Animal Microbiome

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

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Abstract

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

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

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

Keywords Dog, Puppy, Microbiota, Parturition, Colostrum, Neonatal health, Mortality

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Background

Currently, the dynamic interaction between microbial communities (collectively known as the microbiota) and their host organisms offers valuable insights into host health. The most extensively studied microbiota is the gut microbiota (and more specifically the fecal), as its role in shaping its host's health has gained increasing evidence [1, 2]. However, many more microbial communities play crucial roles in host health, although they remain less explored. This includes the vaginal and colostrum microbiotas, which have many essential functions within the host (the dam), such as immune regulation, metabolic equilibrium and protection against pathogens [3–5]. Moreover, all of these microbiotas have emerged as central players in reproductive health and performance [5, 6]. In particular, the maternal vaginal and colostrum microbiotas have been shown in humans to be crucial for enhancing the newborn's immune system by promoting the colonization of the intestine by beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, as they induce specific immune stimulation of the intestinal environment through the production of short-chain fatty acids (SCFAs) and interactions with Treg cells [5, 7, 8].

Given the complex topic of the maternal microbiota and its importance during parturition, maternal factors may have crucial interrelationships with the microbial composition. These factors encompass a spectrum of maternal attributes, such as physiological status, consumption of medications, or diet, as observed in humans [9]. For example, pregnant women exposed to antibiotics presented lower abundances of *Actinobacteria* and greater alpha diversity [10], whereas overweight and obese women presented lower maternal alpha diversity and taxonomic differences in the maternal gut microbiota comapred with the controls [11]. Understanding how these maternal factors interact with microbial diversity has the potential to improve the understanding of the relationship between the host's physiological state and the microbial communities that inhabit it.

Although the importance of the maternal microbiota during parturition for neonates' health trajectory has been demonstrated in human medicine [7, 12, 13], it is currently poorly investigated in canine species. Neonatal puppies are inherently vulnerable, with approximately 13% of puppies dying during their first weeks of life [14]. The transition from the protected intrauterine environment to the external world places them at a critical juncture where among other factors, their ability to establish a resilient microbial ecosystem is paramount. In the case of perturbed microbial seeding, as shown with puppies born from C-sections, less diverse microbiota and delayed growth were observed [15, 16]. Other neonatal events can also impact the proper setting of the microbial communities of newborn puppies. For example, birth weight is associated with microbiota alterations, with low-birth-weight (LBW) puppies exhibiting an increased abundance of opportunistic bacteria in their fecal microbiota during the first three weeks of life compared with normal-weight puppies [17]. For these reasons, understanding the vertical transmission of bacteria from mothers to offspring seems crucial for improving puppies' health.

This study aims to evaluate whether maternal fecal, vaginal and colostral microbial profiles of bitches one day after whelping are associated with neonatal outcomes in terms of LBW, early growth rate, stillbirth and neonatal mortality (defined as deaths occurring during the first three weeks of life) ratios (Fig. 1). Improving the understanding of the interactions among maternal physiological factors, the microbiota and reproductive performance would provide crucial insights into the ability of the modulation of the maternal microbiota to increase the survival and well-being of newborn puppies.

Methods

Animal enrollment and housing

Thirty-six bitches from medium-sized (> 15 and ≤ 25 kg) and large-sized (> 25 kg) breeds were recruited from one breeding kennel in France. Dams were followed from ovulation (G0) up to 24 h postpartum (P1), with a pregnancy diagnosis performed on their 28th day of gestation (G28). They were fed two manufactured dry pet

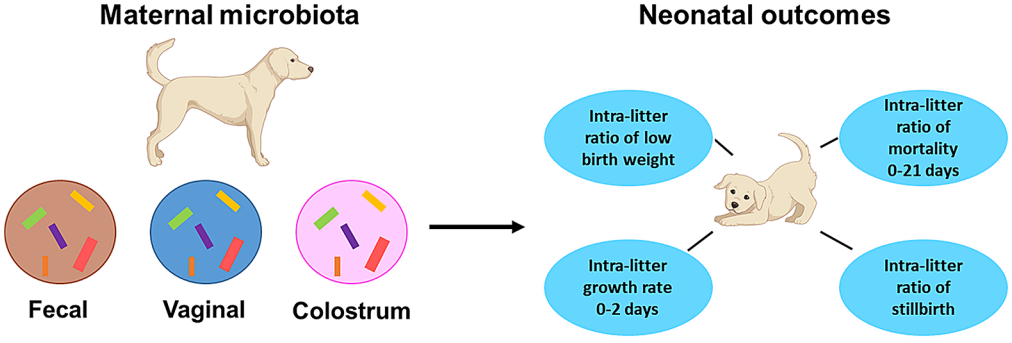

Fig. 1 Summary of the studied relationship between maternal microbiota and neonatal outcomes

food diets. From G0 until G28, the bitches received Diet 1, then Diet 2 from G28 to the end of the experiment according to the National Research Council (NRC, 2006) recommendations [18]. The nutritional values of both diets are available in Supplementary Table S1. During the experiment, all dogs had *ad libitum* access to water. All the dams were vaccinated and dewormed before mating, following the veterinary protocol in place at the breeding facility. They were housed in individual outdoor pens until around the 56th day of gestation (G56), when they were moved to individual pens in the maternity building to prepare for whelping. The pens were equipped with a floor heating system, heating lamps, and wood shavings as bedding material. All puppies were born by natural delivery (i.e., no cesarean section) and remained in the same pens as their mothers during the study period.

Sampling and measures

Both dam weights (digital scale Soehnle model 7859.70.002, Soehnle GmbH, Backnang, Germany, maximum capacity of 200 kg, precision ± 0.1 kg) and body condition scores (BCSs, 9-point scale, with 1 being the thinnest and 9 the fattest [19]) were recorded on G0, G28, G56 and P1. Gestational relative gain weight (GWG) was calculated as the percentage of weight gained or lost by the dams between G0 and P1 according to the following formula: ((weight P1– weight G0) / weight G0 * 100%).

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

Dams' fecal scores (FS) were recorded on three consecutive days, from one to three days postpartum, to assess the gut health of dams following parturition via a 5-point scale fecal scoring system (with 1 being the driest stool and 5 being the most liquid [20]). The mean of the three scores was then calculated and attributed to the P1 time point.

For each dam, reproductive performance was evaluated through the number of puppies born alive, the number of stillborn puppies, the number of puppies dying during the neonatal period (from birth to 21 days post parturition), and the number of low-birth-weight puppies (LBWs). Stillborn, dead and LWB puppies were recorded as a ratio to the number of puppies alive at birth per litter. To determine LBW, puppies were weighed during the first 12 h after birth (D0) via a digital scale (EB3 Series, Ohaus, Parsippany, NJ, USA; maximum capacity, 5 kg; precision, ± 0.1 g). To address interbreed variability, quartiles of birth weight were calculated for each breed on the basis of the last 4010 birth weights registered in the breeding facility [21, 22] (Supplementary Table S2). The thresholds obtained were used to categorize each puppy into one of the four birth weight quartile groups. The first quartile included the 25% lightest puppies, whereas the 25% heaviest puppies were in the fourth quartile, with all puppies under the first quartile threshold considered LBW. Puppies were weighed again at approximately 48 h after birth (D2), and their growth rate (GR) was calculated as follows: ((weight D2– weight D0)/ weight D0) * 100%.

