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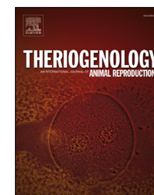
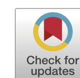


Single layer centrifugation (SLC) for bacterial removal with Porcicoll positively modifies chromatin structure in boar spermatozoa



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A B S T R A C T

The storage of boar semen samples at 17 °C for artificial insemination (AI) doses enables the proliferation of the bacteria, making antibiotics necessary. This can contribute to the development of antimicrobial resistance (AMR). This study tested bacterial presence and sperm chromatin structure after using a low-density colloid (Porcicoll) as an antibiotic alternative to eliminate bacteria. Ejaculates (8 boars, 3 ejaculates each) were split as control and low-density colloid centrifugation (single layer centrifugation, SLC, 20%, and 30% Porcicoll) into 500 ml tubes. Analyses were carried out at days 0, 3, and 7 (17 °C) for microbial presence and sperm chromatin structure analysis: %DFI (DNA fragmentation) and %HDS (chromatin immaturity), monobromobimane (mBBr; free thiols and disulfide bridges), and chromomycin A3 (CMA3; chromatin compaction). Besides comparing bacterial presence (7 species identified) and chromatin variables between treatments, the associations between these sets of variables were described by canonical correlation analysis (CCA). Results showed a significant decrease of some bacteria or a complete removal after SLC (especially for P30). SLC also caused a decrease of %HDS and an increase of disulfide bridges and low and medium mBBr populations, suggesting the removal of immature sperm (poor chromatin compaction). CCA showed an association pattern compatible with the degradation of sperm chromatin parameters with bacterial contamination, especially Enterobacteria, *P. aeuriginosa*, and *K. variicola*. In conclusion, bacterial contamination affects sperm chromatin beyond DNA fragmentation; SLC with low-density colloid not only removes bacteria from boar semen, but also chromatin structure is enhanced after selection.

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1. Introduction

Artificial insemination (AI) with stored semen at 16–18 °C is a critical technique for the pig industry [1] since it allows storage of the semen for several days while avoiding cold shock to the spermatozoa. AI has many advantages, especially in disease control; however, bacteria from healthy animals contaminate the ejaculate during semen collection and the artificial insemination process. Therefore, antibiotics are added to semen doses for artificial insemination to avoid complications from these pathogens in inseminated females and even to avoid deteriorating sperm quality during storage. It could be that the use of these antibiotics is contributing to the spread of antibiotic resistance in inseminated females and workers [2]. The World Health Organisation (WHO) has reported that antimicrobial resistance, especially methicillin-resistant strains, is leading to a crisis in health care in veterinary and human medicine due to infections being difficult or even impossible to treat with antibiotics [3,4]. In animals, antimicrobial use is threefold that of humans [4], and genes for antimicrobial resistance are passed easily between different bacteria and

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reservoirs in humans and animals [5]. Even a small use of antibiotics can lead a considerable antibiotic resistance development [6]. Therefore, the use of antibiotics should be restricted when strictly necessary for therapeutic purposes, choosing the appropriate antibiotics for their pharmacokinetic properties and testing the specific bacteria for sensitivity to the proposed therapeutic agent.

A physical method to remove bacteria from semen could be a good alternative to adding antibiotics. Single layer centrifugation (SLC), a colloid centrifugation technique using a colloid of density 1.104 g/ml, removed or substantially reduced the bacteria in boar semen samples [7], although part of the sperm population was lost because only robust spermatozoa were selected from the rest of the ejaculate [8]. A low-density Porcicoll colloid (1.052 g/ml) allows most spermatozoa to be recovered while separating them from seminal plasma. Although it was assumed that the low-density colloid does not select good-quality spermatozoa [9], since most of the spermatozoa pass through the colloid, sperm quality remained high during storage for one week at 16–18 °C.

Lower densities of colloid (20% and 30% Porcicoll at a density of 1.026 g/ml and 1.039 g/ml, respectively) were checked using 50 ml centrifuge tubes, and almost all of the spermatozoa were recovered [10]. In addition, another study with the same densities of colloid (20% and 30%) was conducted to evaluate the effectiveness of separating sperm from bacteria using 500 ml tubes, obtaining a considerable reduction of some bacteria by both colloids. Thus, the conclusion was that contaminating bacteria in boar semen could be controlled by low-density colloids [11]. However, the ratio of spermatozoa/bacteria at which adverse effects appear is significantly different depending on the bacterial species [12]. Moreover, the bacteria present in sperm samples or doses are usually gram-negative and included in the Enterobacteriaceae family [13], although other bacteria present in seminal doses belong to anaerobes [14].

The present study evaluates the effect of these low-density Porcicoll concentrations (20% and 30%) in 500 ml tubes on sperm chromatin status using canonical correlation analysis (CCA), a useful multivariate technique. This technique produces linear composites from an independent and dependent set of experimental variables, termed canonical variates. Then, it develops a canonical function maximizing the correlation between the two sets, pair to pair, of canonical variates. CCA shares some similarities to the more widely used principal components analysis (PCA) and MANOVA, enabling the assessment of the relationship between metric-independent variables and multiple dependent measures. Therefore, CCA facilitates the study of linear interrelationships between these two sets of variables [11,15]. Additionally, CCA yields as many functions as variables in the smaller variable set; each function is independent of the others, representing different relationships among the sets of dependent and independent variables [16]. Nonetheless, it is still underutilized in many fields, possibly due to a lack of familiarity and the complex computations involved, a drawback before the availability of powerful personal computers [17].

Therefore, the objective of this study was to evaluate whether 20% and 30% low-density colloid Porcicoll used on an industrial scale in large-volume tubes enable sperm samples with better chromatin quality to be obtained and to check the relationship between the presence of bacteria and the quality of the sperm chromatin, identifying if some bacteria have more negative effects on sperm samples than others using CCA models.

2. Materials and methods

2.1. Reagents

General reagents and the fluorescent probes monobromobimane (mBBr) and chromomycin A3 (CMA3) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Consumables and solutions for flow cytometry were purchased from Beckman Coulter (Brea, CA, USA) and Thermo Fisher (Waltham, MA, USA).

2.2. Experimental design

Semen samples were collected as usual at boar stations but with diminished attention to the standard hygienic protocol to try to achieve bacterial contamination that might occur under normal collection conditions. Ejaculates were extended without antibiotics. A part of the ejaculates was prepared by SLC using low-density Porcicoll colloid (20% and 30%; P20 and P30, respectively), and the remainder was used as a control (CTL). Semen samples were stored at 17 °C for 7 days. Sperm chromatin status was evaluated by sperm chromatin structure assay (SCSA), mBBr, and CMA3 after being frozen using TNE (Tris/NaCl/EDTA) buffer on day 0, day 3, and day 7 of storage (17 °C). Microbiological analyses were performed at the same time points.

2.3. Semen extender

Modified Beltsville Thawing Solution (BTS) was used as an antibiotic-free extender. This medium contains 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). Bovine serum albumin (BSA) at 0.5% was added to the BTS used for analysis to prevent aggregation and stickiness of the spermatozoa.

