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# Low-density colloid centrifugation removes bacteria from boar semen doses after spiking with selected species

E. Lacalle [a,b], S. Martínez-Martínez [c], E. Fernández-Alegre [a], C. Soriano-Úbeda [b,d,*], J.M. Morrell [e], F. Martínez-Pastor [b,d]

[a] Bianor Biotech SL, León, Spain
[b] INDEGSAL, Universidad de León, León, Spain
[c] Department of Animal Health, Universidad de León, León, Spain
[d] Molecular Biology (Cell Biology), Universidad de León, León, Spain
[e] Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

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ABSTRACT

Single-layer centrifugation (SLC) with a low-density colloid is an efficient method for removing contaminating microorganisms from boar semen while recovering most spermatozoa from the original sample. This study tested the performance of this technique, using 50-ml tubes, by spiking commercial semen doses prepared without antibiotics with selected bacterial species followed by storage at 17 °C. The doses were spiked up to $10^2$/ml CFU (colony forming units) of the bacteria *Burkholderia ambifaria*, *Pseudomonas aeruginosa*, and *Staphylococcus simulans*. The semen was processed by SLC (15 ml of sample and 15 ml of colloid) with the colloid Porcicoll at 20% (P20) and 30% (P30), with a spiked control (CTL) and an unspiked control (CTL0), analyzing microbiology and sperm quality on days 0, 3 and 7. SLC completely removed *B. ambifaria* and *S. simulans*, considerably reducing *P. aeruginosa* and overall contamination (especially P30, ~$10^4$ CFU/ml of total contamination on day 7, median). Sperm viability was lower in P20 and P30 samples at day 0, with higher cytoplasmic ROS. Still, results were similar in all groups on day 3 and reversed on day 7, indicating a protective effect of SLC (possibly directly by removal of damaged sperm and indirectly because of lower bacterial contamination). Sperm chromatin was affected by the treatment (lower DNA fragmentation and chromatin decondensation) and storage (higher overall condensation on day 7 as per chromomycin A3 and monobromobimane staining). In conclusion, SLC with low-density colloids can remove most bacteria in a controlled contamination design while potentially improving sperm quality and long-term storage at practical temperatures.

## 1. Introduction

Global efforts are underway to replace antibiotics in all human activities, with serious efforts in animal husbandry due to evidence of widespread antimicrobial resistance (AMR) (Van Boeckel et al., 2019). There are many research lines regarding sperm work and artificial insemination (AI) (Santos and Silva, 2020; Schulze et al., 2020) to avoid antibiotics in semen extenders and processing media. Avoiding contamination during semen collection is difficult since bacteria and other microorganisms colonize the distal part of the reproductive tract, and, despite cleaning and strict hygiene, contamination of the ejaculate may occur (Paschoal et al., 2021; Nitsche-Melkus et al., 2020).

Semen contamination is a critical problem in the swine industry since storage temperatures are usually at ranges that enable bacterial growth, although at a reduced rate (16–18 °C). Microorganisms can modify extender characteristics and affect spermatozoa directly (Martínez-Pastor et al., 2021; Sepúlveda et al., 2016; Lopez Rodriguez et al., 2017), potentially lowering reproductive performance (Kuster and Althouse, 2016; Maroto Martín et al., 2010). Moreover, sows can be affected if inseminated with doses containing pathogenic bacteria (Morrell and Wallgren, 2014). Thus, antibiotics are a usual component of semen extenders for the swine industry, preventing critical economic losses. Moreover, AMR has been detected in boar semen (Bresciani et al., 2014), compromising the use of these antimicrobials. A challenge for the industry is to remove antibiotics from the extenders while preventing bacterial growth.

* Corresponding author at: INDEGSAL, Universidad de León, León, Spain.
*E-mail address:* c.soriano.ubeda@unileon.es (C. Soriano-Úbeda).

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In previous studies, we tested removing contaminant bacteria from boar semen instead of adding substances to limit their growth. Single layer centrifugation (SLC) using the Porcicoll colloid enabled most spermatozoa in the pellet to be recovered while retaining bacteria and debris in the upper phase, however, at the cost of some spermatozoa. A further refinement utilized a low-density colloid (1.052 g/ml), demonstrating the removal of bacteria while recovering more spermatozoa (Deori et al., 2020). The storage of selected spermatozoa without antibiotics after processing the sample in 50-ml tubes showed that SLC processing considerably reduced bacterial presence even after many days (Morrell et al., 2019). Moreover, in a recent study, we presented a proof-of-concept for large-volume processing (in 500-ml bottles) using semen purposely collected in a manner that would promote contamination (Martínez-Pastor et al., 2021). Despite detecting bacteria at relatively high concentrations, the SLC was highly efficient at removing them, thus controlling their growth throughout the storage time, and semen quality was noticeably improved.

Previous studies used semen samples that had been naturally contaminated, therefore containing a wide range of bacterial species with no control over these contaminants. However, these accidental contaminants are of little interest to the stud centers and can be largely prevented by following hygienic collection protocols (Morrell et al., 2019). Some bacterial genera or species are of particular interest because of being fastidious or due to their negative effects on semen doses. This study was designed to test the ability of SLC with low-density colloids to remove specific bacterial species isolated by a stud center. We hypothesized that, under controlled conditions, samples spiked with cultured bacteria could be successfully cleaned and stored using SLC.

## 2. Methods

### 2.1. Experimental design

The experimental design is shown in Fig. 1. Boar semen doses ($n = 7$) prepared in BTS (Beltsville Thawing Solution, without antibiotics) were spiked with bacterial cultures at a final concentration of $10^2$ CFU/ml (colony-forming units). Three bacterial strains were selected by the same pig company donating the semen doses (isolates from their farms): *Burkholderia ambifaria*, *Pseudomonas aeruginosa*, and *Staphylococcus simulans*.

Previously to spiking, an aliquot was separated as an unspiked control (CTL0). After spiking, the samples were split among spiked control (CTL), SLC through 20% colloid (Porcicoll, P20), and 30% colloid (P30). After SLC, the pellets were reconstituted in BTS. All samples were stored at 17 °C for seven days. On days 0, 3, and 7, the samples were analyzed for sperm motility, quality, and chromatin structure by flow cytometry and bacterial growth (for the three spiked species and total CFU/ml).

### 2.2. Semen extender

The semen extender was prepared at our laboratory and consisted of a modified Beltsville Thawing Solution (BTS) (Morrell et al., 2019). The composition was glucose (205.4 mM), tri-sodium citrate (20.4 mM), sodium hydrogen carbonate (14.9 mM), sodium EDTA (3.4 mM), and potassium chloride (10.1 mM). The medium contained no antibiotics and was filtered (0.2 μm pore) and stored refrigerated until use.

### 2.3. Animals and semen collection

Semen doses were produced at a commercial pig station (AIM Ibérica; Topigs-Norsvin Spain, Campo de Villavidel, León, Spain) from seven boars (3 Landrace, 4 Large White). The boars were kept under standard husbandry conditions, according to national and international regulations on the housing and care of animals. The boars were adults 1–2.5 years of age and tested for good fertility (routine semen production). In this pig station, the boars are kept in individual pens at 18–22 °C and constant photoperiod 12 h/d, with 2.5 kg/d of a 13% protein diet, water *ad libitum*. Semen collection was performed twice weekly by the gloved-hand technique to prepare commercial semen doses for AI. AIM Ibérica was responsible for all the procedures and donated the semen doses to the researchers, with no experimental procedures carried out on animals.