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

DNA extraction and 16 S rRNA gene amplification and sequencing

Metagenomic DNA was extracted from swabs via a Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. For colostrum DNA extraction, the same kit and instructions were used with minor adaptations on the basis of the methodology of Derakhshani et al. [23]. The extracted DNA was quantified via a fluorometric method with a Quant-iT™ PicoGreen® dsDNA assay kit (Life Technologies, Carlsbad, CA, USA) via a QuantStudio™3 Real Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The V3-V4 region of the 16 S rRNA gene was amplified via PCR via the universal primers 341 F (CCTACGGGAGGCAGCAG) [24] and 806R (GGACTACNVGGGTWTCTAAT) [25]. The PCR amplicons were purified with a HighPrep PCR system (Magbio Genomics, Gaithersburg, MD, USA) and used for library construction with an Illumina NEXTflex PCR-Free DNA Sequencing Kit (Bioo Scientific Corp., Austin, TX, USA). Amplicon libraries were sequenced on an Illumina MiSeq 2500 platform (Illumina, San Diego, CA, USA) at the GeT-PlaGe INRAE Platform (Toulouse, France) for paired-end fragment sizes of 250 bp. All the reagents used were of molecular grade.

Analysis of sequencing data

Raw fastq files were imported, demultiplexed, quality filtered and dereplicated through high-resolution sample inference with DADA2 [26] in QIIME 2 (version 2020.2) [27]. This allowed the identification of amplicon sequence variants (ASVs) under default parameters, excluding primer length. ASV de novo alignment and phylogenetic analysis were performed with MAFFT and FastTree2, respectively [28, 29]. Rarefaction curves were checked for full community sampling depth. Taxonomy was assigned to the resulting 16 S rRNA gene marker genes against Greengenes (gg-13-8-99-nb-classifier)/SILVA (v138) via the sklearn classifier method according to Bokulich et al. [30]. The raw data were rarefied by removing the ASVs present in fewer than 5 samples with an abundance lower than 0.005% of the total abundance.

Data analysis

All the statistical analyses were conducted in R version 4.1.0 (R Core Team, 2021), with resulting p values less than 0.05 considered statistically significant, and box plots and histogram figures were drawn with RStudio (version 2022.02.3 Build 492). Other plots were created via MicrobiomeAnalyst [31]. All clinical data are expressed as the means and SDs (±).

Before compositional, multivariate and principal component analysis (PCA), the ASV abundances were centered log-ratio (CLR) transformed following the replacement of zero with the geometric Bayesian model (GBM) of the zCompositions package [32].

Overall microbiota analysis

Beta diversity Multidimensional scaling (MDS) plots generated via the phyloseq package were generated to visualize the beta diversities among the three microbiota. To highlight potential differences in the fecal, vaginal and colostrum microbiota structures, permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations was performed on the basis of Bray–Curtis distances with the Adonis function available in the "vegan" package v2.6-2 of R software [33].

Alpha diversity The alpha diversity was calculated with observed ASVs and the Shannon [34] index via the Phyloseq package v1.38.0 [35], and ANOVA coupled with Tukey's test was used to evaluate differences among the three microbiotas and between clusters.

Core microbiota and clustering

Definition of the core microbiota To describe and illustrate the main genera and families comprising the different maternal microbiotas, the core microbiotas of the dams were defined as all the ASVs at the genus level present in at least 32 dams (89% of samples) for fecal and colostrum microbiotas and 27 dams (75%) in vaginal microbiota, with a minimum of 1% mean relative abundance [36]. This definition, combining prevalence and abundance, allowed to identify ubiquitous and ecologically significant taxa of each studied site [37]. The thresholds chosen maximize the number of ASVs and their abundance, and are proportional to the bacterial richness of the studied microbiota.

Clustering of the dams The clustering of the dams was performed via Dirichlet Multinomial Mixtures (DMM) clustering via the DirichletMultinomial 1.44.0 package in R [38]. The optimal number of clusters was determined via the Laplace approximation by analyzing model complexity. The contributions of the main ASVs to each microbiota cluster were then determined via linear discriminant analysis effect size (LEfSe).

Relationships between microbiota profiles and neonatal health

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

Neonatal outcome ~ Fecal microbial cluster + Vaginal microbial cluster + Colostral microbial cluster.

Chi-square (for the interlitter LBW, stillbirth, and neonatal mortality ratio) and deviance (for the interlitter GR 0–2 days) tests were used to assess the goodness-of-fit of the final models. The overdispersion of the models was also assessed by comparing the residual deviance to the degrees of freedom.

Results

Description of the population

The 36 dams studied belong to 9 different medium and large breeds: Australian Shepherd ($n = 8$), Golden Retriever ($n = 8$), German Shepherd ($n = 1$), Labrador Retriever ($n = 6$), White Swiss Shepherd ($n = 3$), Pyrenean Mountain Dog ($n = 2$), Bernese Mountain Dog ($n = 6$), Boxer ($n = 1$) and Dalmatian ($n = 1$). Five bitches were primiparous, 8 gave birth once before the study, and 23 had whelped at least 2 times (mean = 2.7 ± 1.8). The mean age of the dams was 3.3 ± 1.2 years. At G0, the dams weighed between 18.5 and 39.5 kg (mean = 29.7 kg ± 5.1) and had BCSs ranging from 3 to 8 (mean = 5.4 ± 1.3) (Table 1). At P1, the weights of the dams and their BCSs decreased (mean = 27.7 kg ± 5.1 and mean = 4.1 ± 1.1, $p < 0.001$, respectively). The mean GWG was − 6.5 ± 7.0%, ranging from − 22 to 6%. The mean FS of the dams at

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
Ratio of Stillbirth	0.1	0.1	0	0.5	1.0
Ratio of Neonatal mortality	0.1	0.2	0	1.0	0

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

parturition was 2.6 ± 0.6. A total of 284 puppies were born in our study, with litter sizes ranging from 4 to 11 puppies (mean = 7.9 ± 1.9). A total of 30 puppies were stillborn (10.6%), with a mean of 0.8 ± 1.1 puppies per litter. Among the 254 puppies born alive, 60 were LBW (23.6%), 8 lost weight during the first two days after birth (3.1%) and 25 (9.8%) died during the first three weeks. Half of the dams (17 out of 36 dams) delivered no LBW puppies, whereas the other half (19 dams) had between 1 and 8 LBW per litter (mean ratio = 0.2 ± 0.3).

Comparison of the fecal, vaginal and colostrum microbiotas one day after parturition

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

Beta diversity The microbial community composition significantly differed depending on location on the basis of the PERMANOVA results obtained via Bray–Curtis distances ($p < 0.001$) and pairwise analysis (p-adjusted = 0.003 for each pair) Fig. 2). The fecal microbiota had greater dissimilarity than the vaginal and colostrum microbiota, which shared more bacterial communities, as seen with the overlapping ellipses. The size of the ellipses also allowed us to highlight greater interindividual variation in the composition of the vaginal microbiota than the other two, where ellipses were smaller. The Bray–Curtis distances were also added to a heatmap to specify the previous results Fig. 3). As anticipated with the MDS plot, approximately half of the vaginal and colostrum ASVs were almost absent from the fecal microbiota, explaining the interdissimilarity.

