Original Article

The use of ethyl lauroyl arginate encapsulated in organic nanoparticles within boar semen extenders to control bacterial growth and preserve sperm quality during short-term storage without antibiotics

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Abstract

The aim of the present study was to investigate the effect of adding ethyl lauroyl arginate (ELA), encapsulated in organic nanoparticles, to a semen extender without antibiotics on sperm quality and bacterial load during short-term storage. Three ejaculates from Duroc boars were divided into four equal portions and diluted with Beltsville Thawing Solution to produce semen doses containing 3 billion spermatozoa in 80 ml. The extended semen did not include antibiotics but instead incorporated different concentrations of ELA nanoparticles into the extenders: 0 ppm, 5 ppm, 10 ppm, and 15 ppm. The semen doses were stored for 4 days, with daily evaluations of semen quality and bacterial counts conducted on days 0, 1, 2, and 3. During storage, sperm motility, viability, acrosome integrity, sperm membrane integrity, mitochondrial activity, and total bacterial count (CFU/ml, $\log_{10}$) were evaluated. Total sperm motility and motion parameters, including straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), and average path velocity (VAP, µm/sec), were assessed using a computer-assisted sperm analyzer (CASA). During the 3 days of semen preservation, the bacterial load in extended boar semen without ELA (0 ppm group) tended to be higher than in semen extended with 5 ppm ELA (3.9 vs. 3.4 CFU/ml, $P = 0.096$) and was significantly higher than in semen extended with 10 ppm (3.3 CFU/ml, $P = 0.045$) or 15 ppm ELA (3.2 CFU/ml, $P = 0.010$). No significant differences were observed in total sperm motility, VSL, sperm viability, or sperm membrane integrity between the 0 ppm and 5 ppm ELA groups ($P > 0.05$). However, total sperm motility in boar semen without ELA supplementation (69.6 ± 6.7%) was higher than in semen extended with 10 ppm (58.6 ± 6.7%, $P = 0.031$) or 15 ppm ELA (55.1 ± 6.7%, $P = 0.006$). In addition, sperm plasma membrane integrity in the 0 or 5 ppm ELA treatments was higher than in semen supplemented with either 10 ppm or 15 ppm ELA ($P < 0.05$). No differences in sperm acrosome integrity were found across the four groups ($P > 0.05$). In conclusion, our findings suggest an alternative antimicrobial agent for reducing antibiotic use in the boar artificial insemination industry. Encapsulated in organic nanoparticles at concentrations of 10 to 15 ppm, ELA effectively controlled bacterial growth during short-term storage without the need for antibiotics but negatively affected some sperm quality parameters. Therefore, using 5 ppm of ELA encapsulated in organic nanoparticles is recommended, as it reduces the bacterial load from 3.9 to 3.4 $\log_{10}$ CFU/ml without adversely impacting sperm characteristics during short-term storage, making it a viable option for antibiotic-free semen extenders.

Keywords: artificial insemination, antibiotic, boar, nanoparticle, semen

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Received September 12, 2024
Accepted November 15, 2024

Introduction

In current pig artificial insemination practices, antibiotics are added to boar semen extenders to prevent bacterial growth and reduce the adverse effects of bacteriospermia on sperm quality (Ngo *et al*., 2023). Due to the global issue of antimicrobial resistance, beta-lactam antibiotics such as penicillins and cephalosporins, along with mixtures of undeclared antibiotics, are no longer recommended for inclusion in boar semen extenders (Waberski *et al*., 2019). Instead, broad-spectrum aminoglycosides, such as gentamicin and neomycin, are still in use (Gączarzewicz *et al*., 2016; Ngo *et al*., 2023). However, the supplementation of antibiotics in boar semen extenders used in routine artificial insemination in the pig industry is clearly not intended for therapeutic purposes. This practice may contribute to the global rise of antibiotic-resistant bacterial strains in artificial insemination facilities and modern pig farming (Morrell *et al*., 2024; Ngo *et al*., 2024). Consequently, there is significant interest in replacing conventional antibiotics in extenders with alternative antimicrobial substances (Schulze *et al*., 2016). There are two key requirements for alternatives to antibiotics in semen extenders: they should effectively inhibit bacterial growth without adversely affecting sperm quality (Keeratikunakorn *et al*., 2023; Ngo *et al*., 2023).

