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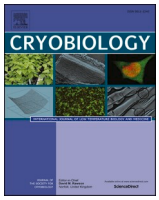

Bacterial survival below zero: Impact of storage time on bacterial viability in bull semen

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ABSTRACT

Although freezing methods have been optimized for preserving sperm integrity, their effectiveness in sustaining bacterial viability is unknown. Therefore, culturing thawed semen samples might not give an accurate picture of the bacteria in the original sample. The aim of this study was to assess how cryopreservation and storage duration influence bacterial populations and the survival of distinct bacterial species. Semen samples were collected from 14 bulls, samples were diluted in equal proportions of antibiotic-free semen extender and transported to the laboratory at 6 °C overnight. Aliquots of semen were cultured within 24 h after semen collection on Plate Count Agar to calculate number of bacteria, and blood agar plates (5 % bovine blood) for identification of bacterial species. The remaining samples were diluted 1:1 in Brain Heart Infusion (BHI) broth with 30 % glycerol and stored at −80 °C. The frozen samples were thawed and cultured for quantification of bacteria as described for fresh semen, after 6 and 13 days at −80 °C. The isolated bacteria were re-cultured on blood agar, incubated for one day at 37 °C before identification by Matrix assisted laser desorption ionization-time of flight mass spectrometry. Total bacterial counts remained consistent across fresh and cryopreserved samples regardless of storage duration. A total of 31 bacterial species were identified, with 20 detected in fresh samples, 16 present after 6 days of storage, and 18 observed after 13 days. Ten species persisted across all time points, while others were unique to a specific sampling day, including nine species on day 1, two species on day 6, and five species on day 13. These findings suggest that while cryopreservation does not alter the overall bacterial load, the survival of individual species varies depending on storage conditions.

1. Introduction

Temperature represents a critical factor for growth and survival of various bacterial species. Most microbial organisms manifest optimal growth conditions at distinct temperature ranges, reflecting their evolutionary adaptations to specific environmental niches [4]. However, when subjected to low temperatures, especially freezing conditions, bacterial survival rates differ significantly [20]. Different bacterial species present variable responses to freezing temperatures, influencing their viability and persistence in cryopreserved samples. In the realm of bacteriology, methods for preserving pure cultures at ultra-low temperatures (e.g., −80 °C) have been developed and standardized, such as freezing in brain heart infusion with glycerol [21]. The efficacy of such preservation techniques in maintaining the viability of bacteria within original samples with mixed bacterial flora remains largely unexplored. Previous studies, mostly focusing on bacteria used in food processing or those used for pharmaceutical products, found that bacterial resistance to freezing and frozen storage depends on 12 different factors, of which two were temperature and duration of the cryoprotection step [11]. Consequently, a notable gap exists in understanding bacterial survival dynamics in their native habitats. One such complex environment is represented by semen samples. In beef farming, where natural mating predominates, reproductive challenges that can manifest in decreased sperm quality and reproductive disorders in females can arise from bacteria present in semen samples. The presence of bacteria in these samples may directly or indirectly affect fertility outcomes [3,6].

Bacteria present in semen samples from healthy animals primarily originate from the mucosa of the reproductive tract, which is colonized by microbes from both the environment and the animals themselves. The cleanliness of the environment where animals are maintained significantly influences the extent of bacterial contamination [18]. Further contamination may arise during semen processing as a result of

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contact with individuals working with semen or from equipment [13]. The presence of bacteria in semen samples matters due to their direct and indirect influences on sperm quality parameters. Metabolic byproducts and toxins produced by bacteria can cause a decline in semen quality by affecting spermatozoa and competing for nutrients, as the semen extender used to maintain sperm viability also serves as a nutrient medium for bacterial growth. The production of metabolic byproducts by bacteria further impacts sperm quality, underscoring the importance of avoiding bacterial contamination in semen samples [8]. Conversely, the absence of bacteria in semen is crucial for ensuring optimal sperm quality and successful artificial insemination procedures [14]. Studying bacteria in frozen bull semen samples is essential for ensuring the health and fertility of livestock [6]. Bacterial contamination in semen can reduce sperm viability, cause infections in cows after insemination, and compromise the success of artificial insemination programs. Identifying opportunistic or environmental bacteria helps improve semen processing and storage practices, leading to higher reproductive efficiency and healthier offspring. Therefore, it is important to consider the differences and complexities between bacterial species when designing sampling and preservation protocols.

The aim of this study was to determine the influence of cryopreservation and length of storage of the semen samples at −80 °C on bacterial count and survival of different bacterial species.

