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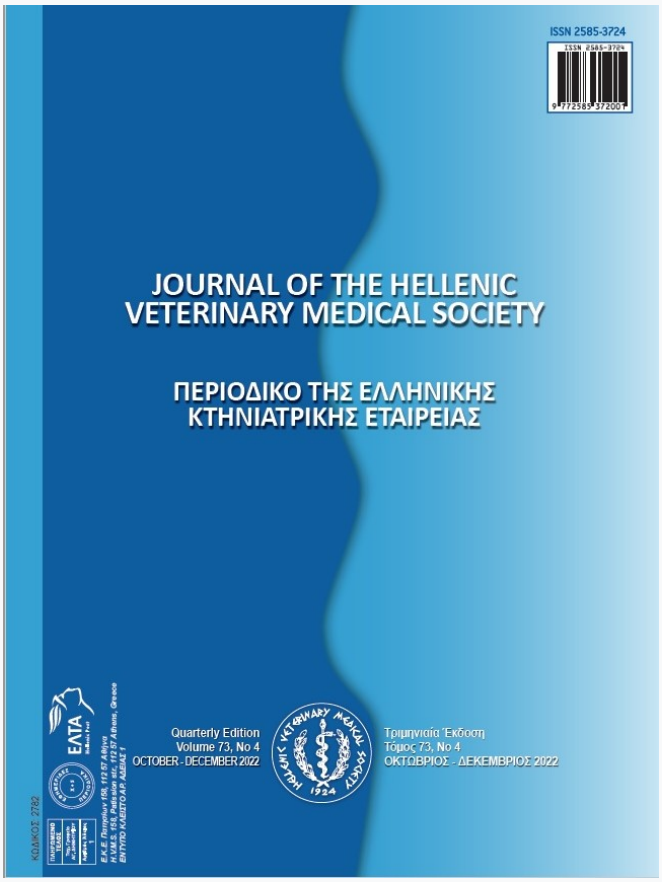

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Bacteriospermia assessment and its relationship with conventional seminal parameters in stud dogs ejaculates (*Canis familiaris*)

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ABSTRACT: The objective of this work was to determine the frequency of bacteriospermia and its effect on the seminal quality in dogs. According to weight, dogs were divided into small dogs between 1 and 10 kg (Group 1) and dogs with more than 10 kg (Group 2). The foreskin was disinfected in each animal (n=15), and the semen sample was collected by the gloved hand method. Sperm motility, morphology, viability, and concentration were evaluated using a 40x microscope. A 10µL of semen drop was cultured by diffusion method on blood agar and MacConkey agar. Colony-forming units (CFU) were quantitatively evaluated, and biochemical identification was carried out after 48 hours at 37°C. Conventional PCR was performed on the semen samples to evaluate the presence/absence of 11 bacteria. Bacterial growth was found in all samples. The CFU/mL in blood agar were 34042.8 for group 1 and 107714.3for group 2, while on MacConkey agar were 142.9 CFU/mL, and 21328.6 CFU/mL, respectively. *Coagulase-negative Staphylococcus* was the most frequent bacteria isolated by conventional culture (64.3%), and *Staphylococcus aureus* and *Klebsiella spp.* were the most common bacteria found by conventional PCR. A statistically significant difference was found between both groups in normal sperm morphology. A negative correlation was observed between sperm viability and morphologyandthe number of CFU. One dog was ruled out because he had azoospermia. Canine seminal bacteriospermia is relatively frequent and could alter its quality. The presence of Gram-negative bacteria was associated with more remarkable alteration in the semen analysis.

Keywords: Semen; Bacteriospermia; Male infertility; Reproduction; Seminal parameters.

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INTRODUCTION

Semen quality assessment allows value the reproductive potential of a male and their ability to be used as a semen donor during assisted reproduction procedures, such as artificial insemination and cryopreservation of semen and others, in most animal species, including canines. (Gradil et al., 2006; Morales et al., 2011). Morphological and physiological diversity in canine sperm has been described because of breeds number (Chłopik and Wysokińska, 2020). Despite the above, performing bacteriological analysis is infrequent, favoring the transmission of diseases through semen (Goericke-Pesch et al., 2011).