After collection, the samples were analyzed at the AIM laboratory, assessing subjective motility, normal morphology, and sperm

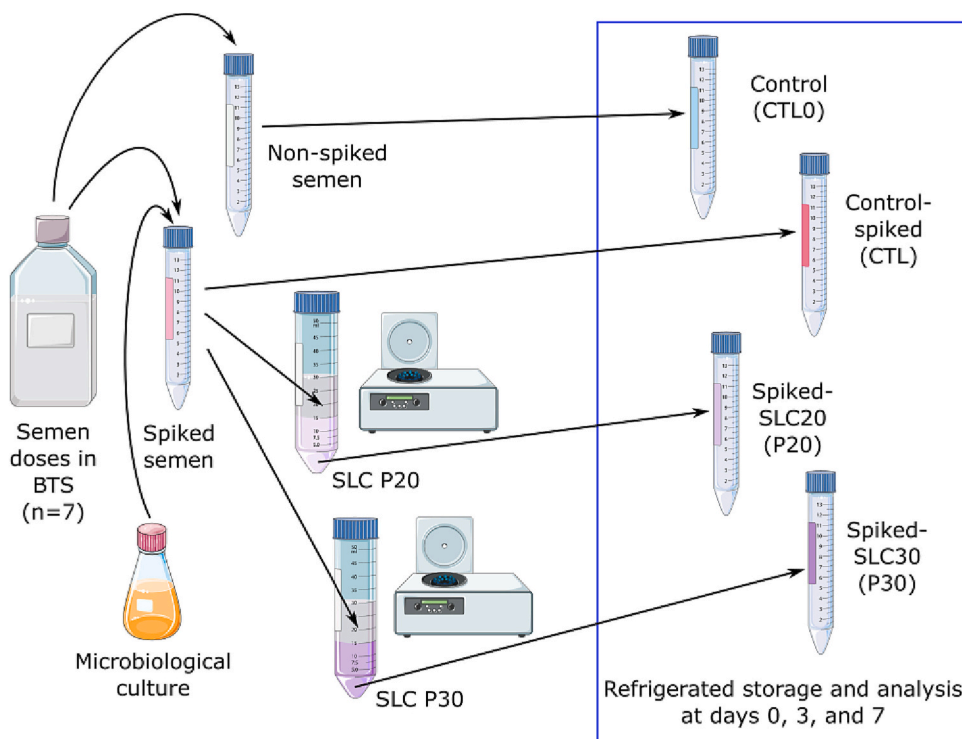


**Fig. 1.** Schematics of the experimental design. Seven semen doses (Beltsville Thawing Solution, BTS, without antibiotics) were split into non-spiked and spiked samples ($10^2$ CFU/ml of *Burkholderia ambifaria*, *Pseudomonas aeruginosa*, and *Staphylococcus simulans*). The spiked samples were processed by SLC (Single Layer Centrifugation) through 20% (P20) and 30% Porcicoll colloid (P30), and the pellets were resuspended. The resulting four treatments (Control, non-processed: CTL0; Control, spiked non-SLC: CTL; Spiked and SLC with 20% Porcicoll: P20; and spiked and SLC with 30% Porcicoll: P30) were stored at 17 °C and assessed at days 0 (after processing), 3, and 7 by CASA, microscopy for morphology, and flow cytometry (physiology and chromatin status).

concentration (Garcia et al., 2010). The ejaculates selected for the present study were normozoospermic with ≥80% motile spermatozoa, ≥75% morphologically normal, and ≥ 95% normal acrosomes (following the center's guidelines for commercial semen doses). Then, the ejaculates were extended with the antibiotic-free BTS (provided by the researchers) at 32.5 °C and 100 × $10^6$ $ml^{-1}$ and transported in an insulated container (18 °C, ~20 min) to the researchers' laboratory at the Institute of Animal Health and Cattle Development (INDEGSAL, University of León, Spain).

### *2.4. Bacterial cultures preparation*

The Microbiology group at the University of León provided the starters for the bacterial cultures (*Burkholderia ambifaria*, *Pseudomonas aeruginosa*, and *Staphylococcus simulans*). First, the bacteria were grown in solid medium (TSA, tryptone-soya-agar) at 37 °C in aerobiosis. Colonies were homogeneously suspended in PBS to a concentration between $10^6$ and $10^7$ CFU/ml (nephelometry).

### *2.5. Single layer centrifugation (SLC) with Porcicoll*

The Porcicoll colloids are silane-coated silica formulations for boar semen processing. The low-density formulations were prepared at a density of 1.039 g/ml for 30% (P30) and at 1.026 g/ml for 20% (P20). For spiking, 50 ml of sample received $10^2$ CFU/ml of each bacterial species (500 μl per bacterial species, with CTL0 receiving 450 μl of culture medium). Then, the spiked sample was divided into a 15-ml CTL portion, and the remaining volume was used for SLC (Fig. 1).

SLC was carried out in 50-ml conical tubes (Morrell et al., 2019) by layering 15 ml of colloid and 15 ml of sample. Then, the tubes were centrifuged at 300 × *g* for 20 min and no brake. The supernatant was removed, and the sperm pellet was resuspended in sterile BTS up to 100 × $10^6$ $ml^{-1}$ (concentration assessed with a haemocytometer, Bürker chamber, by duplicate). All samples were stored at 17 °C in 15-ml tubes, removing 1.5 ml of sample on days 0, 3, and 7 for analysis. All procedures except centrifugation were carried out in a laminar-flow bench to prevent environmental contamination.

### *2.6. Bacterial counts and other microbiological analyses*

For each analysis, sample aliquots were sent to the microbiology laboratory to assess the presence and number of microorganisms in the samples. Procedures were carried out as previously (Morrell et al., 2019), following validated protocols (Moretti et al., 2009; Oviaño et al., 2017). Briefly, the samples (0.1 ml) were cultured for 24 h at 37 °C on a microaerophilic atmosphere in various media: Blood Columbia agar, Cystine-Lactose-Electrolyte-Deficient (Cled) agar, McConkey agar and tryptone soy agar (TSA; OXOID, Hampshire, UK).

For the bacterial count, dilutions were made from −1 to −6, followed by seeding 100 μl in Agar TSA. The number of colonies was counted, and the results were expressed as colony-forming units/ml. Plates were incubated for a further 24 h and read again.

Bacteria were characterized using different methods depending on type: Gram stain, oxidase, and catalase activity and biochemical test (API 20E, API 20NE, API Staph, API Strep; Bio Merieux Inc., Durham, NC), according to the manufacturer's instructions.

Samples were analyzed using Bruker Daltonics UltrafleXtreme MALDI-TOF/TOF equipment controlled with the FlexControl software v. 3.0 (Brucker Daltonics, Bremen, Germany) to acquire mass spectra. Microbial identification was carried out with the Biotyper Real-Time Classification software v3.1 (Brucker Daltonics), comparing the spectra with the corresponding database provided by the manufacturer (MALDI Biotyper database, 5989 entries, Bruker Daltonics). This similarity is expressed as a score ranging from 0 to 3, displaying the top 10 matching results with the highest scores. The reliability of the identification was evaluated according to standard interpretative criteria: 2.300–3.000, high species identification probability; 2.000–2.290, high genus identification probability; 1700–1.999, presumable species identification; 1.700–1.999 presumable genus identification; 0.000–1.699 unreliable identification.