2. Material and methods

2.1. Sampling of semen

Semen samples were collected from 14 dairy bulls (Holstein Friesian and Swedish Red) aged 1–4 years, with no sign of diseases, housed at VikingGenetics - Skara, Sweden. Semen collection was performed by artificial vagina following standard husbandry procedures where the bulls were stimulated by false mounting. After semen collection, the previously sterile collection tube containing the ejaculate was directly transferred to the laboratory. Thereafter, semen samples were diluted 1:1 with Andromed semen extender without antibiotics (AndroMed® CSS one-step 200 mL, Minitüb GmbH, Tiefenbach, Germany). Approximately 5–10 mL of diluted semen was transported overnight to the laboratory at the Swedish University of Agricultural Sciences at 6 °C in an insulated box with cold packs. One aliquot of each semen sample was used for bacteriology analyses performed direct after arrival at the laboratory. The remaining samples were diluted 1:1 in Brain Heart Infusion (BHI) broth with 30 % glycerol and stored at −80 °C, in duplicate. After 6 and 13 days, the samples were thawed and cultured as described below.

2.2. Bacteriological analyses

For bacterial analyses, 1 mL of sperm sample was mixed with the same amount of peptone diluent (1 g peptone and 8.5 g NaCl per liter Milli-Q H_2O_2; autoclaved at 121 °C for 15 min). Subsequently, 0.1 mL of this mix was transferred to cattle blood agar plates (Swedish Veterinary Agency - SVA, Uppsala, Sweden), which were incubated at 37 ± 1 °C and examined for bacterial growth after 24 h. Bacterial colonies of different macromorphology were recultured on new blood agar plates and incubated for 24 h at 37 ± 1 °C with 5 % CO_2, to obtain a pure culture. One isolated colony from each pure culture was identified to species level by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Billerica, MA, USA). The mass spectrum of bacterial isolates was compared with those of known bacterial strains in the database (Bruker Daltonics, Billerica, MA, USA). Score values between 2.0 and 3.0 were considered accurate at both genus and species levels, whereas score values between 1.7 and 2.0 were considered reliable only at the genus level. If colonies could not be identified at the first attempt, a second attempt was performed. If identification to genus level was still not possible, they were recorded as belonging to the "no identification possible" group, suggesting their absence in the database.

2.3. Quantitative analyses

The total number of viable aerobic bacteria in the semen samples was analysed according to NMKL 86, 5 Ed., 2013, with slight modifications. In brief, 1 mL of each sperm sample was transferred to 1 mL of diluent (the same diluent as used for bacteriological analyses). The samples were homogenized using a vortex (Saveen and Werner, Malmö, Sweden). A 1.0 mL of diluted sample was pour plated into a Petri dish measuring 9 cm in diameter and mixed with 10–15 mL plate count agar (PCA) (Oxoid, Basingstoke, UK) melted in boiling water and thereafter placed in a 48 °C water bath to keep it liquid until required. When the agar had solidified, a further 10–15 mL of PCA was added to each dish as an overlay to avoid swarming and facilitate enumeration. After solidification of the agar, the plates were incubated at 30 °C for 72 h. The total number of colony forming unit (CFU) was counted using a colony counter (Gerber instruments, Im Langhag, Switzerland). The standard ISO 7218:2007/A1:2013 formula was used for calculating CFU/mL, the number of bacteria was expressed as Log CFU/ml, with a detection limit of 2.0 Log CFU/mL.

2.4. Statistical analyses

Data analysis was performed by Linear mixed model, (RStudio R-4.3.3), assessing the fixed effects of length of storage and their interaction on bacterial count, while considering random effects of individual bulls. Descriptive statistics were used to summarize and describe the characteristics of the data such as identifying bacterial species in different storage times. This method was also used to analyze differences in CFU/mL between different time points due to the methods margin of error, allowing us to analyze central tendencies, variability, and distribution patterns without assuming the accuracy.

3. Results

Differences in bacterial species were found between storage times as well as in total bacteria count between individual bulls. In total 31 macro-morphologically different bacterial isolates were observed from all 14 semen samples. While 28 of the isolates could be identified by MALDI-TOF MS, only 26 could be identified to species level, while two isolates could be identified to genus level (*Bacillus* sp. and *Corynobacterium* sp.) and three morphologically different isolates were classified as "no identification possible". The majority of identified bacteria were environmental organisms, with some of them described as opportunistic pathogens in cattle that can lead to infections under certain conditions, such as *Escherichia coli, Histophilus somni* and *Trueperella pyogenes*. Bacteria isolated after different storage periods differed in both number and diversity, with some species detected only on day 1, others only found after 6 days of storage and still others detected only after 13 days of storage. Differences in bacterial species at different storage times are shown in Fig. 1.