2.4. Animals and semen samples

Semen samples were donated by AIM Ibérica (Campo de Villavidel, León, Spain). Boars (Large White, Landrace, Duroc, and synthetic breeds) were kept following routine protocols for AI centers, were between 12 and 22 months old, and were subjected to semen collection weekly. They were kept under controlled conditions with a regulated temperature between 18 and 23 °C and light/darkness hour cycles (12/12). AIM Ibérica provided 24 ejaculates from 8 boars (3 replicates/boar obtained in three consecutive weeks). The sperm-rich fraction of the ejaculate was collected and extended at 100×10^6 ml^{-1} with BTS. Ten-milliliter aliquots were transported refrigerated (17–18 °C) and kept until analyses at 17 °C in an IF450 incubator (Memmert GmbH, Schwabach, Germany) until the tests were carried out at the Institute of Animal Health and Cattle Development (INDEGSAL; University of León, Spain).

2.5. Sample preparation

The colloid is based on a silane-coated silica formulation for boar semen (Porcicoll, patent applied for), prepared at a density of 1.039 g/ml (P30) and 1.026 g/ml (P20). The extended semen was split into an uncentrifuged control (CON), SLC with P30, and SLC with P20. SLC was prepared as 200 ml extended semen on 150 ml colloid in 500 ml centrifuge bottles, centrifuged at 300×*g* for 20 min. The supernatant was removed by a water pump, and the pellet was resuspended in sterile BTS up to 200 ml. The sperm suspensions were transferred to 15 ml tubes for storage at 17 °C.

2.6. Microbiological analyses

Bacterial count, bacterial culture, and species identification were carried out using standard protocols [18,19]. A small volume (0.1 ml) was cultured at dilutions −1 to −6 for 24 h at 37 °C in a microaerophilic atmosphere on different media: Tryptone soy agar (TSA), McConkey agar, Cystine-Lactose-Electrolyte-Deficient (Cled)

agar and Blood Columbia agar (OXOID, Hampshire, UK). Then, colonies were counted and expressed as colony-forming units/ml (CFU/ml). Plates were incubated for 24 h and then read again.

The bacteria characterization was realized using various methods depending on type: Gram stain, oxidase, and catalase activity and different biochemical tests (API 20E, API 20NE, API Staph, API Strep; Bio Merieux Inc., Durham, NC), according to the instructions of the manufacturer.

A Bruker Daltonics Ultraflextreme MALDI-TOF/TOF controlled by the FlexControl software v. 3.0 (Brucker Daltonics, Bremen, Germany) was used for analyzing the samples acquiring their mass spectra. The Biotyper Real-Time Classification software v3.1 (Brucker Daltonics) was used for microbial identification, comparing the spectra obtained from our results with the corresponding database provided by the manufacturer (MALDI Biotyper database, 5989 entries, Bruker Daltonics). This software generates a score (0–3) depending on the similarity between a given sample and a reference spectrum. Some ranges were established to interpret the reliability of the results: 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. Assessment of sperm chromatin by flow cytometry

2.7.1. SCSA (Sperm Chromatin Structure Assay)

Chromatin stability was assessed by SCSA (Sperm Chromatin Structure Assay) [20]. The technique is based on the denaturalization of broken DNA and the shift of acridine orange fluorescence (AO; a DNA intercalating fluorochrome) from green (dsDNA) to red (ssDNA, resulting from fragmented DNA). Aliquots of samples were diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM Na_2EDTA, pH 7.4) to 2×10^6 ml^{-1}. The analysis was carried out by mixing 200 μl of the sample with 0.4 ml of acid-detergent solution (0.08 M HCl, 0.15 M NaCl, and 0.1% Triton X-100, pH 1.2). After 30 s, the spermatozoa were stained with 1.2 ml of staining solution (6 μg/ml AO in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM Na_2EDTA, and 0.15 M NaCl, pH 6.0). The tube was kept on ice for 3 min before flow cytometry analysis and run through a FACScalibur flow cytometer (Becton Dickinson) controlled by the acquisition software CellQuest v.3. We analyzed 5000 spermatozoa per sample, exciting the acridine orange with an Ar-ion 488 nm laser and using a 530/30 filter for the green fluorescence of dsDNA-bound AO, and a 650 long-pass filter for the red fluorescence of ssDNA-bound AO. Data were saved in flow cytometry standard (FCS) v. 2 files, which were processed using the R statistical environment [4]. We calculated the DNA Fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence to total fluorescence (red + green). From the DFI values, we obtained the percentage of spermatozoa with high fragmentation index (%DFI, DFI>250) and with high DNA stainability (%HDS), defined as those events with green fluorescence above channel 650.

2.7.2. Monobromobimane labeling (mBBr)

Monobromobimane is a fluorescent probe for staining sulfhydryl groups, the functional group of thiols resulting from breaking disulfide bonds. A higher number of free sulfhydryls indicate a lower number of disulfide bridges and, therefore, less chromatin compaction. In the mBBr technique, two 96-well plates with the same semen samples were used to evaluate free sulfhydryls and disulfide bonds (control and positive control plates). The plate used as a positive control contained DTT (1 mM), reducing disulfide bonds to thiols. DTT was incubated with the sperm (10 min, 37 °C), and then, samples of this plate were centrifuged at 4 °C (1000×g, 11 min). Then, samples of both plates were incubated with mBBr fluorochrome (500 μM; 10 min, 37 °C). After this time, sperm samples were washed using PBS and centrifuged at the conditions above. Finally, iodide propidium was used to stain cells. Sperm samples were kept at 4 °C for 24 h and analyzed in a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). We analyzed 10000 spermatozoa per sample, exciting the mBBr with a 405 nm laser and using a 450/50 nm filter for the blue fluorescence of mBBr bound to sulfhydryl groups. Data were saved in flow cytometry standard (FCS) files, which were processed using the R statistical environment. Disulfide bridges were obtained by subtracting free thiols from total thiols and dividing by 2 [21]. In addition, mBBr low, mBBr moderate, and mBBr high populations (as %) were obtained from the different treatments and males for the sampling days. The mBBr low population encompasses spermatozoa with very little presence of free thiol groups (SH-) and a large number of disulfide bridges (S–S), therefore, being able to present an optimal state thanks to the correct compaction; mBBr moderate includes cells with a moderate Mean Fluorescence Intensity (MFI); mBBr high includes cells with MFI emissions that exceeded the upper threshold, emitted by spermatozoa with highly decompacted chromatin, presenting a large number of free thiol groups (SH-) and a lower number of disulfide bridges (S–S).

2.7.3. CMA3 (chromomycin A3)

The level of sperm chromatin compaction was evaluated using the CMA3 technique. CMA3 has affinity for DNA but cannot access it if it is bound to protamines and disulfide bonds have been established between them, so CMA3 competes with protamine for DNA. An excess of histones or a lack of disulfide bonds increases the CMA3 signal. Thus, it is typically used for detecting protamine deficiency in sperm chromatin. Semen samples were aliquoted in a 96-well plate using PBS and centrifuging (1000×g, 11 min). Then, semen samples were incubated with CMA3 staining (0.05 mM; 20 min, 25 °C) and washed with PBS, followed by centrifuging as described previously. Hoechst 3342 was used to stain cells, and after 15 min, samples were read using MACSQuant Analyzer 10 flow cytometer analyzing 10000 spermatozoa per sample. CMA3 is excited with a blue 488 nm laser, and the emission was collected through a 585/40 nm filter. Data were saved as FCS files processed by R statistical environment. As with mBBr, we obtained the CMA3 MFI and CMA3 low, CMA3 moderate, and CMA3 high populations. CMA3 low includes sperm with a high level of protamines and, thus, correct chromatin compaction, avoiding CMA3 binding; CMA3 moderate contains cells with a moderate MFI; and CMA3 high represents the low-quality sperm population which has poor chromatin compaction.