### *2.7. Sperm morphology*

A 5-μl aliquot of each sample was fixed in PBS with 0.5% formaldehyde and kept at 5 °C. A 5-μl drop on a slide was covered with a coverslip and observed at ×400 with negative contrast optics (Nikon E400, Tokyo, Japan), assessing at least 200 cells. Abnormalities were classified according to their location (head, midpiece, and principal piece). The presence of a proximal or distal cytoplasmic droplet was also recorded. Counts were converted to proportions.

### *2.8. Sperm motility analysis*

Since SLC-processed samples usually present a high degree of sperm stickiness, compromising the accuracy of automated systems, only subjective motility assessment was carried out in this study by the same experienced technician. Motility was estimated by first diluting 5 μl of sample in 10 μl of BTS with 1% of polyvinyl alcohol (to delay stickiness) at 37 °C and then passing a 5-μl drop into a modified Makler counting chamber (20 μm depth; Haifa Instruments, Israel) and a phase contrast microscope (Nikon E400 with warmed stage at 37 °C; ×10 negative contrast optics). At least two drops were evaluated, estimating the proportion of motile and progressive sperm (following an overall straight path) immediately after mounting the Makler chamber to avoid artifacts due to stickiness.

### *2.9. Flow cytometry assessment of sperm quality and chromatin structure*

Sperm quality was assessed as described previously (Fernández-Gago et al., 2013; Tamargo et al., 2019). Fluorescent probes were prepared in BTS supplemented with 0.5% bovine serum albumin (BSA) at the following final concentrations: Hoechst 33342 (H342, 4.5 μM) to facilitate discrimination of debris; Hoechst 33258 (H258, 4.5 μM) or propidium iodide (PI; 1.5 μM) for viability; YO-PRO-1 (YP; 100 nM) for apoptotic-like changes; PNA-Alexa 647 (PNA; 1 μg/ml) for assessing the acrosomal status; $H_2DCFDA$ (CFDA; 5 μM) for detecting cytoplasmic reactive oxygen species (ROS); merocyanine 540 (M540; 2 μM) for assessing capacitation-like changes; MitoSOX (MX; 1 μM) for detecting mitochondria-produced superoxide; Mitotracker deep red (MT; 100 nM) for assessing mitochondrial activity. These probes were combined as H342/YP/M540/PI/PNA (viability, apoptosis, capacitation, viability, and acrosomal status) and H258/CFDA/MX/MT (viability, cytoplasmic ROS, mitochondrial superoxide, and mitochondrial activity). After adding the spermatozoa ($10^6$ $ml^{-1}$), the mixture was incubated in the dark for 15 min at 37 °C and run through a MACSQuant Analyzer 10 (Miltenyi Biotek, Bergisch Gladbach, Germany). We used three lasers for exciting the fluorochromes (violet at 405 nm, blue at 488 nm, and red at 635 nm; V, B, and R, respectively). The filters in each line were: 450/50 nm as V1 for H342; 525/50 nm as B1 for YP, 585/40 nm as B2 for M540, and 655–730 nm as B3 for PI and MX; and 655–730 nm R1 for PNA-Alexa647 and MT. The cytometer was suited with the MACSQuantify™ software. The spermatozoa were identified with appropriate gates in FSC/SSC (forward/side scatter) and H342/SSC cytograms ($H342^+$ events as spermatozoa), with a minimum of 10,000 events acquired and two technical replicates per sample. Data was saved as FSC v.3 files and analyzed by Weasel v. 3.7 (Frank Battye, WEHI, Victoria, Australia). The variables obtained were: Viable sperm as $PI^-$ or $H258^-$, respectively; $YP^-$ spermatozoa as viable-non apoptotic; the ratio of non-apoptotic within viable ($YP^+$ within the $PI^-$ population); acrosome-damaged spermatozoa as $PNA^+$; the ratio of the acrosome-damaged spermatozoa in the live population ($PNA^+$ within $PI^-$); the ratio of capacitated sperm within viable-non apoptotic ($M540^+$ within the $YP^-$ population);

spermatozoa with active mitochondria as $H258^{-}MT^{+}$; the ratio of sperm with high cytoplasmic ROS production within the viable population ($CFDA^{+}$ within $H258^{-}$); and the ratio of high mitochondrial-superoxide production within the viable population ($MX^{+}$ within the $H258^{-}$ population).

### 2.10. Flow cytometry assessment of chromatin structure

Chromatin status was assessed using the Sperm Chromatin Structure Assay (SCSA), as described previously (Fernández-Gago et al., 2017). Briefly, samples were stored at −80 °C in TNE ($2 \times 10^{6}$ $ml^{-1}$; 0.01 M Tris-HCl, 0.15 M NaCl, 1 mM $Na_2EDTA$; pH 7.4). After thawing, 200 μl of sample were mixed with 0.4 ml of acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100; pH 1.2). After 30 s, 1.2 ml of staining acridine orange (AO) solution (6 μg/ml AO in 0.1 M citric acid, 0.2 M $Na_2HPO_4$, 1 mM $Na_2EDTA$, 0.15 M NaCl; pH 6.0) were added, incubating for 3 min. The samples were run through a FACScalibur flow cytometer with the acquisition software CellQuest v. 3 (Becton Dickinson, Franklin Lakes, NJ, USA), acquiring at 200 cells/s and at least 5000 spermatozoa; AO was excited with the Ar-ion 488 nm laser, detecting green fluorescence with a 530/30 filter (dsDNA-bound AO), and the red fluorescence with a 650 long-pass filter (ssDNA-bound AO). Data were saved in flow cytometry standard (FCS) v. 2 files and processed using the R statistical environment (R Core Team 2019) to obtain the proportion of DNA-damaged spermatozoa (%DFI) and the proportion of spermatozoa with immature chromatin (%HDS).

Sperm chromatin structure was also tested by chromomycin A3 staining (CMA3), a method for testing DNA accessibility related to the protamine/histone balance and overall protamination (Zubkova et al., 2005). The spermatozoa were diluted at $2 \times 10^{6}$ $ml^{-1}$ in McIlvaine's medium (17 mM citric acid, 166 mM $Na_2HPO_4$, 8.3 mM $MgCl_2$) with 0.2 mM CMA3 (20 min, 25 °C), washed and resuspended with PBS with H342 (4.5 μM). The samples were run through the MACSQuant Analyzer 10, described in 2.9, using the V1 photodetector for the H342 fluorescence and the B2 for the yellow fluorescence of CMA3. The proportion of cells with high CMA3 fluorescence and the mean fluorescence intensity of CMA3 were recorded.

Finally, the levels of disulfide bridges (protamine compaction) were estimated by monobromobimane (mBBr) staining. The samples were diluted in PBS to $2 \times 10^{6}$ $ml^{-1}$ and divided into two aliquots. One of them (reference) was incubated for 10 min at 37 °C with 1 mM dithiothreitol (DTT) and washed. Both tubes were incubated with 500 μM mBBr for 10 min at 37 °C in the dark, washed, and resuspended in PBS with 2 μM PI. The samples were run through the MACSQuant Analyzer 10, using the V1 photodetector for the blue fluorescence of sulfhydryl-bound mBBr and the B3 for the PI fluorescence (in this case, for discriminating debris). The proportion of cells with high mBBr fluorescence in the samples and the mean fluorescence intensity of mBBr in samples and references were recorded. Disulfide bridges levels were estimated by subtracting the MFI value of the references from their respective samples and dividing by 2 (Zubkova et al., 2005).