Composition At the three sampling sites, *Firmicutes*, *Proteobacteria*, *Fusobacteria*, *Bacteroidetes* and *Actinobacteria* were the five most abundant microbial phyla, except the vaginal phyla, where *Tenericutes*, exclusively composed of *Mycoplasma*, was more abundant than *Actinobacteria* and was significantly more abundant than in feces and colostrum Fig. 4A and Table 2). The relative abundance of *Firmicutes* remained similar among the sampling sites, although the family composition of this phylum varied from one site to another. The fecal microbiota had the most heterogeneous *Firmicutes* composition, with *Clostridiaceae*, *Lachnospiraceae* and *Acidaminococcaceae* being the most abundant families of this phylum. The vaginal and colostrum microbiotas, on the other hand, had only one abundant *Firmicutes* family: *Streptococcaceae* and *Staphylococcaceae*, respectively Fig. 4B). More *Bacteroidetes* (dominated by *Bacteroidaceae* and *Prevotellaceae*) and *Actinobacteria* (dominated by *Corynebacteriaceae*) were present in the fecal microbiota than in the vaginal and colostrum microbiotas (Table 2). In contrast, *Proteobacteria* was the dominant phylum in both the vaginal and colostrum microbiotas, with *Enterobacteriaceae* and *Moraxellaceae* having the highest relative abundances in the vaginal and colostrum microbiotas, respectively.

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

Characteristics of the core microbiota clusters

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

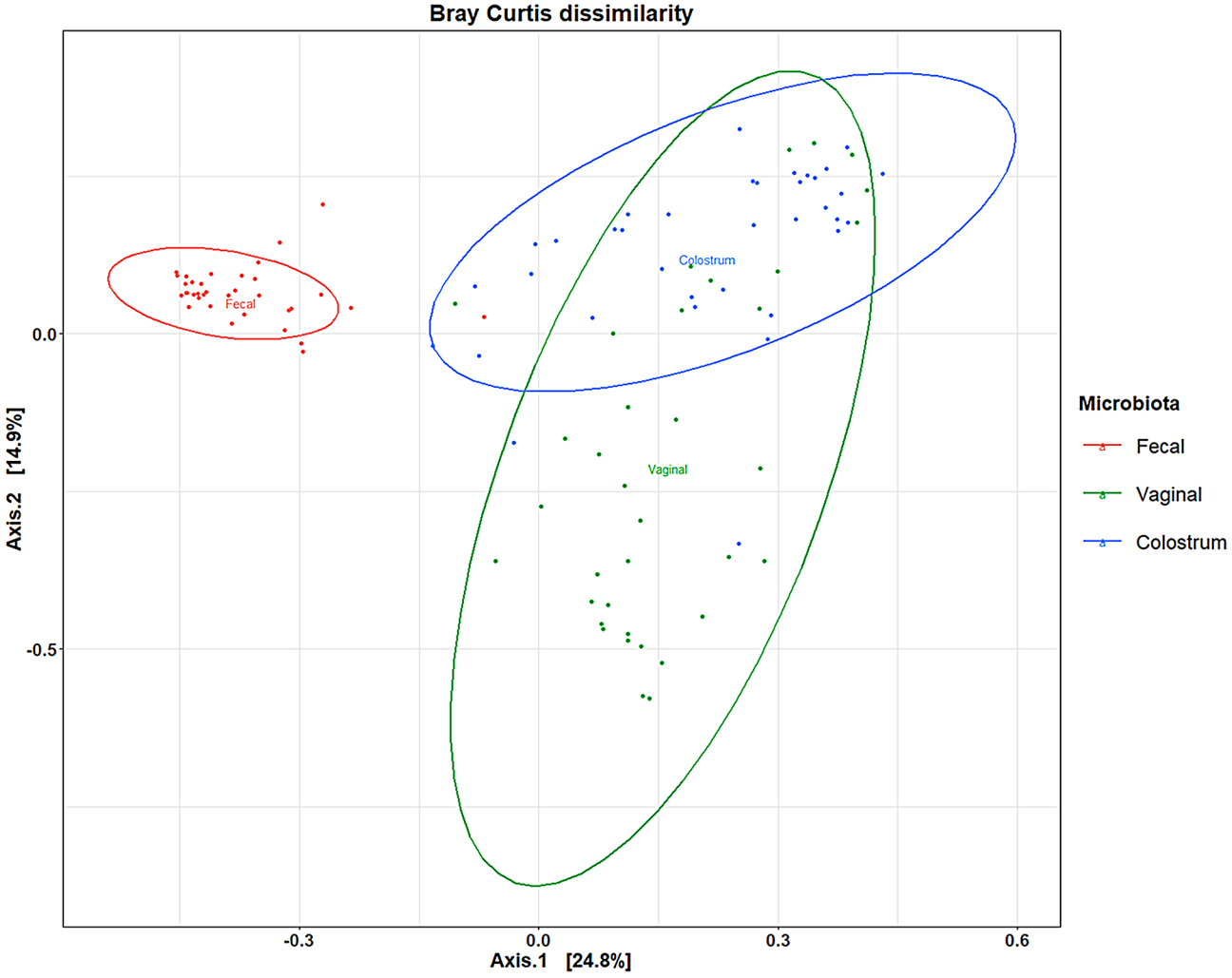

Fig. 2 Beta diversity of the 36 dams' three types of microbiota (fecal in red; vaginal in green and colostral in blue) one day after parturition. The MDS plot shows the dissimilarities between dams' fecal, vaginal and colostrum microbiotas one day after parturition, which were calculated via Bray–Curtis distances. Each point represents an individual dam sample for one specific microbiota, positioned on the plot on the basis of the similarity of its microbiota communities with other samples. Ellipses are based on 95% confidence intervals and standard errors. The similarity of bacterial communities is evaluated on the basis of taxon similarity and abundance ($p < 0.001$)

