ol	C10H20O		+	+		
4-methyl-2,4-dihydro-3H-1,2,4-triazol-3-thion	C3H5N3S		+		+	
2-methylhexadecan-1-ol	C17H36O		+	+		

1-ethyl-5-methyltetrazole	C9H10N4		+		+	
3-methylcinnoline	C9H8N2		+		+	
tetradecanal	C14H28O	Meerkats [8,19]; Giant panda [17]	+		+	
(Z)-(N)-(2-methylpyridin-1-ium-1-yl)benzenecarboximidate	C13H12N2O		+		+	
phenyl carbamate	C7H7NO2		+		+	
4-amino-(N)-(3-morpholin-4-ylpropyl)-1,2,5-oxadiazole-3-carboxamide	C10H17N5O3		+		+	
pentadecyl acetate	C17H34O2		+		+	
3,5-di-(tert)-butylphenol	C14H22O		+		+	
2-hexyldecan-1-ol	C16H34O		+		+	
decyl decanoate	C20H40O2		+			+
(n)-pentyldecanamide	C18H36O2		+			+
n-hexadecanoic acid	C16H32O2	Lion [27] ; Meerkats, [19]; Giant panda [17]	+		+	
octadecanoic acid	C18H36O2	Lion [27]; Giant panda [13,17]; Brown bear [16]	+		+	
(Z)-docos-13-enoic acid	C22H42O2	Giant panda [13,17]	+		+	

303 The presence of the compound is indicated by a '+' in the column corresponding to each sample: domestic  
 304 cat anal sac secretion chemical profile ("Anal sac") and headspace solid phase microextraction (SPME) for  
 305 cultures of *B. fragilis* UCD-AAL1 ("*Bf*"), *Tessaracoccus* sp UCD-MLA ("*Tess*"), and *F. magna* UCD-  
 306 MLG ("*Fm*").

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## 309 Conclusion

310 To our knowledge, this is the first study examining either the VOC profile of domestic feline anal  
311 sacs or the VOC profiles of associated bacteria. We show that these particular feline anal sacs are dominated  
312 by only a few taxa, most of which are easily culturable under anaerobic conditions. These bacteria produce  
313 the majority of the identified volatiles in the total anal sac scent profile. Our preliminary identification of  
314 these volatiles is supported by the existence of known bacterial metabolic pathways for the majority of  
315 these compounds. Together these results support the fermentation hypothesis and suggest that further  
316 characterization of the anal sac microbial community, as well as the VOC's produced therein could  
317 potentially shed light on the potentially symbiotic relationship between these microbes and their host.

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319

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## 332 **Animal Use and Care**

333 No laboratory animals were used in this research. The owner of a male bengal cat volunteered to have her  
334 cat's anal sacs manually expressed by a veterinarian at the Berkeley Dog and Cat Hospital in Berkeley, CA.

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## 337 **Availability of Supporting Data**

338 All raw sequencing data has been deposited at NCBI under BioProject PRJNA533859. All data analysis,  
339 supporting files and intermediate analysis files are available at DASH (doi:10.25338/B8JG7X).

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## 542 Supporting information

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544 **S1 Table. Tentatively identified compounds in the anal gland secretion of headspace solid phase**  
545 **microextraction (SPME) and liquid extraction tert-butyl dimethylsilyl (TBDMS) derivative of domestic cat.**

Compounds	match %	Retention Index (RI)	extraction method
A alkane compound		923	SPME headspace
A fatty acid		923	SPME headspace
A fatty acid		960	SPME headspace
Toluene	87.52	973	SPME headspace
(Methyl disulfanyl)methane	83.11	1013	SPME headspace
1,3-xylene	83.91	1092	SPME headspace
butyl propanoate	72.45	1111	SPME headspace
2-amino-2-cyanoacetamide	81.71	1123	SPME headspace
butan-1-ol	88.53	1124	SPME headspace
(E)-pent-2-enoic acid	63.5	1124	SPME headspace
2-Heptanone	60.5	1153	SPME headspace
dimethyl bicyclo[2.2.1]heptane-2,3-dicarboxylate	55.2	1181	SPME headspace
1,3-Diazine	92.87	1185	SPME headspace
Azetidine	73.34	1186	SPME headspace

