Colloid centrifugation reduces bacterial load in chilled dog semen

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ABSTRACT

Conventional semen extenders contain antibiotics to prevent bacterial growth. Finding alternatives would be beneficial to minimize the development of bacterial resistance mechanisms. The aim of this study was to determine the effect of Single Layer Centrifugation (SLC) with Canicoll of dog semen on microbial load and sperm quality during cooled storage. Twenty-four ejaculates were obtained from healthy dogs by digital manipulation. Samples were diluted in Tris-citrate-fructose extender without antibiotics and divided into two treatment groups: SLC-selected samples and unselected samples. Sperm motility (CASA), viability and acrosome integrity (PI/FITC-PNA) as well as bacterial load of each microorganism species (colony-forming units/mL) were assessed at 0 and 48h of storage at 4ºC. Results indicate SLC-selected dog spermatozoa have greater percentages of motility, viability and acrosome integrity ($P<0.05$). Bacterial growth in SLC sperm samples was less ($P<0.05$) than unselected samples. Removal of individual bacterial species varied from 91% to 98% for *Escherichia coli* (91.62%), *Streptococcus* spp. (98.18%), *Staphylococcus* spp.(95.33%) *and Pseudomonas* spp. (92.50%). In conclusion, the use of SLC with Canicoll has the potential to decrease bacterial load in chilled dog semen.

Keywords

Single Layer Centrifugation (SLC);Bacteria;Dog spermatozoa; Selection; Cold storage

1. Introduction

In recent years, artificial insemination is one of the most frequently used reproductive biotechnologies for dog breeding (Linde-Forsberg, 2001). Semen samples must be diluted in specific commercial or non-commercial dog semen extenders for long-term cooled storage prior to conducting artificial insemination of the bitch during the fertile period. The extender is a medium that contains protective compounds that allow sperm survival outside the reproductive tract, maintaining the sperm fertilizing capacity and controlling microbial growth (Pinto et al., 1999).

The ejaculate is not a sterile secretion because it contains physiological microbial flora from the dog's urogenital tract (Bjurström and Linde-Forsberg, 1992; Osborne and Lees, 1995). These bacteria have a protective action by inhibiting pathogenic growth of microorganisms. The urethra is the main source of contamination, although the foreskin and prostate are also a source of contamination (Ling et al., 1990). In addition, secondary contamination is also possible due to a lack of proper hygienic practices during semen collection and handling procedures (Althouse and Lu, 2005).

Regardless of semen quality, there is bacterial growth in the different seminal fractions of dog semen. There, however, are greater concentrations in the first fraction due to the appearance of bacteria, such *as Escherichia coli, Staphylococcus aureus, Klebsiella* spp., *Mycoplasma canis, Pseudomonas aeruginosa and Streptococcus* spp., which mainly come from the urethra (Bjurström and Linde-Forsberg, 1992; Goericke-Pesch et al., 2011). In dog semen, bacterial growth of less than 10^5colony forming units (CFU)/mL is considered physiological, because a greater concentration represents a potential risk for the maintenance of sperm quality (Johnston, 1991; Goericke-Pesch et al., 2011). Apart from *Brucella canis,* however, concentration of pathological bacteria in dog semen has not yet been determined because it depends on bacteria species and the sperm-bacterial ratio (Althouse and Lu, 2005).

The harmful effects of bacteria on sperm occur as a result of direct contact, competition of nutrients from the environment, detritus production or by the secretion of different extracellular factors (lipopolysaccharides, α- and β-hemolysins, or soluble sperm cytotoxic factors) (Villegas

et al., 2005; Schulz et al., 2010). Bacterial contamination in an ejaculate can lead to alterations in sperm motility and plasma membrane integrity, exocytosis of the acrosomal content, decrease in mitochondrial activity, DNA damage, apoptotic-like changes or agglutination. Furthermore, bacteria may induce uterine pathologies, septicaemia, fertilisation failure, abortions, or contribute to a decrease in the litter size (Maes et al., 2008).

