MICROBIOLOGICAL QUALITY OF BOAR SEMEN DURING HYPOTHERMIC STORAGE

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Abstract

The study aimed to evaluate the effects of biologically active compounds on the microbiological stability of boar semen during hypothermic storage. Semen samples were diluted with the basal GHȚS extender (control) and four experimental formulations supplemented at different concentrations. Storage periods were five days for Extenders 1, 3, and 4, and 96 hours for Extender 2. Microbiological analyses included total viable count (TVC), Escherichia coli, Staphylococcus spp., selected bacterial groups, and fungi.Microbial counts varied with extender composition and supplement concentration. In Extender 1, supplementation at 0.6–0.8% resulted in lower TVC and fungal counts than the control. After five days, Extenders 3 and 4 supplemented at 0.9% showed TVC values of 1.0 × 10² and 1.5 × 10² CFU/mL, respectively, compared with 3.3 × 10² and 3.6 × 10² CFU/mL in the corresponding controls. At 0.9%, E. coli was undetectable, while fungal counts reached 1.0 × 10² and 1.1 × 10² CFU/mL, respectively. However, not all supplemented treatments showed lower microbial counts than the controls.These findings suggest that selected biologically active supplements may improve the microbiological stability of boar semen during hypothermic storage, depending on extender formulation and concentration.

Key words: *boar semen, preservation, semen extenders, fungi*

INTRODUCTION

The application of modern reproductive biotechnologies represents an important approach to genetic improvement and increased efficiency in pig production. In this context, boar semen preservation is an essential component of artificial insemination, enabling the efficient use of genetically valuable breeding boars and the transmission of desirable genetic traits. Maintaining semen quality during storage depends on the composition of the semen extender, the balance of its nutritional and protective components, and its ability to prevent structural and functional alterations in spermatozoa [8, 16].

Early fundamental research on semen preservation and the development of semen extenders contributed to the advancement of modern assisted reproductive technologies. Milovanov's pioneering studies established the foundations for using nutrient-based extenders to maintain sperm viability outside the reproductive tract, while subsequent investigations by Serdiuk and Nauc provided further insights into sperm physiology, biochemistry, and semen preservation methods [11, 12]. Later studies highlighted the importance of extender composition and storage conditions in maintaining the functional characteristics of boar spermatozoa [2, 14].

During storage, spermatozoa are exposed to various stress factors that may promote the accumulation of reactive oxygen species (ROS) and enhance lipid peroxidation. The sperm plasma membrane, which is rich in polyunsaturated fatty acids,

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is particularly susceptible to oxidative damage. These processes may result in reduced sperm motility, impaired plasma membrane and acrosomal integrity, and decreased fertilizing capacity [5, 7, 17]. Therefore, the incorporation of antioxidants and other protective compounds into semen extenders represents an important strategy for improving boar semen preservation.

Recent literature has demonstrated the beneficial effects of supplementing semen extenders with antioxidant compounds on boar sperm quality during both storage at room temperature and cryopreservation. Various substances, including glutathione, vitamin E, coenzyme Q10, L-carnitine, and resveratrol, have been investigated, with their effectiveness depending on concentration, extender composition, and storage conditions [13,20]. Recent studies have reported improvements in sperm motility, viability, and plasma membrane integrity, including following cryopreservation [15]. Furthermore, the protective effects of reduced glutathione on post-thaw boar spermatozoa emphasize the importance of antioxidant defense mechanisms in maintaining the functional integrity of reproductive cells.

Another critical factor affecting the quality of stored semen is the microbiological stability of the preservation medium. Semen can provide a favorable environment for microbial proliferation, and bacterial contamination may lead to nutrient depletion, changes in pH, production of potentially toxic metabolites, and increased oxidative stress, thereby adversely affecting sperm function [1, 4]. Prolonged storage may also be associated with deterioration in sperm quality and reductions in certain functional parameters [21]. Consequently, microbiological monitoring and the incorporation of components with antimicrobial properties are important considerations in modern semen preservation technologies [18, 19].

In this context, biologically active compounds and preparations capable of exhibiting antioxidant, antimicrobial, and cell-protective properties are of particular interest. Research conducted in the Republic of Moldova under the supervision of Academician V. Rudic has highlighted the biological potential of the BioR preparation and other microbial-derived compounds through their effects on metabolic processes and cellular protective mechanisms. Furthermore, previous investigations have demonstrated the potential of biologically active substances to improve semen preservation characteristics. Experience gained from semen preservation studies in other animal species, including rams, provides a scientific basis for investigating similar preparations in boar semen [3,10].

