

Effects of Sustained Semen Collection on Semen Quality, Semen Metabolites, and Semen Microbiota in Ganders

Fuli Huang

Sichuan Agricultural University

Zhaoyan Chen

Sichuan Agricultural University

Yang Song

Sichuan Agricultural University

Xiaopeng Li

Sichuan Agricultural University

Zhiqiang Mo

Sichuan Agricultural University

Haoran Li

Sichuan Agricultural University

Jiwei Hu

Sichuan Agricultural University

Xin Yuan

Sichuan Agricultural University

Xiao Hu

Sichuan HengWang Animal Husbandry Co., Ltd

Qiang Cao

Sichuan HengWang Animal Husbandry Co., Ltd

Chunchun Han

Sichuan Agricultural University

Hua He

Sichuan Agricultural University

Liang Li

Sichuan Agricultural University

Hehe Liu

Sichuan Agricultural University

Jiwen Wang

Sichuan Agricultural University

Shenqiang Hu

shenqiang.hu@sicau.edu.cn

Sichuan Agricultural University

Research Article

Keywords: gander, sustained semen collection, semen quality, bacterial taxa, metabolites

Posted Date: August 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10384511/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Effects of Sustained Semen Collection on Semen Quality, Semen Metabolites, and Semen Microbiota in Ganders

Fuli Huang^{1,2,3#}, Zhaoyan Chen^{1,2,3#}, Yang Song^{1,2,3}, Xiaopeng Li^{1,2,3}, Zhiqiang Mo^{1,2,3},
Haoran Li^{1,2,3}, Jiwei Hu^{1,2,3}, Xin Yuan^{1,2,3}, Xiao Hu⁴, Qiang Cao⁴, Chunchun Han^{1,2,3},
Hua He^{1,2,3}, Liang Li^{1,2,3}, Hehe Liu^{1,2,3}, Jiwen Wang^{1,2,3}, and Shenqiang Hu^{1,2,3*}

¹ State Key Laboratory of Swine and Poultry Breeding Industry, College of Animal Science and Technology, Sichuan Agricultural University, Chengdu 611130, Sichuan, P. R. China.

² Farm Animal Germplasm Resources and Biotech Breeding Key Laboratory of Sichuan Province, College of Animal Science and Technology, Sichuan Agricultural University, Chengdu 611130, Sichuan, P. R. China.

³ Key Laboratory of Livestock and Poultry Multi-omics, Ministry of Agriculture and Rural Affairs, College of Animal Science and Technology, Sichuan Agricultural University, Chengdu 611130, Sichuan, P. R. China.

⁴ Sichuan HengWang Animal Husbandry Co., Ltd, Guangyuan 628400, Sichuan, P. R. China.

[#] These authors have contributed equally to this study.

^{*} Corresponding author: shenqiang.hu@sicau.edu.cn, +86-18782985268

Abstract

Background Semen collection is a crucial step in artificial insemination of geese, directly affecting semen quality and fertilization success. To investigate the effects and regulatory mechanisms of sustained semen collection on semen quality in ganders, 36-week-old healthy ganders that pre-trained for semen collection were randomly allocated to either a sustained semen collection (SC) group (semen collection every three days) or a non-semen collection (NSC) group. At 66 weeks, we compared semen quality, semen microbiota, and metabolites between the SC and NSC groups.

Results Compared with the NSC group, the SC group exhibited significantly higher ejaculate volume, sperm density, viability, motility, and semen quality factor (SQF) ($P<0.01$), along with higher sperm acrosomal integrity ($P<0.05$), and a lower sperm deformity rate ($P<0.05$). 16S rRNA sequencing showed that Proteobacteria was the most abundant phylum in gander semen, with *Acinetobacter* and *Oceanivirga* being the predominant genera in the SC and NSC groups, respectively. The differential microbial taxa in the SC group were Bacillales, Corynebacteriaceae, *Corynebacterium1*, *Megamonas*, *Pasteurellaceae bacterium20215_4*. Whereas those in NSC group were Burkholderiaceae, *Ralstonia*, *Hydrogenophaga*, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*, *Arthrobacter*, and *Escherichia_Shigella*. Metabolome analysis revealed that the metabolites in gander semen were mainly lipids and lipid-like molecules. Furthermore, 38 differential expressed metabolites (DEM) were identified between the SC and NSC groups, especially the five metabolites, including PG (16:0/20:4 (5Z,8Z,11Z,14Z)), PI 40:6, 3-hydroxyoctanoyl carnitine,

crotonic acid, and isobutyrylglycine, being the most significant. The online Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that the DEM were significantly enriched in 20 metabolic pathways. Among these, lipoarabinomannan (LAM) biosynthesis and glycerophospholipid metabolism were associated with immunity and sperm motility, respectively. Integrated analysis further showed that the differential microbial taxa in the SC group (*Bacillales* and *Megamonas*) were significantly positively correlated with several lipids and amino acid derivatives. In the NSC group, the differential microbial taxa (*Escherichia_Shigella*, *Burkholderiaceae*, and *Hydrogenophaga*) were significantly negatively correlated with CAR8:0

Conclusions In conclusion, sustained semen collection contributes to a stable seminal milieu, alleviates oxidative stress, facilitates sperm metabolism, and consequently improves semen quality. In contrast, extended periods without semen collection may compromise spermatogenesis and sperm maturation.

Keywords: gander; sustained semen collection; semen quality; bacterial taxa; metabolites

Background

Driven by the rapid development of the goose industry, the transformation of rearing systems, and the widespread adoption of artificial insemination, goose farming has undergone substantial scaling up. However, despite many conveniences brought about by technological advances, the reproductive efficiency of breeder geese still faces

certain bottlenecks, with poor semen quality of ganders being a key factor restricting fertility [1]. Compared with other poultry, ganders have relatively lower semen volume, semen viability, which is one of the reasons for the low fertilization rate in geese. In roosters, the ejaculate volume typically ranges from 0.3-0.8 mL, with sperm viability and motility reaching up to 96.8% and 86%, respectively [2]. However, the ejaculate volume of ganders is only 0.14-0.5 mL, with sperm viability ranging from 30% to 80% and sperm motility ranging from 20% to 60% [3, 4]. Therefore, in-depth studies and further improvement in gander semen quality are of great significance for improving reproductive efficiency and economic benefits in goose production.

Gander semen quality is influenced by multiple factors such as breed, age, reproductive season, feeding practices, nutrition, libido, semen collection frequency, as well as semen microbiota and metabolites [5-9]. Of them, the method and frequency of semen collection, as an important part of artificial insemination, are particularly critical in influencing semen quality. In several studies on camels and chickens, it has been shown that higher frequency of semen collection has a significant effect on semen quality, with a higher number of viable spermatozoa, higher sperm concentration, higher sperm viability, but a lower semen volume [10-12]. However, studies on the effects of semen collection frequency on semen quality in ganders are still lacking.

Semen is not a sterile environment but rather a complex microbial ecosystem containing diverse microbiota that have complex biological functions, with the presence of both harmful microbiota that may contribute to inflammation in the reproductive tract and beneficial microbiota that maintain environmental homeostasis

66 and defend against viruses and bacteria [13, 14]. Many studies have shown that male
67 infertility, hypospermia, and oligospermia are often closely associated with specific
68 potentially harmful microbial taxa in the reproductive tract. For example, some bacteria
69 such as *Escherichia coli*, *Chlamydia trachomatis*, *Mycoplasma hominis*, and
70 *Staphylococcus aureus* have been reported to decrease semen quality and male fertility
71 in humans [15]. In contrast, a study in humans suggests that the relative abundance of
72 *Prevotella* was significantly higher in the abnormal semen group, whereas both the
73 relative abundance of *Staphylococcus* and *Lactobacillus* were significantly higher in
74 the normal semen group, and these three genera were associated with sperm viability
75 and morphological defects [16]. A previous study in rats showed a significant negative
76 correlation between the abundance of *Prevotella* and sperm motility [17]. In addition,
77 the *Firmicutes* and *Actinobacteria* are directly associated with asthenozoospermia and
78 infertility in humans, and isoprene produced by *Actinobacteria* causes sperm DNA
79 fragmentation, which is a straightforward factor of the decline in semen quality [18,
80 19]. In male poultry, semen quality is a crucial factor affecting the efficiency of artificial
81 insemination. However, bacteria naturally interact with semen in the male reproductive
82 tract, and the process of semen collection and storage may alter this interaction, leading
83 to changes in sperm structure and motility [20]. Also, not all bacteria in semen are
84 harmful, but there are also some beneficial bacteria that inhibit harmful bacteria and
85 increase sperm viability and motility through the metabolites they produce. For
86 example, *Lactobacillus*, commonly present in semen, produces lactic acid which may
87 help maintain an acidic environment that inhibits pathogenic growth [21, 22]. Therefore,

the semen microbiota is complex and its impact on sperm quality depends on microbial species, diversity and quantity [23, 24].

Semen metabolites are considered relevant biomarkers of semen quality, playing important roles in sperm motility and sperm capacitation, helping maintain the structural integrity and normal function of sperm, and reflecting the composition and function of seminal microbiota [23, 25]. To date, few studies have investigated the effects of semen collection, especially prolonged abstinence from collection, on semen quality, seminal microbiota, and seminal metabolites in poultry. Therefore, this study took ganders as the experimental subjects and integrated 16S rRNA sequencing and metabolomic analysis to reveal the differences in semen between sustained collection and non-collection regimes. The aim was to identify key microbiota and metabolites, thereby providing a scientific basis and technical support for optimizing semen collection methods and improving reproductive efficiency in geese.

Materials and Methods

Experimental Animals and Sample Collection

A total of 100 healthy newly hatched ganders from Tianfu Meat geese II, which were hatched at the same batch and provided by the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University (Ya'an, Sichuan, China), were used as our experimental subjects. They were reared under similar environmental and feeding conditions. In detail, the ganders were reared in indoor net brooders from 0~3 weeks of age and subsequently shifted to the net-floor mixed rearing system, where they were

reared in indoor floor pens with a size of 13 m length $\times$ 6 m width, consisting of a 60 m² plastic net at a height of 1 m above the ground level and an 18 m² deep litter floor. Thereafter, the ganders at 10 weeks of age were kept in single cages, with a size of 350 mm width $\times$ 550 mm length $\times$ 700 mm height. After reaching sexual maturation (around 32 weeks of age), the ganders showing normal body condition and external genital development were artificially trained by an experienced technician for semen collection according to the methods introduced in our previously published studies [26, 27]. At 36 weeks of age, the ganders that had been artificially massaged to be able to ejaculate were randomly divided into two experimental groups, including the sustained semen collection group (SC) and the non-semen collection group (NSC). During the period from 37 to 66 weeks of age (corresponding to the annual laying period of the same batched female geese), ganders in the SC group underwent semen collection every three days for artificial insemination, whereas those in the NSC group received no semen collection at all. Starting from the end of 66 weeks of age, semen samples were collected from the SC (n=16) and NSC (n=26) groups of ganders for 5 times at an interval of three days. At each sampling time point, part of the freshly collected semen from each gander was used for semen quality analysis, and the remaining was stored at -80°C after rapidly frozen in liquid nitrogen until 16S rRNA sequencing and metabolome analysis.

