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1 **Composition of the fecal, vaginal and colostrum microbiotas of**
2 **dams at parturition and their relationship with neonatal outcomes**
3 **in dogs**

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21 Keywords: dog; puppy; microbiota; parturition; colostrum; gestation;
22 neonatal health; mortality

23 **Abstract:**

24 **Background:** Microbial seeding in early life is critical for the host's short- and long-term
25 health, and the mother is the first source of bacteria for the newborn. The objective of this study
26 was to characterize the maternal fecal, vaginal, and colostrum microbiotas in the canine species
27 one day after parturition and to evaluate the relationship between the microbial profiles of 36
28 dams and the neonatal outcomes of 284 newborns.

29 **Results:** The first part of the study revealed the presence of 2 fecal, 3 vaginal, and 2 colostrum
30 microbial clusters on the basis of the core microbiota of the dams. Among these three maternal
31 microbiotas, only the vaginal microbiome was found to be associated with neonatal outcomes.
32 Compared with those in the other clusters, females in Cluster 1, with the lowest stillbirth and
33 neonatal mortality ratios, presented a greater abundance of *Moraxellaceae* in their vaginal
34 microbiota; Cluster 2, with a greater abundance of *Pasteurellaceae*, mostly from the
35 *Haemophilus* genus; and Cluster 3 (with the highest stillbirth and neonatal mortality ratios), a
36 greater abundance of *Enterobacteriaceae*, mostly *E. coli*. Moreover, Cluster 3 dams presented
37 significantly lower species richness according to the Shannon index than did dams from the
38 other clusters.

39 **Conclusions:** This study underscores the strong association between maternal microbiota,
40 particularly the vaginal microbiota, and newborn health. The results of this study call for further
41 research to gain a deeper understanding of the optimal vaginal microbiota composition in canine
42 species and the ways to modulate it to improve neonatal outcomes.

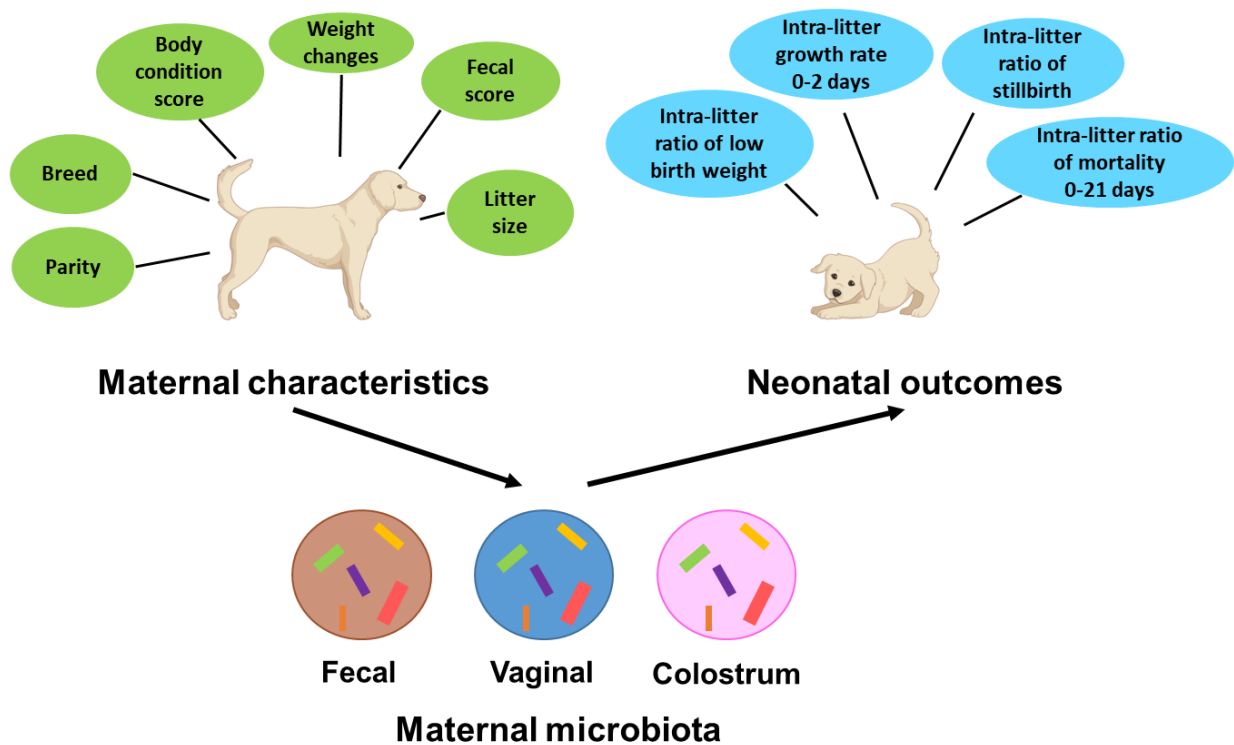
43 **1. Background**

44 Currently, the dynamic interaction between microbial communities (collectively known as the
45 microbiota) and their host organisms offers valuable insights into host health. The most
46 extensively studied microbiota is the gut (and more specifically the fecal), as its role in shaping
47 its host's health has gained increasing evidence^{1,2}. However, many more microbial communities
48 play crucial roles in host health, although they remain less explored. This includes the vaginal
49 and colostrum microbiotas, which have many essential functions within the host (the dam), such
50 as immune regulation, metabolic equilibrium and protection against pathogens³⁻⁵. Moreover,
51 all of these microbiotas have emerged as central players in reproductive health and
52 performance^{5,6}. In particular, the maternal vaginal and colostrum microbiotas have been shown
53 in humans to be crucial for enhancing the newborn's immune system by promoting the
54 colonization of the intestine by beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*,
55 as they induce specific immune stimulation of the intestinal environment through the
56 production of short-chain fatty acids (SCFAs) and interactions with Treg cells^{5,7,8}.

57 Given the complex topic of the maternal microbiota and its importance during parturition,
58 maternal factors may have crucial interrelationships with the microbial composition. These
59 factors encompass a spectrum of maternal attributes, such as physiological status, consumption
60 of medications, or diet, as observed in humans⁹. For example, pregnant women exposed to
61 antibiotics presented lower abundances of *Actinobacteria* and greater alpha diversity¹⁰,
62 whereas overweight and obese women presented lower maternal alpha diversity and taxonomic
63 differences in the maternal gut microbiota¹¹. Understanding how these maternal factors interact
64 with microbial diversity has the potential to improve the understanding of the relationship
65 between the host's physiological state and the microbial communities that inhabit it.

66 Although the importance of the maternal microbiota during parturition for neonates' health
67 trajectory has been demonstrated in human medicine^{7,12,13}, it is currently poorly investigated in
68 canine species. Neonatal puppies are inherently vulnerable, with approximately 13% of puppies
69 dying during their first weeks of life¹⁴. The transition from the protected intrauterine
70 environment to the external world places them at a critical juncture where among other factors,
71 their ability to establish a resilient microbial ecosystem is paramount. In the case of perturbed
72 microbial seeding, as shown with puppies born from C-sections, less diverse microbiota and
73 delayed growth were observed^{15,16}. Other neonatal events can also impact the proper setting of
74 the microbial communities of newborn puppies. For example, birth weight is associated with
75 microbiota alterations, with low-birth-weight (LBW) puppies exhibiting an increased
76 abundance of opportunistic bacteria in their fecal microbiota during the first three weeks of life
77 compared with normal-weight puppies¹⁷. For these reasons, understanding the vertical
78 transmission of bacteria from mothers to offspring seems crucial for improving puppies' health.

79 This study aims to evaluate whether maternal fecal, vaginal and colostrum microbial profiles of
80 bitches one day after whelping are associated with neonatal outcomes in terms of LBW, early
81 growth rate, stillbirth and neonatal mortality (defined as deaths occurring during the first three
82 weeks of life) ratios. Second, the degree to which maternal factors, such as body condition score
83 (BCS), gestational weight changes, fecal scores, parity, and litter size, are correlated with
84 variations in maternal microbial composition related to neonatal outcomes was assessed
85 (**Figure 1**). Improving the understanding of the interactions among maternal physiological
86 factors, the microbiota and reproductive performance would provide crucial insights into the
87 ability of the modulation of the maternal microbiota to increase the survival and well-being of
88 newborn puppies.



89

90 **Fig. 1.** Summary of all maternal factors and neonatal outcomes studied and their potential
 91 interactions with the three maternal microbiota.

92

93 2. Methods

94 2.1. Animal enrollment and housing

95 Thirty-six bitches from medium-sized (> 15 and ≤ 25 kg) and large-sized (> 25 kg) breeds were
 96 recruited from one breeding kennel in France. Dams were followed from ovulation (G0) up to
 97 24 hours postpartum (P1), with a pregnancy diagnosis performed on their 28th day of gestation
 98 (G28). They were fed two manufactured dry pet food diets. From G0 until G28, the bitches
 99 received Diet 1, then Diet 2 from G28 to the end of the experiment. The nutritional values of
 100 both diets are available in Supplementary **Table S1**. During the experiment, all dogs had *ad*
 101 *libitum* access to water. All the dams were vaccinated and dewormed before mating, following
 102 the veterinary protocol in place at the breeding facility. They were housed in individual outdoor

103 pens until around the 56th day of gestation (G56), when they were moved to individual pens in
104 the maternity building to prepare for whelping. The pens were equipped with a floor heating
105 system, heating lamps, and wood shavings as bedding material. All puppies were born by
106 natural delivery (i.e., no cesarean section) and remained in the same pens as their mothers
107 during the study period.

108 2.2. Sampling and measures

109 Both dam weights (digital scale Soehnle model 7859.70.002, Soehnle GmbH, Backnang,
110 Germany, maximum capacity of 200 kg, precision ± 0.1 kg) and body condition scores (BCSs,
111 9-point scale, with 1 being the thinnest and 9 the fattest¹⁸) were recorded on G0, G28, G56 and
112 P1. Gestational relative gain weight (GWG) was calculated as the percentage of weight gained
113 or lost by the dams between G0 and P1 according to the following formula: ((weight P1 –
114 weight G0) / weight G0 * 100%).

115 Every day, individual food intake was recorded by measuring the amount of food provided in
116 the morning and normalizing it on the basis of the remaining food in the bowl the following
117 day.

118 Dams' fecal scores (FS) were recorded on three consecutive days, from one to three days
119 postpartum, to assess the gut health of dams following parturition via a 5-point scale fecal
120 scoring system (with 1 being the driest stool and 5 being the most liquid¹⁹). The mean of the
121 three scores was then calculated and attributed to the P1 time point.

122 For each dam, reproductive performance was evaluated through the number of puppies born
123 alive, the number of stillborn puppies, the number of puppies dying during the neonatal period
124 (from birth to 21 days post parturition), and the number of low-birth-weight puppies (LBWs).
125 Stillborn, dead and LWB puppies were recorded as a ratio to the number of puppies alive at
126 birth per litter. To determine LBW, puppies were weighed during the first 12 hours after birth

127 (D0) via a digital scale (EB3 Series, Ohaus, Parsippany, NJ, USA; maximum capacity, 5 kg;
128 precision, ± 0.1 g). To address interbreed variability, quartiles of birth weight were calculated
129 for each breed on the basis of the last 4010 birth weights registered in the breeding facility^{20,21}
130 (Supplementary **Table S2**). The thresholds obtained were used to categorize each puppy into
131 one of the four birth weight quartile groups. The first quartile included the 25% lightest puppies,
132 whereas the 25% heaviest puppies were in the fourth quartile, with all puppies under the first
133 quartile threshold considered LBW. Puppies were weighed again at approximately 48 h after
134 birth (D2), and their growth rate (GR) was calculated as follows: $((\text{weight D2} - \text{weight}$
135 $\text{D0})/\text{weight D0}) * 100\%$.

136 All samples for maternal microbiota analyses were collected at P1 (108 total samples). Sterile
137 swabs (175 mm, Viscose knob, COPAN, Brescia, Italy) were used to collect fecal and vaginal
138 microbiota samples. Moreover, vaginal swabs were introduced via a sterile plastic speculum to
139 avoid contamination with the skin and vestibule bacterial flora. For colostrum collection,
140 intramuscular administration of 1 or 2 UI of oxytocin was performed before collection. The
141 fourth pair of mammary glands (with the 1st pair being the most cranial and the 5th pair being
142 the most caudal) was cleaned with chlorhexidine soap on a sterile compress, after which 0.1
143 mL of colostrum was collected from each gland into a sterile Eppendorf tube (0.2 mL in total).
144 The swabs and colostrum were stored at -20°C immediately after collection for a maximum of
145 1 month and then at -80°C for the rest of the time until further processing.

146 2.3. DNA Extraction and 16S rRNA Gene Amplification and 147 Sequencing

148 Metagenomic DNA was extracted from swabs via a Quick-DNA Fecal/Soil Microbe Miniprep
149 Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. For
150 colostrum DNA extraction, the same kit and instructions were used with minor adaptations on

151 the basis of the methodology of Derakhshani *et al*²². The extracted DNA was quantified via a
152 fluorometric method with a Quant-iT™ PicoGreen® dsDNA assay kit (Life Technologies,
153 Carlsbad, CA, USA) via a QuantStudio™3 Real Time PCR System (Thermo Fisher Scientific
154 Inc., Waltham, MA, USA). The V3-V4 region of the 16S rRNA gene was amplified via PCR
155 via the universal primers 341F (CCTACGGGAGGCAGCAG)²³ and 806R
156 (GGACTACNVGGGTWTCTAAT)²⁴. The PCR amplicons were purified with a HighPrep
157 PCR system (Magbio Genomics, Gaithersburg, MD, USA) and used for library construction
158 with an Illumina NEXTflex PCR-Free DNA Sequencing Kit (Bioo Scientific Corp., Austin,
159 TX, USA). Amplicon libraries were sequenced on an Illumina MiSeq 2500 platform (Illumina,
160 San Diego, CA, USA) at the GeT-PlaGe INRAE Platform (Toulouse, France) for paired-end
161 fragment sizes of 250 bp. All the reagents used were of molecular grade.