Historically, the leading causes of infertility in dogs have been cryptorchidism, azoospermia, benign prostatic hypertrophy, prostatic cysts, and prostatitis due to bacterial infections of the reproductive tract (Memon, 2007). In addition, semen provides the ideal conditions for bacterial growth, which causesirreversible damage to the male reproductive tract and sperm, affecting animal fertility (Dragonetti et al., 2005; Puerta Suárez et al.,2015). Specifically, the damage mentioned above may be due to the direct effect of the bacteria on the sperm plasmatic membrane or by indirect effects induced by bacterial metabolism's soluble factors products (Puerta Suárez et al., 2015).

Previous research in humans has shown adverse effects on semen quality due to bacteria; for example, the soluble factors of *S. aureus* decreased sperm motility and affected all functional parameters except the chromatin integrity, suggesting a detrimental reproductive function (Galarzo Pardo et al., 2015). In addition, it has been described that *Neisseria gonorrhoeae* can bind to human sperm and decreaseits viability (84.5% vs. 66.5%, $p<0.05$). However, without affecting the functional sperm parameters (Puerta Suárez and Cardona Maya, 2016).It has been reported that *Escherichia coli, Enterococcus faecalis*, and the soluble factors of their metabolism alter sperm motility and some of the functional parameters,although not statistically significant. These microorganisms create interactions with the sperm cell favoring their diffusion into the female reproductive tract after ejaculation during natural coupling or artificial insemination (Cano-Cháves et al., 2017; Guerrero-Hurtado et al., 2018).

Specifically, in canines, previous studies have shown a high frequency of bacteriospermia in ejaculates, 89.1% (Goericke-Pesch et al., 2011) and 86.2% (Goericke-Pesch et al., 2006), relating sperms abnormalities with bacteria presence (Angrimani et al., 2014; Goericke-Pesch et al., 2011; Goericke-Pesch et al., 2006). However, thereis not enough evidence on the bacterial agents present in semen and their influence on the spermparameters of reproductive canines. Therefore, the present work aimed to determine the bacteriospermia frequency, the associated bacterial species, and their effect on seminal quality in stud dogs.

MATERIAL AND METHODS

General clinical and andrological evaluation

Overall, fifteen adult stud dogs between 2 and 8 years of age, not neutered, from dog breeders were included; alldogs were diagnosed as healthy during the general clinical and andrological examination. Physiological constants such as heart and respiratory rates, pulse, temperature, and the appearance of the mucous membranes were evaluated in each dog. In addition, the external parts from the reproductive tract, including the penis and testicle conformation, were assessed to rule out problems such as penis adhesions with the foreskin or testicle adhesions with the scrotum(Angrimani et al., 2014).One animal was discarded from the study because it presented azoospermia. Particular characteristics and findings of that stud dog were described by our research group (Agudelo-Yepes et al., 2020). The Ethics Committee approved this work for Animal Experimentation from the University of Antioquia (Act number 120 of October 9, 2018).

Semen samples obtaining and assessment

The preputial and penis region were washed twice with chlorhexidine, and a saline solution (0.9%) was infused to stimulate debris removal and possible pollutants from the intra-preputial space. Later, the semen sample was taken using the gloved hand method (no artificial vagina was used), and contact between the mucous membrane of the penis and the sterile collection container was avoided to reduce the risk of contamination of the sample of semen (Freshman, 2002).