A aldehyde		1186	SPME headspace
tert-butyl prop-2-enoate	78.02	1186	SPME headspace
Pentan-1-ol	65.58	1188	SPME headspace
Ethenylbenzene	91.95	1229	SPME headspace
A fatty acid		1230	SPME headspace
2-methylpyrazine	98.19	1237	SPME headspace
2-Methylcyclopentanone	73.63	1256	SPME headspace
methyl 3-amino-2-methylpropanoate	61.84	1257	SPME headspace
Cyclohexanone	83.59	1260	SPME headspace
A fatty acid		1263	SPME headspace
2,5-dimethylpyrazine	96.32	1290	SPME headspace
2-ethylpyrazine	88.37	1302	SPME headspace
dimethyltrisulfane	75.44	1348	SPME headspace
A fatty acid		1361	SPME headspace
(E)-hept-2-en-1-ol	74.5	1361	SPME headspace
Nonanal	90.99	1368	SPME headspace
(E)-1-propan-2-yloxyprop-1-ene	61.25	1368	SPME headspace

A fatty acid		1373	SPME headspace
2-ethyl-5-methylpyrazine	90.83	1468	SPME headspace
2-ethylhexyl formate	81.31	1474	SPME headspace
2,5-dimethyl-3-(2-methylpropyl)pyrazine	70.56	1495	SPME headspace
7-methyl-2H-[1,2,4]triazolo[4,3-a]pyridine-3-thione	55.7	1508	SPME headspace
Octan-1-ol	92.56	1545	SPME headspace
Cycloocta-1,5-diene	64.2	1548	SPME headspace
(2Z)-2-pyrrolidin-2-ylideneacetonitrile	83.21	1550	SPME headspace
(2E,6E)-nona-2,6-dien-1-ol	62.34	1562	SPME headspace
A secondary amin compound		1578	SPME headspace
A chain aldehyde compound		1591	SPME headspace
N,N,2,2-tetramethylpropan-1-amine oxide	67.44	1597	SPME headspace
1-methoxy-3,5-dimethylbenzene	62.12	1619	SPME headspace
(E)-N-methyl-1-methylsulfanyl-2-nitroethenamine	60.55	1619	SPME headspace
Methyl benzoate	91.95	1623	SPME headspace
1-(1,3-thiazol-2-yl)ethanone	61.18	1647	SPME headspace
1-phenylethanone	72.3	1650	SPME headspace

2-hexylaziridine		1653	SPME headspace
methyl (Z)-N-hydroxybenzenecarboximidate	59	1653	SPME headspace
Decan-1-ol	76.19	1663	SPME headspace
Non-2-enal	58.48	1705	SPME headspace
2-Decen-1-ol	66.77	1706	SPME headspace
4-methyl-1H-1,2,4-triazole-5-thione	63.22	1751	SPME headspace
Benzyl alcohol	71.73	1763	SPME headspace
2-methylhexadecan-1-ol	66.65	1817	SPME headspace
tetradecan-2-one	55.56	1857	SPME headspace
1-Hydroxy-5-phenyl-tetrazol; 5-phenyl-tetrazol-1-ol	58.57	1887	SPME headspace
3-methylcinoline	80.63	1975	SPME headspace
1-(2-hydroxypropoxy)propan-2-ol	60.43	1975	SPME headspace
A aldehyde compound		1975	SPME headspace
A Ketone compound		>2000	SPME headspace
Phenyl carbamate	74.99	>2000	SPME headspace
decyl decanoate	74.29	>2000	SPME headspace
(Z)-octadec-9-enamide	75.69	>2000	SPME headspace

(17S)-3-methoxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-17-ol	79.33	>2000	SPME headspace
1-Pentadecanol acetate	74.43	>2000	SPME headspace
2-hexyldecan-1-ol	75.8	>2000	SPME headspace
Tetradecanal	57.87	>2000	SPME headspace
3,5-ditert-butylphenol	68.02	>2000	SPME headspace
(Z)-N-(2-methylpyridin-1-ium-1-yl)benzenecarboximidate	57.36	>2000	SPME headspace
1-methyl-3-phenylbenzene	88.31	>2000	SPME headspace
N-Benzoylalanine	65.22	>2000	SPME headspace
Nonanoic acid	69.57	>2000	SPME headspace
Indole	88.85	>2000	SPME headspace
Erucic acid	76.55	>2000	SPME headspace
octyl decanoate	67.75	>2000	SPME headspace
n-Pentyldecanamide	86.68	>2000	SPME headspace
Octadecanoic acid	73.42	>2000	SPME headspace
n-Hexadecanoic acid	95.41	>2000	SPME headspace

