Theriogenology 216 (2024) 1–7

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

Characterization of the semen microbiota of healthy stud dogs using 16S RNA sequencing

P. Banchi [a,b,*], L. Bertolotti [b], L. Spanoghe [a], H. Ali Hassan [a], J. Lannoo [a], G. Domain [a], K.S. Henzel [c], V. Gaillard [c], A. Rota [b], A. Van Soom [a]

[a] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, 9820, Merelbeke, Belgium
[b] Department of Veterinary Science, University of Torino, 10095, Grugliasco, Italy
[c] Royal Canin Research Center, 30470, Aimargues, France

ARTICLE INFO

Keywords:
Sperm
Reproductive microbiota
Canine
Male
16S rRNA sequencing

ABSTRACT

The reproductive microbiota of male dogs has never been investigated using culture-independent sequencing techniques. The purpose of the present study was to get seminal knowledge on the microbiota of the ejaculate. Specifically, factors as the fraction of the ejaculate, the sperm quality (normospermia, teratozoospermia), and the living environment were evaluated. The sperm-rich and the prostatic fractions of the ejaculate were collected from healthy stud dogs. Following the sperm analysis, samples from twenty animals (normospermic n = 10 and teratozoospermic n = 10) were stored at – 80 °C until further processing including DNA extraction and 16S rRNA sequencing. Alpha- (Shannon index) and beta- (Bray-Curtis, Unweighted UniFrac) diversities were assessed and compared (PERMANOVA) based on the group of samples (biological samples from the ejaculate and controls), the fraction of the ejaculate (sperm-rich and prostatic fractions), the animal group (normospermia and teratozoospermia), and the living environment of the animal (kennel or pet living in-house). The most abundant bacterial phyla in canine semen samples were Proteobacteria, Firmicutes, and Actinobacteria. Overall, the dominant bacterial family was that of *Pasteurellaceae* The genus *Mycoplasma* was never detected. No differences in terms of bacterial composition were found based on the fraction of the ejaculate and based on the animal group ($P > 0.05$). On the other hand, differences in alpha and beta diversities were highlighted based on the living environment ($P = 0.001$).

Overall, the results of the present study provide preliminary insights on dog semen microbiota, opening a new chapter in the field of canine andrology. Our results suggest that the environment may play a role in influencing the reproductive microbiota of male dogs and that the prostatic fraction of the ejaculate can be used for further research as a representative of the semen microbiota.

1. Introduction

The spread and development of sequencing techniques, led to a shift in the paradigm of sterility of inner organs and fluids in humans and animals. Therefore, research on the presence of a resident microbiota in healthy organs has developed enormously in the last two decades [1]. As a natural consequence, researchers started focusing on unveiling possible associations between the microbiota and various conditions, including fertility problems [2]. Therefore, research on the reproductive microbiome has blossomed, targeting both male and female organs [3]. In dogs as in humans, the underlying cause of up to 50 % of subfertility cases can be attributed to the male [4–6]. Although bacterial infections represent only one element in the long list of underlying causes that can affect fertility in dogs, empirical treatment with antimicrobials is often attempted by veterinarians to restore reproductive efficiency [7]. However, antibiotics are not the solution to every problem and their effect can also be detrimental [8], altering the resident flora in multiple organs and possibly causing unbalances and antimicrobial resistance, which is not desirable in a *One Health* perspective.

The lower reproductive tract of men and women presents with a characteristic microbiome [9–11] and recent studies have demonstrated an association between semen quality and bacterial communities in the

* Corresponding author. Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, 9820, Merelbeke, Belgium.
E-mail address: penelope.banchi@ugent.be (P. Banchi).

https://doi.org/10.1016/j.theriogenology.2023.12.018
Received 28 August 2023; Received in revised form 13 December 2023; Accepted 13 December 2023
Available online 19 December 2023

ejaculate of healthy and subfertile men. Furthermore, acute and chronic infections of the genito-urinary tract account for up to 15 % of infertility cases in men, possibly also affecting semen parameters [12–14]. The ejaculate conveys bacteria possibly residing in any part of the canine genito-urinary tract, being the less invasive and most immediate substrate to analyze. Specifically, breeding male dogs are routinely subjected to reproductive examination to ensure optimal performances, which is of economic importance. Nevertheless, research on seminal bacteria is very limited and a description of the characteristic microbiota has never been provided. Canine semen contains up to 10^5 bacteria/mL and it has been suggested that a higher overall concentration of bacteria and the presence of potentially pathogenic microorganisms might be associated with lower semen quality [15]. However, previous research on canine semen microbial composition was only based on culture, thereby potentially missing some pathogens. Next generation sequencing (NGS) techniques allow for the description of novel microbiomes without prior knowledge of sequencing information or specific culture requirements. These techniques have been recently used to investigate the seminal microbiome of humans and domestic animals with compelling results [16–18]. To date, no data are available on the healthy dog semen microbiome although this is essential to investigate possible associations between seminal microbial populations and abnormalities in semen parameters, infertility, and reproductive conditions. Therefore, the aim of the present study is to explore the seminal microbiota of male dogs and to assess possible differences based on various factors (i.e., fraction of the ejaculate, semen characteristics, living environment).