Ethyl lauroyl arginate (ELA) is a low-toxicity, amino acid-based cationic surfactant synthesized from L-arginine, lauric acid, and ethanol. It is considered to be one of the most effective antimicrobial substances among new food additives due to its broad-spectrum activity against various bacteria, yeasts, and filamentous fungi (Kim *et al*., 2017; Ma *et al*., 2020; Demircan and Özdestan Ocak, 2021; Ma *et al*., 2023). In two short-term toxicity studies on rats, ELA was found to have no impact on white blood cell characteristics and exhibited very low toxicity in mammals (Ruckman *et al*., 2004; EFSA, 2019). However, no studies were conducted to determine the appropriate ELA supplementation level for sperm preservation, particularly in controlling bacterial growth and assessing its toxicity to sperm cells. A biocompatible and biodegradable organic nanoparticle prototype encapsulating ELA is now available (Naive Innova, a spin-off company from the Faculty of Veterinary Science, Chulalongkorn University). ELA typically appears as a milky, white, cloudy liquid. These organic nanoparticles show greater promise than inorganic nanoparticles, which have high toxicity and negative environmental impacts. Nano-encapsulation enhances the ability of antimicrobial agents to penetrate bacteria or protect them from bacterial alterations, increasing the efficacy of antimicrobial drugs against resistant bacteria. In this way, nanoparticles can help overcome bacterial resistance (Arana *et al*., 2021). This synergistic effect has sparked the hypothesis that ELA encapsulated in organic nanoparticles could reduce bacterial loads without causing sperm cell toxicity at specific concentrations. The present study aims to investigate the effect of adding ELA, encapsulated in organic nanoparticles, to a semen extender without antibiotics on sperm quality and bacterial load during short-term storage.

Materials and Methods

Animals: The study was conducted in accordance with university guidelines and approved by the Institutional Animal Care and Use Committee under protocol number 2331067. Boar semen was obtained from a boar stud located in western Thailand and transported to the semen laboratory at the Department of Obstetrics, Gynaecology, and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. Ejaculates were collected from three Duroc boars, all proven sires aged between 1 and 3 years. The boars were housed individually in pens (9 m^2 per boar) within a closed facility equipped with an evaporative cooling system. They were fed 2.5–3.2 kg of commercial feed daily as part of a standard diet, with water provided *ad libitum* via water nipples.

***Experimental design*:** To evaluate the efficacy of different doses of ELA encapsulated in organic nanoparticles for controlling bacterial growth in extended boar semen during short-term storage without antibiotics, three ejaculates with a bluish-white color, a pH of 7.2 to 7.5, and an average total motility of 78.9 ± 5.1% (ranging from 67.9% to 84.9%) were included in the experiment. Each ejaculate was divided into four portions and extended with Beltsville Thawing Solution (BTS) containing different concentrations of ELA encapsulated in organic nanoparticles to produce semen doses containing 3 billion spermatozoa in 80 ml. The extended semen incorporated varying concentrations of ELA nanoparticles: 0 ppm, 5 ppm, 10 ppm, and 15 ppm. The diluted semen was preserved for 4 days, including the collection day (day 0) and the first, second, and third days of storage (i.e., days 1, 2, and 3). During storage, sperm motility, viability, acrosome integrity, sperm membrane integrity, mitochondrial activity, and total bacterial count (CFU/ml, $\log_{10}$) were evaluated daily starting at 11:00 h. Antibiotics were excluded from all extended boar semen groups to allow full bacterial growth during storage and to assess the efficacy of different concentrations of ELA in the semen extender. The potential toxicity of ELA on sperm cells was also evaluated over a storage period of 0 to 3 days, simulating the practical use of extended boar semen for artificial insemination under field conditions.

Semen collection and processing: The gloved-hand method was used to collect the sperm-rich portion of the three ejaculates, with collections occurring routinely every 5 to 7 days. Immediately after collection, semen volume (237.3 ± 54.3 ml) and pH (7.4 ± 0.2) were measured. Sperm concentration (230.3 ± 28.3 × 10^6 sperm/ml) was evaluated using a Spermacue® (Minitube, Tiefenbach, Germany). The total sperm per ejaculate (54.2 ± 10.3 × 10^9 sperm) was calculated by multiplying semen volume by sperm concentration (Rungruangsak *et al*., 2021a). Subjective sperm motility and viability were evaluated microscopically at 200× magnification (Ngo *et al*., 2022). The semen samples were diluted in a sterile BTS extender with different concentrations of ELA encapsulated in organic nanoparticles to produce semen doses. The ingredients of the BTS semen