A total of 20 bacterial species were identified in fresh samples at Day 1, 16 after 6 days of storage, and 18 after 13 days in −80 °C. Ten species were consistently detected at all time points, while others were exclusive to specific time intervals: nine species on day 1, two species on day 6, and five species on day 13.

The bacterial species detected at each storage time were consistent with those found in semen samples from the majority of bulls. The distribution of bulls contributing to the identification of each bacterial species on specific days, along with the total number of bulls from which each species was isolated, is presented in Table 1.

The distribution of total aerobic bacteria semen between individual bulls was wide (Fig. 2). The mean number of viable bacteria at Day 1 was 1.8 log CFU/mL with a range of 0.9–2.9, whereas the mean at Day 6 was

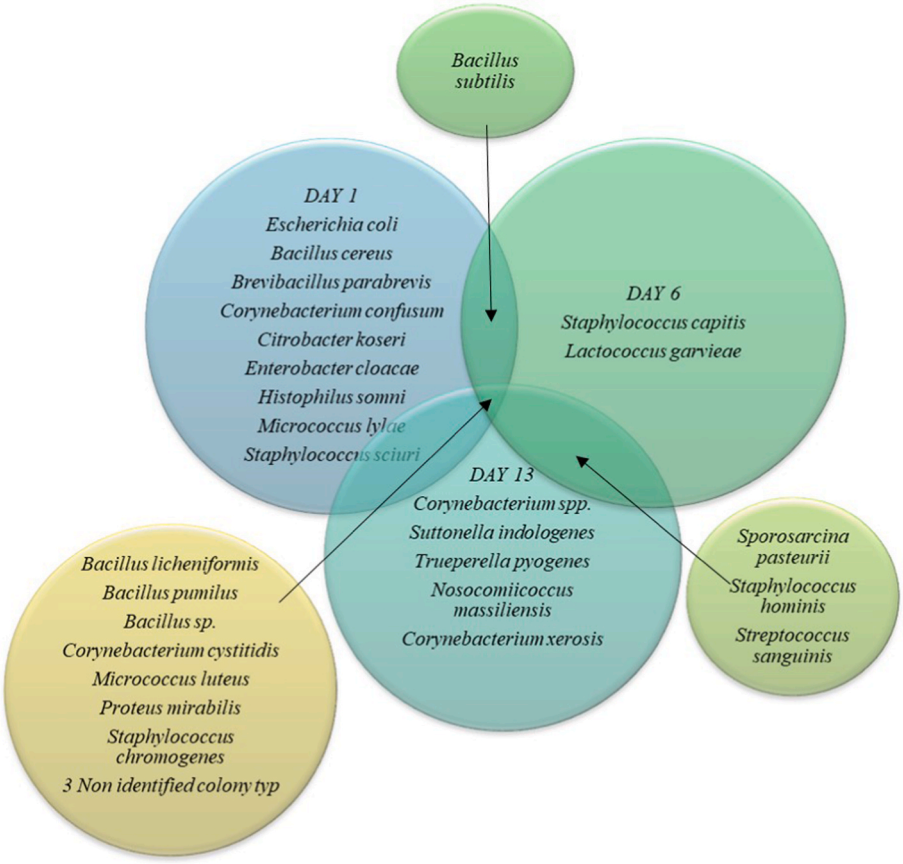

Fig. 1. Bacterial species isolated from semen sampled from 14 Swedish bulls without symptoms of disease analysed after one day of storage in the refrigerator (Day 1) and after 6 and 13 days of storage in −80 °C.

Table 1
Five most common bacterial species isolated from semen samples from 14 Swedish bulls without clinical signs of disease analysed, after one day of storage at 5 °C (Day 1) and after 6 and 13 days of storage in −80 °C.

Bacterial species	No. of bulls Day 1	No. of bulls, Day 6	No. of bulls Day 13	Total No. of bulls
Bacillus licheniformis	9,10,12,13	3,9,10,11,12	1,4,9,12	7
Bacillus pumilus	4,9,10,12,14	4,9,12	4,5,6	7
Corynebacterium cystitidis	6,7,8,11,13,14	1,2,5,7,10,11,12,13,14	7,8,10,11,12,13,14	11
Micrococcus luteus	6,7,8,14	1,6,7,8,11,12,13	2,6,7,8,10,11,14	10
Staphylococcus chromogenes	2,4,10,12,13,14	1,2,4,5,10,12,13,14	1,2,4,9,11,12	10

2.0 log CFU/mL with a range of 1.3–2.8 and Day 13 was 1.9 log CFU/mL with a range of 1.2–2.9. In addition, no significant differences were observed when comparing the total number of viable aerobic bacteria in semen samples across the three storage durations, combining data from all 14 bulls. (Table 2).