It is a legal requirement for all commercial extenders to contain different antibiotics to control bacterial growth. Many countries are trying to reduce the general use of antibiotics to decrease the development of bacterial antibiotic resistances, and some alternatives have been studied (Morrell and Wallgren, 2011). Different sperm selection techniques such as colloid centrifugation have been used in semen processing. Specifically, Single Layer Centrifugation (SLC) through species-specific colloid formulations (Morrell, 2012), including Canicoll for dog semen processing (Morrell et al., 2008), is a suitable method for selecting the most viable spermatozoa with fertilizing capacity. In addition, a decrease in the microbial load of the ejaculates after colloidal centrifugation has been reported for different animal species. In effect, Morrell and Wallgren (2011) reported that boar ejaculates processed through SLC had markedly reduced bacterial contamination, which was also possible when using SLC with a low-density colloid (Morrell et al., 2019). There were similar results for stallion fresh spermatozoa, because colloidal centrifugation resulted in a decrease microbial load between 70% and 90% in seminal doses (Morrell et al., 2014). The purpose of the present study, therefore, was to determine whether the use of SLC could be conducted to separate bacterial contaminants from dog spermatozoa in the ejaculate. In addition, there were analyses of the semen characteristics of SLC-treated and non-treated samples proportioned from the same ejaculates during 48h of cooled storage.

2. Material and methods

2.1. Reagents and media

All chemicals were obtained from Sigma-Aldrich Quimica, S.A. (Madrid, Spain), unless otherwise indicated. The basic extender used to dilute dog sperm was Tris-citrate-fructose

(TCF) extender containing 259 mM Trizma base, 80 mM citric acid and 69 mM fructose at 6.8 pH and an osmolality of 300/330 mOsm/Kg.

2.2. Animals and semen collection

The study was performed following approval by the Veterinary Ethical Committee, University of Zaragoza(PD04/18NE). Care and use of animals were according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. Four clinically healthy beagle dogs, ranging between 4 and 5 years of age, were included in this study. The dogs were individually housed, fed a balanced diet and had access to water *ad libitum.* Twenty-four ejaculates (six per dog) were collected by digital manipulation into a pre-warmed calibrated conical tube. Sperm concentration was determined with a photometer (SpermaCue, Minitub GmbH, Tiefenbach, Germany).Sperm morphology was examined by light microscopy evaluation (Olympus® BH12, Olympus Optical Co., Europe) of smears stained with Diff-Quick® (MICROPTIC S.L., Barcelona, España) staining. At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology.Only sperm samples with concentration $\geq 200\times10^6$ spermatozoa/mL, total motility ≥80%, and normal morphology of ≥75%, were included in the study. All ejaculates obtained met these thresholds.

2.3. Sperm processing

Ejaculates were diluted in TCF extender at room temperature to a final sperm concentration of 100×10^6/mL and were proportioned into two aliquots. One aliquot (unselected sample) was centrifuged at 700×*g* for 3 min (Linde-Forsberg, 2001), and the supernatant was discarded. The sperm pellet was resuspended in TCF extender (50×10^6 sperm/mL). The second aliquot (SLC-selected sample) was processed through Canicoll (Swedish University of Agricultural Sciences – SLU, Uppsala, Sweden) as described by Morrell et al. (2010) with a few modifications. First, 4 mL of colloid (equilibrated at room temperature) were placed in the centrifuge tube (17x120 mm), and 4.5 mL of sperm sample (containing approximately 100×10^6 sperm/ml) was layered on top. Then, samples were centrifuged at 300×*g* for 20 min, and the pellet was diluted in TCF extender to a final concentration of 50×10^6 sperm/mL. Sperm samples, both unselected and

SLC-selected, were cooled gradually in glass beakers containing water and placed in the refrigerator at 4ºC. Cooled extended semen was stored at 4ºC until sperm analysis at 0, 24 and 48h.

2.4. Sperm quality evaluation

2.4.1. Computer-assisted sperm motility analysis

Values for motion variables were determined using a computer-assisted sperm analysis (CASA) system (ISAS®; PROISER; Valencia; Spain). Samples were analysed at a concentration of 20×10^6 sperm/mL. The variables evaluated were total: motile spermatozoa (%TM), progressively motile spermatozoa (%PM), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), and linearity (LIN;VSL/VCL×100, %) and straightness indexes (STR; VSL/VAP×100, %). A 5-µL aliquot of each sperm sample was placed onto a pre-warmed Makler counting chamber (Sefi Medical Instruments; Haifa, Israel). Settings were as follows: 25 frames/s in which spermatozoa had to be present in at least 15 frames to be counted. Percentages of total motile spermatozoa used in statistical analysis include those motile spermatozoa with VCL ≥ 20 µm/s. Three microscopic fields were analysed in each sample using a phase-contrast microscope (Olympus® BH12, Olympus Optical Co., Europe). The total number of spermatozoa evaluated per sample ranged between 150 and 200.