extender included 205 mM glucose ($C_6H_{12}O_6$), 20.4 mM sodium citrate ($Na_3C_6H_5O_7$), 10.0 mM potassium chloride (KCl), 15 mM sodium bicarbonate ($NaHCO_3$), and 3.36 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); the pH was adjusted to 7.2 (Ngo *et al*., 2023). The original ELA solution, encapsulated in organic nanoparticles, had a concentration of 50,000 ppm. Four 80 ml bottles of BTS extender were prepared for the experiment. In the first bottle, no ELA nanoparticles were added, resulting in a concentration of 0 ppm. To the second, third, and fourth bottles, ELA nanoparticles were added in amounts of 8, 16, and 24 µl to achieve final concentrations of 5, 10, and 15 ppm, respectively. The bottles were thoroughly mixed before being combined with fresh semen to produce semen doses, each containing 3 billion sperm in 80 ml.

Semen evaluation: Total sperm motility and motion characteristics of boar spermatozoa, including straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), and average path velocity (VAP, µm/sec), were assessed using a computer-assisted sperm analyzer (CASA) (SCA® CASA System, MICROPTIC S.L., Barcelona, Spain). The system was configured for boar sperm, operating at a frame rate of 50 frames per second with a box size of 100 pixels. The object area was set to range from a minimum of 10 µm² to a maximum of 80 µm². Motile spermatozoa were classified into static (< 10 µm/s), slow-medium (< 25 µm/s), and progressively motile (> 45 µm/s) groups. The diluted semen was placed in a chamber and analyzed on a heated stage (TOKAI HIT, Shizuoka-ken, Japan) at 37 °C using a phase-contrast microscope (BX41, Olympus, Shinjuku, Japan). The proportion of motile sperm was determined by examining 1,500 sperm cells across five randomly selected fields for each sample (Suwimonteerabutr *et al*., 2020).

Sperm viability was assessed using the SYBR-14/EthD-1 staining method (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, the Netherlands). A 10-µl aliquot of the diluted semen sample was combined with 1 µl of 14-µM EthD-1 (Molecular Probes Inc., OR, USA) in 1 ml of PBS and 2.7 µl of 0.38-µM SYBR-14 (Dead/Alive Kit, Molecular Probes Inc.) in 1 ml of dimethyl sulfoxide (DMSO). The mixture was then incubated at 37°C for 15 minutes. Finally, 200 sperm cells were analyzed by fluorescence microscopy (CX-31; Olympus, Tokyo, Japan) at 1000× magnification. Sperm cells stained only green were classified as live with intact plasma membranes, while those stained red or both green and red were considered dead or as having damaged plasma membranes. Sperm viability was determined by calculating the proportion of live sperm with intact plasma membranes (Ngo *et al*., 2023).

Acrosome integrity was evaluated using EthD-1 (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, Netherlands) in combination with fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA) staining (Sigma-Aldrich Co. Ltd., St Louis, MO, USA) (Figure 1A). A 10-µl aliquot of the diluted semen sample was incubated at 37°C for 15 minutes with 10 µl of 14-µM EthD-1 (Molecular Probes Inc., OR, USA). After incubation, an 8-µL drop of the mixture was smeared onto a slide and air-dried at room temperature. The slide was then immersed in 95% ethanol for 30 seconds before staining with 15 µl of FITC-PNA solution (FITC-PNA in PBS, 1:10, v/v) at 4°C for 30 minutes in a humid chamber. After staining, the slide was washed with cold PBS at 4°C and air-dried at room temperature. The acrosome status of 200 sperm per sample was examined using fluorescence microscopy (CX-31, Olympus, Tokyo, Japan) at 1000× magnification. The proportion of sperm with intact acrosomes, indicated by a green-stained (positive) acrosome cap, was calculated. Sperm cells showing orange staining, lacking an acrosome cap, exhibiting a green band at the equatorial segment, or displaying a disrupted patch-like appearance of the acrosome cap were classified as having acrosome damage (Rungruangsak *et al*., 2021b).