2. Materials and methods

2.1. Animals and sample collection

This prospective study included purebred healthy client-owned stud dogs undergoing breeding soundness examination at the Faculty of Veterinary Medicine of Ghent University (Merelbeke, Belgium) between December 2021 and March 2022. The study was conducted in accordance with the EU Directive 86/609/CEE for the care and use of animals and informed consent was obtained from the owners of the enrolled dogs. The dogs could be either in-house pets or animals from breeding facilities. A maximum of two dogs living in the same kennel or in the same household were included, to avoid cohabitation as a confounding factor. Dogs were at least 1 year of age and 10 kg of body weight and had not received any antimicrobial within the previous 6 months. All dogs underwent a general clinical exam and breeding soundness examination (visual assessment of the scrotum, palpation of the testes, examination of the penis and prepuce, ultrasound examination of the prostate) and were deemed healthy. Semen samples were collected by digital manipulation in the absence of a teaser bitch (i.e., a bitch in heat) and the sperm-rich fraction was used to assess semen quality. Dogs that presented both more than 80 % morphologically abnormal spermatozoa and progressive motility lower than 40 % were excluded from the study, as the same animals subsequently participated in a clinical trial with these criteria. The animals that were finally enrolled following the semen analysis, received a standard diet for adult dogs (provided by Royal Canin SAS) for at least 60 days (estimated duration of a full spermatogenic cycle in dogs) [19]. No dog received antimicrobial treatment during this period. Afterwards, the dogs underwent further clinical examination and ejaculates were collected again using sterile plastic funnels. The operators wore gloves, and the prepuce was wiped with a clean gauze before collection. After the ejaculation of the pre-sperm fraction, the second (sperm-rich) and the third (prostatic) fractions were collected separately and immediately carried to the semen laboratory next to the collection room. Based on the estimated volume of the ejaculate, aliquots of 100–500 μL of each fraction were moved into two sterile 1.5 mL Eppendorf tubes (Safe-Lock tubes 1.5 mL PCR-clean, Eppendorf, Germany) using sterile tips and stored at – 80 °C for further analyses. The rest of the sperm-rich fraction was used to assess semen quality.

Sampling controls (n = 2) included sterile saline collected using a tip from the same batch of those used to collect the semen samples and a gauze stored with those used to clean the prepuce, and were stored at – 80 °C.

Ten dogs that presented with normospermia (i.e., 60 % or more morphologically normal spermatozoa) and ten dogs that presented with teratozoospermia (i.e., less than 60 % of morphologically normal spermatozoa) were retrospectively selected based on the result of the semen analysis and the samples were thawed and processed by 16S sequencing, together with the sampling controls.

2.2. Fresh semen analysis

The sperm rich fraction of the ejaculate was used to assess semen parameters. The sperm concentration was measured using the Nucleocounter-SP100® (ChemoMetec, A/S, Allerød, Denmark) using a 10 μL aliquot of semen diluted into 1 mL lysis reagent S100 (ChemoMetec, A/S, Allerød, Denmark), according to the manufacturer's instruction [20]. The semen was then diluted to a concentration of 40 × 10^6 spermatozoa/mL into warm and sterile saline solution (NaCl 0.9 %) and 4 μL were loaded in a pre-warmed ISAS®D4C20 disposable counting chamber to assess motility by the ISAS®v1 system (Proiser, Valencia, Spain) equipped with a heated stage set at 37 °C and a 10 × negative phase-contrast objective [21]. The recorded kinematic parameters were total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), straightness (STR, %), and linearity (LIN, %).

Finally, eosin-nigrosin stain was used to evaluate the morphology and the viability of spermatozoa under bright-field microscopy at 1000 × magnification (Olympus BX51TF, Tokyo, Japan). The percentage of living spermatozoa and the percentage of normal spermatozoa was determined by counting 200 spermatozoa. Specifically, spermatozoa were classified as dead when penetration of the staining into the sperm cell was observed (i.e., damaged plasma membrane). Morphological abnormalities were assessed and included abnormal heads, abnormal midpiece/tails, proximal protoplasmic droplets, and distal protoplasmic droplets.

2.3. Amplification and sequencing of bacterial 16S rRNA

After internal testing of the extraction kit on test samples of different volumes and extraction negative controls, QIAamp® DNA Microbiome (Qiagen, Germany) was chosen to achieve DNA extraction on the 42 included samples (i.e., second and third fractions of 20 dogs and two controls) and on two laboratory blanks (i.e., lysis buffer from the extraction kit). Therefore, 44 samples were further processed. Specifically, 100 μL aliquots were used to achieve bacterial DNA extraction. Host DNA was depleted as for the instructions provided with the extraction kit. Afterwards, bacterial DNA was lysed following the QIAmp® DNA Microbiome (Qiagen, Germany) protocol. The concentration of the extracted DNA was assessed using a Nanodrop 2000 (Thermo Fisher Scientific, USA) spectrophotometer and samples were sent for sequencing of 16S rRNA to an external laboratory (BMR Genomics, Padua, Italy). After 30 cycles of amplification by RT-PCR targeting the hypervariable regions V3–V4 of the bacterial DNA, sequencing was performed using the Illumina MiSeq platform (Illumina, San Diego, CA).