Semen Quality Analysis

After collecting fresh semen from the ganders, several indices of semen quality,

namely ejaculate volume, sperm density, sperm viability, sperm motility, sperm deformity rate, and acrosomal integrity were assessed according to the previously introduced methods [26]. In detail, the volume of ejaculate was measured using a syringe with a range of 0.1~1 ml, while the sperm density, sperm viability, sperm motility, and sperm deformity rate were measured using a computer-assisted sperm analysis system (Mailang, Guangxi, China). Then, sperm acrosomal integrity (%) was defined as the ratio of sperms with intact acrosomes to the total sperm counts, where the Aniline blue staining solution (Sangon Biotech, Shanghai, China) allowed discrimination between sperm with preserved versus disrupted acrosomes, visualized under a phase contrast microscope (Olympus, Tokyo, Japan). Lastly, the semen quality factor (SQF) value was computed as follows: $SQF = \text{ejaculate volume (mL)} \times \text{sperm density } (\times 10^6/\text{mL}) \times \text{sperm viability } (\%) \times (1 - \text{sperm deformity rate } (\%))$.

DNA Extraction and 16S rRNA Sequencing

For 16S rRNA sequencing, four ganders were randomly selected from each of the SC and NSC groups, and semen sample for each gander were mixed by pooling equal volumes of semen collected from 5 times. The semen microbial DNA was obtained using the Bacterial Genome DNA Extraction Kit (DP302-02, Tiangen, China) according to the manufacturer's instructions. The total DNA was quantified using a Qubit Fluorometer (Invitrogen, USA) and was amplified by PCR using the following universal primer 341F/805R (341F: 5'-CCTACGGGGNGGCWGCAG-3'; 805R: 5'-GACTACHVGGGTATCTAATCC-3'). The PCR amplification conditions were: pre-denaturation at 98 °C for 30 s, followed by 32 cycles of denaturation at 98 °C for 10 s,

annealing at 54 °C for 30 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR product was purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and accurately quantified for concentration using a Qubit Fluorometer (Invitrogen, USA). The fragment size distribution of the purified products was assessed on an Agilent 2100 Bioanalyzer (Agilent, USA), and the concentration of the effective library fragments containing intact adapters was determined using Illumina library quantitative kits (Kapa Biosciences, Woburn, MA, USA), which were further pooled and sequenced on an Illumina NovaSeq 6000 (PE250) (LC-Bio Technology Co., Ltd., Hangzhou, China).

Quality Control and Analysis of Microbial Sequencing Data

After removing adapter and barcode sequence from the paired-end sequencing data, DADA2 was called for length filtering and denoising via qiime2 dada2 denoise-paired to obtain the amplicon sequence variants (ASV) features sequences and ASV abundance tables [18, 28]. The filtering criteria were as follows: 1) sequences with feature less than 30 were removed; 2) low abundance sequences were removed at 0.005% of total frequency; 3) singletons ASV were removed [29]. The ASV sequence files were annotated with species according to the Silva database (<https://www.arb-silva.de>, v138) using the classify-sklearn algorithm of the feature classifier plugin, and the abundance of each stratum of species in each sample was counted according to the ASV abundance table [30]. Based on the obtained ASV feature sequences and ASV abundance tables, alpha and beta diversity analyses were conducted. Alpha diversity was evaluated by four indices: shannon, simpson, chao1, and Pielou [13]. Principal coordinate analysis

(PCoA) based on the Bray-Curtis distance matrix was performed using the cmdscale function and ggplot (version 3.5.1) in R (v4.2.2) [31]. Wilcoxon rank-sum test was performed to compare the differences to identify the differentially abundant bacteria, and statistical differences were calculated using linear discriminant analysis effect size (LEfSe) analysis, with a linear discriminant analysis (LDA) score of > 3 and a filtering threshold of $P < 0.05$ [32].

Metabolite Extraction and LC-MS/MS Analysis

Semen samples of 5 ganders were randomly selected for metabolomic sequencing from each of the SC and NSC groups, and semen sample for each gander consisted of equal volumes of semen collected for 5 times. The collected samples were thawed on ice, and metabolites were extracted with 80% methanol buffer. Briefly, 100 μ L of sample was extracted with 400 μ L of precooled methanol. The extraction mixture was then stored in 30 min at -20°C . After centrifugation at 20,000 g for 15 min, the supernatants were transferred into new tubes and dried under vacuum. The samples were redissolved with 100 μ L 80% methanol and stored at -80°C prior to the liquid chromatography-tandem mass spectrometry/mass spectrometry (LC-MS/MS) analysis. All chromatographic separations were performed using an UltiMate 3000 UPLC System (Thermo Fisher Scientific, Bremen, Germany). An ACQUITY UPLC T3 column (Waters, Milford, MA, USA) was used for the reversed phase separation. Separation was performed with the column oven at 45°C and a flow rate of 0.3 mL/min. The mobile phase consisted of solvent A (5 mM ammonium acetate and 5mM acetic acid) and solvent B (acetonitrile). A high-resolution tandem mass

spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) was used to detect metabolites eluted from the column. The Q Exactive was operated in both positive and negative ion modes. Precursor spectra (70–1050 m/z) were collected at 70,000 resolution with an automatic gain control (AGC) target of 3e6 and a maximum injection time of 100 ms. A top 3 configuration to acquire data was set in data-dependent acquisition mode. Fragment spectra were collected at 17,500 resolution to reach an AGC target of 1e5 with a maximum injection time of 80 ms.

Analysis of Metabolomic Data

The acquired mass spectrometry data were preprocessed using XCMS software for peak picking, peak grouping, retention time (RT) correction, secondary peak grouping, and annotation of isotopes and adducts [33]. LC-MS/MS raw data files were converted to mzXML format and then processed by the XCMS, CAMERA, and metaX toolboxes implemented with the R software [34, 35]. Each ion was identified by combining RT and m/z data. The intensity of each peak was recorded and a three-dimensional matrix containing arbitrarily assigned peak indices (retention time-m/z pairs), sample names (observations) and ion intensity information (variables) was generated [36]. The online Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Metabolome Database (HMDB) were used to annotate the metabolites by matching the exact molecular mass data (m/z) of samples with those from database [37]. If a mass difference between the observed and database value was less than 10 ppm, the metabolite would be annotated and the molecular formula of the metabolite would further be identified and validated by isotopic distribution measurements [38, 39]. The

three conditions of $P < 0.05$, fold change (FC) > 1.2 obtained by t test and variable important for the projection (VIP) calculated by partial least squares discriminant analysis (PLS-DA) simultaneously met the screening of the differential expressed metabolites (DEM). Hypergeometric-based enrichment analysis with KEGG Pathway was performed to identify significantly enriched metabolic pathways.

Statistical analysis

Semen quality parameters were compared between the two groups by independent samples t -test (SAS 9.4, SAS Institute Inc., Cary, NC). The P value < 0.05 was considered statistically significant, and the results were visualized using the GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA). Visualization of microbial taxonomic profiles was performed using the R package ggplot2 (version 3.5.1), vegan (version 2.6.10), ggforce (version 0.4.2), readxl (version 1.4.3), reshape2 (version 1.4.4), agricolae (version 1.3.7), ggsci (version 3.2.0), ggprism (version 1.0.5), scales (version 1.3.0). Differential abundance analysis of metabolites was performed using the DESeq2 Bioconductor package (version 1.44.0) [40], and the differential metabolite results were visualized using the ggplot2, ggrepel (version 0.9.5), RColorBrewer (version 1.1.3), and tidyverse (version 2.0.0) packages in R. Heatmap analysis was conducted using Spearman's correlation coefficient, and the heatmap was generated using the ComplexHeatmap (version 2.20.0), circlize (0.4.16), and RColorBrewer R package.

Results

Differences in the Gander Semen Quality between SC and NSC

As shown in Fig 1 and Supplementary Table S1, compared with the NSC, ejaculate volume (A), sperm density (B), sperm viability, sperm motility (C), and SQF (D) was significantly higher in the SC ($P<0.01$), acrosomal integrity (E) was also higher in the SC ($P<0.05$). While, sperm deformity rate (F) was significantly lower in the SC ($P<0.05$).

Differences in the Gander Semen Microbial Compositions between SC and NSC

A total of 496 ASV were identified from the eight samples, which were categorized into 12 phyla, 19 classes, 35 orders, 65 families, and 109 genera. The 12 phyla and the top 20 genera with the highest relative abundance were selected to plot the species composition stacked map. As shown in Fig 2A and Table 1, these 12 phyla included Actinobacteria, Bacteroidetes, Epsilonbacteraeota, Firmicutes, Fusobacteria, Kiritimatiellaeota, Patescibacteria, Planctomycetes, Proteobacteria, Synergistetes, Tenericutes, and Verrucomicrobia. In the SC, the most abundant phylum was Proteobacteria (39.68%), similarly to the NSC where Proteobacteria accounted for 35.69%. As shown in Fig 2B, these 20 genera included *Acinetobacter*, *Bacteroides*, *Campylobacter*, *Erysipelothrix*, *Fastidiosipila*, *Fusobacterium*, *Gallibacterium*, *Hydrogenophaga*, *Jeotgalibaca*, *Lachnoclostridium* 12, *Neisseria*, *Oceanivirga*, *Pasteurellaceae bacterium 19987/1*, *Pasteurellaceae bacterium 20215/4*, *Phreatobacter*, *Porphyromonas*, *Psychrobacter*, *Sphingomonas*, *Streptobacillus*, and *Varibaculum*. In the SC, the most abundant genus was *Acinetobacter* (14.93%), whereas in the NSC, the most abundant genus was *Oceanivirga* (10.61%) (Supplementary Table S2). The results of alpha diversity showed that there were no significant difference

between the SC and the NSC in terms of Chao1, Shannon, Simpson, and Pielou indices (Fig 2C and Supplementary Table S3). The result of PCoA indicated that there were differences in the semen microbial communities between NSC and SC, which distinguished the microbial community of the two groups. However, the samples were more dispersed in both groups, and the intra-group differences were greater in the SC than in the NSC (Fig 2D).