### 2.11. Statistical analysis

Results are presented as means ± SEM unless otherwise specified. Data were analyzed in the R statistical environment (R Core Team, 2021). Variables were first tested for homogeneity of variances and normality with Levene's test and QQ-plots and transformed with the Box-Cox. Models were analyzed with linear mixed-effects models with treatments and storage time as fixed effects and boar and ejaculate as random effects. Pairwise comparisons were adjusted by Tukey's method. The analysis of associations between bacterial load and sperm quality was carried out by Pearson correlations, with $P$ values corrected by the false discovery method (FDR). $P < 0.05$ was used as the threshold for significance.

## 3. Results

### 3.1. Recovery rate and bacterial growth

The mean (± SD) recovery rate after SLC was 90.4% ± 13.6 for P20 and 82.8% ± 22.9 for P30, showing very low retention of spermatozoa in the colloid.

The results of the microbiology analyses are summarized in Tables 1 and 2. Spiked bacteria *B. ambifaria* and *P. aeruginosa* were absent from the unspiked control even on day 7 (Table 1), but *S. simulans* was found at low concentrations as an environmental contaminant. Its presence increased on day 3, and on day 7, it was not significantly different from the spiked control. The spiked control showed a fast growth with time, especially for *B. ambifaria*. Regarding the spiked species, both colloids efficiently removed the bacteria from the samples, with no detectable *B. ambifaria* and *S. simulans* at day 7. However, *P. aeruginosa* was present at low concentrations after SLC and increased with time ($P < 0.001$), reaching concentrations lower than CTL but non-significantly different at day 7 ($P > 0.05$).

Considering overall contamination (Table 2), bacteria were found in all treatments, but SLC-processed samples showed the lowest, even when compared with the unspiked control ($P < 0.05$). Contamination increased notably in the unspiked control and P20 (although lower in the latter than in the former); it remained low on day 3, and on day 7, only two samples presented contamination within the range of the spiked control at day 0.

### 3.2. Sperm quality

#### 3.2.1. Sperm motility and morphology

Motility and morphology results are displayed in Fig. 2. After the SLC, total and progressive motility decreased slightly but significantly (Figs. 2a and b). However, motility evaluation was affected by the tendency of processed spermatozoa to stick to the glass of the microscopic chambers (which increased with storage in all samples). Although there were no differences in total motility at day 7, the control samples tended to present lower values.

The proportion of spermatozoa with abnormalities or cytoplasmic droplets presented minor differences between treatments and days (Figs. 2c–i). Considering total abnormalities (excluding droplets; Fig. 2c), the SLC-treated samples showed a higher proportion on day 7, mainly influenced by the combination of abnormal midpieces and principal pieces (Figs. 2f and g) and due to a higher observation of bent

**Table 1**
Median and first and third quartiles (parentheses) for each spiked bacterial species ($\times 10^{3}$ CFU/ml) in controls (CTL0: non-spiked; CLT: Spiked, no SLC) and samples after Single Layer Centrifugation through 20% (P20) and 30% Porcicoll (P30). Latin letters vary among significantly different treatments within each day ($P < 0.05$), and Greek letters differ among days within each treatment for $P < 0.05$.

| Day | Treatment | *Burkholderia ambifaria* | *Pseudomonas aeruginosa* | *Staphylococcus simulans* |
|---|---|---|---|---|
| 0 | CTL0 | $0^{b}$ (0,0) | $0^{c}$ (0, 0) | $0.2^{b\alpha}$ (0.1, 0.4) |
| | CTL | $8.2^{a\alpha}$ (2.5, 32.5) | $8.3^{a\alpha}$ (5.8, 15.6) | $20^{a\alpha}$ (4, 52) |
| | P20 | $0^{b}$ (0, 0) | $0.2^{b\alpha}$ (0.2, 0.4) | $0^{c}$ (0, 0) |
| | P30 | $0^{b}$ (0, 0) | $0.2^{b\alpha}$ (0.1, 0.2) | $0^{c}$ (0, 0) |
| 3 | CTL0 | $0^{b}$ (0, 0) | $0^{c}$ (0, 0) | $8.1^{b\beta}$ (7.2, 42.3) |
| | CTL | $270^{a\beta}$ (146, 545) | $47.5^{a\beta}$ (10.9, 244.5) | $73^{a\beta}$ (25.5, 165) |
| | P20 | $0^{b}$ (0, 0) | $5.5^{b\beta}$ (3.2, 7.1) | $0^{c}$ (0, 0) |
| | P30 | $0^{b}$ (0, 0) | $0.8^{b\beta}$ (0.4, 0.9) | $0^{c}$ (0, 0) |
| 7 | CTL0 | $0^{b}$ (0, 0) | $0^{c}$ (0, 0) | $75^{a\gamma}$ (18.8, 660) |
| | CTL | $1600^{a\gamma}$ (840, 2400) | $535^{a\gamma}$ (237.5, 2280) | $710^{a\gamma}$ (445, 965) |
| | P20 | $0^{b}$ (0, 0) | $195^{ab\gamma}$ (101.5, 597.5) | $0^{b}$ (0, 0) |
| | P30 | $0^{b}$ (0, 0) | $8.6^{b\gamma}$ (7.3, 26.4) | $0^{b}$ (0, 0) |

**Table 2**
Total bacterial counts for each boar ($\times 10^3$ CFU/ml) in controls (CTL0: non-spiked; CLT: Spiked, no SLC) and samples after Single Layer Centrifugation through 20% (P20) and 30% Porcicoll (P30). Lower rows show the median and the first and third quartiles. Superscripts in the Median row show significant differences, with Latin letters varying among significantly different treatments within each day ($P < 0.05$), and Greek letters differing among days within each treatment for $P < 0.05$.

| | Day 0 | | | | Day 3 | | | | Day 7 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Boar | CTL0 | CTL | P20 | P30 | CTL0 | CTL | P20 | P30 | CTL0 | CTL | P20 | P30 |
| 1 | 0.6 | 151.4 | 0.2 | 0.2 | 7.9 | 942.7 | 2.9 | 0.3 | 954.7 | 2472.8 | 130.2 | 32.1 |
| 2 | 0.8 | 122.4 | 0.4 | 0.2 | 71.7 | 537.8 | 7.5 | 1 | 895.8 | 3838.2 | 129 | 9.9 |
| 3 | 0.5 | 49.6 | 0 | 0 | 98.4 | 1004.8 | <0.1 | <0.1 | 289.8 | 13,711 | 0.6 | <0.1 |
| 4 | 0.9 | 52.8 | 0.8 | 0.3 | 54.8 | 259.6 | 4.7 | 1.1 | 362.5 | 2163 | 333 | 16 |
| 5 | 0.9 | 5.1 | 0 | 0 | 97.4 | 303.1 | <0.1 | <0.1 | 558 | 3710.4 | 0.2 | <0.1 |
| 6 | 0.3 | 46.6 | 0.2 | 0.2 | 62.7 | 840.9 | 6.8 | 1 | 836.7 | 5104.8 | 710.3 | 9.8 |
| 7 | 0.3 | 38.4 | 0.2 | 0.3 | 76.6 | 100.9 | 9.3 | 3.5 | 775.1 | 672.8 | 750.1 | 74 |
| Median | $0.6^{a\alpha}$ | $49.6^{b\alpha}$ | $0.2^{c\alpha}$ | $0.2^{c\alpha}$ | $71.7^{a\beta}$ | $537.8^{b\beta}$ | $4.7^{c\beta}$ | $1^{c\beta}$ | $775.1^{ab\gamma}$ | $3710.4^{a\gamma}$ | $130.2^{bc\gamma}$ | $9.9^{c\gamma}$ |
| Q1, Q3 | 0.4, 0.9 | 42.5, 87.6 | <0.1, 0.3 | <0.1, 0.25 | 58.8, 87 | 281.4, 891.8 | 1.5, 7.15 | 0.2, 1.05 | 460.3, 866.3 | 2317.9, 4471.5 | 64.8, 521.7 | 5.0, 24.1 |

