https://doi.org/10.17221/79/2025-CJAS

Cryopreservative and antimicrobial properties of kaempferol on the post-thaw quality of turkey spermatozoa

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Citation: Benko F., Slanina T., Ďuračka M., Kačániová M., Tvrdá E. (2025): Cryopreservative and antimicrobial properties of kaempferol on the post-thaw quality of turkey spermatozoa. Czech J. Anim. Sci., 70: 404–413.

Abstract: At present, the low post-thaw quality of poultry semen presents a challenge to develop new strategies for its cryopreservation. The purpose of this research was to assess the impact of kaempferol (KAE) on post-thaw turkey sperm characteristics (motility, membrane and acrosome integrity, mitochondrial function), oxidative and microbial profile. Turkey semen (n = 40) was diluted and cryopreserved in modified Beltsville extender with 5, 10, and 25 µM of KAE or without it (cryopreserved control – $Ctrl_C$), while fresh semen served as negative control ($Ctrl_N$). Following thawing, parameters were evaluated including sperm motility, membrane and acrosome integrity, mitochondrial functionality, DNA fragmentation index, apoptosis status, global reactive oxygen species (ROS) generation, lipid peroxidation (LPO) and protein oxidation. Bacterial identification was performed by matrix-assisted laser desorption/ionisation mass spectrometry. Our data suggest that motility, membrane and acrosome integrity, mitochondrial activity continuously increased correspondingly to KAE concentration versus $Ctrl_C$ ($P < 0.05$) while cell apoptosis, ROS generation, LPO and protein oxidation were significantly decreased in KAE treated groups versus $Ctrl_C$ ($P < 0.05$). Bacterial growth was suppressed in all KAE-treated groups, which acted synergistically with penicillin to eradicate most bacterial strains from cryopreserved samples versus $Ctrl_N$. Finally, our results suggest that KAE possesses strong antioxidant and antimicrobial properties which may be used to improve commercially available extenders for more effective preservation of turkey spermatozoa.

Keywords: extender; flavonoid; freezing; poultry; reproduction

Supported by the Slovak Research and Development Agency (Grants Nos. APVV-21-0095, APVV-20-0058) and by the VEGA (Grant No. 1/0067/24).

https://doi.org/10.17221/79/2025-CJAS

The poultry account for almost 40 percent of worldwide meat production, which include nearly 5 percent of turkey meat. The development of artificial insemination and new breeding strategies for poultry during the 1940s have increased the production of turkey meat, which has become more available to the consumers (Kalman and Szollosi 2023). At present, the turkey industry primarily depends on artificial insemination. Since then, due to the intensive selective breeding for heavier muscles and wider breast area turkey males have lost their ability to reproduce naturally and mate with the hens since they are too heavy (Bakst and Dymond 2013).

Poultry spermatozoa present a different shape and membrane composition than their mammalian counterparts. A higher content of polyunsaturated fatty acids makes them more vulnerable to oxidative and cryo-induced damage during the freezing procedure, which requires a stronger antioxidant protection (Ciftci and Aygun 2018). Turkey semen is usually characterised by a higher sperm concentration and reduced volume of the seminal plasma when compared to mammals. Turkey seminal plasma is by and large composed of inorganic ions (Na^+ and Cl^-), carbohydrates (inositol), lipids (cholesterol, polyunsaturated fatty acids, particularly arachidonic and docosatetraenoic acid), hormones, amino acids and proteins (albumin), which protect the sperm membrane against premature destabilisation during the fertilisation process. However, in common practice turkey semen must be diluted, which decreases the protective ability of the already limited seminal plasma and its overall potential for cryopreservation (Santiago-Moreno and Blesbois 2020). At the same time, turkey spermatozoa are more sensitive to thermal and osmotic stress, which induces the generation of reactive oxygen species, and associated peroxidative damage to the fatty acids present in the cell membrane (Ansari et al. 2019).

In order to prolong the lifespan of poultry semen for cryopreservation purposes, the standardisation of new protocols or optimisation of the existing ones has been extensively studied. At present, the cryopreservation of poultry semen is still problematic mainly because of poor post-thaw sperm motility and viability due to cryoshock and oxidative stress. The cryosensitivity of turkey semen is due to a combination of structural fragility (filiform thread-like head shape), lack of cytoplasmic protection (low cytoplasm volume and buffering capacity), high content of membrane polyunsaturated fatty acids and poor tolerance to osmotic changes (Zong et al. 2023). For the minimisation of sperm cryodamage, bioactive molecules with antioxidant properties have recently become more popular in the development of modern cryoprotectants and diluents (Partyka and Nizanski 2021).