Although numerous studies have investigated the effects of antioxidant and biologically active compounds on sperm functional characteristics, their influence on the microbiological stability of boar semen during hypothermic storage remains insufficiently characterized, particularly with regard to the concentration-dependent effects of experimental extender formulations. Further investigations are therefore needed to determine whether supplementation with biologically active preparations can contribute to controlling microbial contamination during semen preservation.

The aim of the present study was to evaluate the microbiological stability of boar semen preserved in experimental extenders supplemented with biologically active compounds at different concentrations during 96 hours of hypothermic storage [6, 9]. The study focused on changes in total viable count (TVC), *Escherichia coli*, *Staphylococcus spp.*, and fungal contamination, together with other microbial groups where assessed. It was hypothesized that the microbiological response would depend on the composition of the extender and the concentration of the biologically active supplement. The findings indicated that selected

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experimental formulations, particularly at higher supplementation concentrations, were associated with lower microbial counts and favorable microbiological characteristics, suggesting their potential application in improving boar semen preservation technologies.

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MATERIAL AND METHOD

Experimental investigations were conducted using semen collected from breeding boars maintained under commercial production conditions at Nivelina LLC, Republic of Moldova. Semen was collected manually using the gloved-hand technique, in accordance with standard procedures applied in swine reproductive management. Ejaculates meeting the quality requirements for semen preservation were selected for the experimental investigations.

Following collection, semen samples were diluted using the GHȚS basal extender, which served as the control treatment, and four experimental extender formulations supplemented with biologically active preparations possessing antioxidant properties. The biologically active preparations were incorporated into the GHȚS extender at different experimental concentrations ranging from 0.1% to 1.0% to evaluate their effects on the microbiological stability of boar semen during hypothermic storage.

The diluted semen samples were stored under hypothermic conditions for 96 hours at the recommended temperature for liquid boar semen preservation. Samples for microbiological examination were collected at the beginning of the storage period (0 h) and after 96 hours. The microbiological results obtained for the experimental treatments were compared with those of the control group.

Microbiological evaluation of the stored semen and semen extenders included the assessment of the overall microbial contamination level and the detection of selected microbial groups relevant to the microbiological quality and hygienic status of the samples.

The following microbiological parameters were evaluated:

- Total viable count (TVC): determined to assess the overall culturable microbial load of the samples, with results expressed as colony-forming units per millilitre (CFU/mL).
- Indicator bacteria: detection of *Escherichia coli* and *Staphylococcus spp.* as indicators of microbial contamination.
- Other selected bacterial groups: detection of *Clostridium spp.*, *Bacillus spp.*, *Bifidobacterium spp.*, *Enterococcus spp.*, and other microbial groups identified according to the experimental treatment.
- Fungal contamination: assessment of fungal counts in the analyzed samples.

Microbiological analyses were performed using culture-based methods on appropriate selective or differential culture media, followed by microbial identification and enumeration in accordance with standard microbiological procedures used for evaluating biological materials of animal origin. Quantitative results were expressed as colony-forming units per millilitre (CFU/mL).

To assess the effects of biologically active preparations on the microbiological stability of boar semen, the results obtained for the experimental treatments were compared with those of the GHȚS control extender. This comparative approach was used to evaluate the potential of the supplemented extenders to limit the proliferation of contaminating microorganisms and maintain the microbiological quality of boar semen during hypothermic storage.

RESULTS AND DISCUSSIONS

Maintaining the microbiological quality of boar semen is essential for the effectiveness of hypothermic storage and the microbiological safety of semen doses used in swine reproduction. During storage, the nutrient components of semen extenders

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may provide a favorable environment for the proliferation of contaminating microorganisms. Through their metabolic activity, these microorganisms may alter the physicochemical properties of the extender and adversely affect semen stability.

The microbial populations associated with semen may originate from the reproductive tract of the breeding boar or from external sources of contamination during semen collection, handling, and processing. An increase in microbial load may lead to the depletion of nutrients in the storage medium, the production of potentially toxic metabolites, changes in pH, and increased oxidative stress, thereby compromising the overall biological quality of stored semen.

The use of semen extenders supplemented with biologically active compounds is of particular interest because of their potential contribution to maintaining the microbiological stability of the storage medium. Compounds with antioxidant and other biological properties may help limit the proliferation of contaminating microorganisms and support the preservation of semen quality during hypothermic storage.

