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Original Research Article

Reduced bacterial load in stallion semen by modified single layer centrifugation or sperm washing

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ABSTRACT

The presence of bacteria poses a significant challenge to the quality of stallion semen used in artificial insemination. The bacterial content of insemination doses arises from various sources, such as the healthy stallion, environment, and collection equipment, and is implicated in fertility problems as well as reduced sperm quality during storage. The conventional approach of adding antibiotics to semen extenders raises concerns about antimicrobial resistance and potential negative effects on sperm characteristics, and may not be effective in inhibiting all bacteria. The objective of this study was to determine whether an innovative alternative to antibiotic usage – centrifugation through a single layer of a low density colloid (SLC) – could reduce the bacterial load in stallion semen, and to compare sperm characteristics in samples arising from this procedure, or simple extension of the ejaculate in semen extender, or from sperm washing, i.e. adding extender and then centrifuging the sample to allow the removal of most of the seminal plasma and extender. Eighteen semen samples were collected from six stallions. The semen samples were split and extended prior to washing or SLC, or received no further treatment other than extension. After preparation aliquots from each type of sample were sent for bacteriological examination; the remaining samples were stored for up to 72 h, with daily checks on sperm quality. The low density colloid SLC outperformed sperm washing or extension for bacterial reduction, effectively removing several bacterial species. The bacterial load in the samples was as follows: extended semen, $16 \pm 6.7 \times 10^5$; washed, $5.8 \pm 2.0 \times 10^5$; SLC, $2.3 \pm 0.88 \times 10^5$, $p < 0.0001$. In addition, SLC completely removed some bacterial species, such as *Staphylococcus xylosus*. Although there is no selection for robust spermatozoa with the low density colloid, sperm motility, membrane integrity, and DNA fragmentation were not different to washed sperm samples. These findings suggest that SLC with a low density colloid offers a promising method for reducing bacterial contamination in stallion semen without resorting to antibiotics.

1. Introduction

Bacteria are frequently transferred to the ejaculate during semen collection and processing [1]. The penis and prepuce of the stallion, the environment, and the equipment used during semen handling are all sources of bacteria [2]. These bacteria might be a cause of fertility problems in inseminated mares [3], and could decrease sperm characteristics during storage before artificial insemination [4–8] Antibiotics are added to the semen extender to inhibit their growth; however, antimicrobial resistance might result from this non-therapeutic use of antibiotics [9,10]. Moreover, in a previous study, sperm quality characteristics declined during cooled storage when the semen extender contained antibiotics [11]. The DNA fragmentation index i.e. single stranded DNA breaks, was observed to be higher in sperm samples with antibiotics than in the corresponding samples without antibiotics [12].

Physically removing bacteria from semen would be an alternative approach to adding antibiotics to semen extenders [13]. Previously it was shown that robust sperm could be selected from stallion ejaculates using Single Layer Centrifugation (SLC) with a high density colloid, resulting in improved sperm motility, morphology and chromatin

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integrity [14]. Subsequently, boar sperm were separated from bacteria by SLC through a high density colloid [15]. Further studies described a modification of SLC, in which an inner tube was included to facilitate harvesting of the sperm pellet, reduced bacterial count in stallion semen and selected robust spermatozoa [12,16]. However, some of the good quality spermatozoa were lost during sperm preparation. To improve sperm yield, SLC was carried out with a low density colloid to separate bpar spermatozoa from bacteria, i.e. without selecting robust spermatozoa, in an attempt to retrieve more of the sperm population. With a low-density colloid, approximately 85 % of the boar spermatozoa could be retrieved, and sperm characteristics were not adversely affected [17].

Although SLC with a high density colloid has been used for stallion semen in several studies [12,16,18], no study has yet investigated the effect of SLC with a low density colloid on stallion spermatozoa. Therefore, the current study was designed to compare SLC using a low density colloid with two other common methods of preparing stallion semen: i) simple extension, and ii) washing the sperm by centrifugation in extender. Either one or the other of these methods may be used when preparing insemination doses. Bacterial count was evaluated after these three procedures as well as sperm characteristics using Computer Assisted Sperm Analysis (CASA) of sperm motility and membrane integrity, and Flow cytometry (FC) for the sperm chromatin structure assay (SCSA). Our hypothesis was that SLC with a low density colloid could be used to separate stallion spermatozoa from seminal plasma and bacteria without adversely affecting sperm quality.