Sperm plasma membrane integrity was assessed using the short hypo-osmotic swelling test (sHOST) (Figure 1B). A 10-µl aliquot of the diluted semen sample was combined with 200 µl of citrate buffer (75 mOsm) and incubated in the dark at 37°C for 30 minutes in a 1.5-ml Eppendorf tube. After incubation, 175 µl of a hypo-osmotic solution containing 5% formaldehyde (75 mOsm) was added. An 8-µl drop of the semen sample was then placed on a glass slide. The tails of 200 sperm cells were examined under a light microscope (400×) and categorized as positive (coiled tail) or negative (straight tail). The proportion of positive sperm reflects the presence of a functional sperm membrane (Ngo *et al*., 2023).

Mitochondrial activity was evaluated using the fluorochrome JC-1 (5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethyl benzimidazole-carbocyanine iodide; Molecular Probes Inc., Eugene, OR, USA) (Figure 1C). A 12.5-µl aliquot of diluted semen was mixed with 25 µl of JC-1 solution, which included 1.6 µl of 0.153 mM JC-1, 1 µl of 0.02 mM SYBR-14, and 1.6 µl of 2.4 mM propidium iodide (PI) in 100 µl of HEPES-buffered medium. The mixture was incubated at 37°C for 30 minutes. Following incubation, an 8-µl drop of the stained semen sample was placed on a glass slide, and 200 sperm cells were examined using a fluorescence microscope (CX-31; Olympus, Tokyo, Japan) at 1000× magnification. Sperm cells with yellow-orange fluorescence in the midpiece region were classified as positive, indicating high mitochondrial membrane potential, while those with reduced or absent green fluorescence in the midpiece were classified as negative, indicating low mitochondrial membrane potential (Ngo *et al*., 2023).

Bacterial culture: Total aerobic bacterial contents in semen samples were quantified through bacterial culture on sheep blood agar (Ngo *et al*., 2023). Briefly, 1 ml of extended semen was diluted in tubes containing 9.0 ml of PBS (0.1 M phosphate buffer with 0.15 M NaCl, pH 7.3) to prepare serial dilutions ranging from 10^0 to 10^6. For each dilution, 1.5 ml was plated onto three agar plates (0.5 ml per plate) and incubated aerobically at 37°C for 24 hours. Plates with 30 to 300 CFU/ml were selected for bacterial counting. The total bacterial count was determined as the average number of colonies on the three plates, and this value was then

subjected to logarithmic transformation (CFU/ml, log_{10}).

Statistical analysis: The statistical analyses were performed using SAS statistical software version 9.4 (SAS Inst. Inc., Cary, NC, USA). Data on boar sperm parameters, including sperm motility, sperm kinematics, sperm viability, acrosome integrity, sperm plasma membrane integrity, mitochondrial activity, and total bacterial count (CFU/ml, log_{10}), were analyzed using multiple analyses of variance with the mixed model procedure of SAS (MIXED). The statistical models included fixed effects for treatment groups (0 ppm, 5 ppm, 10 ppm, and 15 ppm), storage days (days 0, 1, 2, and 3), and their two-way interactions. Boar identity was included in the model as a random effect to account for repeated measurements. Least-squares means were obtained for each factor level and compared using the least-significant difference test. Differences with $P < 0.05$ were considered statistically significant.

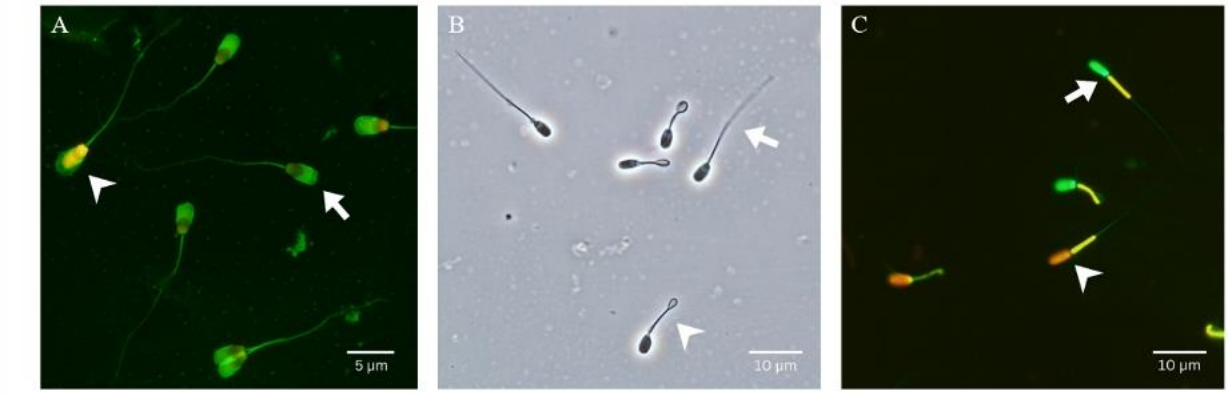

Figure 1 Boar semen evaluation: (A) Acrosome integrity, (B) Sperm plasma membrane integrity, and (C) Mitochondrial activity. The arrow indicates an intact sperm, while the arrowhead points to a damaged one.