To evaluate the effects of experimental extender formulations on the microbiological stability of boar semen, samples stored for 96 hours were examined. The GHȚS basal extender was used as the control treatment, while four experimental formulations were supplemented with biologically active preparations at different concentrations. The microbiological parameters evaluated included the total viable count (TVC), selected indicator microorganisms (*Escherichia coli, Staphylococcus spp.*, and *Enterococcus spp.*), as well as other microbial groups and fungi detected in the analyzed samples.

The results provided a basis for assessing the ability of the experimental extenders to maintain the microbiological stability of boar semen compared with the control extender. The microbiological parameters recorded for the first experimental formulation are presented in Table 1.

Table 1. Microbiological composition of boar semen samples (CFU/mL)

Microbiological parameter		Sampling time			
		No. 1 Control (GHȚS)	No. 1 Control (GHȚS)	No. 1 Control (GHȚS)	No. 1 Control (GHȚS)
Total viable count (TVC)	inițial	2.90×10^5	1.40×10^5	9.6×10^4	5.20×10^4
	Day 5	1.10×10^5	6.20×10^4	1.36×10^4	1.11×10^4
Escherichia coli	inițial	4.0×10^3	0	0	0
	Day 5	6.0×10^2	0	0	0
Enterococcus spp	inițial	0	0	0	0
	Day 5				
Staphylococcus spp	inițial	0	0	0	0
	Day 5				
Clostridium spp.	inițial	4.1×10^3	3.6×10^3	2.1×10^3	1.1×10^3
	Day 5	4.30×10^3	1.27×10^3	9.0×10^2	9.0×10^2
Lactobacillus spp.	inițial	0	0	0	0
	Day 5				
Bifidobacterium spp	inițial	8.3×10^4	2.10×10^4	1.93×10^4	1.8×10^4
	Day 5	4.70×10^4	6.60×10^3	3.1×10^3	3.0×10^3
Bacillus spp.	inițial	6.3×10^4	3.4×10^4	1.19×10^3	1.10×10^3
	Day 5	1.24×10^3	4.00×10^2	0	0
Fungi	inițial	2.4×10^4	3.06×10^3	1.2×10^3	1.00×10^3
	5-a zi	3.6×10^3	6.0×10^2	2.0×10^2	2.00×10^2

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Microbiological evaluation of stored boar semen is an important criterion for assessing the effectiveness of semen extenders, as microbial proliferation may adversely affect the structural and functional integrity of spermatozoa. In this context, changes in the main microbiological parameters were investigated in semen samples stored in the GHȚS control extender and in experimental formulations supplemented with LB/MP at concentrations of 0.4%, 0.6%, and 0.8%, both at the beginning of storage and after five days.

The results indicated that the incorporation of the biologically active supplement LB/MP was associated with a reduction in the total viable count (TVC) compared with the control sample. The initial TVC in the control sample was 2.90 × 10^5 CFU/mL, whereas lower counts were recorded in the experimental treatments, ranging from 1.40 × 10^5 CFU/mL at 0.4% LB/MP to 5.20 × 10^4 CFU/mL at 0.8% LB/MP. After five days of storage, this trend was maintained, with a TVC of 1.10 × 10^5 CFU/mL in the control sample, compared with 6.20 × 10^4, 1.36 × 10^4, and 1.11 × 10^4 CFU/mL in the treatments supplemented with 0.4%, 0.6%, and 0.8% LB/MP, respectively. The lowest microbial counts were observed at concentrations of 0.6% and 0.8%, indicating a more pronounced reduction in the overall culturable microbial load.

The assessment of selected indicator microorganisms revealed a more favorable microbiological profile in the experimental samples. *Escherichia coli* was initially detected in the control sample at a concentration of 4.0 × 10^3 CFU/mL, which decreased to 6.0 × 10^2 CFU/mL after five days of storage. In contrast, *E. coli* was not detected in any of the LB/MP-supplemented treatments, either at the beginning or at the end of the storage period. These findings suggest a potential contribution of the biologically active supplement to limiting *E. coli* contamination in stored boar semen.

No positive counts of *Enterococcus spp.*, *Staphylococcus spp.*, or *Lactobacillus spp.* were recorded in the reported initial assessments. The absence of detectable *Staphylococcus spp.* in these samples represents a favorable microbiological finding, although the overall microbiological safety of stored semen cannot be established solely on the basis of this parameter.