Flavonoids are one of the largest and best-known groups of plant-based bioactive molecules. Flavonoids are polyphenolic substances characterised by strong antioxidant properties stemming from their unique structure and the presence of functional hydroxyl groups that can scavenge free radicals (Jamalan et al. 2016). Kaempferol (3,4',5,7-tetrahydroxyflavone) is a natural flavonoid bio-phenolic compound, which may be found at high concentrations in green leafy vegetables and chives, broccoli, onion, beans or berries (Periferakis et al. 2022). It was observed that kaempferol is able to inhibit apoptosis, mitochondrial dysfunction and nuclear condensation of different cell types which is coupled with notable antimicrobial properties against selected pathogenic bacteria (Calderon-Oliver and Ponce-Alquicira 2018).

Hence, the focus of this research was to evaluate the potential of kaempferol to offer protection to turkey spermatozoa during cryopreservation, leading to an improved post-thaw quality, lower risk for bacterial contamination of the frozen-thawed samples and its possible use as a cryoprotective agent in the future.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals and reagents used in this study were obtained from the Sigma-Aldrich company (St. Louis, MO, USA) unless otherwise stated. A list of chemicals is provided below: Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium chloride – D8537; Dimethyl sulfoxide (DMSO) – D8418; Kaempferol – 60010; Beltsville Turkey Semen Extender (Agtech, Inc., Manhattan, KS, USA) – P2-7450; Soybean lecithin (Thermo Fisher Scientific, Kandel, Germany) – Z1209728; Penicillin – P3032; Glycerol – G5516; Eosin Y – E4009; Nigrosin – 198285; Glucose – G8270; Sodium citrate – W302600; Fast green FCF – F7252; (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) – M2128; Isopropanol; Halomax kit (Halotech DNA,

https://doi.org/10.17221/79/2025-CJAS

Madrid, Spain) – #HT-MG40; Annexin V-FLUOS (AV) kit (Roche, Basel, Switzerland) – 11828681001; Propidium iodide (PI) – P4170; DAPI dihydrochloride – D9542; Luminol – A8511; RIPA lysis buffer (Millipore Corp., Billerica, MA, USA) – 3318781; Total protein kit (DiaSys, Holzheim, Germany) – 60139108; Acetic acid – 338826; Sodium hydroxide – 567530; Sodium dodecyl sulphate (SDS) – L3771; Thiobarbituric acid (TBA) – T5500; Trichloroacetic acid (TCA) – T4885; 2,4-Dinitrophenylhydrazine (DNPH) – 04732; Guanidine hydrochloride (GuaHCl) – G4505.

Animals, sampling and cryopreservation

For the experiments we used semen samples obtained by cloacal massage that were collected from adult Big 6-line turkey males (n = 40) from the local breeding facility Branko Nitra, a.s. (Nitra, Slovakia). All animals were cared for under the ethical guidelines of the Slovak Animal Protection Regulation RD 377/12, conforming to the European Directive 2010/63/EU causing no harm or distress to animals.

Each sample was divided into five equal parts as follows: (*i*) negative control: fresh semen diluted in the DPBS; (*ii*) positive (cryopreserved) control: diluted in fresh extender with 0.1% DMSO without KAE treatment; (3,4,5) experimental groups: diluted in fresh extender enriched with 5, 10 or 25 µM of KAE dissolved in DMSO.

Commercially available Beltsville turkey semen extender (pH ~ 7.0/320–350 mOsm/kg) which contained sodium glutamate, potassium and magnesium acetate, glucose and polyvinylpyrrolidone, with added soybean lecithin (1% w/v), penicillin (300 µg/ml) and glycerol (3% v/v) served as the extender for cryopreservation. Following the dilution, the samples were cooled at 5 °C for 2 h, subsequently transferred into cryovials (vol. 1.5 ml), equilibrated for 10 min in nitrogen vapours and stored in liquid nitrogen at –196 °C for one month. Before analysis, cryopreserved samples were thawed in water bath at 37 °C for 90 s and immediately processed.

Qualitative parameters of spermatozoa

Collected samples were diluted in the DPBS (1 : 100) and counted in the Makler counting chamber (depth 10 µm; Sefi Medical Instruments, Haifa, Israel). The proportion of motile spermatozoa (MOT %) was assessed by the CASA (computer-assisted sperm analysis) system (v14.0 TOX IVOS II; Hamilton-Thorne Biosciences, Beverly, CA, USA) and the Animal motility program (Hamilton-Thorne Biosciences, Beverly, MA, USA).

All semen samples were pre-screened to meet the following minimum quality thresholds: volume ≥0.3 ml, sperm concentration ≥5.0 × 10^9/ml, and total motility ≥75 %.

For the evaluation of membrane integrity we used a combined eosin/nigrosin staining protocol (Slanina et al. 2018). The prepared slides were observed under a light microscope at 400× magnification (Nikon Eclipse E100LED MV R; Nikon, Tokyo, Japan) by counting 300 cells per slide.