Differences in the Gander Semen Microbial Abundance between SC and NSC

LEFSe analyses were performed to confirm unique taxonomic biomarkers on semen of ganders in SC and NSC groups. As shown in Fig 3A, there were differences in evolutionary branching of microbial taxa in SC and NSC groups, with a relative concentration in the NSC and a relative dispersion in the SC. As shown in Fig 3B and Table 2, compared with the NSC, *Pasteurellaceabacterium20215_4*, *Megamonas*, *Corynebacteriaceae*, *Corynebacterium1*, and Bacillales were significantly higher in the SC; *Escherichia_Shigella*, *Ralstonia*, *Arthrobacter*, *Hydrogenophaga*, *Sphingomonas*, *Sphingomonadales*, *Sphingomonadaceae*, and *Burkholderiaceae* were significantly lower in the SC (LDA > 3, $P < 0.05$).

Differences in the Gander Semen Metabolic Compositions between the SC and

NSC

The metabolomic analysis showed that a total of 14447 features were generated in positive (7449) and negative (6998) ion modes. Secondary mass spectrometry (MS2) analysis showed that a total of 1088 metabolites were matched against the HMDB, of which 635 metabolites were confirmed in positive ion mode and 454 metabolites in

negative ion mode. Of all annotated metabolites, 1086 metabolites were shared between the SC and NSC groups, while only two metabolites (2,6-Dimethylpyridine and 2,4-Dimethylpyridine) were exclusively detected in the SC group. Among the annotated metabolites, lipids and lipid-like molecules, organic acids and derivatives, and organoheterocyclic compounds were the top three superclass in the HMDB-based metabolite classification (Fig 4A). The identified metabolites were performed KEGG pathway annotation, a total of 241 pathways were annotated in both the SC and NSC groups, and the top 20 pathways with the highest number of metabolites were selected for display, and the results were shown in Fig 4B. The metabolism pathway (KEGG level 1) and the metabolic pathways (KEGG level 3) both exhibited the highest numbers of enriched metabolites.

Differences in the Gander Semen Metabolic Expression between the SC and NSC

DEM were screened by using FC values of metabolites between groups, *p*-values obtained by log₂transformation of metabolite abundance followed by t tests, and the VIP values of each metabolite obtained by multivariate statistical PLS-DA analysis, with the DEM satisfying the following criteria: $FC \geq 1.2$ or $FC \leq 0.83$, *P* value < 0.05, and $VIP \geq 1$. The results were shown in Fig 5A, 30 metabolites were significantly up-regulated and 8 metabolites were significantly down-regulated in the SC group compared with the NSC. The PLS-DA plot was shown in Fig 5B, which indicated differences in the semen metabolomes between the SC and NSC groups. Samples 1 and 5 in the SC group were relatively dispersed, while samples 1 and 3 in the NSC group showed greater dispersion. Heat maps of the DEM were presented (Fig 5C), 30

306 metabolites highly expressed in the SC group: PC(18:1(9Z)/18:1(9Z)), crotonic acid, 3-
 307 Methyl-L-Histidine, Isobutyrylglycine, N-(2-Hydroxyethyl)acrylamide, Proline, D-
 308 chiro-Inositol, CAR 8:0, 3-hydroxyoctanoyl carnitine, SM 31:1;2O/5:0,
 309 hydroxyphenyllactate, LPS 18:1, LysoPC(22:5(7Z,10Z,13Z,16Z,19Z)/0:0),
 310 LysoPC(22:4(7Z,10Z,13Z,16Z)/0:0), PG(16:0/20:4(5Z,8Z,11Z,14Z)), Niranthin, 5-O-
 311 Methylvisammioside, sphingosin C16 (Artifact)-emf, b-Guanidinopropionate,
 312 Carbamoyl-DL-Aspartic acid, Propionylcarnitine, 3-Hydroxyisovaleryl carnitine,
 313 Methyl nicotinate, 2,3,4,5-Tetrahydro-2-pyridinecarboxylic acid,
 314 Methylenecyclopropylglycine, 2-(4-Methyl-5-thiazolyl)ethyl formate, 4-
 315 Aminosalicylic Acid, PI 38:5, PI 38:4, and PI 40:6 were highly expressed in the SC
 316 group. Eight metabolites highly expressed in the SC group: taurohyodeoxycholic acid,
 317 Trideca-7,9,11-trienoylcarnitine, 2-Acetyl-3-ethylpyrazine, Methyl jasmonate, N-
 318 Dodecanoyl-N-methylglycine, 8-methyl-5-propyl-1,2,3,5,8,8a-hexahydroindolizine,
 319 Molybdenum, and PC 16:0_18:1.

320 **Functional Enrichment Analysis of the Identified DEM in Gander Semen**

321 DEM were used for differential enrichment analysis, and the results of KEGG
 322 enrichment analysis showed that a total of 31 metabolic pathways were enriched
 323 (Supplementary Table S4), of which 5 belonged to cellular processes, 2 belonged to
 324 environmental information processing, 3 belonged to human diseases, 20 belonged to
 325 metabolism, and 1 belonged to organismal systems. Among these, 20 pathways showed
 326 significant enrichment ($P < 0.05$) and were drawn as the KEGG bar graph (Fig 6A). 31
 327 pathways were taken for the bubble graphs, and the results are shown in Fig 6B. DEM

were highly significantly enriched into the pathways lipoarabinomannan (LAM) biosynthesis (rich factor=1.5, $P < 0.001$), glycerophospholipid metabolism (rich factor=0.16, $P < 0.001$), choline metabolism in cancer (rich factor=0.36, $P < 0.001$).

Correlation Analysis between the DEM and Differentially Abundant Microbiota in Gander Semen

The results of correlation analysis between the 38 DEM and differential microbiota are presented in Supplementary Table S5, and the top 15 DEM with the lowest P values were selected for heatmap presentation. As shown in Fig 7, Bacillales was significantly and strongly positively correlated with PG (16:0/20:4(5Z,8Z,11Z,14Z)) ($r = 0.74$, $P = 0.0364$), PI 40:6 ($r = 0.75$, $P = 0.032$), Methylenecyclopropylglycine($r = 0.77$, $P = 0.027$), Isobutyrylglycine($r = 0.81$, $P = 0.0144$), Proline ($r = 0.72$, $P = 0.0425$), SM 31:1;2O/5:0 ($r = 0.99$, $P < 0.001$) and LPS 18:1($r = 0.88$, $P = 0.004$). *Hydrogenophaga* was significantly and strongly negatively correlated with CAR 8:0($r = -0.72$, $P = 0.0445$). *Megamonas* was significantly and strongly positively correlated with PI 40:6($r = 0.83$, $P = 0.0108$), Methylenecyclopropylglycine($r = 0.85$, $P = 0.0069$), Isobutyrylglycine($r = 0.91$, $P = 0.0015$), PC(18:1(9Z)/18:1(9Z)) ($r = 0.75$, $P = 0.0331$), Proline($r = 0.78$, $P = 0.0232$), SM 31:1;2O/5:0($r = 0.92$, $P = 0.0011$), and LPS 18:1($r = 0.95$, $P = 0.0003$). *Pasteurellaceabacterium20215_4* was significantly and strongly positively correlated with PI 40:6 ($r = 0.79$, $P = 0.0193$), crotonic acid ($r = 0.91$, $P = 0.0018$), and 3-Hydroxyisovalerylcarnitine ($r = 0.89$, $P = 0.0029$). Burkholderiaceae was significantly and strongly negatively correlated with CAR 8:0 ($r = -0.72$, $P = 0.0456$). *Corynebacterium1* was significantly and strongly positively correlated with 3-

hydroxyoctanoyl carnitine ($r = 0.73$, $P = 0.0388$). *Escherichia Shigella* was significantly and strongly negatively correlated with 3-hydroxyoctanoyl carnitine ($r = -0.75$, $P = 0.031$) and CAR 8:0 ($r = -0.81$, $P = 0.0148$). *Arthrobacter* was significantly and strongly positively correlated with 2-Acetyl-3-ethylpyrazine ($r = 0.8$, $P = 0.0184$).

Discussion

Semen collection, as a key part of artificial insemination, has a significant impact on reproductive efficiency of ganders. In this present study, sperm viability, sperm motility, sperm density, SQF, acrosomal integrity and ejaculate volume were higher in the SC compared with the NSC. Previous studies have shown that a reduction in the frequency of semen collection is associated with a progressive increase in the single ejaculation volume and sperm density, yet a decline in sperm motility and viability. When the frequency of semen collection is once every 3 days, it has the best semen quality in poultry [41, 42]. A study on turkeys found that semen collected once or twice a week had higher semen volume, sperm progressive motility, sperm density, total sperm per ejaculate and percentage of normal sperm compared with semen collected three or four times a week [43]. Another study on broiler breeders demonstrated that a prolonged interval between semen collection increases semen volume, but the indicators of sperm viability and motility decreased [44]. This is somewhat similar to the present study, in which the semen quality of ganders in the NSC was significantly reduced after prolonged periods of no semen collection. The reason for this may lie in the changes in the environment of the reproductive tract of ganders in terms of microbial communities, metabolites, as well as the structure and function of testes and

epididymides. It has been shown that semen collection leads to changes in protein and ionic balance in epididymal fluid, which interferes with sperm maturation process and may contribute to an increased rate of sperm malformations [45]. The above studies suggest that prolonged absence of sperm collection in ganders can lead to a decrease in semen quality.

Regarding microbial taxa, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria are the four common phyla identified in avian semen [13, 46], with Proteobacteria being the most abundant phylum, which is similar to the finding on drakes [47]. And at the genus level, the common bacterial genera identified in semen include *Bacteroides*, *Fastidiosipila*, *Fusobacterium*, *Oceanivirga*, and *Porphyromonas*, which are also commonly found in bull semen [48]. *Acinetobacter*, which has a highest abundance in the SC, it is associated with sperm DNA damage and is significantly more abundant in human semen with high DNA fragmentation index [49]. Nevertheless, a study has demonstrated that semen microbial communities harboring *Acinetobacter* in humans achieve the highest success rates in vitro fertilization and the intracytoplasmic sperm injection, relative to those without this bacterium [50]. A study of boars found that higher relative abundance of *Acinetobacter* was associated with low sperm quality and could be used as a biomarker for differences in semen quality [51]. Collectively, these findings indicate that *Acinetobacter* exerts species-dependent effects on sperm morphology, thereby potentially compromising reproductive fitness. While the highest abundance of *Oceanivirga* in the NSC group, presented in the semen of goats during the breeding season, as well as in bovine semen, whereas its specific function has not

been previously reported and remains unclear [48, 52]. Surprisingly, *Lactobacillus* which is more common in the semen of other animals such as boars, cows and chickens, was only present in the NSC with low abundance, and it has not been identified in semen of drakes [53]. *Lactobacillus* in the human reproductive tract was positively correlated with bacterial vaginosis-associated bacteria [50]. These studies suggest that prolonged periods without semen collection may increase the risk of reproductive tract diseases in waterfowl.