2. Materials and methods

2.1. Animals

Semen was collected from six stallions (3 adult Shetland ponies, 1 Belgian Warmblood, 1 American Quarter Horse, and 1 Westphalian Warmblood stallion) aged between 4 and 19 years. The stallions were housed under standard husbandry conditions at the Center for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria. Stallions were fed hay and mineral supplements, and water was available at all times. All of them had daily access to an outdoor paddock for several hours. Semen collection was approved according to European Union regulations (Directive 65/92 EEC). The stallions were scheduled for regular semen collections to ensure stable semen characteristics for cryopreservation, which was to take place after this study. Semen had previously been collected several times in the two weeks prior to the start of this study.

2.1.1. Semen collection

Three ejaculates were collected from each stallion during December using a sterilized Hannover artificial vagina (Minitube, Tiefenbach, Germany) fitted with an inline filter for removal of the gel fraction after the stallions had mounted a phantom with an estrous mare by the side. The penis was not washed prior to semen collection; such procedures are not routinely used at this facility.

The study design is shown in Fig. 1. An aliquot of the raw ejaculates (1 mL) was removed for bacteriology. Each ejaculate was then extended in EquiPlus without antibiotics (Minitube) and divided into three for extended, washed, and SLC groups. After preparation in each group, a further sample was taken for bacteriology and the sperm concentration was adjusted to 25×10^6/mL before evaluation of sperm characteristics immediately (0 h) and at 24, 48 and 72 h.

2.2. Bacteriology

Raw and diluted samples (1 mL) were sent for bacteriological examination 1–5 h after collection. Two hundred μl of each sample were added to 1.8 mL 2-SP medium (0.2 mol/L sucrose in 0.02 mol/L phosphate buffer, supplemented with 10 % fetal calf serum) and serially diluted up to 1×10^{-8}. Dilutions (0.1 mL) were then plated, in triplicate, onto Columbia Agar with 5 % sheep blood, Schaedler Agar with vitamin K1 and 5 % sheep blood (both BBL™, BD Diagnostics, Schwechat, Austria), and PPLO (Pleuropneumonia-Like-Organism) Agar (Difco™, BD Diagnostics, Schwechat, Austria) supplemented with 20 % horse serum (Gibco™, Thermo Fisher Scientific, Vienna, Austria). Columbia Agar plates were incubated in ambient air at 37 °C, PPLO Agar at 37 °C under microaerobic conditions for the isolation of mycoplasmas, and Schaedler Agar at 37 °C in an anaerobic jar with gas packs (BD Diagnostics, Schwechat, Austria). The plates were examined daily for up to 96 h, and bacterial colonies were counted. Single colonies displaying different colony morphology were identified at the species level using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF: Bruker Daltonics, Billerica, MA, USA). The mean total colony counts per sample and microbial isolate were calculated.

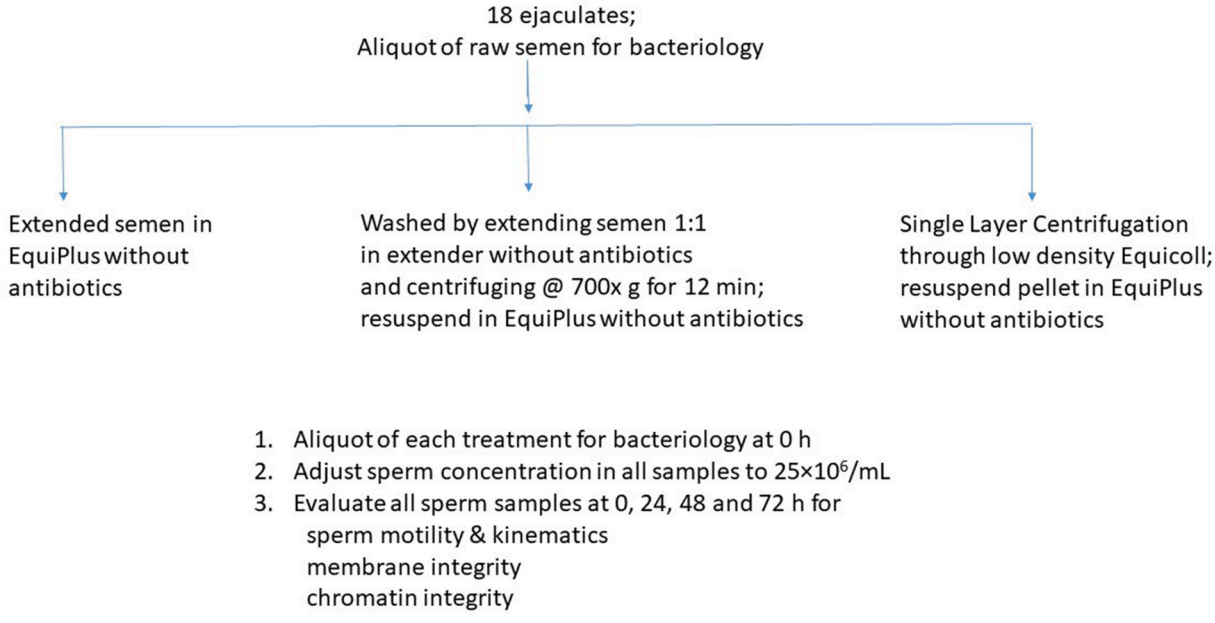

Fig. 1. Study design.