2.4.2. Sperm plasma membrane integrity

Sperm plasma membrane integrity was evaluated using the LIVE/DEAD® sperm viability kit (Thermo Fisher Scientific, Hennigsdorf, Germany). A volume of 100 µL of diluted sperm was mixed with 5 µL SYBR-14 solution (10 µL/ml) and incubated at 37ºC in a dark room for 10 min. Then, the samples were mixed with 5 µLpropidium iodide (PI) solution (2.4 mM) and incubated at 37ºC for 10 min. At least 200 sperm were observed (two replicates per sample) at x400 magnification using a fluorescence microscope (Leica® DM2500 LED, l´Hospitalet del Llobregat, Spain). The percentage of viable cell with an intact plasma membrane was determined in the spermatozoa that had green fluorescence, whereas the cells with damaged plasma membrane had red fluorescence.

2.4.3. Acrosome membrane integrity

Acrosome membrane integrity was assessed using peanut agglutinin conjugated to fluorescein isothiocyanate (PNA-FITC) and PI staining. An aliquot of 500 µL sperm suspension from each treatment group was added with 10 µL of FITC-PNA solution (200µg/mL stock solution) and 4 µL PI solution (500 µg/mL stock solution), incubated at 38ºC for 5 min, and finally fixed with paraformaldehyde (4%, v/v) in saline solution. At least 200 spermatozoa were examined, determining the percentages of viable spermatozoa (intact plasma and acrosome membranes; PNA−/PI−) under a fluorescence phase-contrast microscope.

2.5. Bacterial culture and identification

Sixteen sperm samples from each treatment were aliquoted for bacteriological analysis after 0 h and 48 h of cooled storage at 4 ºC. A volume of 0.1 mL of each sample was seeded using a spread-plate, onto 5% sheep blood agar (bioMérieux; Marcy l'Etoile, France) and on MacConkey agar (bioMérieux, Marcy l'Etoile, France) and incubated at 37ºC for 48 h. Bacterial load of each isolated microorganism was calculated by counting the CFU/mL. The identification of the bacteria was performed according to colony morphology, Gram staining, and standard biochemical test using the catalase andoxidase reactions, coagulase test and haemolysin production (Barrow and Feltham, 1993) and using the API identification system (bio-Mérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. The proportion of bacteria removed by SLC was calculated as follows: (number of bacteria in SLC samples/ number of bacteria in control samples) x 100.

2.6. Statistical analyses

Statistical analyses were performed using SPSS version 22.0 for Windows (SPSS. PC software, Chicago, Il, USA). Kolmogórov-Smirnov tests were used to verify the normality of the data.Differences in mean values for motility variables, viability and acrosome integrity between groups were determined using an analysis of variance utilising a linear mixed model (intra-subjects factor: cooled storage time; inter-subjects factor: SLC compared with Unselected sperm) In those cases where there were differences, values were compared using the least significant difference pairwise multiple comparisons *post hoc* test (Tukey HSD test). The contamination results and bacterial elimination were analysed by the Wilcoxon test. The data

were expressed as mean value ± standard deviation (SD). There were considered to be mean differences when there was a $P< 0.05$.

3. Results

3.1. Bacterial growth

Data for bacteria load in the different ejaculates are provided in Table 1. Microbial contamination (CFU/mL) in each sperm sample varied between dogs and ejaculates from each animal. The SLC-selected samples had a lesser bacterial load than unselected sperm samples ($P<0.05$). The effectiveness of bacteria removal depended on the quantity of the microbial load present in the ejaculates. The mean value of bacteria eliminated was greater in sperm samples with relatively greater bacterial contamination than in sperm samples with lesser bacterial contamination. The SLC method, therefore, was more effective in Dog 2 than Dog 4 (94±2.33% compared with 71±4.54%). The microbial load was greater in unselected samples than SLC-selected samples after 48h of cooling in all dogs utilised in the study, Dog 1 (4800±909.21 CFU/mLcompared with 475.21±187.88 CFU/mL), Dog 2 (5450±932.74 CFU/mL compared with 375±104.73 CFU/mL), Dog 3 (5150±1405.94 CFU/mL compared with 390±66.84 CFU/mL) and Dog 4 (465±220.48 CFU/mL compared with 122.5±25.10 CFU/mL).