The hypo-osmotic swelling test (HOST) was performed for the detection of spermatozoa with intact plasma membrane (%). Sperm cell suspension was incubated with the hypo-osmotic solution (~100 mOsm/l) at 37 °C for 20 min (Rezaie et al. 2021) and immediately checked under a light microscope at 400× magnification (Nikon Eclipse E100LED MV R; Nikon, Tokyo, Japan; 300 cells/slide) for the presence of swollen flagellum as a characteristic sign of the functional membrane.

The integrity of the acrosome was evaluated with the help of the fast green stain (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 10 µl of diluted samples were mixed with 10 µl of fast green solution and incubated for 70 seconds. Prepared dried slides were examined under a light microscope at 400× magnification (Nikon Eclipse E100LED MV R; Nikon, Tokyo, Japan; 300 cells/slide) and the proportion of spermatozoa with intact acrosomes was expressed in percentage (%).

Mitochondrial functionality was assessed through the MTT assay, which follows the transformation of MTT tetrazolium salt to formazan by the succinate-coenzyme Q reductase of intact mitochondria. Diluted samples were incubated with 10 µl of MTT solution for 20 min at 37 °C. After that the samples were treated with 40 µl of isopropanol and transferred to clear 96-well microplates, and mitochondrial activity was measured by using the combined spectro-fluoro-luminometer Glomax Multi+ (570/620 nm; Promega Corporation, Madison, WI, USA), and expressed in percentage of the negative control (set to 100%).

The DNA fragmentation index (IF %) was evaluated using the commercially available Halomax

https://doi.org/10.17221/79/2025-CJAS

kit according to the manufacturer's instructions and visualised under a fluorescence microscope Leica DM IL TED at 400× magnification (Leica Microsystems, Wetzlar, Germany).

The number of sperm apoptotic cells (%) was processed using the Annexin-V kit with added PI as an indicator of necrotic cells and analysed with the Glomax Multi+ spectro-fluoro-luminometer (Promega Corporation, Madison, WI, USA). First, washed sperm cells were incubated with 100 µl of Annexin-V solution which included 10 µl of PI for 20 min at 37 °C under dark conditions. Before measurement, 10 µl of DAPI were added to the samples. Totally three types of cells were identified: (*i*) viable (AV^-/PI^-); (*ii*) apoptotic (AV^+/PI^-), and (*iii*) necrotic (AV^-/PI^+) (Najafi et al. 2024).

The global generation of ROS by spermatozoa was quantified by the chemiluminescent method with the use of luminol (Sigma-Aldrich, St. Louis, MO, USA) as a probe following a previous publication (Lenicky et al. 2021). The amount of ROS expressed by relative light units (RLU/s/10^6 spermatozoa) was measured with the Glomax Multi+ spectro-fluoro-luminometer (Promega Corporation, Madison, WI, USA).

Oxidative damage of lipids and proteins

Before the assessment of existing oxidative damage, spermatozoa underwent lysis. All samples were washed in phosphate saline buffer, centrifuged (300 × *g*/10 min) and cell pellets were treated with the RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) with added protease inhibitor for overnight lysis. The next day, the samples were centrifuged again (5 000 × *g*/10 min) and the amount of total proteins from lysates was measured with a commercially available Total Protein kit (DiaSys, Holzheim, Germany) and the Monza photometric analyser (Randox Laboratories, Crumlin, UK) (Banas et al. 2023).

The extent of lipid peroxidation (LPO) was assessed through the thiobarbituric acid-reactive substances (TBARS) assay and quantification of malondialdehyde (MDA) as the main indicator of LPO (µmol MDA/g protein). Samples and standards were treated with 100 µl of 5% SDS dissolved in 20% acetic acid and 4 ml of TBA working solution (50 ml of 20% acetic acid + 37.5 ml of 1% NaOH + 12.5 ml of dH_2O → pH~3.5 + 530 mg of TBA) and boiled in water bath for 1 h at 100 °C. Following the cooling on ice and centrifugation, the absorbance was measured on a combined spectro-fluoro-luminometer Glomax Multi+ (Promega Corporation, Madison, WI, USA) at 560/450 nm.

The amount of protein carbonyls (PC) served as the indicator of protein oxidation and it was evaluated by the traditional dinitrophenylhydrazine (DNPH) method. Standardised samples were treated with 1 ml of 20% TCA, cooled in the fridge for 10 min and centrifuged. Then, 1 ml of DNPH working solution was added to the pellets and incubated for 1 h at 37 °C, and 1 ml of 20% TCA was added again (10 min/fridge). Following the centrifugation, pellets were washed three times with ethyl alcohol/ethyl acetate solution and incubated with 1 ml of Gua-HCl for 15 min at 37 °C. Oxidative damage to proteins (nmol PC/mg protein) was determined using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) at 360 nm.