2.4. Data analysis

Data analysis of the raw sequences was performed using Quantitative Insights into Microbial Ecology tool (QIIME2-2019.10) together with Greengenes database [22,23]. Paired-end forward and reversed sequencing reads were assembled and assigned to their original sample based on the barcode. After removing primer sequences and barcodes,

the denoising procedure using the Deblur method implemented in Qiime2 was applied. The sequences were classified in OTUs (Operational Taxonomic Unit) at 97 % identity threshold against the Greengenes reference database (ver. 13-8-99-515-806) for taxonomy assignment. Alpha (i.e., intra-sample bacterial diversity) and beta (i.e., in-between sample diversity) diversities were assessed by Shannon-index and Bray-Curtis and Unweighted UniFrac, respectively. Differences in number of reads per sample based on different groups (i.e., type of sample, fraction of the ejaculate, normospermia and teratozoospermia groups, living environment) were assessed using Mann-Whitney *U* test. To understand the differences in the variation of samples, Wilcoxon test was used to compare the alpha diversity distribution values showed by groups of samples (biological samples from the ejaculate and controls), the fraction of the ejaculate (second and third fractions), the normospermia and teratozoospermia groups, and the living environment of the animal (kennel or pet living in-house).

A permutational multivariate analyses of variance (PERMANOVA) was run for each based on Bray-Curtis dissimilarity to assess differences based on sample type (biological sample from the ejaculate and controls), fraction of the ejaculate (sperm-rich and prostatic fractions), living environment (in-house pet and kennel dogs), and group (normospermic and teratozoospermic dogs).

Data were analyzed using R ver. 4.2.2 (Vienna, Austria). Significance was considered for $P < 0.05$. Differences among samples were calculated using alpha and beta diversity estimation with Qiime pipeline.

3. Results

All included animals were healthy at both clinical examinations, and semen collection was always successful. Dogs were aged 1–10 years (median 3.3, IQR 2–4.4 years), weighted between 10.7 and 70.1 kg (median 25, IQR 15.75–28.75), and belonged to 11 different breeds (Table 1). Eight animals came from breeding kennels, whereas the remaining twelve lived in households as pets. None of the dogs had mated in the previous week. Results from the semen analyses are summarized in Table 2 as a mean and standard deviation. The mean percentage of morphologically normal spermatozoa was 76 % (SD 22.4) in dogs classified as normospermic and 38.4 % (SD 22.2) in dogs classified as teratozoospermic.

DNA concentration per sample was between 3.7 and 42.1 ng/μL. The number of reads per sample is reported in Table 1. The most abundant bacterial phyla in canine semen samples were Proteobacteria, Firmicutes, and Actinobacteria. Relative abundances for each sample are reported in Fig. 1. Overall, the dominant bacterial family was *Pasteurellaceae* (phylum Proteobacteria), being identified in every sample, except in those collected from one normospermic dog and in controls and laboratory blanks. *Clostridiaceae* (7 dogs), *Lactobacillaceae* (5 dogs), and *Lachnospiraceae* (5 dogs) were the most prevalent families belonging to the phylum Firmicutes, whereas *Micrococcaceae* (7 dogs) and *Streptomycetaceae* (4 dogs) were the most represented ones belonging to the phylum Actinobacteria. Although most sequences were assigned only to higher taxonomic level (i.e., order, family), some relevant genera were identified. Specifically, the genus *Clostridium* and *Lactobacillus* were detected in the second and third fractions of the ejaculate of six and three dogs, respectively. Interestingly, these bacteria were found in pairs of dogs sharing the same living environment.

The alpha-diversity, calculated by the Shannon index, did not differ between semen samples and controls, between the second and third fraction of the ejaculate (F2 and F3, respectively), and based on the group (i.e., teratozoospermia or normospermia) (Wilcoxon test $P > 0.05$). A significant difference in alpha diversity was shown when living environments (i.e., pet or kennel) were compared (Wilcoxon test $P < 0.05$).

Bacterial population structure of dog semen was investigated

Table 2
Semen characteristics of twenty healthy dogs included in the present study.

	Mean	Standard deviation	Median	Interquartile range
Volume of the 2nd fraction of the ejaculate (mL)	2.07	1.09	1.80	1.55–2.20
Concentration (x 10^6 spermatozoa/mL)	305.23	190.61	274.35	139.47–436.95
Total Sperm Output (x 10^6 spermatozoa/ejaculate)	578.41	423.25	563.64	248.90–804.50
Total motility (%)	77	13.50	83	65.75–89.25
Progressive motility (%)	65.30	16.60	68.50	53.75–78.75
Viability (%)	92.50	5.98	95.50	90.75–96
Morphology (% of normal spermatozoa)	57.20	22.38	58.50	36.75–75.25
Abnormal heads (%)	10.90	7.90	9.50	5.50–14.25
Abnormal midpieces (%)	18.40	21.50	3	1–7.25
Abnormal tails (%)	13.35	13.60	8	7.75–15.25

Table 1
Individual characteristics of twenty healthy dogs included in the present study.

Animal	Breed	Age (months)	Body weight (kg)	Group[a]	Living environment[b]	Number of reads
A	Basset fauve de Bretagne	44	11.8	N	K	597
B	Basset fauve de Bretagne	50	13.7	N	K	749
C	Border collie	24	20.1	T	P	1483
D	American Staffordshire terrier	38	28.6	T	K	788
E	Border collie	41	22.5	T	P	3150
F	Basset fauve de Bretagne	33	14.4	N	K	1486
G	Great Dane	49	62.6	N	P	4589
H	Bassett hound	97	25.2	T	P	555
I	Viszla	15	28.5	N	K	757
L	Border collie	63	25.6	T	P	5053
M	Pug	23	10.7	T	P	9781
N	Border collie	36	24.4	N	P	7677
O	Border collie	48	26.4	N	P	15,602
P	German shepherd	15	34.6	N	P	6141
Q	English Springer Spaniel	28	24.8	T	P	6471
R	Rottweiler	79	39.3	T	P	9644
S	Bassett fauve de Bretagne	125	16.2	T	K	7040
T	Bassett fauve de Bretagne	20	11.7	N	K	3568
U	American Staffordshire terrier	23	29.2	N	K	1909
Z	Bullmastiff	61	70.1	T	P	1322

[a] Normospermia (N) or teratozoospermia (T).
[b] Pet (P) or Kennel (K).