LEFSe analysis showed that Bacillales, Corynebacteriaceae, *Corynebacterium*, *Megamonas*, and *Pasteurellaceae bacterium20215_4* exhibited significantly higher relative abundances in the SC compared with the NSC. Interestingly, Bacillales is an estrus-related microorganism in bulls, with its abundance increasing significantly during estrus and being notably higher in the testis than in semen [54, 55]. *Megamonas* is highly abundant in the human prostate, and its abundance is positively correlated with serum testosterone levels, suggesting that it may be involved in androgen metabolism [56]. While in NSC group, Burkholderiaceae, Sphingomonadales, Sphingomonadaceae, *Ralstonia*, *Hydrogenophaga*, *Sphingomonas*, *Arthrobacter*, and *Escherichia-Shigella* exhibited significantly higher relative abundance compared with the SC. Burkholderiaceae, which is considered a beneficial bacterial family, is associated with high sperm density, sperm morphology and DNA adsorption; however, *Ralstonia*, a genus under this classification, although positively correlated with semen volume, is negatively correlated with sperm concentration and sperm motility, and its presence has been detected in the rat testis, epididymis, vas deferens, seminal vesicles,

and prostate, suggesting that it may be associated with the development of asthenozoospermia [17, 57]. A higher abundance of Sphingomonadaceae, particularly of its genus *Sphingomonas*, was significantly positively correlated with the extent of sperm DNA damage in humans [57]. *Escherichia_Shigella* is considered to be a deleterious bacterium causing DNA replication defects, which are negatively correlated with sperm motility in humans [58]. From the above results, it can be seen that the abundance of harmful bacteria was higher in the NSC ganders, which may be a direct cause of their poor semen quality.

The metabolites identified in gander semen were mainly lipids and lipid-like molecules, which play an important role in protecting sperm plasma membrane integrity, energy metabolism, spermatogenesis, and fertilization [59-61]. Studies have shown that phosphatidylcholine (PC) and sphingomyelin (SM) are the most important components of the sperm plasma membrane, serving as key indicators for assessing sperm fertilization potential, and their abundance are associated with sperm motility and viability; however, in chicken semen, high levels of PC lead to the decrease of chicken fertility [62, 63]. Thirty-eight DEM were identified between the SC and NSC groups, of which five metabolites, PG (16:0/20:4 (5Z,8Z,11Z,14Z)), PI 40:6, 3-hydroxyoctanoyl carnitine, crotonic acid and Isobutyrylglycine showed the most significant differences, all with higher abundance in the SC. PG(16:0/20:4(5Z,8Z,11Z,14Z)) and PI 40:6 are glycerophospholipids, which play important roles in regulating sperm motility and semen preservation. Specifically, phosphatidylinositol phosphate produced by phosphatidylinositol (PI) modification

plays a key role in spermatogenesis, capacitation and fertilization, where spermatogenesis has a clear role as a central regulator [64]. Studies in chickens have shown that PI abundance is significantly increased in the high sperm viability group, and its involvement in phosphatidylinositol signaling has been identified as essential for sperm viability and sperm storage tubule metabolic processes in poultry [65, 66]. As a key carrier in mitochondrial fatty acid β -oxidation, 3-hydroxyoctanoyl carnitine has typically been detected at higher levels in groups of abnormal sperm motility and poor semen quality in bulls [67, 68]. This finding is somewhat inconsistent with the results of the present study, which showed that 3-hydroxyoctanoyl carnitine was higher in semen of ganders in the SC group, where semen quality was superior, a discrepancy that may be attributable to species differences. DEM were enriched for a total of 31 metabolic pathways, with highly significant enrichment for LAM biosynthesis and glycerophospholipid metabolism. LAM is an important non-peptide factor regulating host immune response, which is mainly produced by microbiota such as *Corynebacteriaceae* and *Corynebacterium*1, which can bind to macrophages and inhibit their activation, thereby reducing local immune activity [69]. It is speculated that LAM may regulate immune microenvironment homeostasis and immune privilege (a local active tolerance mechanism that protects spermatozoa from inflammatory damage) in the gander reproductive tract, alleviate local inflammatory response, reduce immune clearance and oxidative damage of sperm, so as to maintain sperm motility. Meanwhile, it helps to protect the structural integrity of seminiferous tubules, sustain the physiological function of Sertoli cells and the normal secretion of testosterone [70]. On

the other hand, sustained semen collection may change the reproductive tract microecology of ganders, increase the abundance of LAM-producing microbiota in semen, and further activate the LAM biosynthesis pathway.

Combined analysis of differential microbial taxa with the DEM, the differential microbial taxa in the SC, Bacillales and *Megamonas*, both showed significant positive correlations with the DEM, including lipids, amino acids, and their derivatives, such as PG(16:0/20:4(5Z,8Z,11Z,14Z)), PC(18:1(9Z)/18:1(9Z)), PI 40:6, SM 31:1;2O/5:0, Isobutyrylglycine, LPS 18:1, and Proline. phosphatidylglycerol (PG), PC, and PI play important roles in spermatogenesis and sperm energy metabolism. Proline, as an antioxidant in semen cryopreservation, helps to improve sperm membrane integrity, sperm motility, and ATP levels, effectively protecting sperm from oxidative stress. Moreover, proline metabolism plays a key role in regulating sperm motility [71-73]. This suggests that the two microbial taxa, Bacillales and *Megamonas*, may indirectly influence spermatogenesis, potentially through their associations with the levels of metabolites such as PG, PI, PC, and proline, which are critical for maintaining sperm plasma membrane integrity and providing energy for sperm. Isobutyrylglycine is an acylglycine, a secondary metabolite of fatty acids, which is commonly used as a biomarker of the immune response, reflecting the degree of tissues exposure to infection [74]. LPS 18:1, a component of the outer membrane of Gram-negative bacteria, can induce testicular inflammation, thereby impairing spermatogenesis and steroidogenesis in rats [75]. *Corynebacterium 1* and *Pasteurellaceae bacterium 20215_4* showed a significant positive correlation with 3-hydroxyoctanoyl carnitine. It is speculated that

these two microbial taxa may be involved in the fatty acid β -oxidation process. On the one hand, they may regulate carnitine content to provide acyl carriers for fatty acid oxidation in organisms; on the other hand, this may also reflect dysregulation of the fatty acid oxidation pathway, thereby leading to metabolic disturbances [76, 77]. This indicates that sustained semen collection, while promoting beneficial microbiota and their metabolites, also increases the risk of testicular or reproductive tract inflammation and metabolic disorders. Interestingly, the differential microbial taxa in the NSC, *Hydrogenophaga*, *Escherichia*, *Shigella*, and Burkholderiaceae were significantly negatively correlated with CAR8:0. As a derivative of carnitine, acylcarnitine (CAR) is a product of cellular energy and metabolism. It plays an important role in facilitating the entry of long-chain fatty acids into mitochondria for β oxidation and in reducing oxidative damage to sperm. On the other hand, carnitine is an essential substance for sperm maturation, and the increased sperm motility in the epididymis is often accompanied by an increase in carnitine levels [78, 79]. It is hypothesized that during long-term abstinence from semen collection, some harmful microbiota suppress carnitine production, which subsequently disrupts fatty acid β -oxidation. Consequently, spermatogenesis is indirectly impaired, leading to decreased sperm motility.

Conclusion

This study elaborates on the differences between the SC and NSC ganders in semen quality, semen microbiota, and metabolites. In terms of semen quality, the SC group exhibited better semen quality compared with the NSC; in terms of semen microbiota, most of the differential bacteria in SC group favored the stability of the

semen environment, whereas some of the differential bacteria in NSC group negatively affected the semen quality; in terms of metabolome, most of the DEM were up-regulated in SC group, and the LAM pathway was an important metabolic pathway. Integrated analysis further showed that the differential microbial taxa in the SC group were significantly positively correlated with several lipids and amino acid derivatives, suggesting that their potential roles in maintaining sperm plasma membrane integrity and providing energy for spermatozoa. Yet in the NSC group, the differential microbial taxa may suppress carnitine synthesis, thus impairing fatty acid β -oxidation and spermatogenesis, and consequently reducing sperm motility and semen volume. In conclusion, sustained semen collection contributes to a stable seminal milieu, alleviates oxidative stress, facilitates sperm metabolism, and consequently improves semen quality. In contrast, extended periods without semen collection may compromise spermatogenesis and sperm maturation.

Ethics Statement

All experimental procedures involving the manipulation of geese in this study were conducted in concordance with the “Guidelines for Experimental Animals” of the Ministry of Science and Technology (Beijing, China). The animal use protocol has been reviewed and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee (Approval No.: 20240411).

Abbreviations

SC sustained semen collection

- 525 NSC non-semen collection group
- 526 KEGG Kyoto Encyclopedia of Genes and Genomes
- 527 LAM lipoarabinomannan
- 528 DEM differential expressed metabolites
- 529 SQF semen quality factor
- 530 ASV amplicon sequence variants
- 531 PCoA Principal coordinate analysis
- 532 LEfSe linear discriminant analysis effect size
- 533 LDA linear discriminant analysis
- 534 LC-MS/MS liquid chromatography-tandem mass spectrometry/mass spectrometry
- 535 AGC automatic gain control
- 536 RT retention time
- 537 HMDB Human Metabolome Database
- 538 FC fold change
- 539 VIP variable important for the projection
- 540 PLS-DA partial least squares discriminant analysis
- 541 MS2 Secondary mass spectrometry

542 PC phosphatidylcholine

543 SM sphingomyelin

544 PI phosphatidylinositol

545 CAR acylcarnitine

546 **Consent for publication**

547 Not applicable.

548 **Data availability**

549 The 16S rRNA sequence data generated by this study was submitted to the Sequence

550 Read Archive (SRA) at the National Center for Biotechnology Information (NCBI)

551 with accession number PRJNA1472836

552 (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1472836?reviewer=3ejfho825tmqva>

553 [rk75a49vdk1k](https://dataview.ncbi.nlm.nih.gov/object/PRJNA1472836?reviewer=3ejfho825tmqva)).

554 **Authors' contributions**

555 Fuli Huang: writing – original draft, investigation, data curation, formal analysis, and

556 visualization. Zhaoyan Chen: writing – original draft, investigation, and data curation.

557 Yang Song: investigation and data curation. Xiaopeng Li: data curation and

558 visualization. Zhiqiang Mo: data curation. Haoran Li: investigation. Jiwei Hu:

559 resources. Xin Yuan: investigation. Xiao Hu: resources. Qiang Cao: resources.

