USE OF LYSOZYME AND NISIN AS BACTERIOSTATIC AGENTS IN DOG SEMEN PRESERVATION – PRELIMINARY STUDIES

Oksana Pulkowska-Bluj[1], Monika Trzcińska[1], Paweł Stefanowicz[2]

[1]National Research Institute of Animal Production, Department of Reproductive Biotechnology and Cryoconservation, 32-083 Balice near Kraków, Poland
[2]Private veterinary practice: RETINA Veterinary Clinic, 3 A. Szyllinga St., 31-444 Kraków, Poland
E-mail: oksana.pulkowska@iz.edu.pl

ORCID
Oksana Pulkowska-Bluj: https://orcid.org/0009-0002-3149-1976
Monika Trzcińska: https://orcid.org/0000-0001-9758-1304

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Abstract

The aim of the study was to evaluate the bacteriostatic efficacy of nisin (Niz) and lysozyme (Liz) and their effects on the quality of dog semen during short-term storage. Collected ejaculates were diluted in a Tris-citrate-fructose extender supplemented with hen egg yolk (TCF-EY). Antibiotics (benzylpenicillin 0.06 g/100 ml + streptomycin 0.1 g/100 ml) were added to TCF-EY to obtain the control group (Antb), while bacteriostatic agents were added to obtain two experimental groups: Niz (nisin 50 µg/ml + EDTA 1.25 µg/ml) and Liz (lysozyme 500 µg/ml). Semen was stored at 4°C, and after 48 and 96 hours the following parameters were analyzed: motility, morphology, plasma membrane integrity, and chromatin structure. Bacterial culture and antibiogram tests were also performed for samples from each ejaculate. Nisin and lysozyme showed bacteriostatic activity comparable to that of the routinely used antibiotics. Enterobacteriaceae isolated from the samples were resistant to amoxicillin with clavulanic acid. In terms of semen quality, the Niz and Liz treatments did not significantly affect motility, morphology, plasma membrane integrity, or sperm chromatin structure after 48 and 96 hours of storage. The results suggest that nisin and lysozyme may constitute effective alternatives to conventional antibiotics for short-term storage of dog semen.

Keywords: canine semen, cooling, nisin, lysozyme, canine semen preservation, bacteriostatic agents

Introduction

Semen cooling is one of the most commonly used and effective methods for maintaining the fertilizing capacity of spermatozoa during short-term storage. To preserve appropriate sperm quality parameters, semen is diluted in suitable extenders which, in addition to protective, stabilizing and energy-providing components, also contain antibiotics intended to limit bacterial proliferation during semen storage (Schäfer-Somi et al., 2022).

Bacteriospermia in dogs is common and is observed in more than 80% of examined ejaculates, including those from clinically healthy individuals (Agudelo-Yepes et al., 2023).

The main sources of microbial contamination of semen are the external environment and urethral microflora. Contamination may be further increased by insufficient hygiene during ejaculate collection, which clearly emphasizes the importance of maintaining appropriate standards during semen collection (Agudelo-Yepes et al., 2023). Antibiotics are used in semen extenders to ensure the microbiological safety of stored biological material intended for insemination, which is also frequently traded internationally (Peña et al., 2006). The mechanism of action of antibiotics used in semen preservation is based mainly on bacteriolysis resulting from disruption of bacterial cell-wall synthesis. This process leads to bacterial lysis and the release of endotoxins, including lipopolysaccharides, which may adversely affect sperm quality parameters (Santos and Silva, 2020; Ros-Santaella et al., 2024). Moreover, adding antibiotics to semen may disturb the microbiome of the female reproductive tract, which plays a key role in maintaining local protection against infection (Agudelo-Yepes et al., 2023).

The increasing resistance of bacteria to antibiotics remains a major challenge in veterinary medicine. This phenomenon may also be intensified by the excessive use of antibiotics in semen preservation. Consequently, current research focuses on evaluating the effects of probiotics and natural antimicrobial substances, including antimicrobial peptides and proteins (AMPPs), on semen quality and their potential use in the preservation of semen from livestock and companion animals (Miao et al., 2024; Ros-Santaella et al., 2024).