2.8. Data analysis

Data analysis was performed in the R statistical environment v. 4.0.5 [4]. The effects of the SLC and storage time were analyzed by linear mixed-effects models (lmerTest package [5]), with the SLC treatment and the storage time as fixed effects factors and the boar as the grouping factor in the random part of the model. The relationship between the microbiological content of the samples and the chromatin parameters was analyzed using canonical correlations, with the CFU/ml for the bacterial species as the independent variable set and the chromatin parameters as the dependent variable set. The analysis produced two sets of canonical variates, and we recorded the loadings and cross-loadings between the original variables and the new canonical variates. Additionally, we performed a canonical discriminant analysis with MANOVA to test the discriminant effect of the factors on the canonical variates. The

significance threshold was adjusted at $p \leq 0.05$, using the false discovery rate for adjusting the p-values in multiple testing.

3. Results

3.1. Effect of SLC and time on bacterial content

Seven bacterial species were detected in the sperm samples: *Aeromonas caviae*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus simulans*, *Citrobacter koseri*, and *Bacillus licheniformis*. *A. caviae*, *C. koseri*, and *E. coli* are Gram-negative bacillus in the Enterobacteriaceae family. *A. caviae* is motile, ubiquitous, typical of aquatic reservoirs, heterotrophic, facultative anaerobes, and producers of various virulence factors; they are present in the environment in fresh or brackish water and can also be present in spoiled food. *C. koseri* is aerobic and frequently found in water, soil, food, and vegetation, and as a saprophytic microbiota in the intestinal tract of many animals; it is frequently related to urinary tract infections. *E. coli* is a facultative anaerobe with a preferred growth temperature is 37 °C, fimbriated, and commonly motile by peritrichous flagella; It is part of the microbiota of the gastrointestinal tract of animals and man, with some strains causing gastroenteritis. *E. faecalis* is a Gram-positive coccus in the Enterococcaceae family; it is an immotile, facultatively anaerobic, glucose-fermenting commensal in the digestive tract of animals, and it is an indicator of fecal contamination; It can cause bladder infections. *P. aeruginosa* is a Gram-negative in the Pseudomonadaceae family; it is an aerobic, opportunistic pathogen that can infect the lungs and respiratory tract, urinary tract, and other tissues and causes other sepsis. *S. simulans* is a Gram-positive coccus in the Staphylococcaceae family; it is part of the normal microbiota of the skin, being pathogenic in some cases (e.g., after stress), producing skin infections. *B. licheniformis* is a Gram-positive bacillus in the Bacillaceae family; It is a facultatively anaerobic, sporulating bacteria, typically inhabiting water and soil; It produces important enzymes related to antibacterial or antifungal activity, although some strains of this species are also related to infection.

The evolution of the detected bacteria as CFU/ml is shown in Fig. 1. The SLC treatments (P20 and P30) significantly decreased overall bacterial presence (Fig. 1a), except for *A. caviae* (Fig. 1b). Some bacterial species were detected only in the CTL samples (Fig. 1f–h), apparently increasing by day 7 but not reaching significance because of being present in only some samples. *E. coli* and *P. aeruginosa* (Fig. 1c and e, respectively) were removed almost entirely by SLC; *E. faecalis* was reduced better by P30 (Fig. 1d).

3.2. Effect of SLC and time on chromatin structure

Whereas SLC did not modify the DNA fragmentation (%DFI; Fig. 2b), P30 reduced chromatin heterogeneity (SD-DFI; $P = 0.044$; Fig. 2a) and chromatin immaturity (%HDS; $P < 0.001$, Fig. 2c), but only the for the first day ($P = 0.008$). The chromatin structure evaluated by CMA3 was not affected by the SLC treatment (Fig. 3). However, the intensity of CMA3 staining increased by day 3 and then decreased by day 7 in all treatments, especially P30, although not significantly different from day 0 ($P = 0.066$; Fig. 3a). No significant differences were detected between treatments in the CMA3 intensity populations (CMA3-low, CMA3-moderate, and CMA3-high) ($p > 0.05$; Fig. 3b – d).

The mBBr analysis (Fig. 4) showed changes both across treatments (CTL, P20, and P30) and with time (days 0, 3, and 7). The estimation of disulfide bridges showed a decrease with time; however, it is more pronounced in the CTL samples by day 3 (Fig. 4a). Considering the populations defined by the mBBr fluorescence, mBBr low (fewer thiol groups available) followed the opposite trend (Fig. 4b), and mBBr moderate mirrored it (Fig. 4c); mBBr high (highly-stained spermatozoa with increased availability of thiol groups) tended to diminish with analysis day, with no differences among treatments (Fig. 4d).

3.3. Canonical correlation analysis (CCA) of the microbial charge and sperm chromatin structure

The descriptive statistics of the bacterial and sperm chromatin parameters are shown in Tables 1 and 2 as overall means ± SD for reference (with trends already shown in Figs. 1–4). Since there are many variables in both groups, explaining relationships in terms of correlations (in supplementary material, Tables S1–S3) is complex and could easily lead to false relationships, even correcting P values for multiple testing. CCA facilitates the analysis by reducing the number of correlations and provides some statistics contributing further information about the explanatory power of the studied relationship.

In our analysis, we intended to establish if there were a relationship between the bacterial presence in our samples and the chromatin structure of spermatozoa, especially if the individual bacterial species has a specific effect. Entering the bacterial presence variables (explanatory, X-set) and the chromatin analysis variables (response, Y-set) in the CCA, we obtained three significant canonical correlation coefficients (Table 1), accounting for nearly 80% of the variability in the relationship between both sets. The first two correlations were especially high (0.86 and 0.76, with $P < 0.001$).

Table 2 shows the standardized canonical coefficients for the three first canonical variates in each set. The canonical coefficients show the relative contribution of each initial variable to the respective variate (optimal linear combinations of the initial variables defining the canonical variates). That is, for X_1, the presence of *P. aeruginosa, B. licheniformis,* and *K. variicola* increases it, but the presence of *A. caviae* and enterobacteria decreases it, other variables having a minor impact. The same interpretation follows for X_2, positively related mainly to *E. coli* and less to *P. aeuriginosa*, *B. licheniformis*, and *P. vulgaris*, and strongly negatively to *E. faecalis*, and X_3 positively to *C. koseri* and *B. licheniformis* and negatively to *A. caviae*, *P. aeuriginosa*, *E. coli*, and *P. vulgaris*. Similarly, Y_1 is positively influenced by the SD-DFI and %HDS from SCSA, disulfide bridges, and the mBBr low population levels and negatively by % DFI, moderate and high mBBr populations, and the CMA3 MFI. Y_2 basically opposites Y_1, whereas Y_3 coefficients are similar to Y_2, with a sign change for the moderate and high mBBr population levels and CMA3 MFI.

Canonical loadings inform the contribution (correlation) of the individual starting variables to the multivariate relationship between bacterial presence and sperm chromatin status (Table 3). Therefore, most X variables influenced X_1, although enterobacteria showed the highest (negative) contribution (X_1 was only positively related to *P. aeuriginosa*, *S. simulans*, and *K. variicola*), whereas X_2 was influenced mainly by *P. aeuriginosa*, *E. coli*, and *P. vulgaris*, and A. caviae and K. variicola presence mainly influenced X_3. For the Y set, SCSA variables, disulfide levels, and the low CMA3 population contributed the most to Y_1. Y_2 was affected primarily by the high mBBr population (with some influence of CMA3 MFI and the high CMA3 population), and Y_3 by %DFI, %HDS (different sign), and the low mBBr population.