560 Chunchun Han: investigation. Hua He: investigation. Liang Li: investigation. Hehe Liu:

investigation. Jiwen Wang: resources and funding acquisition. Shenqiang Hu: writing
– review & editing, methodology, supervision, and funding acquisition.

Acknowledgements

The authors express their sincere thanks to the researchers and laboratories that
contributed to this study.

Funding

This study was financially supported by the Program for Waterfowl Industry
Technology System Innovation Team of Sichuan Province (SCCXTD-2024-25), the
Key Agricultural Technology Breakthrough Program of Sichuan Provincial Department
of Agriculture and Rural Affairs (2025NYGJ04-02), the Independent Research Fund of
State Key Laboratory of Swine and Poultry Breeding Industry (2025ZQQZ-G55), the
Key Technology Support Program of Sichuan Province (2025YFYZ0006), the China
Agricultural Research System (CARS-42-4), and the Sichuan HengWang Animal
Husbandry Co., Ltd. - Sichuan Agricultural University Cooperative Research Project
(20244447).

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial
or financial relationships that could be construed as a potential conflict of interest.

Reference

1. Akhtar MF, Shafiq M, Ali I, Improving gander reproductive efficacy in the context of
globally sustainable goose production. *Animals*. 2022; 12(1):44.

- 582 2. Ayeneshet B, Taye M, Esatu W, Tsefa A, Comparative analysis of semen quality and
583 fertility in diverse rooster breeds: a systematic review. *Worlds Poult Sci J.* 2024;
584 80(3):947-975.
- 585 3. Łukaszewicz E, Kowalczyk A, Jerysz A, Characteristics of semen collected from
586 gander included in the genetic resources conservation program. *Poult Sci.* 2021;
587 100(9):101314.
- 588 4. Hu S, Song Y, Chen J, He X, Li G, Liu T, He Z, Li X, Wang Z, Hu J, Transcriptome
589 analysis of testis and epididymis identifies key genes and pathways regulating gander
590 sperm motility. *Poult Sci.* 2025; 104(4):105012.
- 591 5. Boz MA, Baş H, Sarica M, Erensoy K, The effects of natural mating and artificial
592 insemination on reproductive traits of 1-and 2-year-old domestic Turkish geese. *Vet*
593 *Res Commun.* 2021; 45(4):211-221.
- 594 6. Ďuračka M, Belić L, Tokárová K, Žiarovská J, Kačániová M, Lukáč N, Tvrdá E,
595 Bacterial communities in bovine ejaculates and their impact on the semen quality. *Syst*
596 *Biol Reprod Med.* 2021; 67(6):438-449.
- 597 7. He Z, Liu T, He X, Song Y, Li G, Li X, Ouyang Q, Hu S, He H, Li L, Molecular
598 mechanisms of libido influencing semen quality in geese through the hypothalamic-
599 pituitary-testicular-external genitalia axis. *Poult Sci.* 2025; 104(2):104756.
- 600 8. Łukaszewicz E, Effects of semen filtration and dilution rate on morphology and fertility
601 of frozen gander spermatozoa. *Theriogenology.* 2001; 55(9):1819-1829.
- 602 9. Łukaszewicz E, Artificial insemination in geese. *Worlds Poult Sci J.* 2010; 66(4):647-
603 658.

- 604 10. Al-Bulushi S, Manjunatha B, Bathgate R, Rickard J, De Graaf S, Effect of semen
605 collection frequency on the semen characteristics of dromedary camels. *Anim. Reprod.*
606 *Sci.* 2018; 197:145-153.
- 607 11. Noirault J, Brillard J, Effects of frequency of semen collection on quantitative and
608 qualitative characteristics of semen in turkey breeder males. *Poult. Sci.* 1999;
609 78(7):1034-1039.
- 610 12. Riaz A, Aleem M, Ijaz A, Saeed M, Latif A, Effect of collection frequency on the semen
611 quality of broiler breeder. *Br. Poult. Sci.* 2004; 45(6):823-827.
- 612 13. Jiang X, Zhang B, Gou Q, Cai R, Sun C, Li J, Yang N, Wen C, Variations in seminal
613 microbiota and their functional implications in chickens adapted to high-altitude
614 environments. *Poult. Sci.* 2024; 103(8):103932.
- 615 14. Tvrdá E, Petrovičová M, Benko F, Ďuračka M, Kováč J, Slanina T, Galovičová L,
616 Žiarovská J, Kačániová M, Seminal bacterioflora of two rooster lines: Characterization,
617 antibiotic resistance patterns and possible impact on semen quality. *Antibiotics.* 2023;
618 12(2):336.
- 619 15. Farsimadan M, Motamedifar M, Bacterial infection of the male reproductive system
620 causing infertility. *J. Reprod. Immunol.* 2020; 142:103183.
- 621 16. Baud D, Pattaroni C, Vulliemoz N, Castella V, Marsland BJ, Stojanov M, Sperm
622 microbiota and its impact on semen parameters. *Front. Microbiol.* 2019; 10:234.
- 623 17. Li G, Shen Q, Gao Y, Ma C, Song B, Wang C, Tang D, He X, Cao Y, The microbiota
624 continuum along the upper reproductive tract of male rat and its relation to semen
625 parameters. *Heliyon.* 2024; 10(12):e32556.

- 626 18. Chen H, Jiang W, Application of high-throughput sequencing in understanding human
627 oral microbiome related with health and disease. *Front. Microbiol.* 2014; 5:508.
- 628 19. Chen H, Luo T, Chen T, Wang G, Seminal bacterial composition in patients with
629 obstructive and non-obstructive azoospermia. *Exp Ther Med.* 2018; 15(3):2884-2890.
- 630 20. Tvrdá E, Petrovičová M, Ďuračka M, Benko F, Slanina T, Galovičová L, Kačániová M,
631 Short-Term Storage of Rooster Ejaculates: Sperm Quality and Bacterial Profile
632 Differences in Selected Commercial Extenders. *Antibiotics.* 2023; 12(8):1284.
- 633 21. Barbonetti A, Cinque B, Vassallo MRC, Mineo S, Francavilla S, Cifone MG,
634 Francavilla F, Effect of vaginal probiotic lactobacilli on in vitro–induced sperm lipid
635 peroxidation and its impact on sperm motility and viability. *Fertil. Steril.* 2011;
636 95(8):2485-2488.
- 637 22. Lenický M, Kačániová M, Ďuračka M, Zajacová Ž, Muráňová K, Tvrdá E, Bovine
638 spermatozoa and Lactobacilli interactions: deterioration or improvement of the sperm
639 vitality? *Anim. Reprod. Sci.* 2022; 247:107131.
- 640 23. Ďuračka M, Petrovičová M, Benko F, Kováčik A, Lukáč N, Kačániová M, Tvrdá E,
641 Lohmann Brown Rooster Semen: Intrinsic Bacteria and Their Impact on Sperm
642 Progressive Motility and Seminal Biochemical Parameters—A Preliminary Study.
643 *Stresses.* 2023; 3(2):424-433.
- 644 24. Lenický M, Slanina T, Kačániová M, Galovičová L, Petrovičová M, Ďuračka M, Benko
645 F, Kováč J, Tvrdá E, Identification of bacterial profiles and their interactions with
646 selected quality, oxidative, and immunological parameters of turkey semen. *Animals.*
647 2021; 11(6):1771.

- 648 25. Diamandis EP, Arnett WP, Foussias G, Pappas H, Ghandi S, Melegos DN, Mullen B,
649 Yu H, Srigley J, Jarvi K, Seminal plasma biochemical markers and their association
650 with semen analysis findings. *Urology*. 1999; 53(3):596-603.
- 651 26. Hu S, Li X, Qing E, Wang J, Chen Q, Song Y, Chen J, Hu J, Li L, Wang J, Molecular
652 mechanisms underlying age-dependent effects of rearing system on the goose testicular
653 development and semen quality. *Poult. Sci*. 2025; 104(1):104589.
- 654 27. Li X, He X, Li G, Wang Z, Huang F, Chen J, Song Y, Liu T, Chen Z, Wang X,
655 Identification of the crucial circ-mi-mRNA interaction networks regulating testicular
656 development and spermatogenesis in ganders. *Poult. Sci*. 2025; 104(3):104863.
- 657 28. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP, DADA2:
658 High-resolution sample inference from Illumina amplicon data. *Nat. Methods*. 2016;
659 13(7):581-583.
- 660 29. Hernandez-Segura A, Rubingh R, Demaria M, Identification of stable senescence-
661 associated reference genes. *Aging Cell*. 2019; 18(2):e12911.
- 662 30. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA,
663 Gregory Caporaso J, Optimizing taxonomic classification of marker-gene amplicon
664 sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*. 2018; 6:1-17.
- 665 31. Wen C, Yan W, Mai C, Duan Z, Zheng J, Sun C, Yang N, Joint contributions of the gut
666 microbiota and host genetics to feed efficiency in chickens. *Microbiome*. 2021; 9:1-23.
- 667 32. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C,
668 Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011; 12:1-18.
- 669 33. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G, XCMS: processing mass

670 spectrometry data for metabolite profiling using nonlinear peak alignment, matching,
671 and identification. *Anal. Chem.* 2006; 78(3):779-787.

672 34. Kuhl C, Tautenhahn R, Bottcher C, Larson TR, Neumann S, CAMERA: an integrated
673 strategy for compound spectra extraction and annotation of liquid
674 chromatography/mass spectrometry data sets. *Anal. Chem.* 2012; 84(1):283-289.

675 35. Wen B, Mei Z, Zeng C, Liu S, metaX: a flexible and comprehensive software for
676 processing metabolomics data. *BMC Bioinform.* 2017; 18:1-14.

677 36. Dieterle F, Ross A, Schlotterbeck G, Senn H, Probabilistic quotient normalization as
678 robust method to account for dilution of complex biological mixtures. Application in
679 ¹H NMR metabonomics. *Anal. Chem.* 2006; 78(13):4281-4290.

680 37. Han Q, Li H, Jia M, Wang L, Zhao Y, Zhang M, Zhang Q, Meng Z, Shao J, Yang Y,
681 Age-related changes in metabolites in young donor livers and old recipient sera after
682 liver transplantation from young to old rats. *Aging Cell.* 2021; 20(7):e13425.

683 38. Chen Y, Yang L, Lu Y, Liu N, Ma W, Fan H, Hu Q, Han X, Gan W, Li D, Up-regulation
684 of NMRK2 mediated by TFE3 fusions is the key for energy metabolism adaption of
685 Xp11. 2 translocation renal cell carcinoma. *Cancer Lett.* 2022; 538:215689.

686 39. Li R, Huang X, Liang X, Su M, Lai KP, Chen J, Integrated omics analysis reveals the
687 alteration of gut microbe–metabolites in obese adults. *Brief. Bioinform.* 2021;
688 22(3):bbaa165.