The semen samples were macroscopically (volume, color, and consistency), and microscopic analysis (concentration, viability, motility, and sperm morphology) was evaluated (Kolster, 2018) by the same expert technician. Briefly, sperm concentration was performed using the Makler chamber (Sefi-Medical Instruments, Israel) and reported as millions/mL (Betancur et al., 2009);viability was determined by

mixing 10μL of eosin/nigrosine, and 0.5% sodium citrate (Merck-Chemicals, Germany) and 10μL of the seminal sample, counting in duplicate 200 sperm between alive (unstained) and dead (stained red) (Rijsselaere et al., 2005). Motility was performed by counting 200 sperm, discriminating them into three types of motility: progressive motility, non-progressive motility, and non-motility (Root Kustritz, 2007). Finally, morphology was assessed using an extended sample (10μL of semen) on a slide and stained with Giemsa (Novalab®, Medellín, Colombia). A total of 200 sperm were evaluated per slide according to sperm characteristics (Hesser et al., 2017). Conventional seminal parameters that were taken as a reference are progressive motility (≥ 75%), viability (≥ 75%), abnormal morphology (≤ 20%), and sperm concentration (450 to 1900 x10^6/mL) (Goericke-Pesch et al., 2006).

Bacteriological examination and conventional culture

In order to assess the presence of aerobic bacteria, 10μL from each semen sample was seeded quantitatively (by confluence) on blood agar (Merck-Chemicals, Germany) and MacConkey agar (Merck-Chemicals, Germany). In addition, another 10μL from each semen sample was seeded in brain and heart infusion medium (BHI, Merck-Chemicals, Germany). Later, samples on BHI were re-seeded on blood agar and MacConkey agar and subsequently identified. All the media were incubated for 18-24 hours at 37°C (Goericke-Pesch et al., 2011).

The colonies were counted and expressed as the number of colonies per milliliter of sample. Identification of bacteria was carried out according to the morphology of the colony, their morphology in Gram staining, and biochemical tests. The differentiation of *Staphylococcus spp*. was performed using the coagulase test. *Streptococcus spp*. were grouped according to their hemolysis behavior on blood agar(Alpha, Beta, or Gamma). Gram-negative bacteria were identified by biochemical series (Puerta Suárez et al., 2015).

Molecular testing

A molecular test was performed by conventional PCR after DNA extraction from each semen sample using the Phenol-Chloroform-Isoamyl Alcohol method (Ríos Sánchez et al., 2016).Eleven different microorganisms were evaluated for each sample using the commercial PCR Master Mix kit (Thermo Scientific™, USA). Conventional PCR products were revealed by electrophoresis on a 3% agarose gel with SYBR (Safe Invitrogen from Thermo Scientific™, USA) in TAE buffer (Tris, acetic acid, and EDTA) for 15 minutes at 100 Volts. The molecular weight marker used in each gel was 100 base pairs (Hyperladder II 100 lines, Bioline, Life Science Company, London, United Kingdom) and visualized by photo-documenter (Molecular Image Gel Doc TM XR Bio-Rad, CA, USA) (Moreno and Agudelo-Flórez, 2010).

Each reaction was a final volume of 25uL containing 12.5uL of Master Mix, 0.025 U/μL of Taq DNA polymerase, 2mM of $MgCl_2$ and 0.2mM of each dNTP (dATP, dCTP, dGTP and dTTP) diluted in reaction buffer. For each reaction, 0.6μL of each primer, 9.3μL of water, and 2μL of DNA were added. UNDER SPECIFIC CONDITIONS, the PCR was run in a thermal cycler (T3000, Whatman, Biometric, Goettingen, Germany) under specific conditions (Table 1). Positive PCR products were used as a positive control, while water was used as a negative control.

Animals were grouped according to the weight in two groups: Group 1, small dogs between 1 and 10kg; and Group 2,medium and large dogs with more than 10 kg (Table 3). In addition, animals were grouped according to the bacteria amount detected by PCR in the semen: the average in the whole group was 4.2 bacteria. Therefore, they were divided into greater and below the number of bacteria averages.

Statistical analysis

An unpaired t-test was performed for data with a Gaussian distribution or a Mann-Whitney test for the results that did not meet a normal distribution. Correlations were made between all the parameters evaluated. A comparison analysis of proportions was performed for the data obtained by PCR. All analyzes were performed using the GraphPad Prism 8 statistical program (GraphPad Software Inc. La Jolla, CA, USA). Values of $p<0.05$ were considered statistically significant.

Additionally, a multivariate ordering method of principal component analysis (PCA) was used using the statistical program R (http://www.R-project.org/).