AMPPs are a group of molecules capable of immunostimulation and constitute a first line of defense against pathogens, including both Gram-positive and Gram-negative bacteria and some viruses. In recent years, the mechanisms of action of several substances naturally present in mammalian semen have been characterized, including lysozyme, lactoferrin, phospholipase A2 (PLA2), and secretory leukocyte protease inhibitor (SLPI). These substances exhibit bacteriostatic activity and have a positive effect on semen quality (Miao et al., 2024). Lysozyme (also known as muramidase) was the first antibacterial enzyme to be discovered. It occurs in body fluids such as tears, milk, semen and saliva, as well as in various tissues and cells, and is part of the innate mammalian defense against pathogens (Miao et al., 2024; Ros-Santaella et al., 2025). Nisin is a polypeptide synthesized by *Lactococcus lactis* subsp. *lactis*, and its bacteriostatic activity has been used since the 1950s for food preservation, mainly in meat and dairy products. This bacteriocin is also the subject of biomedical research on cancer therapy and bacterial infections (Ros-Santaella et al., 2024). In a study on boar semen, both peptides significantly reduced bacterial counts without adversely affecting sperm quality during 48 hours of storage at 17°C (Ros-Santaella et al., 2024).

These findings prompted the authors of the present study to investigate the usefulness of selected AMPPs for short-term storage of dog semen. The main objective was to assess the bacteriostatic activity of nisin and lysozyme and their effects on sperm quality parameters during storage of dog semen at 4°C for 48 and 96 hours.

Materials and methods

Semen collection and preservation

The experiment was conducted on three ejaculates collected from three stud dogs of different breeds: dog no. 1 – Yakutian Laika, 6 years old; dog no. 2 – Polish Greyhound, 2 years old; dog no. 3 – Border Collie, 2 years old. All dogs came from private breeding kennels and were registered with the Polish Kennel Club. The animals were fed a complete, balanced commercial dry diet and had not received oral or topical antibiotics for at least 30 days before semen collection. The inclusion criterion was proven male fertility, documented by previous successful matings or inseminations resulting in multiple-pup pregnancies.

Before semen collection, the penile area was thoroughly cleaned with chlorhexidine solution and the prepuce was then rinsed with sterile saline to minimize the risk of microbial

contamination. Olfactory stimulation of the males was performed using vaginal swabs collected from bitches in estrus, which elicited a physiological erectile response. Ejaculates were collected by approximately one minute of massage of the *bulbus glandis* while wearing sterile disposable gloves. The sperm-rich fraction of the ejaculate was collected directly into a sterile tube connected to a sterilized funnel, ensuring aseptic conditions throughout the procedure. Ejaculates were included in the study if they met the following quality criteria: total sperm motility ≥90%, sperm concentration ≥200×10^6/ml, and normal sperm morphology ≥70%. Ejaculates were centrifuged (720 × g, 4 min), and spermatozoa were then diluted in a Tris-citrate-fructose extender supplemented with hen egg yolk (TCF-EY) containing: 3.025 g TRIS; 1.7 g citric acid; 1.25 g fructose; and 20% hen egg yolk (Peña et al., 2006). Each diluted ejaculate was divided into three groups:

1. Control (Antb): TCF-EY supplemented with routinely used antibiotics — benzylpenicillin (0.06 g/100 ml) and streptomycin (0.1 g/100 ml),
2. Liz: TCF-EY supplemented with lysozyme (500 µg/ml),
3. Niz: TCF-EY supplemented with nisin (50 µg/ml) and EDTA (1.25 µg/ml).

EDTA was added to the Niz group to increase bacteriostatic efficacy against Gram-negative bacteria (Ros-Santaella et al., 2024). After preparation, the samples were placed in a refrigerated chamber and stored at 4°C for 96 hours.

Methods for semen quality assessment

Semen was analyzed immediately after collection (0 h) and after 48 and 96 hours of storage at 4°C. Sperm motility and concentration were assessed using a CASA system (Computer Assisted Semen Analysis, S.C.A. V5.1, Microptic, Barcelona, Spain). The percentage of all motile spermatozoa (total motility, TM) and the percentage of spermatozoa showing normal progressive movement (progressive motility, PM) were determined. Sperm morphology was evaluated in at least 200 spermatozoa from each sample. Analyses were performed with a light microscope (Nikon Eclipse 200, Tokyo, Japan) at 100× magnification under immersion. Smears were prepared manually and stained using the Diff-Quick® method (RAL Diagnostics, Martillac, France). Assessment was conducted according to the morphological classification of sperm defects proposed by Brito et al. (2025).

Plasma membrane integrity and sperm chromatin structure were assessed by flow cytometry (CytoFLEX, Beckman Coulter, USA) according to the methodology described by Gogol and Trzcińska (2020). Plasma membrane integrity was analyzed using the Live/Dead Sperm Viability Kit (Thermo Fisher Scientific, USA). Sperm chromatin structure was assessed using the Sperm Chromatin Structure Assay (SCSA), which enables detection of abnormalities in sperm chromatin condensation and stability.

