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Comparative study: Efficacy of egg-yolk *vs* soy lecithin-based diluent in preservation of chilled bovine semen – Bacteriology and sperm quality

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Abstract: This study compared the efficacy of the egg yolk (EY) and soy lecithin (SL) semen extender during 72-h storage at 4 °C in the presence/absence of streptomycin, penicillin, lincomycin, and spectinomycin. Bull semen samples (*n* = 12) were obtained and stored in either ANDROMED® (SL extender) or TRILADYL CSS® (EY extender). After 24, 48, and 72 h of storage at 4 °C, bacterial colonies (CFU – colony forming units) were grown and counted on tryptic soy and blood agar, and the bacterial isolates were identified using the MALDI-TOF MS Biotyper. Sperm motility, mitochondrial membrane potential (MMP), membrane and acrosome integrity, sperm DNA fragmentation, and reactive oxygen species (ROS) production were analysed. The results showed that the type of semen diluent could be crucial for the efficacy of used antibiotics, since significantly decreased bacterial occurrence following 48 h or no bacterial growth after 72 h was recorded in the SL-based groups. Even the SL medium itself did not favour bacterial growth, as significantly decreased bacterial load was observed after 48 h ($P < 0.01$) and 72 h ($P < 0.000\ 1$). In contrast, the bacterial load in the EY medium without antibiotics significantly increased ($P < 0.05$). In contrast, an improved ability to preserve sperm quality parameters was observed in EY-extended semen. In particular, sperm motility was significantly higher ($P < 0.000\ 1$ and $P < 0.001$) after 24 h in each EY-based group in comparison to the control (Ctrl), whereas among the SL-based groups, only the spectinomycin-supplemented group presented with a significant motility improvement ($P < 0.01$) when compared to the Ctrl. Although the EY semen extender provided enhanced sperm quality preservation during the 72-h storage, in the future, the SL medium composition should be improved to match the sperm preservation ability of the EY medium while maintaining its microbial safety.

Keywords: bacteria; bull semen extender; cooled spermatozoa; liquid semen storage

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Semen extenders are crucial in cattle breeding to maintain sperm quality and maximise bull breeding potential. They prolong spermatozoa survival during cryopreservation and intrauterine insemination, overcoming changes in osmotic pressure, pH, and reactive oxygen species (ROS) production (Phillips 1939; Raheja et al. 2018).

Insemination doses can be stored in liquid form for three days or in frozen form, with quality maintained even after several years. Egg yolk, 1–2.5% of the extender, offers the best survivability for sperm for up to 72 h at 5 °C (Sahni and Mohan 1988). Membrane stabilisers, such as cholesterol-containing cyclodextrins, soy lecithin, and docosahexaenoic acid, are used to improve sperm quality after thawing. However, egg yolks carry risks of microbial contamination and are difficult to standardise owing to variations among producers (van Wagtendonk-de Leeuw et al. 2000). Soy lecithin, a primary source of lipoproteins and fatty acids, could become a preferred substitute for egg yolk in semen extender formulations (Gadea 2003; Oke et al. 2010; Simonik et al. 2019; Miguel-Jimenez et al. 2020). This comparative study aimed to determine the effectiveness of egg yolk (EY)-based *vs* soy lecithin (SL)-based semen extenders for 72 h in liquid semen stored at 4 °C with or without antibiotic supplementation.

MATERIAL AND METHODS

Semen collection

Samples (n = 12) were collected from 12 sexually mature Holstein-Friesian breeding bulls on a regular collection schedule at a nearby insemination station. Sample collection was conducted in accordance with institutional and government regulations (Regulation of the government No. 377/2012). A sterilised artificial vagina was used to avoid contamination during collection. Immediately after collection, the samples were transferred to the laboratories of the AgroBioTech Research Centre, SUA in Nitra (Nitra, Slovakia).

Study design

The effectiveness of the selected semen extenders ANDROMED® (without antibiotics; MiniTube, Tiefenbach, Germany) and TRILADYL CSS® (without antibiotics; MiniTube, Tiefenbach, Germany) was tested for 72 hours. Both types of media are appropriate for long- and short-term storage. Both media were prepared according to the manufacturer's recommendations. TRILADYL medium was enriched with dried EY (Sigma-Aldrich, St. Louis, MO, USA). The control group contained spermatozoa diluted with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). The experimental groups were administered the following antibiotics: 500 IU/mL streptomycin, 500 IU/mL penicillin, 150 µg/mL lincomycin, and 300 µg/mL spectinomycin. Antibiotics and their dosages were used following the valid EU directive 88/407 (EC 1988)to control the bacterial growth of preserved semen. All antibiotics were purchased from Sigma-Aldrich. Fresh semen samples from each group were diluted at a ratio of 1 : 40. The motility, mitochondrial membrane potential, membrane and acrosome integrity, DNA integrity, ROS production, and bacterial composition of the sperm samples were analysed after 24, 48, and 72 h of incubation at 4 °C.