2.3. Semen preparation

Extended samples were adjusted to a sperm concentration of 25 × 10^6/mL without any further manipulation. Washed samples were extended 1:1 (v/v) with EquiPlus and centrifuged at 700×*g* for 12 min. The supernatant was removed, and the sperm pellet was resuspended with EquiPlus to a sperm concentration of 25 × 10^6/mL. The SLC samples were prepared under aseptic conditions as described in a previous study [12] with the modification that a low density colloid was used instead of the high density colloid. This colloid formulation was prepared by one of us (JMM), who is the inventor of Equicoll, to provide a colloid of density of 1.0325 g/mL. Briefly, the low-density Equicoll (15 mL) was poured into a 50 mL sterile tube and a sterile 5 mL plastic tube (Cytology Brush; Minitube, Celadice, Slovakia) was inserted through a hole in the middle of the lid [12,16]. Extended semen (15 ml) was gently pipetted on top of the colloid through a second small hole at the edge of the lid. The tube was centrifuged in a bench centrifuge at 300×*g* for 20 min using a swing-out rotor [16]. The sperm pellet was then recovered using a long Pasteur pipette passed through the central tube insert and was then resuspended with EquiPlus to 15 ml at a sperm concentration of 25 × 10^6/mL.

2.4. Analysis of semen characteristics

2.4.1. Sperm concentration

Sperm concentration was measured using a Nucleocounter-SP 100 (Chemometec, Allerød, Denmark) as described [19]. Briefly, 50 μL samples were mixed with 5 mL reagent S100 (Chemometic, Allerød, Denmark), and this mixture was loaded into a cassette containing propidium iodide (PI). The cassette was inserted into the fluorescence meter, which measured the fluorescence and converted it to sperm concentration.

2.4.2. Computer-assisted sperm analysis (CASA)

Samples were equilibrated to room temperature before motility analysis. Sperm motility evaluation was performed utilizing a SpermVision analyzer (Minitube) attached to an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a heated stage (38 °C) as previously described [20,21]. After incubation of the sample at room temperature for 15 min, one drop (7 μL) of semen was placed on a pre-warmed glass slide and covered with a pre-warmed glass coverslip. Thirty frames per field were evaluated. At least seven fields per sample with approximately 100 cells per field were evaluated. Spermatozoa with an average orientation change <8 μm were considered immotile. Spermatozoa with curvilinear velocity >10 μm/s, distance straight line >6 μm, and radius >15 μm were considered progressively motile. The proportion of motile sperm (total motility) and progressively motile sperm was calculated by the SpermVision software. Sperm motility was analyzed in eight fields, including at least 800 spermatozoa.

2.4.3. Membrane integrity

Sperm membrane integrity was analyzed as described [20]. Briefly, aliquots of 3 μl staining mixture containing SYBR-14/PI were frozen at −20 °C for later use. For staining, 100 μl of diluted semen samples were added to the vials, which were then incubated at room temperature for 10 min. A drop of the stained semen was placed on a glass slide and examined under a fluorescence microscope at 400 × magnification. The microscope used had phase contrast objectives and specific filters for excitation and suppression (Olympus AX70, Olympus Optical Co., Ltd., Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). Viable spermatozoa appeared as bright green, while damaged membranes were stained red. Each sample was evaluated once, with 4–8 representative fields assessed per sample. The software provided by SpermVision was used to calculate the mean.

2.4.4. Sperm chromatin structure assay (SCSA)

Sperm samples were mixed with an equal volume of buffer solution containing 0.01 M Tris-HCl, 0.15 M sodium chloride, and 1 mM EDTA (pH 7.4, TNE). The mixture was then rapidly frozen in liquid nitrogen and stored at −80 °C until further analyses of chromatin integrity using a FACSVerse™ flow cytometer (BD Biosciences, Becton Dickinson and Company, San Jose, CA, USA). Samples were taken at different time points (0, 24, 48, and 72 h) after semen collection for the SCSA analysis. For analysis [12], the samples were thawed on crushed ice just before staining. A mixture of 80 μL of TNE and 20 μL of semen was prepared, followed by the addition of 200 μL of a low-pH detergent solution containing 0.17 % Triton X-100, 0.15 M sodium chloride, and 0.08 M hydrochloric acid (pH 1.2). After 30 s, 600 μL of acridine orange (AO) solution (6 μg mL^{-1} in 0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0) was added. Spermatozoa with single-stranded DNA emitted red fluorescence, while those with normal double-stranded DNA emitted green fluorescence. The ratio of red to (green + red) fluorescence was used to determine the proportion of spermatozoa with damaged DNA (%DFI) in the population. The FACSVerse™ flow cytometer was used to measure the green and red fluorescence, as well as forward and side scatter. The collected data was analyzed using FCSExpress version 2 software (DeNovo Software, Thornhill, ON, Canada), which calculated the ratio for each cell and generated a histogram to determine %DFI.