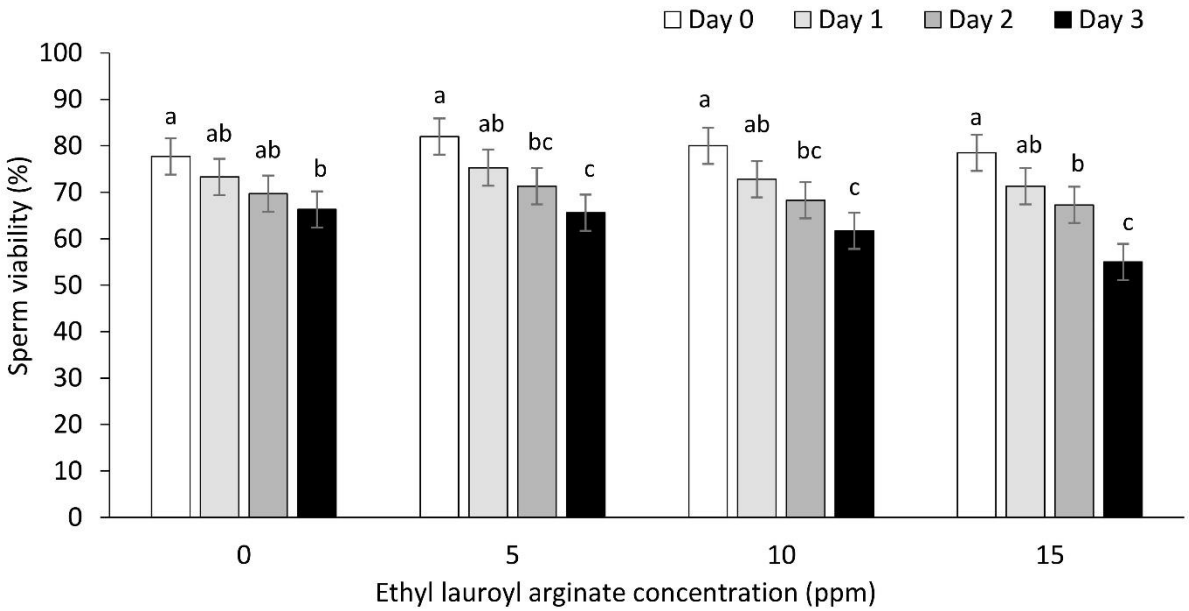

Figure 2 Sperm viability over 3 days of storage with four different concentrations of Ethyl lauroyl arginate (ELA) (n = 3 per subgroup). [a,b,c] Different letters within each ELA concentration indicate significant differences ($P < 0.05$).

Result

Effect of ethyl lauroyl arginate: During the 3 days of semen preservation, the bacterial load (Table 1) in extended boar semen without ELA (0 ppm group) tended to be higher than in semen containing 5 ppm ELA (3.9 vs. 3.4 CFU/ml, $P = 0.096$) and was significantly higher than in semen with 10 ppm ELA (3.3 CFU/ml, $P = 0.045$) or 15 ppm ELA (3.2 CFU/ml, $P = 0.010$).

Over the 3 days of storage, there were no significant differences in total sperm motility, VSL, or sperm membrane integrity between the 0 ppm and 5 ppm groups ($P > 0.05$) (Table 1). However, total sperm motility in boar semen without ELA supplementation (69.6 ± 6.7%) was higher than in semen extended with 10 ppm (58.6 ± 6.7%, P = 0.031) or 15 ppm ELA (55.1 ± 6.7%, $P = 0.006$). Additionally, total sperm motility in the 15 ppm group (55.1 ± 6.7%) was significantly lower compared to the 5 ppm group (64.7 ± 6.7%, $P = 0.051$) (Table 1). Sperm plasma membrane integrity in semen supplemented with 0 or 5 ppm ELA was also higher than in semen supplemented with 10 ppm or 15 ppm ELA ($P < 0.05$). No significant differences in sperm mitochondrial activity were observed among the 0

ppm, 5 ppm, and 10 ppm groups ($P > 0.05$), and there were no differences in sperm acrosome integrity across the four groups ($P > 0.05$).