162 2.4. Analysis of Sequencing Data

163 Raw fastq files were imported, demultiplexed, quality filtered and dereplicated through high-
164 resolution sample inference with DADA2²⁵ in QIIME 2 (version 2020.2)²⁶. This allowed the
165 identification of amplicon sequence variants (ASVs) under default parameters, excluding
166 primer length. ASV de novo alignment and phylogenetic analysis were performed with MAFFT
167 and FastTree2, respectively^{27,28}. Rarefaction curves were checked for full community sampling
168 depth. Taxonomy was assigned to the resulting 16S rRNA gene marker genes against
169 Greengenes (gg-13-8-99-nb-classifier)/SILVA (v138) via the sklearn classifier method
170 according to Bokulich *et al.*²⁹. The raw data were rarefied by removing the ASVs present in
171 fewer than 5 samples with an abundance lower than 0.005% of the total abundance.

172 2.5. Data analysis

173 All the statistical analyses were conducted in R version 4.1.0 (R Core Team, 2021), with
174 resulting p values less than 0.05 considered statistically significant, and box plots and histogram

175 figures were drawn with RStudio (version 2022.02.3 Build 492). Other plots were created via
176 MicrobiomeAnalyst³⁰. All clinical data are expressed as the means and SDs (\pm).

177 Before compositional, multivariate and principal component analysis (PCA), the ASV
178 abundances were centered log-ratio (CLR) transformed following the replacement of zero with
179 the geometric Bayesian model (GBM) of the zCompositions package³¹.

180 2.5.1. Overall microbiota analysis

181 **Beta diversity.** Multidimensional scaling (MDS) plots generated via the phyloseq package were
182 generated to visualize the beta diversities among the three microbiota. To highlight potential
183 differences in the fecal, vaginal and colostrum microbiota structures, permutational multivariate
184 analysis of variance (PERMANOVA) with 9999 permutations was performed on the basis of
185 Bray–Curtis distances with the Adonis function available in the “vegan” package v2.6-2 of R
186 software³².

187 **Alpha diversity.** The alpha diversity was calculated with observed ASVs and the Shannon³³
188 index via the Phyloseq package v1.38.0³⁴, and ANOVA coupled with Tukey’s test was used to
189 evaluate differences among the three microbiotas and between clusters.

190 2.5.2. Core microbiota and clustering

191 **Definition of the core microbiota.** To describe and illustrate the main genera and families
192 comprising the different maternal microbiotas, the core microbiotas of the dams were defined
193 as all the ASVs at the genus level present in at least 32 dams (89% of samples) for fecal and
194 colostrum microbiotas and 27 dams (75%) in vaginal dams, with a minimum of 1% mean
195 relative abundance³⁵.

196 **Clustering of the dams.** The clustering of the dams was performed via Dirichlet Multinomial
197 Mixtures (DMM) clustering via the DirichletMultinomial 1.44.0 package in R³⁶. The optimal

198 number of clusters was determined via the Laplace approximation by analyzing model
199 complexity. The contributions of the main ASVs to each microbiota cluster were then
200 determined via linear discriminant analysis effect size (LEfSe).

201 **2.5.3. Relationships between microbiota profiles and neonatal health**

202 The associations between the microbial clusters identified in the dams' feces (Cluster 1/Cluster
203 2), vagina (Cluster 1/Cluster 2/Cluster 3), and colostrum (Cluster 1/Cluster 2) and the neonatal
204 outcomes in their litter (the ratio of LBW and GR between 0 and 2 days of life, the ratio of
205 stillbirth, and the ratio of neonatal mortality) were evaluated via generalized linear models
206 (using the `glm` function of the R *stats* package). The four following models were constructed to
207 address these questions:

208 *Neonatal outcome ~ Fecal microbial cluster + Vaginal microbial cluster + Colostral*
209 *microbial cluster*

210 Chi-square (for the interlitter LBW, stillbirth, and neonatal mortality ratio) and deviance (for
211 the interlitter GR 0–2 days) tests were used to assess the goodness-of-fit of the final models.

212 The overdispersion of the models was also assessed by comparing the residual deviance to the
213 degrees of freedom.

214 **2.5.4. Relationships between microbiota profiles and maternal factors**

215 In the final part of the study, only the microbiota associated with neonatal outcomes were
216 investigated. Eight factors were chosen for their biological relevance to study the association
217 between maternal factors and microbial profiles: breed, age, parity, food intake, GWG, BCS,
218 FS at P1, and litter size (Figure 1). On the basis of correlation matrices (using Kendall's tau),
219 parity and age were significantly correlated ($\rho > 0.4$; $p < 0.001$); thus, out of the two, only parity
220 was included in further analyses. Similar results were observed for food intake and GWG;

221 therefore, only GWG was used for further analysis. A multivariate logistic regression model
222 was subsequently built using the *glm* function to evaluate the associations between maternal
223 parameters (breed as a nominal variable and parity, GWG, BCS, FS, and litter size as
224 continuous variables) and the clusters (cluster name) of microbiota correlated with neonatal
225 health. Different models, including the six mentioned fixed effects, were tested, and the final
226 model was built on the basis of the lowest Akaike criterion as follows:

227
$$\text{Microbial clusters} \sim \text{Parity} + \text{Litter_size} + \text{Breed}$$

228 The overdispersion of the model was assessed by comparing the residual deviance to the
229 degrees of freedom.

230 **3. Results**

231 **3.1. Description of the population**

232 The 36 dams studied belong to 9 different medium and large breeds: Australian Shepherd (n=8),
233 Golden Retriever (n=8), German Shepherd (n=1), Labrador Retriever (n=6), White Swiss
234 Shepherd (n=3), Pyrenean Mountain Dog (n=2), Bernese Mountain Dog (n=6), Boxer (n=1)
235 and Dalmatian (n=1). Five bitches were primiparous, 8 gave birth once before the study, and
236 23 had whelped at least 2 times (mean = 2.7 ± 1.8). The mean age of the dams was 3.3 ± 1.2
237 years. At G0, the dams weighed between 18.5 and 39.5 kg (mean = $29.7 \text{ kg} \pm 5.1$) and had
238 BCSs ranging from 3 to 8 (mean = 5.4 ± 1.3) (**Table 1**). At P1, the weights of the dams and
239 their BCSs decreased (mean = $27.7 \text{ kg} \pm 5.1$ and mean = 4.1 ± 1.1 , $p < 0.001$, respectively). The
240 mean GWG was $-6.5 \pm 7.0\%$, ranging from -22 to 6%. The mean FS of the dams at parturition
241 was 2.6 ± 0.6 . A total of 284 puppies were born in our study, with litter sizes ranging from 4 to
242 11 puppies (mean = 7.9 ± 1.9). A total of 30 puppies were stillborn (10.6%), with a mean of 0.8
243 ± 1.1 puppies per litter. Among the 254 puppies born alive, 60 were LBW (23.6%), and 25

244 (9.8%) died during the first three weeks. Half of the dams (17 out of 36 dams) delivered no
 245 LBW puppies, whereas the other half (19 dams) had between 1 and 8 LBW per litter (mean
 246 ratio = 0.2 ± 0.3).

247 **Table 1.** Characteristics of the dams (n=36) and their litters included in the study.

Parameter	Mean	Standard error	Minimum	Maximum	Median
Weight at G0 (kg)	29.7	5.2	19	40	30.9
Weight at P1 (kg)	27.7	5.13	19	38	28.2
BCS at G0	5.4	1.3	3	8	6.0
BCS at P1	4.1	1.2	2	7	4.0
Parity	2.7	1.9	0	6	3.0
Fecal score at P1	2.6	0.6	1.7	4.5	2.4
Litter size	7.9	1.9	4	11	3.6
Sex ratio	1.5	1.0	0	4	1.0
Ratio of LBW (%)	0.2	0.3	0	0.9	0.1
GR 0-2 days (%)	8.1	8.2	-5.6	25.1	8.0
Stillbirth (%)	0.8	1.1	0	4	1.0
Neonatal mortality (%)	0.4	0.5	0	1	0

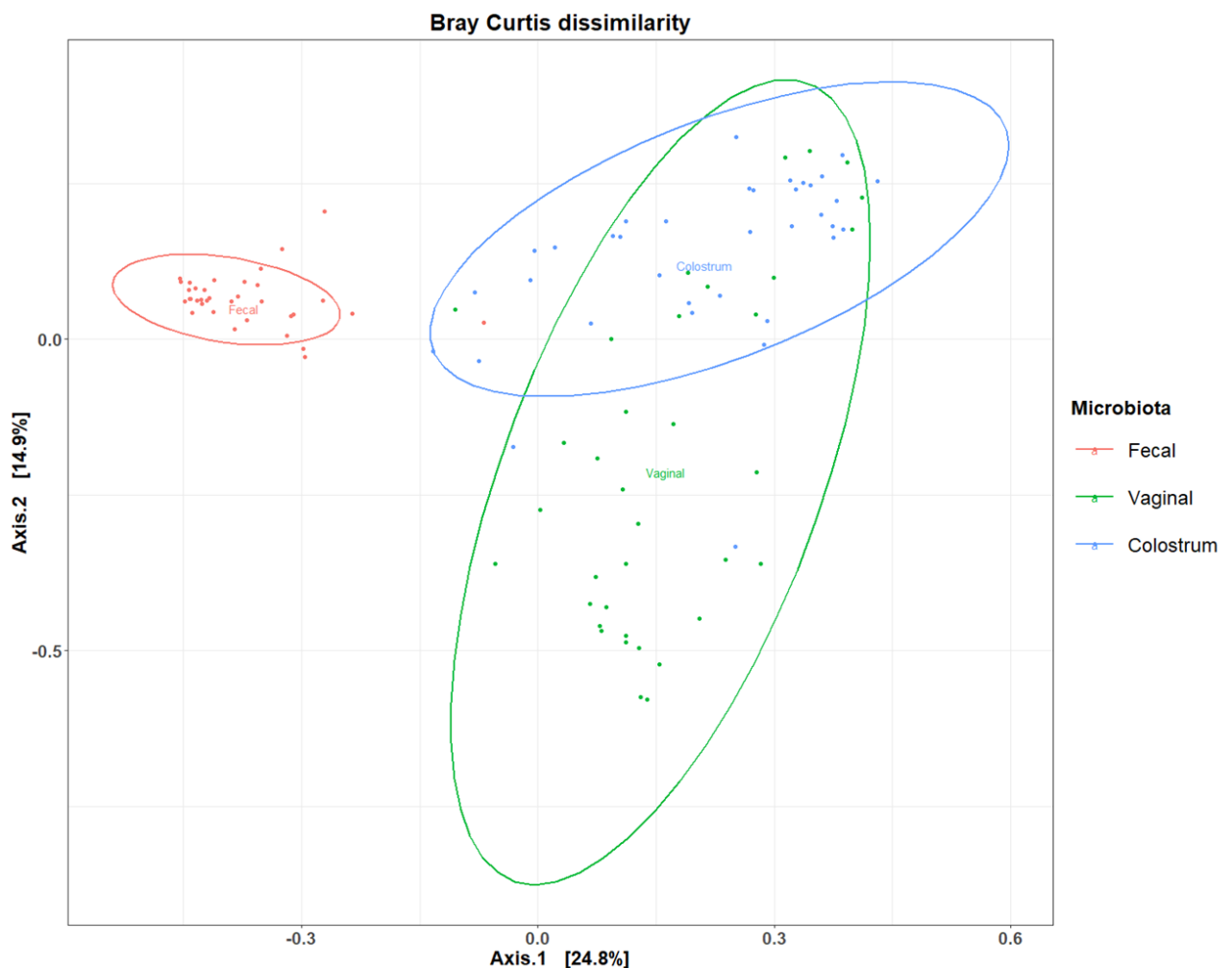
248 *G0* – ovulation day; *P1* – postpartum day 1; *BCS* – body condition score; *LBW* – low birth weight; *GR* – growth
 249 rate

250 3.2. Comparison of the fecal, vaginal and colostrum microbiotas 251 one day after parturition

252 A total of 3,087,414 sequences were used for the analysis. After the cleaning, filtration, and
 253 affiliations of the sequencing reads, 235 ASVs were identified, including 11 phyla, 22 classes,
 254 40 orders, 79 families, and 128 genera.

255 *Beta diversity.* The microbial community composition significantly differed depending
 256 on location on the basis of the PERMANOVA results obtained via Bray–Curtis distances ($p <$

257 0.001) and pairwise analysis (p-adjusted = 0.003 for each pair) (**Figure 2**). The fecal microbiota
258 had greater dissimilarity than the vaginal and colostrum microbiota, which shared more
259 bacterial communities, as seen with the overlapping ellipses. The size of the ellipses also
260 allowed us to highlight greater interindividual variation in the composition of the vaginal
261 microbiota than the other two, where ellipses were smaller. The Bray–Curtis distances were
262 also added to a heatmap to specify the previous results (**Figure 3**). As anticipated with the MDS
263 plot, approximately half of the vaginal and colostrum ASVs were almost absent from the fecal
264 microbiota, explaining the interdissimilarity.

