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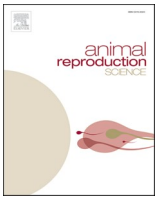

Metagenomic identification of bull semen microbiota in different seasons

Aleksandar Cojkic [a,*], Adnan Niazi [b], Jane M. Morrell [a]

[a] Swedish University of Agricultural Sciences (SLU), Department of Clinical Sciences, Uppsala 75007, Sweden
[b] SLU Global Bioinformatics Centre, Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala 75007, Sweden

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ABSTRACT

A seasonal effect on sperm quality parameters was observed previously. Although identification of the bull semen microbiota by 16S rRNA sequencing was performed previously, it has not been carried out in commercial semen samples from different seasons, and its connection with sperm quality parameters has not been evaluated yet. The objectives in this study were; (i) to evaluate diversity of bull semen microbiota and sperm quality parameters in different seasons, and (ii) to find if specific bacteria were associated with seasonal differences in specific sperm quality parameters. Bull semen microbiota was identified in 54 commercial bull semen samples from 3 seasons (winter, spring, summer). Sperm quality was analysed by Computer Assisted Sperm Analyses (CASA) and Flow Cytometry (FC). From 28 phyla in all samples, six phyla were identified in samples from all seasons, with observed seasonal differences in their distribution. At genus level, 388 genera were identified, of which 22 genera had a relative abundance over 1 % and showed seasonal differences in bacterial diversity, and 9 bacteria genera were present in all seasons. Differences between spring and summer ($P < 0.05$) were observed for live hydrogen peroxide positive sperm cells. A trend towards significance ($0.10 > P > 0.05$) was observed for some CASA kinematics (VCL and LIN) and FC parameters (High respiratory activity, and live hydrogen peroxide positive sperm cells) between seasons. Nevertheless, associations between sperm quality parameters and specific bacteria were observed in spring.

1. Introduction

Spermatogenesis can be affected positively or negatively by many factors, and is reflected in semen quality and quantity (Colenbrander and Kemp, 1990). These factors can be classified as animal-based, resource-based (environment), or management-based, according to their origin. For the bull industry, negative factors should to be identified and prevented wherever possible, because they may lead to bull subfertility or infertility, equating to economic losses. One of the environmental factors contributing to variation in sperm quality and possibly male fertility is the season of the year (Foote, 1978). A seasonal effect on sperm quality has been previously evaluated in many animal species; boars (Ciereszko et al., 2000), rams (Martí et al., 2012), stallions (Hoffmann and Landeck, 1999) and dogs (Lojkić et al., 2022), but the highest number of studies in the past few decades was conducted on bull semen. The effect of season and high environmental temperatures on fertility in dairy cattle was established 40 years ago

* Corresponding author.
E-mail addresses: aleksandar.cojkic@slu.se (A. Cojkic), adnan.niazi@slu.se (A. Niazi), jane.morrell@slu.se (J.M. Morrell).

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(Cavestany et al., 1985). The connection between seasonal effect and sperm quality in the cattle industry first started with beef bulls (Fields et al., 1979; Rahman et al., 2011; Seifi-Jamadi et al., 2020). Thereafter, studies were carried out on dairy bull semen in different climates (Valeanu et al., 2015; Sabes-Alsina et al., 2017), and retrospective analyses were made of sperm quality in dairy bull semen between climatic zones, in North and South Europe (Sabes-Alsina et al., 2019). It was concluded that dairy bull semen quality is affected by climate even in temperate zones and that the timing of heat stress during spermatogenesis determines which sperm quality parameter would be affected (Sabes-Alsina et al., 2019). Seasonal effect on sperm quality in other bull breeds in the tropics was also investigated (Nongbua et al., 2020). Furthermore, seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls, raised under the same climate condition (Nichi et al., 2006), was evaluated.

The reason for this extensive research on bull semen is due to their use in the artificial insemination (AI) industry, where the semen samples with the best quality are required to maintain fertility after cryopreservation.

Another factor negatively influencing sperm quality is bacteriospermia (Koziol et al., 2022; Tvrdá et al., 2022). Different bacteria present in semen samples can have a negative effect on spermatozoa either by competing for the nutrients or by producing metabolic byproducts and toxins that can have deleterious effect on sperm quality (Morrell and Wallgren, 2014), e.g. producing phospholipases and damaging phospholipids in sperm membranes (Schmiel and Miller, 1999). Until recently, isolation and identification of bacteria in bull semen was reserved only for cases when clinical signs of reproductive disease were present or when the sperm quality of a specific bull decreased over a long period of time (Prince et al., 1949). The reasons for not carrying out extensive studies of the seminal microbiota of healthy bulls, or looking for possible relationships with sperm quality, are many. The first and most important is that antibiotics are added to semen extenders as defined by national and international regulations (EUR-Lex, Council Directive 88/407/EEC of 14 June, 1988) for semen trade, and is was previously assumed that the antibiotics would suppress bacterial growth and proliferation. It is now known, however, that some bacteria in bull semen doses are resistant to these antibiotics (Goularte et al., 2020). An additional reason for not studying the seminal microbiota is that isolation and identification of bacteria by traditional culture-depended methods is time-consuming and not all bacteria can be identified by such methods (Woo et al., 2008). In the last decade, 16S rRNA sequencing has played an important role in the accurate identification of bacteria and the discovery of novel bacterial species in clinical microbiology. Furthermore, several studies have been conducted of the microbiome of the healthy bull prepuce (Koziol et al., 2017; Wickware et al., 2020), and semen microbiota (Medo et al., 2021) and their correlation with sperm quality (Medo et al., 2021; Koziol et al., 2022) or fertility results (Cojkic et al., 2021). It has also been found that sperm quality and seminal bacterial load are affected by season in rams (Azawi and Ismaeel, 2012), goats (Gangwar et al., 2021), and buffalo (Sannat et al., 2015), as well as different breeds of cattle (Sannat et al., 2016). Identification of bacteria in these studies was done by traditional culture-dependent methods where bacteria that are difficult to culture may be missed, leaving us with an incomplete knowledge of the bull semen microbiota.

The aims of this study were: (i) to identify bull semen microbiota by 16S rRNA sequencing in three seasons and to establish if there are seasonal differences in microbial abundance; and (ii) to determine possible differences in bull semen quality between seasons and specific bacteria.

2. Materials and methods

2.1. Experimental design

Semen samples from 18 bulls, collected in three seasons (winter, spring and summer), were used for bacteriological identification by 16S rRNA sequencing, with sperm quality evaluation by CASA and Flow cytometry. More details about the samples and analyses are provided in the specific sections of the Materials and methods.

