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# Double semen collection at a 1-h interval in dogs decreases the bacterial contamination of canine ejaculates

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ABSTRACT

Semen extenders usually contain antibiotics with the aim to minimize bacterial growth, but the indiscriminate use of antibiotics increases the emergence of multidrug-resistant bacteria. A limiting factor of semen processing in dogs is the low total sperm count that limits the number of insemination doses that can be obtained from one ejaculate. Therefore, two ejaculates collected at a short interval can be combined to increase the number of AI doses. In this study, semen was collected from dogs either once or the same dogs (n = 28) were submitted to dual semen collection 1 h apart. All ejaculates were submitted to bacteriological analysis. We hypothesized that bacterial contamination of semen is low but that a dual semen collection might increase contamination. A sample for bacteriological examination was taken from raw semen immediately after semen collection. Bacteria including mycoplasmas were isolated using conventional cultivation procedures and isolates were identified to the species level by matrix-assisted laser desorption ionization – time of flight (MALDI-ToF) mass spectrometry. In total, 22 bacterial species were identified in the 84 ejaculates with *Mycoplasma cynos*, *Streptococcus canis* and *Canicola haemoglobinophilus* being most frequent. Bacterial growth was sporadic in 16 and absent in 10 ejaculates. The overall bacterial growth was lower in the second than in the first ejaculate of dual semen collections ($p < 0.05$). The percentage of motile and membrane-intact spermatozoa in frozen-thawed ejaculates was not associated with the degree of bacterial contamination of raw semen. In conclusion, there was only limited microbial contamination in dog semen and the microorganisms isolated are considered part of the normal genital bacterial flora. Repeated semen collection reduced bacterial contamination in the second in comparison to the first ejaculate. The use of antibiotics in canine semen should be questioned.


## 1. Introduction

The dog is the first mammalian species were a pregnancy from artificial insemination (AI) has been reported [1], but only during the last decades AI with cryopreserved semen is increasingly used in dogs [2]. In AI in dogs (e.g. Refs. [3,4]), as in other animal species (e.g., bull [5]; stallion [6,7]), semen extenders usually contain antibiotics aimed at minimizing bacterial growth in extended semen. Furthermore, addition of antibiotic substances to semen for AI is often required by animal health regulations [8,9]. The indiscriminate and often inappropriate use of antibiotics has led to an increasing emergence and spread of multidrug-resistant bacteria. Without action to reverse these trends, the spectrum of antibiotics to effectively treat infectious disease in humans and in animals will increasingly dwindle [10]. Except for *Brucella canis*, bacterial pathogens only sporadically cause fertility problems in dogs and the requirement to add antibiotics to semen extenders should thus be questioned. Most bacterial infections of the reproductive tract are endogenous in origin and the bacteria etiologically involved are part of the physiological urogenital microflora. Bacterial reproductive disease is therefore mainly opportunistic and requires predisposing factors to develop (reviewed by Ref. [11]).

A limiting factor of AI in dogs is the low number of insemination doses obtained per ejaculate [2,3,12,13]. When two ejaculates were collected from dogs at a 1-h interval, there was no difference in sperm quality [14] and when the two ejaculates were combined and cryopreserved the number of AI doses increased by 70%

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compared to cryopreservation of only one ejaculate [2]. On the other hand, in stallions, bacterial numbers in semen were higher with more than one intromission before ejaculation [15]. Repeated semen collections may thus also increase bacterial contamination of a combined ejaculate in dogs.

In the present study, semen was collected from dogs either once or the same dogs were submitted to a dual semen collection at a 1-h interval [2]. Samples from all ejaculates were submitted to bacteriological examination. We hypothesized that dog semen in general is free from obligatory pathogenic bacteria and also contamination with opportunistic bacteria is low. A dual semen collection might, however, increase the bacterial contamination of dog semen.

## 2. Material and methods

The study was approved by the Ethics and Animal Welfare Committee of Vetmeduni Vienna (study number ETK-014/01/2020). Informed consent was obtained from all dog owners before their animals participated in the study.