There are data included in Table 2 for the different bacteria isolated in the ejaculates, at 0 and 48h of cooled storage. There was a mixed microorganism population of different species in each sample collectedin this study: *E. coli, Streptococcus* spp.*, Staphylococcus* spp*. and Pseudomonas* spp. No pathogenic bacteria wereobserved. A mixed microorganism population of two to four different species was detected in each sample. The removal of individual species varied from 91.62% for *E.coli* to 98.18% for *Streptococcus* spp. The SLC technique was effective in reducing ($P<0.05$) the mean values of bacteria in sperm samples, especially *Streptococcus* spp*.* and *Staphylococcus*spp. The number of bacteria of all species of microorganisms was greater in both experimental groups after 48h of cooled storage in comparison to values at the 0h.

3.2. Sperm quality

202 The data for sperm motility variables after cooled storage at 4 °C for 48 h are summarised in
203 Table 3.Total and progressive motility, VAP, VSL, and STR did not differ between unselected
204 and SLC-selected spermatozoa after 24 h of cooled storage. The SLC-selected samples,
205 however, had a greater proportion of total and progressive motile spermatozoa than the
206 unselected samples after 48 h of storage at 4°C (Figure 1;P<0.05).
207 Values for all kinematics variables increased (P<0.05) after 48h in SLC as compared with
208 the control samples, but only VCL and LIN values were enhanced (P<0.05) after 24h of storage
209 at 4°C. In addition, the values of total and progressive motility, VCL and VAP were greater at
210 0h as compared with 48h of storage (P<0.05). In contrast, values for STR were greater after
211 cold storage (P<0.05). With regard to sperm plasma and acrosome membrane integrity, there
212 were similar results because acrosome integrity of unselected and SLC-selected spermatozoa
213 were not different at 0 and 24 h of cooled storage, but membrane integrity percentages were
214 greater (P<0.05) in SLC-selected than in unselected spermatozoa after 48 h of cold storage
215 (Figure 2).
216
217 **4. Discussion**
218 The European legislative framework regulates the use of antibiotics to reduce the spread of
219 antimicrobial resistances in the veterinary field; thus, it is necessary to develop alternatives to
220 decrease its use, including semen extenders. In the present study, there was determination of
221 whether SLC is a suitable technique to separate sperm from bacterial contaminants in canine
222 species. It was determined that SLC-selected sperm samples had a lesser bacterial load than
223 unselected samples. These results are consistent with those from different studies that indicated
224 SLC was an effective procedure for reduction of the bacterial load in boar and stallion semen
225 (Morrell and Wallgren, 2011; Morrell et al., 2014; Guimaraes et al., 2015; Morrell et al., 2019).
226 Dog semen is rarely free of bacterial contamination because of physiological bacterial flora
227 from the urogenital tract (Bjurström and Linde-Forsberg, 1992; Osborne and Lees, 1995). In the
228 present study, a bacterial load $<10^5$ CFU/mL in dog semen was considered to be physiological
229 (Johnston, 1991; Goericke-Pesch et al., 2011). Greater mean bacteria values may be a risk for

the maintenance of sperm quality, because of the lesser motility, viability, mitochondrial activity and, finally, fertilisation rates with greater bacterial loads (Althouse and Lu, 2005). Bacteria concentrations that occurred in the present study did not affect seminal quality at 0 and 24 h of cooled storage in SLC-selected and unselected samples. Only some sperm motility variables and plasma membrane integrity in unselected samples were affected ($P<0.05$) compared with SLC-selected samples after 48h of cooled storage.

The centrifugation of dog sperm using SLC before cooling resulted in an increase on sperm motility after cold storage (Morrell et al., 2008). Dorado et al. (2013) determined that with Canicoll use, there was selection of a sperm subpopulation with greater motility, membrane integrity and acrosome integrity when there was processing of frozen–thawed dog semen. Similar results have been reported for different animal species (Johannisson et al., 2009; Martínez-Alborcia et al., 2013; Nongbua et al., 2017; Malo et al., 2018). In addition, results from previous studies on stallion semen, indicated that with use of SLC, there was an increase in the percentages of total and progressive motility, and there was greater retained sperm viability than with unselected spermatozoa at 48 h of cooled storage similar to what was observed in the present study. It was not determined whether this effect was due to selection of spermatozoa with greater viability, or was the result of an exclusion of dead and dying spermatozoa from the samples, or both (Varela et al., 2018).The findings in the present study indicate that SLC can be used to improve the quality of dog sperm quality.