4. Discussion

The results showed a variation in identified bacterial species between individual bulls and ejaculates at different storage times, as well as differences in total bacterial count between bulls. However, no differences was found in the number of total viable bacteria at different storage times.

The variation of the number of bacteria and identified bacterial species between individual bulls and individual ejaculates is in agreement with previous studies [7,9]. Unexpectedly not all bacterial species could be detected from fresh semen on Day 1; some species were only detected after storage at −80 °C for 13 days, which according to our knowledge has not been reported previously. The differences in identified bacterial species observed during different storage times could potentially be attributed to interbacterial synergism or antagonism [17]. This concept involves bacteria actively inhibiting the growth or survival of other bacteria through various mechanisms. Traditionally, the study of bacteria interacting with their environment has primarily focused on strategies for acquiring nutrients and withstanding environmental stresses. However, there has been a recent argument suggesting that this focus has somewhat overlooked another crucial aspect of bacterial life: the constant threat posed by antagonistic interactions with other bacteria [17]. Some of these interactions are production of antimicrobial compounds, competition for resources and quorum sensing where bacteria use signaling molecules to communicate with each other and coordinate group behaviors, including the production of toxins or enzymes that target competing species [16]. These interbacterial antagonism pathways highlight the intricate dynamics within bacterial communities and emphasize the importance of considering not only how bacteria interact with their environment but also with each other in shaping microbial populations and ecosystem dynamics. An additional factor contributing to the observed differences in bacterial

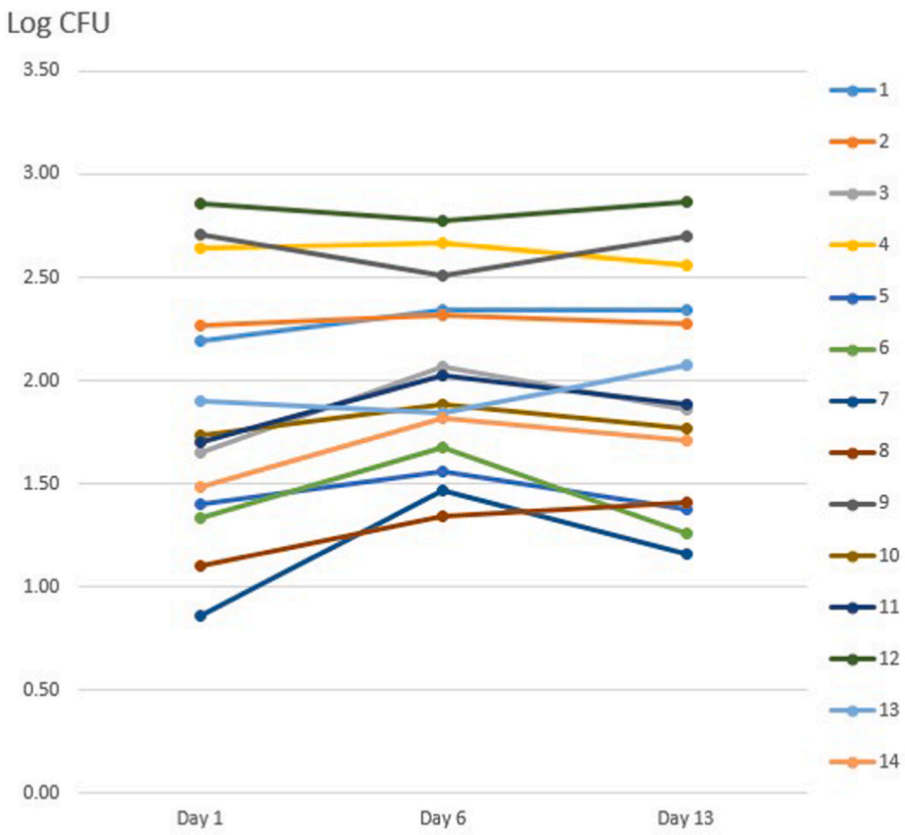

Fig. 2. Distribution of total count of aerobic bacteria presented as logarithmic (Log) values per mL semen from bulls (1–14) analysed after one day of storage in the refrigerator (Day 1) and after 6 and 13 days of storage in −80 °C.

Table 2
Total number of viable aerobic bacteria (CFU/mL) in the semen samples from all 14 bulls sampled after one day of storage in the refrigerator (Day 1) and after 6 and 13 days of storage in −80 °C.