Similarly, cross-loadings (Table 4) show the correlation of each variable set to the other canonical variates. The general distribution of cross-loadings resembles the previous canonical loadings, which is expected given the relatively high canonical correlations found initially, especially for the first two variates (Table 1).

Considering the canonical loadings and the canonical

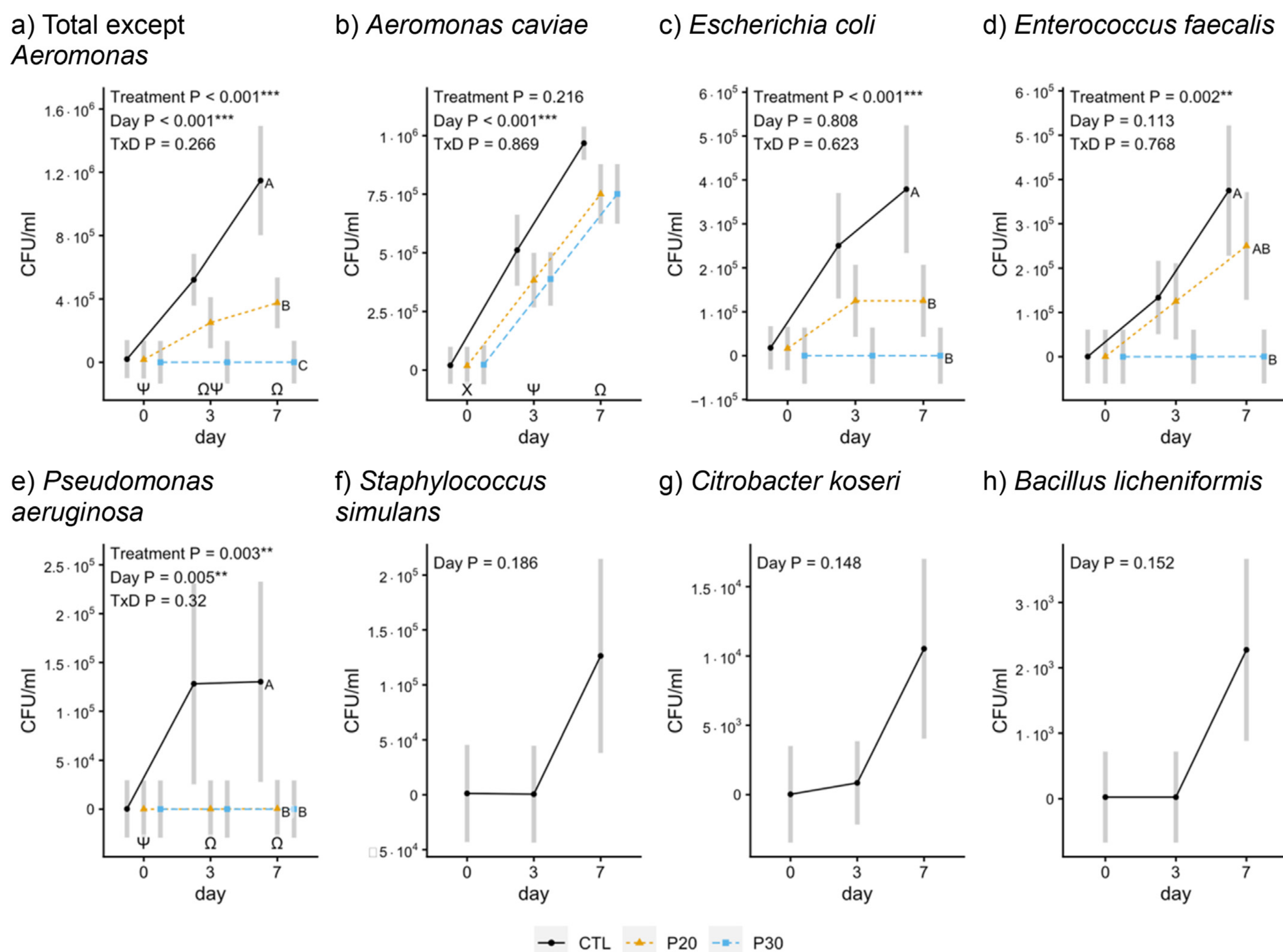


Fig. 1. Bacterial presence in the semen samples as CFU/ml, control (CTL) and treated by SLC (P20: 20% Porcicoll; P30: 30% Porcicoll), and sampled at 0 (day of processing), 3 and 7 days at 5 °C. The plots show the mean ± CI95% for each treatment × time combination, with lines joining treatments, except for *S. simulans*, *C. koseri*, and *B. licheniformis*, which were only detected in some CTL samples (P < 0.05 for day in *C. koseri* and *B. licheniformis* if samples with no presence at any time are removed). Insets show the significance of the effects of the treatment (SLC) and refrigeration time. Capital Latin letters show differences among treatments, and Greek letters show differences among times (only main effects, no significant interactions). *Klebsiella variicola* appeared only in three samples, in the limit of detection (~100 CFU/ml) and not changing among treatments or days. *Proteus vulgaris* appeared only in one control sample, increasing from 10^2 on day 0–10^6 CFU/ml on day 7.

correlations, there was a clear relationship between both sets of variables. Interpreting the first pair of variates X_1Y_1 (Table 1), the presence of most bacterial species was associated with lower chromatin status (notice the signs in Table 3), defined by SCSA variables %DFI and %HDS, and disulfide levels. X_2Y_2 highlighted the possible negative role of some bacteria (*P. aeuriginosa* and *E. coli*) on chromatin structure as defined by mBBr and CMA3. The last pair X_3Y_3, even if showing the lowest correlation, indicated an influence of the *A. caviae* and *K. variicola* species on the chromatin compaction (suggesting an increase), as defined by SCSA %HDS and the low mBBr population.

Whereas canonical correlations are useful statistics, they do not provide information on the amount of variable variance accounted for by the other set of variables. Thus, the redundancy index indicates the amount of variance in one of the canonical variates explained by the other canonical variate in each canonical function. Table 5 shows the proportions of variance extracted by each of the canonical variates for both the X and Y-sets and the respective redundancy indexes. Although the R^2 values shown in Table 1 are relatively high, we found that the X canonical variates accounted only for 18.1%, 4.5%, and 4.1% of the respective Y canonical variates, and the Y canonical variates accounted for 20.9%, 5% and 2.1% of the X counterparts.

3.4. Canonical discriminant analysis of the microbial charge and sperm chromatin structure

Plotting the canonical variates (supplementary material, Figs. S1 and S2) showed that the first pair captured mainly the variability among samples (boars) (Figs. S1a and S2a). In contrast, the second pair better discriminated among the control and SLC treatments (Figs. S1e and S2e), and the third one among the sampling days (Figs. S1i and S2i).