2.2. Semen samples

The frozen bull semen samples used in this experiment were commercial semen doses, kindly donated by Viking Genetics (Skara, Sweden). Semen was collected from 18 Swedish Red dairy bulls, aged between four and seven years old, and processed with semen extender containing antibiotics. The straws of frozen semen were selected from each bull in three consecutive seasons (winter, spring and summer), one straw per season. The bulls were housed at Viking Genetics bull stud and the samples were collected during a one-month period for each season, between 2010 and 2012. The semen samples were collected using a sterile artificial vagina at a predetermined thermal threshold to avoid temperature shock. The semen straws (250 μL) were thawed in a water-bath, at 37 °C for 12 s before further analyses were performed.

2.3. DNA extraction

AllPrep DNA/RNA/miRNA Universal Kit Cat No./ID 80224 was used following the manufacturer's protocol: Simultaneous Purification of Genomic DNA and Total RNA, to extract DNA from semen samples in the Clinical Sciences Laboratory of the Swedish University of Agricultural Sciences (SLU). Volumes of 50 μL sperm sample were centrifuged, the supernatant was removed, and only pelleted cells were used. The purity and concentration of the DNA was tested using Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Eugene, Oregon, USA) with the Quantitation range of 0.1 – 120 ng. The DNA samples were stored at −80°C until further preparation. Before sending for 16S rRNA amplification and sequencing, all semen samples were adjusted to 0.4 ng/μL of DNA with elution buffer.

2.4. 16S rRNA amplification and sequencing

The complete library preparation for V3-V4 regions of 16S rRNA included two PCRs followed by two bead-based purifications of the PCR products. Both PCR set-ups and bead clean-ups were performed with Agilent NGS workstation Bravo (Agilent Technologies, USA) in a 96-well plate format. The first PCR (PCR1) was performed to amplify the 16S region of the bacterial DNA content. A full PCR1 reaction mix (25 μL) contains 4 ng of samples, 12,5 μL KAPA HiFi HotStart ReadyMix (Cat no: 07958935001 Roche), 0.5 μg / μL BSA (Cat no: B14, Thermo Scientific; 50 mg/mL), 1.25 μL Primer mix (7.5 μM solution, contains 341F and 805R, forward and reverse primers), and 0.5 μL dimethyl sulfoxide (DMSO). Bead clean-up was performed after PCR1 using MagSi-NGS prep plus (Cat no: MDKT00010075 Tataa). In this step, free primers were removed and the amplicons were purified for the second PCR by binding the DNA to magnetic beads, washing and releasing the DNA in elution buffer (EB) (Cat no:19086; QIAGEN). A full PCR2 reaction mix (20 μL) includes 6 μL of sample, 10 μL KAPA HiFi HotStart ReadyMix (Cat no: 07958935001 Roche), and 4 μL of indexing primer mix (i5 and i7 indexing primer, 2.5 μM). Conditions and primers used to amplify V3-V4 regions of 16S rRNA are presented in Supplementary Table 1. (Table S1). Final bead clean-up was performed after PCR2 using MagSi-NGS prep plus (Car no: MDKT00010075, Tataa). In this step, free primers were removed and the amplicons were purified by binding the DNA to magnetic beads, washing and releasing the DNA in EB. The quality of the adapter-ligated libraries was checked Caliper GX LabChip GX/HT DNA high sensitivity kit (Cat no: CLS760672, PerkinElmer). Libraries were normalised and pooled before sequencing on an Illumina MiSeq v3–600 flowcell, with a 301–10–10–301 read setup.

2.5. Controls for potential sampling- and DNA extraction-associated contamination

To address the possibility of contamination, a negative PCR control for water used in DNA extraction and amplification PCR mix was performed. The negative control for DNA extraction kit was tested on Qubit before the samples were sent for sequencing; no DNA was present. The *E. coli* genomic DNA (0.25 ng/μl) was used as positive control in a PCR reaction. As no bands were present after negative PCR control, sequencing of negative and positive controls (DNA extraction kits and PCR amplification mix) was not performed in the commercial laboratory, since this is not part of their standard operating procedure for clinical samples.

2.6. 16S profiling

Analysis of 16S rRNA sequencing data was performed using the Nextflow pipeline ampliseq v2.2.0 (https://github.com/nf-core/ampliseq). Briefly, raw sequencing reads were quality checked initially using FastQC (Andrews, 2010), followed by trimming of primer sequences from the reads using cutadapt v3.4 (Martin, 2011). Sequencing reads were denoised, dereplicated, and filtered for chimeric sequences using DADA2 (Callahan et al., 2016). Denoised paired-end reads were truncated from position 279 (forward) and 206 (reverse), whereas all other reads shorter than 50 bp were removed. The truncated sequences were merged with minimum 12 bp overlap, resulting in a total of 1939 amplicon sequence variants (ASVs) which were used for downstream analysis. These ASVs were taxonomically classified from phylum to species level using the SILVA v138 prokaryotic SSU database (Quast et al., 2012) by applying Naive Bayes classifier implemented in QIIME 2 (Bolyen et al., 2019), trained on the preprocessed database. Following taxonomic classification of ASVs classified as Mitochondria or Chloroplast were removed. The ASVs with a minimum read frequency $\geq$5 in at least one sample were maintained for a further analysis.

2.7. Computer-assisted sperm motility analyses (CASA)

Sperm motility was assessed by computer-assisted sperm analysis (CASA) using the SpermVision™ (Minitűb, Tiefenbach, Germany) with an Olympus BX51 microscope (Olympus, Japan) as described previously (Cojkic et al., 2023b). Briefly, 5 μL aliquots of the semen sample were pipetted on a warm slide and covered with an 18 × 18 mm cover slip (VMR, Leuven, Belgium). Particles with an area ranging from 20 to 100 μm^2 were identified as cells and were included in the analysis The following parameters were measured: total motility (%), progressive motility (%), velocity average trajectory (VAP, mm/s), velocity curved line (VCL, mm/s), velocity straight line (VSL, mm/s), straightness (STR, VSL/VAP, %), linearity (LIN, VSL/ VCL, %), wobble (WOB, VAP/VCL, %), amplitude of lateral head displacement (ALH, mm), and beat cross frequency (BCF, Hz). Spermatozoa were considered as immotile if the area under curve (AOC) was $<$ 5, VSL $<$ 0.2, and BCF $<$ 0.2; they were considered to be locally motile if spermatozoa covering a straight-line distance (DSL) was $<$ 4.5. Approximately 400 cells were evaluated in 8 fields.