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

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

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

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

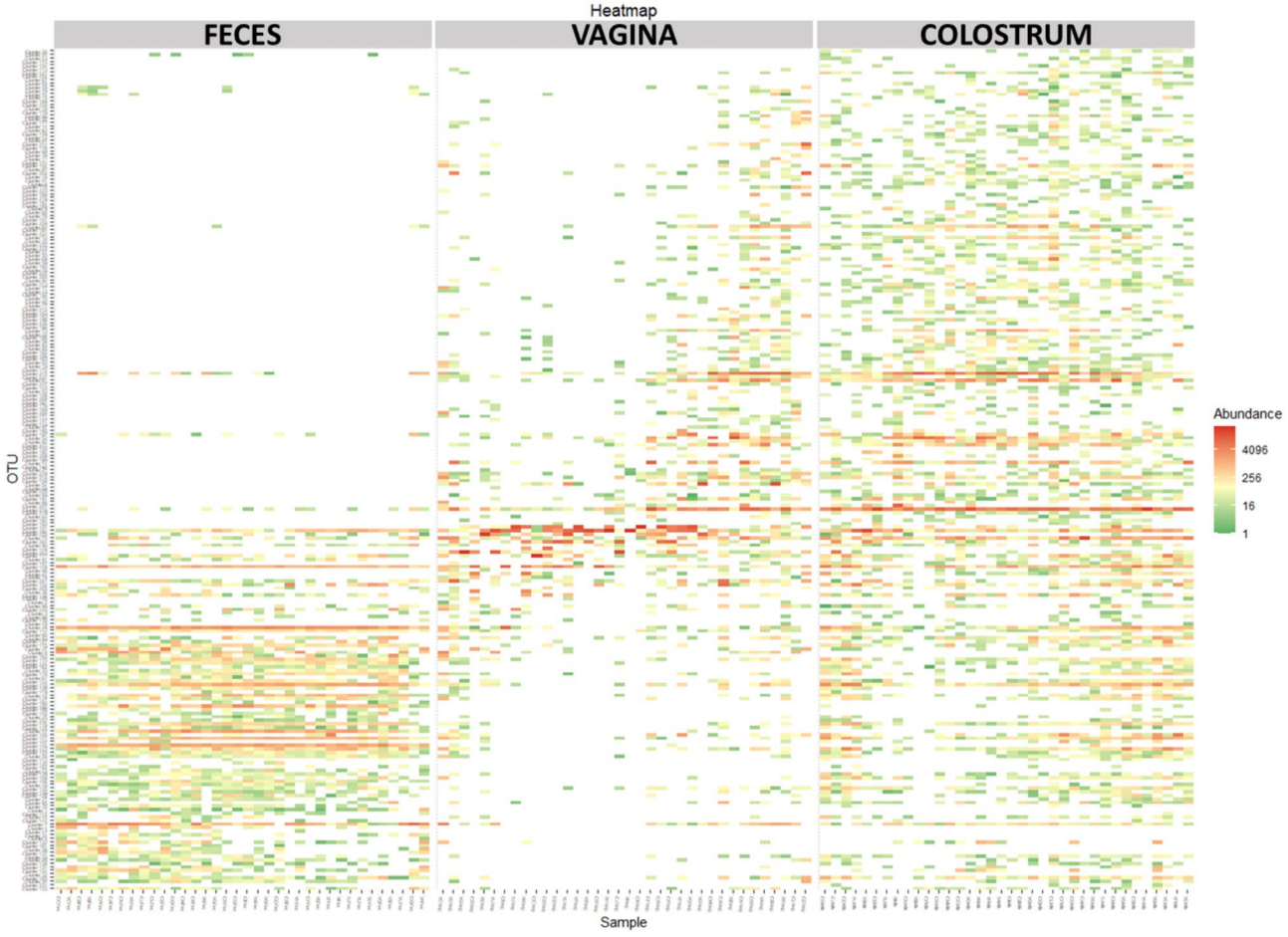

Fig. 3 Heatmap of dam microbiota one day after parturition at the family level between the three studied sites (fecal, vaginal and colostral). The color scale is based on the relative abundance of the amplicon sequence variant (ASV)

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

Core microbiota clusters and reproductive performance

On the basis of the linear models assessing the LBW ratio, GR 0–2 days, stillbirth ratio, and neonatal mortality ratio, with fecal, vaginal, and colostral microbiotas as the studied factors, only the vaginal microbiota was consistently associated with reproductive outcomes, except for the LBW ratio (Table 3).

Litter GR over the first 2 days of life tended to be 2 times greater in puppies from Cluster 1 bitches than in those from Clusters 2 and 3 (Table 4; Fig. 10A). In contrast, the interlitter ratio of stillbirth and neonatal mortality was over 4 times greater in Cluster 3 litters than in Cluster 1 litters (Table 4; Fig. 10B and C). No significant difference was observed in neonatal outcomes among dams with different microbial fecal or colostral clusters.

Discussion

Limitations of the study

To the best of our knowledge, this study is one of the few in the literature and the first in canine species to provide results concerning the composition of the maternal microbiota of the vagina, feces and colostrum concomitantly at parturition and how they are associated with early-life outcomes. While these results are promising for understanding how maternal factors influence the microbiota at parturition, several elements must be considered. A total of 36 bitches and their 284 puppies were included in our study. Although this is an important number of animals, the number of animals per group after clustering has decreased to a more limited population (between

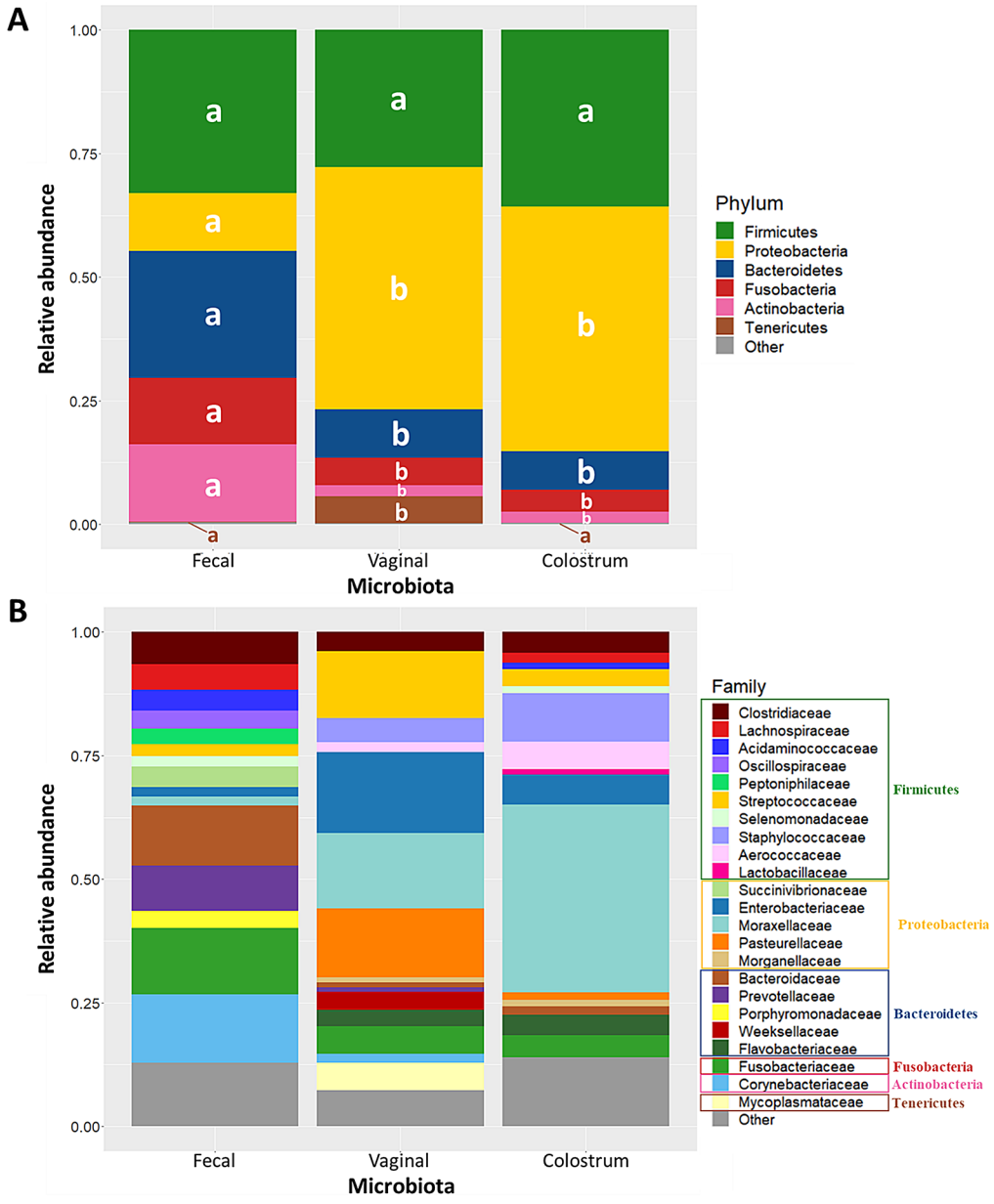

Fig. 4 Mean relative abundance of the bacterial taxa of the three studied microbiotas in 36 bitches one day after parturition at the (**A**) phylum and (**B**) family levels. The 15 most abundant families at each site are highlighted. All the remaining taxa are summed in the "Other" category. Significant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

10 and 23 dams among different clusters), lowering the statistical power of the models. Factors such as type of parturition, diet and breed have a strong influence on gut microbial communities [16] but were not investigated in the present study. Indeed, the included dams originated from one kennel with the same nutrition, kennel conditions and management. Thus, further studies are needed to determine the maternal microbiota composition and role depending on the mentioned characteristics.