or coiled flagella. The proportion of these abnormalities was only slightly higher than for CTL0 and not critical for sperm quality. The average proportion of proximal droplets was higher at day 0 for CTL0 than for P30 ($P = 0.009$), being the only difference detected for treatment or time.

### 3.2.2. *Flow cytometry analysis of sperm physiology*

The most relevant parameters regarding sperm physiology are shown in Fig. 3. The SLC negatively influenced sperm viability at day 0 (Figs. 3a and b; $P < 0.001$), but this effect tended to dissipate with the storage time, with a general trend to remain higher in the processed samples by day 7. A similar change was observed for acrosomal damage and sperm capacitation (Figs. 3d–f). Interestingly, the acrosomal damage (Figs. 3d and e) seemed to decrease with time for the SLC samples. Combined with the information from the viability and merocyanine probes, this could be due to an increased membrane permeability after the SLC rather than to an actual loss of membrane and acrosomal integrity.

The proportion of spermatozoa with active mitochondria was not affected by the treatments and decreased with storage time, but only by day 7 (Fig. 3g). However, the production of free radicals, both cytoplasmic (Fig. 3h) and mitochondrial (Fig. 3i), was affected both by treatment and time. Interestingly, we observed a higher proportion of live spermatozoa with high cytoplasmic ROS production by day 0 in the SLC samples than the controls ($P < 0.001$). These values dropped to the same levels by day 3, but on that day, the proportion of live spermatozoa with elevated mitochondrial superoxide was higher in P20 and P30 than in the controls ($P < 0.001$). However, by day 7, both parameters were higher for the controls, especially for the spiked one (CTL), and P30 showed the lowest values ($P < 0.001$ for all the significant comparisons).

### 3.2.3. *Flow cytometry analysis of the chromatin structure*

Results of the analyses of the chromatin structure are shown in Fig. 4. The SCSA technique yielded variables %DFI as the estimator of DNA fragmentation and %HDS as the estimator of DNA compaction or maturity. At days 3 and 7, %DFI was significantly lower for the SLC-treated samples (Fig. 4a). %HDS showed the same trend with lower values for P20 and P30 (Fig. 4b), and, interestingly, demonstrated a complex dynamic for chromatin compaction, significantly decreasing by day 3 but returning to initial values on day 7.

The sperm populations obtained from the CMA3 analysis (Fig. 4c–e) showed only small changes considering the treatments and time. At day 0, the control samples showed a lower proportion for the population with moderate CMA3 fluorescence, compared with the SLC-treated samples, with similar values for days 3 and 7.

Considering the analysis of the free thiols and disulfide bridges among protamines, the mBBr staining suggested a dynamic rearrangement of the sperm chromatin with time. The sperm populations derived from the mBBr staining (Figs. 4f–h) indicated a lower availability of free thiols in the successive days of analysis, with an increase of the low-mBBr fluorescence population, whereas the moderate and high-mBBr decreased. The treatments significantly varied on days 0 and 3, with the controls showing a lower proportion of low-mBBr cells by day 0 but a higher one by day 3, and the opposite pattern was observed for moderate-mBBr cells. The overall amount of disulfide bridges (Fig. 4i), estimated from the fluorescence difference of DTT-treated and neat samples, did not differ among treatments, but it notably increased on day 7 ($P < 0.001$).

## 3.3. *Correlations between bacterial presence and sperm parameters*

Fig. 5 displays a heatmap with the significant correlations between the bacterial concentration and the sperm parameters described in 3.2. The total bacterial count (as CFU/ml) was negatively correlated with abnormal midpieces, total motility, and the high-mBBr fluorescence population, and positively with the DNA fragmentation as %DFI, the overall presence of disulfide bridges, and the low-mBBr fluorescence population. When considering the spiked bacteria, *P. aeruginosa* did not significantly correlate with the sperm parameters, whereas *B. ambifaria* and *S. simulans* were positively correlated with the apoptotic ratio. *S. simulans* was also positively correlated with %DFI and %HDS. An additional analysis within each treatment (tables S2–4 in the supplementary material) yielded consistent associations of bacterial loads with sperm quality and the high-mBBr fluorescence population (negative) and with the low-mBBr fluorescence population and disulfide bridges (positive).

## 4. Discussion

Previous studies have tested the suitability of the SLC with low-density colloid (20% and 30%) for processing boar semen in medium and large volumes and challenging it with moderate bacterial contamination (Martínez-Pastor et al., 2021; Deori et al., 2020; Morrell et al., 2019; Morrell and Wallgren, 2011). However, the method had not been tested in more controlled conditions considering the type and abundance of bacteria in the samples. Thus, the present study contributes towards validating this method by confirming its good performance in removing microbiological contamination and improving sperm quality in stored boar doses.

As reported previously (Martínez-Pastor et al., 2021), the SLC not only contributed to preserving sperm quality by removing bacteria, but this procedure seemed to affect the spermatozoa directly and could positively contribute to its long-term preservation. The SLC altered the sperm viability and acrosomal integrity shortly after the procedure (on day 0), but our results confirm a subsequent recovery, suggesting that there is not real cell damage but rather a transient modification of the sperm membranes. The sperm plasmalemma is considerably modified during its passage through the epididymis and mixing with seminal plasma. Sperm handling procedures alter the plasmalemma composition, especially the peripheral proteins coating the sperm head and modulating its physiology (Leahy and Gadella, 2011; Kruse et al., 2011).