Microbial analysis

Bacteria from semen samples were quantified through inoculation of the samples onto selected growth media that included tryptone soya agar (TSA) (Soyabean casein digest agar; Merck, Darmstadt, Germany) and blood agar (BA) (Blood agar base No. 2; Merck, Darmstadt, Germany), followed by incubation and purification. Purified bacteria from the samples were identified with the help of matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) Biotyper mass spectrometry (Brucker Daltonics, Bremen, Germany), Microflex LT instrument, the flexControl software v3.4. Microbial identification was performed using a Bruker MALDI Biotyper system with MBT Compass Reference Library v11.0 (4.1, V11.0.0.0_10833).

Statistics

For the data analysis we used the GraphPad Prism program (v10.1.1 for Mac; GraphPad Software, La Jolla, CA, USA) and one-way ANOVA with Greenhouse-Geisser correction and Tukey's test. The groups were compared as follows: cryopreserved control ($Ctrl_C$) vs experimental groups treated with KAE. Differences between groups were considered as significant at $P < 0.05$.

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RESULTS

Sperm quality

As the results from Table 1 suggest, the post-thaw motility (MOT) of turkey spermatozoa significantly decreased ($P < 0.05$) in the cryopreserved control ($Ctrl_C$) as well as in the experimental groups treated with KAE ($P < 0.05$) compared to the fresh sperm motility (Table 2).

However, there was a significant improvement ($P < 0.05$) in the sperm motility in the experimental groups treated with 10 and 25 µm of KAE against the non-treated $Ctrl_C$.

Similarly to the motility, KAE administration significantly increased the percentage of spermatozoa with intact membranes ($P < 0.05$) and HOS positivity ($P < 0.05$) in the experimental groups supplemented with 10 and 25 µM of KAE when compared to $Ctrl_C$ or with the data listed in Table 2.

A negative impact of cryopreservation was also observed in the acrosomal integrity (Table 2) of post-thaw spermatozoa. A significant decrease ($P < 0.05$) of spermatozoa with intact acrosomes was observed in $Ctrl_C$ but there was a significant continuous increase ($P < 0.05$) of spermatozoa with intact acrosomes in the groups enriched with 10 and 25 µM of KAE when compared to $Ctrl_C$.

In the mitochondrial functionality (Table 1), significant differences ($P < 0.05$) were observed between $Ctrl_C$ and groups enriched with KAE. Following the KAE supplementation, a significant improvement ($P < 0.05$) in mitochondrial activity was recorded in all KAE treated groups (from 5 µM to 25 µM of KAE) when compared to $Ctrl_C$.

Detrimental effects of cryopreservation on the DNA fragmentation (Table 1) were recorded in all cryopreserved groups with or without KAE. Nevertheless, a significant decline ($P < 0.05$) of DNA fragmentation index was observed in all experimental groups supplemented with KAE when compared to $Ctrl_C$. Differences between the values listed in Table 2 and post-thaw results indicate the extent of cryodamage.

A significantly higher proportion ($P < 0.05$) of spermatozoa exhibiting an apoptotic or necrotic state (Table 1) was recorded in the $Ctrl_C$. Apoptotic and necrotic cryoinjury significantly decreased ($P < 0.05$) in all groups supplemented with KAE (from 5 to 25 µM of KAE) against $Ctrl_C$.

Oxidative profile

Table 3 summarises the potential oxidative cryodamage caused by the cryopreservation procedure of turkey spermatozoa. The highest global generation of ROS was observed in $Ctrl_C$ against KAE groups or fresh turkey spermatozoa (Table 2). In the groups enriched with KAE, there was a significant dose-dependent decline ($P < 0.05$) of ROS generation against the untreated $Ctrl_C$, especially in the doses of 10 and 25 µM of KAE.

At the same time, the highest levels of protein oxidation (Table 3) were observed in $Ctrl_C$ associated with a significant increase ($P < 0.05$) of protein

Table 1. Qualitative parameters of cryopreserved turkey spermatozoa in the absence or presence of kaempferol (KAE)

Parameter	Control	Experimental groups		
	$Ctrl_C$	5 µM KAE	10 µM KAE	25 µM KAE
MOT (%)	35.7 ± 4.9	47.3 ± 2.5	53.3 ± 3.8*[a]	61.3 ± 6.9*[a]
MI (%)	51.3 ± 2	59.6 ± 4.2	69.3 ± 2.3*[a]	70.3 ± 4.7*[a]
HOS (%)	50 ± 3.5	60.6 ± 3.3	63.3 ± 6.2*[a]	65.2 ± 5.3*[a]
AI (%)	47.2 ± 4.8	58.3 ± 2.7	64.2 ± 1.9*[a]	65.6 ± 1.8*[a]
MTT (%)	46 ± 4.5	57.7 ± 11.1*[a]	66.3 ± 5.8*[a]	66.3 ± 2.6*[a]
DNA (%)	44.3 ± 1.2	30.6 ± 1.2*[a]	26.6 ± 1.7*[a]	22 ± 4.3*[a]
AN-V (%)	49 ± 6.1	39.3 ± 2.3*[a]	36.3 ± 1.2*[a]	29.6 ± 2.0*[a]
PI (%)	17 ± 2.4	10.7 ± 1.9	8.1 ± 2.8[a]	7 ± 1.0*[a]