Several bacteria (e.g., *E.coli*, *S.aureus*, *Klebsiella* spp., *Mycoplasma canis*, *P.aeruginosa*and *Streptococcus* spp.) are the physiological flora isolated in the ejaculates of clinically healthy dogs (Bjurström et al., 1992; Goericke-Pesch et al., 2011). In the present study, there were similar bacterial florain all sperm samples, mainly *E. coli*, but also *Streptococcus* spp*., S. aureus and Pseudomonas* spp. These bacteria are not pathological but rather are saprophytes of the urinary and genital tract of animals (from the prostate, urethra and/or foreskin), as described by Ling et al. (1990). In an attempt to prevent the contamination of semen samples, the prepuce area was cleaned before seminal collection in the present study (Althouse and Lu, 2005). The type and load of bacterial species isolated in the ejaculates in the present study, therefore, would

not be expected to influence conception and whelping rates (Johnston, 1991; Bjurström et al., 1992). Nevertheless, standard criteria to differentiate between physiological and pathological flora do not exist (except for *Brucella canis*), although Goericke-Pesch et al. (2007) reported that there was a tendency for large a bacterial load in ejaculates in dogs with andrological clinical symptoms or abnormal semen samples.

The SLC procedure is a sperm selection method for separating spermatozoa with greater motility, morphology, viability and DNA integrity from the other contents of the ejaculate, including bacteria (Morrell et al., 2010). In the present study, SLC-selected samples had a lesser bacteria load than unselected samples, especially in the case of *Streptococcus* spp. and *Staphylococcus* spp. Findings in the present study in this regard are consistent with the results reported by Morrell and Wallgren (2011) and Morrell et al. (2014), on boar and stallion sperm, respectively. In these previous studies, with the use of Androcoll-E and Androcoll-P, there was removal of 78% to 100% of bacteria in manually infected stallion ejaculates (97% of *E. coli* and 93% of *Streptococcus equi*), and 95% of *E. coli* in the case of boar semen. The overall reduction of contaminating bacteria in the present study ranged from 91% to 98%. The separation of sperm and bacteria was more effective for some bacteria than others, which might not only be related to the capacity of specific bacterial types to adhere to the sperm surface (including the flagellum) but also the capacity of specific bacteria to induce sperm agglutination (Morrell et al., 2014; Guimaraes et al., 2015). In addition, Morrell et al. (2014) described that bacteria had a lesser capacity to multiply after passing through the colloid, even though there was a lack of antibiotics in the colloid formulation.

Environmental factors influence rate of bacterial growth such as acidity (pH), water activity, macro and micro-nutrients, oxygen concentrations, toxins, and especially temperature (Hills and Wright, 1994).All metabolic reactions of bacteria are enzyme-catalysed and the rate of these reactions is dependent on temperature. The combined effects of hygienic semen collection and processing protocols, the use of extenders with antibiotics and seminal dose storage at 4ºC minimise growth of the contaminating bacteria. In different studies, however, results indicated that some bacteria could survive even when the semen was frozen-thawed (Morrell et al., 2014;

286 Guimaraes et al., 2015). In the present study, bacterial growth increased slightly in both
287 experimental groups, although sperm quality decreased after cooling to 4ºC for 48 h. Varela et
288 al. (2018) reported that bacteria increased the total production of ROS in stored samples and
289 consequently induced a redox imbalance or oxidative stress responsible for peroxidative damage
290 of sperm membranes and a decrease in sperm quality. Another hypothesis is that selected sperm
291 could be more resistant to bacterial effects than unselected sperm, therefore, are viable for
292 longer periods in storage.
293
294 **5. Conclusion**
295 In conclusion, with the use of SLC with Canicoll, there was a reduction in the bacterial load
296 and improved sperm motility and viability of chilled dog semen. The results from the present
297 study indicate the control of bacterial contamination in dog semen may be possible without the
298 use of antibiotics, although the effects of longer storing periods and different storage
299 temperatures need to be investigated.
300
301 **Funding**
302 This research was supported by the Government of Aragon (Ref. Group A17_17R-RAySA) co-
303 funded with FEDER 2014-2020 "Building Europe from Aragon.
304
305 **Author Contribution Statement**
306 Victoria Luño: Conceptualization, Investigation, Data curation, Formal analysis,
307 Methodology, Writing -original draft. Noelia González: Investigation, Validation, Visualization,
308 Writing - review & editing. Felisa Martínez: Conceptualization, Investigation, Writing - review
309 & editing. Ana Revert: Investigation, Methodology, Writing - original draft. Jane Morrell:
310 Conceptualization, Writing- review & editing. Lydia Gil: Conceptualization, Funding
311 acquisition, Writing - review & editing.
312
313 **Competing Interests Statement**

The authors have not conflict of interest to declare.