RESULTS

We found bacterial growth on 100% of the collected seminal samples from different breeds (Table 2). Regarding the seminal parameters, we observed

Table 1. PCR primers and conditions.

Bacteria	Primers	Fragment sizes	Initial denaturation temperature	Cycles	Final elongation temperature
Escherichia coli *d-1-deoxyxylulose 5-phosphate synthase gene (dxs) Chromosomal DNA*	F: 5'CGAGAAACTGGCGATCCTTA 3' R: 5'CTTCATCAAGCGGTTTCACA 3'	113 bp	95°C 5 min	40 cycles 94°C:30s 52°C:30s 72°C:30 min	72°C 10 min
Staphylococcus aureus *sa442*	F: 5'TCGGTACACGATATTCTTCAC 3' R: 5'ACTCTCGTATGACCAGCTTC 3'	179 bp	94°C 5 min	40 cycles 96°C:30s 50°C:30s 72°C:1min	72°C 8 min
Neisseria gonorrhoeae *Ngu3 y Ngu4*	F: 5´CGTTCATCGGCGTAGGGTAA 3´ R: 5´CACTTCTCGGTGTTAAGAAA 3´	200 bp	94°C 5 min	40 cycles 94°C:30s 52°C:30s 72°C:1min	72°C 10 min
Klebsiella pneumoniae *ntrA*	F: 5'CATCTCGATCTGCTGGCCAA 3' R: 5'GCGCGGATCCAGCGATTGGA 3'	90 bp	95°C 5 min	40 cycles 94°C:30s 52°C:30s 72°C:30 min	72°C 10 min
Pseudomonas aeruginosa *lasa*	F: 5'AGTTGTCGCGGCGCTACTAC 3' R: 5'GCTCACCTGGATCTGGTCCA 3´	125 bp	95°C 5 min	35 cycles 94°C:30s 55°C:30s 72°C:30s	72°C 5 min
Staphylococcus epidermidis *DHFR gene*	F: 5'GGGAAACCATTGCCAAATAGAC 3´ R: 5'CGAATAACGTTTGTCCTCCAAATA 3´	145 bp	94°C 5 min	42 cycles 94°C:30s 50°C:30s 72°C:1min	72°C 10 min
Enterococcus faecalis *16S rRNA E16S 72f E16S 210r*	F: 5'CCGAGTGCTTGCACTCAATTGG 3' R: 5'CTCTTATGCCATGCGGCATAAAC 3'	138 bp	95°C 5 min	40 cycles 94°C:30s 52°C:30s 72°C:30 min	72°C 10 min
Streptococcus agalactiae *CFBSb CFBAb*	F: 5'ATGATGTATCTATCTGGAACTCTAGTG 3´ R: 5'CGCAATGAAGTCTTTAATTTTTC 3´	272 bp	94°C 5 min	42 cycles 94°C:30s 50°C:30s 72°C:1min	72°C 12 min
Chlamydia trachomatis *OMP1*	F: 5´GCTCGGATGCCTTGTTAACAC 3´ R: 5´TCCAAAATGTGCTCCGGATTT 3´	100 bp	95°C 5 min	35 cycles 94°C:30s 55°C:30s 72°C:30s	72°C 5 min
Haemophilus spp bexA	F: 5´ATCGAAAGTTTAGAGGCAA 3´ R: 5´TTCTTTCGATGGATGTGGTT 3´	84 bp	94°C 5 min	35 cycles 94°C:30s 55°C:30s 72°C:30s	72°C 5 min

bp: base pairs; F: Forward; R: Reverse; min: minutes; s: seconds; ºC: Celsius degrees

Table 2. Stud dogs included in the two study groups.

Breed	Group 1. small (between 1 - 10 kg)	Group 2. Median to big (>10 kg)
Weight, kg, Mean ± SD	6.1 ± 2.45	18.7 ± 9.54
Miniature pinscher	1	
Chihuahua	2	
Pomeranian	1	
French bulldog	3	3
Schnauzer		1
Siberian Husky		2
Bernese Mountain Dog		1

SD: standard deviation.