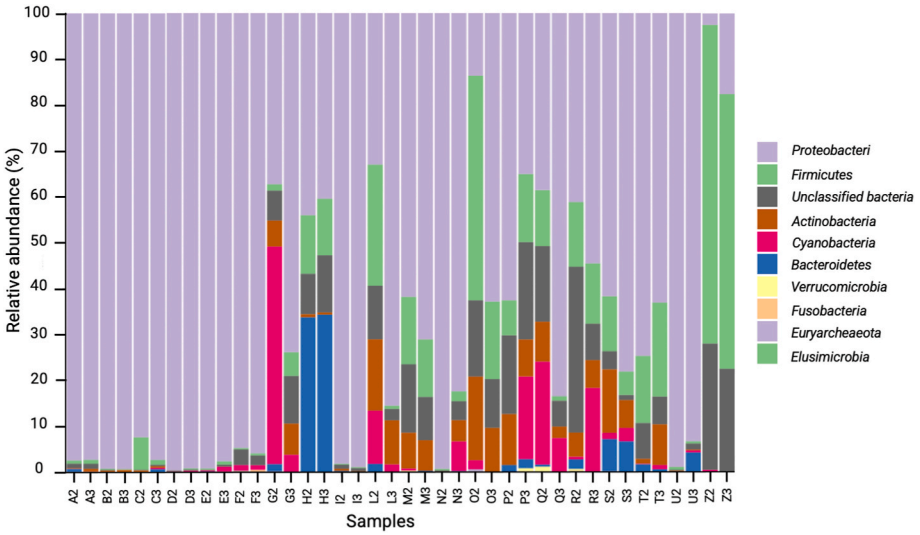

Fig. 1. Phylum distribution as a percentage of the total number of identified sequences in individual samples. The same letter indicates samples collected from the same dog and ejaculate ("2" for the sperm-rich fraction and "3" for the prostatic fraction of the ejaculate, respectively).

evaluating beta-diversity assessed by Bray-Curtis dissimilarity and Unweighted UniFrac algorithms. Results are summarized in Table 3. The only comparisons showing significant differences using both algorithms were i) between biological samples (i.e., dog ejaculates) and controls and ii) between pet and kennel dogs (PERMANOVA $P < 0.05$ in both cases) (Fig. 3). No differences based on the fraction of the ejaculate or based on the group were found.

Two dimensions scaling plots were extrapolated for both algorithms and clustering by Bray-Curtis is represented in Fig. 2.

The semen of pet dogs was enriched in *Pasterurellaceae*, *Bacillaceae*, and *Sphingomonadaceae* (*Sphingomonas*) and presented a lower bacterial load compared to kennel dogs, whose semen was mainly enriched in *Pasteurellaceae*. Interestingly, kennel dogs presented a higher bacterial load compared to pet dogs (median 9566 reads/sample, IQR 11769–8258.75 and 6296, IQR 7694–3985.25; $P = 0.003$).

The structure of bacterial communities did not differ significantly based on breed, fraction of the ejaculate, and group ($P > 0.05$).

4. Discussion

In the present study, 16S rRNA sequencing was used to explore the microbiota of dog semen. Specifically, we described the most common bacterial populations in a group of overall healthy dogs housed either in breeding facilities or in a domestic environment.

Bacterial genetic material was sequenced in all the processed samples, although the bacterial load was generally low, and intra-sample diversity (i.e., alpha diversity) did not differ between semen samples and controls. However, bacterial composition of the semen was different when compared with controls in terms of beta-diversity (i.e., inter-sample diversity). Although these results must be carefully interpreted as the number of semen samples was higher compared to that of controls, our purpose was to confirm that bacterial presence was not caused by contamination during sampling and laboratory procedures. The prepuce was cleaned using a sterile gauze before collection, although few bacteria could remain on the prepuce. Future research should aim to compare the microbiome of the prepuce with that of the ejaculate, to detect which bacteria tend to swim up the urethra. Bacterial viability was not assessed, as growth in culture remains a gold standard to confirm viability, but majority of bacteria does is considered unculturable [24]. This obstacle is overcome by sequencing techniques, that detect bacterial sequences belonging to unculturable microorganisms. Furthermore, the presence of bacteria in the ejaculate of healthy dogs has been previously described using culture-dependent methods [15]. Specifically, bacterial growth was previously found to be positive in all three fractions of the canine ejaculate (i.e., pre-sperm, sperm-rich, and prostatic). The pre-sperm fraction was indicated as the most contaminated one, presenting bacterial growth in 89.1 % of cases, probably exerting the function of cleaning the urethra. We did not perform any analysis on the pre-sperm fraction, as it is always discarded when semen is collected for routine analyses or for ARTs. We focused on the second and third fractions, that mirror the fluid in which the spermatozoa are suspended and the status of the prostate, respectively. We found no differences in terms of bacterial populations between these two fractions. This means that analyses for microbiome studies in dogs can be performed on the third fraction of the ejaculate in dogs that present no clinically detectable prostatic disease. The advantage of using only the third fraction for the semen microbiome is twofold: it is making the whole second sperm rich fraction available for semen quality assessment and other procedures (e.g., artificial insemination, cryopreservation), and it is avoiding performing the extraction on a substrate highly

Table 3
Comparisons of beta diversities between/among sample features determined with Bray-Curtis and Unweighted UniFrac algorithms are reported.