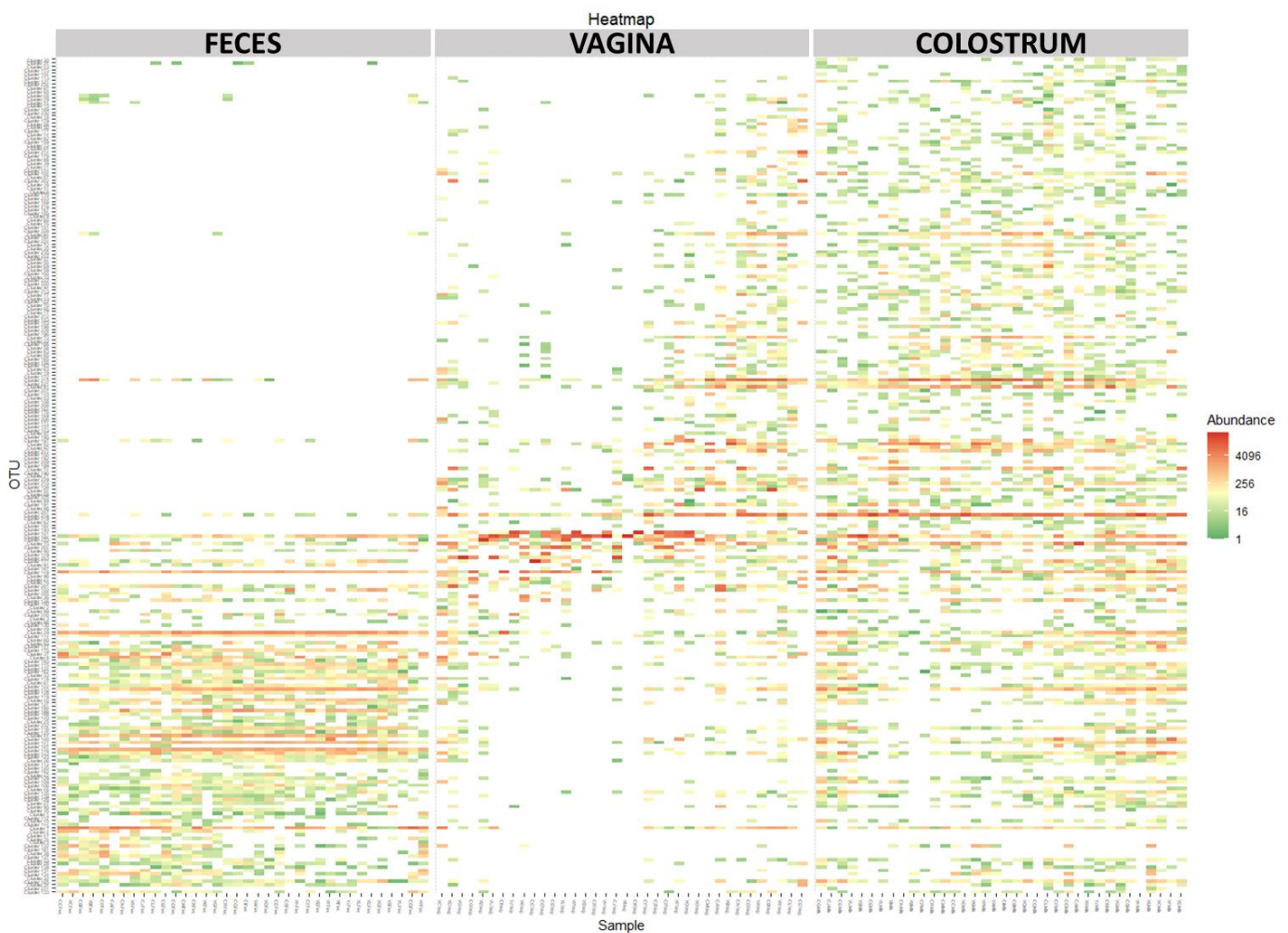


265

266 **Fig. 2.** Beta diversity of the 36 dams' three types of microbiota (fecal in red; vaginal in green
267 and colostrum in blue) one day after parturition. The MDS plot shows the dissimilarities between
268 dams' fecal, vaginal and colostrum microbiotas one day after parturition, which were calculated

269 via Bray–Curtis distances. Each point represents an individual dam sample for one specific
270 microbiota, positioned on the plot on the basis of the similarity of its microbiota communities
271 with other samples. Ellipses are based on 95% confidence intervals and standard errors. The
272 similarity of bacterial communities is evaluated on the basis of taxon similarity and abundance.
273 ($p < 0.001$)

274

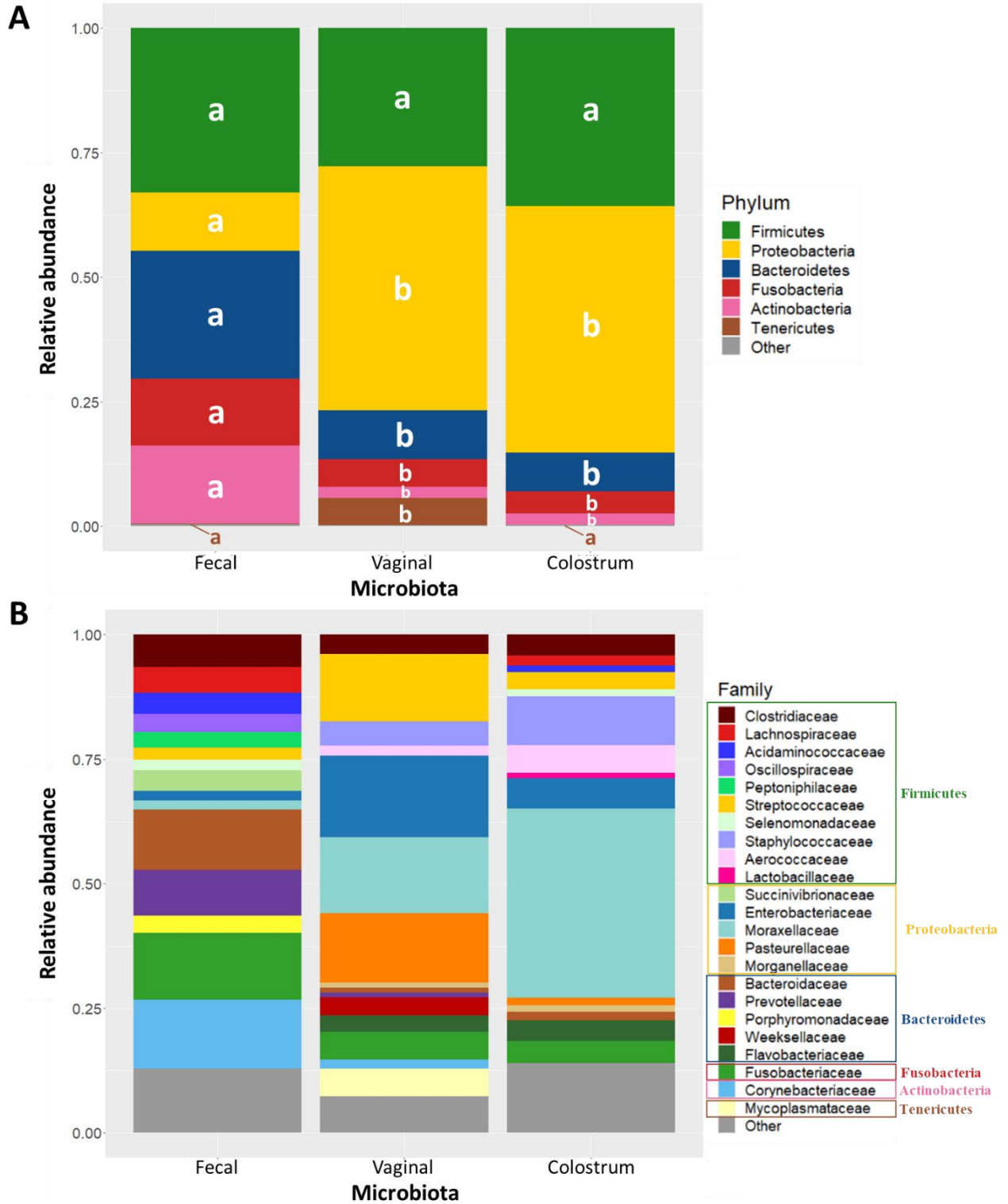


276 **Fig. 3.** Heatmap of dam microbiota one day after parturition at the family level between the
277 three studied sites (fecal, vaginal and colostrum). The color scale is based on the relative
278 abundance of the amplicon sequence variant (ASV).

279 *Composition.* At the three sampling sites, *Firmicutes*, *Proteobacteria*, *Fusobacteria*,
280 *Bacteroidetes* and *Actinobacteria* were the five most abundant microbial phyla, except the
281 vaginal phylum, where *Tenericutes*, exclusively composed of *Mycoplasma*, was more abundant
282 than *Actinobacteria* and was significantly more abundant than in feces and colostrum (**Figure**
283 **4A and Table 2**). The relative abundance of *Firmicutes* remained similar among the sampling
284 sites, although the family composition of this phylum varied from one site to another. The fecal
285 microbiota had the most heterogeneous *Firmicutes* composition, with *Clostridiaceae*,
286 *Lachnospiraceae* and *Acidaminococcaceae* being the most abundant families of this phylum.
287 The vaginal and colostrum microbiotas, on the other hand, had only one abundant *Firmicutes*
288 family: *Streptococcaceae* and *Staphylococcaceae*, respectively (**Figure 4B**). More
289 *Bacteroidetes* (dominated by *Bacteroidaceae* and *Prevotellaceae*) and *Actinobacteria*
290 (dominated by *Corynebacteriaceae*) were present in the fecal microbiota than in the vaginal
291 and colostrum microbiotas (**Table 2**). In contrast, *Proteobacteria* was the dominant phylum in
292 both the vaginal and colostrum microbiotas, with *Enterobacteriaceae* and *Moraxellaceae*
293 having the highest relative abundances in the vaginal and colostrum microbiotas, respectively.
294
295 **Table 2.** Mean relative abundance of each phylum and prominent family in the three
296 microbiotas of the dams one day after parturition (n=36).

Phylum	Relative abundance (%) (mean±SD)			Family	Relative abundance (%) (mean±SD)		
	Fecal	Vaginal	Colostrum		Fecal	Vaginal	Colostrum
<i>Actinobacteria</i>	15.9±14.5	2.2±4.8	2.2±2.0	<i>Corynebacteriaceae</i>	14.2±14.2	1.9±4.3	1.1±1.3
<i>Bacteroidetes</i>	25.4±8.7	10.3±16.0	8.0±8.0	<i>Bacteroidaceae</i>	12.0±4.5	1.1±5.2	1.6±1.9
				<i>Prevotellaceae</i>	9.0±5.4	1.2±4.1	1.0±1.4
<i>Firmicutes</i>	32.9±9.9	26.2±25.4	35.3±19.5	<i>Staphylococcaceae</i>	0.8±1.6	4.4±8.5	10.0±12.1
				<i>Streptococcaceae</i>	2.4±5.3	13.1±21.9	3.2±4.8
				<i>Clostridiaceae</i>	6.4±4.3	3.4±15.7	4.1±4.7
				<i>Lachnospiraceae</i>	5.1±2.7	0.4±1.0	2.0±2.6
				<i>Acidaminococcaceae</i>	4.2±2.3	0.1±0.5	1.2±1.7
<i>Fusobacteria</i>	13.5±4.8	7.0±14.2	4.3±4.6	<i>Fusobacteriaceae</i>	13.5±4.8	7.0±14.0	4.3±4.6

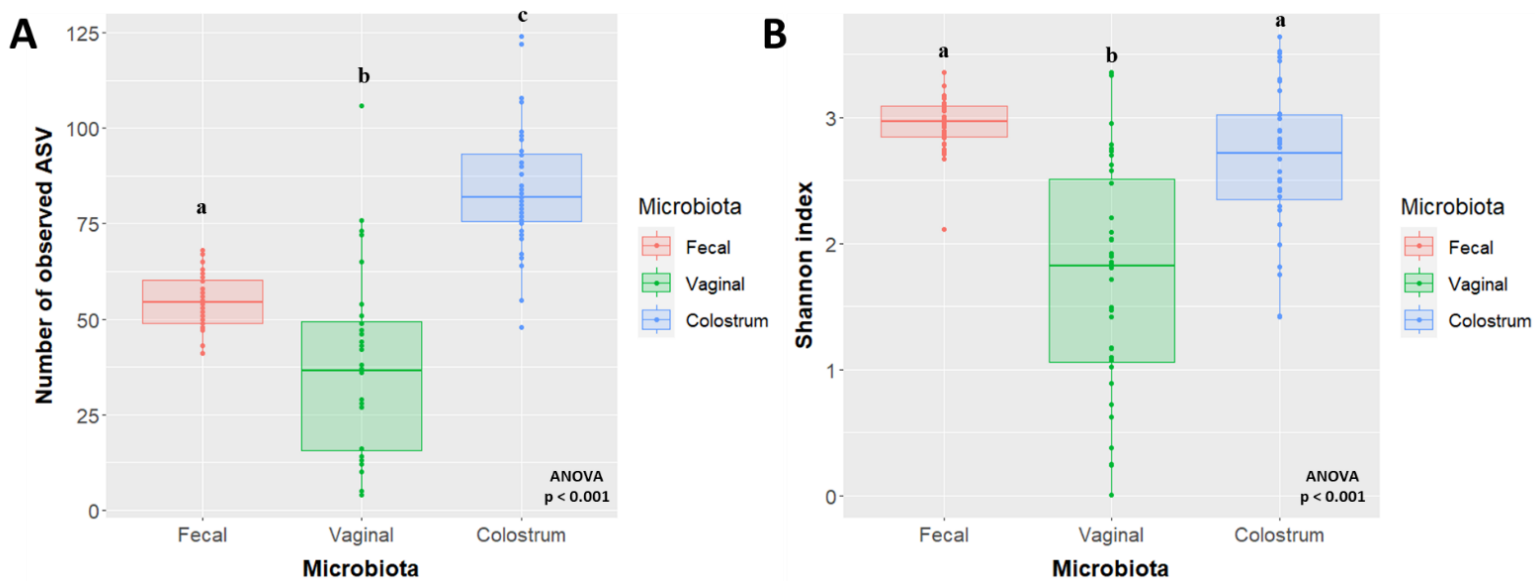
<i>Proteobacteria</i>	11.5±7.4	48.7±27.1	49.7±21.4	<i>Succinivibrionaceae</i>	4.1±5.1	0.0±0.1	0.2±0.3
				<i>Enterobacteriaceae</i>	1.9±1.9	16.2±25.2	5.6±11.2
				<i>Pasteurellaceae</i>	0.6±0.8	14.3±23.6	1.7±5.7
				<i>Moraxellaceae</i>	1.9±4.7	14.4±18.9	38.6±20.2
<i>Tenericutes</i>	0.1±0.3	5.3±15.7	0.1±0.2	<i>Mycoplasmataceae</i>	0.04±0.2	5.3±15.7	0.05±0.2



298 **Fig. 4.** Mean relative abundance of the bacterial taxa of the three studied microbiotas in 36
 299 bitches one day after parturition at the (A) phylum and (B) family levels. The 15 most abundant
 300 families at each site are highlighted. All the remaining taxa are summed in the “Other” category.
 301 Significant differences between phyla are identified with Tukey’s HSD letters.

302 *Alpha diversity.* The microbiotas from the vagina, colostrum and feces presented
 303 significantly different numbers of observed ASVs and Shannon diversity indices ($p < 0.001$).
 304 The colostrum microbiota had a significantly greater number of observed ASVs (mean = 84.9
 305 ± 17.0) compared to the fecal one (mean = 54.3 ± 7.4) which itself was significantly greater
 306 than the vaginal one (mean = 37.5 ± 23.6) (**Figure 5A**). According to the Shannon index, the
 307 fecal and colostrum microbiota did not significantly differ (mean = 2.9 ± 0.2 and mean = $2.7 \pm$
 308 0.6), implying that the evenness of the taxa in the fecal microbiota is greater (**Figure 5B**). The
 309 alpha diversity of the microbiota from both locations was significantly greater than that of the
 310 vaginal microbiota (mean = 1.7 ± 0.9).