### 2.1. Animals

Dogs to be included into the study were recruited from breeders who are regular clients of Vetmeduni Vienna or members of a local kennel club. Out of initially 46 dogs, 11 were excluded for azoospermia (n = 3), general health problems (n = 1), prepubertal age (n = 1), or because semen collection was not possible (n = 6). The remaining 35 dogs were 5.1 ± 3.3 (mean ± SD) years old (range 1.2–13.2 years) and belonged to 19 different breeds as described previously [2]. The most frequent breeds were Beagle (n = 9) and English Cocker Spaniel, Border Collie and Australian Shepherd (n = 3 each). Before they were included into the study, dogs had at least one week of sexual rest. To the best of our knowledge, none of the dogs received antibiotics during the last two months before semen collection, based on owner questionnaire. Clinical and ultrasound examination of the genital organs was performed to ascertain general and genital health of the dogs before proceeding with semen collections. Dual semen collection was successful in 28 out of 35 dogs and results were included only for the 28 dogs where all semen collections were successfully performed.

### 2.2. Experimental design

All dogs were submitted to two semen collection sessions one week apart. At one occasion, only one ejaculate was collected, processed immediately after collection and the sperm rich fraction cryopreserved within 1 h. On the other occasion, two ejaculates were collected at an approximate 1-h interval (57 ± 6 min, range 45–73 min), the two sperm rich fractions were processed for cryopreservation and combined before filling the processed semen into straws. Aliquots from all ejaculates were submitted for bacteriological analysis. Results on ejaculate and semen characteristics and the number of AI doses collected have been published previously [2].

### 2.3. Semen collection and analysis

Semen was collected manually in three fractions (pre-sperm, sperm-rich, third fraction) as described previously [2]. The person performing the semen collection was wearing sterile latex gloves. Erection and emission of the dog's penis was stimulated manually without prior cleaning or disinfection of the prepuce. When the dog started friction movements, a sterile, pre-warmed tulip-glass was held in place to collect the ejaculate with exchange of the glass after each ejaculate fraction. The sperm-rich-fraction was used for the study and a sterile swab (Aptaca S.p.A, Canelli, AT, Italy) was taken immediately after semen collection, placed in Amies agar transport medium, and brought to the microbiological laboratory within 60 min. Thereafter, the sperm-rich fraction was analyzed for volume, pH and sperm concentration and the total sperm count was calculated as described previously [2]. Semen was then cryopreserved with a computer-controlled rate freezer (Ice Cube 14 M, Sylab, Purkersdorf, Austria). After centrifugation and re-dilution of ejaculates before cryopreservation and after freezing-thawing. the percentage of motile, progressively motile and membrane-intact spermatozoa was determined by computer-assisted sperm analysis (CASA; SpermVision, Minitube, Tiefenbach, Gemany). For assessment of membrane integrity, SYBR-14/PI (Minitube) was used [16–18]. Data on sperm characteristics have been published previously [2].

**Table 1**
Incidence and intensity of bacterial growth in canine ejaculates collected only once (single collection) or twice at an 1 h interval (dual collection, first and second ejaculate).