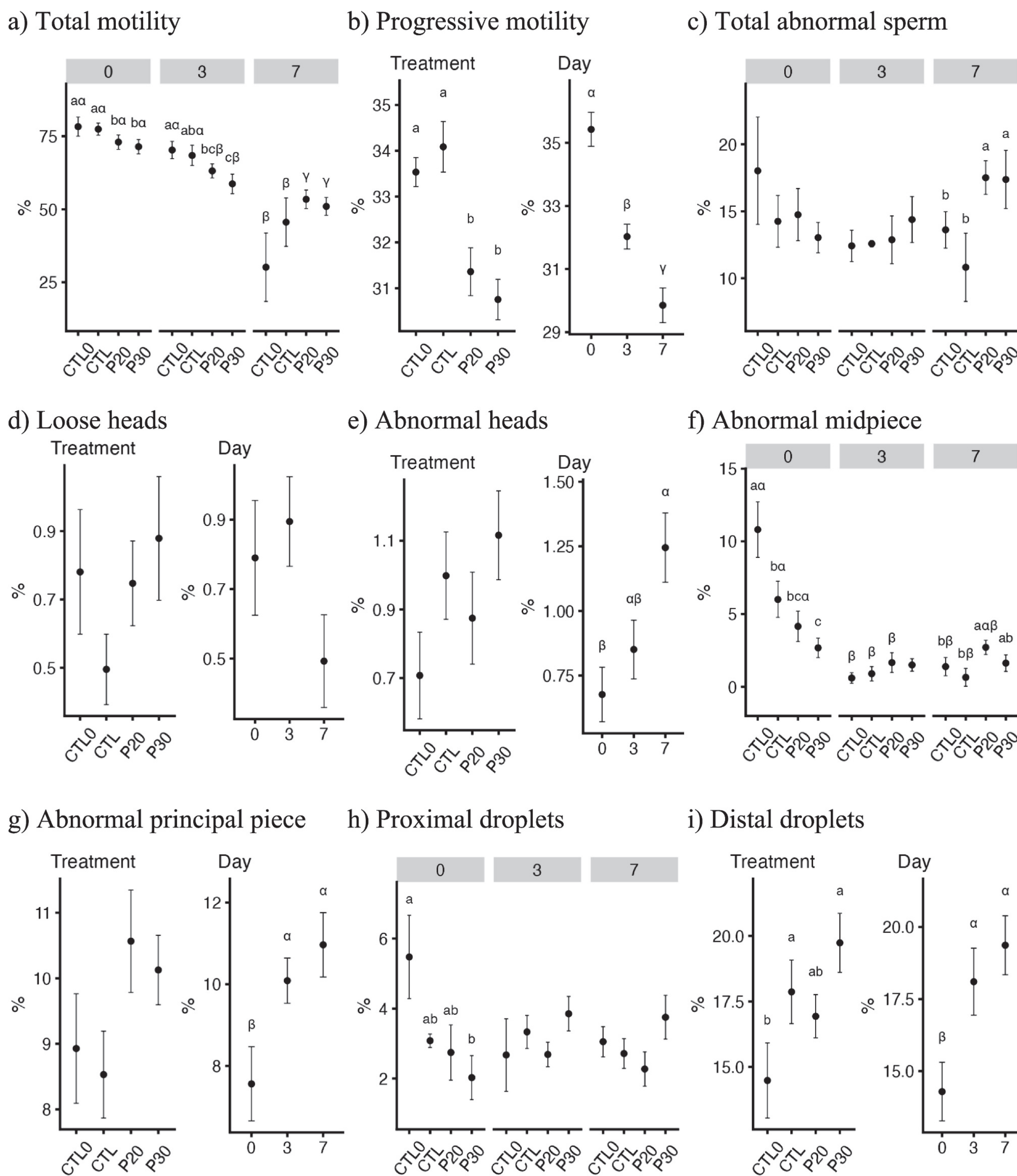


**Fig. 2.** Effect of the colloid centrifugation on sperm motility and abnormalities (CTL0: Unspiked control; CTL: Spiked control; P20 and P30: Spiked and submitted to SLC with Porcicoll 20% and 30%, respectively). The plots show mean ± SEM for the variables for each combination of treatment and storage day. Results from models with a significant interaction between factors are presented in a single panel, with different lowercase Latin letters indicating $P < 0.05$ between treatments within days, and different Greek letters indicate $P < 0.05$ between days within treatments. When the interaction was not significant, the factors were studied as main effects (double panel, b, d, e, g, i).

This dynamic was also observed in previous studies using SLC with boar semen (Martínez-Pastor et al., 2021; Morrell et al., 2019), and it could indicate that an adaptation period follows these procedures until membrane permeability is restored. The incidence of these processes on sperm fertility should be tested.

The increase of $ROS^+$ spermatozoa observed in SLC-treated samples on day 0 could be related to this adaptation process as a stress response (O'Brien et al., 2021). In fact, carrying out the SLC seemed to protect the spermatozoa against increasing ROS production observed in the controls on day 7. Whereas bacterial contamination appears to play a role in this

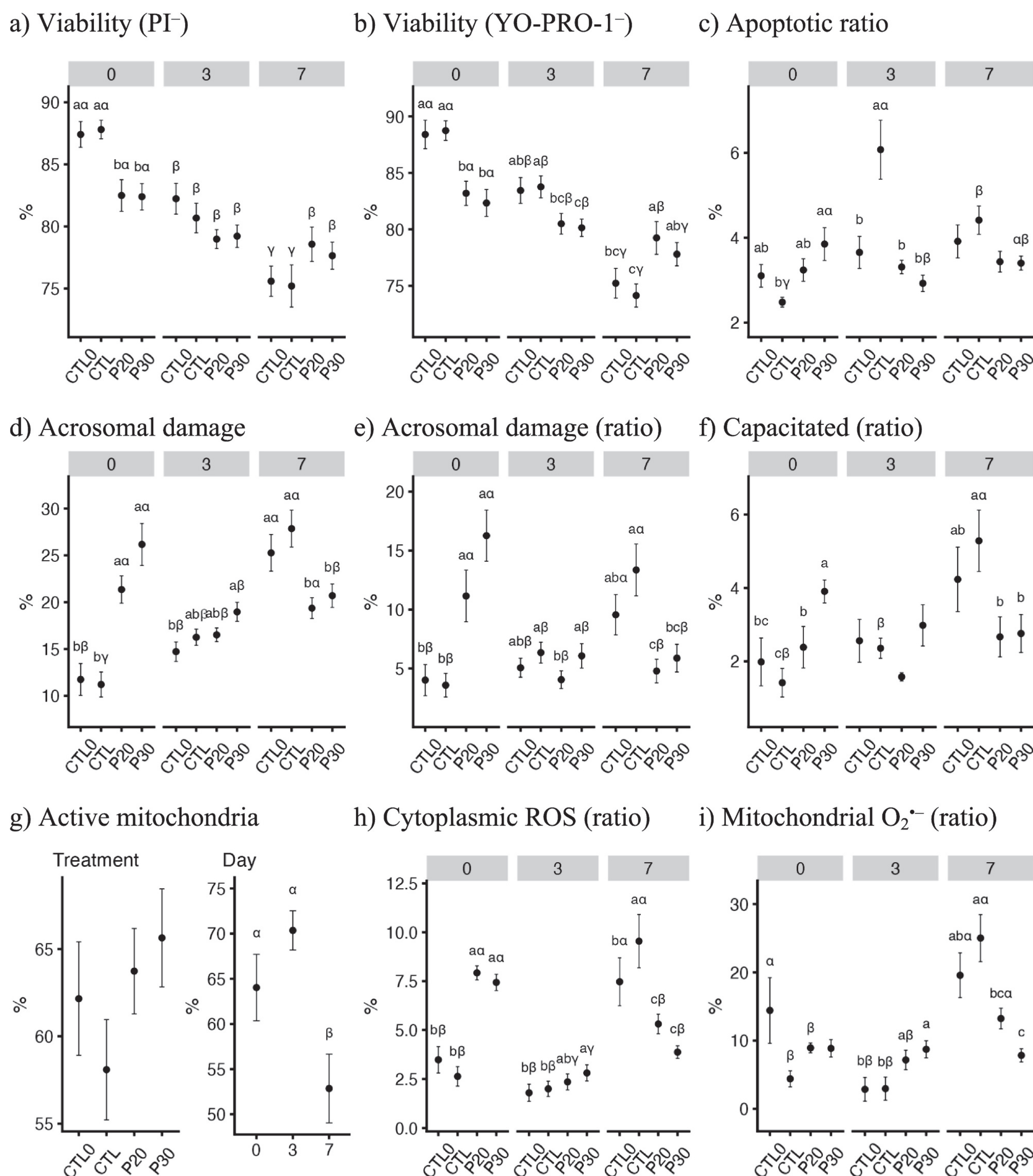


**Fig. 3.** Effect of the colloid centrifugation on flow cytometry parameters (CTL0: Unspiked control; CTL: Spiked control; P20 and P30: Spiked and submitted to SLC with Porcicoll 20% and 30%, respectively). Parameters defined as ratios correspond only to the subpopulation considered as viable. The plots show mean ± SEM for the variables for each combination of treatment and storage day. Results from models with a significant interaction between factors are presented in a single panel, with different lowercase Latin letters indicating $P < 0.05$ between treatments within days, and different Greek letters indicate $P < 0.05$ between days within treatments. When the interaction was not significant, the factors were studied as main effects (double panel, g).