311



313 **Fig. 5.** Alpha diversity of 36 samples of maternal fecal, vaginal and colostrum microbiotas one
 314 day after parturition using (A) observed species (bacterial richness) and (B) the Shannon index

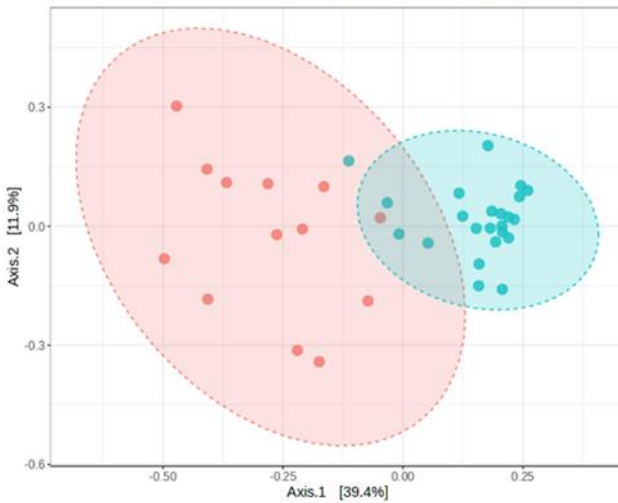
315 (bacterial richness and evenness). Significant differences are identified with Tukey Honest
316 Significant Difference (HSD) letters. Boxes with different letters are significantly different (p
317 < 0.05).

318 3.3. Characteristics of the core microbiota clusters

319 The core fecal, vaginal and colostrum microbiotas were composed of 15, 6, and 14
320 ASVs, respectively, at the genus level. The composition of the core microbiota is presented in
321 the supplementary data (**Table S3**). Using DMM community clustering, two clusters were
322 identified in the fecal and colostrum microbiota, and three were identified in the vaginal
323 microbiota, which is consistent with the previous Bray–Curtis results, highlighting greater
324 intradissimilarity in the vaginal microbiota than in the other two microbiotas (**Figure 6**).

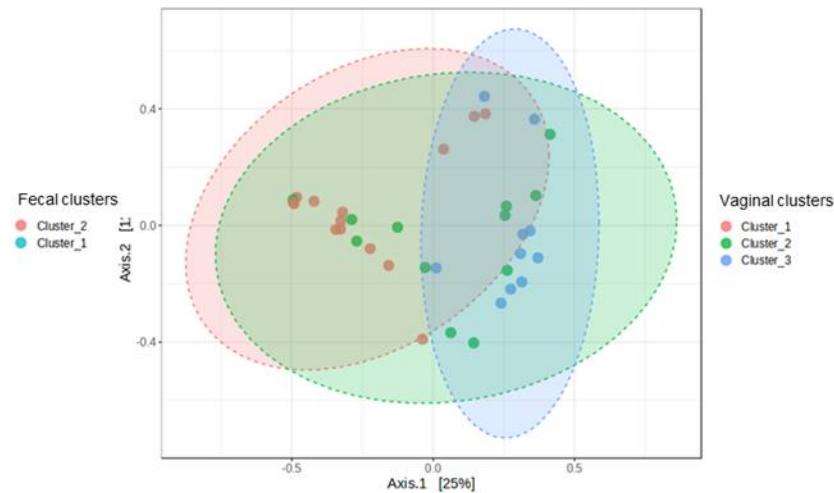
A. Fecal microbial clusters

[PERMANOVA] F-value: 15.293; R-squared: 0.31024; p-value: 0.001



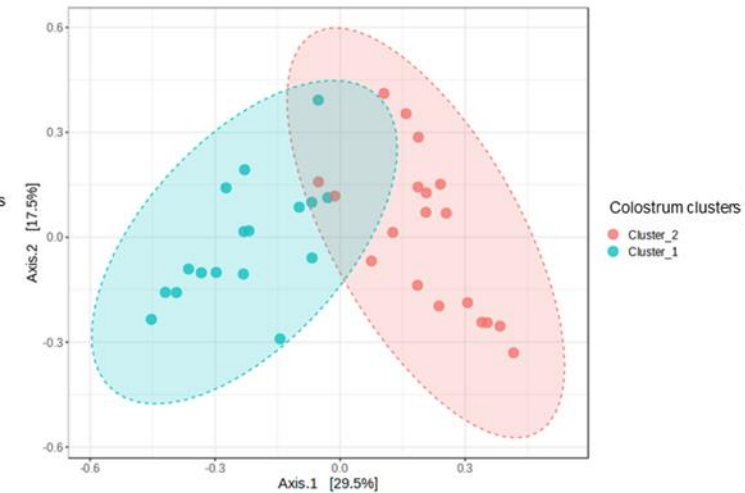
B. Vaginal microbial clusters

[PERMANOVA] F-value: 3.2877; R-squared: 0.16615; p-value: 0.001



C. Colostral microbial clusters

[PERMANOVA] F-value: 9.9819; R-squared: 0.22696; p-value: 0.001



326 **Fig. 6.** Beta diversity plots showing the differences in the fecal (A), vaginal (B) and colostrum (C) microbiota structures between the clusters
327 identified in the 36 dams one day after parturition via Dirichlet Multinomial Mixtures (DMM) clustering analysis. The MDS plot shows the
328 dissimilarities between clusters, which were calculated via Bray–Curtis distances. Each point represents an individual dam sample for one specific
329 microbiota, positioned on the plot on the basis of the similarity of its microbiota communities with other samples. Ellipses are based on 95%
330 confidence intervals and standard errors. The similarity of bacterial communities is evaluated on the basis of taxon similarity and abundance. ($p <$
331 0.001).

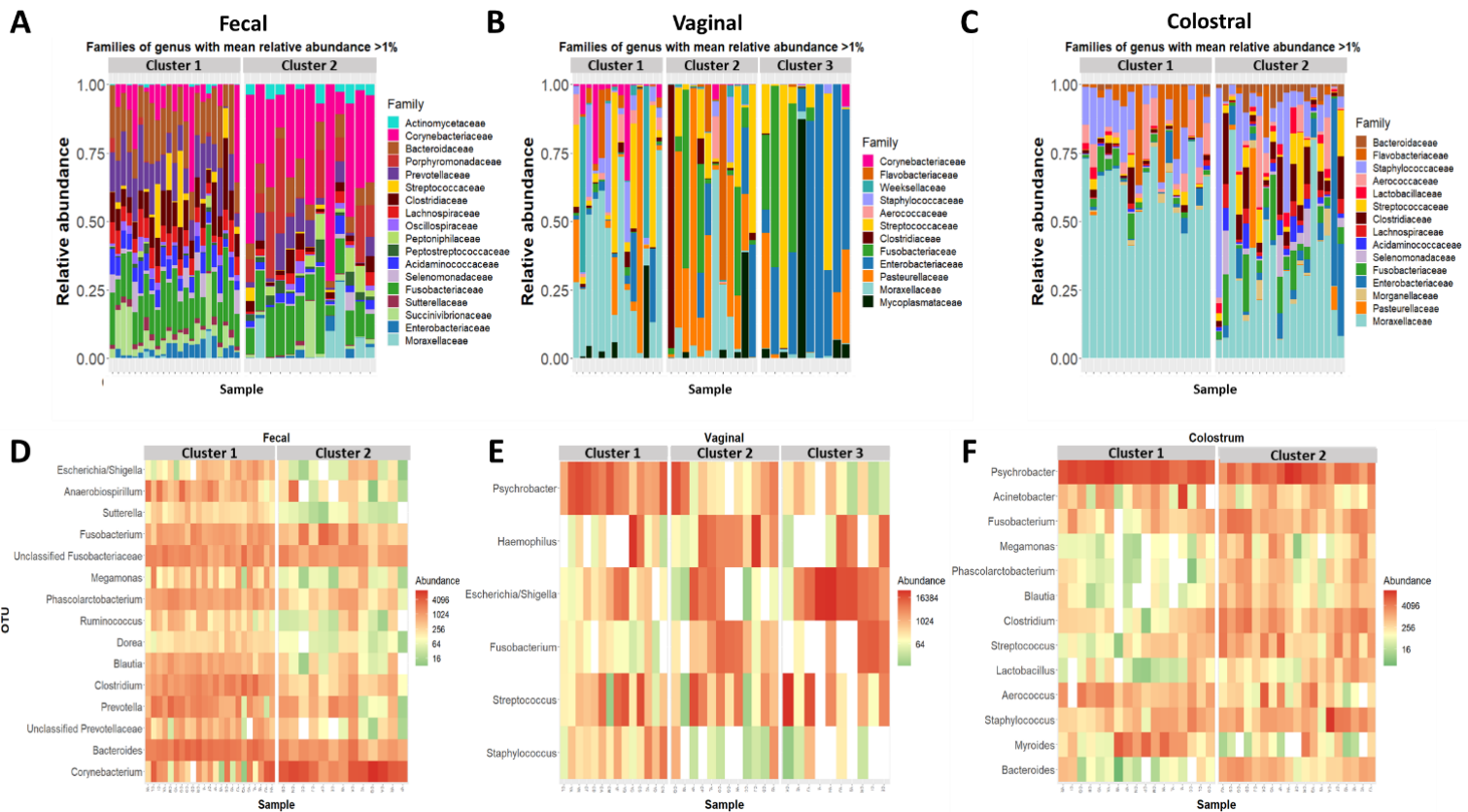
332

333 *Composition.* In feces, the clusters were separated on the basis of the relatively high relative
334 abundances of *Clostridiaceae* and *Selenomonadaceae* in Cluster 1 (means of 9% and 3% vs 3%
335 and 1%, respectively) and the relatively high relative abundances of *Corynebacteriaceae*
336 (namely, *Corynebacterium* and *Porphyromonadaceae*), mostly *Bacteroides*, in Cluster 2
337 (means of 26% and 9% vs 7% and 0.5%, respectively) (**Figure 7A, Figure 8A**).

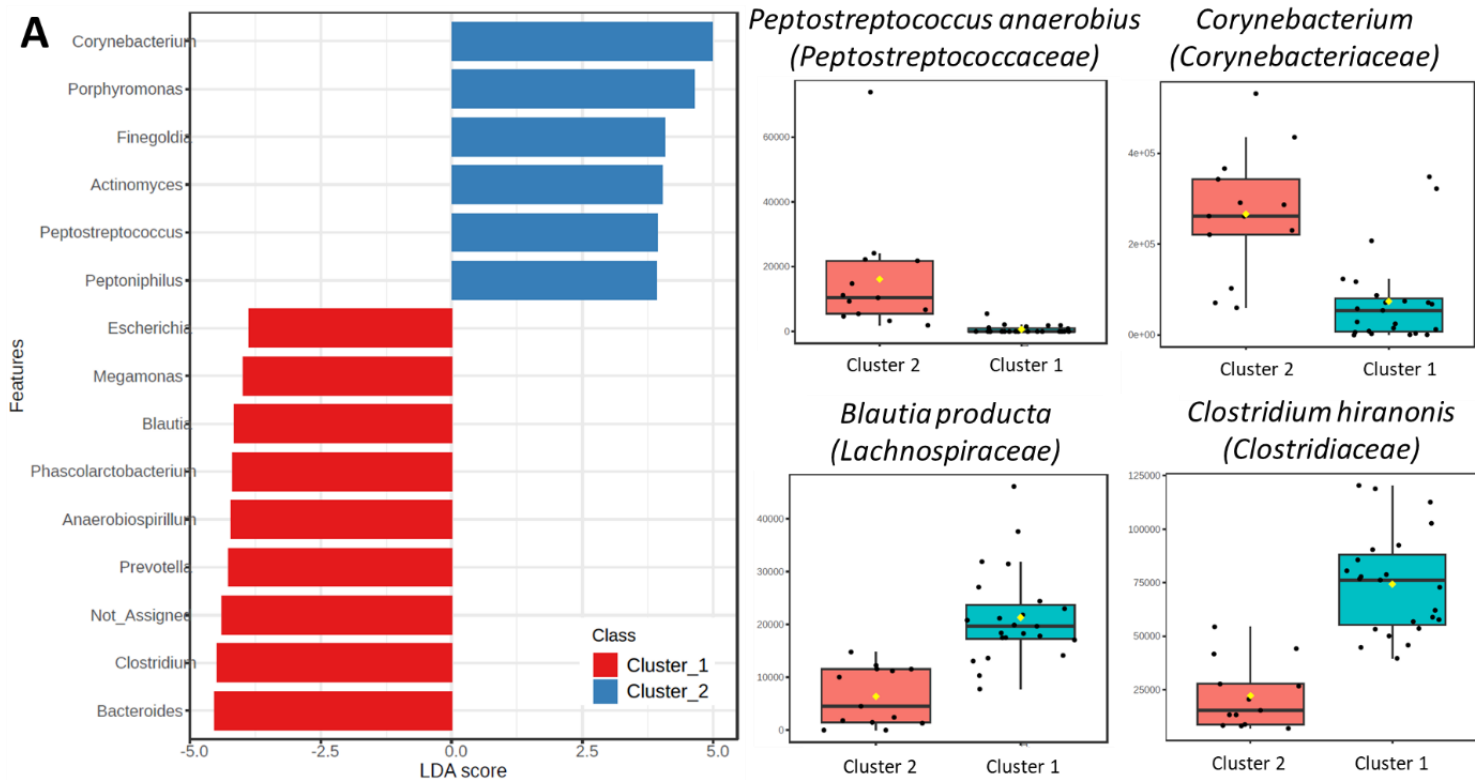
338 In the vagina, Cluster 1 was separated by a relatively high relative abundance of *Moraxellaceae*
339 (28% vs 10% and 1%), mostly *Psychrobacter* and *Acinetobacter* (**Figure 7E and Figure 8B**),
340 and a relatively low relative abundance of *Fusobacteriaceae* (0.5% vs 9% and 13%). Cluster 2
341 was separated by a relatively high relative abundance of *Pasteurellaceae* (23% vs 10% and
342 0.9%), mostly *Haemophilus*. Cluster 3 was separated by a relatively high relative abundance of
343 *Enterobacteriaceae* (41% vs 5% and 8%), mostly *Escherichia*, and a relatively low abundance
344 of *Flavobacteriaceae* (0.1% vs 3% and 6%). The abundance of the *Mycoplasma* genus did not
345 differ among the three clusters.