Importance of the maternal microbiota for newborns

Early colonization of the gut and development of the intestinal microbiota are crucial steps for newborns, which can shape many vital functions in the short and long term [39, 40]. More specifically, the expression of some genes in newborns is influenced by maternal microbial status, even before birth, affecting the later development of the immune system, neurophysiology, or energy metabolism [41]. It is now widely known that maternal factors and the microbiota play crucial roles in the establishment of bacterial communities in newborns in the constant presence of their mothers during parturition

Table 2 Mean relative abundance of each phylum and prominent family in the three microbiotas of the dams one day after parturition ($n = 36$)

	Relative abundance (%) (mean ± SD)				Relative abundance (%) (mean ± SD)		
Phylum	**Fecal**	**Vaginal**	**Colostrum**	**Family**	**Fecal**	**Vaginal**	**Colostrum**
Actinobacteria	15.9 ± 14.5	2.2 ± 4.8	2.2 ± 2.0	*Corynebacteriaceae*	14.2 ± 14.2	1.9 ± 4.3	1.1 ± 1.3
Bacteroidetes	25.4 ± 8.7	10.3 ± 16.0	8.0 ± 8.0	*Bacteroidaceae*	12.0 ± 4.5	1.1 ± 5.2	1.6 ± 1.9
				Prevotellaceae	9.0 ± 5.4	1.2 ± 4.1	1.0 ± 1.4
Firmicutes	32.9 ± 9.9	26.2 ± 25.4	35.3 ± 19.5	*Staphylococcaceae*	0.8 ± 1.6	4.4 ± 8.5	10.0 ± 12.1
				Streptococcaceae	2.4 ± 5.3	13.1 ± 21.9	3.2 ± 4.8
				Clostridiaceae	6.4 ± 4.3	3.4 ± 15.7	4.1 ± 4.7
				Lachnospiraceae	5.1 ± 2.7	0.4 ± 1.0	2.0 ± 2.6
				Acidaminococcaceae	4.2 ± 2.3	0.1 ± 0.5	1.2 ± 1.7
Fusobacteria	13.5 ± 4.8	7.0 ± 14.2	4.3 ± 4.6	*Fusobacteriaceae*	13.5 ± 4.8	7.0 ± 14.0	4.3 ± 4.6
Proteobacteria	11.5 ± 7.4	48.7 ± 27.1	49.7 ± 21.4	*Succinivibrionaceae*	4.1 ± 5.1	0.0 ± 0.1	0.2 ± 0.3
				Enterobacteriaceae	1.9 ± 1.9	16.2 ± 25.2	5.6 ± 11.2
				Pasteurellaceae	0.6 ± 0.8	14.3 ± 23.6	1.7 ± 5.7
				Moraxellaceae	1.9 ± 4.7	14.4 ± 18.9	38.6 ± 20.2
Tenericutes	0.1 ± 0.3	5.3 ± 15.7	0.1 ± 0.2	*Mycoplasmataceae*	0.04 ± 0.2	5.3 ± 15.7	0.05 ± 0.2

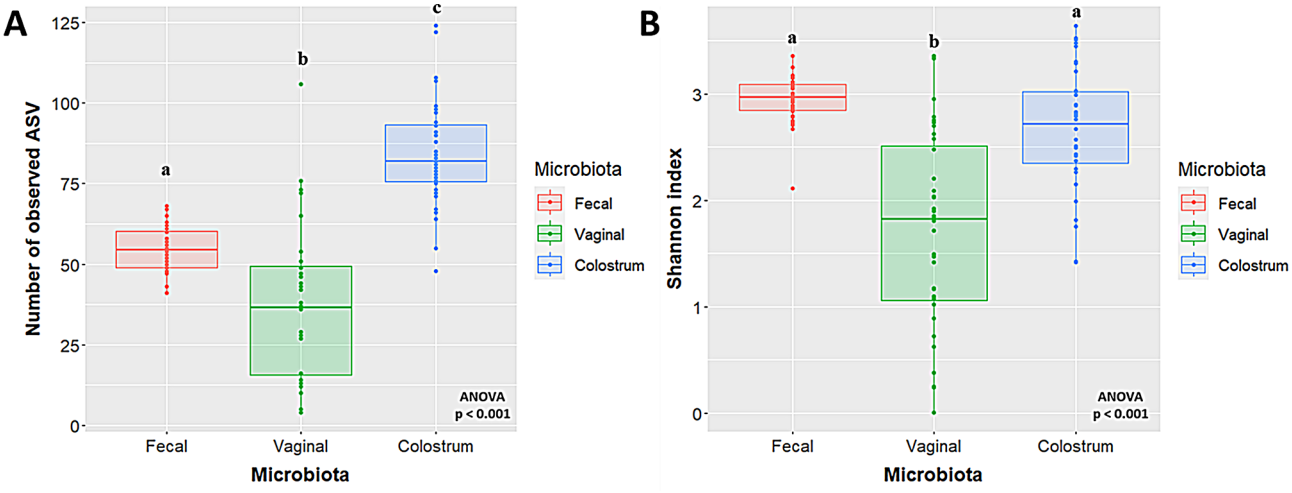

Fig. 5 Alpha diversity of 36 samples of maternal fecal, vaginal and colostrum microbiotas one day after parturition using (**A**) observed species (bacterial richness) and (**B**) the Shannon index (bacterial richness and evenness). Significant differences are identified with Tukey Honest Significant Difference (HSD) letters. Boxes with different letters are significantly different ($p < 0.05$)

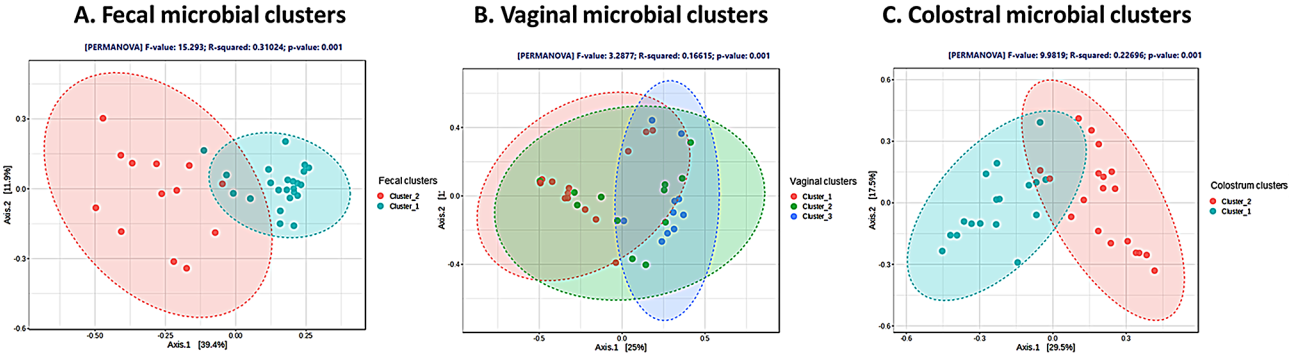

Fig. 6 Beta diversity plots showing the differences in the fecal (**A**), vaginal (**B**) and colostral (**C**) microbiota structures between the clusters identified in the 36 dams one day after parturition via Dirichlet Multinomial Mixtures (DMM) clustering analysis. The MDS plot shows the dissimilarities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