*[a]Significant in comparison with $Ctrl_C$; *$P < 0.05$

5, 10 and 25 µM of KAE = cryopreserved groups treated with KAE, $n = 40$; AI = acrosome integrity; AN-V = cell apoptosis; $Ctrl_C$ = cryopreserved control without KAE treatment; DNA = DNA fragmentation; HOS = hypoosmotic test; MI = membrane integrity; MOT = motility; MTT = mitochondrial functionality; PI = cell necrosis

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Table 2. Mean values of fresh turkey spermatozoa (n = 40) qualitative parameters including the oxidative profile and bacterial load

Parameter	Value (mean ± SD)
MOT (%)	82.8 ± 5.4
MI (%)	78.2 ± 5.5
HOS (%)	71.3 ± 2
AI (%)	70.9 ± 4.1
MTT (%)	100 ± 5.1
DNA (%)	12 ± 1.1
AN-V (%)	16.3 ± 3.8
PI (%)	4.6 ± 0.1
ROS (RLU/s/10^6 sperm)	4.8 ± 0.51
PO (nmol PC/mg protein)	1.8 ± 0.04
LPO (μM MDA/g protein)	1.2 ± 0.31
Bacterial load (log CFU/ml)	15.4 ± 1.2

AI = acrosome integrity; AN-V = cell apoptosis; CFU = colony-forming units; DNA = DNA fragmentation; HOS = hypoosmotic test; LPO = lipid peroxidation; MDA = malondialdehyde; MI = membrane integrity; MOT = motility; MTT = mitochondrial functionality; PC = protein carbonyls; PI = cell necrosis; PO = protein oxidation; RLU = relative light units; ROS = reactive oxygen species

carbonyls. A significant decline ($P < 0.05$) of protein oxidation was observed in the experimental groups exposed to 10 and 25 μM of KAE against the untreated $Ctrl_C$.

In the case of LPO (Table 3) a similar phenomenon was recorded. The highest content of MDA was found in $Ctrl_C$. Despite that there was a continuous decline of MDA in all groups supplemented with KAE, only at the dose of 25 μM of KAE a significant difference ($P < 0.05$) was found in comparison with $Ctrl_C$ or the data listed in Table 2, which reflect the cryo-induced oxidative damage due to the cryopreservation procedure.

Microbial profile

A total of 15 bacterial species (15.4 ± 1.2 log CFU/ml) were isolated and identified from fresh turkey semen (Table 4) including *Acinetobacter baumannii*, *Bacillus cereus*, *Citrobacter braakii*, *Empedobacter brevis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus chromogenes*,

Table 3. Oxidative profile of cryopreserved turkey spermatozoa in the absence or presence of kaempferol (KAE)

Parameter	Control	Experimental groups		
	$Ctrl_C$	5 μM KAE	10 μM KAE	25 μM KAE
ROS (RLU/s/10^6 sperm)	20.6 ± 1.2	17.6 ± 2	11.1 ± 1.6*[a]	8.5 ± 1.2*[a]
PO (nmol PC/mg protein)	9.9 ± 0.42	7.8 ± 0.12	6.6 ± 0.20*[a]	5.8 ± 0.12*[a]
LPO (μM MDA/g protein)	3.3 ± 0.27	3 ± 0.37	2.4 ± 0.47*[a]	2.1 ± 0.54*[a]

*[a]Significant in comparison with $Ctrl_C$; *$P < 0.05$

$Ctrl_C$ = cryopreserved control without KAE treatment; 5, 10 and 25 μM of KAE = cryopreserved groups treated with KAE, n = 40; LPO = lipid peroxidation; MDA = malondialdehyde; PC = protein carbonyls; PO = protein oxidation; RLU = relative light units; ROS = reactive oxygen species

Table 4. Microbial profile of turkey spermatozoa

Bacterial profile	Control	Experimental groups		
	$Ctrl_C$ + PEN	5 μM KAE + PEN	10 μM KAE + PEN	25 μM KAE + PEN
Bacterial load (log CFU/ml)	5 ± 3.1	4.1 ± 0.42	3.9 ± 0.31	2.1 ± 0.19
Species	*E. brevis* *E. faecium* *E. coli* *S. lentus*	*E. brevis* *E. faecium* *E. coli* *S. lentus*	*E. brevis* *E. faecium* *E. coli* *S. lentus*	*E. faecium* *E. coli* *S. lentus*

$Ctrl_C$ + PEN = cryopreserved control without KAE treatment plus penicillin; 5, 10 and 25 μM of KAE + PEN = cryopreserved groups treated with different concentrations of KAE plus penicillin, n = 40; CFU = colony-forming units; KAE = kaempferol

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Staphylococcus epidermidis, *Staphylococcus lentus* and *Streptococcus alactolyticus.* Penicillin-treated cryopreserved samples ($Ctrl_C$ and KAE treated groups) showed much fewer bacterial species compared to fresh samples, but not even the presence of penicillin was able to fully eradicate some of the resilient bacterial strains including *Empedobacter brevis*, *Enterococcus faecium*, *Escherichia coli* and *Staphylococcus lentus*. Nevertheless, a continuous dose-dependent decline of the bacterial load was observed in the groups supplemented with KAE from 5 to 25 µM. What is more, it seems that 25 µM of KAE successfully eradicated *Empedobacter brevis* from the samples.