2.5. Statistical analysis

The data analysis was performed using two-way repeated measure ANOVA, with Tukey test for multiple comparisons, in the R Software (R, 4.3.0) after assessing the normality of the data. Stallions and ejaculates were considered as random factors, while treatments, time and treatment × time interaction were variable factors in the analysis. The results are reported as Least Squares Means ± Standard Error. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Bacteriology

The bacterial count in the extended samples was reduced compared to the raw ejaculate (extended = 1.60.67 × 10^6; raw = 76 ± 20 × 10^6, p = 0.0018). The bacterial count was higher in the extended samples (1.6 ± 0.67 × 10^6) and washed samples (0.58 ± 0.20 × 10^6), than in the SLC samples (0.23 ± 0.088 × 10^6). The differences between groups were significant ($p < 0.0001$).

Overall, thirteen bacterial species were identified from semen samples (Table 1). *Finegoldia magna* and *Peptoniphilus* spp. were isolated in all semen samples. After semen had been extended, four bacterial species could no longer be detected, namely *Brevibacterium paucivorans, Campylobacter sputorum*, *Mobiluncus porci,* and *Pantoea agglomerans*.

Washing or SLC could completely separate sperm from 6 species of bacteria, namely *Brevibacterium paucivorans*, *Campylobacter sputorum*, *Corynebacterium* spp. A, *Mobiluncus porci*, *Pantoea agglomerans*, and *Tessaracoccus* spp. However, *Staphylococcus xylosus* was completely removed only by SLC. Neither washing nor low density SLC could completely remove *Corynebacterium* sp. B, *Cutibacterium avidum*, *Finegoldia magna*, *Mycoplasma subdolum*, *Peptoniphilus* spp., or *Proteiniphilum* spp.; however, the bacterial count (CFU) was decreased after these treatments compared with the extended semen.

3.2. Sperm characteristics

3.2.1. Sperm motility

Total motility was different between extended and washed samples; and extended and SLC only at 72 h (Fig. 2). There were differences between treatments for some other kinematics (Table 2): between SLC and

Table 1
Number of samples containing each bacterial species and the mean bacterial load (colony forming units, CFU/mL × 10^4) isolated before (Raw) and after treatment (Extended, Washed, SLC) (n = 18).

Bacterial species	Raw	Semen treatment method		
		Extended	Washed	SLC
Brevibacterium paucivorans	3 (1.2)	0	0	0
Campylobacter sputorum	3 (0.009)	0	0	0
Corynebacterium spp. A	18 (4.7)	12 (0.053)	0	0
Corynebacterium spp. B	6 (330)	6 (34)	6 (8.5)	6 (8.2)
Cutibacterium avidum	14 (1800)	14 (30)	14 (14)	14 (4.6)
Finegoldia magna	18 (3600)	18 (37)	18 (13)	18 (3.8)
Mobiluncus porci	6 (0.076)	0	0	0
Mycoplasma subdolum	12 (4.8)	12 (0.82)	12 (0.16)	12 (0.36)
Pantoea agglomerans	6 (0.029)	0	0	0
Peptoniphilus spp.	18 (2200)	18 (18)	18 (8.8)	18 (5.8)
Proteiniphilum spp.	6 (3600)	6 (300)	6 (95)	6 (42)
Staphlococcus xylosus	13 (0.054)	3 (0.045)	1 (0.014)	0
Tessaracoccus spp.	9 (5.7)	8 (0.019)	0	0

extended samples for VAP, VCL, and STR at 0 h, for VAP, VCL, VSL, STR, and ALH at 24 h, and for PM, VAP, VCL, VSL, STR, WOB, and ALH at 48 h; between SLC and washed samples for VAP, VCL, VSL, and BCF at 48 h, for BCF at 72 h; and between washed and extended samples for VAP and BCF at 0 h, for VAP, VCL, VSL, WOB, and BCF at 24 h, for VAP, VCL, VSL, WOB, and BCF at 48 h, for VAP, VCL, VSL, and BCF at 72 h ($P \leq 0.05$). Lower values were observed for VAP, VCL, VSL and ALH in the SLC samples than in extended samples.