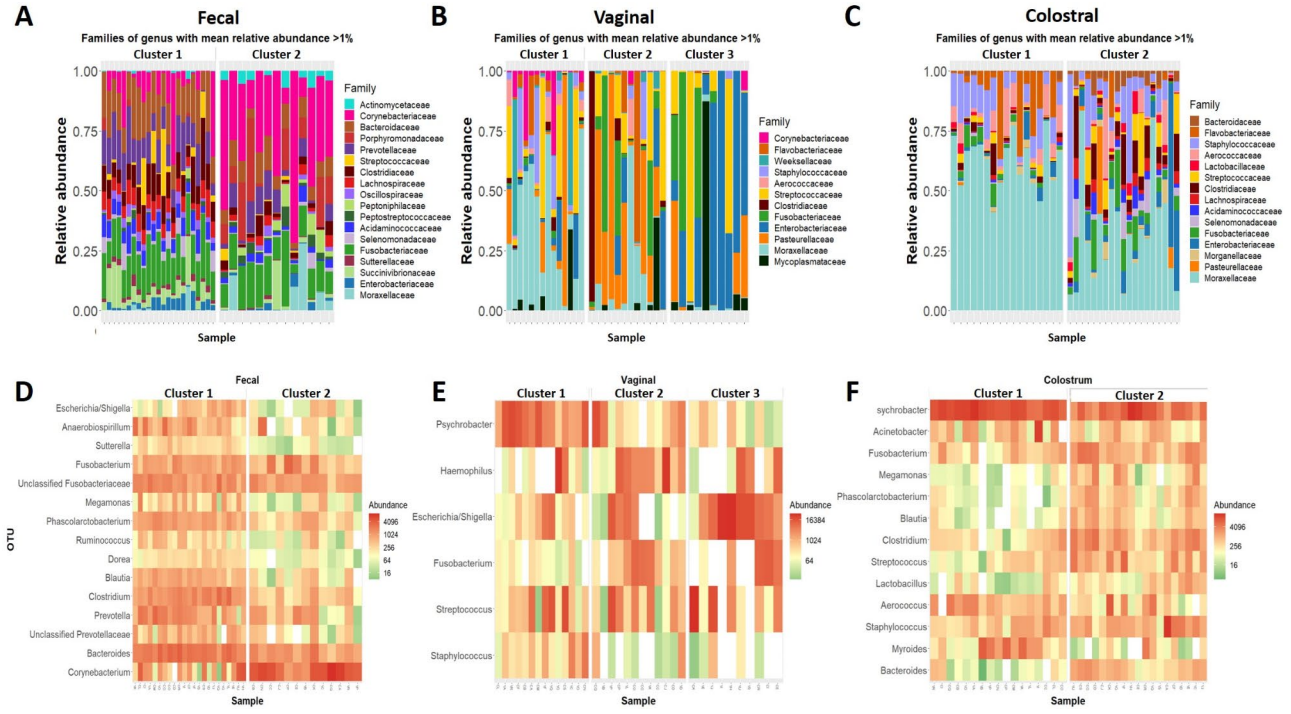

Fig. 7 Microbiota profiles in determined clusters on different body sites of dams one day after parturition. Relative abundance of the core families with > 1% relative abundance for each cluster of the fecal (**A**), vaginal (**B**) and colostrum (**C**) microbiotas and heatmaps of the core genera of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

[13, 42, 43]. Among the maternal microbiota, the fecal microbiota is by far the most studied, although many recent studies have shown that the vaginal and colostrum microbiota play crucial roles in shaping the microbiota of newborns [5, 44]. Each of these microbiota intervenes at different times in the development of the newborn's microbiota, with different effects. In humans, the vaginal environment is the first in contact with the newborn, even before exposure to the external environment; hence, it is the one shaping initial bacterial colonization [45]. It is then expanded by the colostrum microbiota, which is rapidly ingested by the newborn, shaping its gut microbiota [46]. Finally, animals at birth might come into contact with their mother's feces, which induces another vertical transfer of bacteria from the mother to the offspring. Thus, to obtain a full picture of the association between the maternal microbiota and offspring health, several microbiota have been studied rather than one.

Composition of the fecal microbiota

First, some important differences between the fecal, vaginal, and colostral microbiota were demonstrated in our study. Compared with those of the other two microbiota, the fecal microbiota at parturition had the most uniform composition among the dams, with no clear dominance of any specific taxa. The fecal microbiota has been extensively studied in recent decades in dogs, and the composition presented in our study did not differ much from previously published data [47–50]. In some taxa, such as *Lactobacillus*, the relative abundance in the fecal microbiota was very low (0.9% of total abundance) compared with that in nonpregnant animals. Indeed, in previous results concerning the evolution of the fecal microbiota during pregnancy, we reported that the abundance of lactobacilli decreased to almost zero at parturition [51]. One hypothesis for this disappearance was that lactobacilli translocate from the gut to the mammary glands; this phenomenon is known as the "entero-mammary pathway" [52]. Moreover, the increase in estrogen levels during pregnancy has been linked to a decrease in vaginal pH, fostering the growth of lactobacilli and reducing pathogen colonization [53]. However, these hypotheses, as well as the role of *Lactobacillus* in newborn dogs, remain to be investigated. More generally, other studies are needed to understand the role of the maternal fecal microbiota in newborn seeding.

Although the same diet was provided to all the dams in this study, two bacterial profiles were distinguished in the fecal microbiota *postpartum*. The first profile (Cluster 1) was characterized by a relatively high abundance of *Bacteroides* and *Clostridium* and mostly *Clostridium hiranonis.* This species affects secondary bile acid production and inhibits the growth of pathogenic bacteria such as *C. perfringens* [54, 55]. On the other hand, *Corynebacterium*, which is overrepresented in Cluster 2, is associated with a lower risk of food-responsive diarrhea or IBD in