DISCUSSION

Recently, natural substances of plant origin like kaempferol have become more popular for their strong antioxidant properties and ability to protect spermatozoa during cryopreservation. In the case of kaempferol there is still a lack of studies, especially in poultry about its cryoprotective effects.

One of the main problems with cryopreserved poultry semen is that spermatozoa are highly sensitive to morphological damage following the freeze-thaw procedure because of a higher content of polyunsaturated fatty acids (PUFA), especially docosatetraenoic (22:4n-6) and arachidonic (20:4n-6) acid, which increases the risk of lipid peroxidation. In general, the avian spermatozoa have higher chances of mitochondrial and midpiece ultrastructure abnormalities than the bovine ones. Another difference lies in the sperm length; avian spermatozoa are longer than most of the mammalian gametes which makes them more vulnerable to mechanical injury during the sample processing (Siari et al. 2022).

Usually, prior to cryopreservation, the motility of turkey spermatozoa ranges between 60% and 80%, which is consistent with our findings (Table 2) as well as with previous studies (Ogbu et al. 2015; Lenicky et al. 2021).

In the field of poultry production, KAE is popular as a nutrition supplement. A basal diet enriched with KAE alone (0.4 g/kg) or with the combination of KAE with vitamin E significantly improved the egg production rate and increased the serum levels of reproductive hormones including oestradiol, follicle-stimulating hormone or luteinising hormone of late-laying hens (Zhang et al. 2024). According to recent findings, the flavonoid nutritional treatment of aged broiler breeder males with naringin and hesperidin improved most of the sperm characteristics including the ejaculate volume, overall sperm motility, sperm concentration and it led to a decline of dead or abnormal sperm cells as well as sperm DNA fragmentation (Mawlood and Taha 2024).

Additionally, the administration of semen extenders with flavonoid compounds like quercetin and naringenin improved the post-thaw quality of rooster semen. In the case of quercetin, 10 and 15 µM added into Beltsville extender significantly increased ($P < 0.05$) the post-thaw motility and kinetic parameters, membrane integrity as well as mitochondrial activity of rooster spermatozoa. What is more, the level of lipid peroxidation significantly decreased ($P < 0.05$), when compared to untreated groups (Siari et al. 2022). Flavonoid treatment was also successful against the development of lipid peroxidation, supplementation of the semen diluent with quercetin led to a significant decline of malondialdehyde content in rooster spermatozoa after 24-h treatment at cooling conditions (Najafi et al. 2022). Similarly, Mehdipour et al. (2020) suggested that the supplementation of naringenin (100 µM) improved the viability, membrane functionality and percentage of active mitochondria followed by a decline of lipid peroxidation, which led to better post-thaw rooster semen quality. A potential use of flavonoids for prolonging the rooster sperm cryosurvival was also studied by Najafi et al. (2022). Spermatozoa treated with 100 µmol/l apigenin presented a significant improvement ($P < 0.001$) of motility, membrane integrity, mitochondrial function and total antioxidant capacity. In red jungle fowl (*Gallus gallus murghi*), the inclusion of quercetin (15 µM) in cryoprotective media enhanced total and progressive motility, chromatin condensation, antioxidant capacity (measured via FRAP), and decreased ROS production and lipid peroxidation (Rakha et al. 2020). Our motility evaluation revealed that a rising concentration of KAE from 5 µM to 25 µM ensured the maintenance of sperm motility after thawing with the best results obtained at 25 µM of KAE supplemented into the semen extender.

From a microbiological point of view, studies show that KAE presents the antibacterial activity

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against a wide range of bacteria, including Gram-positive and Gram-negative bacteria, especially *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp. and others (Shamsudin et al. 2022).

It seems that KAE is one of the most effective flavonoids in damaging the cell membrane of *E. coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) inhibiting the bacterial DNA gyrase, which plays a pivotal role in the bacterial replication (Wu et al. 2013; He et al. 2014).

It is well known that *E. coli* belongs to the most common colonisers of the gastrointestinal tract and the presence of urogenital infections is nothing unusual especially in the avian cloacal system (Pormohammad et al. 2019). The study of Li et al. (2022) confirmed that KAE possessed bacteriostatic properties by controlling the bacterial energy metabolism and destruction of cellular integrity leading to the leakage of bacterial content and potential cell death.