Table 3. Bacteria evaluated by PCR

Bacteria (PCR)	Number, percentage of positivity
Escherichia coli	3, 21.42%
Staphylococcus aureus	10, 71.42%
Neisseria gonorrhoeae	9, 64.28%
Klebsiella spp.	10, 71.42%
Pseudomonas spp.	4, 28.57%
Staphylococcus epidermidis	9, 64.28%
Enterococcus faecalis	0, 0%
Streptococcus agalactiae	9, 64.28%
Chlamydia trachomatis	3, 21.42%
Haemophilus spp.	0, 0%
Streptococcus pneumoniae	3, 21.42%

72.1% and 58.5% sperm motility, 88.8% and 65.2% ($p < 0.05$) normal morphology, 81.9% and 63.7% viability, and 196.4x10^6 sperm/mL and 86.1 x10^6 sperm/mL concentration for group 1 and group 2, respectively.

The average CFU on blood agar was 34042.8 CFU/mL for group 1 and 107714.3 CFU/mL for group 2, and on MacConkey agar was 142.9 CFU/mL and 21328.6 CFU/mL, respectively. Conventional techniques allowed the identification of *coagulase-negative Staphylococcus, gamma-hemolytic Streptococcus, alpha-hemolytic Streptococcus, E. coli, Proteus spp.,* and *Proteus mirabilis*, with *Staphylococcus coagulase-negative* being the most frequent isolate (64.3%).

A negative correlation was found between sperm viability ($r=-0.95$, $p<0.05$) and normal morphology ($r=-0.92$, $p<0.05$) with CFU in McConkey agar. Similarly, normal morphology and CFU in Blood agar ($r=-0.72$, $p<0.05$). Besides,the dog's bodyweight negatively correlated with both sperm viability ($r=-0.68$, $p<0.05$)and normal sperm morphology ($r=-0.66$, $p<0.05$).

Progressive sperm motility was found in 50% (7/14) of the dogs and sperm viability above the cut-off point (>75%). Also, 50% of the dogs presented abnormal sperm morphology below the cut-off point (<20%). Finally, 57.1% (8/14) of the dogs were above the established range of sperm concentration.

In identifying the bacterial species by conventional PCR, 14 samples were analyzed overall, and 11 different bacteria were evaluated (Table 3). Both *Haemophilus* and *E. faecalis* were not found in any of the samples.

On the other hand, we classified the stud dogs into two groups,High and Low, based on the diversity of bacteria found in their semen samples by PCR. For "High number of bacteria" group (n=7) and "Low number of bacteria" (n=7). Althoughthere was no statistical significance in bacterial genus between groups, the "Low number of bacteria" group trend to exhibit better seminal parameters, including a higher percentage of motility, viability, sperm concentration, and normal morphology (Figure 1).

Using the PCA and considering the variability explained by the data demeanor, we found two principal components (Figure 2), allowing a two-dimensional graph to visualize data and variables. The principal component 1 (PC1), x-axis, was considered as the main axis because it presented the highest coefficient of variability within the system (44.2), compared to principal component 2 (PC2), y-axis, representing 24.8% of the variability of the data. When considered together, both variables represent 69% of data variability (Figure 2A). The vectors represent the sums of variables in the total animals; the similar lengths of the vectors in the graph suggest the same contribution of the variables in data distribution. The angles of the vectors allowed establishing the relationships between the results, as follows: the 90° angles between the variables indicated that they were not correlated. Angles greater and lower than 90° indicated that the correlation was positive or negative, respectively. If the angle of the variable and the principal component selected as the axis is close to 90°, the variable does not exert any weight in the correlation with the other variables(Mar and Sharma, 2010; Rodríguez et al., 2011).