| Bacterial species | Single collection | | | | | | Dual collection, first ejaculate | | | | | | Dual collection, second ejaculate | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | neg | 0.5 | 1 | 2 | 3 | total | neg | 0.5 | 1 | 2 | 3 | total | neg | 0.5 | 1 | 2 | 3 | total |
| *Mycoplasma arginini* | 24 | 0 | 1 | 1 | 1 | 3 | 26 | 0 | 1 | 0 | 0 | 1 | 26 | 1 | 0 | 0 | 0 | 1 |
| *Mycoplasma canis* | 25 | 0 | 1 | 1 | 0 | 2 | 25 | 1 | 0 | 1 | 0 | 2 | 26 | 0 | 1 | 1 | 0 | 2 |
| *Mycoplasma cynos* | 20 | 3 | 0 | 1 | 3 | 7 | 17 | 1 | 2 | 5 | 2 | 10 | 19 | 1 | 0 | 5 | 2 | 8 |
| *Mycoplasma edwardii* | 25 | 0 | 0 | 1 | 1 | 2 | 26 | 0 | 0 | 1 | 0 | 1 | 26 | 0 | 0 | 1 | 0 | 1 |
| *Mycoplasma* species novum | 25 | 0 | 0 | 1 | 1 | 2 | 22 | 0 | 0 | 4 | 1 | 5 | 22 | 1 | 1 | 2 | 1 | 5 |
| *Mycoplasma spumans* | 25 | 0 | 1 | 1 | 0 | 2 | 26 | 0 | 1 | 0 | 0 | 1 | 26 | 0 | 1 | 0 | 0 | 1 |
| *Ureaplasma canigenitalium* | 24 | 0 | 1 | 2 | 0 | 3 | 23 | 0 | 1 | 2 | 1 | 4 | 24 | 0 | 1 | 1 | 1 | 3 |
| *Canicola haemoglobinophilus* | 19 | 3 | 1 | 3 | 1 | 8 | 20 | 6 | 1 | 0 | 0 | 7 | 22 | 4 | 1 | 0 | 0 | 5 |
| *Streptococcus canis* | 20 | 3 | 0 | 4 | 0 | 7 | 22 | 2 | 0 | 3 | 0 | 5 | 22 | 4 | 0 | 1 | 0 | 5 |
| *Staphylococcus chromogenes* | 27 | 0 | 0 | 0 | 0 | 0 | 26 | 1 | 0 | 0 | 0 | 1 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Staphylococcus haemolyticus* | 27 | 0 | 0 | 0 | 0 | 0 | 26 | 1 | 0 | 0 | 0 | 1 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Staphylococcus pseudintermedius* | 24 | 4 | 0 | 0 | 0 | 4 | 25 | 2 | 0 | 0 | 0 | 2 | 26 | 1 | 0 | 0 | 0 | 1 |
| *Mammaliicoccus sciuri* | 26 | 1 | 0 | 0 | 0 | 1 | 27 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Lactobacillus* species | 26 | 1 | 0 | 0 | 0 | 1 | 24 | 2 | 1 | 0 | 0 | 3 | 26 | 1 | 0 | 0 | 0 | 1 |
| *Pseudomonas aeruginosa* | 26 | 1 | 0 | 0 | 0 | 1 | 27 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Pasteurella dagmatis* | 27 | 0 | 0 | 0 | 0 | 0 | 26 | 1 | 0 | 0 | 0 | 1 | 27 | 0 | 0 | 0 | 0 | 0 |
| Anaerobes (unspecified) | 26 | 1 | 0 | 0 | 0 | 1 | 27 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Escherichia coli* | 26 | 1 | 0 | 0 | 0 | 1 | 26 | 1 | 0 | 0 | 0 | 1 | 26 | 1 | 0 | 0 | 0 | 1 |
| haemolytic *Escherichia coli* | 26 | 1 | 0 | 0 | 0 | 1 | 25 | 0 | 0 | 2 | 0 | 2 | 25 | 1 | 1 | 0 | 0 | 2 |
| *Enterococcus faecium* | 27 | 0 | 0 | 0 | 0 | 0 | 25 | 2 | 0 | 0 | 0 | 2 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Bacteroides fragilis* | 26 | 0 | 0 | 0 | 1 | 1 | 27 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 0 | 0 |
| *Enterococcus hirae* | 27 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 0 | 0 | 26 | 1 | 0 | 0 | 0 | 1 |

Intensity of bacterial growth: 0 = negative, 0.5 = sporadic, 1 = slight, 2 = moderate, 3 = pronounced.

### 2.4. Bacteriological examination

Bacteria including *mycoplasmas* were cultivated from swab samples as described previously [19] with slight modifications. Briefly, swabs were plated onto two Columbia agar III with 5% sheep blood, and one MacConkey II agar plates (BD Diagnostics, Vienna, Austria) using the three-phase streaking methods. Plates were incubated aerobically (Columbia agar, MacConkey agar) or anaerobically (Columbia agar) at 37 °C for 48 h. Microbial growth was semi-quantitatively graded as sporadic, slight, moderate, or pronounced depending on the occurrence and number of isolated colonies in streaking sections (sporadic <5, slight 5–30, moderate 31–100, pronounced >100 colonies). For the isolation of mycoplasmas and ureaplasmas, swab samples were placed into 1 ml 2SP medium, vortexed, and 100 µl of suspension plated onto SP4 agar and Ureaplasma agar medium. Plates were incubated at 37 °C under 6% $CO_2$ atmosphere for up to 7 days and checked daily for the presence of *mycoplasma* and *ureaplasma* colonies. For semi-quantification, colonies were counted and mycoplasma/ureaplasma growth graded as described above. Bacterial and *mycoplasma* isolates were identified to the species level using matrix-assisted laser desorption ionization – time of flight (MALDI-ToF) mass spectrometry as described previously [19,20]. *Ureaplasma* isolates (*Ureaplasma canigenitalium*) were identified based on their characteristic colony morphology and on urease activity leading to a colour change of the agar medium.