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Figure legends

Fig. 1. Percentages of total motility (A) and progressive motility (B)in dog ejaculates subjected to single layer centrifugation (SLC) or not (Control) and evaluated after 0, 24 and 48h of cooled storage(*n*=24);Data are presented as mean ± S.D.; * $P< 0.05$

Fig. 2. Percentages of plasma membrane integrity (A) and acrosome integrity (B)in dog ejaculates subjected to single layer centrifugation (SLC) or not (Control) and evaluated after 0, 24 and 48h of cooled storage (*n*=24);Data are presented as mean ± S.D.; * $P< 0.05$

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451 **Tables**

452 Table 1

453 Effects of single layer centrifugation (SLC) of bacteria load of ejaculated dog semen;Values are

	Dog 1 (CFU/ml)	Dog 2 (CFU/ml)	Dog 3 (CFU/ml)	Dog 4 (CFU/ml)
Control	3300±697.61[a]	4100±1067.71[a]	3525±991.21[a]	432.5±346.73[a]
SLC	225±54.47[b]	245±95.39[b]	220±58.87[b]	127.5±106.89[b]
Proportion removed (%)	93	94	94	71

454 expressed as mean ± S.D;(n = 16)

455 CFU/mL: Colony-forming units/mL

456 [ab]Values within column at each dog with different superscripts differ ($P \leq 0.05$)

457

458

Table 2

Percentage of isolations(%) and mean microorganism load (CFU/mL)of different species isolated in dog semencollected subjected to single layer centrifugation (SLC) or not (Control), determined after chilling storage at 0 and 48hours;Values are expressed as mean ± S.D.; (n = 16);.

Isolated species	Time (h)	Control frequency of isolation (%)	SLC frequency of isolation (%)	Control bacteria load (CFU/ml)	SLC bacteria load (CFU/ml)	Proportion removed (%)
E. coli	0	93.75 (15/16)	93.75 (15/16)	2355.34±1209.34[a]	197.34±70,56[b]	91.62
	48	93.75 (15/16)	93.75 (15/16)	3330.67±1725.89[a]	343.34±152.16[b]	
Streptococcus spp.	0	25 (4/16)	12.5 (2/16)	1100±336.65[a]	20±23.09[b]	98.18
	48	25 (4/16)	0 (0/16)	1575±623.83[a]	0±0[b]	
Staphylococcus spp.	0	18.75 (3/16)	6.25 (1/16)	1500±608.27[a]	70±10.0[b]	95.33
	48	18.75 (3/16)	6.25 (1/16)	1600±346.41[a]	56.67±51.31[b]	
Pseudomonas spp.	0	6.25 (1/16)	6.25 (1/16)	1200±0	90±0	92.50
	48	6.25 (1/16)	6.25 (1/16)	2400±0	130±0	

[ab]Values within row at each bacteria with different superscripts differ ($P \leq 0.05$)

Table 3

Effect of single layer centrifugation (SLC) on sperm kinematic parametersafter storage at 4ºC for 48h in ejaculated spermatozoa of dogs;Values are expressed as mean ± S.D.; (n = 16)

Time (h)	Groups	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)
0	Control	148.61±6.93	86.47±3.63	103.04±5.97	58.97±3.83	84.07±4.06
	SLC	151.30±2.81	87.88±3.88	102.83±6.34	57.46±6.76	85.67±4.85
24	Control	141.87±3.03[a]	83.50±2.76	95.21±3.46	58.88±2.36[a]	87.82±4.78
	SLC	145.27±4.21[b]	85.23±3.93	96.91±3.97	61.36±2.06[b]	89.03±4.01
48	Control	134.61±3.22[a]	84.89±3.12[a]	88.68±3.84[a]	61.89±1.86[a]	94.04±3.80[a]
	SLC	139.52±2.30[b]	87.74±3.66[b]	92.63±3.36[b]	63.59±1.88[b]	96.61±3.64[b]

VCL: Curvilinear velocity, VSL: Straight velocity, VAP: Average velocity. LIN: Linearity; STR: Straightness

[ab]Values within column at each time with different superscripts differ ($P \leq 0.05$)