2.8. Flow cytometry analyses (FC)

Flow cytometry (FC) analysis was performed using a FACSVerse™ flow cytometer (BD Biosciences, Becton Dickinson and Company, San Jose, CA, USA). The lasers used to excite the fluorescent stains were a blue laser (emitting at 488 nm) and a violet laser (emitting at 405 nm). The band-pass filters used to detect different fluorescence were; for green fluorescence (FL1, 527/32 nm), orange (FL2, 586/42 nm), red (FL3, 700/54 nm) and blue fluorescence (FL5, 528/45 nm). The obtained data for all FC parameters were further analyzed using FCS Express 5 software (De Novo, Glendale, CA, USA). Sperm samples were diluted with Buffer B (patent pending; J.M. Morrell and H. Rodriguez-Martinez) to adjust sperm concentration to approximately 2 × 10^6 spermatozoa/mL (spz/mL). Aliquots of 300 μL of sperm/Buffer B dilution were used for each analysis.

2.8.1. Assessment of membrane integrity and mitochondrial membrane potential

Evaluation of sperm plasma membrane integrity was performed using SYBR14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, Oregon, USA). Each 300 μL aliquot of sperm/Buffer B (sperm aliquots) was stained with 1.2 μL SYBR14 dilution (0.5 μL of 1 mM SYBR14 diluted 50 times with Buffer B). Thereafter, sperm samples were also stained with 3 μL of 2.4 mM PI. The stained aliquots were incubated at 38 °C for 10 minutes before evaluation was performed and the proportions of membrane intact (SYBR Living), membrane damaged (SYBR Dead) and intermediate populations (SYBR Dying) were enumerated. In order to evaluate the mitochondrial potential, 1.2 μL of tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1 stock 3 mM) were mixed with each 300 μL sperm aliquot and incubated for at least 30 min at 38 °C before analysis. Fluorescence from JC-1 was measured in the FL1 and FL2 channels of the flow cytometer. A total number of 10,000 cells was evaluated, and classified as sperm cells with high respiratory activity (JC High) emitting orange florescence, or low respiratory activity (JS Low) emitting green florescence.

2.8.2. Assessment of reactive oxygen species and sperm chromatin structure

To assess reactive oxygen species (ROS), hydroethidine (HE; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) and 20, 70 -dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) were used to detect superoxide (O_2^-) and hydrogen peroxide (H_2O_2), respectively. Hoechst 33258 (HO) was used to permit the simultaneous differentiation of living and dead cells, Aliquots (300 μL) of semen extended to a concentration of approximately 2×10^6 spermatozoa/mL (spz/mL) with Buffer B were stained with 3 μL of HO (40 mM), 3 μL HE (40 mM) and 3 μL DCFDA, (2 mM). The samples were gently mixed and incubated at 38 °C for 30 min before analysis. Using the dot-plots for HO/HE and HO/DCFDA fluorescence, the following populations were quantified: ROS Live O_2^-; ROS Live O_2^+; ROS Dead O_2^+; ROS Dead H_2O_2; ROS Dead $H_2O_2^+$; ROS Live H_2O_2 and ROS Live $H_2O_2^+$.

Sperm chromatin integrity was evaluated using the sperm chromatin structure assay (SCSA) which utilizes the metachromatic dye, acridine orange (AO). An aliquot of each sperm sample was mixed with the same volume of Tris, sodium chloride, ethylene-diaminetetraacetic acid buffer (TNE buffer) and immediately transferred to a liquid nitrogen container for snap-freezing; the samples were stored at −80°C until analysis. On the day of analysis they were thawed on ice and an aliquot (10 μL) was mixed with 90 μL of TNE, and 200 μL of acid-detergent solution. Exactly 30 s later, the sample was stained with 600 μL of AO staining solution, and analyzed within 3–5 min. The DNA fragmentation index (%DFI) is calculated and expressed as the proportion of cells with a high ratio of denatured, single stranded DNA; %DFI = (red fluorescence/ [green fluorescence + red fluorescence]) x 100.

2.9. Statistical analysis

Data analysis was performed using R statistical software (R Core Team, 2022; v 4.2.2). For the sperm quality parameters, analysis of

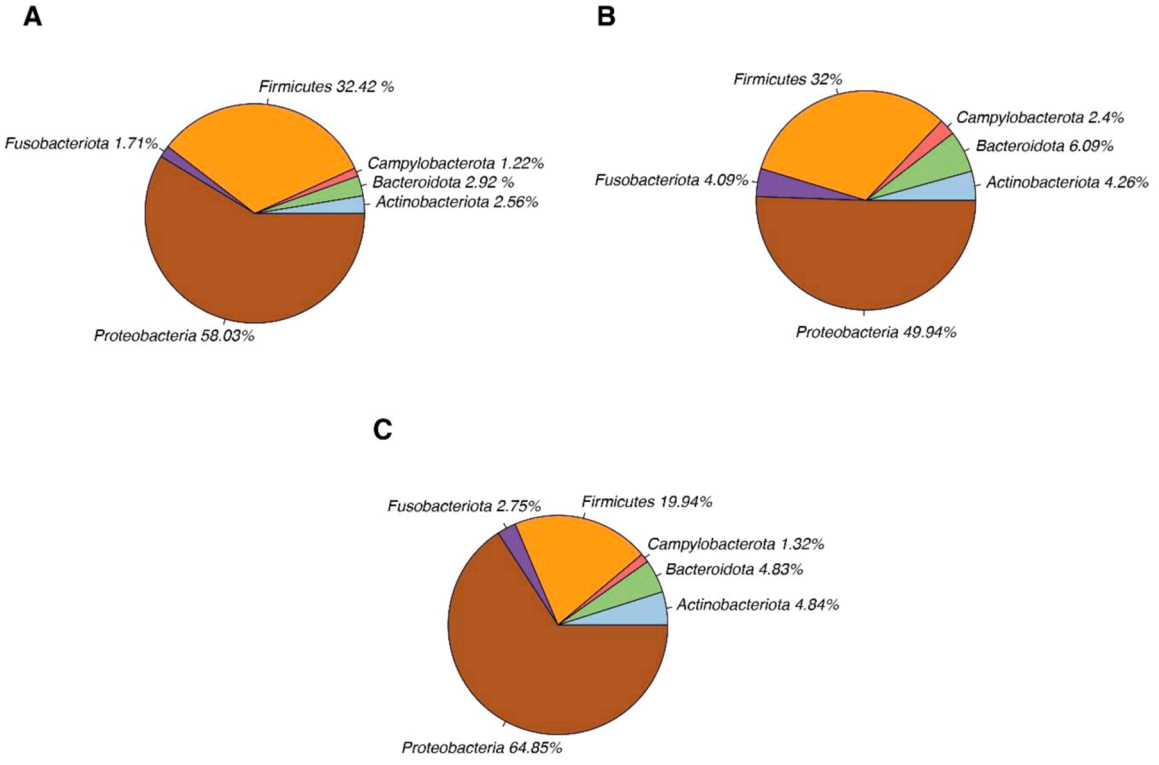

Fig. 1. Seasonal differences in bull semen microbiota on Phylum level between winter (A), spring (B) and summer (C) (percentage).