3.2.2. *Membrane integrity*

Membrane integrity did not differ among extended, washed, and SLC samples at any time points (Fig. 2).

3.2.3. *Sperm chromatin structure assay*

Values for %DFI for extended, washed, and SLC samples were not different among treatments or time points (Fig. 2).

4. Discussion

Stallion sperm preparation commonly involves either simple extension or sperm washing to remove some of the seminal plasma. The objective of this study was to investigate the effect of separating stallion spermatozoa from seminal plasma by SLC through a low-density colloid, or by washing, on bacterial load and sperm characteristics during storage compared to extended samples. Bacterial load was reduced by both treatments but was lower in SLC samples than in washed samples compared to extended samples. Sperm characteristics were, however, similar in SLC and washed samples.

Several studies utilizing colloid centrifugation with a high density colloid demonstrated a decrease in bacterial contamination in several

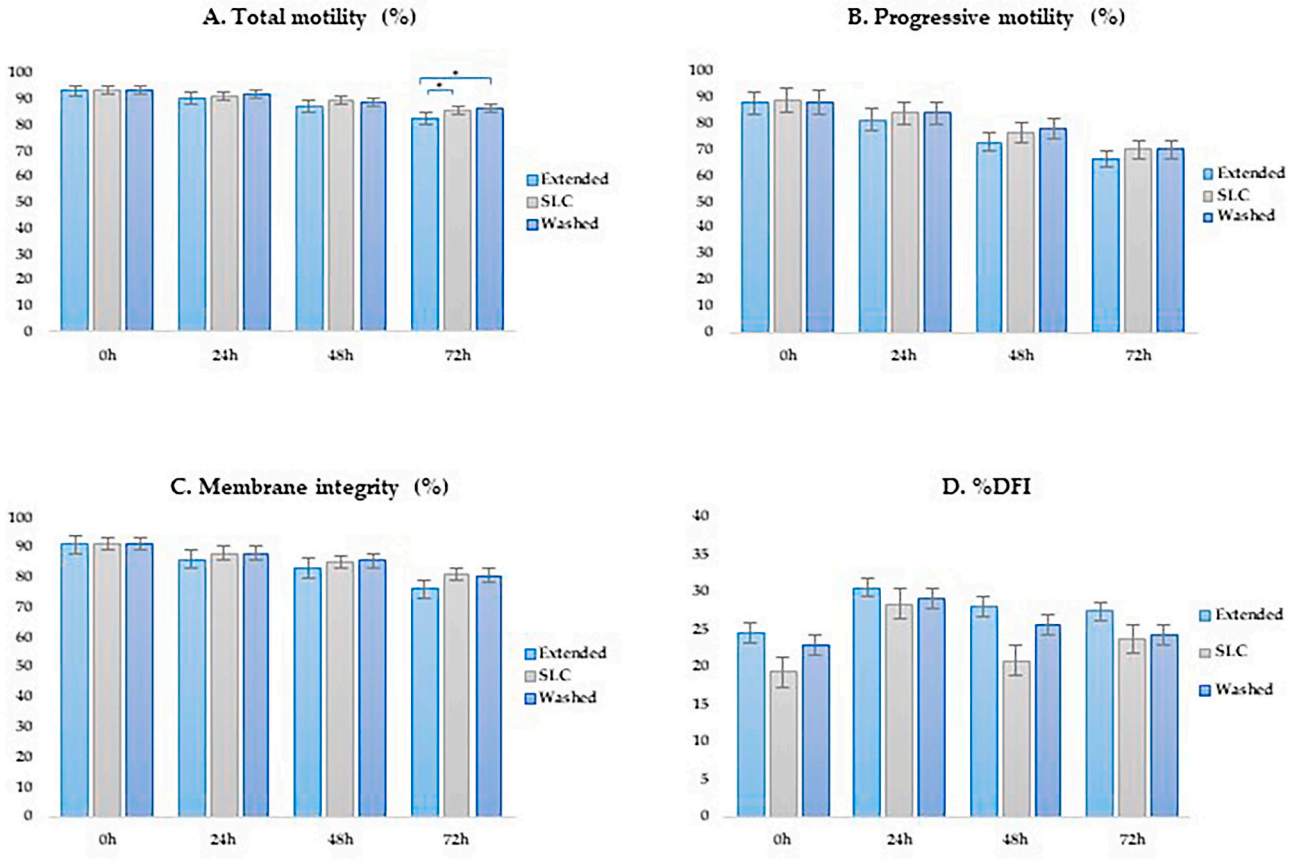

Fig. 2. Total motility, progressive motility, membrane integrity and DNA fragmentation in extended, washed samples, and samples prepared by Single Layer Centrifugation (SLC), during storage for 72 h at 6 °C. Values are Least Square Means ± SE (n = 18)
Note: *p < 0.05. DFI = DNA Fragmentation Index.