When visualizing data from each group of stud dogs (Figure 2B), the circles represent the individuals

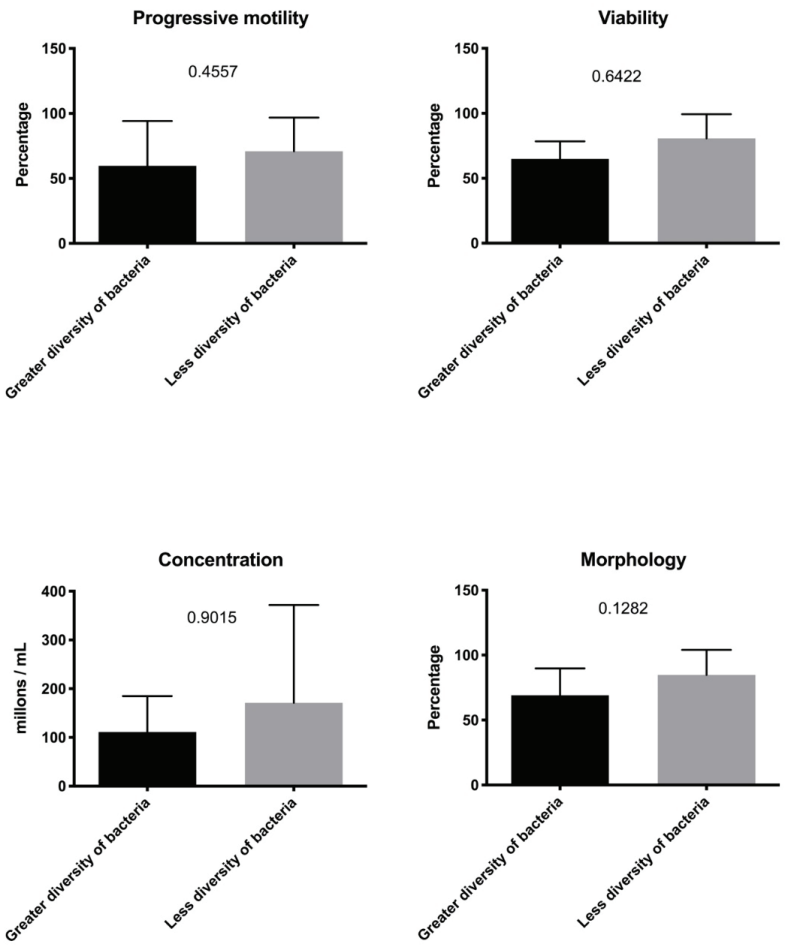

Figure 1. Seminal parameters in stallion groups when grouped according to the diversity of bacteria in the semen.