We carried out a canonical discriminant analysis to further investigate the effects of these factors on the obtained variables. First, a MANOVA for the effects of boar, treatment, and day on the microbiological and chromatin variables showed that the three factors were highly significant (P < 0.001). The results of the subsequent canonical discriminant analyses are detailed in the supplementary material. For the microbiological variables, four significant canonical variates were significant for the boar effect (Table S4) and one for treatment and day (Tables S5 and S6,

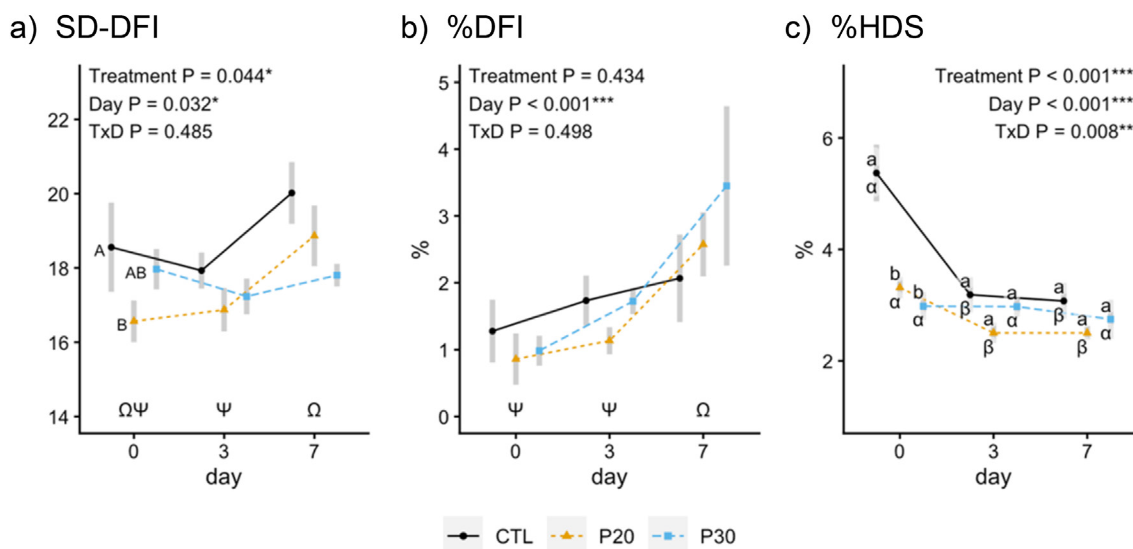


Fig. 2. Effect of SLC and refrigeration time on chromatin parameters yielded by SCSA: DNA heterogeneity (SD-DFI), DNA fragmentation (% DFI), and chromatin immaturity (% HDS). The plots show the mean ± CI95% for each treatment × time combination, with lines joining treatments. The P values for the effects of the factors and their interaction are shown in the insets. Latin letters show differences among treatments, and Greek letters show differences among times (capitals for main effects, overall, lowercase for within-times differences when the interaction is significant).

respectively), whereas for the chromatin status variables, we found five, one, and two significant ones, respectively (Tables S13–S15). Detailed information on canonical coefficients and structure coefficients are shown in Tables S7-S12 and S16-S21.

For brevity, we will describe only the first canonical variate in all cases. For the microbiological variables, the first canonical variate was mainly related to *A. caviae* (0.398), *E. coli* (0.411), and negatively with *K. variicola* (−0.988) for boar effect (Table S8); to total bacteria (0.859) and most species except *P. aeuriginosa* and *K. variicola* for treatment (Table S10); and to total bacteria (0.688), *A. caviae* (0.905), *P. aeuriginosa* (0.323), *E. faecalis* (0.52), and (negatively) *K. variicola* (−0.427) for day (Table S12). The projection of the two first canonical variables shows that the microbiology clearly separated the boars into two groups by the first dimension (Fig. 5a, notably by *K. variicola* presence), with some discrimination for the second dimension (mainly due to *P. aeruginosa* and enterobacteria presence). For the treatments, the first canonical variate clearly showed that both SLC treatments decreased bacterial presence (Fig. 5b). The days of analysis were sequentially aligned along the first canonical variate, evidencing increasing bacterial loads with time (Fig. 5c). In the case of the chromatin status variables, the first canonical variable was mainly associated with the disulfide bond levels, the low and moderate mBBr populations, and (negatively) the SCSA variables for boar (Table S17); to the SCSA variables, low mBBr population, and (negatively) the mBBr moderate population for treatment (Table S19); and (negatively) to % HDS, disulfide levels, the mBBr moderate population, and (positively) the mBBr low population for day (Table S21). Plotting the scores shows how the boars were separated mainly by the SCSA and mBBr variables (Fig. 5d) and how the SLC treatments achieved lower scores for the first canonical variable (lower chromatin alterations, Fig. 5e), and again the days were sorted along the first canonical variate, mainly with varying chromatin compaction, with some effect of the SCSA variables on the second dimension (Fig. 5f).

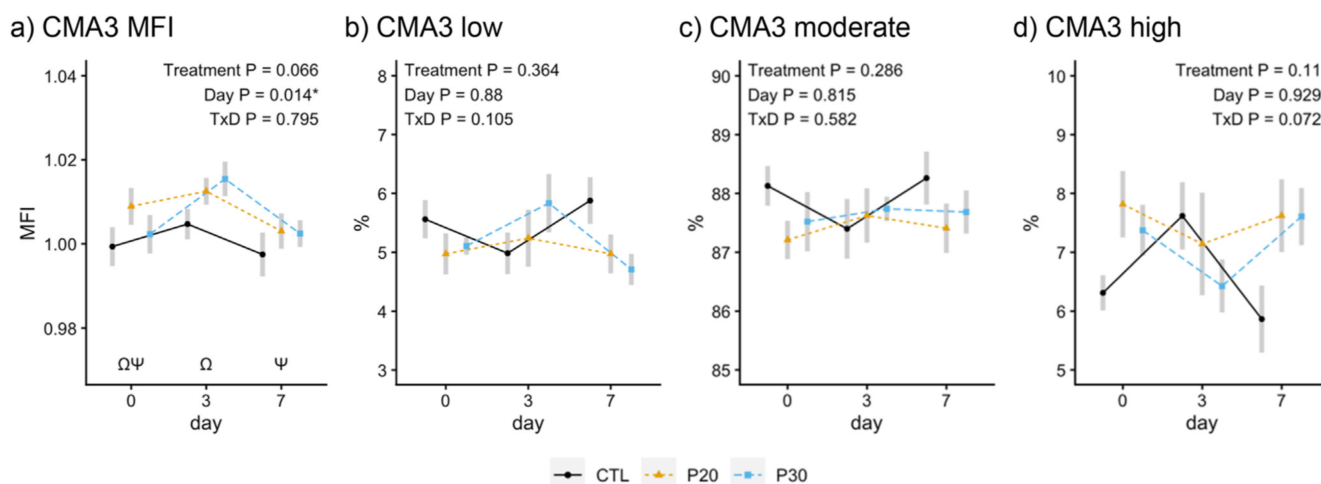


Fig. 3. Effect of SLC and refrigeration time on chromatin parameters yielded by the CMA3 staining. The plots show the mean ± CI95% for each treatment × time combination, with lines joining treatments. P-values for the effects of the factors and their interaction are shown in the insets. Latin letters show differences among treatments, and Greek letters show differences among times (capitals for main effects, overall, lowercase for within-times differences when the interaction is significant).