Comparison	Group 1	Group 2	Bray-Curtis			Unweighted UniFrac		
			PseudoF	P value	PERMANOVA P value	PseudoF	P value	PERMANOVA P value
Biological samples (i.e., dog ejaculates)	Controls	Biological samples	2.90	0.007*	0.006*	1.7716	0.046*	0.058
Fraction of the ejaculate	Second (F2)	Third (F3)	1.045921	0.334	0.029*	1.1255	0.267	0.071
Group	Normospermia	Teratozoospermia	1.9469	0.070	0.011*	0.916570	0.499	0.115
Environment	Kennel	Pet	4.7336	0.001*	0.001*	2.399827	0.012*	0.004*

*Significance for $P < 0.05$.

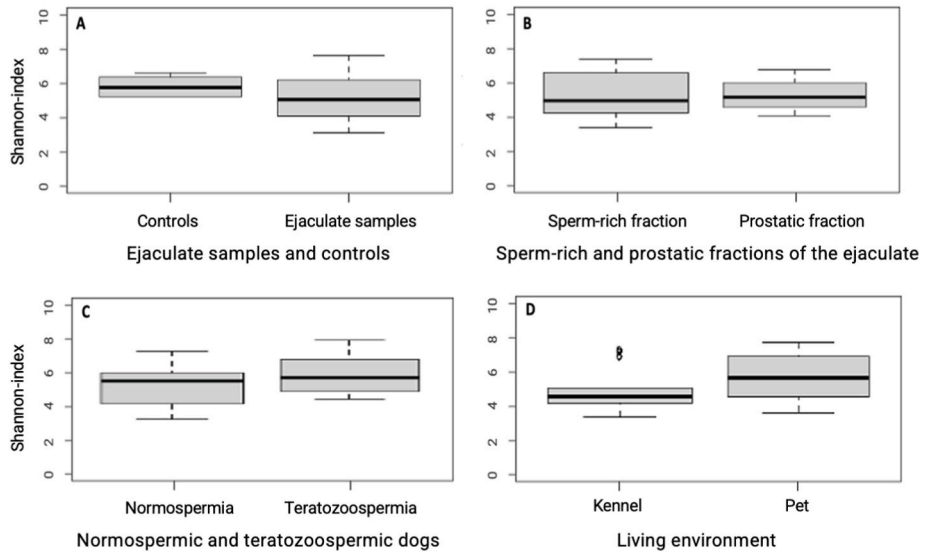

Fig. 2. Alpha-diversities (Shannon index) comparisons based on A) type of sample (biological samples from the ejaculate and controls); B) fraction of the ejaculate (second and third fractions); C) group (normospermia and teratozoospermia); D) living environment of the animal (kennel or pet living in-house).

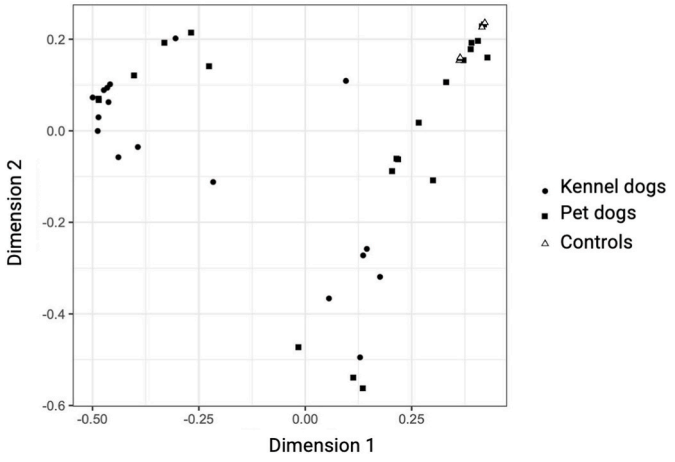

Fig. 3. Two dimensions scaling plot by Bray-Curtis based on the living environment: kennel (black dots), pet (black squares), and controls (white triangles). Majority of kennel dogs clustered together, as majority of pet dogs clustered together, meaning that the bacterial diversity is higher between dogs coming from different type of living environment.

contaminated with eukaryotic DNA (i.e., spermatozoa). Nevertheless, an intriguing target for future research could be the assessment of possible differences between fractions of the ejaculate in cases of prostatic disease. Parallelly, the hypothesis of the sperm-rich fraction mirroring the testicular microbiome and the prostatic one mirroring that of the prostate, should be also confirmed by studies specifically sampling these organs and investigating their microbiota, as done for men testicular tissue [3]. The possibility that the bacterial sequences detected in the present study could derive from ascending migration from the urethra cannot be ruled out, although sperm is never collected bypassing this organ, except for epididymal sperm, which is an extremely different scenario out of the purpose of the present study.