Effect of storage days: Table 2 shows boar sperm characteristics and bacterial loads across different storage days (Days 0, 1, 2, and 3) in BTS semen extender with ELA. With the exception of VCL and acrosome integrity, most sperm parameters declined over the three days of storage ($P < 0.01$). However, no significant differences were observed in VCL, VSL, VAP, sperm membrane integrity, or sperm mitochondrial activity between Day 0 and Day 1 ($P > 0.05$) (Table 2). Additionally, no significant differences were found in total sperm motility, viability, or sperm mitochondrial activity between Day 1 and Day 2 of storage ($P > 0.05$) (Table 2). Importantly, sperm acrosome integrity remained unchanged from Day 0 to Day 3 of storage ($P = 0.169$). The total bacterial count increased over the three days of storage ($P < 0.001$), though no significant difference was observed between Day 0 and Day 1 ($P = 0.666$) (Table 2).

The interaction effect of ethyl lauroyl arginate and storage days: Figure 2 shows sperm viability over 3 days of storage across four different ELA groups. In all groups, sperm viability on Day 3 was significantly lower than on Day 0 ($P < 0.05$). However, no significant differences were observed between Day 1 and Day 2 of storage ($P > 0.05$). On Day 2, sperm viability in the 0 ppm, 5 ppm, 10 ppm, and 15 ppm groups were 69.7%, 71.3%, 68.3%, and 67.3%, respectively.

Figure 3 depicts the total bacterial count over 3 days of storage across four different ELA groups. In all groups, the total bacterial count on Day 3 was significantly higher than on Day 0 ($P < 0.05$). Over the three-day period, the total bacterial count increased by 2.3 CFU/ml in the 0 ppm group, while the increases in the 5 ppm, 10 ppm, and 15 ppm groups were 2.1, 1.9, and 1.6 CFU/ml, respectively. Notably, in the 5 ppm group, no significant differences in total bacterial count were observed between Days 0, 1, and 2 ($P > 0.05$).

Discussion

Alternatives to antibiotics in semen extenders for a sustainable artificial insemination industry for pigs are highly recommended (Schulze *et al*., 2016; Morrell *et al*., 2024). However, any antimicrobial additives must effectively prevent bacterial growth without negatively impacting sperm quality during storage (Schulze *et al*., 2016; Ngo *et al*., 2023). In this context, the concentration of ELA encapsulated in organic nanoparticles was evaluated. At a concentration of 5 ppm, ELA nanoparticles effectively reduced the bacterial load from 3.9 CFU/ml to 3.4 CFU/ml without adversely affecting sperm characteristics, including total motility, viability, and membrane integrity, during the first two days of storage. Stronger antibacterial effects were observed at higher concentrations of 10 ppm and 15 ppm. However, these higher concentrations posed potential toxicity to sperm quality, as indicated by lower sperm motility, sperm viability, and membrane integrity in these 10 ppm and 15 ppm groups. Notably, sperm motility in the 15 ppm group was 14.4% lower than in the 0 ppm group and 9.6% lower than in the 5 ppm group.

Table 1 Boar sperm characteristics and bacterial contamination at varying concentrations of Ethyl lauroyl arginate: 0 ppm, 5 ppm, 10 ppm, and 15 ppm (n = 12 per group) over 3 days of storage. Values shown are least-square means ± SEM.