Earlier reports (Shu-Chen et al. 2020; Seker et al. 2021) provided evidence that KAE treatment was also effective against *Klebsiella pneumoniae* by inhibiting its growth, which agrees with our results. Interestingly, there is evidence that KAE can act synergically in the presence of quercetin, extending the antimicrobial effects of both flavonoids through the disruption of the bacterial cell membrane, activation of apoptosis and fragmentation of DNA in *Micrococcus luteus* (Li et al. 2015). In our case, we observed that *M. luteus* was successfully eradicated in the group supplemented the penicillin exclusively but also in the experimental groups treated with KAE followed by a continuous significant decrease of the bacterial load. Interestingly, *Empedobacter brevis* was completely absent in the group with 25 μM of KAE, indicating a possible selective antimicrobial effect potentially due to the lack of defence mechanisms like biofilm formation or efficient efflux pumps. While promising, these inhibition highlights should be interpreted with caution and need further investigation. For the future, in assessing the antimicrobial properties of KAE, it is important to consider the presence of penicillin in the experimental design. But our goal was to assess whether the additive could offer additional antimicrobial benefits. Observing a further reduction of bacterial growth suggests a potential synergic effect, which is valuable for improving the semen quality.

Summarising the available information, the supplementation of semen extenders with a variety of flavonoids including KAE has a potential in the poultry breeding industry. The advantage of KAE is that it acts as an effective global ROS scavenger. However, careful attention to dosing, experimental controls, and potential interactions with other agents is essential to fully understand and optimise their applications in reproductive and microbiological contexts.

CONCLUSION

Based on the collected data, we may emphasise the beneficial impact of dose-dependent KAE treatment (especially 25 μM of KAE) on the post-thaw quality of turkey spermatozoa including motility and membrane stabilising effects accompanied by its strong antioxidant and antimicrobial properties.

These novel findings may be helpful in the development of new semen extenders, which may improve the cryosurvival of turkey spermatozoa used for artificial insemination.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Ansari MS, Rakha BA, Akhter S, Blesbois E, Santiago-Moreno J. Effect of cryopreservation on lipid peroxidation, antioxidant potential, chromatin integrity and mitochondrial activity of Indian red jungle fowl (Gallus gallus murghi) semen. Biopreserv Biobank. 2019 Aug 12; 17(4):288-95.

Bakst MS, Dymond JS. Artificial insemination in poultry. In: Lemma A, editor. Succesfull in artificial insemination: Quality of semen and diagnostics employed. London, UK: InTech; 2013. p. 175-206.

Banas S, Benko F, Duracka M, Lukac N, Tvrda E. Kaempferol enhances sperm post-thaw survival by its cryoprotective and antioxidant behavior. Stresses. 2023 Sep 28;3(4): 687-700.

Calderon-Oliver M, Ponce-Alquicira E. Fruits: A source of polyphenols and health benefits. In: Grumezescu AM, Holban AM, editors. Natural and artificial flavoring

https://doi.org/10.17221/79/2025-CJAS

agents and food dyes. Handbook of Food Bioengineering. London, UK: Elsevier Inc; 2018. s. 189-228.

Ciftci HB, Aygun A. Poultry semen cryopreservation technologies. Worlds Poult Sci J. 2018 Sep 12;74(4): 699-710.

He M, Wu T, Pan S, Xu X. Antimicrobial mechanism of flavonoids against Escherichia coli ATCC 25922 by model membrane study. Appl Surf Sci. 2014 Jun 30;305(30): 515-21.

Jamalan M, Ghaffari MA, Hoseinzadeh P, Hashemitabar M, Zeinali M. Human sperm quality and metal toxicants: Protective effects of some flavonoids on male reproductive function. Int J Fertil Steril. 2016 Jul-Sep;10(2):215-23.

Kalman A, Szollosi L. Global tendencies in turkey meat production, trade and consumption. Acta Agrar Debreceniensis. 2023 Dec 1;2:83-9.

Li A, He Y, Zhang S, Shi Y. Antibacterial activity and action mechanism of flavonoids against phytopathogenic bacteria. Pestic Biochem Physiol. 2022 Nov;188:105221.

Li XM, Luo XG, Si CL, Wang N, Zhou H, He JF, Zhang TC. Antibacterial active compounds from Hypericum ascyron L. induce bacterial cell death through apoptosis pathway. Eur J Med Chem. 2015;96:436-44.

Lenicky M, Slanina T, Kacaniova M, Galovicova L, Petrovicova M, Duracka M, Benko F, Kovac J, Tvrda E. Identification of bacterial profiles and their interactions with selected quality, oxidative, and immunological parameters of turkey semen. Animals (Basel). 2021 Jun 14;11(6): 1771.

Mawlood AD, Taha AT. Effect of various hesperidin and naringin addiction levels on specific sperm indicators and the integrity of their DNA as measured by the comet assay in aged broiler breeder males. IOP Conf Ser: Earth Environ Sci. 2024 Sep;1302:012052.