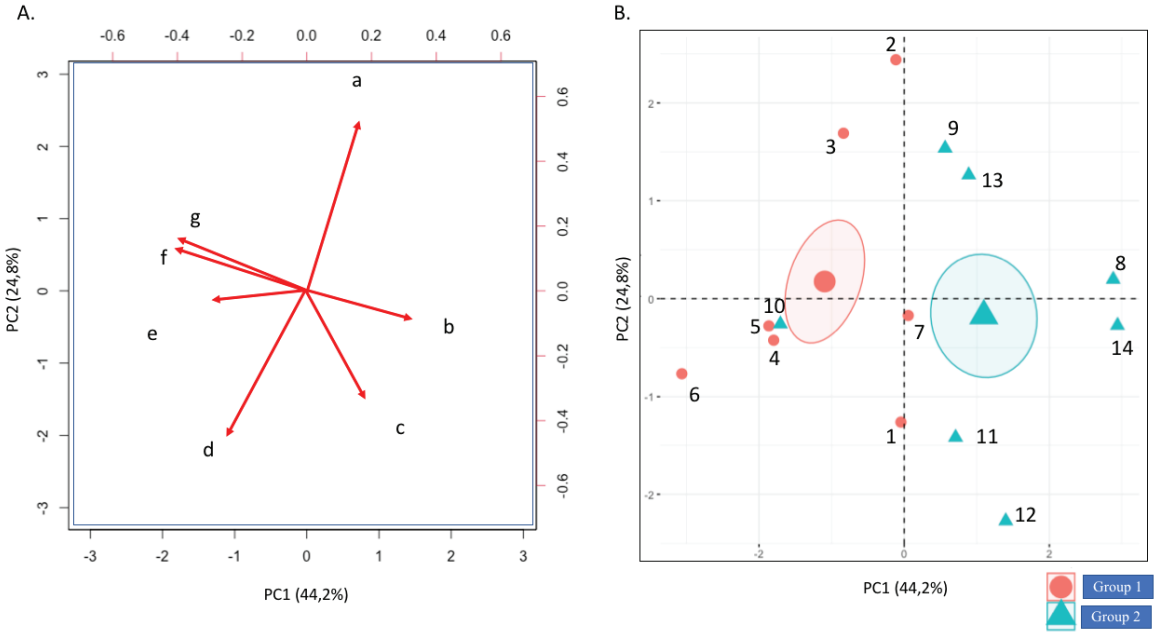

Figure 2. Principal component analysis. A. Spatial distribution of the variables a. Non-progressive motility, b. Weight, c. Blood agar, d. Progressive motility, e. Sperm concentration, f. Viability, g. Normal morphology. B. The general distribution of each group is shown (circle for group 1, triangle for group 2), the circle with shadow shows the consolidated ones for all animals, the number marks each individual

from group 1, small dogs, and the triangles represent the individuals from group 2, medium, and large dogs. The majority of dogs in group 2 were located on the right plane (85.7% -6 of 7-), animals from this group showed a higher percentage of non-progressive motility, a higher concentration of CFU/mL in MacConkey agar, greater weight, and a lower number of CFU/mL in blood agar. In contrast, the animals from group 1 were located on the left plane, presented better normal morphology, a higher percentage of live sperm, higher sperm concentration, and a lower percentage of progressive motility than group 2.

DISCUSSION

In this work, bacteriospermia frequency, the associated bacterial species, and the effect on conventional sperm parameters in canine reproductive males were assessed in a sample of healthy stud dogs. The results showed that the dogs' ejaculate is not sterile (bacteria-free)because 100% of the samples showed bacterial growth. Microbiomes play an essential role in health and disease. Skin, intestines, oral cavity, vagina, and urethra can host microbial communities, and the presence of bacteria in semen does not equate with infection. CFU/mL was negatively correlated with seminal quality parameters such as viability and normal sperm morphology. Accordingly, we found that the fewer bacterial species found in dogs' semen, the better the seminal parameters.

Our results agree with previous studies that reported bacterial growth in more than 80% of semen samples (Goericke-Pesch et al., 2011; Goericke-Pesch et al., 2006). In addition, this work compared stud dogs depending on their body weight, considering this factor as a determining factor for future health and well-being and the prevention of diseases in dogs in the region (Salt et al., 2020). Previous reports performed in other males, such as bulls, had established that genetic correlations between body weight and semen characteristics depend mainly on age: the older the male, the lower semen quality. However, it was shown that a greater average daily weight gain could explain the slight negative genetic trends observed for semen characteristics in young bulls (Olsen, 2020).In the present work, a significant difference in the normal morphology of the sperm in relation to the animal body weight was observed. Similar results have been reported showing a difference in ejaculated volume (Tesi et al., 2017, Zeller et al., 2019), motility (curvilinear velocity), and total sperm output (Rijsselaere et al., 2007, Zeller et al., 2019) between small size dogs (<15 kg), and medium-sized dogs (16-40 kg).

Regarding bacterial growth, a higher number of CFU/mL in both blood agar and MacConkey agar were found for the heavier dogs. However, we must consider that the growth of microorganisms in semen samples alone cannot predict fertility (Lopate, 2012) because fertility depends on several other factors. It is a concept that the male and female cannot be analyzed separately because fertility is an indicator of the percentage of females that become pregnant after intercourse, that is when a female becomes pregnant after mating with the dog (Eilts, 2005; Lopate, 2012).