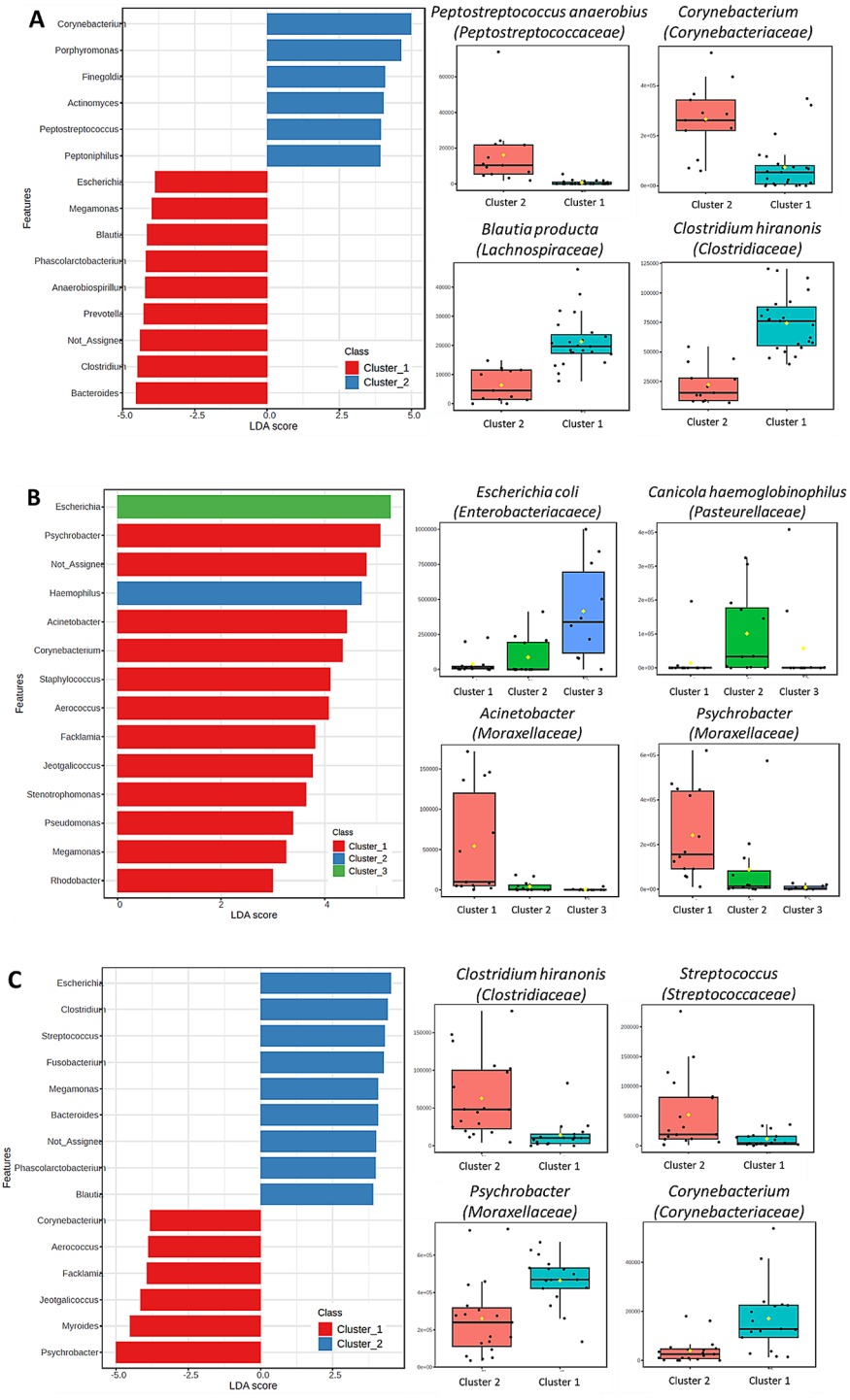

Fig. 8 The main taxon ASVs of the fecal (**A**), vaginal (**B**), and colostrum (**C**) microbiota significantly distinguishing the observed clusters according to linear discriminant analysis effect size (LEfSe) on samples obtained from 36 dams one day after parturition

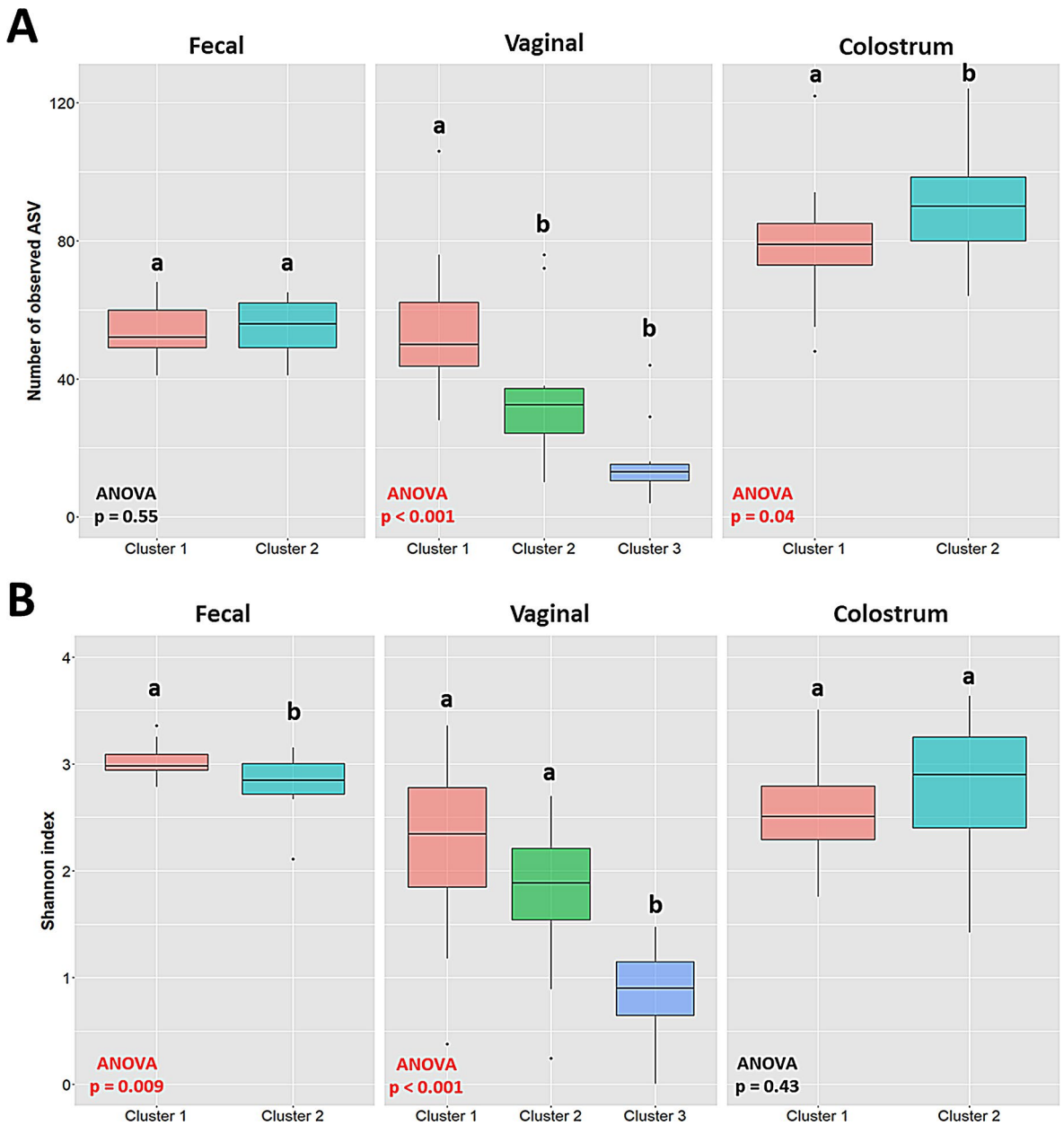

Fig. 9 Bacterial richness of all three microbiota in 36 bitches one day after parturition according to the defined clusters. Significant differences are identified with Tukey's HSD letters. Boxes with different letters are significantly different ($p < 0.05$) according to (**A**) the number of observed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

Table 3 Associations between fecal, vaginal and colostrum microbial clusters one day after parturition and neonatal outcomes: P values of the four linear models. Significant P values (< 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

Table 4 Characteristics of the neonatal outcomes of 36 dams according to their fecal, vaginal or colostral microbiota clusters identified via dirichlet multinomial mixtures (DMM)

	Fecal		Vaginal			Colostrum	
Cluster	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

LBW– low birth weight; GR– growth rate

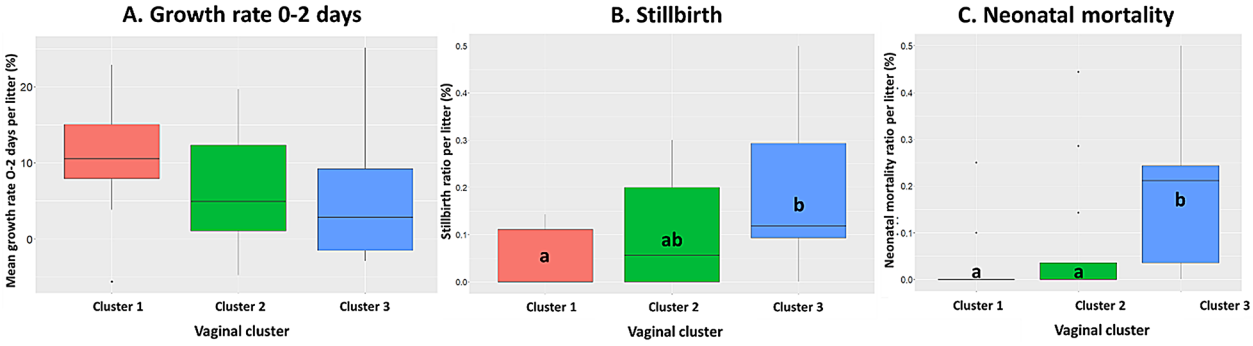

Fig. 10 Neonatal outcomes ((**A**) growth rate 0–2 days; (**B**) stillbirth ratio; (**C**) neonatal mortality ratio) of 36 litters according to the vaginal microbial profile of their dam. Significant differences are identified with Tukey's HSD letters. Boxes with different letters are significantly different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

dogs [56]. Nevertheless, no relationship between the fecal microbiome and neonatal outcomes was observed in this study.