Regarding *Clostridium spp.*, lower bacterial counts were observed in the experimental treatments compared with the control. Initially, the count in the control sample was 4.1 × 10^3 CFU/mL, compared with 3.6 × 10^3, 2.1 × 10^3, and 1.1 × 10^3 CFU/mL in the treatments supplemented with 0.4%, 0.6%, and 0.8% LB/MP, respectively. After five days of storage, the lowest counts were recorded at supplementation levels of 0.6% and 0.8%, both reaching 9.0 × 10^2 CFU/mL, compared with 4.30 × 10^3 CFU/mL in the control sample.

A similar pattern was observed for *Bifidobacterium spp.* The initial count in the control sample was 8.3 × 10^4 CFU/mL, whereas the experimental treatments showed lower values, reaching 1.8 × 10^4 CFU/mL at 0.8% LB/MP. After five days of storage, the count was 4.70 × 10^4 CFU/mL in the control sample, compared with 6.60 × 10^3, 3.10 × 10^3, and 3.00 × 10^3 CFU/mL in the treatments supplemented with 0.4%, 0.6%, and 0.8% LB/MP, respectively.

A pronounced reduction in *Bacillus spp.* counts was also observed in the supplemented treatments. In the control sample, the initial count was 6.3 × 10^4 CFU/mL, decreasing to 1.24 × 10^3 CFU/mL after five days of storage. In the treatments containing 0.6% and 0.8% LB/MP, *Bacillus spp.* were no longer detected at the end of the storage period. These findings indicate a more favorable microbiological profile in the supplemented samples.

Fungal counts were also lower in the experimental treatments. Initially, the fungal count in the control sample was 2.4 × 10^4 CFU/mL, compared with 3.06 × 10^3,

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1.2 × 10^3, and 1.0 × 10^3 CFU/mL in the samples supplemented with 0.4%, 0.6%, and 0.8% LB/MP, respectively. After five days of storage, the fungal count decreased to 3.6 × 10^3 CFU/mL in the control sample, whereas the corresponding counts in the experimental treatments were 6.0 × 10^2, 2.0 × 10^2, and 2.0 × 10^2 CFU/mL, respectively.

Overall, the results indicate that the use of Extender 1 supplemented with LB/MP was associated with improved microbiological stability of boar semen during storage. The most favorable microbiological results were obtained at supplementation concentrations of 0.6% and 0.8%, which were associated with the lowest total viable counts, reduced counts of several bacterial groups, and the absence of detectable *E. coli* in the experimental samples. These findings suggest that the biologically active supplement may contribute to controlling microbial contamination and maintaining the microbiological quality of stored boar semen.

Further microbiological evaluation of stored boar semen was conducted to assess the influence of the second experimental extender formulation on microbial populations during storage. The analysis involved a comparison between the control sample and experimental treatments containing Extender 2 supplemented with different concentrations of a biologically active compound. The main microbiological parameters monitored included the total viable count (TVC), selected microbial indicators of contamination, and other bacterial groups potentially relevant to semen quality during storage (Table 2).

Maintaining microbiological stability is essential for semen preservation because microbial proliferation may alter the physicochemical properties of the storage medium, deplete available nutrients, promote the production of potentially toxic metabolites, and increase oxidative stress. These processes may adversely affect sperm membrane integrity and functional capacity. Therefore, comparing changes in microbial populations among experimental treatments provides valuable information for assessing extender performance and the potential role of biologically active compounds in maintaining the biological quality of stored semen. The corresponding results are presented in Table 2.

Table 2. Microbiological parameters of boar semen supplemented with Extender 2 (CFU/mL)

Microbiological parameter	Control (semen + GHȚS)	Diluent 2,%			
		0,2%	0,4%	0,6%	0,8%
Initial microbiological assessment					
Total viable count (TVC)/ml	6.1×10^3	1.28×10^4	3.8×10^3	2.9×10^3	3.0×10^3
E. coli, UFC/ml	1.52×10	1.57×10^3	1.1×10^3	4.2×10^2	4.0×10^2
Staphylococcus spp., UFC/ml	0	0	0	0	0
Fungal count, UFC/ml	3.4×10^2	2.9×10^2	2.7×10^2	2.2×10^2	1.9×10^2
After 96 hours of storage					
Total viable count (TVC)ml	3.9×10^2	5.4×10^3	1.7×10^3	1.3×10^3	1.06×10^3
Escherichia coli, UFC/ml	3.1×10^2	5.4×10^2	4.8×10^2	4.1×10^2	4.0×10^2
Staphylococcus spp., UFC/ml	0	0	0	0	0
Fungal count, UFC/ml	2.6×10^2	5.7×10^2	5.1×10^2	4.0×10^2	2.0×10^2

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The microbiological analysis of boar semen samples preserved using Extender 2 (Table 2) provided an assessment of its ability to maintain microbiological stability during storage. The evaluation was performed by comparing the control treatment (semen + GHȚS) with experimental treatments supplemented with a biologically active compound at concentrations of 0.2%, 0.4%, 0.6%, and 0.8%. The main microbiological parameters examined were the total viable count (TVC), *Escherichia coli* and *Staphylococcus spp.* counts, and the level of fungal contamination, both initially and after 96 hours of storage.