Bacterial quantification and identification

Aliquots of semen samples were cultured on blood and tryptic soy agar (Oxoid, Basingstoke, UK) under aerobic conditions (37 °C; 24–48 h). The colonies were counted and purified using the four-way streak plate method. Bacterial identification was carried out using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) Biotyper, following the methodology described previously (Duracka et al. 2021).

Assessment of sperm motility

Sperm motility was assessed using computer-assisted sperm analysis (CASA; v.14.0; TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, USA). Briefly, 10 µL were placed on a preheated Makler-counting chamber (37 °C, depth 10 µm; Sefi Medical Instruments, Haifa, Izrael). An objective evaluation of sperm motility and concentration was performed on a minimum of 300 cells per sample.

Membrane integrity

Eosin-nigrosin staining was used to analyse sperm integrity. Diluted spermatozoa were mixed with

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eosin and nigrosin at a ratio of 1 : 2 : 2 and spread over a slide. After drying, the sperm cells were examined under a bright-field microscope, and red-stained sperm heads with damaged membranes and white-stained sperm heads with intact membranes were counted (Figure 1). At least 200 cells were counted in each sample.

Acrosome integrity

Sperm acrosome integrity was assessed using a mixture of fast green and rose bengal dyes (Pope et al. 1991). Diluted spermatozoa were stained at a ratio of 1:1 with the staining solution for 70 seconds. Subsequently, the samples were spread on glass slides. After drying, at least 200 spermatozoa with intact or damaged acrosomes were counted under a bright-field microscope (Figure 2).

Mitochondrial membrane potential

The mitochondrial membrane potential was measured using the lipophilic cytofluorimetric dye JC-1 (Cayman Chemical Company, Ann Arbor, MI, USA) which forms aggregates (red fluorescence) in depolarised mitochondrial membranes or remains in monomeric form (green fluorescence) in polarised membranes. This analysis was conducted using previously established protocols (Duracka et al. 2021).

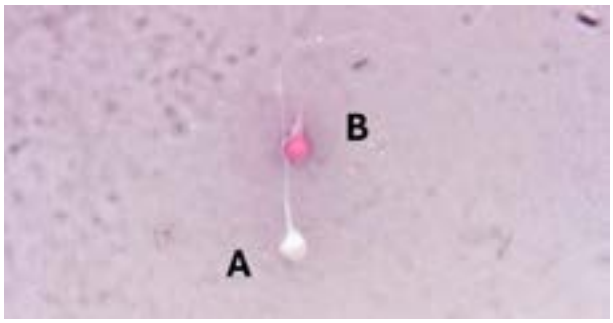

Figure 1. The specific staining pattern of eosin-nigrosin
A = intact sperm membrane; B = damaged sperm membrane

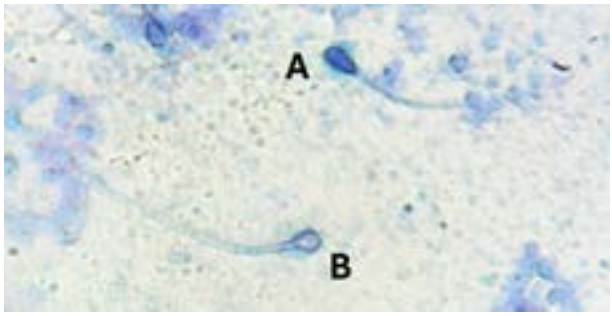

Figure 2. The specific staining pattern of fast green/rose bengal
A = intact sperm acrosome; B = damaged sperm acrosome

Sperm DNA fragmentation assay

Halomax kit (HT-BT40; Halotech DNA, Madrid, Spain) was used to analyse sperm DNA integrity. Twenty million cells per millilitre were diluted in agarose and fixed on a microscopic slide. The samples were incubated for 5 min with lysis solution, washed with distilled water, and fixed with 70, 90, and 100% ethanol for 2 min each. Spermatozoa were stained with SYBR Green in Vectashield, and a minimum of 200 spermatozoa per sample were evaluated for the presence (fragmented DNA) or absence (intact DNA) of a halo (Figure 3).