### 2.5. Statistical analysis

For statistical comparisons, the bacterial growth classifications of all bacteria isolated was summed up for each ejaculate. The sum of scores and the number of bacterial species isolated between ejaculates (single collection, dual collection – first ejaculate and dual collection – second ejaculate) were then compared by Friedman test, taking into account that different ejaculates were obtained from the same animals. For analysing effects of bacterial growth on semen characteristics in frozen-thawed ejaculates, the ejaculates were grouped by degree of bacterial growth as follows: bacterial growth classification <1 (n = 9), bacterial growth ≥1 and < 3 (n = 8) and bacterial growth ≥3 (n = 10). These groups were then compared by Kruskal-Wallis-H test. All statistical comparisons were made with the SPSS statistics software (version 28; IBM-SPSS, Armonk, NY, USA). A p-value <0.05 was considered significant and results are presented as scatterplots with the median and individual values.

## 3. Results

In total, 22 different bacterial species were identified in 84 canine ejaculates with *Mycoplasma cynos*, *Streptococcus canis* and *Canicola haemoglobinophilus* being the most frequent. No bacterial growth was detected in 10 of the 84 ejaculates collected and bacterial growth was classified as sporadic in 16 ejaculates (see Table 1).

When summarizing the growth score of all bacteria in a single ejaculate, a reduced bacterial growth was demonstrated in the second ejaculate collected within 1 h compared to the first ejaculate of the dual semen collection and the ejaculate from the single semen collection ($p < 0.05$; Fig. 1a). The number of bacterial species isolated from semen did not differ significantly among the three different ejaculates collected from each dog. On average 1.5 ± 1.0 bacterial species were isolated per ejaculate with a maximum of 4 different bacterial species in 3 ejaculates (Fig. 1b).

When the percentage of motile, progressively motile and membrane-intact spermatozoa in frozen-thawed ejaculates was compared among ejaculates differing in bacterial contamination, no differences among groups could be demonstrated (Fig. 2).

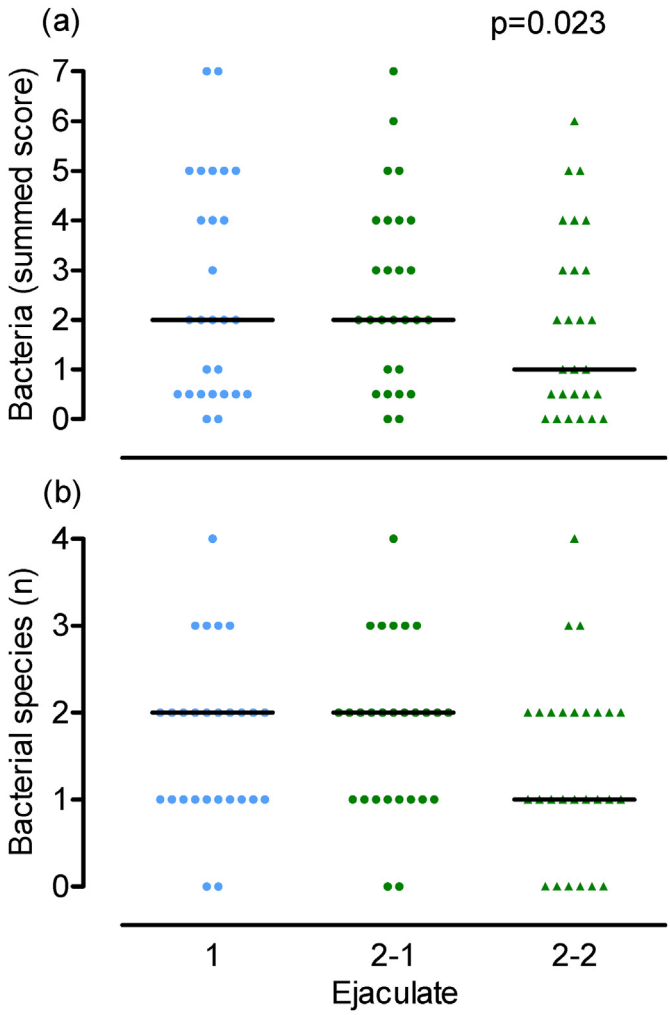


**Fig. 1.** (a) Summarized growth score of all bacteria per ejaculate and (b) number of different bacterial species isolated per ejaculate (ejaculates blue - 1 = single semen collection, green dots - 2-1 = first ejaculate of dual semen collection, green triangle - 2-2 = second ejaculate of dual semen collection). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## 4. Discussion

This study indicates a reduction in bacterial contamination of ejaculates from dogs when dual semen collections were performed. The second ejaculate thus does not pose a higher risk of transmitting bacteria from the male to the female dog and, in contrast, showed less bacterial growth than the first ejaculate. Within one ejaculate, bacterial contamination has been demonstrated to decrease with subsequent fractions [21,22] and our study extends these findings to ejaculates collected at short interval from the same dog.