689 40. Love MI, Huber W, Anders S, Moderated estimation of fold change and dispersion for
690 RNA-seq data with DESeq2. *Genome Biol.* 2014; 15:1-21.

691 41. Bushra, Allah, Rakha, Iftikhar, Hussain, Muhammad, Fahim, Malik, Shamim, Akhter,

692 Impact of ejaculate frequencies on the quality of Red Jungle Fowl (*Gallus gallus*
693 *murghi*) semen. *Avian Biol. Res.* 2015; 8(2):65-78.

694 42. Pimprasert M, Kheawkanha T, Boonkum W, Chankitisakul V, Influence of semen
695 collection frequency and seasonal variations on fresh and frozen semen quality in Thai
696 native roosters. *Animals.* 2023; 13(4):573.

697 43. Ezike J, Ezea J, Machebe S, Onyimonyi A, Impact of different rearing systems and
698 frequency of semen collection on semen characteristics of Turkeys. *Niger. J. Anim. Sci.*
699 2021; 23(3):46-52.

700 44. Shaheen MS, Aslam S, Mehmood S, Tariq M, Abbas Y, Ashfaq H, Ahmad S, Effects of
701 age, body weight, semen collection frequency and holding duration on semen traits of
702 broiler breeder reared under different housing systems. *Trop Anim Health Prod.* 2023;
703 55(1):21.

704 45. Strzeżek J, Torska J, Borkowski K, Glogowski J, Wysocki P, Holody D, The
705 Biochemical Characteristics of Boar Seminal Plasma during High Ejaculation
706 Frequency. *Reprod. Domest. Anim.* 2010; 30(2):77-84.

707 46. Fredriksen S, The ejaculate microbiota in an avian hybrid system across allo-and
708 sympatry. 2017.

709 47. Hu X, Li J, Xin S, Ouyang Q, Li J, Zhu L, Hu J, He H, Liu H, Li L, Genome sequencing
710 of drake semen micobiome with correlation with their compositions, sources and
711 potential mechanisms affecting semen quality. *Poult. Sci.* 2024; 103(4):103533.

712 48. Cojkic A, Niazi A, Guo Y, Hallap T, Padrik P, Morrell JM, Identification of bull semen
713 microbiome by 16S sequencing and possible relationships with fertility.

714 Microorganisms. 2021; 9(12):2431.

715 49. He J, Ma M, Xu Z, Guo J, Chen H, Yang X, Chen P, Liu G, Association between semen
716 microbiome disorder and sperm DNA damage. Microbiol. Spectr. 2024; 12(8):e00759-
717 00724.

718 50. Koort K, Sõsa K, Türk S, Lapp E, Talving E, Karits P, Rosenstein K, Jaagura M,
719 Sekavin A, Sõritsa D, Lactobacillus crispatus-dominated vaginal microbiome and
720 Acinetobacter-dominated seminal microbiome support beneficial ART outcome. Acta
721 Obstet. Gynecol. Scand. 2023; 102(7):921-934.

722 51. Riedel A, Vahjen W, Lautner M, Leiding C, Jung M, Zentek J, Schulze M,
723 Acinetobacter in fresh faecal samples is related to sperm motility: A case report in
724 Piétrain boars housed on different deep-litter bedding materials. Reprod. Domest. Anim.
725 2024; 59(5):e14616.

726 52. Mocé ML, Esteve IC, Pérez-Fuentes S, Gómez EA, Mocé E, Microbiota in goat Buck
727 ejaculates differs between breeding and non-breeding seasons. Front. Vet. Sci. 2022;
728 9:867671.

729 53. Zhang J, Liu H, Yang Q, Li P, Wen Y, Han X, Li B, Jiang H, Li X, Genomic sequencing
730 reveals the diversity of seminal bacteria and relationships to reproductive potential in
731 boar sperm. Front. Microbiol. 2020; 11:1873.

732 54. Retherford SA, Woodruff KL, Harstine BR, Dittoe DK, Jeremy B, The Bull
733 Reproductive Microbiome: A Comparative Analysis of Microbial Communities within
734 Semen and Organs of the Bull Reproductive System. Biol. Reprod. 2026; 114(4):1343-
735 1358.

- 736 55. Sharma R, Singh PK, Onteru SK, Singh D, Faecal microbiome analysis reveals
737 Clostridiales and Bacteroidales as signature gut microbes during estrus of buffalo.
738 Reprod. Biol. 2021; 21(2):100509.
- 739 56. Shin JH, Park YH, Sim M, Kim SA, Joung H, Shin DM, Serum level of sex steroid
740 hormone is associated with diversity and profiles of human gut microbiome. Res.
741 Microbiol. 2019; 170:192-201.
- 742 57. Garcia-Segura S, Del Rey J, Closa L, Garcia-Martínez I, Hobeich C, Castel AB, Vidal
743 F, Benet J, Ribas-Maynou J, Oliver-Bonet M, Seminal microbiota of idiopathic infertile
744 patients and its relationship with sperm DNA integrity. Front. Cell Dev. Biol. 2022;
745 10:937157.
- 746 58. Pan Y, Wang S, Liu L, Liu X, Characteristics of gut microbiota in patients with
747 asthenozoospermia: a Chinese pilot study. BMC Microbiol. 2024; 24(1):22.
- 748 59. An Z, Shi L, Zhou H, Hou G, Xun W, Exploratory Metabolomics and Lipidomics
749 Profiling Contributes to Understanding How Curcumin Improves Quality of Goat
750 Semen Stored at 16° C in Tropical Areas. Int. J. Mol. 2024; 25(18):10200.
- 751 60. Arienti G, Carlini E, Polci A, Cosmi E, Palmerini CA, Fatty acid pattern of human
752 prostatesome lipid. Arch. Biochem. 1998; 358(2):391-395.
- 753 61. Partyka A, Bonarska-Kujawa D, Sporniak M, Strojecki M, Nizański W, Modification
754 of membrane cholesterol and its impact on frozen–thawed chicken sperm
755 characteristics. Zygote. 2016; 24(5):714-723.
- 756 62. Shan S, Xu F, Bleyer M, Becker S, Melbaum T, Wemheuer W, Hirschfeld M, Wacker
757 C, Zhao S, Schütz E, Association of α/β -Hydrolase D16B with bovine conception rate

758 and sperm plasma membrane lipid composition. *Int. J. Mol.* 2020; 21(2):627.

759 63. Shan S, Xu F, Hirschfeld M, Brenig B, Sperm lipid markers of male fertility in
760 mammals. *Int. J. Mol.* 2021; 22(16):8767.

761 64. Brill JA, Yildirim S, Fabian L: Phosphoinositide signaling in sperm development.
762 *Semin Cell Dev Biol.* 2016;59: 2-9.

763 65. Bond A, Mills K, Ferreira CR, Harford I, Flack B, Long JA, Diehl K, Broiler breeder
764 putative lipid biomarkers associated with sperm mobility. *Front. Physiol.* 2024;
765 15:1504557.

766 66. Lemoine M, Dupont J, Guillory V, Tesseraud S, Blesbois E, Potential involvement of
767 several signaling pathways in initiation of the chicken acrosome reaction. *Biol. Reprod.*
768 2009; 81(4):657-665.

769 67. Fang L, Jitian L, Jinzhong T, Relationship between Sperm Motility and Seminal Plasma
770 Carnitine in Simmental Bull Based on Two LC-MS Technology. *Acta Vet. Zootech. Sin.*
771 2019; 50(11):8.

772 68. Houle E, Li YY, Schroder M, Mcritchie SL, Rahil T, Sites CK, Sumner SJ, Pilsner JR,
773 Exploring the internal exposome of seminal plasma with semen quality and live birth:
774 A Pilot Study. *Syst Biol Reprod Med.* 2023; 69(4):296-309.

775 69. Nigou J, Gilleron M, Puzo G, Lipoarabinomannans: from structure to biosynthesis.
776 *Biochimie.* 2003; 85(1-2):153-166.

777 70. Al-Asmakh M, Hedin L, Microbiota and the control of blood-tissue barriers. *Tissue*
778 *barriers.* 2015; 3(3):e1039691.

779 71. Feng C, Zhu Z, Bai W, Li R, Zheng Y, Tian Xe, Wu D, Lu H, Wang Y, Zeng W, Proline

780 Protects Boar Sperm against Oxidative Stress through Proline Dehydrogenase-
781 Mediated Metabolism and the Amine Structure of Pyrrolidine. *Animals*. 2020;
782 10(9):1549.

783 72. Liu C, Feng H, Han J, Zhou H, Yuan L, Pan H, Wang X, Han X, Qiao R, Yang F, Effect
784 of L-proline on sperm quality during cryopreservation of boar semen. *Anim. Reprod.*
785 *Sci.* 2023; 258:107359.

786 73. Ren X, Jiang K, Yin J, Ma Z, Chen Z, Yang K, Liu S, Integrated transcriptomic and
787 metabolomic analysis of goose epididymis reveals molecular markers associated with
788 sperm mobility. *Poult. Sci.* 2025; 104(7):105180.

789 74. Liu K, Jia B, Zhou L, Xing L, Wu L, Li Y, Lu J, Zhang L, Guan S, Ultrapformance
790 liquid chromatography coupled with quadrupole time-of-flight mass spectrometry-
791 based metabolomics and lipidomics identify biomarkers for efficacy evaluation of
792 mesalazine in a dextran sulfate sodium-induced ulcerative colitis mouse model. *J.*
793 *Proteome Res.* 2020; 20(2):1371-1381.

794 75. Zheng X, Li Y, Shang X, Liu R, Is lipopolysaccharide-induced lipid metabolism
795 disorder in testis of rats a consequence of plasma lipid changes? *J Inflamm Res.* 2024;
796 17:765-776.

797 76. Bremer J, Carnitine--metabolism and functions. *Physiol. Rev.* 1983; 63(4):1420-1480.

798 77. Tan G, Wang H, Yuan J, Qin W, Dong X, Wu H, Meng P, Three serum metabolite
799 signatures for diagnosing low-grade and high-grade bladder cancer. *Sci. Rep.* 2017;
800 7(1):46176.

801 78. Enomoto A, Wempe MF, Tsuchida H, Shin HJ, Cha SH, Anzai N, Goto A, Sakamoto

802 A, Niwa T, Kanai Y, Molecular identification of a novel carnitine transporter specific
803 to human testis: insights into the mechanism of carnitine recognition. J. Biol. Chem.
804 2002; 277(39):36262-36271.

805 79. Zhu K, Song Y, He Z, Wang P, Wang X, Liu G, Effect of Seminal Plasma on the
806 Freezability of Boar Sperm. Animals (2076-2615). 2024; 14(24):3656.