ROS analysis

ROS production was analysed *via* chemiluminescence using luminol (5 mM; 5-amino-2,3-dihydro-1,4-phthalazinedione) as a probe in the tested samples and negative and positive controls. The blank, negative, and positive controls contained 100 µL of DPBS. Hydrogen peroxide (30%, 9.8 M) was added to the positive controls. All chemicals were purchased from Sigma-Aldrich (St. Louis, USA). The measurements were performed using the Glomax

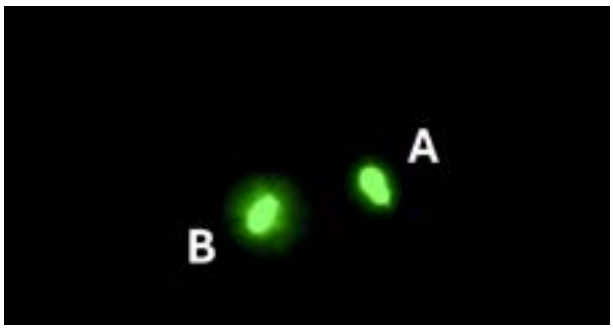

Figure 3. Assessment of Sperm DNA fragmentation *via* Halo Assay Using Halomax Kit and SYBR Green Fluorescence
A = intact sperm DNA without a halo around the sperm head; B = fragmented sperm DNA with a halo around the sperm head

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Multi$^+$ system (Promega, Madison, WI, USA). The results were expressed as relative light units per second per million sperm cells (Duracka et al. 2021).

Statistical evaluation

The data were processed using GraphPad Prism (v.10.1.1; GraphPad Software, La Jolla, USA). One-way analysis of variance (ANOVA) and Dunnett's post-hoc test were performed to compare the data obtained from the experimental groups with those obtained from the control group. The values are presented as the mean ± standard deviation (SD). The significance levels were set at **** ($P < 0.000\ 1$), *** ($P < 0.001$), ** ($P < 0.01$), and * ($P < 0.05$).

RESULTS

Sperm quality following 24 h of storage at 4 °C

The CASA analysis (Table 1) following 24 h showed significantly preserved sperm motility in each EY group, while the highest motility was recorded when spectinomycin (SP) was added (45.60 ± 7.41%; $P < 0.0001$). The spermatozoa stored in EY exhibited nearly double the motility compared to those stored in SL. In the SL group, only the SP-treated group exhibited significantly higher motility ($P < 0.01$) than the control (Ctrl) group. In each experimental group, the mitochondrial membrane potential was significantly preserved compared to that in the control. Among the groups, EY + antibiotics, SL + lincomycin (L), and SL + SP preserved the mitochondrial membrane at the highest levels ($P < 0.000\ 1$). Similar to the results obtained from the JC-1 analysis, sperm membrane integrity was best preserved in the EY + antibiotics, SL + L, and SL + SP groups. The highest percentage of spermatozoa with intact acrosomes was recorded in the EY + SP group ($P < 0.001$). DNA fragmentation was significantly lower when antibiotics were added (except in the SL + P group). The lowest values of DNA fragmentation were observed in the groups administered spectinomycin and lincomycin (only in the EY group). The production of ROS was significantly reduced in each experimental group, whereas the lowest values were recorded in the groups supplemented with spectinomycin and lincomycin and in the EY + ST group.

Bacterial occurrence following 24 h of storage at 4 °C

The prevalence of *Enterococcus faecium* was recorded in the control group after 24 h (Table 2). In addition, *Pseudomonas aeruginosa*, *Neisseria subflava*, *Microbacterium paraoxydans*, and five *Staphylococcus* species were identified.

Table 1. Sperm quality parameters in EY- and SL-based media with/without antibiotics after 24 h at 4 °C

Groups	Motility (%)	MMP (JC-1 units)	Membrane integrity (%)	Acrosome integrity (%)	DNA fragmentation (%)	ROS (RLU/s/10^6 sperm)
Control	8.10 ± 2.37	0.52 ± 0.05	78.00 ± 4.95	83.00 ± 5.43	29.00 ± 4.95	10.36 ± 1.35
EY	37.80 ± 6.10^c	0.68 ± 0.060^c	86.00 ± 2.23^b	90.00 ± 2.40^a	21.00 ± 2.24	8.20 ± 0.86^b
EY + ST	41.30 ± 6.80^c	0.73 ± 0.04^d	88.00 ± 0.83^c	90.75 ± 3.63^a	18.75 ± 2.82^a	7.04 ± 0.63^d
EY + P	44.60 ± 5.03^d	0.71 ± 0.04^d	88.75 ± 0.83^c	91.25 ± 3.96^a	18.28 ± 1.92^a	8.09 ± 0.26^b
EY + L	45.50 ± 5.82^d	0.76 ± 0.06^d	89.25 ± 1.09^c	92.00 ± 3.71^b	17.75 ± 2.09^b	6.51 ± 0.53^d
EY + SP	45.60 ± 7.41^d	0.78 ± 0.05^d	91.25 ± 0.83^c	93.50 ± 3.20^c	15.58 ± 2.30^b	6.35 ± 0.48^d
SL	19.00 ± 4.36	0.63 ± 0.02^a	86.50 ± 2.29	89.00 ± 3.39	20.50 ± 2.29	7.69 ± 0.66^c
SL + ST	21.60 ± 3.01	0.68 ± 0.07^c	87.00 ± 3.08^a	89.60 ± 1.83	20.00 ± 3.08^a	7.67 ± 0.49^c
SL + P	23.50 ± 2.42	0.66 ± 0.06^b	86.25 ± 1.92	88.75 ± 3.35	20.75 ± 2.92	7.89 ± 0.75^c
SL + L	26.60 ± 5.22	0.71 ± 0.04^d	88.00 ± 2.12^c	90.50 ± 2.87^a	19.00 ± 2.12^a	6.65 ± 0.38^d
SL + SP	34.40 ± 4.99^b	0.75 ± 0.04^d	90.25 ± 1.29^c	92.25 ± 4.08^b	16.55 ± 1.29^b	6.42 ± 0.37^d