Table 2
Sperm kinematics for washed, SLC and extended groups without antibiotics at 0–72 h (Least Squares Means ± Standard Error; n = 18).

Time		Washed	Extended	SLC
0h	VAP (μm/s)	96.47 ± 3.70[a]	103.54 ± 4.21[a,b]	92.33 ± 2.86[b]
	VCL (μm/s)	176.55 ± 5.10	183.48 ± 6.55[a]	169.93 ± 4.09[a]
	VSL (μm/s)	83.48 ± 3.27	88.27 ± 3.24	81.17 ± 2.56
	STR%	0.86 ± 0.01	0.85 ± 0.01[a]	0.88 ± 0.01[a]
	LIN%	0.47 ± 0.01	0.48 ± 0.01	0.47 ± 0.01
	WOB%	0.54 ± 0.01	0.56 ± 0.01	0.54 ± 0.01
	ALH (μm)	3.89 ± 0.12	3.96 ± 0.15	3.66 ± 0.13
	BCF (Hz)	34.59 ± 0.92[a]	36.37 ± 0.87[a]	35.51 ± 0.92
24h	PM%	83.64 ± 0.89	81.18 ± 1.77	83.63 ± 1.16
	VAP (μm/s)	80.75 ± 2.92[a]	99.78 ± 3.82[a,b]	87.82 ± 4.36[b]
	VCL (μm/s)	163.40 ± 5.20[a]	194.72 ± 6.21[a,b]	174.11 ± 7.79[b]
	VSL (μm/s)	68.56 ± 2.52[a]	81.43 ± 2.92[a,b]	73.60 ± 3.21[b]
	STR%	0.84 ± 0.01	0.82 ± 0.01[a]	0.84 ± 0.01[a]
	LIN%	0.41 ± 0.01	0.41 ± 0.01	0.42 ± 0.01
	WOB%	0.49 ± 0.01[a]	0.51 ± 0.01[a]	0.50 ± 0.01
	ALH (μm)	3.99 ± 0.10	4.17 ± 0.13[a]	3.80 ± 0.15[a]
	BCF (Hz)	29.27 ± 0.91[a,b]	32.94 ± 0.47[a]	33.12 ± 0.97[b]
48h	PM%	77.53 ± 1.13	72.72 ± 2.42[a]	76.49 ± 2.31[a]
	VAP (μm/s)	75.92 ± 2.47[a,b]	101.19 ± 3.58[a,c]	85.31 ± 3.59[b,c]
	VCL (μm/s)	160.63 ± 4.09[a,b]	200.34 ± 5.90[a,c]	176.98 ± 7.23[b,c]
	VSL (μm/s)	61.68 ± 2.18[a,b]	77.05 ± 2.15[a,c]	69.19 ± 2.41[b,c]
	STR%	0.81 ± 0.01	0.77 ± 0.02[a]	0.81 ± 0.01[a]
	LIN%	0.38 ± 0.01	0.38 ± 0.01	0.39 ± 0.01
	WOB%	0.47 ± 0.01[a]	0.50 ± 0.01[a,b]	0.48 ± 0.01[b]
	ALH (μm)	4.29 ± 0.12	4.78 ± 0.19[a]	4.11 ± 0.13[a]
	BCF (Hz)	26.61 ± 0.79[a,b]	30.16 ± 0.79[a]	31.02 ± 0.96[b]
72h	PM%	70.00 ± 1.61	66.15 ± 2.52	69.96 ± 2.37
	VAP (μm/s)	76.03 ± 3.31[a]	90.70 ± 4.53[a,b]	78.41 ± 3.64[b]
	VCL (μm/s)	159.93 ± 5.90[a]	185.18 ± 6.78[a,b]	164.01 ± 7.01[b]
	VSL (μm/s)	59.83 ± 2.62[a]	69.68 ± 2.97[a]	62.42 ± 2.26
	STR%	0.78 ± 0.01	0.77 ± 0.01[a]	0.80 ± 0.01[a]
	LIN%	0.37 ± 0.01	0.37 ± 0.01	0.38 ± 0.01
	WOB%	0.47 ± 0.01	0.48 ± 0.01	0.47 ± 0.01
	ALH (μm)	4.80 ± 0.16[a]	4.73 ± 0.17[b]	4.16 ± 0.20[a,b]
	BCF (Hz)	24.53 ± 0.65[a,b]	27.73 ± 0.82[a]	29.77 ± 0.85[b]