807 **Table 1.** Mean relative abundance of bacterial phyla identified in the gander semen
808 between the SC and NSC.

Phylum	SC (%)	NSC (%)
Proteobacteria	39.68±4.22	35.69±7.94
Firmicutes	26.67±2.7	20.6±2.16
Actinobacteria	2.3±0.19	6.99±1.95
Patescibacteria	0.91±0.31	1.04±0.38
Bacteroidetes	13.62±1.38	15.37±2.34
Fusobacteria	13.45±2.94	17.45±3.89
Synergistetes	1.77±0.58	0.88±0.38
Epsilonbacteraeota	1.3±0.26	1.34±0.27
Kiritimatiellaeota	0.01±0.01	0±0
Tenericutes	0.28±0.12	0.51±0.23
Planctomycetes	0±0	0.01±0
Verrucomicrobia	0±0	0.12±0.06

809 ^aSC: sustained semen collection; NSC: non-semen collection

Table 2 Differential microbial taxa identified by LEFSe analysis and their relative abundance in the gander semen from the SC and NSC.

Microbial taxa	SC (%)	NSC (%)	LDA score	<i>P value</i>
f__Burkholderiaceae	1.17±0.29	9.57±2.65	4.66	0.0433
g__ <i>Hydrogenophaga</i>	0.77±0.26	8.07±2.12	4.58	0.0433
g__ <i>Sphingomonas</i>	0.25±0.07	7.62±3.31	4.57	0.0433
g__ <i>Ralstonia</i>	0.09±0.02	1.35±0.51	3.82	0.0433
g__ <i>Escherichia_Shigella</i>	0.04±0.01	0.17±0.04	3.21	0.0433
o__Bacillales	0.28±0.07	0.09±0.01	3.77	0.0209
g__ <i>Arthrobacter</i>	0±0	0.01±0	4.28	0.0472
g__ <i>Corynebacterium1</i>	0.21±0.07	0±0	4.00	0.0180
g__ <i>Megamonas</i>	0.03±0.01	0±0	4.22	0.0472
g__ <i>Pasteurellaceabacterium20215_4</i>	6.79±3.38	0±0	4.68	0.0472

^aSC: sustained semen collection; NSC: non-semen collection

Figures

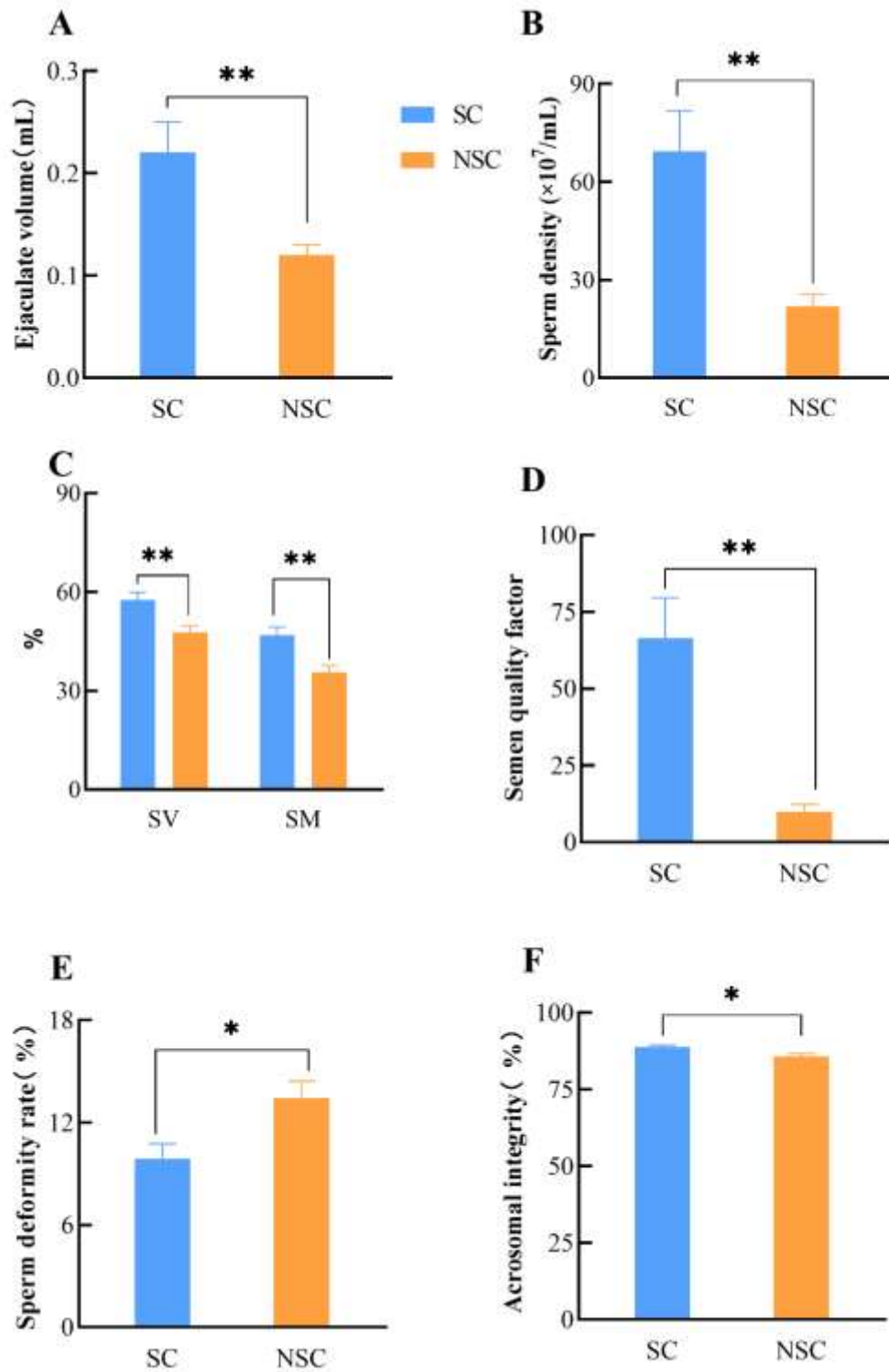


Fig 1. Comparison of the gander semen quality parameters between the sustained semen collection (SC) and non-semen collection (NSC) groups. (A) Ejaculate

of the semen microbiota.

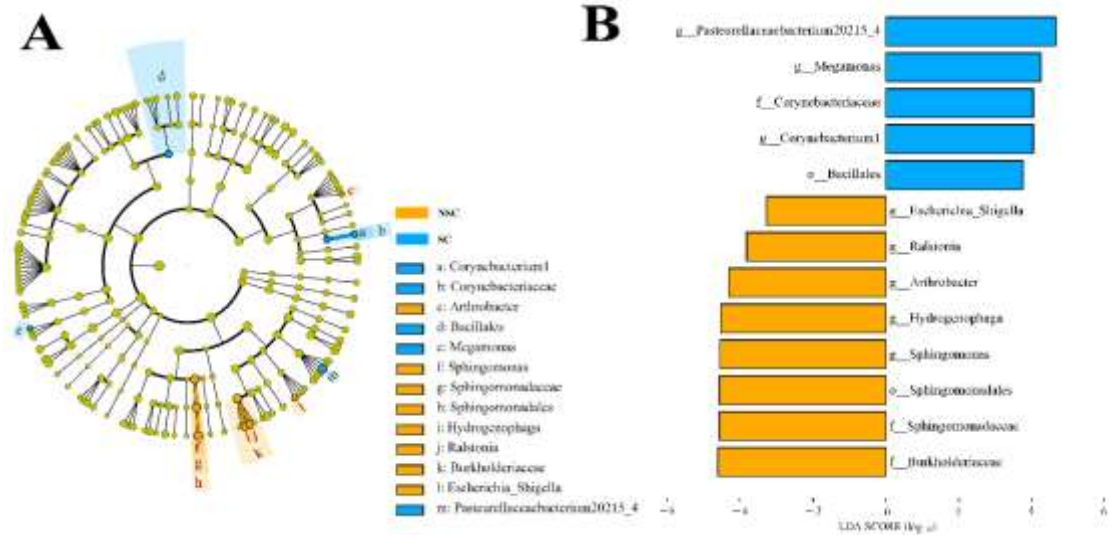


Fig 3. The linear discriminant analysis effect size (LEFSe) analysis was performed on the gander semen to identify differentially abundant bacteria between the sustained semen collection (SC) and non-semen collection (NSC) groups. (A) Cladogram of the LEFSe results (B) A bar graph showing the bacterial taxa that discriminated between the two groups, with a logarithmic LDA score of at least 3.

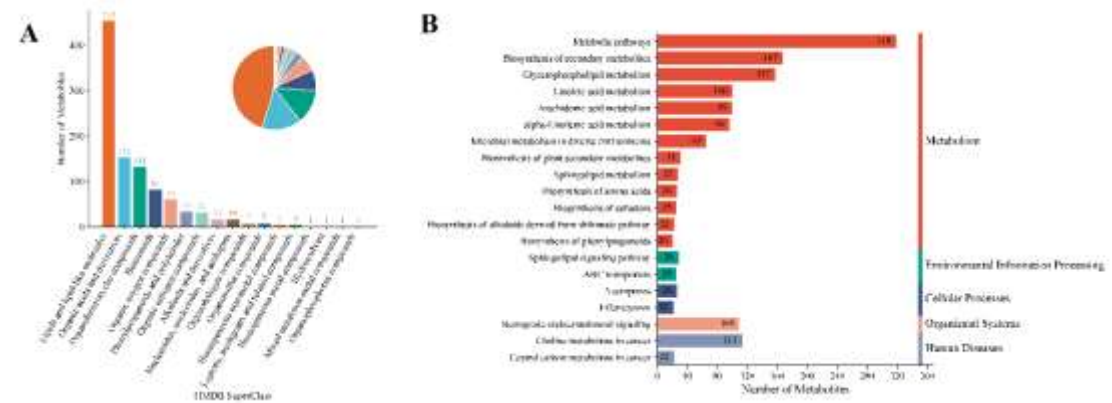


Fig 4 Characterization and function annotation of metabolites in the gander semen

by the secondary mass spectrometry (MS2). (A) The number of the metabolites annotated in the Human Metabolome Database (HMDB). (B) The number of the metabolites in the top 20 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG).