variance was used; the statistical model included the fixed effect of season and the random effect of bull. Least-squares means for season were compared using Student's t-test, with *P*-value <0.05 being considered significant and *P*-values of 0.05< *P* <0.10 being considered to indicate a trend toward significance (Desbiens, 2003). The assumptions of normal distribution and homogeneity of variance for the residuals were checked by residual plots. Alpha diversity significance for bacterial diversity, richness within the samples and species evenness (Pielou) was determined using Kruskal–Wallis test. Beta diversity significance was determined using overall and pairwise PERMANOVA tests with a Bonferroni corrected *P*-value < 0.05. For the microbiome data, differential abundance of ASVs was calculated with DESeq2 v 1.38.3 (Love et al., 2014) using the Wald test with a corrected $P < 0.05$. Pearson correlations were calculated between ASVs in different seasons. Spearman correlation was used to assess associations between bacteria genera and sperm quality parameters that were significantly different between seasons. *P*-values were adjusted using the Benjamini-Hochberg method to correct for multiple testing. Correlations with $|r| > 0.5$ and *P*-value < 0.05 were considered significant.

3. Results

3.1. Metagenomic analysis

A total of 1729 ASVs was identified across all the samples In total, 28 phyla and 388 genera were identified in the 54 bull semen samples in three seasons. In winter, spring and summer, 20, 21, 20 phyla and 234, 211, 233 genera respectively were identified. There was no significant difference ($P > 0.05$) in seminal bacterial diversity (richness; Shannon Index (Supplementary Fig. 1 A) and observed ASVs (Supplementary Fig. 1B)), community evenness (Pielou, data not shown) between the three season groups (summer, spring,

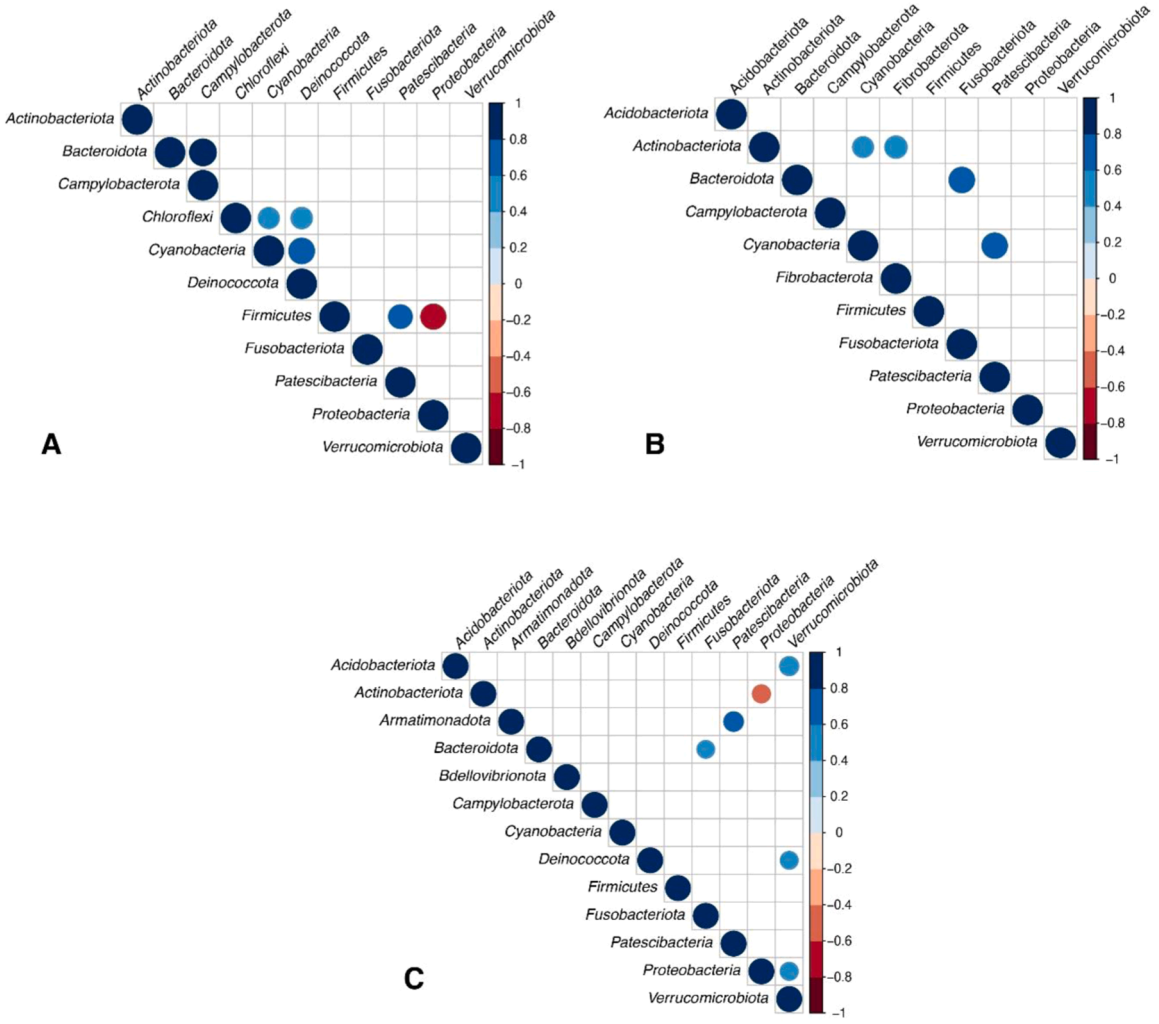

Fig. 2. Correlation plots between different bacterial phyla in winter (A), spring (B) and summer (C). In the correlation matrix, a significant correlation ($|r| > 0.5$ and $P < 0.05$) is presented by blue dots for positive and red dots for negative correlations. Blank cells indicate non-significant correlations.

winter). Similarly, bull semen community structure (beta diversity) did not show a significant difference ($P > 0.05$) in Bray Curtis measurements (Supplementary Fig. 2).