$^{a–d}$Significant difference *vs* control at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.000\ 1$, respectively

EY – yolk extender; L – lincomycin; MMP = mitochondrial membrane potential; P – penicillin; RLU – relative light units; ROS = reactive oxygen species; SL – soy lecithin extender; SP – spectinomycin; ST – streptomycin

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Table 2. Bacterial occurrence in semen stored in EY- and SL-based media with/without antibiotics after 24 h at 4 °C

Groups	Bacterial species	log_{10} CFU/ml (tryptic soy agar)	log_{10} CFU/ml (blood agar)
Control	*P. aeruginosa* (7), *E. faecium* (11), *S. vitulinus* (2), *S. xylosus* (1), *S. simulans* (1) *S. epidermidis* (1), *N. subflava* (1), *M. paraoxydans* (1), *S. haemolyticus* (1)	1.47 ± 0.45	0.89 ± 0.30
EY	*P. aeruginosa* (7), *E. faecium* (6), *S. vitulinus* (1), *S. xylosus* (1), *S. simulans* (1), *S. epidermidis* (1), *S. haemolyticus* (1), *N. subflava* (1), *M. paraoxydans* (1)	1.50 ± 0.29	1.59 ± 0.48
EY + ST	*P. aeruginosa* (3), *E. faecium* (5), *S. haemolyticus* (1), *M. paraoxydans* (1), *N. subflava* (1)	1.12 ± 0.31	0.79 ± 0.34
EY + P	*P. aeruginosa* (4), *E. faecium* (2)	1.85 ± 0.16	0.66 ± 0.30
EY + L	*P. aeruginosa* (7), *E. faecium* (7), *S. vitulinus* (1)	1.43 ± 0.31	0.51 ± 0.23
EY + SP	*P. aeruginosa* (5), *E. faecium* (11), *S. vitulinus* (1)	0.22 ± 0.15[a]	0.41 ± 0.21
SL	*P. aeruginosa* (6), *E. faecium* (3), *S. vitulinus* (1), *S. xylosus* (1), *S. simulans* (1), *S. epidermidis* (1), *N. subflava* (1)	0.46 ± 0.26	0.80 ± 0.32
SL + ST	*P. aeruginosa* (2), *S. haemolyticus* (1)	0.35 ± 0.24[a]	0.27 ± 0.08
SL + P	*P. aeruginosa* (3), *E. faecium* (1)	0.63 ± 0.28	0.34 ± 0.04
SL + L	*P. aeruginosa* (2), *N. subflava* (1)	0.43 ± 0.27	0.27 ± 0.07
SL + SP	*P. aeruginosa* (1), *E. faecium* (1), *S. haemolyticus* (1)	0.11 ± 0.08[b]	0.11 ± 0.08

[a,b]Significant difference *vs* control at $P < 0.05$, $P < 0.01$, respectively

EY – yolk extender; CFU – colony-forming units; L – lincomycin; P – penicillin; SL – soy lecithin extender; SP – spectinomycin; ST – streptomycin; numbers in parentheses indicates the number of samples positive for a bacteria

A significant decrease ($P < 0.05$) in the bacterial load was observed in the EY extender + spectinomycin group compared to that in the Ctrl group. Treatment of the SL extender with spectinomycin ($P < 0.01$) and streptomycin ($P < 0.05$) significantly inhibited bacterial growth.

Sperm quality following 48 h of storage at 4 °C

The analyses after 48 h (Table 3) revealed that sperm motility remained significantly higher in all EY groups except for the penicillin group ($P < 0.001$ for EY + SP and EY + L; $P < 0.01$ for EY and EY + ST). In contrast,

Table 3. Sperm quality parameters in EY- and SL-based media with/without antibiotics after 48 h at 4 °C