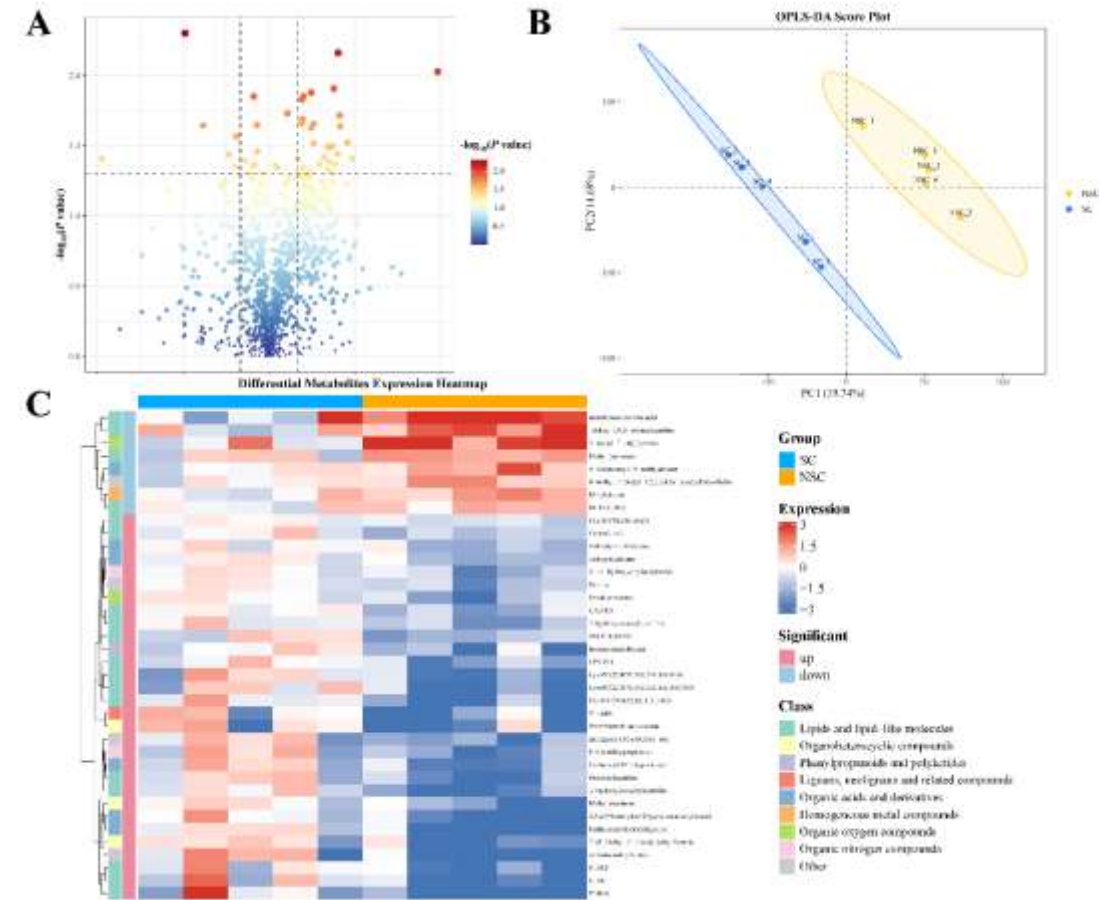


Fig 5 Identification of differentially expressed metabolites (DEM) in the gander semen between the sustained semen collection (SC) and non-semen collection (NSC) groups. (A) Volcano plot for the identified DEM. (B) The orthogonal partial least squares discriminant analysis (OPLS-DA) plot of the semen differential metabolites from 10 samples. (C) Hierarchical clustering heatmap of DEM between the two groups.

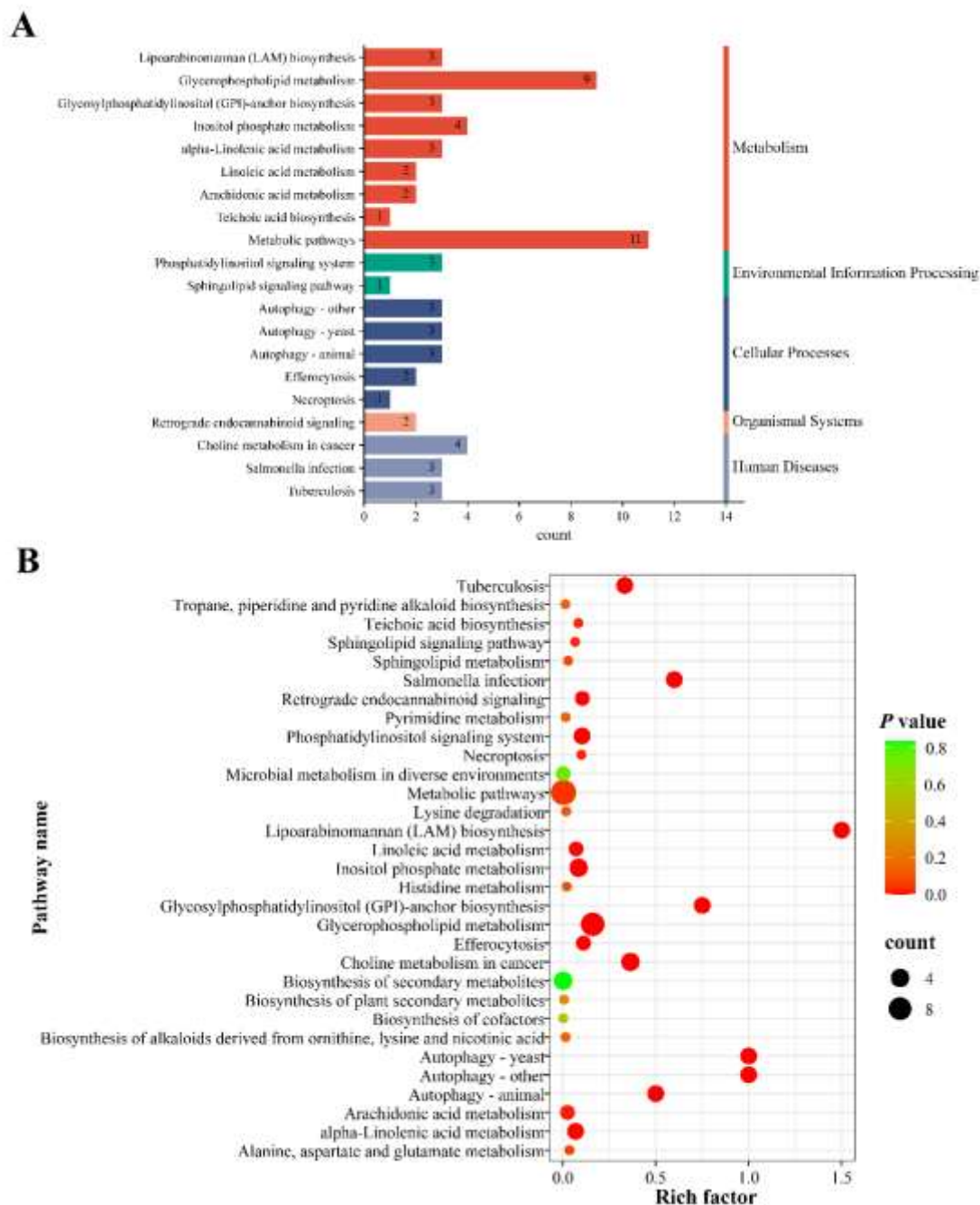


Fig 6. Functional enrichment analysis of differentially expressed metabolites (DEM) in the gander semen between the sustained semen collection (SC) and non-semen collection (NSC) groups. (A) The KEGG enrichment analysis of the semen DEM. (B) KEGG enrichment scatter plot.

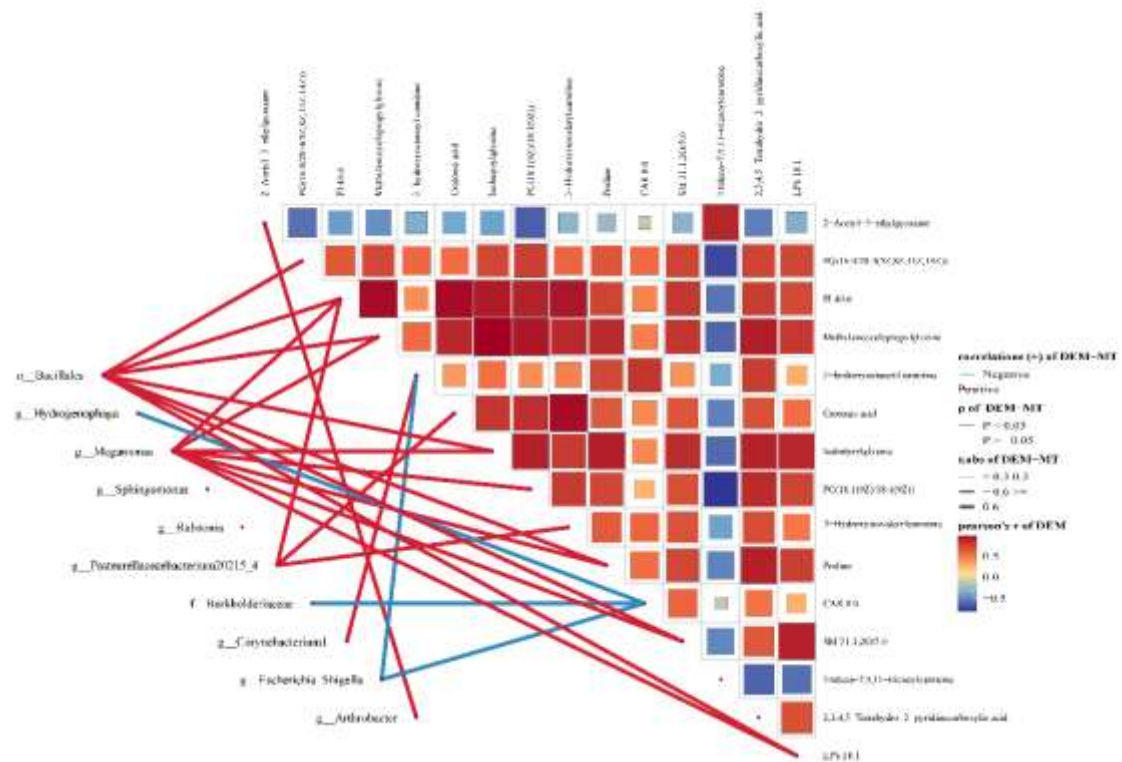


Fig 7. A cluster heatmap showing correlations among the selected 10 differentially abundant bacteria and the top 15 differentially expressed metabolites (DEM) in the gander semen between the sustained semen collection (SC) and non-semen collection (NSC) groups. The color of the lines indicates the positive and negative correlations between microbiota and DEM, the thickness of the lines represents the magnitude of the correlation coefficient between microbiota and DEM, and the color and size of the squares denote the magnitude of the correlation coefficient of DEM. “MT indicates microbial taxa.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1.docx](#)
- [SupplementaryTableS2.docx](#)
- [SupplementaryTableS3.docx](#)
- [SupplementaryTableS4.docx](#)
- [SupplementaryTableS5.docx](#)