Note: Similar letters within rows indicate statistical difference between columns for the same parameter, a,b,c $P < 0.05$. Abbreviations: PM, progressive motility; VAP, velocity of the average path; VCL, curvilinear velocity; VSL, straight line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, lateral head displacement; BCF, beat cross frequency.

species, .e.g. boar [15], stallion [16] and bull [22]. Density gradient centrifugation was effective in improving sperm viability and reducing bacterial contamination in human semen [23]. Considerable reduction in bacterial contamination was reported in boar semen samples that were processed using the SLC technique with a high density colloid [15]. In a similar study with stallion semen, approximately 90 % of the bacterial load could be effectively removed using SLC [12]. However, in a study using a higher g force for centrifugation, only approximately 50 % of the bacterial load could be removed from stallion semen samples [9]. In the present study, a low density colloid was used, specifically to recover as many spermatozoa as possible without selecting for good quality spermatozoa. The SLC treatment significantly reduced bacterial colony count compared to washed and extended samples, indicating its potential as an effective method for bacterial reduction in stallion semen. Since more bacteria could be removed by SLC than by washing without any detrimental effects on sperm characteristics, it could be a useful method of reducing bacterial load in stallion sperm samples for AI.

Numerous other studies on stallion spermatozoa prepared by SLC with a high density colloid have consistently shown improved sperm characteristics compared to control or extended groups [e.g. 12,24,25]. Improvements in motility and a decrease in fragmented DNA were reported in stallion sperm samples processed through SLC compared to extended samples in stored samples [12,25–27]. In donkey semen, SLC samples had higher proportions of viable spermatozoa and normal morphology after 24 h of cooled storage [28]. These findings support the notion that SLC using a high density colloid positively influences viability and DNA integrity in stallion sperm samples. In our present study with a **low density** colloid, where there was no selection for robust spermatozoa, there was no difference in sperm characteristics between extended samples, washed samples and SLC samples for the first 48h. However, SLC sperm samples had better motility than extended or washed samples at 72h after semen collection, suggesting that the deterioration in sperm quality seen in extended or washed samples was not as pronounced in the SLC-samples, as was also previously shown for boar spermatozoa [29].

In SLC samples, the sperm velocities were consistently lower than those of the extended group at all time points, as shown in a previous study [12], possibly as a result of traces of the colloid left in the sample. However, the velocity measurements are influenced by the specific instrument and settings used for analysis, such as the CASA system. With the SpermVision instrument, the SLC-spermatozoa exhibited a slower but straighter motility pattern than the extended samples. They also displayed less pronounced head movements than in the extended samples, as shown in a previous study [12]. On the other hand, BCF (an indirect measure of sperm energy) was higher in both the washed and SLC samples than in the extended samples. In other studies, selected spermatozoa after SLC through a high density colloid demonstrated higher fertility rates than unselected spermatozoa [30], suggesting that mean CASA kinematics may not necessarily be the best indictors of the ability of the spermatozoa to pass through the female reproductive tract.

In a previous study using a high density colloid, both membrane integrity and chromatin integrity were found to be higher in SLC samples than in washed samples during storage for 48 h [27]. However, the extender was different in previous study and the present study, and membrane integrity was evaluated by flow cytometry in the previous study, facilitating evaluation of many more spermatozoa than was possible by microscopy in the present study.

The SLC samples in the present study exhibited a lower bacterial content than both washed and extended samples. This suggests that even though there is no selection for robust spermatozoa, the use of SLC through a low density colloid can provide considerable benefit in reducing the bacterial content of the samples. Similarly, in a study with boar semen, bacterial load was reduced and sperm quality maintained during storage for one week. Furthermore, low-density colloid centrifugation resulted in the complete removal of two out of three bacteria from "spiked" boar semen (intentional addition of bacteria) [31]. In a preliminary artificial insemination trial with boar semen prepared by low density colloid centrifugation, sperm fertility was not adversely affected [32]. These compelling results further endorse the use of low-density colloid centrifugation as a reliable technique for reducing bacterial contamination in semen samples.

It is difficult to provide exact figures for the implications of this processing technique on the bacterial load in insemination doses since there is currently no one "standard" AI doses in equine breeding. The number of sperm to be inseminated and volume of inseminate vary in different countries and between different studs, and depend also on the insemination technique to be used. For conventional AI, sperm doses ranging from e.g. 200×10^6 progressively motile sperm [33], 600×10^6 progressively motile sperm [34] to 1000×10^6 progressively motile sperm [35] have been advocated. However, for the sake of argument, a comparison of the bacterial load after preparation of the AI dose by each of the three preparation techniqes mentioned here for an ejaculate of a given sperm concentration and motility is presented in Supplementary Table 1.