The six phyla, identified in all seasons are presented in Fig. 1. The majority of the samples showed a relative abundance of the phylum Proteobacter with the second most abundant being the phylum Firmicutes. A predominance of the phylum Proteobacter was seen in summer (64.85 %) compared with winter (58.03 %) and spring (49.94 %) (Fig. 1). On the other hand, Firmicutes was lower in summer (19.94) compared with winter (32.42 %) and spring (32.00 %). The Actinobacteriota, Bacteroidota, Campylobacterota and Fusobacteriota were relatively equally distributed between the seasons. The phylum Patescibacterie was present in winter (0.31 %) and summer (0.56 %), and Acidobacteriota only in spring (0.39 %).

The correlations found between bacteria differed according to season (Fig. 2). A negative correlation between bacteria was observed in winter and summer, but not in spring. The phylum Proteobacteria was the only one that negatively correlated ($|r| > 0.5$) in both seasons with Firmicutes (winter) and Actinobacteriota (summer). In spring, Proteobacteria did not show a correlation with other bacterial genera. Positive correlations between bacteria differed between seasons.

All samples contained a high relative ASV abundance of *Escherichia-Shigella* genus. Since this genus was present in such high relative abundance in all samples, it was thought to be a contamination; these reads were removed to enable further analysis of the remaining reads. The top 20 genera for each individual sample after filtering of the data to remove the reads for this *Escherichia-Shigella* genus are presented in Fig. 3. The number of bacteria differing with > 1 % of relative abundance between the seasons are presented in Table 1. A 1 % threshold is an arbitrary cutoff as no values or guidelines are available for interpretation of 16S rRNA gene sequence-based results (Woo et al., 2009). In total, 22 genera had a relative abundance over 1 %. Only nine bacterial genera were present in all seasons, four were present in two seasons (spring-summer) and four genera in only one season, either winter or spring. The *Prevotella* was the only bacterial genus present in just one season (spring) and *Acidocella* was present in the majority of the samples as the genus with the highest relative ASV abundance. The proportion of each genus according to season is shown in Fig. 4. Differences in relative abundance between bacterial genera were present in all seasons. The most abundant genus *Acidocella* was distributed similarly in winter (28.28 %) and spring (27.22 %) compared with summer (19.32 %). Furthermore, a similar distribution of *Bacillus* was seen in spring (3.19 %) and summer (2.79 %), compared to winter (10.39 %). In contrast, *Histophilus* was lower in winter (2.03 %) than in spring (6.97) and summer (6.1 %). Nevertheless, *Burkholderia* had a similar abundance in winter (12.4 %) and spring (12.27 %) compared to summer (7.98 %). The presence of *Streptococcus* differed among seasons. The remaining bacterial genera (*Campylobacter, Geobacillus*, *Lactobacillus* and *Pseudomonas*) had similar abundance (<3 %) in all seasons.

A differential abundance of bacterial ASVs between seasons was observed. There were significant differences in AVSs of some bacteria genera between winter and spring (*Neisseria, Granulicatella, Acidovorax, Enterococus*), and winter and summer (*Neisseria, Granulicatella, Rothia, Acidovorax, UCG-005, Enterococus, Bacillus*), while no significant difference was observed between spring and summer. Just two genera, *Acidovorax* and *Bacillus*, were classified in Top 20 genera and had a relative abundance over 1 % in different seasons (Table 1 and Fig. 4).

3.2. Sperm quality parameters

Sperm CASA measurements are presented in Table 2 for the three seasons. Total and progressive motility did not differ between seasons, although a trend towards significance (P=0.08) was observed for VCL between winter and summer, as well as for LIN (P=0.09) between spring and summer. The remaining kinematic parameters were not different between seasons.

The mean proportion of spermatozoa with high respiratory activity varied in all three seasons, 43.55 % in winter, 41.18 % in

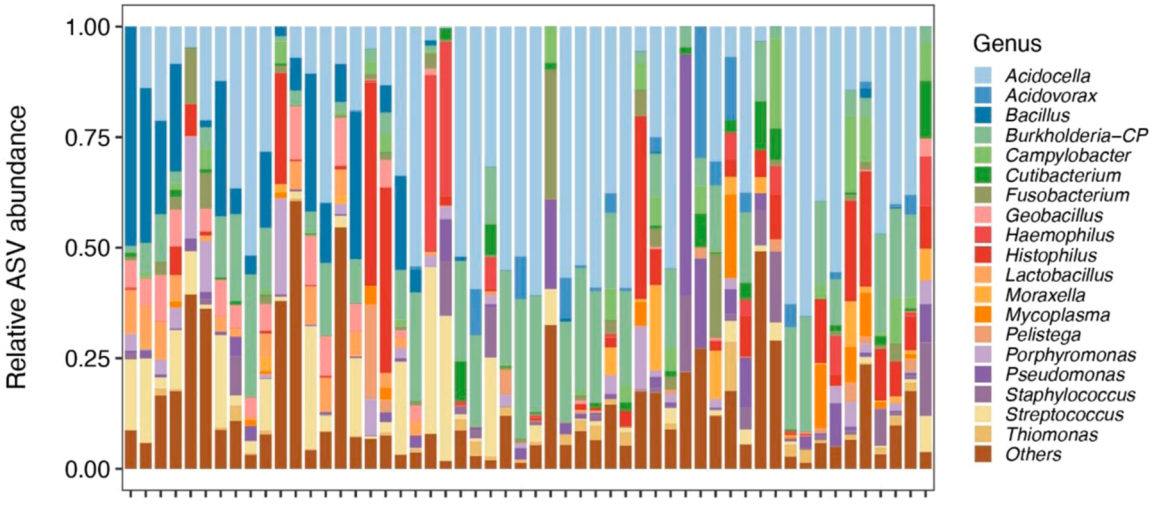

Fig. 3. Distribution of the 20 most abundant bacterial genera in bull semen identified by 16S rRNA sequencing, after removal of the AVS for *Escherichia-Shigella* (n=54).

Table 1
Presence of bacterial genera ($n = 22$) with relative abundance over 1 % in different seasons.

Winter ($n = 13$)	Spring ($n = 14$)	Summer ($n = 17$)
Acidovorax, Limosilactobacillus, Moraxella, Pelomonas	*Prevotella*	*Corynebacterium, Enhydrobacter, Pelistega, Staphylococcus*
	Fusobacterium, Haemophilus, Mycoplasma, Porphyromonas	
Acidocella, Bacillus, Burkholderia, Campylobacter, Geobacillus, Histophilus, Lactobacillus, Pseudomonas, Streptococcus		

*n – represents number of different genera. The black box indicates that the season was not included.