346 Finally, in colostrum, Cluster 1 was separated from Cluster 2 on the basis of the higher relative
347 abundance of *Moraxellaceae* (51% vs 27%), mostly *Psychrobacter*, and *Flavobacteriaceae* (8%
348 vs 1%), and lower relative abundances of *Enterobacteriaceae* (2% vs 9%), *Clostridiaceae* (1%
349 vs 7%) and *Lachnospiraceae* (0.5% vs 3%) (**Figure 7F and Figure 8C**).

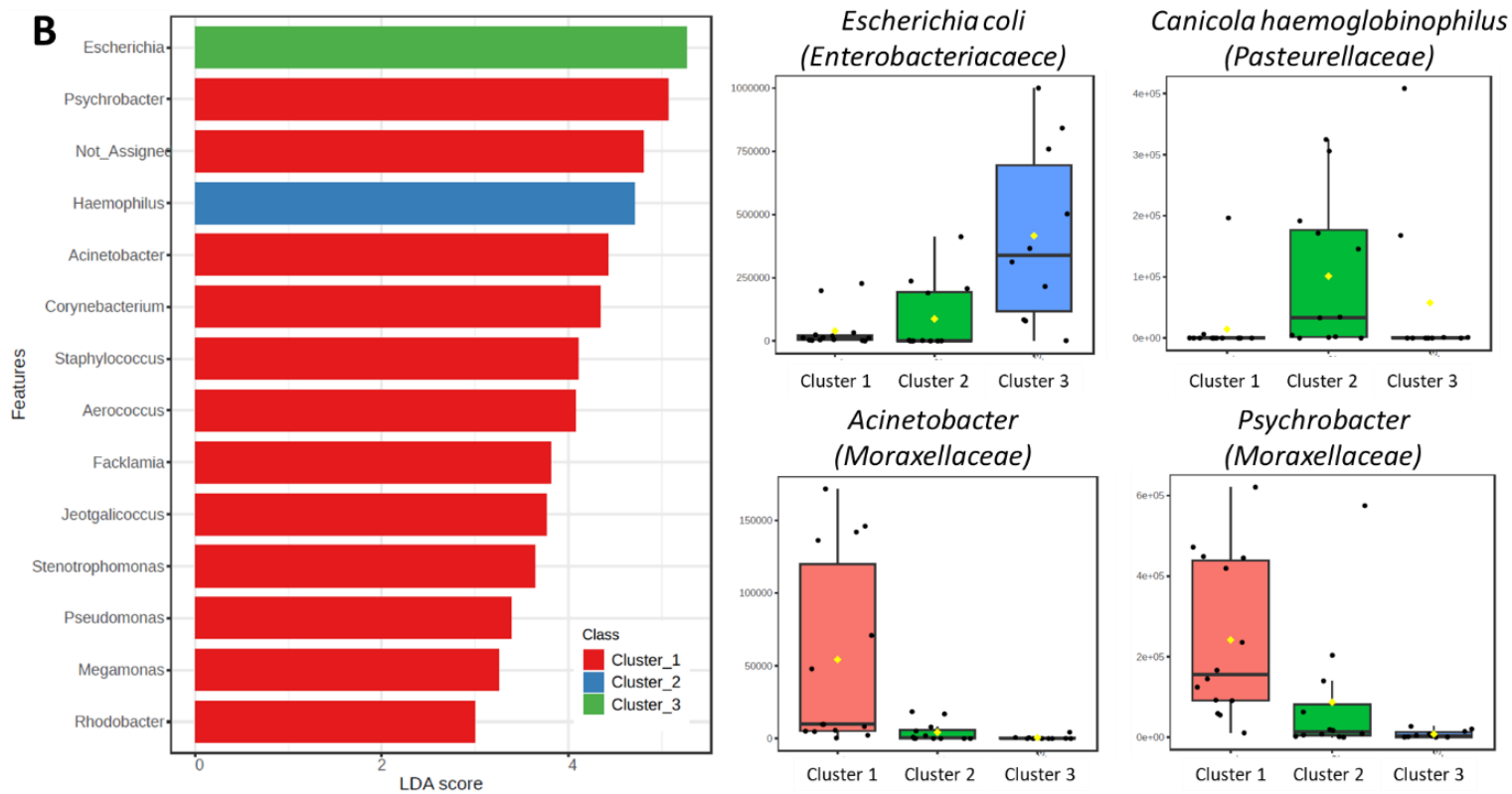
350 All mean relative abundances of the core families of each cluster are compiled in
351 Supplementary **Table S3**.



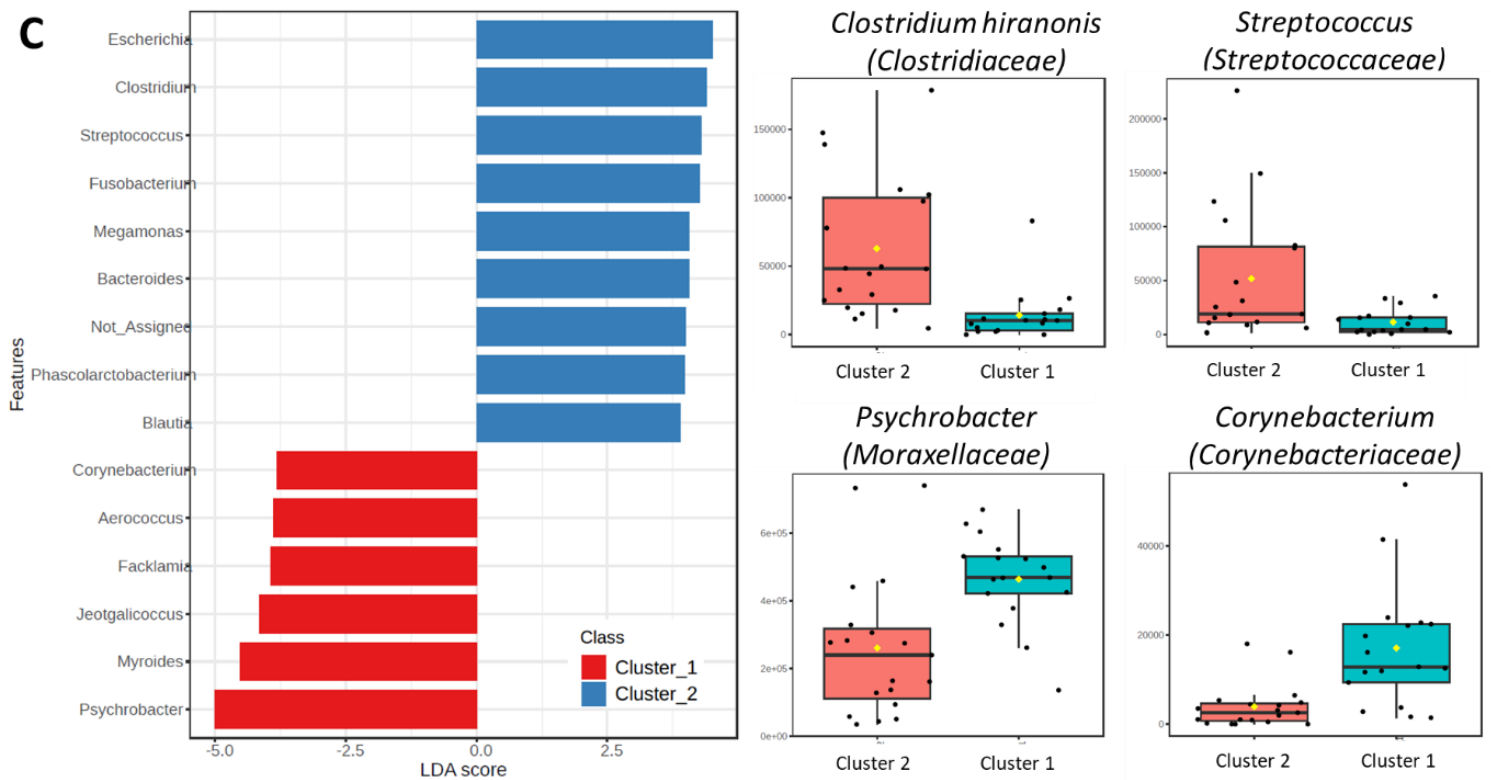
353 **Fig. 7.** Microbiota profiles in determined clusters on different body sites of dams one day after
 354 parturition. Relative abundance of the core families with > 1% relative abundance for each
 355 cluster of the fecal (A), vaginal (B) and colostrum (C) microbiotas and heatmaps of the core
 356 genera of ASVs for the fecal (D), vaginal (E) and colostrum (F) microbiotas.



357



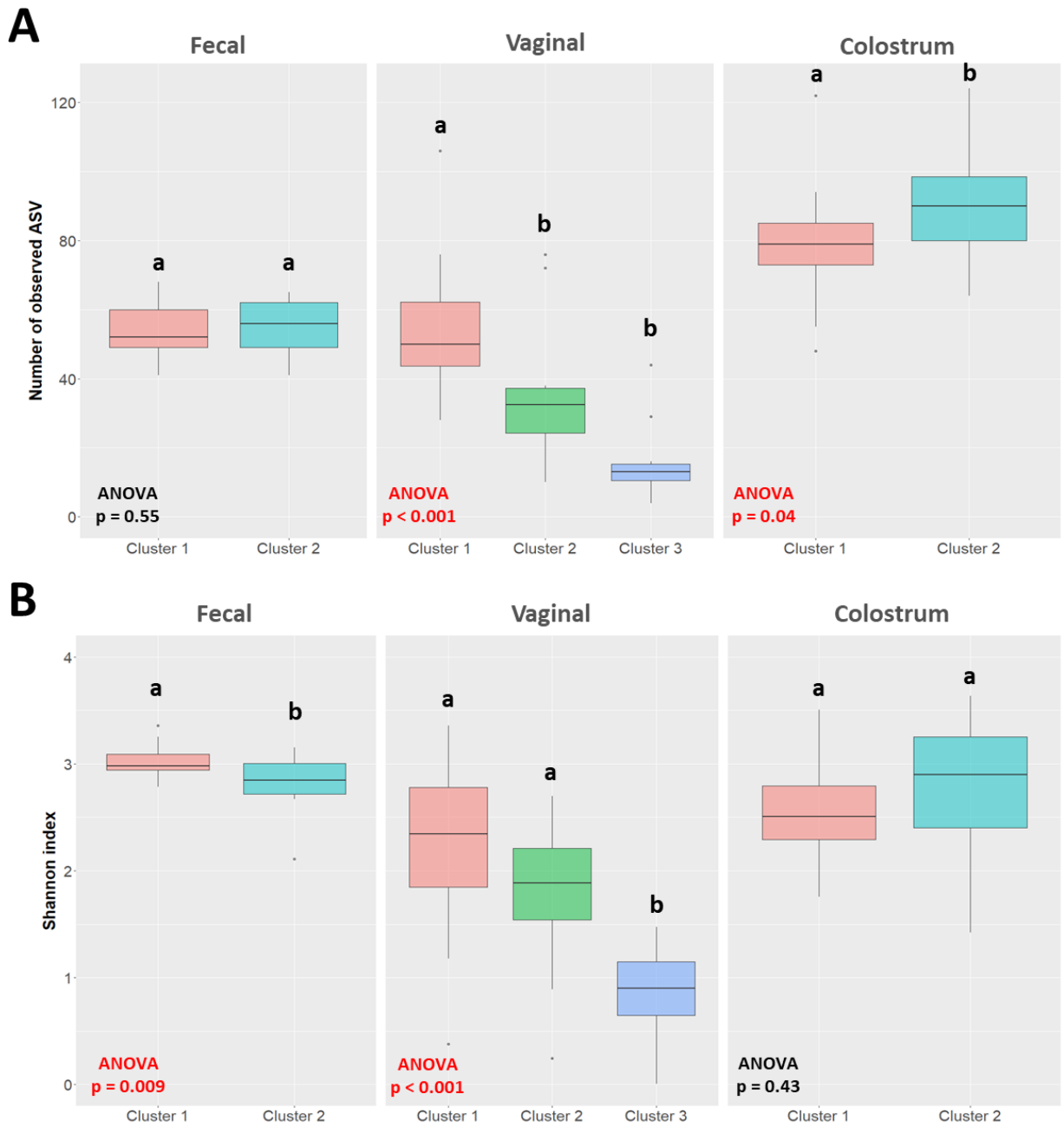
358



360 **Fig. 8.** The main taxon ASVs of the fecal (A), vaginal (B), and colostrum (C) microbiota
 361 significantly distinguishing the observed clusters according to linear discriminant analysis
 362 effect size (LEfSe) on samples obtained from 36 dams one day after parturition.

363

364 *Alpha diversity.* There was a significant difference in the Shannon index between the
 365 two clusters of fecal microbiota, with Cluster 1 having a significantly greater index (**Figure 9**).
 366 For the colostrum microbiota, there was a significant difference between clusters in the
 367 observed ASV only, with Cluster 1 having a lower number of observed ASVs. For the vaginal
 368 microbiota, Cluster 1 had a significantly greater number of observed ASVs than did Clusters 2
 369 and 3, whereas Cluster 3 had a significantly lower Shannon index. All the data are compiled in
 370 Supplementary **Table S4**.



371

372 **Fig. 9.** Bacterial richness of all three microbiota in 36 bitches one day after parturition according
 373 to the defined clusters. Significant differences are identified with Tukey's HSD letters. Boxes
 374 with different letters are significantly different ($p < 0.05$) according to (A) the number of
 375 observed ASVs and (B) the Shannon index.

376

377

378

3.4. Core microbiota clusters and reproductive performance

379

On the basis of the linear models assessing the LBW ratio, GR 0–2 days, stillbirth ratio, and

380

neonatal mortality ratio, with fecal, vaginal, and colostrum microbiotas as the studied factors,

381

only the vaginal microbiota was consistently associated with reproductive outcomes, except for

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the LBW ratio (**Table 3**).

383

384

Table 3. Associations between fecal, vaginal and colostrum microbial clusters one day after

385

parturition and neonatal outcomes: p values of the four linear models. Significant p values (<

386

0.05) and tendencies (<0.1) are highlighted in bold.