The dominant phyla in the ejaculates of healthy dogs fed a standardized diet were Proteobacteria, Firmicutes, and Actinobacteria. These phyla, together with Bacteroidetes, match those that dominate the human semen microbiota [18,25–28]. Although freezing at −80 °C and thawing all the sample at the same time for DNA extraction is a routine procedure [16,25], previous research found that thawed samples have a slightly different microbiome composition in terms of relative abundance in stool samples [29]. This has never been investigated for dog semen. Nevertheless, the present study confirms that the microbiota of the canine semen and that of human semen have a different microbial composition when analyzed at a deeper taxonomic level. For instance, the genus *Lactobacillus* was reported as an indicator of seminal health in men [13,18,25] but it was never sequenced in the dogs included in the present study. However, many bacterial sequences remained unclassified at lower taxonomic levels, not allowing to exclude the presence of any specific bacteria. In dogs, sequences assigned to the bacterial family *Pasteurellaceae* were overall the most abundant and no other family was represented likewise. Interestingly, bacteria belonging to this family were rarely isolated in culture from canine ejaculates [15] and molecular studies in humans highlighted their presence in samples from normospermic men [25,30] and individuals with hypomotile spermatozoa [18]. However, comparisons with previous literature are limited by the lack of studies applying molecular methods to describe the bacterial populations in dog semen. Furthermore, we found that the living environment influences the seminal microbiota, possibly complicating comparisons between different canine populations. Cohabitation is known to be associated with similarities in the gut microbiota [31–33], not only within individuals of the same species, but also between dogs and humans [33,34]. This strengthens the idea that the environment is a key factor in shaping the reproductive microbiota. In this regard, the diet is a possible confounding factor, for it is known to influence the bacterial populations in the gut. Hence, the present research was conducted on dogs that followed the same diet for the two months preceding the assessment of the semen microbiota. This suggests that living in-house or in a kennel influences the semen characteristics in terms of bacterial populations. On the other hand, the standardized diet, together with the fact that all the dogs were healthy, might have mitigated some differences in the seminal microbiota and this should be considered when conducting future research. Semen bacterial communities were not different between normospermic and teratozoospermic dogs, although this does not allow neither to exclude nor confirm that the semen microbiota influences semen parameters or fertility. Furthermore, some animals had a percentage of normal spermatozoa slightly higher or lower compared to the set cut off value of 60 %, this could have reduced the possibility to detect real differences in the bacterial communities within the two groups. As mentioned, only healthy dogs were included in the present study and their fertility in terms of successful mating and litter production was not taken into account. Since the

collection method influences the semen characteristics [35], we standardized our protocol by performing collection in the absence of a teaser bitch. Specifically, the presence of a female in estrus is associated with higher total sperm output, although it is not associated with sperm morphology [35]. Morphological abnormalities of the spermatozoa could lead to a decrease in motility parameters and fertility [36,37] and the inclusion of subfertile and infertile dogs will be a further step in this field of research. Moreover, targeted research is needed to investigate the presence and abundance of certain controversial bacteria, whose relationship with fertility is suspected but remains unclear. One specific example is that of bacteria belonging to the genera *Mycoplasma* and *Ureaplasma* [38]. When culture targeting *Mycoplasma* spp. is used, the prevalence of these bacteria in the genital tract of the canine population reaches almost 89 % [36] and it seems higher in poor quality ejaculates compared to high quality ones [38–40]. Hence, it is possible that only some species have a pathogenic potential on the canine reproductive tract, although it remains unknown which species this would be [36]. Interestingly, sequences belonging to *Mycoplasma* spp. or to the phylum Tenericutes were not found in the present study. However, the presence of these bacteria cannot be completely ruled out because some sequences remained unclassified and because PCR protocols specifically targeting *Mycoplasma* spp. may be needed [41,42]. The relationship between *Mycoplasma* and fertility in dogs should be a focus for future research, because the role of these bacteria may be overestimated in cases of infertility, leading to unnecessary antimicrobial treatments.

In conclusion, the present study aimed to provide seminal knowledge on the bacterial composition of ejaculates from healthy dogs, opening a novel chapter in canine andrology. The living environment affects the semen microbiome of dogs and further studies should include non-healthy animals and target specific bacteria. Further research can be conducted by performing molecular analyses using the third fraction of the ejaculate.

CRediT authorship contribution statement

P. Banchi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **L. Bertolotti:** Data curation, Supervision. **L. Spanoghe:** Writing – review & editing. **H. Ali Hassan:** Investigation. **J. Lannoo:** Investigation. **G. Domain:** Investigation. **K.S. Henzel:** Resources, Writing – review & editing. **V. Gaillard:** Resources, Writing – review & editing. **A. Rota:** Supervision, Writing – review & editing. **A. Van Soom:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

VG and KH were employed by Royal Canin, which produces the dog food supplied to the dogs included in the present study and funded the research. They participated in the final revision of the paper, but their commercial affiliation does not interfere with the full and objective presentation of the results of this work. The other authors have no conflicts of interest to declare.

The authors also thank all the breeders who cooperated in the study.

Acknowledgements

Funding: This work was supported by Royal Canin SAS for the expenses related to sample collection and processing. The same company also provided the food for the enrolled animals.

References

[1] Yagi K, Huffnagle GB, Lukacs NW, Asai N. The lung microbiome during health and disease. Int J Mol Sci 2021;22(19):10872. https://doi.org/10.3390/ijms221910872.

[2] Okwelogu SI, Ikechebelu JI, Agbakoba NR, Anukam KC. Microbiome compositions from infertile couples seeking in vitro fertilization, using 16S rRNA gene sequencing methods: any correlation to clinical outcomes? Front Cell Infect Microbiol 2021;11:709372. https://doi.org/10.3389/fcimb.2021.709372.