Composition of the colostrum microbiota

Similar to that of the fecal microbiota, very limited interindividual diversity was observed between the core colostrum microbiota of dams. The source of the colostrum microbiota is still unclear, and many studies suggest that some of the bacterial communities are derived from contamination from the skin, the external environment and the oral microbiota of the offspring [57]. Nevertheless, *Proteobacteria* was the dominant phylum in the colostrum microbiota, as observed in women's milk *postpartum* [58]. This phylum was represented mainly by the *Moraxellaceae* family, which had the highest relative abundance among all the bacterial families. Interestingly, in a previous study on puppies, the *Moraxellaceae* family had the highest mean relative abundance in puppy feces at birth [59]. This finding reinforces the relationship between the maternal colostral microbiota and the initial development of the intestinal microbiota of the newborn. Indeed, nearly 70% of stool bacteria originate from maternal milk in newborn human babies younger than 6 days of age [58]. However, this hypothesis needs further investigation in dogs.

Two clusters were identified in the colostral microbiota of the dog, with *Enterobacteriaceae* and *Clostridiaceae* dominating in one cluster and *Moraxellaceae* and *Lachnospiraceae* in the second cluster. To date, it remains impossible to determine which colostral microbial profile might be more beneficial for newborn health, as no relationship was observed between these two parameters in the present study. Further investigations are needed to address the presence of these taxa in canine milk, as well as their potential function in the newborn gut [60].

Composition of the vaginal microbiota and its associations with neonatal outcomes

General composition

Similar to colostrum, *Proteobacteria* was the dominant phylum in the vaginal microbiota, with *Enterobacteriaceae*, *Moraxellaceae*, *Streptococcacae*, and *Pasteurellaceae* being the key bacterial taxa observed *postpartum* in our population. These results are consistent with previous studies in which these taxa were also found to be the most abundant in the vaginal samples of canine females [61–63]. The vaginal microbiota showed considerable interindividual diversity, with some dams having a microbiota composed almost entirely of one main bacterial family, whereas others had a more diverse vaginal microbiota.

Three distinct profiles emerged from the clustering of the vaginal microbiota in our study. The first cluster (Cluster 1) consisted of dams dominated by *Moraxellaceae*, the second cluster (Cluster 2) had dams with a relatively high relative abundance of *Pasteurellaceae*, and the third cluster (Cluster 3) had a relatively high relative

abundance of *Enterobacteriaceae.* Both this high interindividual variation between dams and the composition of the vaginal microbiota are quite different from what can be seen in humans. Indeed, the core vaginal microbiota of humans around birth is dominated by *Lactobacillus*, which often accounts for more than half of the relative abundance, followed by *Enterococcus* and *Staphylococcus* [64]. The higher abundance of *Lactobacillus* in the human vaginal microbiota may be due to a lower pH, typically below 4.5, whereas the pH of the canine vagina ranges from 5.0 to 8.1[62]. Lactic acid bacteria can survive in low-pH environments, allowing them to dominate areas where other bacteria cannot survive, such as the human vagina [65]. The high pH in dogs' vaginas leads to greater competition and hence a lower abundance of *Lactobacillus* at the expanse of *Proteobacteria*, which grow at higher pH.

Association with mortality in puppies

One of the most important findings of this study is the association between the vaginal microbiota in dams and the neonatal outcomes of their puppies. The vaginal microbiota in women, cows and sows has been shown to influence the risk of prematurity, morbidity and mortality in newborns [66–68], but this study is one of the first to describe such results in canine species via sequence-based bacterial analysis. Indeed, one of the vaginal microbial profiles (Cluster 3) was significantly associated with a higher risk of stillbirth and neonatal mortality in puppies, and a trend toward a lower growth rate over the first two days of life was observed, with the last parameter being known as a good marker of the maternal colostrum intake [21].

In parallel with the increased stillbirth ratio, Cluster 3 dams also presented lower vaginal bacterial richness than the other clusters did, suggesting that both are significantly correlated. This is a surprising result, as elevated bacterial diversity was observed in human vaginal samples with an increased risk of premature birth [69].

E. coli was found to be the most abundant *Enterobacteriaceae* in Cluster 3 dams. This bacterium is considered opportunistic [70–72] and is the most commonly isolated bacterium from the vagina of healthy female dogs. However, in the case of neonatal mortality, an abundant pure culture of *E. coli* from a vaginal sample from a dam is interpreted as a likely cause of neonatal death [73]. Indeed, *E. coli* is also most often isolated from internal organs of puppies that die during the neonatal period [43, 74–76]. Moreover, over 80% of cases of neonatal septicaemia in dogs occur within the first two days of life, strongly suggesting contamination during late gestation or birth via the uterus or vagina of the dam [77]. In humans, ascending bacterial infection with *E. coli* leading to chorio-amnionitis before or after membrane rupture is usually the most common infectious cause of stillbirth [78]. Our results seem to confirm the potential pathological role of this bacteria, as we have shown that, compared with dams with a vaginal microbiota rich in bacteria from the *Moraxellaceae* family, those with a vaginal microbiota dominated by *E. coli* present a significantly greater risk of stillbirth and neonatal mortality.

Among the most abundant bacteria of Cluster 2 bitches, we identified bacteria from the *Pasteurellaceae* family, more precisely, *Canicola haemoglobinophilus*. Interestingly, these bacteria were found to be associated with urogenital tract infections in dogs [79], but they were also isolated from dead newborn dogs [80]. Thus, one could hypothesize that the vaginal microbiota of bitches from Cluster 2 could be associated with neonatal mortality. However, our data do not allow us to confirm this hypothesis.

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

Conclusions

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

Abbreviations

ASV	Amplicon sequence variant
DMM	Dirichlet Multinomial Mixtures
FS	Fecal Score
GIT	Gastrointestinal tract
GWG	Gestational Weight Gain
LBW	Low birth weight

GR Growth ratio

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-025-00384-1.

Supplementary Material 1

Acknowledgements
We would like to thank Pauline David, Elodie Mansalier and Laetitia Montacq for their invaluable assistance in the collection of samples and analysis of data. We would also like to thank the technical staff of the Lallemand laboratory, particularly Caroline Achard and Elodie Colin, for their help in the extraction and sequencing of the microbial DNA and Amélie Mugnier for manuscript reviewing. Finally, we deeply thank the owner of the kennel for allowing us access to his facilities and dogs during the study.

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

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

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

Declarations

Competing interests
E. A. and A. R. were employed by the company Lallemand SAS. All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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

Received: 9 October 2024 / Accepted: 17 February 2025
Published online: 06 March 2025

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