Groups	Motility (%)	MMP (JC-1 units)	Membrane integrity (%)	Acrosome integrity (%)	DNA fragmentation (%)	ROS (RLU/s/10^6 sperm)
Control	4.60 ± 1.22	0.36 ± 0.06	67.75 ± 3.26	72.75 ± 3.41	42.25 ± 3.27	15.08 ± 2.28
EY	28.50 ± 4.87[b]	0.46 ± 0.09	74.50 ± 2.98[b]	79.50 ± 4.21[a]	35.30 ± 2.96[a]	12.28 ± 1.31
EY + ST	26.90 ± 5.69[b]	0.64 ± 0.07[d]	79.00 ± 1.22[c]	84.00 ± 3.63[a]	31.00 ± 1.22[b]	9.41 ± 1.32[b]
EY + P	20.30 ± 4.45	0.53 ± 0.04[b]	81.00 ± 1.58[c]	86.00 ± 3.39[A]	27.40 ± 1.58[d]	12.96 ± 1.24
EY + L	32.00 ± 7.08[c]	0.67 ± 0.04[d]	80.75 ± 2.38[c]	85.20 ± 3.22[b]	27.25 ± 2.38[d]	9.16 ± 1.27[b]
EY + SP	33.10 ± 6.40[c]	0.69 ± 0.03[d]	86.00 ± 2.92[c]	90.50 ± 2.57[c]	24.00 ± 2.91[d]	8.91 ± 0.68[b]
SL	18.40 ± 3.59	0.40 ± 0.04	68.50 ± 2.54	73.50 ± 5.86	33.50 ± 5.67[a]	10.01 ± 1.14[a]
SL + ST	18.90 ± 3.32	0.59 ± 0.05[d]	73.25 ± 3.11[a]	78.25 ± 3.52	26.75 ± 3.11[d]	9.74 ± 0.94[a]
SL + P	16.70 ± 3.40	0.53 ± 0.04[b]	70.83 ± 2.76	78.40 ± 7.88	26.18 ± 2.76[d]	10.71 ± 2.00
SL + L	21.60 ± 4.41	0.63 ± 0.04[d]	77.49 ± 3.07[c]	82.83 ± 3.67[a]	23.51 ± 3.07[d]	9.37 ± 0.71[c]
SL + SP	33.00 ± 4.81[c]	0.65 ± 0.06[d]	80.25 ± 3.56[c]	85.25 ± 5.93[b]	21.75 ± 3.56[d]	8.49 ± 0.56[c]

[a–d]Significant difference *vs* control at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.000\,1$, respectively

EY – yolk extender; L – lincomycin; MMP = mitochondrial membrane potential; P – penicillin; RLU – relative light units; ROS = reactive oxygen species; SL – soy lecithin extender; SP – spectinomycin; ST – streptomycin

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only the spectinomycin-administered group significantly preserved ($P < 0.001$) sperm motility amongst the SL groups. JC-1 analysis showed a statistically significant ($P < 0.000\ 1$) preservation of mitochondrial activity in each antibiotic-administered group, whereas penicillin was the least effective ($P < 0.01$) in protecting mitochondrial membrane functionality. In both media, the spectinomycin group showed the highest percentage of spermatozoa with intact membranes and acrosomes, supported by the lowest ROS production and the least DNA damage in sperm.

Bacterial occurrence following 48 h of storage at 4 °C

The EY-supplemented groups showed a significant decrease ($P < 0.05$) in the growth of bacterial colonies only in the group that had been administered spectinomycin (Table 4). A significant reduction ($P < 0.01$) in CFU was observed in all groups stored in lecithin-based medium on TS agar, including the group without antibiotic addition. The greatest decrease was observed in the group treated with spectinomycin ($P < 0.001$). A significant decrease ($P < 0.05$) in CFU cultured on BA was recorded in lecithin medium supplemented with antibiotics. No bacterial growth was observed on BA when lincomycin or spectinomycin was used.

Sperm quality following 72 h of storage at 4 °C

As shown in Table 5, the percentage of motile spermatozoa was the highest in the EY + SP group ($P < 0.001$ in comparison with the control). Matched molecular pair (MMP) analysis revealed significantly higher values in groups treated with spectinomycin, lincomycin ($P < 0.000\ 1$), and streptomycin. Sperm membrane integrity was affected considerably in all experimental groups, with a statistical significance of $P < 0.0001$ (except for EY), while only the spectinomycin groups presented over 70% of sperm cells with intact membranes. The antibiotic-administered SL groups and EY + SP group showed significantly less damaged spermatozoa ($P < 0.000\ 1$) than the Ctrl group. The lowest percentage of sperm with damaged DNA was observed in the SL + SP, SL + L, and SL + P groups. The lowest ROS production was recorded in the EY + ST, SL + SP, SL + L, EY + SP, and SL + ST groups, which were significantly lower ($P < 0.000\ 1$) than in the Ctrl group.