increase of sperm-produced ROS, as CTL samples showed the highest values, we cannot discount that SLC could remove other noxious components from the sample, favoring a better performance of long-stored spermatozoa. As a hypothesis, removing the lowest-quality spermatozoa from the sample could contribute to these effects since SLC is an efficient method for separating dead and damaged sperm (Crespo-Félez et al., 2017), and the presence of such spermatozoa has been confirmed as unfavorable for the whole semen dose (Roca et al., 2013). Both SLC colloids enabled a high recovery of spermatozoa, but P30 demonstrated a higher ability to remove bacteria while retaining more spermatozoa. Thus, P30 could also eliminate a higher proportion of damaged spermatozoa, which could negatively influence the whole sperm dose during

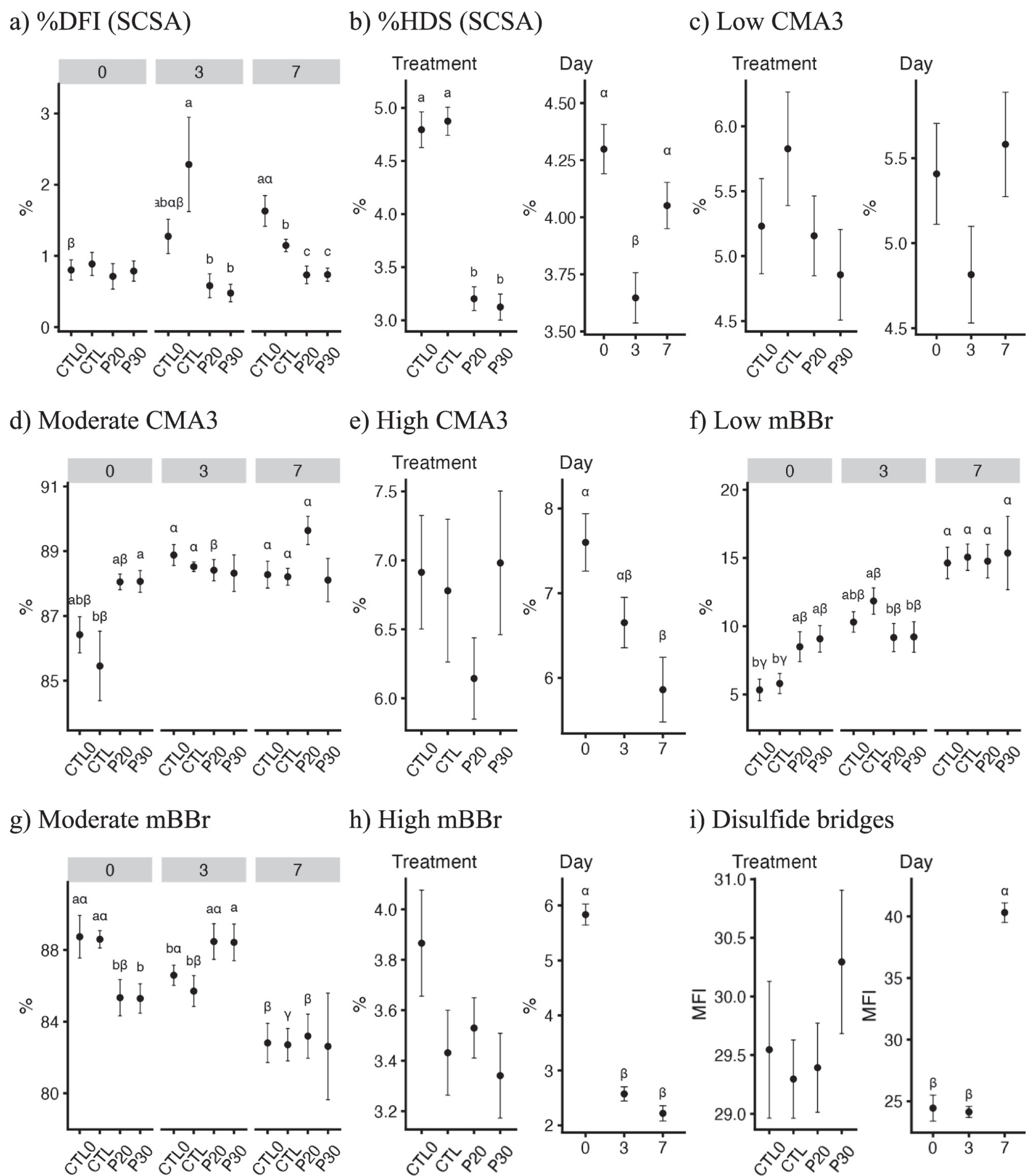


**Fig. 4.** Effect of the colloid centrifugation on the sperm chromatin structure (CTL0: Unspiked control; CTL: Spiked control; P20 and P30: Spiked and submitted to SLC with Porcicoll 20% and 30%, respectively). Figures presents results for the two variables from SCSA (a, b), from the CMA3 stain (c–e), and from the mBBr stain (f–i). The plots show mean ± SEM for the variables for each combination of treatment and storage day. Results from models with a significant interaction between factors are presented in a single panel, with different lowercase Latin letters indicating $P < 0.05$ between treatments within days, and different Greek letters indicate $P < 0.05$ between days within treatments. When the interaction was not significant, the factors were studied as main effects (double panel, b, d, e, h, i).

storage, explaining the lower propensity to increased mitochondrial $O_2^{\bullet-}$ production. A specific study could be designed to test these hypotheses and definitely separate the effects of suboptimal sperm and bacterial contamination after SLC with low-density colloids. Indeed, bacteria were present in the SLC tubes (including the spiked *Pseudomonas*), albeit in low counts and a higher concentration for P20. Even with a small presence, bacteria could negatively affect sperm quality by releasing endotoxins (Okazaki et al., 2010), contributing to the slightly higher ROS-producing spermatozoa values in P20 samples.