Maternal microbiota	Ratio of low-birth-weights	Growth rate 0-2 days	Ratio of stillbirth	Ratio of mortality 0-21 days
Fecal microbiota	0.364	0.670	0.709	0.929
Vaginal microbiota	0.638	0.059	0.005	0.018
Colostrum microbiota	0.294	0.189	0.598	0.622

387

388

Litter GR over the first 2 days of life tended to be 2 times greater in puppies from Cluster 1

389

bitches than in those from Clusters 2 and 3 (**Table 4, Figure 10A**). In contrast, the interlitter

390

ratio of stillbirth and neonatal mortality was approximately twice as high in Cluster 2 litters and

391

over 4 times greater in Cluster 3 litters than in Cluster 1 litters (Table 4; **Figure 10B and 10C**).

392

No significant difference was observed in neonatal outcomes among dams with different

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microbial fecal or colostrum clusters.

394

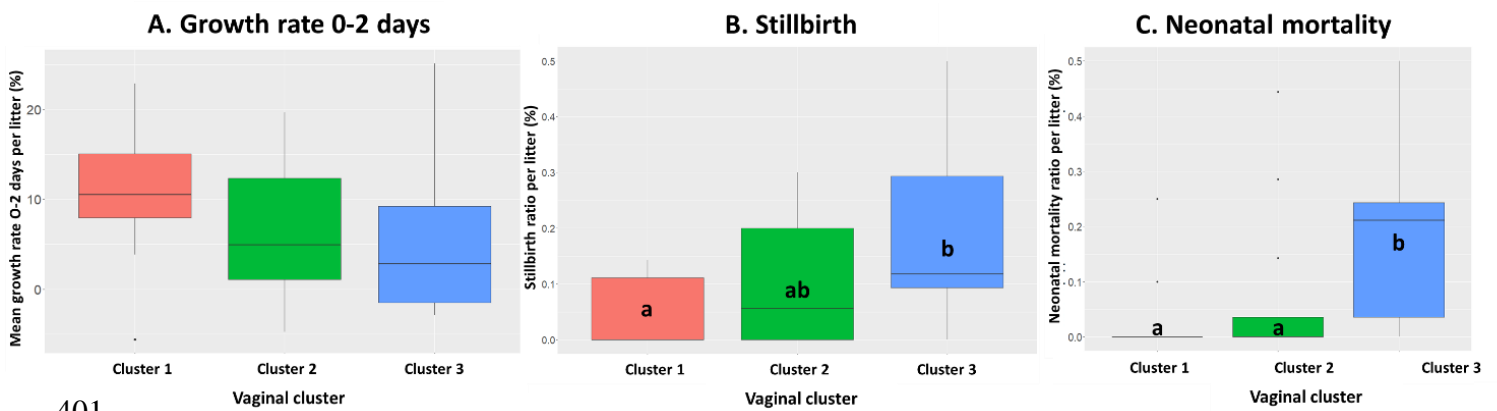
395

396 **Table 4.** Characteristics of the neonatal outcomes of 36 dams according to their fecal, vaginal
 397 or colostral microbiota clusters identified via Dirichlet Multinomial Mixtures (DMM).
 398 Significant differences ($p < 0.05$) and tendencies (< 0.1) are highlighted in bold.

Cluster	Fecal		Vaginal			Colostrum	
	Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 3	Cluster 1	Cluster 2
Number of dams	23	13	14	12	10	17	19
Ratio of LBW (mean±SD ; %)	24.5±30.1	17.8±27.6	26.4±29.8	19.4±29.7	19.2±29.3	27.3±32.9	17.4±24.9
GR 0-2 days (mean±SD; %)	7.8±8.7	8.8±7.4	11.3±7.3	6.4±7.3	5.8±9.5	6.9±6.3	9.2±9.6
Ratio of stillbirth (mean±SD; %)	9.8±10.9	10.5±15.3	4.3±6.0	9.9±11.4	18.3±16.2	10.0±11.0	10.1±13.9
Ratio of neonatal mortality (mean±SD; %)	9.3±13.6	8.9±15.2	4.3±9.2	7.3±14.6	18.2±15.7	9.1±14.2	9.2±14.2

399 *LBW – low birth weight; GR – growth rate*

400



401

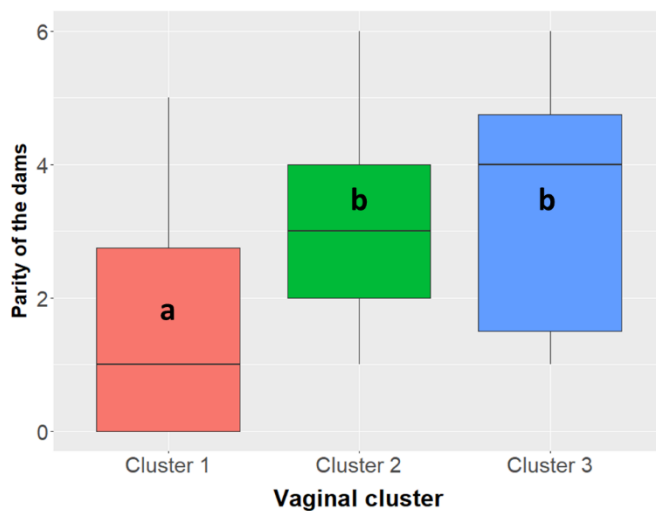
402 **Fig. 10.** Neonatal outcomes ((A) growth rate 0–2 days; (B) stillbirth ratio; (C) neonatal
 403 mortality ratio) of 36 litters according to the vaginal microbial profile of their dam. Significant
 404 differences are identified with Tukey’s HSD letters. Boxes with different letters are
 405 significantly different ($p < 0.05$).

406

407

408 3.5. Factors associated with variation in the core microbiota
409 clusters

410 Among the three studied microbiota, only the maternal vaginal microbial profile was
411 significantly associated with the reproductive performance of the dam (stillbirth and neonatal
412 mortality), and a trend was observed for the GR at 0–2 days. Thus, the vaginal microbial profile
413 was further explored. The results of the final multivariate logistic regression model on the
414 vaginal microbial profile revealed a significant association with parity (0.049) but not with
415 breed or litter size ($p > 0.05$ for all the parameters). Bitches from Cluster 2 and Cluster 3 had a
416 mean parity of 3 and 4, respectively, whereas the mean parity in Cluster 1 was 1 (**Figure 11**).
417 More specifically, all primiparous dams belonged to this cluster. However, no significant
418 differences between nulliparous ($n=5$) and multiparous ($n=31$) dams were found via LEfSe
419 analysis.



420 **Fig. 11.** Parity of the 36 followed bitches according to their vaginal microbial profile.
421 Significant differences are identified with Tukey's HSD letters. Boxes with different letters are
422 significantly different ($p < 0.05$).
423

424

4. Discussion

425

4.1. Limitations of the study

426

To the best of our knowledge, this study is one of the few in the literature and the first in canine

427

species to provide results concerning the composition of the maternal microbiota of the vagina,

428

feces and colostrum concomitantly at parturition and how they are associated with maternal

429

factors and early-life outcomes. While these results are promising for understanding how

430

maternal factors influence the microbiota at parturition, several elements need to be considered.

431

A total of 36 bitches and their 284 puppies were included in our study. Although this is an

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important number of animals, the number of animals per group after clustering has decreased

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to a more limited population, lowering our statistical power in models, specifically those

434

associated with maternal factors. On the basis of previous studies, factors such as type of

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parturition, diet and breed have a strong influence on microbial communities and could

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overshadow the factors investigated in the present study¹⁶; indeed, the included dams originated

437

from one kennel with the same nutrition, kennel conditions and management.

438

4.2. Importance of the maternal microbiota for newborns

439

Early colonization of the gut and development of the intestinal microbiota are crucial steps for

440

newborns, which can shape many vital functions in the short and long term ^{37,38}. More

441

specifically, the expression of some genes in newborns is influenced by maternal microbial

442

status, even before birth, affecting the later development of the immune system,

443

neurophysiology, or energy metabolism³⁹. It is now widely known that maternal factors and the

444

microbiota play crucial roles in the establishment of bacterial communities in newborns in the

445

constant presence of their mothers during parturition^{13,40,41}. Among the maternal microbiota,

446

the fecal microbiota is by far the most studied, although many recent studies have shown that

447 the vaginal and colostrum microbiota play crucial roles in shaping the microbiota of newborns
448 ^{5,42}. Each of these microbiotas intervenes at different times in the development of the newborn's
449 microbiota, with different effects. In humans, the vaginal environment is the first in contact
450 with the newborn, even before exposure to the external environment; hence, it is the one shaping
451 initial bacterial colonization⁴³. It is then expanded by the colostrum microbiota, which is rapidly
452 ingested by the newborn, shaping its gut microbiota⁴⁴. Finally, animals at birth might come into
453 contact with their mother's feces, which induces another vertical transfer of bacteria from the
454 mother to the offspring. Thus, to obtain a full picture of the association between the maternal
455 microbiota and offspring health, several microbiota have been studied rather than one.

456 4.3. Composition of the fecal microbiota

457 First, some important differences between the fecal, vaginal, and colostrum microbiotas were
458 demonstrated in our study. Compared with those of the other two microbiotas, the fecal
459 microbiota at parturition had the most uniform composition among the dams, with no clear
460 dominance of any specific taxa. The fecal microbiota has been extensively studied in recent
461 decades in dogs, and the composition presented in our study did not differ much from previously
462 published data ⁴⁵⁻⁴⁸. In some taxa, such as *Lactobacillus*, the relative abundance in the fecal
463 microbiota was very low (0.9% of total abundance) compared with that in nonpregnant animals.
464 Indeed, in previous results concerning the evolution of the fecal microbiota during pregnancy,
465 we reported that the abundance of lactobacilli decreased to almost zero at parturition⁴⁹. One
466 hypothesis for this disappearance was that lactobacilli translocate from the gut to the mammary
467 glands; this phenomenon is known as the "entero-mammary pathway"⁵⁰. Moreover, the increase
468 in estrogen levels during pregnancy has been linked to a decrease in vaginal pH, fostering the
469 growth of lactobacilli and reducing pathogen colonization⁵¹. However, these hypotheses, as
470 well as the role of *Lactobacillus* in newborn dogs, remain to be investigated. More generally,

471 other studies are needed to understand the role of the maternal fecal microbiota in newborn
472 seeding.

473 Although the same diet was provided to all the dams in this study, two bacterial profiles were
474 distinguished in the fecal microbiota *postpartum*. The first profile (Cluster 1) was characterized
475 by a relatively high abundance of *Bacteroides* and *Clostridium* and mostly *Clostridium*
476 *hiranonis*. This species affects secondary bile acid production and inhibits the growth of
477 pathogenic bacteria such as *C. perfringens*^{52,53}. On the other hand, *Corynebacterium*, which is
478 overrepresented in Cluster 2, is associated with a lower risk of food-responsive diarrhea or IBD
479 in dogs⁵⁴. Nevertheless, no relationship between the fecal microbiome and neonatal outcomes
480 was observed in this study.

481 4.4. Composition of the colostrum microbiota

482 Similar to that of the fecal microbiota, very limited interindividual diversity was observed
483 between the core colostrum microbiota of dams. The source of the colostrum microbiota is still
484 unclear, and many studies suggest that some of the bacterial communities are derived from
485 contamination from the skin, the external environment and the oral microbiota of the
486 offspring⁵⁵. Nevertheless, *Proteobacteria* was the dominant phylum in the colostrum
487 microbiota, as observed in women's milk *postpartum*⁵⁶. This phylum was represented mainly
488 by the *Moraxellaceae* family, which had the highest relative abundance among all the bacterial
489 families. Interestingly, in a previous study on puppies, the *Moraxellaceae* family had the highest
490 mean relative abundance in puppy feces at birth¹⁷. This finding reinforces the relationship
491 between the maternal colostrum microbiota and the initial development of the intestinal
492 microbiota of the newborn. Indeed, nearly 70% of stool bacteria originate from maternal milk
493 in newborn human babies younger than 6 days of age⁵⁶. However, this hypothesis needs further
494 investigation in dogs.

495 Two clusters were identified in the colostrum microbiota of the dog, with *Enterobacteriaceae*
496 and *Clostridiaceae* dominating in one cluster and *Moraxellaceae* and *Lachnospiraceae* in the
497 second cluster. To date, it remains impossible to determine which colostrum microbial profile
498 might be more beneficial for newborn health, as no relationship was observed between these
499 two parameters in the present study. Further investigations are needed to address the presence
500 of these taxa in canine milk, as well as their potential function in the newborn gut⁵⁷.

501 4.5. Composition of the vaginal microbiota and its associations 502 with neonatal outcomes

503 4.5.1. General composition

504 Similar to colostrum, *Proteobacteria* was the dominant phylum in the vaginal microbiota, with
505 *Enterobacteriaceae*, *Moraxellaceae*, *Streptococcaceae*, and *Pasteurellaceae* being the key
506 bacterial taxa observed *postpartum* in our population. These results are consistent with previous
507 studies in which these taxa were also found to be the most abundant in the vaginal samples of
508 canine females^{58–60}. The vaginal microbiota showed considerable interindividual diversity, with
509 some dams having a microbiota composed almost entirely of one main bacterial family, whereas
510 others had a more diverse vaginal microbiota.

511 Three distinct profiles emerged from the clustering of the vaginal microbiota in our study. The
512 first cluster (Cluster 1) consisted of dams dominated by *Moraxellaceae*, the second cluster
513 (Cluster 2) had dams with a relatively high relative abundance of *Pasteurellaceae*, and the third
514 cluster (Cluster 3) had a relatively high relative abundance of *Enterobacteriaceae*. Both this
515 high interindividual variation between dams and the composition of the vaginal microbiota are
516 quite different from what can be seen in humans. Indeed, the core vaginal microbiota of humans
517 around birth is dominated by *Lactobacillus*, which often accounts for more than half of the

518 relative abundance, followed by *Enterococcus* and *Staphylococcus*⁶¹. The higher abundance of
519 *Lactobacillus* in the human vaginal microbiota may be due to a lower pH, typically below 4.5,
520 whereas the pH of the canine vagina ranges from 5.0 to 8.1⁵⁹. Lactic acid bacteria can survive
521 in low-pH environments, allowing them to dominate areas where other bacteria cannot survive,
522 such as the human vagina⁶². The high pH in dogs' vaginas leads to greater competition and
523 hence a lower abundance of *Lactobacillus* at the expense of *Proteobacteria*, which grow at
524 higher pH.