The microbiological stability of the storage medium is important for preserving the functional properties of spermatozoa, as excessive microbial proliferation may adversely affect extender composition, sperm membrane integrity, and fertilizing capacity. In this context, changes in microbial counts during storage provide useful information for evaluating the performance of semen extenders.

The analysis of total viable count (TVC) revealed differences between the control and experimental treatments. Initially, the TVC in the control sample was 6.1×10^3 CFU/mL, whereas values in the Extender 2 treatments ranged from 2.9×10^3 to 1.28×10^4 CFU/mL. The lowest initial microbial count among the experimental treatments was recorded at a supplementation concentration of 0.6% (2.9×10^3 CFU/mL), followed by 0.8% (3.0×10^3 CFU/mL). Both values were lower than that recorded in the control sample.

After 96 hours of storage, a reduction in TVC relative to the initial values was observed in all experimental treatments. The TVC in the control sample decreased to 3.9×10^2 CFU/mL, whereas counts of 5.4×10^3, 1.7×10^3, 1.3×10^3, and 1.06×10^3 CFU/mL were recorded at supplementation concentrations of 0.2%, 0.4%, 0.6%, and 0.8%, respectively. Among the experimental treatments, the lowest final microbial count was observed at 0.8%. However, all supplemented treatments exhibited higher TVC values than the control after 96 hours, indicating that the observed reduction during storage did not translate into a lower final microbial load relative to the control.

Regarding indicator microorganisms, *E. coli* counts generally decreased with increasing concentrations of the biologically active supplement. Initially, the *E. coli* count in the control sample was 1.52×10^3 CFU/mL, compared with 4.0×10^2 CFU/mL in the treatment supplemented with 0.8%. After 96 hours, the count in the control sample decreased to 3.1×10^2 CFU/mL, whereas values in the experimental treatments ranged from 5.4×10^2 to 4.0×10^2 CFU/mL. The lowest *E. coli* count among the supplemented treatments was recorded at 0.8%, although this value remained higher than that of the control at the end of storage.

An important finding was that *Staphylococcus spp.* were not detected in any of the analyzed samples, either initially or after 96 hours of storage. This represents a favorable microbiological observation regarding this bacterial group. Nevertheless, the absence of detectable *Staphylococcus spp.* alone does not establish the overall microbiological safety of the stored semen or exclude the presence of other potentially pathogenic microorganisms.

The analysis of fungal contamination revealed a concentration-dependent pattern across the experimental treatments. Initially, fungal counts ranged from 3.4×10^2 CFU/mL in the control sample to 1.9×10^2 CFU/mL at a supplementation concentration of 0.8%. After 96 hours of storage, the fungal count in the control sample was 2.6×10^2 CFU/mL, compared with 5.7×10^2, 5.1×10^2, 4.0×10^2, and 2.0×10^2 CFU/mL in the experimental treatments supplemented with 0.2%, 0.4%,

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0.6%, and 0.8%, respectively. The lowest final fungal count was recorded at 0.8%, which was also lower than that observed in the control sample. In contrast, the other supplemented treatments exhibited higher fungal counts than the control after 96 hours.

Overall, the results indicate that Extender 2 supplemented with the biologically active compound produced concentration-dependent differences in the microbiological characteristics of stored boar semen. Among the experimental treatments, the 0.8% concentration showed the most favorable overall profile after 96 hours, with the lowest TVC, *E. coli* count, and fungal count, while *Staphylococcus spp.* remained undetected. Nevertheless, the control treatment exhibited lower final TVC and *E. coli* counts. Therefore, the findings suggest that supplementation at 0.8% may offer certain microbiological advantages compared with the other supplemented formulations, but they do not demonstrate an overall improvement over the control across all microbiological parameters.