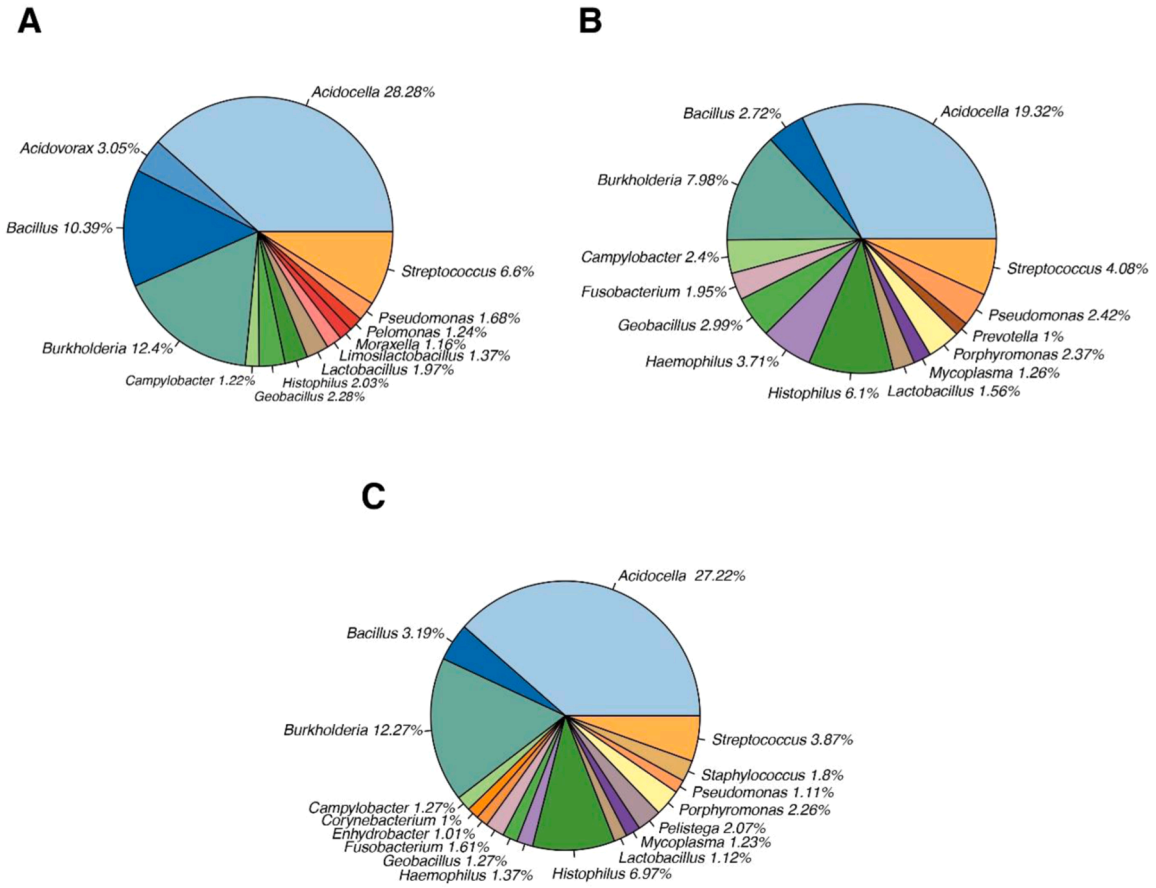

Fig. 4. Seasonal differences in bull semen microbiota on genus level between winter (A), spring (B) and summer (C). Each genus is present as a proportion of the total bacterial genera.

spring, and 49.92 % in summer, with a trend towards significance (P=0.09) being observed between spring and summer (Table 3). The mean values for spermatozoa with low respiratory activity, and of living, dying and dead spermatozoa, and %DFI, are also presented in Table 3 but did not show seasonal differences. Only live, H_2O_2 positive sperm cells were different (P=0.05) between winter and spring and between spring and summer (P<0.05). The remaining ROS sub-populations were not different between seasons (Table 3).

Genera that were significantly correlated with H_2O_2 positive sperm cells were only present in spring season, while they were not significantly correlated in summer and winter. Genera that were positively correlated were *Ruminococcus, Limosilactobacillus, Bacillus, Geobacillus* and *Lactobacillus*, while *Cutibacterium* was the only genus that was negatively correlated with H_2O_2 positive sperm cells (Fig. 5).

4. Discussion

The aim of the present study was to determine whether there could be a seasonal effect on the microbiome of bull semen and on

Table 2
Effect of season on mean values of bull sperm kinematics measured by Computer Assisted Sperm Analysis (18 bulls per season, one ejaculate per bull).

Parameters	Winter	Spring	Summer	RMSE[a]
Total motility (%)	51.36	50.66	51.48	12.03
Progressive motility	48.16	47.55	47.75	11.77
VAP (μm/s)	69.34	66.6	64.37	7.24
VCL (μm/s)	133.87[a]	126.65	123.12[a]	14.81
VSL (μm/s)	51.69	49.79	46.42	8.61
STR (%)	0.74	0.75	0.71	0.06
LIN (%)	0.39	0.40[a]	0.37[a]	0.04
WOB (%)	0.52	0.52	0.53	0.02
ALH (μm)	5.11	4.82	4.84	0.61
BCF (Hz)	24.81	23.91	23.49	3.01

VCL—curvilinear velocity, VAP—average path velocity, VSL—straight-line velocity, LIN—linearity, STR—straightness, WOB—wobble, ALH—amplitude of lateral head displacement, BCF—beat cross frequency. Superscript letter denotes a trend towards significance for VCL (P=0.08) between winter and summer season, and LIN (P=0.09) between spring and summer season.

[a] RMSE = Root mean squared error.

Table 3
Effect of season on proportion (%) (mean ± RMSE) of bull sperm viability parameters measured by Flow cytometry (18 bulls per season, one ejaculate per bull).

Parameters	Winter	Spring	Summer	RMSE[1]
Membrane integrity				
Living	44.72	50.05	50.10	11.50
Dying	4.27	4.84	3.62	2.67
Dead	49.45	43.99	45.26	11.24
Mitochondrial membrane potential				
High respiratory activity	43.55	41.18[a]	49.92[a]	12.05
Low respiratory activity	47.73	50.11	42.73	11.10
% DFI	9.82	7.21	6.53	6.96
Reactive oxygen species category				
Live, SO negative	33.17	38.69	36.98	11.58
Live, SO positive	17.53	17.50	19.14	7.59
Dead, SO positive	44.44	37.89	38.36	9.13
Live, H_2O_2 negative	50.79	55.73	55.09	10.24
Live, H_2O_2 positive	0.058[b]	0.16[b,c]	0.064[c]	0.13
Dead, H_2O_2 negative	49.30	45.28	44.97	11.05
Dead, H_2O_2 positive	0.03	0.06	0.03	0.05

SO = superoxide, H_2O_2= hydrogen peroxide, %DFI = DNA fragmentation index.