525 4.5.2. Association with mortality in puppies

526 One of the most important findings of this study is the association between the vaginal
527 microbiota in dams and the neonatal outcomes of their puppies. The vaginal microbiota in
528 women, cows and sows has been shown to influence the risk of prematurity, morbidity and
529 mortality in newborns⁶³⁻⁶⁵, but this study is one of the first to describe such results in canine
530 species via sequence-based bacterial analysis. Indeed, one of the vaginal microbial profiles
531 (Cluster 3) was significantly associated with a higher risk of stillbirth and neonatal mortality in
532 puppies, and a trend toward a lower growth rate over the first two days of life was observed.

533 In parallel with the increased stillbirth ratio, Cluster 3 dams also presented lower vaginal
534 bacterial richness than the other clusters did, suggesting that both are significantly correlated.
535 This is a surprising result, as elevated bacterial diversity was observed in human vaginal
536 samples with an increased risk of premature birth⁶⁶.

537 *E. coli* was found to be the most abundant *Enterobacteriaceae* in Cluster 3 dams. This
538 bacterium is considered opportunistic⁶⁷⁻⁶⁹ and is the most commonly isolated bacterium from
539 the vagina of healthy female dogs. However, in the case of neonatal mortality, an abundant pure
540 culture of *E. coli* from a vaginal sample from a dam is interpreted as a likely cause of neonatal
541 death⁷⁰. Indeed, *E. coli* is also most often isolated from internal organs of puppies that die

542 during the neonatal period^{41,71-73}. Moreover, over 80% of cases of neonatal septicaemia in dogs
543 occur within the first two days of life, strongly suggesting contamination during late gestation
544 or birth via the uterus or vagina of the dam⁷⁴. In humans, ascending bacterial infection with *E.*
545 *coli* leading to chorio-amnionitis before or after membrane rupture is usually the most common
546 infectious cause of stillbirth⁷⁵. Our results seem to confirm the potential pathological role of
547 this bacteria, as we have shown that, compared with dams with a vaginal microbiota rich in
548 bacteria from the *Moraxellaceae* family, those with a vaginal microbiota dominated by *E. coli*
549 present a significantly greater risk of stillbirth and neonatal mortality.

550 Among the most abundant bacteria of Cluster 2 bitches, we identified bacteria from the
551 *Pasteurellaceae* family, more precisely, *Canicola haemoglobinophilus*. Interestingly, these
552 bacteria were found to be associated with urogenital tract infections in dogs⁷⁶, but they were
553 also isolated from dead newborn dogs⁷⁷. Thus, one could hypothesize that the vaginal
554 microbiota of bitches from Cluster 2 could be associated with neonatal mortality. However, our
555 data do not allow us to confirm this hypothesis.

556 In future studies, data on a large number of animals with maternal vaginal bacteria and bacteria
557 isolated from stillborn puppies or puppies dying during the neonatal period are needed to
558 identify the existence of any specific pathogens responsible for neonatal losses in the dog.

559 4.6. Vaginal microbiota and parity

560 Parity was the only maternal factor correlated with the vaginal microbial profile in female dogs.
561 Bitches from Cluster 3 presented lower bacterial richness and evenness associated with higher
562 parity. As parity in this study increased proportionally with the age of the female, the alpha
563 diversity of the canine vagina may have decreased with age, similar to what can be observed in
564 the feces⁷⁸. While similar results have been reported in the vaginal microbiota of baboons⁷⁹,
565 opposite results have been reported in human and canine populations, where vaginal bacterial

566 diversity increases with age^{80,81}. As Cluster 3 dams presented a greater risk of mortality in their
567 puppies together with higher parity, one could hypothesize that the vaginal microbiota
568 undergoes some significant changes with age and/or parity of the host, leading to potential
569 vaginal dysbiosis.

570 **5. Conclusions**

571 The present results demonstrated the association of puppy health with the maternal microbiota,
572 particularly the vaginal microbiota. Low vaginal microbiota richness was associated with an
573 increased risk of stillbirth. A specific vaginal microbial profile was identified in this study and
574 found to be associated with an increased risk of stillbirth and neonatal mortality in newborns.
575 A promising avenue for future research in canine species lies in understanding how hormonal
576 changes affect the physicochemical parameters of environments such as the gut, vaginal
577 mucosa, and colostrum. Hormones such as estrogen, progesterone, and cortisol alter pH levels,
578 mucosal secretions, and immune functions, which in turn create dynamic niches for microbial
579 communities. The practical implications of this research are vast. By understanding the
580 hormonal modulation of these microbial ecosystems and their interactions (called together
581 holomicrobiome), interventions could be developed to optimize the maternal microbiota during
582 pregnancy, potentially reducing the risk of complications such as stillbirth and infections.
583 Moreover, this knowledge could inform the development of probiotic, prebiotic, or dietary
584 strategies to support a healthy holomicrobiome, with cascading benefits for both maternal and
585 neonatal health.

586 **Supplementary materials**587 **Supplementary Table S1:** Analysis of the nutritional values of the feeds used in the study.

Parameter	Feed	
	Diet 1	Diet 2
Moisture, %	6.3	5.7
Crude ash, % as fed	9.3	8.7
Crude protein, % as fed	28.4	30.8
Ether extract, % as fed	16.8	15.1
Crude fiber, % as fed	<2	2.7
Carbohydrates, %	37.7	37.0
Vitamin E, mg/kg as fed	114	124
Vitamin A, IU as fed	7 330	7 670
Vitamin D, IU as fed	1 000	1 080
Metabolizable energy, kcal/100 g	408.6	385.8

588

589

590 **Supplementary Table S2:** Thresholds of birth weight used to create the four quartiles for each breed and the number of birth weights used to
 591 calculate the threshold:

Birth weight category	LBW	NBW		HBW	Number of birth weight values used to calculate thresholds
Breed	Q1	Q2	Q3	Q4	
Australian Shepherd	< 325 g	325-378 g	378-426 g	> 426 g	663
White Swiss Shepherd	< 389 g	389-438 g	438-498 g	> 498 g	249
Golden Retriever	< 336 g	336-382 g	382-425 g	> 425 g	1094
Labrador Retriever	< 364 g	364-410 g	410-455 g	> 455 g	713
German Shepherd	< 426 g	426-488 g	488-541 g	> 541 g	153
Bernese Mountain Dog	< 406 g	406-453 g	453-499 g	> 499 g	489
Boxer	< 393 g	393-438 g	438-487 g	> 487 g	269
Dalmatian	< 296 g	296-344 g	344-396 g	> 396 g	175
Pyrenean Mountain Dog	< 464 g	464-508 g	508-566 g	> 566 g	130

592

593

Fecal			Vaginal				Colostrum		
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	Cluster 3		Cluster 1	Cluster 2
<i>Moraxellaceae</i>	0.56	4.14	<i>Moraxellaceae</i>	27.78	10.10	0.89	<i>Moraxellaceae</i>	51.06	27.43
<i>Enterobacteriaceae</i>	2.47	1.00	<i>Enterobacteriaceae</i>	5.08	8.33	41.20	<i>Enterobacteriaceae</i>	1.91	8.91
<i>Streptococcaceae</i>	3.40	0.54	<i>Streptococcaceae</i>	11.67	10.25	18.64	<i>Streptococcaceae</i>	1.13	5.14
<i>Clostridiaceae</i>	8.64	2.52	<i>Clostridiaceae</i>	0.94	9.00	0.14	<i>Clostridiaceae</i>	1.40	6.51
<i>Fusobacteriaceae</i>	14.25	12.13	<i>Fusobacteriaceae</i>	0.47	9.49	13.06	<i>Fusobacteriaceae</i>	2.37	6.10
<i>Bacteroidaceae</i>	14.37	8.97	<i>Mycoplasmataceae</i>	3.30	3.52	10.25	<i>Bacteroidaceae</i>	0.44	2.68
<i>Actinomycetaceae</i>	0.31	2.60	<i>Flavobacteriaceae</i>	3.15	6.11	0.09	<i>Flavobacteriaceae</i>	7.91	1.42
<i>Prevotellaceae</i>	11.16	5.18	<i>Staphylococcaceae</i>	8.65	2.64	0.39	<i>Staphylococcaceae</i>	8.75	11.08
<i>Lachnospiraceae</i>	6.75	2.23	<i>Weeksellaceae</i>	8.24	1.10	0.00	<i>Lachnospiraceae</i>	0.46	3.29
<i>Peptostreptococcaceae</i>	0.87	2.36	<i>Pasteurellaceae</i>	10.26	23.02	9.35	<i>Pasteurellaceae</i>	0.29	2.96
<i>Corynebacteriaceae</i>	7.38	26.25	<i>Corynebacteriaceae</i>	4.00	0.38	0.79	<i>Morganellaceae</i>	1.07	1.40
<i>Peptoniphilaceae</i>	0.91	7.60	<i>Aerococcaceae</i>	3.68	1.75	0.01	<i>Aerococcaceae</i>	7.53	4.00
<i>Selenomonadaceae</i>	2.82	0.94					<i>Selenomonadaceae</i>	0.19	2.45
<i>Acidaminococcaceae</i>	5.35	2.24					<i>Acidaminococcaceae</i>	0.24	2.10
<i>Succinivibrionaceae</i>	5.33	2.00					<i>Lactobacillaceae</i>	0.55	1.61
<i>Porphyromonadaceae</i>	0.46	8.97							
<i>Oscillospiraceae</i>	4.60	1.77							

595

596 **Supplementary Table S4:** Mean number (\pm SD) of observed ASVs and the Shannon index of each dam for each cluster of each microbiota site.

	Cluster	Mean observed ASV	Mean Shannon index
Fecal	Cluster 1	53.74 \pm 7.72	3.01 \pm 0.14
	Cluster 2	55.31 \pm 7.23	2.82 \pm 0.27
Vaginal	Cluster 1	55.00 \pm 19.74	2.24 \pm 0.84
	Cluster 2	35.00 \pm 20.55	1.79 \pm 0.75
	Cluster 3	15.90 \pm 12.00	0.86 \pm 0.48
Colostrum	Cluster 1	78.88 \pm 16.00	2.58 \pm 0.45
	Cluster 2	90.37 \pm 16.81	2.73 \pm 0.70

597

598 **6. Abbreviations**

599 **ASV:** Amplicon sequence variant

600 **DMM:** Dirichlet Multinomial Mixtures

601 **FS:** Fecal Score

602 **GIT:** Gastrointestinal tract

603 **GWG:** Gestational Weight Gain

604 **LBW:** Low birth weight

605 **GR:** Growth ratio

606 **7. Declarations**

607 7.1. Ethics approval

608 The animal study was reviewed and approved by the local ethical committee (Comité d'Éthique
609 en Expérimentation Animale, Science et Santé Animale n°115; reference number: SSA_2020-
610 004, Toulouse, France). All applicable guidelines for the care and use of animals were followed.
611 Written informed consent was obtained from the kennel owner for his participation in this study.

612 7.2. Data availability

613 All the data generated or analyzed during this study are included in this published article [and
614 its supplementary information files].

615 7.3. Conflict of interest

616 E. A. and A. R. were employed by the company Lallemand SAS.

617 All the authors declare that the research was conducted in the absence of any commercial or
618 financial relationships that could be construed as potential conflicts of interest.

619 7.4. Funding

620 The study was co-founded by Occitania Region (La region Occitanie Pyrénées – Méditerranée,
621 France) and Lallemand SAS. The employees of Lallemand SAS have participated in the study
622 design, sample analyses and manuscript writing.

623 7.5. Author Contributions

624 S.C., H.M., E.A. and Q.G. designed the study and protocols. Q.G. and N.R. collected the
625 samples. Q.G. performed the DNA extraction with the supervision of E.A. A.R. and Q.G.
626 performed the DNA-based and statistical analyses. Q.G., S.C., H.M. and E.A. participated in
627 the investigation and interpretation of the results. Q.G. and H.M. drafted the manuscript,
628 designed the figures and wrote the final manuscript after reviewing. H.M., S.C. and E.A.
629 reviewed and contributed to the writing of the final manuscript.

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637

8. References

- 639 1. Lynch, S. V. & Pedersen, O. The Human Intestinal Microbiome in Health and Disease. *N.*
640 *Engl. J. Med.* **375**, 2369–2379 (2016).
- 641 2. Blake, A. B. & Suchodolski, J. S. Importance of gut microbiota for the health and disease
642 of dogs and cats. *Anim. Front.* **6**, 37–42 (2016).
- 643 3. Yoo, J. Y., Groer, M., Dutra, S. V. O., Sarkar, A. & McSkimming, D. I. Gut Microbiota
644 and Immune System Interactions. *Microorganisms* **8**, 1587 (2020).
- 645 4. Chee, W. J. Y., Chew, S. Y. & Than, L. T. L. Vaginal microbiota and the potential of
646 Lactobacillus derivatives in maintaining vaginal health. *Microb. Cell Factories* **19**, 203
647 (2020).
- 648 5. Toscano, M. *et al.* Impact of delivery mode on the colostrum microbiota composition. *BMC*
649 *Microbiol.* **17**, 205 (2017).
- 650 6. France, M., Alizadeh, M., Brown, S., Ma, B. & Ravel, J. Towards a deeper understanding
651 of the vaginal microbiota. *Nat. Microbiol.* **7**, 367–378 (2022).
- 652 7. Reyman, M. *et al.* Impact of delivery mode-associated gut microbiota dynamics on health
653 in the first year of life. *Nat. Commun.* **10**, 4997 (2019).
- 654 8. Saturio, S. *et al.* Role of Bifidobacteria on Infant Health. *Microorganisms* **9**, 2415 (2021).
- 655 9. Sinha, T., Brushett, S., Prins, J. & Zhernakova, A. The maternal gut microbiome during
656 pregnancy and its role in maternal and infant health. *Curr. Opin. Microbiol.* **74**, 102309
657 (2023).
- 658 10. Su, Y. *et al.* Effect of exposure to antibiotics on the gut microbiome and biochemical
659 indexes of pregnant women. *BMJ Open Diabetes Res. Care* **9**, e002321 (2021).
- 660 11. Stanislawski, M. A. *et al.* Pre-pregnancy weight, gestational weight gain, and the gut
661 microbiota of mothers and their infants. *Microbiome* **5**, 113 (2017).
- 662 12. Sanidad, K. Z. *et al.* Maternal gut microbiome-induced IgG regulates neonatal gut
663 microbiome and immunity. *Sci. Immunol.* **7**, eabh3816 (2022).
- 664 13. Funkhouser, L. J. & Bordenstein, S. R. Mom Knows Best: The Universality of Maternal
665 Microbial Transmission. *PLOS Biol.* **11**, e1001631 (2013).
- 666 14. Chastant-Maillard, S. *et al.* Reproductive performance and pre-weaning mortality:
667 Preliminary analysis of 27,221 purebred female dogs and 204,537 puppies in France.
668 *Reprod. Domest. Anim.* **52**, 158–162 (2017).
- 669 15. Garrigues, Q., Apper, E., Chastant, S. & Mila, H. Gut microbiota development in the
670 growing dog: A dynamic process influenced by maternal, environmental and host factors.
671 *Front. Vet. Sci.* **9**, (2022).
- 672 16. Zakošek Pipan, M., Kajdič, L., Kalin, A., Plavec, T. & Zdovc, I. Do newborn puppies have
673 their own microbiota at birth? Influence of type of birth on newborn puppy microbiota.
674 *Theriogenology* **152**, 18–28 (2020).
- 675 17. Garrigues, Q. *et al.* Composition and evolution of the gut microbiota of growing puppies is
676 impacted by their birth weight. Preprint at <https://doi.org/10.21203/rs.3.rs-2604924/v1>
677 (2023).
- 678 18. Laflamme, D. Development and validation of a body condition score system for dogs.
679 *Canine Pract.* **22**, 10–15 (1997).
- 680 19. Royal Canin. Royal Canin Faecal Score Guide. [Online]
681 [https://vetportal.royalcanin.co.uk/wp-](https://vetportal.royalcanin.co.uk/wp-content/uploads/2020/05/FINAL_Faecal_Score_Guide_digital_version.pdf)
682 [content/uploads/2020/05/FINAL_Faecal_Score_Guide_digital_version.pdf](https://vetportal.royalcanin.co.uk/wp-content/uploads/2020/05/FINAL_Faecal_Score_Guide_digital_version.pdf).
- 683 20. Mila, H., Grellet, A., Feugier, A. & Chastant-Maillard, S. Differential impact of birth
684 weight and early growth on neonatal mortality in puppies. *J. Anim. Sci.* **93**, 4436–4442
685 (2015).