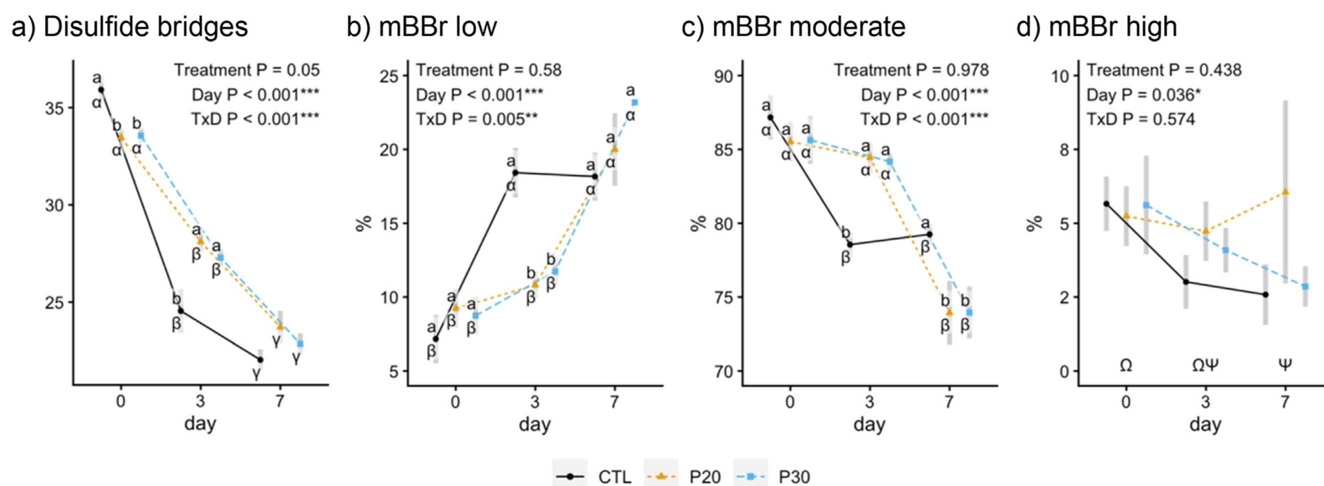


Fig. 4. Effect of SLC and refrigeration time on chromatin parameters yielded by the monobromobimane (mBBr) staining. The plots show the mean ± CI95% for each treatment × time combination, with lines joining treatments. P-values for the effects of the factors and their interaction are shown in the insets. Latin letters show differences among treatments, and Greek letters show differences among times (capitals for main effects, overall, lowercase for within-times differences when the interaction is significant).

4. Discussion

The success of AI depends critically on the correct structure and status of the sperm chromatin, with defects hampering fertility and the number of piglets farrowed [22,23]. A recognized threat for AI doses is bacterial contamination, controlled by highly hygienic procedures in the stud centers and by antibiotic supplementation in the extenders. However, deviations from optimal procedures can occur, and the current trend towards lower or no use of antibiotics aggravates this situation. Our study tested replacing antibiotics with an alternative technique for bacterial removal, namely SLC with low-density Porcicoll (20% and 30%). Previous studies have tested the suitability of SLC using low-density colloid in small (1.5 ml; [24]), medium (50 ml; [9,10]), and large volumes (500 ml; [11]), achieving a considerable reduction or even complete removal of bacteria, and evaluating its impact on some sperm quality parameters, such as motility, viability apoptotic-like changes, acrosomal status, reactive oxygen species (ROS), capacitation, and mitochondrial activity [9,11]. However, for the sperm chromatin assessment, only DNA fragmentation has been considered regarding SLC effects and bacterial presence [10].

In a previous study [11], some sperm quality parameters, such as the apoptotic ratio, damaged acrosomes, and ROS production, were more affected by the presence of bacteria, these parameters being lower in control samples than in the low-density Porcicoll treatments on day 7 of storage. However, there was an increase of ROS + spermatozoa in SLC-treated samples on day 0; it could be regarding a stress response [25]. Actually, SLC removes the lowest-quality sperm from the sample described as unfavorable for the whole semen doses [26].

Semen samples were collected at boar stations to check the bacterial contamination that might occur under normal collection conditions. The contamination in CTL shows that if the collection procedure does not strictly adhere to recommended protocols for hygienic collection, bacterial growth is a problem if antibiotics are not added to semen extenders [27], with severe consequences for AI centers [28]. Even small numbers of bacteria could negatively affect sperm quality by releasing endotoxins [29]. In a previous study, the bacterial presence was minimal when following stringent protocols, but it was appreciable in several ejaculates, especially after storage [9].

In addition, the degree of alteration for sperm parameters depends on several factors: bacterial species, infective dose, and storage time [30]. About the infective dose, some findings suggest that a threshold exists for 10^7 CFU/ml in the case of *E. coli* and *C. perfringens*, although under this threshold, changes in sperm parameters throughout the storing period were very similar between bacterial species and infective doses [30,31]. Our results show that after 7 days of storage at 17 °C, the bacterial concentration reached 10^5 CFU/ml, except for *C. koserii* and *B. licheniformis* (10^4 and 10^3 CFU/ml, respectively). Moreover, other studies indicated that in seminal doses infected with *E. coli* from 10^1 to 10^6 CFU/ml, the adverse effects of bacterial contamination on sperm quality were significant from days 4 and 3, respectively, with a decrease in sperm motility before that of sperm viability [30,32,33]. In our study, we observed a significant increase of *E. coli* for 10^5 CFU/ml, prevented by P30. In addition, sperm motility and sperm viability decreased simultaneously, obtaining significant differences from the control after 3 days of storage at 17 °C [11]. *E. coli*, as well as other gram-negative bacteria such as *P. aeruginosa* (a usual contaminant and prevalent in our study), also induce disturbances in the acrosome integrity in boar sperm samples [31], agreeing with our results [11]. *P. aeruginosa* also produces soluble quorum-sensing molecules inducing acrosomal exocytosis [34].

This study shows that SLC contributed to achieving a significant reduction in some bacteria (*E. coli*, *E. faecalis*, and *P. aureginosa*) and

Table 1
Summary results for the canonical correlation analysis and test of H~0 (Wilks Lambda): The canonical correlations in the current row and all that follow are zero. The first (significant) three canonical correlations are shown.

Variate	Canonical R	Canonical R^2	Eigenvalues	Percent	Cum. Percent	Wilks Lambda	d.f.	P
X_1Y_1	0.855	0.732	2.726	44.0	44.0	0.021	110	<0.001
X_2Y_2	0.764	0.583	1.398	22.6	66.6	0.077	90	<0.001
X_3Y_3	0.663	0.440	0.785	12.7	79.3	0.184	72	0.01

Table 2
Standardized canonical coefficients for canonical variates. Only canonical variates from the first (significant) three canonical correlations are shown.