Table 4. Bacterial occurrence in semen stored in EY- and SL-based media with/without antibiotics after 48 h at 4 °C

Groups	Bacterial species	$\log_{10}$ CFU/mL (Tryptic soy agar)	$\log_{10}$ CFU/mL (Blood agar)
Control	*P. aeruginosa* (7), *E. faecium* (11), *S. vitulinus* (2), *S. xylosus* (1), *S. simulans* (1), *S. epidermidis* (1),*N. subflava* (1), *M. paraoxydans* (1), *S. haemolyticus* (1)	1.55 ± 0.47	1.09 ± 0.35
EY	*P. aeruginosa* (7), *E. faecium* (6), *S. vitulinus* (1), *S. simulans* (1), *S. epidermidis* (1), *S. haemolyticus* (1), *N. subflava* (1), *M. paraoxydans* (1)	1.77 ± 0.22	1.74 ± 0.47
EY+ST	*P. aeruginosa* (3), *E. faecium* (5), *S. haemolyticus* (1), *M. paraoxydans* (1), *N. subflava* (1)	1.19 ± 0.32	0.49 ± 0.26
EY+P	*P. aeruginosa* (2), *E. faecium* (2)	1.65 ± 0.28	0.44 ± 0.06
EY+L	*P. aeruginosa* (7), *E. faecium* (3), *S. vitulinus* (1)	1.22 ± 0.28	0.26 ± 0.18
EY+SP	*P. aeruginosa* (5), *E. faecium* (9), *S. vitulinus* (1)	0.55 ± 0.25[a]	0.17 ± 0.07
SL	*P. aeruginosa* (6), *E. faecium* (3)	0.21 ± 0.10[b]	0.45 ± 0.07
SL+ST	*P. aeruginosa* (2), *S. haemolyticus* (1)	0.17 ± 0.09[b]	0.12 ± 0.03[a]
SL+P	*P. aeruginosa* (3), *E. faecium* (1)	0.15 ± 0.83[b]	0.11 ± 0.04[b]
SL+L	*P. aeruginosa* (2), *N. subflava* (1)	0.22 ± 0.09[b]	0.00 ± 0.00[a]
SL+SP	*P. aeruginosa* (1), *E. faecium* (1), *S. haemolyticus* (1)	0.08 ± 0.09[c]	0.00 ± 0.00[a]

[a–c]Significant difference *vs* control at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively

EY – yolk extender; CFU – colony-forming units; L – lincomycin; P – penicillin; SL – soy lecithin extender; SP – spectinomycin; ST – streptomycin; numbers in parentheses indicates the number of samples positive for a bacteria

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Table 5. Sperm quality parameters in EY- and SL-based media with/without antibiotics after 72 h at 4 °C

Groups	Motility (%)	MMP (JC-1 units)	Membrane integrity (%)	Acrosome integrity (%)	DNA fragmentation (%)	ROS (RLU/s/10^6 sperm)
Control	4.10 ± 1.62	0.26 ± 0.04	41.25 ± 3.98	46.25 ± 2.51	58.75 ± 5.98	23.74 ± 1.06
EY	25.20 ± 6.68^{a}	0.40 ± 0.04	54.00 ± 2.91^{c}	59.00 ± 2.95	46.00 ± 2.91^{a}	17.04 ± 0.63^{b}
EY + ST	23.60 ± 3.94^{a}	0.53 ± 0.09^{d}	61.00 ± 3.74^{d}	66.00 ± 7.00^{c}	39.00 ± 3.74^{c}	13.31 ± 2.82^{d}
EY + P	16.10 ± 3.78	0.36 ± 0.04	56.00 ± 2.23^{d}	61.00 ± 5.47^{a}	38.20 ± 3.36^{c}	17.16 ± 0.76^{b}
EY + L	28.70 ± 5.21^{b}	0.58 ± 0.05^{d}	59.00 ± 3.43^{d}	64.00 ± 6.63^{b}	36.00 ± 3.43^{c}	15.60 ± 3.09^{c}
EY + SP	31.80 ± 5.60^{c}	0.59 ± 0.05^{d}	75.25 ± 3.89^{d}	70.25 ± 5.26^{d}	34.75 ± 3.89^{c}	14.76 ± 1.42^{d}
SL	18.33 ± 4.61	0.38 ± 0.04	56.50 ± 1.18^{d}	61.75 ± 4.46^{a}	43.50 ± 4.80^{b}	16.21 ± 1.97^{c}
SL + ST	17.60 ± 3.49	0.46 ± 0.04^{b}	64.75 ± 3.11^{d}	69.75 ± 6.09^{d}	35.25 ± 3.11^{c}	15.01 ± 2.82^{d}
SL + P	15.80 ± 4.62	0.40 ± 0.04	68.50 ± 1.18^{d}	73.50 ± 4.03^{d}	34.50 ± 3.11^{d}	16.84 ± 1.64^{b}
SL + L	20.50 ± 4.77	0.53 ± 0.04^{d}	65.75 ± 2.89^{d}	74.75 ± 6.75^{d}	31.25 ± 2.86^{d}	14.01 ± 2.21^{d}
SL + SP	26.60 ± 4.19^{b}	0.55 ± 0.07^{d}	72.00 ± 1.18^{d}	77.00 ± 3.78^{d}	30.00 ± 4.02^{d}	13.76 ± 2.30^{d}