[1] RMSE = Root mean squared error

[a] P = 0.09 Superscript letter denotes towards significance for Mitochondrial membrane potential for High respiratory activity between winter, spring and summer.

[b] P = 0.05 Superscript letter denotes significant differences for Live, H_20_2 positive sperm cells between winter and spring.

[c] P < 0.05 Superscript letter denotes significant differences for Live, H_20_2 positive sperm cells between spring and summer.

sperm quality, and if the two could be linked. Briefly, seasonal differences in bacterial relative abundance were observed, as well as differences in some sperm quality parameters. Additionally, correlations between some bacteria and selected sperm quality in different seasons were observed.

The over-representation of the *Escherichia-Shigella* genus in all semen samples indicated that it was likely to be a contamination, since the relative abundance was equally distributed in all samples. Despite the fact that *Escherichia-Shigella* was present in all samples, the diversity in community type cannot be disregarded since the bacteria present or absent in different samples were not observed in the negative PCR control, and/or DNA presence could not be identified by Qubit. Furthermore, the five phyla present in each season are in accordance with the bacteria identified in the prepuce of healthy bulls, which belonged to Actinobacteria, Bacteriodetes, Firmicutes, Fusobacteria and Proteobacteria (Wickware et al., 2020). Similar results were found in Holstein Friesian bull semen samples in the Slovak Republic where the predominant phyla were Bacteroidetes (12 %), Actinobacteria (13 %), Fusobacteria (18 %), Proteobacteria (22 %) and Firmicutes (31 %) (Medo et al., 2021). Our study showed the same pattern of top five phyla in each season starting with the highest abundance, Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria. The findings in these studies in different countries lead us to conclude that the seminal microbiota on phylum level is not specific to geographic areas. Interestingly, the same five dominant phyla were found as a part of microbiota in human seminal plasma (Yang et al., 2020), which raises the question of interspecies similarity in semen microbiota. Furthermore, the ratio of top five phyla presented in this study differ between the seasons. To our knowledge, this is the first metagenomics study performed on bull semen microbiota from commercial bull semen samples, evaluating seasonal differences in sperm quality and its connection with bacteria in semen. Nevertheless, a seasonal effect on bacterial load in different breeds of cattle was reported previously (Sannat et al., 2016). Interestingly, in a study on the goat

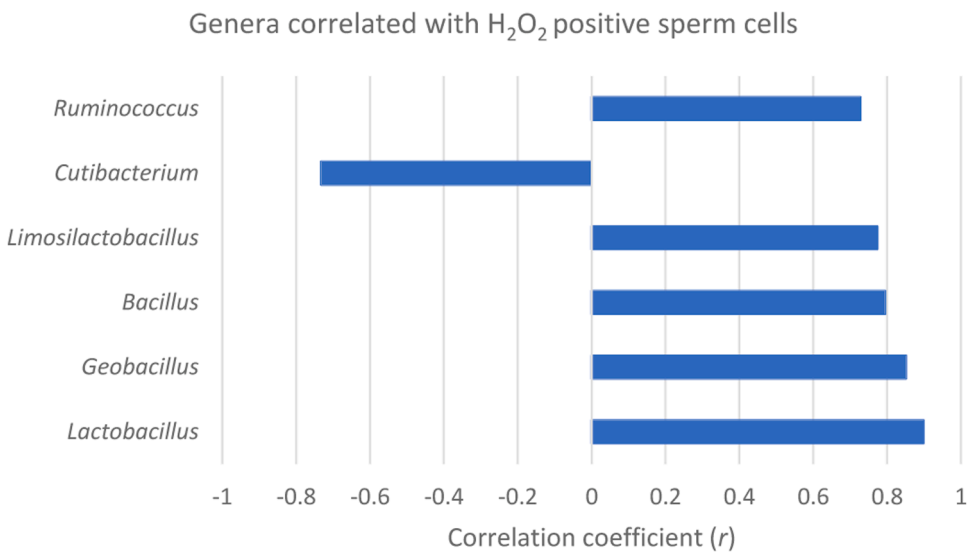

Fig. 5. Correlation coefficient between different bacterial genera and H_20_2 positive sperm cells in spring ($|r| > 0.5$ and $P < 0.05$).

semen microbiota (Moce et al., 2022) in Spain, the five dominant phyla differed slightly between the breeding season (November--December) and non-breeding season (February-Match), and showed similarities to our results with bull semen. In detail, the five dominant phyla were Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria, and Bacteroidetes during the breeding season, followed by Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria during the non-breeding season.

In contrast to the results at phylum level, which were similar between countries, the top 20 genera at the genus level, identified in this study differed by almost 80 % from the top 20 genera found in bull semen samples from Estonian bulls that we described previously (Cojkic et al., 2021). The genera, which were present in semen samples in bulls from both countries are *Camilobacter, Cutinobacterium, Fusobacterium, Histophilus, Porphyromonas* and *Stapylococcus*. The source of the named genera was already discussed (Cojkic et al., 2021). Even the study of preputial microbiota, which was conducted on another continent (USA), showed similar results at genus level; five of their top 20 genera are also present in our study; *Bacillus, Fusobacterium, Histophilus, Mycoplasma* and *Porphyromonas*. Our present results are similar at the genus level to another study in the USA on bull semen microbiota (Koziol et al., 2022): *Bacillus, Fusobacterium, Histophilus, Lactobacillus* in addition to *Escherichia-Shigella,* which we characterized as a potential contaminant. Only two genera were identified in all of these studies, *Fusobacterium* and *Histophilus.* The reasons for the differences on genus level can be many. First, sampling time; our samples are 10–12 years old and it is likely that the bull semen microbiota could change with time in living animals. Second, instead of raw ejaculate we used commercial semen samples where the semen was already extended, which would lead to low bacterial density and diversity in the samples. At the same time, semen samples were manipulated during semen processing at the bull station that could lead to cross-contamination (between samples, and from personnel to semen samples). Another reason is geographical, where animals in different countries and even different animal facilities could have their own microbiome.

In previously published studies, the bull semen microbiome was identified by metagenomic analyses from raw semen samples (Wickware et al., 2020; Medo et al., 2021; Koziol et al., 2022). In the present study, frozen bull semen samples stored for 10–12 years were used to evaluate seasonal effects on bull semen microbiota. Such “historical samples” were used because the length of time that bulls are kept at semen collection stations nowadays is usually limited (covering no longer than two seasons). Conducting a study involving a large number of bulls and longer time interval, therefore, would not be possible. Nevertheless, identification of bacteria in commercial semen samples has additional practical aspects to its purpose here, both to determine how to avoid spread of infection through insemination but also potentially to reduce antimicrobial use during semen preservation.