	Day 1	Day 6	Day 13
Mean	167	170	176
Median	52	91	75
Minimum	7	22	15
Maximum	727	593	735
Standard deviation	226	173	216
Standard error	60	40	58

growth among species across various storage times could be the human factor. Specifically, during the preparation of samples for freezing, the original sample was divided into three aliquots. This process may have introduced variability, as bacteria present in minimal concentrations in the original sample could have been unevenly distributed, potentially leading to their presence in only one aliquot. This sampling variation could impact the representation and subsequent growth dynamics of certain bacterial species. One of the bacteria identified in all time points of incubation was *Micrococcus luteus*, the only bacterium found producing antimicrobial metabolites and exhibiting good probiotic characteristics [2]. Nevertheless, *Micrococcus luteus* showed antibacterial activity against foodborne pathogens, *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli*. The bacterium was also found to be active against *Klebsiella pneumoniae* and *Staphylococcus aureus* [2]. The presence of *Micrococcus lutes* as a predominant species in frozen bull semen samples was previously reported [1,9,22]. Besides the described biochemical activities [1], this species has the potential to cause liver abscesses in cattle [23] and recurrent bacteremia [24], although no effect on sperm quality has been described.

The imperative of preserving samples until assessment is paramount in bacteriology, particularly when logistical constraints hinder immediate processing. Factors such as geographical position of collection sites and/or inadequate laboratory equipment, and the need to transport samples to distant facilities necessitate detailed sample preservation methodologies. Furthermore, the preservation of original samples for further bacteriological analyses is crucial for comprehensive microbial characterization. Efficient transport of semen samples from the places where animals are kept to laboratories for bacteriological analysis, requires systematic preservation protocols to ensure the integrity of microbial communities. As such, explaining the complexities of bacterial survival below zero not only contributes to fundamental scientific understanding but also serves as the basis for practical methodologies for sample handling and analysis in diverse contexts. Psychrophiles produce specialized proteins such as cold-adapted proteins, cold-acclimation proteins, cold-shock proteins, ice-binding proteins or other molecules, for example antifreeze and osmolyte. They showed potential to enable the cell to survive under low-temperature conditions. Nevertheless, these bacteria have developed mechanisms to limit metabolic activity by entering a dormant state [5].

Furthermore, certain genera of bacteria isolated from milk are both psychrotrophic and thermoduric, e.g., *Bacillus, Clostridium, Microbacterium, Micrococcus*, and *Corynebacterium* [15]. The majority of bacterial species present on every storage day in our study were members of these genera and also isolated in all bull ejaculates in a previous study [9], where their origin and effect on sperm quality was described. The *Bacillus* species are highly diverse and can occupy a wide range of ecological niches as well as in bull semen [9,10]. Different species were identified in bull semen samples that were divided into several group according to their motility [10], suggesting that *Bacillus* could have an effect on sperm quality in bulls.

Bacterial species that were isolated only on Day 13 were *Suttonella*

indologenes, Trueperella pyogenes, Nosocomiicoccus massiliensis and *Corynebacterium xerosis*. Despite the fact that these bacteria are part of the biota of skin and mucous membranes, they are also classified as opportunistic pathogens. The bacteria from the species *Trueperella pyogenes* caused reproductive disorders such as metritis and mastitis, besides pneumonia and abscesses [19]. Furthermore, *Corynebacterium xerosis* has also been associated with bovine mastitis [12]. Both bacterial species cause significant economic losses in cattle breeding, causing a reduction of meat and milk yield, as well as decreasing reproductive efficiency and/or excluding sick animals from production. Nevertheless, *Nosocomiicoccus massiliensis* and *Suttonella indologenes* have not previously been identified in semen samples, therefore their influence on sperm quality or cattle reproduction health is unknown. The fact that these bacteria could not be identified in semen samples in previous time points suggests the possibility of interbacterial antagonism and supports the importance of adequate handling of semen sample for bacteriological assessment.

In conclusion, there are differences in total bacterial count in semen samples between bulls and identified bacterial species in different time points. Cryopreservation of semen does have a variable effect on bacterial survival depending on species. Thus, there is a need for optimization of semen cryopreservation processes to ensure bacterial survival in semen samples used for bacteriological assessment.

CRediT authorship contribution statement

Aleksandar Cojkic: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ingrid Hansson:** Writing – review & editing, Visualization, Validation, Methodology. **Jane M. Morrell:** Writing – review & editing, Visualization, Validation, Resources, Project administration, Funding acquisition.

Ethics approval

Ethical approval is not required in Sweden for the collection of bull semen using an artificial vagina. The bulls were housed at a commercial stud under standard husbandry conditions for the species.

Funding sources

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Declarations of competing interest

None.

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