$^{A–D}$Significant difference *vs* control at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.000\,1$, respectively

EY – yolk extender; L – lincomycin; MMP = mitochondrial membrane potential; P – penicillin; RLU – relative light units; ROS = reactive oxygen species; SL – soy lecithin extender; SP – spectinomycin; ST – streptomycin

Table 6. Bacterial occurrence in semen stored in EY- and SL-based media with/without antibiotics after 72 h at 4 °C

Groups	Bacterial species	$\log_{10}$ CFU/ml (tryptic soy agar)	$\log_{10}$ CFU/ml (blood agar)
Control	*P. aeruginosa* (7), *E. faecium* (11), *S. vitulinus* (2), *S. xylosus* (1), *S. simulans* (1), *S. epidermidis* (1), *N. subflava* (1), *M. paraoxydans* (1), *S. haemolyticus (1)*	1.64 ± 0.21	1.12 ± 0.31
EY	*P. aeruginosa* (7), *E. faecium* (6), *S. vitulinus* (1), *S. epidermidis* (1), *S. haemolyticus* (1), *N. subflava* (1), *M. paraoxydans* (1)	2.00 ± 0.38	2.00 ± 0.43^{a}
EY + ST	*P. aeruginosa* (3), *E. faecium* (5), *N. subflava* (1)	0.98 ± 0.32	0.16 ± 0.08^{b}
EY + P	*E. faecium* (2)	1.42 ± 0.26	0.23 ± 0.08^{b}
EY + L	*P. aeruginosa* (1), *E. faecium* (3)	1.01 ± 0.32	0.08 ± 0.03^{b}
EY + SP	*P. aeruginosa* (1), *E. faecium* (9)	0.79 ± 0.31	0.08 ± 0.07^{b}
SL	*P. aeruginosa* (6), *E. faecium* (3)	0.08 ± 0.03^{d}	0.12 ± 0.03^{b}
SL + ST	–	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
SL + P	–	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
SL + L	–	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
SL + SP	–	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}

$^{a–d}$Significant difference *vs* control at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.000\,1$, respectively

EY – yolk extender; CFU – colony-forming units; L – lincomycin; P – penicillin; SL – soy lecithin extender; SP – spectinomycin; ST – streptomycin; numbers in parentheses indicates the number of samples positive for a bacteria

Bacterial occurrence following 72 h of storage at 4 °C

Samples cultured for 72 h in the EY medium did not show significant differences compared to the control group on tryptic soy (TS) agar (Table 6). However, a significantly higher bacterial concentration was detected on blood agar ($P < 0.05$) within the same group. The storage of semen in the SL extender significantly reduced bacterial growth ($P < 0.001$), whereas the addition of any of the antibiotics completely halted bacterial growth on both agar plates.

DISCUSSION

Although deep-frozen semen belongs to a standard concept of livestock reproduction, it requires a much greater investment in equipment and

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machinery, including service fees. Cooled bovine semen accounts for a significant proportion of stored reproductive cells, while refrigerated samples can be preserved for up to 2.5 to 3 days (Vishwanath and Shannon 2000). In particular, countries with seasonal grass-based systems, such as New Zealand or Ireland, utilise traditional liquid-stored semen because of the short breeding season (Verberckmoes et al. 2005).

Bacterial occurrence in the EY medium was slightly higher after 24 and 48 h and significantly higher after 72 h than in the Ctrl group, indicating a suitable environment for the growth of seminal bacteriocenoses. On the other hand, the SL medium most likely did not provide a hospitable environment for bacterial growth, as evidenced by a descending trend of bacterial occurrence on both tryptic soy and blood agar. Spectinomycin was revealed to be the most effective antibiotic in both types of storage media since it inhibited the overall growth of bacteria, besides having bactericidal effects on most *Staphylococcus* isolates coupled with *N. subflava* and *M. paraoxydans*. Nevertheless, the effectiveness of antibiotics depends on the type of storage medium. Our results showed that the effectiveness of antibiotics in the SL medium was higher than in the EY medium. *P. aeruginosa* and *S. haemolyticus* were present in SL+ST, while *E. faecium*, *M. paraoxydans*, and *N. subflava* were identified in EY + ST. Significant effects of antibiotics were noticed in all SL groups following 48 h of storage. Moreover, after 72 h, no growth was observed in the SL groups supplemented with any antibiotic.