Taking into account all bacteriological results, our study demonstrates only limited microbial contamination in a total of 84 ejaculates collected from 28 different dogs. In general, most of the microorganisms isolated are considered part of the normal bacterial flora residing in the oral cavity and urogenital tract of healthy dogs that only occasionally cause opportunistic infections [11,23]. Infection of the canine male reproductive tract is usually associated with cytologic evidence of inflammation and decreased semen

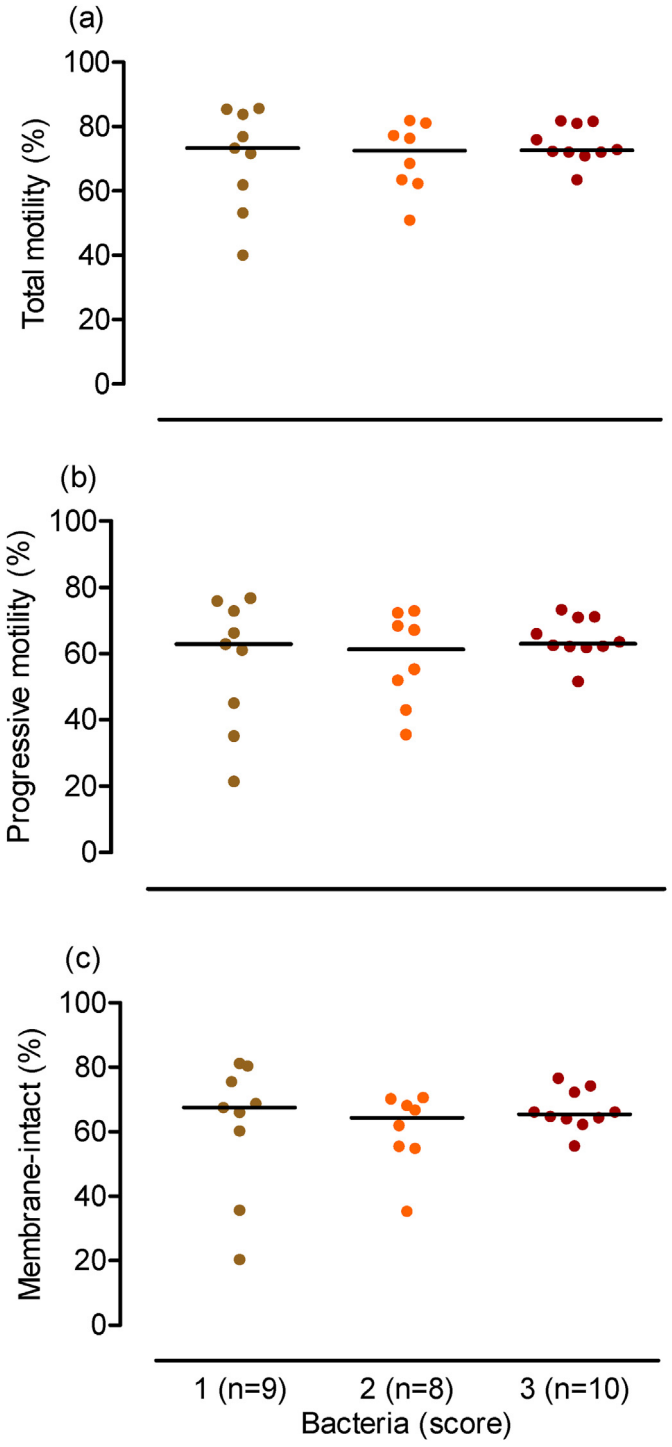


**Fig. 2.** (a) Total motility, (b) progressive motility and (c) membrane-intact spermatozoa in frozen-thawed semen differing in the degree of bacterial contamination. There are no significant differences among groups. Degree of bacterial growth is classified from summarized growth of all bacteria per ejaculate (brown - score 1: bacterial growth <1, orange - score 2: bacterial growth ≥1 and < 3, red - score 3: bacterial growth ≥3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

quality [24]. In our study, the bacteria detected had neither an influence on semen characteristics nor were they associated with abnormal findings on clinical examination of the genital tract including the prostate gland [2]. The presence of bacteria in semen therefore can be considered a non-pathological contamination without negative effects on semen quality.