[3] Alfano M, Ferrarese R, Locatelli I, Ventimiglia E, Ippolito S, Gallina P, Cesana D, Canducci F, Pagliardini L, Viganò P, Clementi M, Nebuloni M, Montorsi F, Salonia A. Testicular microbiome in azoospermic men-first evidence of the impact of an altered microenvironment. Hum Reprod 2018;33(7):1212–7. https://doi.org/10.1093/humrep/dey116.

[4] Mason SJ. An update on male canine infertility. Vet Clin North Am Small Anim Pract 2023;21(23):S0195–5616. https://doi.org/10.1016/j.cvsm.2023.04.006.00068-2.

[5] Brandão P, Gonçalves-Henriques M, Ceschin N. Seminal and testicular microbiome and male fertility: a systematic review. Porto Biomed J 2021;6(6):e151. https://doi.org/10.1097/j.pbj.0000000000000151.

[6] Farahani L, Tharakan T, Yap T, Ramsay JW, Jayasena CN, Minhas S. The semen microbiome and its impact on sperm function and male fertility: a systematic review and meta-analysis. Andrology 2021;9(1):115–44. https://doi.org/10.1111/andr.12886.

[7] Tompson AC, Mateus ALP, Brodbelt DC, Chandler CIR. Understanding antibiotic use in companion animals: a literature review identifying avenues for future efforts. Front Vet Sci 2021;8:719547. https://doi.org/10.3389/fvets.2021.719547.

[8] Zdunczyk S, Domoslawska A. Effect of drugs on fertility in male dogs: a review. Reprod Domest Anim 2022;57(9):949–56. https://doi.org/10.1111/rda.14173.

[9] Toson B, Simon C, Moreno I. The endometrial microbiome and its impact on human conception. Int J Mol Sci 2022;23(1):485. https://doi.org/10.3390/ijms23010485.

[10] Altmäe S, Franasiak JM, Mändar R. The seminal microbiome in health and disease. Nat Rev Urol 2019;16(12):703–21. https://doi.org/10.1038/s41585-019-0250-y.

[11] Lyman CC, Holyoak GR, Meinkoth K, Wieneke X, Chillemi KA, DeSilva U. Canine endometrial and vaginal microbiomes reveal distinct and complex ecosystems. PLoS One 2019;14(1):e0210157. https://doi.org/10.1371/journal.pone.0210157.

[12] Schuppe HC, Pilatz A, Hossain H, Diemer T, Wagenlehner F, Weidner W. Urogenital infection as a risk factor for male infertility. Dtsch Arztebl Int 2017;114(19):339–46. https://doi.org/10.3238/arztebl.2017.0339.

[13] Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, Yang T, Yang TL, Liu CY, Wu WY, Chang YA, Chang TH, Huang HD. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. PLoS One 2014;9(10):e110152. https://doi.org/10.1371/journal.pone.0110152.

[14] Weidner W, Pilatz A, Diemer T, Schuppe HC, Rusz A, Wagenlehner F. Male urogenital infections: impact of infection and inflammation on ejaculate parameters. World J Urol 2013;31(4):717–23. https://doi.org/10.1007/s00345-013-1082-7.

[15] Goericke-Pesch S, Weiss R, Wehrend A. Bacteriological findings in different fractions of canine ejaculates showing normospermia, teratozoospermia or azoospermia. Aust Vet J 2011;89(8):318–22. https://doi.org/10.1111/j.1751-0813.2011.00808.x.

[16] Koziol JH, Sheets T, Wickware CL, Johnson TA. Composition and diversity of the seminal microbiota in bulls and its association with semen parameters. Theriogenology 2022;182:17–25. https://doi.org/10.1016/j.theriogenology.2022.01.029.

[17] Quiñones-Pérez C, Hidalgo M, Ortiz I, Crespo F, Vega-Pla JL. Characterization of the seminal bacterial microbiome of healthy, fertile stallions using next-generation sequencing. Anim Reprod 2021;18(2):e20200052. https://doi.org/10.1590/1984-3143-AR2020-0052.

[18] Baud D, Pattaroni C, Vulliemoz N, Castella V, Marsland BJ, Stojanov M. Sperm microbiota and its impact on semen parameters. Front Microbiol 2019;10:234. https://doi.org/10.3389/fmicb.2019.00234.

[19] Soares JM, Avelar GF, França LR. The seminiferous epithelium cycle and its duration in different breeds of dog (Canis familiaris). J Anat 2009;215(4):462–71. https://doi.org/10.1111/j.1469-7580.2009.01122.x.

[20] Domain G, Banchi P, Ali Hassan H, Eilers A, Lannoo J, Wydooghe E, Niżański W, Van Soom A. Sperm gone smart: a portable device (iSperm®) to assess semen concentration and motility in dogs. Animals (Basel) 2022;12(5):652. https://doi.org/10.3390/ani12050652.

[21] Rijsselaere T, Van Soom A, Maes D, Nizanski W. Computer-assisted sperm analysis in dogs and cats: an update after 20 years. Reprod Domest Anim 2012;47(6):204–7. https://doi.org/10.1111/rda.12057.

[22] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7(5):335–6. https://doi.org/10.1038/nmeth.f.303.

[23] DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006;72(7):5069–72. https://doi.org/10.1128/AEM.03006-05.

[24] Kaeberlein T, Lewis K, Epstein SS. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 2002;296(5570):1127–9. https://doi.org/10.1126/science.1070633.