Among the six bacterial species that were still present after SLC, only *Mycoplasma subdolum* is considered to be a potential pathogen of the equine genital tract, since it has occasionally been implicated in reproductive tract pathologies in horses. However, since it has also been isolated from the genital tract of healthy mares and stallions [36] the pathogenicity of this bacterium is uncertain. Adhesion or even invasion of mycoplasmas into spermatozoa, as reported previously for other

Mycoplasma species [37,38], could have been responsible for the failure to separate *Mycoplasma subdolum* by SLC in our case. The other bacteria in this study that were still present after processing were possibly contaminating bacteria originating from humans, other animals and the environment.

The results of this study hold potential importance in reducing the reliance on antibiotics in semen extenders to inhibit bacterial growth. However, further research is required to establish the specific conditions and scenarios in which antibiotics can be safely excluded. For example, insemination studies are necessary to understand the implications and feasibility of excluding antibiotics from semen extenders, while maintaining optimal sperm characteristics and minimizing bacterial contamination. Such studies are currently underway, and will examine the effect on pregnancy rate as well as the future fertility of the mare in a subsequent insemination cycle.

The advantages of using a low density colloid, as in the present experiment, compared to the high density colloid used before is in the number of sperm available after centrifugation. With the high density colloid, selection is made for sperm with certain characteristics, but the number of sperm in the pellet after SLC will then depend on the quality of the original ejaculate. With the low density colloid, there is little or no selection, and nearly all the sperm would be expected to pass into the sperm pellet. Conventional insemination, as opposed to endoscopic or deep uterine insemination, uses several hundred million sperm. Therefore, one would assume that more insemination doses could be obtained with the low density colloid sperm preparation than the corresponding one with the high density colloid. What has not been established yet is how many sperm would actually be needed for conventional insemination after selection, when the sperm sample is considered to be highly fertile [30].

An interesting point to note is seminal plasma is removed when using SLC, at least with the high density colloid [39]. Several authors have observed a beneficial effect on sperm survival during storage from removing most of the seminal plasma from stallion semen [40–42]. However, others consider that a small amount of seminal plasma is necessary for the correct function of the uterus, but in our previous insemination studies, SLC-prepared stallion sperm samples were highly fertile, more so than the control samples that had not been prepared by SLC [30]. Therefore, the absence of seminal plasma did not affect the ability of these mares to become pregnant. In studies with mares that were prone to show an exaggerated post-breeding inflammatory response, there was a suggestion that the uterine response was much less if SLC-prepared sperm samples were inseminated [43]. However, since bacteria would also have been removed from the inseminated samples, it is not clear whether the lack of an exaggerated response was due to the absence of seminal plasma or to the absence of bacteria. At any rate, there is currently no suggestion that removing the seminal plasma by SLC is detrimental to fertility in the mare.

In this study, the penis of the stallion was not washed prior to semen collection. Washing the penis before semen collection may reduce the bacterial load in semen [44], although others (e.g.]2, 7]) did not find a reduction in colony forming units in semen from penis washing or not prior to semen collection. Where the penis was routinely washed with water before semen collection, the normal flora was replaced by potential pathogens [45]. Furthermore, *Pseudomonas* spp. (and, presumably, other water-borne bacteria) could be transferred to the penis from the water supply during washing [46] Therefore, on many studs, the penis is not washed routinely prior to semen collection unless it is clearly dirty [47].

5. Conclusion

Bacterial count in stallion semen samples was significantly reduced by SLC through a low-density colloid and there was no adverse effect on sperm characteristics compared to washed samples and controls (extended semen). These results have implications for reducing antibiotic usage in semen extenders. However, further research is needed to determine the conditions under which antibiotics can be safely excluded from insemination doses, and an insemination trial would be advantageous to ascertain that the fertilizing capacity of the sperm samples is retained.

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Ethical approval

Semen was collected from stallions with an artificial vagina which is a normal husbandry procedure in this species. Since no procedures were carried out on the animals themselves, no specific ethical approval was required for the study. The animals were housed and cared for according to national and international regulations for breeding equids.

Data availability

All data are presented in the manuscript.

CRediT authorship contribution statement

Pongpreecha Malaluang: Writing – original draft, Investigation, Formal analysis, Data curation. **Lisa Helène Wagner:** Investigation. **Aleksandar Cojkic:** Methodology. **Joachim Spergser:** Methodology, Investigation. **Christine Aurich:** Supervision, Methodology, Conceptualization. **Jane M. Morrell:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

JMM is the inventor and one of the patent holders of the colloid used in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.12.034.

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