Pentadecanoic acid	86.66	>2000	SPME headspace
6-octyloxan-2-one	64.26	>2000	SPME headspace
4-amino-N-(3-morpholin-4-ylpropyl)-1,2,5-oxadiazole-3-carboxamide	86.12	>2000	SPME headspace
A aromatic acid		>2000	SPME headspace
A aromatic compound		>2000	SPME headspace
A aromatic nitrogen compound		>2000	SPME headspace
A fatty acid		>2000	SPME headspace
A fatty acid		>2000	SPME headspace
A secondary amin compound		>2000	SPME headspace
A fatty acid		810	TBDMS liquid extraction
Butyric acid TBDMS	93.42	864	TBDMS liquid extraction
A fatty acid		870	TBDMS liquid extraction
A fatty acid		914	TBDMS liquid extraction
Hymexazole, TBDMS derivative	72.42	951	TBDMS liquid extraction



Propane-1,2-diol, 2TBDMS derivative	89.06	984	TBDMS liquid extraction
D-2-Aminobutyric acid, 2TBDMS derivative	97.26	1033	TBDMS liquid extraction
Methylsuccinic acid, 2TBDMS derivative	63.67	1043	TBDMS liquid extraction
L-Valine, 2TBDMS derivative	87.14	1045	TBDMS liquid extraction
Urea	85.13	1047	TBDMS liquid extraction
Benzenepropanoic acid, TBDMS derivative	70.77	1048	TBDMS liquid extraction
L-Leucine, 2TBDMS derivative	97.13	1055	TBDMS liquid extraction
2-Octanol, TBDMS derivative	56.26	1073	TBDMS liquid extraction
A fatty acid		1073	TBDMS liquid extraction
Cinnamic acid, (E)-, TBDMS derivative	82.7	1081	TBDMS liquid extraction
2-Ethylhexanoic acid, TBDMS derivative	57.22	1089	TBDMS liquid extraction

5-Aminovaleric acid, 2TBDMS derivative	94.67	1094	TBDMS liquid extraction
Indole-3-carboxylic acid, 2TBDMS derivative	55.58	1103	TBDMS liquid extraction
L-Phenylalanine, 2TBDMS derivative	69.25	1140	TBDMS liquid extraction
Phthalic acid, 2TBDMS derivative	62.75	1154	TBDMS liquid extraction
2-Thiobarbituric acid, TBDMS derivative	64	1166	TBDMS liquid extraction
A fatty acid		1172	TBDMS liquid extraction
Oxalacetic acid, enol-2TBDMS derivative	58.08	1183	TBDMS liquid extraction
L-Lysine, 3TBDMS derivative	64.9	1192	TBDMS liquid extraction
10-Heptadecenoic acid, (Z)-, TBDMS derivative	60.4	1193	TBDMS liquid extraction
L-Dihydroorotic acid, TBDMS derivative		1217	TBDMS liquid extraction
Nonadecanoic acid, TBDMS derivative	63.24	1226	TBDMS liquid extraction

11-Eicosenoic acid, (Z)-, TBDMS derivative	88.79	1243	TBDMS liquid extraction
A fatty acid		1253	TBDMS liquid extraction
13-Docosenoic acid, (Z)-, TBDMS derivative	63.18	1280	TBDMS liquid extraction
A fatty acid		1304	TBDMS liquid extraction
A fatty acid		1334	TBDMS liquid extraction
17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol	97.51	1361	TBDMS liquid extraction
5-Docosyldihydrofuran-2(3H)-one	71.42	1372	TBDMS liquid extraction
1-Heptacosanol, TBDMS derivative	68.16	1407	TBDMS liquid extraction
Cholesta-3,5-diene	82.89	1407	TBDMS liquid extraction
Hexacosanoic acid, TBDMS derivative	85.31	1412	TBDMS liquid extraction
1-Octacosanol, TBDMS derivative	56.45	1471	TBDMS liquid extraction

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546 Compounds were tentatively identified by calculating their Kovats Retention Index in comparison to  
547 reported literature values and by comparison of extracted mass spectra to the NIST 2014 mass spectral  
548 library.  
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