[25] Monteiro C, Marques PI, Cavadas B, Damião I, Almeida V, Barros N, Barros A, Carvalho F, Gomes S, Seixas S. Characterization of microbiota in male infertility cases uncovers differences in seminal hyperviscosity and oligoasthenoteratozoospermia possibly correlated with increased prevalence of

infectious bacteria. Am J Reprod Immunol 2018;79(6):e12838. https://doi.org/10.1111/aji.12838.
[26] Chen H, Luo T, Chen T, Wang G. Seminal bacterial composition in patients with obstructive and non-obstructive azoospermia. Exp Ther Med 2018;15(3):2884–90. https://doi.org/10.3892/etm.2018.5778.
[27] Mändar R, Punab M, Korrovits P, Türk S, Ausmees K, Lapp E, Preem JK, Oopkaup K, Salumets A, Truu J. Seminal microbiome in men with and without prostatitis. Int J Urol 2017;24(3):211–6. https://doi.org/10.1111/iju.13286.
[28] Mändar R, Punab M, Borovkova N, Lapp E, Kiiker R, Korrovits P, Metspalu A, Krjutškov K, Nõlvak H, Preem JK, Oopkaup K, Salumets A, Truu J. Complementary seminovaginal microbiome in couples. Res Microbiol 2015;166(5):440–7. https://doi.org/10.1016/j.resmic.2015.03.009.
[29] Chen A, Hu Y, Zhang Y, Li Z, Zeng Y, Pang X. Cryopreservation of stool samples altered the microbial viability quantitively and compositionally. Arch Microbiol 2022;204(9):557. https://doi.org/10.1007/s00203-022-03169-1.
[30] Hou D, Zhou X, Zhong X, Settles ML, Herring J, Wang L, Abdo Z, Forney LJ, Xu C. Microbiota of the seminal fluid from healthy and infertile men. Fertil Steril 2013;100(5):1261–9. https://doi.org/10.1016/j.fertnstert.2013.07.1991.
[31] Finnicum CT, Beck JJ, Dolan CV, Davis C, Willemsen G, Ehli EA, Boomsma DI, Davies GE, de Geus EJC. Cohabitation is associated with a greater resemblance in gut microbiota which can impact cardiometabolic and inflammatory risk. BMC Microbiol 2019;19(1):230. https://doi.org/10.1186/s12866-019-1602-8.
[32] Mosites E, Sammons M, Otiang E, Eng A, Noecker C, Manor O, Hilton S, Thumbi SM, Onyango C, Garland-Lewis G, Call DR, Njenga MK, Wasserheit JN, Zambriski JA, Walson JL, Palmer GH, Montgomery J, Borenstein E, Omore R, Rabinowitz PM. Microbiome sharing between children, livestock and household surfaces in western Kenya. PLoS One 2017;12(2):e0171017. https://doi.org/10.1371/journal.pone.0171017.
[33] Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, Caporaso JG, Knights D, Clemente JC, Nakielny S, Gordon JI, Fierer N, Knight R. Cohabiting family members share microbiota with one another and with their dogs. Elife 2013;2:e00458. https://doi.org/10.7554/eLife.00458.
[34] Zhao R, Hao J, Yang J, Tong C, Xie L, Xiao D, Zeng Z, Xiong W. The Co-occurrence of antibiotic resistance genes between dogs and their owners in families. iMeta 2022;1:e21. 10.102/imt2.21.
[35] Kustritz MV, Hess M. Effect of administration of prostaglandin F2alpha or presence of an estrous teaser bitch on characteristics of the canine ejaculate. Theriogenology 2007;67(2):255–8. https://doi.org/10.1016/j.theriogenology.2006.07.009.
[36] Tesi M, Sabatini C, Vannozzi I, Di Petta G, Panzani D, Camillo F, Rota A. Variables affecting semen quality and its relation to fertility in the dog: a retrospective study. Theriogenology 2018;118:34–9. https://doi.org/10.1016/j.theriogenology.2018.05.018.
[37] Mickelsen WD, Memon MA, Anderson PB, Freeman DA. The relationship of semen quality to pregnancy rate and litter size following artificial insemination in the bitch. Theriogenology 1993;39(2):553–60. https://doi.org/10.1016/0093-691x(93)90397-n.
[38] Domrazek K, Kaszak I, Kanafa S, Sacharczuk M, Jurka P. The influence of Mycoplasma species on human and canine semen quality: a review. Asian J Androl 2023;25(1):29–37. https://doi.org/10.4103/aja2021124.
[39] Johnston SD, Kustritz MV, Olson PN. Canine and feline theriogenology. first ed. London: Saunders; 2001.
[40] Laber G, Holzmann A. Experimentally induced mycoplasmal infection in the genital tract of the male dog. Theriogenology 1977;7:177–88. https://doi.org/10.1016/0093-691X(77)90214-X.
[41] Chalker VJ, Brownlie J. Taxonomy of the canine Mollicutes by 16S rRNA gene and 16S/23S rRNA intergenic spacer region sequence comparison. Int J Syst Evol Microbiol 2004;54(Pt 2):537–42. https://doi.org/10.1099/ijs.0.02869-0.
[42] Hemmatzadeh F, Niap F, Bennett BA, Trott DJ, Peaston AE. A novel quantitative polymerase chain reaction to monitor urinary tract mycoplasma infection in a dog. Lett Appl Microbiol 2019;68(5):409–14. https://doi.org/10.1111/lam.13139.