Microbiological Evaluation of Boar Semen Stored in Extender 3

To further investigate the effects of experimental extender formulations on boar semen quality, microbiological analyses were performed on semen samples stored in Extender 3. Microbiological assessment is an important component of evaluating semen preservation systems, as controlling microbial contamination may contribute to maintaining sperm structural integrity and functional characteristics during storage.

During hypothermic storage, changes in the nutrient composition of the extender and the presence of organic substances may create conditions favorable for microbial proliferation. The metabolic activity of bacteria and fungi may alter pH, deplete available nutrients, promote the accumulation of potentially toxic metabolites, and intensify oxidative processes. These changes may adversely affect sperm motility, plasma membrane integrity, and acrosomal structure.

For this reason, the development of semen extenders supplemented with biologically active compounds aims not only to protect spermatozoa against structural damage but also to support the microbiological stability of stored semen. Monitoring microbiological parameters makes it possible to assess the extent to which experimental extender formulations limit the proliferation of undesirable microorganisms and provide suitable conditions for semen preservation.

In the present study, the microbiological characteristics of boar semen samples stored in Extender 3 were evaluated in comparison with the control treatment. The analysis focused on changes in total viable count (TVC), the occurrence of selected indicator microorganisms, and variations in fungal counts during the storage period.

The results presented in Table 3 provide a basis for assessing the effects of different supplementation concentrations on the microbiological stability of the samples and identifying the experimental formulation associated with the most favorable microbiological conditions for boar semen preservation.

The evaluation of microbiological parameters in boar semen stored in an extender supplemented with biologically active compounds revealed differences in microbial contamination levels among the experimental treatments. The main microbiological parameters investigated were the total viable count (TVC), *Escherichia coli*, *Staphylococcus spp.*, and fungal counts, assessed both initially and after five days of storage.

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Table 3. Microbiological composition of boar semen and the preservation medium (CFU/mL)

Microbiological parameter	Diluant	MP+AN	GHȚS control	Semen	GHȚS + Diluant 3,%				
					0,2	0,4	0,6	0,8	0,9
Initial microbiological assessment (fresh semen)									
Total viable count (TVC),UFC/ml	0	3.4×105	5.8×103	4.1×104	6.2×104	5.8×103	3.9×103	3.0×103	2.0×102
E. coli, UFC/ml	0	0	1.1×103	3.2×104	1.7×103	1.1×103	4.2×102	2.0×102	0
Staphylococcus spp., UFC/ml	0	0	0	0	0	0	0	0	0
Fungal count, UFC/ml	0	1.9×104	4.1×102	1.6×103	2.9×102	2.7×102	2.2×102	1.9×102	1.0×102
Microbiological assessment on Day 5									
Total viable count, UFC/ml	0	2.2×105	3.3×102	2.6×104	5.4×103	3.7×103	2.3×103	1.1×103	1.0×102
E. coli, UFC/ml	0	0	2.9×102	6.6×103	4.1×102	4.8×102	3.0×102	0	0
Staphylococcus spp., UFC/ml	0	0	0	0	0	0	0	0	0
Fungal count, UFC/ml	0	1.1×105	2.3×102	1.1×103	2.8×102	2.2×102	2.1×102	2.0×102	1.0×102

Regarding TVC, the control semen sample exhibited an initial microbial count of 5.8×10^3 CFU/mL, which decreased to 3.3×10^2 CFU/mL after five days of storage. In the experimental treatments, microbial counts generally decreased with increasing concentrations of the biologically active supplement, although this trend was not uniform across all concentrations. The most favorable results were obtained at a concentration of 0.9%, where TVC was 2.0×10^2 CFU/mL initially and 1.0×10^2 CFU/mL after five days of storage. These findings indicate that the highest supplementation concentration was associated with the lowest total microbial counts among the evaluated semen samples.

The analysis of *E. coli* revealed differences between the control and experimental treatments. In fresh semen, the control sample exhibited a count of 1.1×10^3 CFU/mL, whereas *E. coli* was not detected in the treatment supplemented with 0.9%. After five days of storage, the count in the control sample decreased to 2.9×10^2 CFU/mL, while *E. coli* was not detected in the treatments containing 0.8% and 0.9% of the biologically active supplement. These results suggest that higher supplementation concentrations may contribute to limiting detectable *E. coli* contamination during semen storage.

No detectable *Staphylococcus spp.* were recorded in any of the analyzed treatments, either initially or after the storage period. This finding indicates that contamination by this bacterial group remained below the detection limit of the applied microbiological method throughout the experimental period.