X var.	X_1	X_2	X_3	Y var.	Y_1	Y_2	Y_3
Total	−0.194	0.050	0.110	SD-DFI	0.506	−1.131	−0.447
A. caviae	−0.268	0.059	−0.746	%DFI	−0.525	0.940	1.149
P. aeuriginosa	0.	0.362	−0.523	%HDS	1.335	−2.054	−3.588
S. simulans	0.047	−0.043	0.076	Disulfide levels	1.978	−1.720	−2.783
E. coli	−0.311	0.904	−0.412	Low mBBr fl.	0.888	−1.811	−1.389
E. faecalis	−0.258	−1.150	−0.165	Moderate mBBr fl.	−0.633	−0.734	0.599
B. licheniformis	0.214	0.364	0.376	High mBBr fl.	−0.713	−0.689	1.093
C. koseri	−0.122	0.174	0.900	CMA3 MFI	−1.046	−1.289	1.357
P. vulgaris	0.064	0.393	−0.537	Low CMA3 fl.	−0.381	0.509	−0.266
K. variicola	0.282	−0.062	−1.117	Moderate CMA3 fl.	−0.127	−0.635	−0.291
				High CMA3 fl.	0.025	0.055	0.252

MFI: Median fluorescence intensity; fl.: Fluorescence.

a complete removal of other bacteria (*S. simulans*, *C. koseri,* and *B. licheniformis*) after 7 days of storage at 17 °C. Only the presence of *A. caviae* did not show significant differences in CTL samples concerning SLC treatments in sperm samples after 7 days of storage. In addition, chromatin heterogeneity (SD-DFI), chromatin immaturity (%HDS), and disulfide bridges were enhanced in the SLC samples. Disulfide bridges were maintained with significant differences in both SLC treatments for CTL treatment. Finally, the mBBr low population (sperm with a large number of disulfide bridges and then a high chromatin compaction) increased with SLC treatments with respect to the CTL treatment showing a better sperm population after storage using SLC.

No differences were observed between SLC and CTL treatments for sperm protamination assessed by CMA3. However, CMA3 might not be a good reference for decondensation, leading to wrong interpretations [35]. CMA3 is inappropriate to establish the degree of protamination showing an absence of correlation between %HDS and CMA3, and the difference $\Delta = \text{CMA3} - \text{TUNEL}$ seems to be a more precise method of evaluating the structure and should be compared with HDS due to a part of the fluorescence of CMA3 is relating its interaction with DNA [36], existing a strong correlation ($r = 0.956$) between TUNEL and CMA3 [37]. Our analyses yielded a considerable number of variables, frequently assessed when using modern techniques for sperm evaluation. Whereas the analysis with linear models produced useful information on the SLC and semen storage, a multivariate approach, both in the explanatory and response sides of the problem, helps interpret complex relationships. CCA seems to be a good option when several variables are used. Thus, CCA revealed that not all bacterial isolates have the same impact on boar sperm, according to other studies [38]. Although there was no great redundancy for the variable sets (possibly because of a combination of factors involved, including treatments and refrigeration storage), we obtained high canonical R^2 values and sensible X−Y relationships.

Semen storage at 17 °C is an ideal environment for the growth of antimicrobial-resistant microorganisms (AMR). However, Sone et al. [39] reported that the survival of boar sperm within the first two days of storage was affected mainly when *E. coli* and *Pseudomonas* were present (isolated in 80.4% of all cases). Then, these bacteria were related to the poor quality of boar sperm. In contrast, *Alcaligenes* sp., *Actinomyces* sp., *Streptococcus* sp., and *Staphylococcus* sp. had no negative influence on semen storage [39]. However, it is necessary to consider the number of bacteria present in a sperm sample to determine the impact [40], the ratio of bacteria-to-sperm for inducing agglutination and reducing motility being higher in *E. coli* (1:1) than in other bacteria, with a ratio as low as 1:100 ratio is enough to reduce the sperm quality [32,33,40,41].

The results of this study agree with previously published results, showing a decrease in sperm quality when *E. coli* is present [32,41]: for the bacterial presence (X-set), X_1 is related to the non-presence of these bacteria, showing a particularly antagonistic relationship with *E. faecalis* and *E. coli*. The variable Y_1 pertains to high sperm quality parameters, showing a strong relationship with SCSA variables (%DFI and %HDS) and a strong relationship with disulfide levels. This means that an absence of *E. coli* and *E. faecalis* in the sperm samples is related to samples with better chromatin structure showing less fragmentation and immaturity and a high level of protamination (more disulfide bridges). The same conclusion, but from a different point of view, can be obtained by relating the variables X_2 to Y_2. X_2 is strongly associated with the presence of *P. aeruginosa* and *E. coli,* and Y_2 is mainly negatively associated with the low mBBr population. This supports the idea that bacterial contamination by *E. coli* and *P. aeruginosa* is primarily related to poor quality of sperm chromatin since they have a strong negative relationship with the sperm population with a better degree of chromatin compaction. Finally, the variate X_3Y_3 indicates that the presence of *K. variicola* is closely related to a decrease in sperm immaturity (%HDS), defined as the proportion of spermatozoa with

Table 3
Canonical loadings of the original variables with their canonical variates. Only canonical variates from the first (significant) three canonical correlations are shown.

X var.	X_1	X_2	X_3	Y var.	Y_1	Y_2	Y_3
Total	−0.58	0.27	−0.28	SD-DFI	−0.64	−0.26	−0.23
A. caviae	−0.56	0.06	−0.38	%DFI	−0.76	−0.08	0.37
P. aeuriginosa	0.44	0.47	−0.1	%HDS	−0.73	0.05	−0.53
S. simulans	0.25	0.18	−0.05	Disulfide levels	0.81	0.25	0.3
E. coli	−0.79	0.42	−0.05	Low mBBr fl.	0.01	−0.63	0.45
E. faecalis	−0.85	−0.15	−0.1	Moderate mBBr fl.	0.13	0.11	−0.14
B. licheniformis	−0.26	0.14	−0.01	High mBBr fl.	0.14	0.26	0.11
C. koseri	−0.21	0.4	0.1	CMA3 MFI	−0.18	−0.28	0.02
P. vulgaris	−0.48	0.35	−0.11	Low CMA3 fl.	−0.64	−0.08	−0.23
K. variicola	0.52	−0.15	−0.47	Moderate CMA3 fl.	−0.26	−0.22	0.4
				High CMA3 fl.	0.02	−0.31	0.09

MFI: Median fluorescence intensity; fl.: Fluorescence.

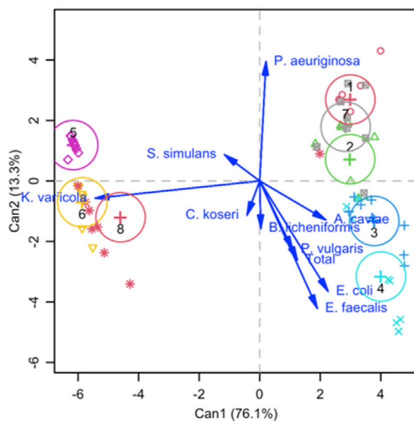
Table 4
Cross-loading of the original variables with the opposite canonical variates. Only canonical variates from the first (significant) three canonical correlations are shown.