The number of bacterial genera identified with a relative abundance of >1 % differs slightly between the seasons: summer (n=17), spring (n=14) and winter (n=13). Until now, no studies have been conducted evaluating bacterial differences in bull semen between seasons. The only study similar to this was an evaluation of a seasonal effect on cultured bacterial load in bull semen (Sannat et al., 2016), showing that differences between season exist. The three seasons included in their evaluation were winter, rainy season and summer in India. A higher bacterial load was isolated in semen samples collected during the rainy season, compared with winter and summer. Apart from the nine bacterial genera identified in all seasons, four genera were present in spring and summer, leaving winter as the season with different genera. This finding, together with the differential abundance of bacterial ASVs between winter other seasons, suggests that the bacteria colonising the mucosa of the reproductive tract could depend on the environmental temperature, or even the season.

Both positive and negative correlations between different bacterial ASVs present in two seasons were found in this study. The phylum Proteobacteria showed negative correlations ($|r| > 0.5$) in two seasons, winter and summer. The bacteria that were correlated in this study were also from the same phyla as those identified in semen samples from men with azoospermia (obstructive and non-

obstructive), which showed an increase in Firmicutes while the number of Proteobacteria and Actinobacteriota was low (Chen et al., 2018). Likewise, in our study Proteobacteria was negatively correlated with Firmicutes (winter) but also with Actinobacteriota (summer). A negative effect of these bacteria on sperm quality was not seen in bull semen and, to our knowledge, there are no previously published data about this problem in bulls, although such an effect could be masked by the addition of antibiotics to bull insemination doses. Nevertheless, the bacteria that were negatively correlated in a previous study (Cojkic et al., 2021) were not the same as those that were enriched in groups of bulls with low fertility, and did not correspond with the results of similar studies in humans.

An effect between the genera *Ruminococcus* and *Lactobacillus* semen samples and fertility results (Cojkic et al., 2021) or sperm quality parameters (Closa Gil et al., 2022) were described previously. The *Bacillus* species are highly diverse and can occupy a wide range of ecological niches as well as in bull semen (Ďuračka et al., 2021; Cojkic et al., 2023a). Different species were identified in bull semen samples that were divided into several group according to their motility rate (Ďuračka et al., 2021), suggesting that *Bacillus* could have effect on sperm quality in bulls.

On the other hand, genomic diversity of the genera *Limosilactobacillus,* formerly known as *Lactobacillus* was reviewed (Ksiezarek et al., 2022), showing that *Limosilactobacillus* genus is commonly found in various environments, often associated with fermented foods, and in the human microbiome, including the gastrointestinal tract, oral cavity, vaginal tract, and skin, but not in semen samples. Furthermore, *Geobacillus* species, obligatory thermophilic bacteria with growth occurring in the temperature range 37–75°C, are used extensively in different industries and biotechnology applications, making them excellent candidates for future research. Their presence and positive influence on bull semen quality parameters have not been described previously. Nevertheless, bacteria from *Cutiobacterium* genus were commonly found on human skin and mucous membranes contributing to skin health, besides occasionally acting as opportunistic pathogens, particularly in conditions such as acne (Mayslich et al., 2021). These genera have been found to correlate negatively with the proportion of fragmented cells in a study of human semen (Closa Gil et al., 2022), and it was the only genus that negatively correlated with H_2O_2 positive sperm cells in this study. In the same study of human sperm (Closa Gil et al., 2022) *Bacillus* showed positive correlation with semen volume and sperm with poor protamination, while *Lactobacillus* had a negative correlation with fragmented cells.

Interestingly, in this study, the presence of *Limosilactobacillus* genera with relative abundance over 1 % was seen in winter, while *Bacillus, Geobacillus* and *Lactobacillus* were identified in all seasons. The presence of *Ruminococcus* and *Cutibacterium* was not identified in high abundance in any season (Table 1). Furthermore, all genera that were correlated with specific sperm quality parameters in spring belonged to the bacterial phylum Firmicutes.

Seasonal differences in sperm quality parameters in this study were, at some level, comparable with the previous published results for bulls housed in similar conditions. In a previous study (Valeanu et al., 2015), the proportion of living sperm cells was lower in summer compared with winter, and the %DFI was higher in summer than in spring. In our study, the only difference between the seasons was for live hydrogen peroxide-producing cells, which were higher in spring than in winter or summer. There was a tendency for higher respiratory activity mitochondrial membrane potential cells in winter then in spring, VCL in winter compared with summer, and LIN in spring then in summer; which is in accordance with other studies (Sabes-Alsina et al., 2017). Different climate factors such as temperature, humidity, and photo-period have been evaluated separately (Brito et al., 2002) and combined, as the temperature-humidity index (Llamas-Luceno et al., 2020). Nevertheless, even when these factors were evaluated in the same geographic location but at a different time, contradictory outcomes were obtained. Thus, in the first study (Brito et al., 2002) sperm production and quality were not affected by ambient temperature and humidity, compared with a study conducted 3–4 years later (Nichi et al., 2006) where season had an effect; even though the average temperatures in both studies were similar. This suggests that other factors apart from climatic conditions could influence sperm quality. In addition, the over-representation of the *Escherichia--Shigella* genus in all semen samples suggests that 16S rRNA sequencing should be re-evaluated as a diagnostic tool for clinical samples, specifically bacterial identification of samples with low diversity and density. There is a risk of misinterpreting such results, leading to incorrect or inadequate treatment recommendations in a clinical setting. It should be noted that the presence of bacterial DNA does not mean that these bacteria are viable. In cases where bacterial diversity is low but one genus is present in high relative abundance, recommendations for antimicrobial therapy should be treated with caution, unless every step of sample preparation (DNA extraction, PCR amplification and library preparation) for 16S sequencing is evaluated to exclude possible contamination. The importance, however, of 16S sequencing for genomic identification of pathogenic bacteria with challenging culture properties should not be neglected.

5. Conclusion

There were seasonal differences in the relative abundance of the top six phyla found in bull semen, as well as in the number and phyla that are positively or negatively correlated. Furthermore, the number of genera and their amount in different seasons vary. We observed only small differences in sperm quality between seasons, and connections between specific bacteria and sperm quality were detected.

CRediT authorship contribution statement

Aleksandar Cojkic: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adnan Niazi:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation. **Jane M Morrell:** Writing – review & editing, Validation, Supervision, Resources, Project

administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

All authors contributed to the study and have approved the manuscript for submission. The authors confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Authors state that there are no interests to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2024.107569.

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