Bacteriological examination

Samples of fresh semen and semen stored for 48 and 96 h were collected using swabs with charcoal transport medium and sent to a commercial laboratory (Vetlab, Poland). Samples were inoculated onto the following microbiological media: Columbia agar with 5% sheep blood, MacConkey agar, Columbia CNA agar with 5% sheep blood, and chocolate agar. Incubation was carried out at 35–37°C for 48 hours under aerobic conditions (Columbia agar with 5% sheep blood, MacConkey agar and Columbia CNA agar with 5% sheep blood) or in an atmosphere with an elevated CO2 concentration (chocolate agar), using a CO2 atmosphere generator (Gen Compact, bioMérieux, Marcy l'Etoile, France). Bacterial growth was evaluated after 24 and 48 hours of incubation. The resulting bacterial colonies were then identified using a MALDI-TOF Biotyper Sirius IV system (Billerica, MA, USA).

Results

The collected ejaculates had concentrations sufficient to obtain more than 100×10^6 spermatozoa in each of the extenders tested. The basic parameters of microscopic and macroscopic assessment of the ejaculates are presented in Table 1.

Table 1. General characteristics of the sperm-rich fraction of ejaculates qualified for the study

Dog no.	Semen characteristic			
	pH	Concentration ($\times10^6$/ml)	Volume (ml)	Total sperm count ($\times10^6$)
1	6,2	215,16	2,5	537,90
2	6,7	325,28	1,5	487,92
3	6,5	407,25	4,5	7832,63

Semen motility analysis showed total motility (TM) exceeding 80% in ejaculates from each individual, regardless of storage time and in all extenders tested. Progressive motility (PM) showed a clear downward trend as sample cooling time increased. This parameter also showed a larger standard deviation, indicating greater variability among the individuals examined: 52.06–74.70% in fresh semen, 31.55–53.69% after 48 h of storage, and 10.54–68.15% after 96 h of storage. The highest percentage of dead spermatozoa (33.48%) was recorded after 48 hours of storage in the Liz sample from dog no. 3. Among morphological defects, single and multiple coiling of the sperm tail predominated after both 48 and 96 hours of storage. Analysis of individual results showed that a decline in the percentage of morphologically normal spermatozoa (below 70%) was observed only in dog no. 1 after 96 hours of storage in the Antb and Niz groups. The effects of lysozyme and nisin on motility, morphology, plasma membrane integrity, and chromatin structure during storage of dog semen at 4°C are presented in Table 2.

Table 2. Semen quality parameters in dogs: fresh semen and semen preserved with antibiotics, lysozyme, or nisin

Cooling time	Extender	Dog no.	Motility %		SYBR+/PI− %	SCSA %	% of morphologically normal spermatozoa
			TM	PM	Live	DFI	
0 h	NN	1	98,82	60,65	85,04	2,74	88,15
		2	92,88	52,06	86,12	2,66	85,49
		3	99,78	74,70	85,48	5,01	78,09
		(mean ± SD)	97,16±3,74	62,47±11,43	85,55±0,54	3,38±1,41	83,91±5,21
48 h	Antb	1	89,68	36,88	72,01	2,59	87,93
		2	98,45	53,69	73,35	1,37	85,71
		3	94,17	31,55	48,73	2,25	80,47
		(mean ± SD)	94,10±4,39	40,71±11,56	64,70±13,84	2,07±0,63	84,70±3,83
	Liz	1	83,24	36,07	79,92	2,12	85,34
		2	93,36	50,79	79,70	1,22	80,87
		3	93,61	33,62	62,27	2,26	83,70
		(mean ± SD)	90,07±5,92	40,16±9,29	73,96±10,13	1,87±0,56	83,31±2,26
	Niz	1	88,54	33,98	73,29	2,80	82,73
		2	93,36	50,79	79,72	1,05	83,54
		3	94,94	42,56	48,58	2,74	72,91
		(mean ± SD)	92,28±3,33	42,44±8,41	67,20±16,44	2,20±0,99	79,73±5,92
96 h	Antb	1	97,63	23,56	68,84	2,47	66,44
		2	99,21	45,80	72,12	1,00	81,63
		3	87,44	31,35	50,34	2,50	77,66
		(mean ± SD)	94,76±6,39	33,57±11,28	63,77±11,74	1,99±0,86	75,24±7,88
	Liz	1	87,80	10,54	67,00	2,75	70,77
		2	97,81	68,15	75,28	2,54	77,39
		3	85,90	21,80	53,71	2,35	81,62
		(mean ± SD)	90,50±6,40	33,50±30,53	65,33±10,88	2,55±0,20	76,60±5,47
	Niz	1	87,56	14,54	70,19	2,61	60,23
		2	98,77	52,90	71,60	0,85	82,35
		3	92,57	31,92	52,58	2,23	72,91
		(mean ± SD)	92,97±5,62	33,12±19,21	64,79±10,60	1,90±0,93	71,83±11,10