*Mycoplasma cynos* was the microorganism most frequently isolated from semen in our study, confirming previous reports [25]. Despite considerable research, negative effects of mycoplasmas on canine fertility remain doubtful and to the best of our knowledge have not been demonstrated convincingly so far [4,11,26–29]. In contrast, *Ureaplasma canigenitalium* has been suggested to potentially reduce fertility in dogs [25] but was rarely detected in the present study. Unlike *M. cynos*, which appears to be host-specific for canines and is associated with respiratory disease [30–32], *M. canis* shows a broader host range and has been isolated from the lower respiratory tracts of humans with pneumonia [33] and both healthy and pneumonic cattle [34]. *Mycoplasma canis*, however, was isolated only from the ejaculates of two dogs in the present study.

The beta-hemolytic *Streptococcus canis* is potentially pathogenic and has been linked to diseases of the reproductive tract but beta-hemolytic streptococci have also been isolated frequently from the urogenital tracts of clinically healthy male and female dogs [11]. Although there may be some evidence for zoonotic potential of *Streptococcus canis* [35], clinical signs associated with its isolation from the reproductive tract of dogs are not evident [36]. *Canicola haemoglobinophilus*, formerly known as *Haemophilus haemoglobinophilus* [37] was among the most frequent bacterial isolates from semen in our study, corroborating other studies [38]. *Canicola haemoglobinophilus* belongs to the *Pasteurellaceae* family [38] and has been associated with neonatal mortality in puppies [39] but not reproductive failure. *Staphylococcus pseudointermedius* is a frequent colonizer of the skin and mucous membranes of dogs [11,40] and its infrequent detection in semen can be considered an occasional contamination. *Escherichia coli* can cause opportunistic infections of the genital tract in dogs [11], but was only recovered from semen of one dog in our study. *Escherichia coli* usually originates from the intestinal microflora and its near absence in semen indicates that contamination during semen collection can successfully be avoided with appropriate collection techniques.

With regard to the physiological microbial flora in dog semen, our study confirms and extends previous reports. Based on other studies, the urethra is considered the main source of contamination, with additional contribution from the prepuce and prostate gland. Secondary contamination may occur during semen collection but can be controlled by adequate hygienic procedures [21,41,42]. Our study adds further emphasis on questioning the routine use of antibiotics in canine semen extenders. Neither were pathogenic bacteria detected in semen nor were post-thaw semen characteristics negatively affected by the degree of bacterial contamination, at least in the range as was determined in our study. Post-thaw semen characteristics and the presence of bacteria, however, should be directly compared in semen frozen both with and without addition of antimicrobial substances in further studies.

It has been suggested that dog semen should not contain more than 10.000 bacteria per mL [24] and given adequate hygienic procedures are followed at semen collection and preservation, this can easily be achieved in healthy dogs. For a reduction in the bacterial load of semen, alternatives to semen extenders with antibiotics have been suggested. In dogs, these include colloid centrifugation of semen [42] but also repeated semen collections as in the present study. Reducing the use of antibiotics may help to slow the development of antibiotic resistance in animals and humans. With regard to semen quality, the addition of certain antibiotics in higher concentrations has also been shown to negatively affect fertility of preserved semen [43].

The present study may have two limitations, one being the fact that bacteria were detected by conventional cultivation procedures that fail to identify a majority of organisms inhabiting a given

ecosystem. Further studies on the microbiome in canine semen using culture-independent techniques such as 16S rRNA gene sequence analysis may thus be justified. Furthermore, with the present study restricted to laboratory-based parameters, insemination trials are needed to confirm that the omission of antibiotics in semen extender does not impair fertility when such semen is employed with the aim to produce offspring.

In conclusion, our study showed only limited microbial contamination in canine ejaculates collected for semen preservation. The routine use of antibiotics in dog semen should thus be questioned. Repeated semen collections resulted in a reduction of bacterial contamination in canine ejaculates suggesting this as a procedure for semen production in dogs with higher bacterial load in the genital tract.

## Credit author statement

Dominik Lechner: designed the project, performed the experimental animal work, the semen analysis, compiled the data, wrote and edited the manuscript. Jörg Aurich: compiled the data, wrote and edited the manuscript. Joachim Spergser: the bacteriological examination. Christine Aurich: designed the project, the semen analysis, wrote and edited the manuscript.

## Declaration of competing interest

None of the authors has any conflict of interest to declare.

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