In this work, the bacterial microbiota in the semen of stud dogs was similar to that described by other authors (Bjurström and Linde-Forsberg, 1992; Goericke-Pesch et al., 2011, 2006).In addition, we found some bacterial species that only due to their presence cannot be considered harmful except for *β-hemolytic streptococci*, which are of particular importance as pathogens in the genital tract of the canine male but were not isolated in this study. The negative correlation between viability and normal morphology with CFUs on MacConkey agar indicates that Gram-negative microbiota in dogs' ejaculate negatively influences seminal quality as described in humans (Diemer et al., 1996; Moretti et al., 2009).We found a negative correlation between normal morphology and CFU in blood agar, considering that this is an enriched culture medium that allows the growth of many bacterial species. It could be due to the growth of Gram-negative microorganisms in blood agar. The above allows us to suggest that bacteria growing in seminal fluid, especially multiple infections associated with Gram-negative bacteria, could produce direct alterations on the sperm (Diemer et al., 1996; Moretti et al., 2009). In addition, we found that the higher the number of CFU/mL, the lower the percentage of sperm with normal morphology. Therefore, we insist that not only the bacteriospermia should be evaluated qualitatively but especially quantitatively.

The most frequent bacteria found in the canine semen were *Staphylococcus aureus and Klebsiella spp*. Works performed in humans show that incubating spermatozoa with *K. pneumonia* considerably reduces progressive motility, and their soluble factors increase the number of necrotic spermatozoa and induce its apoptosis (Zuleta-González et al., 2019). Moreover, *Staphylococcus aureus* has been reported as a negative factor that worsens seminal quality and affects male fertility in humans. These bacteria and

their metabolism products mainly affect the progressive motility of sperm (Esmailkhani and Akhi, 2018; Gupta and Prabha, 2012). About the PCR bacterial identification, we can only report as presence or absence because molecular tests only detect the DNA of the microorganism.For the above, we unknown if the bacteria were alive or not and can be misinterpreted with past infection. In addition, for bacterial assessment, the conventional PCR technique should be replaced for a qPCR because bacteriospermia determination should ideally be assessed quantitatively.

When we short the stud dogs into two groups according to the number of bacteria detected by PCR, above and below the mean, no statistically significant differences were found. Still, there was a trend in which stud dogs with fewer bacterial species exhibited better seminal parameters. Although these results were qualitative, they show that a greater number of bacterial species present in canine semen could be altering the seminal parameters, perhaps because more species generate greater metabolic products and wastes that can affect the sperm membrane and because bacteria could interact directly with sperm causing irreparable damage. In this work, the multivariate analysis of main components allowed us to establish that the individuals in group 1 (small dogs, 1-10kg) presented better normal morphology, a higher percentage of live sperm, a higher sperm concentration, and a lower percentage of progressive motility. For group 2 (medium to large dogs, > 10kg), most animals were located on the right plane, the stud dogs from this group have a higher percentage of non-progressive motility, a higher concentration of CFU/mL in MacConkey agar, greater body weight, and a lower number of CFU/mL in blood agar. We have shown that lower body weight dogspresented better sperm parameters. However, they presented a low percentage in progressive motility. To the best of the authors' knowledge, the present study is the first to compare the bodyweight with sperm values and bacteriospermia presence (CFU/mL). We found the most common bacterial species found by PCR were *S. aureus* and *Klebsiella spp.* in 85.7% (6/7) from stud dogs of group 1. Both bacterial have been described as affecting mainly progressive motility (Esmailkhani and Akhi, 2018; Gupta and Prabha, 2012), which could explain the decrease in the progressive motility for group 1.

Our work had limitations. First, the number of stud dogs analyzed. Second not having determined the *in vitro* effects of the interaction between canine sperm and bacterial species or soluble metabolism products found more frequently. Finally, to determine if high bacteriospermia levels negatively influence canine fertility, further studies must test if fertile female dogs mated to stud dogs treated or not with specific antibiotic-sensitive bacteria becomes pregnant and the resulting litter size.

CONCLUSIONS

The findings of this work indicate that canine ejaculate is not sterile (bacterial-free). We observed that the greater the number of bacterial species present in canine semen, the worse the seminal quality parameters. A negative correlation between the CFU/mL and the conventional seminal parameters was found. Finally, this work strengthens the evidence on the importance of including quantitative bacteriological examination during routine seminal analysis. A complete reproductive evaluation will guarantee time and money savings in production system models for pets.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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