NN – fresh raw semen, DFI – DNA fragmentation index, SD – standard deviation

Table 3. Details of culture test results and antibiograms performed for the analyzed semen

Dog no.	Group (storage time)	Cultured bacteria	Growth on the medium	Antibiogram resistance
1	0h Antb (48 h) Niz (48 h) Liz (48 h) Antb (96 h) Niz (96 h) Liz (96 h)	*Escherichia coli*	(+)	amoxicillin + clavulanic acid
3	Liz (48 h)*	*Pseudomonas tolaasii*	(+)	none
3	Antb (96 h) Niz (96 h)	*Pseudomonas synxantha*	(+)	ceftazidime
3	Liz (96 h)	*Pseudomonas extremorientalis*	(+)	none

Interpretation of bacterial growth intensity: (+) scanty, (++) moderate, (+++) abundant.
*Growth was obtained after incubation in an enrichment medium; no bacterial growth was observed from the direct culture without prior enrichment.

The culture results are presented in Table 3. In cultures of material collected from dog no. 2, no aerobic bacterial growth was observed either in fresh semen samples or in samples cooled in the different extenders. Consequently, no antibiogram was performed for any sample from this individual.

Discussion

The use of extenders during semen preservation is crucial for maintaining fertilizing capacity. Each extender component plays an important role in mitigating the adverse effects of cold shock, providing optimal osmotic pressure and protecting the sperm plasma membrane. The addition of proteins to newly developed extenders requires evaluation of their effects on semen quality during preservation. The lack of an adverse effect of lysozyme on sperm assessment parameters may result from the physiological presence of this enzyme in mammalian body fluids (Miao et al., 2024). Nisin, in contrast, is a bacteriostatic agent synthesized by the prokaryote *Lactococcus lactis* subsp. lactis, and its cytotoxicity has been excluded in numerous in vivo studies (Ros-Santaella et al., 2024; Shin et al., 2016). The results presented here suggest that the proposed concentrations of the tested bacteriostatic agents do not adversely affect semen quality parameters and are effective in exerting bacteriostatic activity.

The microbiological analysis of fresh semen from the studied dogs showed that no bacterial growth was observed in one of the three ejaculates analyzed. For comparison, in a study conducted on a larger population, 36.5% of samples also showed no bacteria, confirming substantial individual differences in the presence and composition of the normal canine semen microflora (Sorkytė et al., 2024). By contrast, studies by Agudelo-Yepes et al. (2023) and Lechner et al. (2023) demonstrated the consistent presence of microorganisms in all analyzed samples from dogs.

In fresh semen from healthy dogs, the microorganisms most frequently isolated and considered part of the physiological microflora of the reproductive tract include *Staphylococcus spp.*, *Streptococcus spp.*, and *Escherichia coli* (Sorkytė et al., 2024). The presence of these bacteria is not always associated with pathology, and their detection in an ejaculate may reflect the microflora naturally inhabiting the urethra or preputial surface. In one male (dog no. 3), bacteria of the genus *Pseudomonas* were detected; these are rarely isolated from canine semen (Sorkytė et al., 2024). Such bacteria may be potentially pathogenic because they produce toxins and lipopolysaccharides (LPS), which can damage sperm plasma membranes, induce oxidative stress, and reduce sperm fertilizing capacity (Ros-Santaella et al., 2024). The dog in whose semen this bacterial genus was detected showed no deterioration in any semen parameter, either immediately after collection or during storage. This observation confirms that scant (+) growth

of *Pseudomonas* in semen does not necessarily result in clinical signs or reduced ejaculate quality. According to current scientific reports, dogs may act as reservoirs and sources of transmission of bacteria resistant to β-lactam antibiotics, including strains belonging to the order *Enterobacterales* (de Mendieta et al., 2024). This represents a significant risk, particularly where companion animals are in close contact with humans. The finding emphasizes the need for continued monitoring of antimicrobial resistance among microorganisms isolated from semen and for consideration of alternative antimicrobial agents such as AMPPs in order to limit selection of drug-resistant strains.