Regarding fungal contamination, lower counts were observed in several supplemented treatments compared with the control. The initial fungal count in the control semen sample was 4.1×10^2 CFU/mL, decreasing to 2.3×10^2 CFU/mL after five days of storage. The lowest fungal counts were recorded at a supplementation concentration of 0.9%, reaching 1.0×10^2 CFU/mL both initially and after storage. These findings indicate a more favorable fungal contamination profile at the highest supplementation concentration.

Overall, the results suggest that Extender 3 supplemented with biologically active compounds may contribute to maintaining the microbiological quality of boar semen during hypothermic storage. Among the evaluated experimental treatments, supplementation concentrations of 0.8–0.9% were associated with favorable

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microbiological characteristics, particularly lower total viable counts and reduced or undetectable *E. coli* counts. The 0.9% treatment exhibited the lowest total viable and fungal counts, while *Staphylococcus spp.* remained undetected in all samples throughout the storage period. These observations support further investigation of the potential protective role of the biologically active supplement in boar semen preservation.

Microbiological Evaluation of Boar Semen Stored in Extender 4

As a continuation of the investigations into the effects of experimental extender formulations on boar semen quality, the microbiological characteristics of semen samples preserved in Extender 4 were also evaluated. Microbiological assessment complements the evaluation of sperm functional and structural parameters, as maintaining a favorable microbiological environment is an important prerequisite for preserving semen quality during storage.

During hypothermic storage, spermatozoa are exposed to changes in the extracellular environment, while the presence and proliferation of microorganisms may accelerate semen quality deterioration through nutrient depletion, the production of potentially toxic metabolites, changes in pH, and increased oxidative stress. These processes may adversely affect plasma membrane integrity, acrosomal integrity, and sperm motility, thereby reducing the fertilizing potential of stored semen.

In this context, the use of semen extenders supplemented with biologically active compounds aims not only to provide cellular protection but also to limit the proliferation of undesirable microorganisms. Monitoring microbiological parameters allows the performance of experimental extender formulations to be assessed and helps identify treatments that provide favorable conditions for prolonged semen storage (Table 4).

Table 4. Microbiological composition of boar semen and the semen preservation medium (CFU/mL)

Microorganisme	Diluant	GHȚS+IMB-2(%)	Martor GHȚS	GHȚS + Diluant 4-2(%)				
				0,2	0,4	0,6	0,8	0,9
Initial assessment (fresh semen)								
Total viable coun, UFC/ml	0	3.2×10^5	6.1×10^3	2.0×10^4	6.5×10^3	6.9×10^3	4.1×10^3	2.5×10^2
E. coli, UFC/ml	0	0	1.3×10^3	3.5×10^3	1.8×10^3	1.0×10^2	3.8×10^2	0
Staphylococcus spp., UFC/ml	0	0	0	0	0	0	0	0
Fungal count, UFC/ml	0	2.1×10^4	4.5×10^2	1.0×10^2	3.0×10^2	2.8×10^2	2.3×10^2	1.2×10^2
After five days of storage								
Total viable coun, UFC/ml	0	2.2×10^5	3.6×10^2	5.9×10^3	5.2×10^3	3.5×10^3	2.2×10^3	1.5×10^2
E. coli, UFC/ml	0	0	3.1×10^2	7.5×10^2	4.0×10^2	4.5×10^2	2.8×10^2	0
Staphylococcus spp., UFC/ml	0	0	0	0	0	0	0	0
Fungal count, UFC/ml	0	1.1×10^5	2.5×10^2	7.8×10^2	3.0×10^2	2.3×10^2	2.0×10^2	1.1×10^2

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For Extender 4, the main microbiological parameters of stored boar semen samples were evaluated in comparison with the control treatment. The investigation focused on changes in total viable count (TVC), the dynamics of selected indicator microorganisms, the occurrence of *Staphylococcus spp.*, and the level of fungal contamination. The results provide a basis for assessing the influence of different supplementation concentrations on the microbiological stability of boar semen and identifying the experimental formulation associated with the most favorable conditions for maintaining semen quality during storage (Table 4).

The analysis of microbiological parameters in boar semen stored in an extender supplemented with biologically active compounds revealed differences in microbiological stability among the experimental treatments. The main microbiological parameters evaluated were the total viable count (TVC), *Escherichia coli*, *Staphylococcus spp.*, and fungal counts, assessed both initially and after five days of storage.