Although dried EY is a microbiologically safer variant than fresh EY, its composition appears to promote the growth of viable bacteria (Brackett and Beuchat 1991). We hypothesised that the use of SL medium instead of EY-based medium could reduce the overuse of antibiotics, which is directly related to antibiotic resistance. In a study published by Fernandez-Novo et al. (2021), lecithin-based media had a significantly lower bacterial load after 24 h at 5 °C than milk-based media and provided stable microbiological conditions throughout the storage period. Our study showed that penicillin was the least effective antibiotic, regardless of the extender used. In the study by Goularte et al. (2020), all 55 microorganisms isolated from bull semen were resistant to penicillin; however, all but one microorganism was resistant to lincomycin. Akhter et al. (2007) showed that the combination of streptomycin and penicillin was not sufficiently bactericidal for bovine semen bacteriocins.

Singh et al. (2018) found that bacterial contamination in bovine insemination doses was highest with EY-based semen extenders and significantly lower with SL-based extenders. Similarly, Bousseau et al. (1998) found that samples diluted with semen extenders containing animal products (TRILADYL) and EY exhibited moderate bacterial or mycoplasma contamination, whereas no contamination was observed when using a lecithin diluent. Thus, the use of non-yolk semen extenders can significantly contribute to the microbial quality of insemination doses (Singh et al. 2018).

Although the microbial occurrence was maintained at a lower level in the SL extender, significantly higher motility was observed only in the spectinomycin group. In contrast, except for the penicillin group, significantly higher motility was recorded in all EY groups, including the EY group without antibiotics. Swelum et al. (2019) found EY as the best cryoprotectant for long-term sperm storage, while studies comparing EY and lecithin sperm extenders found both effective in maintaining bovine spermatozoa's motility in liquid (Rehman et al. 2014; Rahimizadeh et al. 2021) or frozen states (Naz et al. 2018; Miguel-Jimenez et al. 2020).

Judicious application of antibiotics at semen doses may achieve the desired antimicrobial effect without substantially impacting sperm quality parameters. In our study, the selected antibiotics helped preserve MMP, especially during the first 48 hours. Penicillin did not improve the MMP following 72 h, which agrees with the findings of Boonthai et al. (2016), who reported a decrease in the MMP in fish spermatozoa when penicillin was combined with gentamicin.

The groups treated with spectinomycin and lincomycin exhibited the most pronounced changes in ROS production. This observation may correlate with the efficacy of these antibiotics against the present bacterial species, as aerobic bacteria inherently generate ROS. However, our findings suggest that lincomycin and spectinomycin may not disrupt the oxidative balance. Recent research has shown that bactericidal antibiotics, such as penicillin, can stimulate ROS production in bacteria by intensifying respiration. This hyperactivation of the electron transport chain leads to increased superoxide radical formation, potentially damaging

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iron-sulphur clusters. The resulting destabilised ferrous cations can then participate in the Fenton reaction with hydrogen peroxide, forming hydroxyl radicals (van Acker and Coenye 2017). In contrast, bacteriostatic antibiotics, including spectinomycin, decelerate bacterial respiratory chains. This property may create more favourable conditions for liquid sperm storage.

Sperm DNA integrity is closely related to ROS production and the presence of bacteria (Sabeti et al. 2016). Our study showed that liquid semen stored in SL media had a lower proportion of spermatozoa with damaged DNA than liquid semen stored in EY media. In particular, the spectinomycin- and lincomycin-treated groups exhibited the lowest levels of sperm DNA damage. Bactericidal antibiotics, such as penicillin and streptomycin, may impair sperm quality parameters, starting with mitochondrial dysfunction, leading to ROS overproduction and sperm DNA damage (Santos et al. 2021). This finding is supported by our finding that penicillin supplementation did not preserve mitochondrial membrane function and that ROS were highly produced in these groups, regardless of the storage media.

EY-based storage media in bovine semen preservation leads to the overuse of antibiotics. The use of lecithin semen extenders could improve antibiotic efficacy and reduce antibiotic resistance in the eminal microflora. *E. faecium* and *P. aeruginosa* had the highest antibiotic resistance, with the longest survival times. This aligns with the findings of Goularte et al. (2020) that *Enterococcus* and *Pseudomonas* isolates are resistant to penicillin, streptomycin, lincomycin, and spectinomycin. These species harm sperm physiology and function, thereby reducing the fertility potential. In the future, it will be essential to create an environment in which the full potential of antimicrobial substances can be exploited.

CONCLUSION

We can conclude that the antibiotics used in liquid bovine semen storage could be selectively effective, depending on the type of semen extender. Among the bacteria tested, *E. faecium* and *P. aeruginosa* exhibited the longest survival times. The lecithin medium exhibited greater microbiological safety than the egg yolk medium, which could reduce the overuse of antibiotics during semen storage. However, it seems that the composition of the lecithin extender is not reliable enough to ensure at least the same sperm quality as that of the yolk extender. Although the EY extender effectively preserved sperm quality parameters, the advantages of using yolk-free extenders in terms of biosafety should not be overlooked. However, further studies are required to increase the potential for their use during semen storage.

Conflict of interest

The authors declare no conflict of interest.

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