Mehdipour M, Daghigh Kia H, Najafi A, Mohammadi H, Alvarez-Rodriguez M. Effect of crocin and naringenin supplementation in cryopreservation medium on post-thaw rooster sperm quality and expression of apoptosis associated genes. PLoS One. 2020 Oct 29;15(10):e0241105.

Najafi A, Daghigh Kia H, Mehdipour M, Mohammadi H. Improving the quality of rooster sperm during storage at 4 °C by adding quercetin in the form of nano-liposomes and NLC in a diluting medium. Res Anim Prod. 2022 Oct 3;13(36):104-13.

Najafi A, Mohammadi H, Sharifi SD, Rahimi A. Apigenin supplementation substantially improves rooster sperm freezability and post-thaw function. Sci Rep. 2024 Feb 24; 14(1):4527.

Ogbu NN, Ogbu CO, Ugwu SOC. Effects of selenium and zinc on biochemical constituents and quality of indigenous turkey semen. IJAIR. 2015 Oct 26;4:866-71.

Partyka A, Nizanski W. Supplementation of avian semen extenders with antioxidants to improve semen quality – Is it an effective strategy? Antioxidants (Basel). 2021 Nov 30;10(12):1927.

Periferakis A, Periferakis K, Badarau IA, Petran EM, Popa DC, Caruntu A, Costache RS, Scheau C, Caruntu C, Costache DO. Kaempferol: Antimicrobial properties, sources, clinical, and traditional applications. Int J Mol Sci. 2022 Nov 30;23(23):15054.

Pormohammad A, Pouriran R, Azimi H, Goudarzi M. Prevalence of integron classes in Gram-negative clinical isolated bacteria in Iran: A systematic review and meta-analysis. Iran J Basic Med Sci. 2019 Feb;22(2):118-27.

Rakha BA, Qurrat-Ul-Ain, Ansari MS, Akhter S, Akhter A, Awan MA, Santiago-Moreno J. Effect of quercetin on oxidative stress, mitochondrial activity, and quality of indian red jungle fowl (Gallus gallus murghi) sperm. Biopreserv Biobank. 2020 Aug;18(4):311-20.

Rezaie FS, Hezavehei M, Sharafi M, Shahverdi A. Improving the post-thaw quality of rooster semen using the extender supplemented with resveratrol. Poult Sci. 2021 Sep;100(9): 101290.

Santiago-Moreno J, Blesbois E. Functional aspects of seminal plasma in bird reproduction. Int J Mol Sci. 2020 Aug 7; 21(16):5664.

Seker ME, Ay E, Aktas Karacelik A, Huseyinoglu R, Efe D. First determination of some phenolic compounds and antimicrobial activities of Geranium ibericum subsp. jubatum: A plant endemic to Turkey. Turk J Chem. 2021 Feb 17;45(1):60-70.

Shamsudin NF, Ahmed QU, Mahmood S, Ali Shah SA, Khatib A, Mukhtar S, Alsharif MA, Parveen H, Zakaria ZA. Antibacterial effects of flavonoids and their structure-activity relationship study: A comparative interpretation. Molecules. 2022 Feb 9;27(4):1149.

Shu-Chen G, Rui G, Yi-Teng X, Ting-Xia D, Huai-You W, Wah-Keung Karl T. [Quantitative analysis of fermented aerial part of Bupleurum chinense and prediction of their antimicrobial activity]. Zhongguo Zhong Yao Za Zhi. 2020 Sep;45(17):4238-45. Chinese.

Siari S, Mehri M, Sharafi M. Supplementation of Beltsville extender with quercetin improves the quality of frozen-thawed rooster semen. Br Poult Sci. 2022 Apr;63(2):252-60.

Slanina T, Miskeje M, Tirpak F, Blaszczyk M, Formicki G, Massanyi P. Caffeine strongly improves motility parameters of turkey spermatozoa with no effect on cell viability. Acta Vet Hung. 2018 Mar;66(1):137-50.

Wu T, Zang X, He M, Pan S, Xu X. Structure-activity relationship of flavonoids on their anti-Escherichia coli activity and inhibition of DNA gyrase. J Agric Food Chem. 2013 Aug 28;61(34):8185-90.

https://doi.org/10.17221/79/2025-CJAS

Zhang J, Zhang J, Li K, Fu X, Liang Y, Zhang M, Zhuang S, Gao Y. Kaempferol and vitamin E improve production performance by linking the gut-uterus axis through the reproductive hormones and microbiota of late-laying hens. Animals (Basel). 2024 Dec 25;15(1):15.

Zong Y, Li Y, Sun Y, Mehaisen GMK, Ma T, Chen J. Chicken sperm cryopreservation: Review of techniques, freezing damage, and freezability mechanisms. Agriculture. 2023 Feb 14;13(2):445.

Received: June 4, 2025
Accepted: August 13, 2025
Published online: September 26, 2025