An effect not realized in SLC-treated samples was the removal of

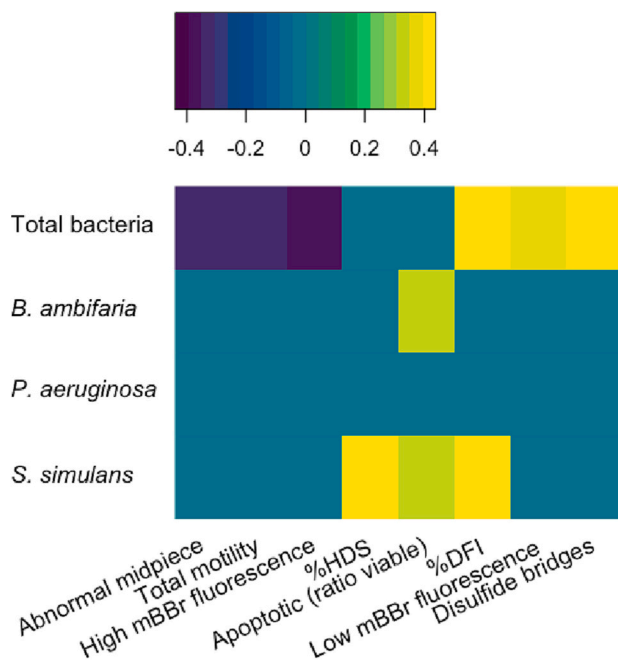


**Fig. 5.** Correlation matrix (heatmap, Pearson correlations) for associations between the bacterial contamination (total and spiked) and the sperm parameters. Correlations with $P > 0.05$ have been excluded (plain background; $P$ values corrected for multiple testing by the false discovery rate method). The scale shows the strength of the correlations (r value). The correlation coefficients and P values are displayed in the Table S1 (Supplementary Material).

abnormal spermatozoa. Previously, we found some decrease in abnormal heads for SLC-treated samples but an increase in detached heads for P30 (Martínez-Pastor et al., 2021). The main reason could be that these parameters were very low initially. Interestingly, whereas SLC enabled better sperm quality by day 7, abnormalities were higher, mainly due to bent flagella. This storage effect on the SLC-treated samples was not observed in previous studies and could be related to osmotic processes, which would not affect sperm viability or functionality.

Interestingly, the sperm recovery rates in this experiment after SLC were intermediate between those found in a first study using 50-ml tubes (Deori et al., 2020) and the previous one using 500-ml tubes (Martínez-Pastor et al., 2021), with a slightly higher sperm loss. Whereas experimental and sample variability could explain part of these differences, it is possible that the tube geometry and other physical factors could play an important role, suggesting that the design of specific bottles for large-volume processing could improve both the workflow and the recovery results, in line with the swine industry requirements (Martinez-Alborcia et al., 2013).

The main objective of SLC with a low-density colloid was removing bacteria from the samples so that samples could be stored at practical temperatures (15–18 °C). In this experiment, three species relevant to the stud center were spiked, and two of them, *B. ambifaria* and *S. simulans* (isolated from the semen in a previous study (Morrell et al., 2019)) were undetectable on day 7. Interestingly, *S. simulans* was detectable in CTL0, showing its relevance as an environmental contaminant. The SLC method could not remove all *P. aeruginosa* or overall contamination, but their levels were considerably reduced compared with the controls, especially P30. The contamination present in CTL0 on days 3 and 7 shows that even following the strict protocols of modern stud centers, bacterial growth can be problematic if antibiotics are not used in extenders and doses are kept at normal storage temperatures. In fact, any failure in the adherence to the protocols could result in critical bacterial contamination, as shown previously (Martínez-Pastor et al., 2021), and other authors have warned that the occurrence of such events is not unusual (Nitsche-Melkus et al., 2020; Schulze et al., 2015) and of their consequences for AI centers (Kuster and Althouse, 2016; Ubeda et al., 2013).

We conducted a simple association analysis of bacterial contamination with sperm quality, finding some relevant findings, such as increased DNA damage or chromatin compaction (disulfide bridges). However, the specific bacteria used in the spiking were not clearly associated with large changes in sperm quality. Other authors identified that enterobacteria were especially associated with decreasing sperm quality (Ubeda et al., 2013; Bussalleu et al., 2011; Prieto-Martínez et al., 2014). Nevertheless, *P. aeruginosa* could be problematic, considering that the SLC could not remove it entirely from the samples, and previous reports indicate a negative effect on sperm viability and functionality (Sepúlveda et al., 2016; Sepúlveda et al., 2014). Nevertheless, the concentrations in these studies were three orders of magnitude higher than those found in P20 and five from P30. It is essential to consider that, even in the presence of antibiotics, contamination can develop quickly in the semen doses (Bresciani et al., 2014; Gączarzewicz et al., 2016), an event more frequent with widespread AMR (Costinar et al., 2021). Storing the semen doses at low temperatures (4–5 °C) effectively delays bacterial growth (de Menezes et al., 2020), but it is impractical considering current AI protocols in swine.

The study on sperm chromatin deserves a deeper analysis. Its alterations, especially those related to DNA damage, directly affect the fertility of spermatozoa in a non-compensable manner (Didion et al., 2009; Boe-Hansen et al., 2008). To attain a complete picture of the structure of the sperm chromatin, we used three techniques: SCSA for assessing DNA fragmentation and chromatin compaction, also termed sperm decondensation index (SDI); CMA3 as a complementary method for evaluating the SDI, although its interpretation is complex (Ménézo, 2021); and the monobromobimane (mBBr) method for assessing the thiol groups and disulfide bridges among protamines. Whereas DNA fragmentation (%DFI) showed low values, as is typical for pig spermatozoa (Didion et al., 2009), SLC maintained these values at baseline levels even for the 7-day storage and, at the same time, improved SDI as well (%HDS). These results agree with previous reports indicating that colloid centrifugation improved these parameters (Morrell et al., 2009; de Mateo et al., 2011; Larson et al., 1999). In our earlier studies using large-volume SLC (Martínez-Pastor et al., 2021), we did not detect a change in %DFI, although %HDS improved similarly. Therefore, the use of different volumes or vessel shapes could affect not only sperm recovery but also efficiency in removing a particular sperm subpopulation of higher sensitivity to DNA damage. This observation could help improve the more practical large-volume SLC and could prevent the increased DNA damage observed during long-term boar semen storage in some studies (Boe-Hansen et al., 2008; Boe-Hansen et al., 2005).

Considering the tertiary chromatin structure, that is, the protamine-histone-DNA arrangement (Ménézo, 2021), the CMA3 and mBBr were affected by the storage time. This is compatible with our results for %HDS (a parameter related to chromatin compaction (Boe-Hansen et al., 2018)) in the present and previous studies (Fernández-Gago et al., 2017). The sperm chromatin appeared to compact during the storage overall, which could be due to continuous peroxidative activity in the nucleus (Noblanc et al., 2011), with the formation of new disulfide bridges, thus explaining mBBr results and CMA3 exclusion, whereas %HDS could be reporting other aspects of the chromatin compaction.

## 5. Conclusions

SLC with low-density Porcicoll (especially at 30%) was highly efficient in removing contamination after this controlled experiment spiking specific bacteria. This procedure prevented relevant bacterial growth up to eight days of storage at the usual refrigeration temperatures for boar semen doses (17 °C) without antibiotics. The low contamination (total removal of two species, *B. ambifaria,* and *S. simulans*) benefited the samples, which showed a higher quality than

the controls. Moreover, part of the benefits could directly derive from removing damaged spermatozoa from the original samples, and the modulation of sperm chromatin changes could be relevant for the long-term storage of valuable samples. These results encourage improving the method to make it worthwhile for stud centers, at least for genetically important boars.

## Declaration of Competing Interest

The authors state that there is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.03.024.

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