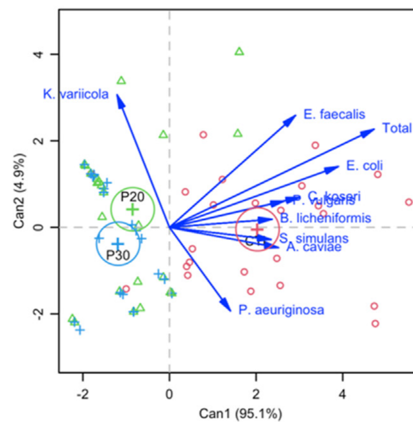
X var.	Y_1	Y_2	Y_3	Y var.	X_1	X_2	X_3
Total	−0.5	0.21	−0.18	SD-DFI	−0.55	−0.2	−0.15
A. caviae	−0.48	0.04	−0.25	%DFI	−0.65	−0.06	0.25
P. aeuriginosa	0.38	0.36	−0.07	%HDS	−0.63	0.04	−0.35
S. simulans	0.21	0.14	−0.03	Disulfide levels MFI	0.7	0.19	0.2
E. coli	−0.67	0.32	−0.03	Low mBBr fl.	0.01	−0.48	0.3
E. faecalis	−0.73	−0.12	−0.07	Moderate mBBr fl.	0.11	0.08	−0.09
B. licheniformis	−0.22	0.1	−0.01	High mBBr fl.	0.12	0.2	0.08
C. koseri	−0.18	0.31	0.07	CMA3 MFI	−0.16	−0.21	0.01
P. vulgaris	−0.41	0.27	−0.07	Low CMA3 fl.	−0.54	−0.06	−0.15
K. variicola	0.44	−0.11	−0.31	Moderate CMA3 fl.	−0.23	−0.17	0.27
				High CMA3 fl.	0.01	−0.23	0.06

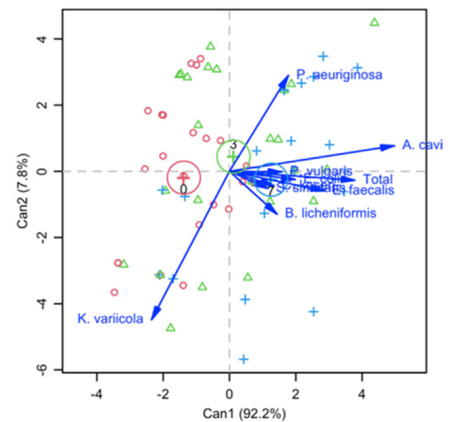
MFI: Median fluorescence intensity; fl.: Fluorescence.

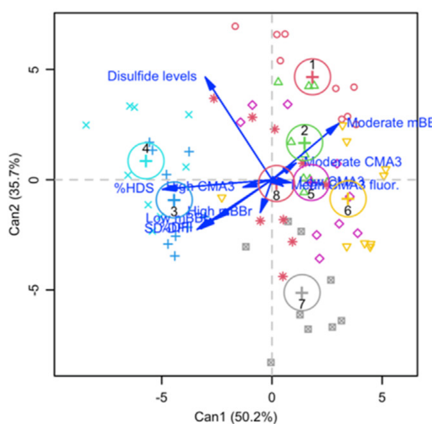
Table 5
Explained total variation ratio by canonical variates for the variable sets and their redundancies. Only canonical variates from the first (significant) three canonical correlations are shown.

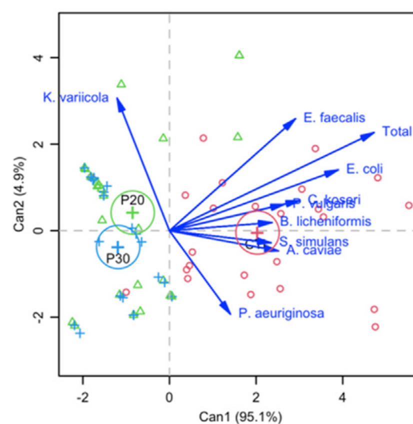
X-variable set				Y-variable set			
Variance extracted		Redundancy		Variance extracted		Redundancy	
X_1	0.286	Y_1	0.181	Y_1	0.248	X_1	0.209
X_2	0.086	Y_2	0.045	Y_2	0.077	X_2	0.05
X_3	0.048	Y_3	0.041	Y_3	0.092	X_3	0.021











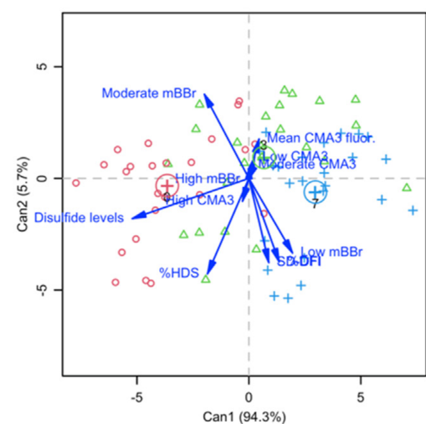


Fig. 5. Graphical representation of the two first canonical dimensions from the canonical discriminant analysis performed on the microbiology and chromatin status variables for the three effects considered: Boar, treatment, and day of storage. The plots represent the association between these parameters.

low chromatin compaction. This lower compaction might be due to many factors, such as retained histones due to the lack of full protamination [42], but more probably due to decreased disulfide bridges between protamines. Indeed, X_3Y_3 is also strongly related to the proportion of sperm with low free thiols, potentially oxidized to disulfide bridges (low mBBr population). Moreover, the CCA interpretation suggests that *E. coli*, *E. faecalis*, *P. aeuriginosa,* and *K. variicola* are the species that most negatively affect sperm chromatin. Since SLC can remove these bacteria, it could be an efficient method for reducing antibiotic use and improving the quality of semen doses throughout storage. Interestingly, even the low-density colloids used here seemed to have a selective capacity for sperm with better chromatin.

The findings on sperm chromatin and its relationship with the presence of specific bacteria must be confirmed with more specific experimental designs and analyses. Even though the use of CCA enables us to investigate multivariate data more effectively, our results allow us to draw associations between the variables considered, but not solid causality. Mechanistic studies more strictly controlling the different variables would allow testing the other relationships proposed in this study (between SLC treatments, bacterial species presence, and sperm chromatin structure) [43]. Another relevant limitation is that the researchers did not control bacterial contamination. We have found seven species at specific concentrations, but the microbiological conditions could greatly vary between stud farms. Moreover, the effects of bacteria on boar spermatozoa depend on the species, as revealed from previous studies describing many others present in boar semen [33,44–46].

Finally, it is interesting how the discriminant analysis showed the separation not only between the SLC treatments and the control but also between groups of boars. Between-boar variability regarding chromatin status has been reported and is probably related to characteristics such as freezability [23]. However, studies associating bacterial variability between ejaculates from different boars are scarce. Nevertheless, some authors have investigated large numbers of ejaculates, finding considerable variability even when using antibiotics, and this microbial variability is possibly related to differences in sperm quality and fertility [14,47]. Considering the different associations between bacterial presence and sperm chromatin and that SLC efficiency could vary among boars, this finding is worth further research. For instance, investigating if the discrimination between groups of boars (due to *K. variicola* and partially *P. aeruginosa* vs. Enterobacteria) depends on the individual boar.

5. Conclusions

Sperm samples processed by SLC treatments using low-density Porcicoll showed reduced overall bacterial presence and improved disulfide bridge levels even after 7 days of storage. Multivariate analysis CCA is a good alternative to conventional analyses in spermatology, using many variables which are difficult to interpret simultaneously.

CRediT authorship contribution statement

Estíbaliz Lacalle: Investigation, Data curation, Writing – original draft. **Estela Fernández-Alegre:** Investigation, Data curation. **Cristina Soriano-Úbeda:** Investigation, Visualization, Writing – review & editing. **Sonia Martínez-Martínez:** Investigation, Writing – review & editing, Data curation. **Juan Carlos Domínguez:** Investigation, Resources, Funding acquisition. **J. Ramiro González-Montaña:** Investigation, Visualization. **Jane M. Morrell:** Supervision, Conceptualization, Methodology, Investigation, Data curation, Funding acquisition. **Felipe Martínez-Pastor:** Supervision, Methodology, Formal analysis, Visualization, Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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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.2023.02.017.

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