- 686 21. Mugnier, A. *et al.* Birth weight as a risk factor for neonatal mortality: Breed-specific
687 approach to identify at-risk puppies. *Prev. Vet. Med.* **171**, 104746 (2019).
- 688 22. Derakhshani, H., Plaizier, J. C., De Buck, J., Barkema, H. W. & Khafipour, E. Association
689 of bovine major histocompatibility complex (BoLA) gene polymorphism with colostrum
690 and milk microbiota of dairy cows during the first week of lactation. *Microbiome* **6**, 203
691 (2018).
- 692 23. Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. Profiling of complex microbial
693 populations by denaturing gradient gel electrophoresis analysis of polymerase chain
694 reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700
695 (1993).
- 696 24. Caporaso, J. G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions of
697 sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.* **108 Suppl 1**, 4516–4522 (2011).
- 698 25. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon
699 data. *Nat. Methods* **13**, 581–583 (2016).
- 700 26. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science
701 using QIIME 2. *Nat. Biotechnol.* **37**, 852–857 (2019).
- 702 27. Katoh, K., Misawa, K., Kuma, K. & Miyata, T. MAFFT: a novel method for rapid multiple
703 sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **30**, 3059–3066
704 (2002).
- 705 28. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2--approximately maximum-likelihood
706 trees for large alignments. *PLOS ONE* **5**, e9490 (2010).
- 707 29. Bokulich, N. A. *et al.* q2-longitudinal: Longitudinal and Paired-Sample Analyses of
708 Microbiome Data. *mSystems* **3**, e00219-18 (2018).
- 709 30. Dhariwal, A. *et al.* MicrobiomeAnalyst: a web-based tool for comprehensive statistical,
710 visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **45**, W180–W188 (2017).
- 711 31. Palarea-Albaladejo, J. & Martín-Fernández, J. A. zCompositions — R package for
712 multivariate imputation of left-censored data under a compositional approach. *Chemom.*
713 *Intell. Lab. Syst.* **143**, 85–96 (2015).
- 714 32. Oksanen, J. *et al.* vegan: Community Ecology Package. (2022).
- 715 33. Shannon, C. E. & Weaver, W. A mathematical theory of communication. *Univ. Ill. Press*
716 *Urbana* **27**, 379–423 (1949).
- 717 34. McMurdie, P. J. & Holmes, S. phyloseq: An R Package for Reproducible Interactive
718 Analysis and Graphics of Microbiome Census Data. *PLOS ONE* **8**, e61217 (2013).
- 719 35. Astudillo-García, C. *et al.* Evaluating the core microbiota in complex communities: A
720 systematic investigation. *Environ. Microbiol.* **19**, 1450–1462 (2017).
- 721 36. Holmes, I., Harris, K. & Quince, C. Dirichlet Multinomial Mixtures: Generative Models
722 for Microbial Metagenomics. *PLOS ONE* **7**, e30126 (2012).
- 723 37. Nowland, T. L., Kirkwood, R. N. & Pluske, J. R. Review: Can early-life establishment of
724 the piglet intestinal microbiota influence production outcomes? *Animal* 100368 (2021)
725 doi:10.1016/j.animal.2021.100368.
- 726 38. Adlerberth, I. & Wold, A. Establishment of the gut microbiota in Western infants. *Acta*
727 *Paediatr.* **98**, 229–238 (2009).
- 728 39. Husso, A. *et al.* Impacts of maternal microbiota and microbial metabolites on fetal intestine,
729 brain, and placenta. *BMC Biol.* **21**, 207 (2023).
- 730 40. Jost, T., Lacroix, C., Braegger, C. P., Rochat, F. & Chassard, C. Vertical mother–neonate
731 transfer of maternal gut bacteria via breastfeeding. *Environ. Microbiol.* **16**, 2891–2904
732 (2014).
- 733 41. Bertero, A. *et al.* Meconium microbiota in naturally delivered canine puppies. *BMC Vet.*
734 *Res.* **20**, 363 (2024).

- 735 42. Mortensen, M. S. *et al.* Modeling transfer of vaginal microbiota from mother to infant in
736 early life. *eLife* **10**, e57051 (2021).
- 737 43. Dominguez-Bello, M. G. *et al.* Delivery mode shapes the acquisition and structure of the
738 initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci.* **107**,
739 11971–11975 (2010).
- 740 44. Ge, Y. *et al.* The Maternal Milk Microbiome in Mammals of Different Types and Its
741 Potential Role in the Neonatal Gut Microbiota Composition. *Anim. Open Access J. MDPI*
742 **11**, 3349 (2021).
- 743 45. Guard, B. C. *et al.* Characterization of microbial dysbiosis and metabolomic changes in
744 dogs with acute diarrhea. *PLOS ONE* **10**, e0127259 (2015).
- 745 46. Handl, S., Dowd, S. E., Garcia-Mazcorro, J. F., Steiner, J. M. & Suchodolski, J. S. Massive
746 parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal
747 communities in healthy dogs and cats. *FEMS Microbiol. Ecol.* **76**, 301–310 (2011).
- 748 47. Kubinyi, E., Bel Rhali, S., Sándor, S., Szabó, A. & Felföldi, T. Gut Microbiome
749 Composition is Associated with Age and Memory Performance in Pet Dogs. *Animals* **10**,
750 1488 (2020).
- 751 48. You, I. & Kim, M. J. Comparison of Gut Microbiota of 96 Healthy Dogs by Individual
752 Traits: Breed, Age, and Body Condition Score. *Anim. Open Access J. MDPI* **11**, 2432
753 (2021).
- 754 49. Mercier, F. Étude expérimentale sur les modifications du microbiote intestinal chez la
755 chienne en gestation et en lactation. (Université Paul Sabatier, Toulouse, 2022).
- 756 50. Rodríguez, J. M. The Origin of Human Milk Bacteria: Is There a Bacterial Entero-
757 Mammary Pathway during Late Pregnancy and Lactation?1234. *Adv. Nutr.* **5**, 779–784
758 (2014).
- 759 51. Farage, M. A., Miller, K. W. & Sobel, J. D. Dynamics of the Vaginal Ecosystem—
760 Hormonal Influences. *Infect. Dis. Res. Treat.* **3**, IDRT.S3903 (2010).
- 761 52. Blake, A. B. *et al.* Developmental stages in microbiota, bile acids, and clostridial species in
762 healthy puppies. *J. Vet. Intern. Med.* **34**, 2345–2356 (2020).
- 763 53. Guard, B. C. *et al.* Longitudinal assessment of microbial dysbiosis, fecal unconjugated bile
764 acid concentrations, and disease activity in dogs with steroid-responsive chronic
765 inflammatory enteropathy. *J. Vet. Intern. Med.* **33**, 1295–1305 (2019).
- 766 54. Pilla, R. & Suchodolski, J. S. The Role of the Canine Gut Microbiome and Metabolome in
767 Health and Gastrointestinal Disease. *Front. Vet. Sci.* **6**, (2020).
- 768 55. Moossavi, S. & Azad, M. B. Origins of human milk microbiota: new evidence and arising
769 questions. *Gut Microbes* **12**, 1667722.
- 770 56. Corona-Cervantes, K. *et al.* Human milk microbiota associated with early colonization of
771 the neonatal gut in Mexican newborns. *PeerJ* **8**, e9205 (2020).
- 772 57. Selma-Royo, M. *et al.* Human milk microbiota: what did we learn in the last 20 years?
773 *Microbiome Res. Rep.* **1**, 19 (2022).
- 774 58. Baba, E., Hata, H., Fukata, T. & Arakawa, A. Vaginal and uterine microflora of adult dogs.
775 *Am. J. Vet. Res.* **44**, 606–609 (1983).
- 776 59. Golińska, E. *et al.* The vaginal microflora changes in various stages of the estrous cycle of
777 healthy female dogs and the ones with genital tract infections. *BMC Vet. Res.* **17**, 8 (2021).
- 778 60. Rota, A. *et al.* Effect of sterilization on the canine vaginal microbiota: a pilot study. *BMC*
779 *Vet. Res.* **16**, 455 (2020).
- 780 61. Rasmussen, M. A. *et al.* Ecological succession in the vaginal microbiota during pregnancy
781 and birth. *ISME J.* **14**, 2325–2335 (2020).
- 782 62. Miller, E. A., Beasley, D. E., Dunn, R. R. & Archie, E. A. Lactobacilli Dominance and
783 Vaginal pH: Why Is the Human Vaginal Microbiome Unique? *Front. Microbiol.* **7**, 1936
784 (2016).

- 785 63. Bicalho, M. L. S. *et al.* Dynamics of the microbiota found in the vaginas of dairy cows
786 during the transition period: Associations with uterine diseases and reproductive outcome.
787 *J. Dairy Sci.* **100**, 3043–3058 (2017).
- 788 64. Sanglard, L. P. *et al.* Vaginal microbiota diverges in sows with low and high reproductive
789 performance after porcine reproductive and respiratory syndrome vaccination. *Sci. Rep.* **10**,
790 3046 (2020).
- 791 65. Bayar, E., Bennett, P. R., Chan, D., Sykes, L. & MacIntyre, D. A. The pregnancy
792 microbiome and preterm birth. *Semin. Immunopathol.* **42**, 487–499 (2020).
- 793 66. Baud, A. *et al.* Microbial diversity in the vaginal microbiota and its link to pregnancy
794 outcomes. *Sci. Rep.* **13**, 9061 (2023).
- 795 67. Santos, T. M. A., Gilbert, R. O. & Bicalho, R. C. Metagenomic analysis of the uterine
796 bacterial microbiota in healthy and metritic postpartum dairy cows. *J. Dairy Sci.* **94**, 291–
797 302 (2011).
- 798 68. Sato, Y. Vulvar Abscess Caused by *Bacteroides* sp. Infection in a Female Dog. *Jpn. J. Vet.*
799 *Dermatol.* **15**, 187–190 (2009).
- 800 69. Lanari, M., Sogno Valin, P., Natale, F., Capretti, M. G. & Serra, L. Human milk, a concrete
801 risk for infection? *J. Matern. Fetal Neonatal Med.* **25**, 67–69 (2012).
- 802 70. Agnew, D. Pathology of Perinatal Disorders. *Vet. Clin. North Am. Small Anim. Pract.* **53**,
803 1147–1159 (2023).
- 804 71. Meloni, T. *et al.* A survey on bacterial involvement in neonatal mortality in dogs. *Vet. Ital.*
805 **50**, 293–299 (2014).
- 806 72. Mila, H., Guerard, C. & Raymond-Letron, I. Guidelines for postmortem examination of
807 newborn dogs. *Anim. Health Res. Rev.* **22**, 109–119 (2021).
- 808 73. Münnich, A. & Küchenmeister, U. Causes, diagnosis and therapy of common diseases in
809 neonatal puppies in the first days of life: cornerstones of practical approach. *Reprod.*
810 *Domest. Anim.* **49**, 64–74 (2014).
- 811 74. Nobre Pacifico Pereira, K. H. *et al.* Neonatal sepsis in dogs: Incidence, clinical aspects and
812 mortality. *Theriogenology* **177**, 103–115 (2022).
- 813 75. Cools, P. The role of *Escherichia coli* in reproductive health: state of the art. *Res. Microbiol.*
814 **168**, 892–901 (2017).
- 815 76. Karthikeyan, R. *et al.* Microbiological and molecular detection of Canicola (Haemophilus)
816 haemoglobinophilus from the urine of a dog. *Indian J Anim Health* **62**, 192–198 (2023).
- 817 77. Maclachlan, G. & Hopkins, G. Early death in pups due to Haemophilus
818 haemoglobinophilus (canis) infection. *Vet. Rec.* **103**, 409–410 (1978).
- 819 78. Mizukami, K. *et al.* Age-related analysis of the gut microbiome in a purebred dog colony.
820 *FEMS Microbiol. Lett.* **366**, fnz095 (2019).
- 821 79. Uchihashi, M. *et al.* Influence of age, reproductive cycling status, and menstruation on the
822 vaginal microbiome in baboons (*Papio anubis*). *Am. J. Primatol.* **77**, 563–578 (2015).
- 823 80. Romero, R. *et al.* The Vaginal Microbiota of Pregnant Women Varies with Gestational
824 Age, Maternal Age, and Parity. *Microbiol. Spectr.* **11**, e03429-22 (2023).
- 825 81. Hu, J. *et al.* Dynamics of vaginal microbiome in female beagles at different ages. *Res. Vet.*
826 *Sci.* **149**, 128–135 (2022).
- 827