The results of the present experiment also showed that adding the bacteriostatic agents at the proposed concentrations did not reduce the values of the analyzed sperm quality parameters. Semen motility results in the Liz and Niz groups were comparable to those obtained in the control group (Antb) at each cooling time. After 48 hours of semen cooling in the commercial CaniPlus Chill LT extender (containing antibiotics), Sugai et al. (2024) recorded more than 70% total and progressive sperm motility, more than 60% spermatozoa with intact plasma membranes (live cells), and more than 40% morphologically normal spermatozoa. Rota et al. (1999) used a TCF-EY extender (containing benzylpenicillin and dihydrostreptomycin sulfate) for short-term semen storage and obtained total motility of 70.4 ± 15.4% after 48 hours and 53.6 ± 20.1% after 96 hours. As in our study, the authors also used fluorescent staining to assess plasma membrane integrity and obtained 78.4 ± 6.4% live spermatozoa after 48 hours and 72.1 ± 10.4% after 96 hours of storage at 4°C.

Comparison of our results with the literature shows similar values for both total sperm motility, 98.45–83.24% after 48 hours and 99.21–85.90% after 96 hours, and the percentage of live spermatozoa with intact plasma membranes, 79.92–48.58% after 48 hours and 75.28–50.34% after 96 hours of cooling. The cited studies did not analyze changes in sperm chromatin structure. Our results showed that in every individual, in each treatment group and at every stage of semen storage, the DNA fragmentation index (DFI) did not exceed 15%, indicating good semen quality (Niżański et al., 2012).

The preliminary results demonstrate the bacteriostatic potential of the proteins used in preserved dog semen. The public-health threat posed by bacterial resistance indicates the need to continue studies using AMPPs in a larger group of animals. Reducing the use of antibiotics in extenders is also justified by the fact that bacterial growth and the type of bacteria detected in samples do not always indicate deterioration of semen quality or disease of the male reproductive tract (Goericke-Pesch et al., 2006; Sorkytė et al., 2024). In view of these issues, the authors plan to continue the research by extending the microbiological analysis to determine the number of viable bacteria capable of forming colonies on solid medium (CFU – Colony Forming Units/ml). The purpose of this quantitative analysis will be to precisely assess the bacteriostatic efficacy of lysozyme and nisin by comparing their effects with those of extenders containing antibiotics.

Conclusions

The addition of lysozyme (500 µg/ml) and nisin (50 µg/ml) combined with EDTA (1.25 µg/ml) to extenders used for cooling dog semen showed bacteriostatic activity comparable to that of commonly used antibiotics — benzylpenicillin (0.06 g/100 ml) and streptomycin (0.1 g/100 ml). The presence of the bacteriostatic peptides did not adversely affect sperm motility or morphology parameters or the results of cytometric analyses, regardless of semen storage time. In conclusion, these preliminary results indicate that nisin and lysozyme may constitute effective alternatives to conventional antibiotics used in extenders for short-term storage of dog semen.

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Oksana Pulkowska-Bluj, Monika Trzcińska, Paweł Stefanowicz

SUMMARY

The present study aimed to evaluate the bacteriostatic effectiveness of nisin (Niz) and lysozyme (Liz), as well as to assess their impact on the quality of short-term stored dog semen. Each ejaculate was evaluated immediately after collection (0 h), diluted in a Tris-citrate-fructose egg yolk (TCF-EY) extender, and divided into the following groups: "Antb" (benzylpenicillin 0.06 g/100 ml + streptomycin 0.1 g/100 ml); "Niz" (nisin 50 µg/ml + EDTA 1.25 µg/ml); and "Liz" (lysozyme 500 µg/ml). Mean values of sperm motility, morphology, plasma membrane integrity and chromatin structure were compared between groups. Bacteriological examination and antibiograms were performed for samples after 48 and 96 hours of storage at 4°C. Microbiological testing showed that: not all ejaculates contained aerobic bacteria, the addition of Niz and Liz exerted bacteriostatic effects comparable to those of conventional antibiotics, bacteria from the *Enterobacteriaceae* family isolated from the samples were resistant to amoxicillin with clavulanic acid. Furthermore, the presence of *Enterobacter* spp. in semen may reduce its ability to maintain quality during cooling. The obtained results suggest that nisin and lysozyme may serve as an alternative to conventional antibiotics for short-term storage of canine semen.

Keywords: canine semen, cooling, nisin, lysozyme, semen preservation, bacteriostatics