Regarding TVC, the control semen sample exhibited an initial microbial count of 6.1×10^3 CFU/mL, which decreased to 3.6×10^2 CFU/mL after five days of storage. A reduction in microbial counts during storage was also observed in the experimental treatments, particularly at higher concentrations of the biologically active supplement. The most favorable results were obtained at a concentration of 0.9%, where TVC was 2.5×10^2 CFU/mL initially and 1.5×10^2 CFU/mL after five days. These findings indicate that the highest supplementation concentration was associated with the lowest total microbial counts among the evaluated semen samples.

The analysis of *E. coli* revealed differences in bacterial contamination among the experimental treatments. In the control semen sample, the initial *E. coli* count was 1.3×10^3 CFU/mL, decreasing to 3.1×10^2 CFU/mL after five days of storage. In the experimental treatments, *E. coli* counts varied according to the supplementation concentration. Notably, *E. coli* was not detected in the treatment containing 0.9% of the biologically active supplement, either initially or after five days of storage. This finding suggests that the highest supplementation concentration may contribute to limiting detectable *E. coli* contamination in stored boar semen.

Regarding *Staphylococcus spp.*, no detectable counts were recorded in any of the analyzed treatments, either initially or after five days of storage. This finding indicates that contamination by this bacterial group remained below the detection limit of the applied microbiological method throughout the experimental period.

The evaluation of fungal contamination also revealed differences among the treatments. In the control semen sample, the initial fungal count was 4.5×10^2 CFU/mL, decreasing to 2.5×10^2 CFU/mL after five days of storage. The lowest fungal counts were recorded at a supplementation concentration of 0.9%, reaching 1.2×10^2 CFU/mL initially and 1.1×10^2 CFU/mL after storage. These results indicate that the highest supplementation concentration was associated with lower fungal contamination compared with the control sample.

Overall, the results suggest that Extender 4 supplemented with biologically active compounds may contribute to maintaining the microbiological quality of boar semen during hypothermic storage. Among the experimental treatments, the most favorable microbiological profile was observed at a supplementation concentration of 0.9%, which was characterized by the lowest total viable and fungal counts, the absence of detectable *E. coli*, and no detectable *Staphylococcus spp.* throughout the storage period. These findings support the potential beneficial role of biologically active supplements in semen

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preservation, although further investigations are required to confirm their antimicrobial activity and effects on sperm functional characteristics.

CONCLUSIONS

1.The microbiological findings indicate that supplementation of semen extenders with biologically active compounds may contribute to maintaining the microbiological stability of boar semen during 96 hours of hypothermic storage. The experimental formulations exhibited differences in microbial contamination levels depending on the extender composition and supplementation concentration. Several supplemented treatments showed favorable microbiological characteristics, suggesting the potential protective role of biologically active compounds in semen preservation.

2.The assessment of total viable count (TVC) revealed that the effectiveness of the experimental semen extenders varied according to the concentration of the biologically active supplements. The most favorable results were generally observed at higher supplementation concentrations (0.6–0.9%). In selected experimental treatments, TVC values as low as 1.0×10^2–2.5×10^2 CFU/mL were recorded, indicating a comparatively low microbial load. However, the extent of microbial reduction differed among the experimental formulations and relative to their respective controls.

3.Supplementation with biologically active compounds was associated with differences in the counts of selected indicator microorganisms, particularly *Escherichia coli*, as well as in fungal contamination levels during storage. In certain experimental treatments, *E. coli* was not detected after the storage period, suggesting that some supplemented formulations may help limit bacterial contamination. These observations indicate potential antimicrobial properties that require further experimental confirmation.

4.Staphylococcus spp. were not detected in the analyzed samples, while *Enterococcus spp.* were not detected in the reported assessments. Lower counts of *Bacillus spp.*, *Clostridium spp.*, and fungi were observed in selected experimental treatments, indicating that the effects of biologically active supplementation differed among microbial groups. These findings support further investigation of supplemented semen extenders as a potential approach to improving microbiological control during boar semen preservation for reproductive biotechnology applications.

5.Overall, the microbiological evaluation identified supplementation concentrations of 0.6–0.9% as promising for maintaining the microbiological quality of stored boar semen, depending on the extender formulation. Selected treatments exhibited TVC values of 1.0×10^2–2.5×10^2 CFU/mL, reduced or undetectable *E. coli* counts, and comparatively low fungal contamination, while *Staphylococcus spp.* remained undetected. The results suggest that biologically active compounds may contribute to microbiological stability during hypothermic storage, although additional studies are necessary to establish their antimicrobial mechanisms and effects on sperm viability, functional integrity, and fertilizing capacity.

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