Variables	Ethyl lauroyl arginate concentrations (ppm)				*P* value
	0	5	10	15	
Total sperm motility (%)	69.5 ± 6.7^{a}	64.7 ± 6.7^{ac}	58.6 ± 6.7^{bc}	55.1 ± 6.7^{b}	0.026
Progressive motility (%)	51.9 ± 7.0^{a}	45.9 ± 7.0^{ab}	39.5 ± 7.0^{bc}	36.6 ± 7.0^{c}	0.011
- Curvilinear velocity (VCL, µm/sec)	63.9 ± 5.0^{a}	57.4 ± 5.0^{a}	57.5 ± 5.0^{a}	57.5 ± 5.0^{a}	0.337
- Straight-line velocity (VSL, µm/sec)	12.8 ± 1.7^{a}	12.3 ± 1.7^{ab}	11.2 ± 1.7^{bc}	10.6 ± 1.7^{c}	0.008
- Average path velocity (VAP, µm/sec)	28.2 ± 3.5^{a}	27.9 ± 3.5^{a}	25.5 ± 3.5^{a}	25.4 ± 3.5^{a}	0.125
Sperm viability (%)	71.7 ± 2.8^{ab}	73.6 ± 2.8^{a}	70.7 ± 2.8^{ab}	68.0 ± 2.8^{b}	0.113
Acrosome integrity (%)	85.5 ± 2.7^{a}	86.3 ± 2.7^{a}	86.0 ± 2.7^{a}	81.8 ± 2.7^{a}	0.595
Sperm membrane integrity (%)	47.1 ± 4.7^{a}	44.5 ± 4.7^{a}	37.5 ± 4.7^{b}	35.4 ± 4.7^{b}	0.002
Sperm mitochondrial activity (%)	65.8 ± 2.9^{ab}	69.8 ± 2.9^{a}	65.7 ± 2.9^{ab}	63.3 ± 2.9^{b}	0.044
Total bacterial count (CFU/ml, $\log_{10}$)	3.9 ± 0.4^{a}	3.4 ± 0.4^{ab}	3.3 ± 0.4^{b}	3.2 ± 0.4^{b}	0.061

^{a,b,c,d} Different letters in each row indicate significant differences ($P < 0.05$) following the least-significant different test. *P* value (upper right) indicates the overall significant effect of the main factors using analysis of variance (*F* statistic).

Table 2 Boar sperm characteristics and bacterial contamination in relation to different storage days in BTS semen extender over 4 concentrations of ELA: day 0 (collection day), day 1 (first day of storage), day 2 (second day of storage), and day 3 (third day of storage) (n = 12 per group). Values shown are least-square means ± SEM.

Variables	Preservation day				*P* value
	0	1	2	3	
Total sperm motility (%)	78.9 ± 6.7^{a}	63.7 ± 6.7^{b}	55.3 ± 6.7^{bc}	50.5 ± 6.7^{c}	< 0.001
Progressive motility (%)	59.8 ± 7.0^{a}	46.0 ± 7.0^{b}	35.3 ± 7.0^{c}	32.8 ± 7.0^{c}	< 0.001
- Curvilinear velocity (VCL, µm/sec)	57.9 ± 5.0^{ab}	65.3 ± 5.0^{a}	56.9 ± 5.0^{b}	56.2 ± 5.0^{b}	0.137
- Straight-line velocity (VSL, µm/sec)	12.5 ± 1.7^{a}	13.7 ± 1.7^{a}	10.6 ± 1.7^{b}	10.1 ± 1.7^{b}	< 0.001
- Average path velocity (VAP, µm/sec)	27.3 ± 3.5^{ab}	29.9 ± 3.5^{a}	25.5 ± 3.5^{bc}	25.4 ± 3.5^{c}	0.004
Sperm viability (%)	79.5 ± 2.8^{a}	73.2 ± 2.8^{b}	69.2 ± 2.8^{b}	62.2 ± 2.8^{c}	< 0.001
Acrosome integrity (%)	85.8 ± 2.7^{ab}	86.8 ± 2.7^{ab}	87.4 ± 2.7^{a}	79.7 ± 2.7^{b}	0.169
Sperm membrane integrity (%)	48.3 ± 4.7^{a}	46.1 ± 4.7^{a}	37.4 ± 4.7^{b}	32.8 ± 4.7^{b}	< 0.001
Sperm mitochondrial activity (%)	72.1 ± 2.9^{a}	68.4 ± 2.9^{ab}	64.3 ± 2.9^{b}	59.8 ± 2.9^{c}	<0.001
Total bacterial count (CFU/ml, $\log_{10}$)	2.7 ± 0.4^{a}	2.8 ± 0.4^{a}	3.8 ± 0.4^{b}	4.6 ± 0.4^{c}	< 0.001

^{a,b,c,d} Different letters in each row indicate significant differences ($P < 0.05$) following the least-significant different test. P value (upper right) indicates the overall significant effect of the main factors using analysis of variance (F statistic).

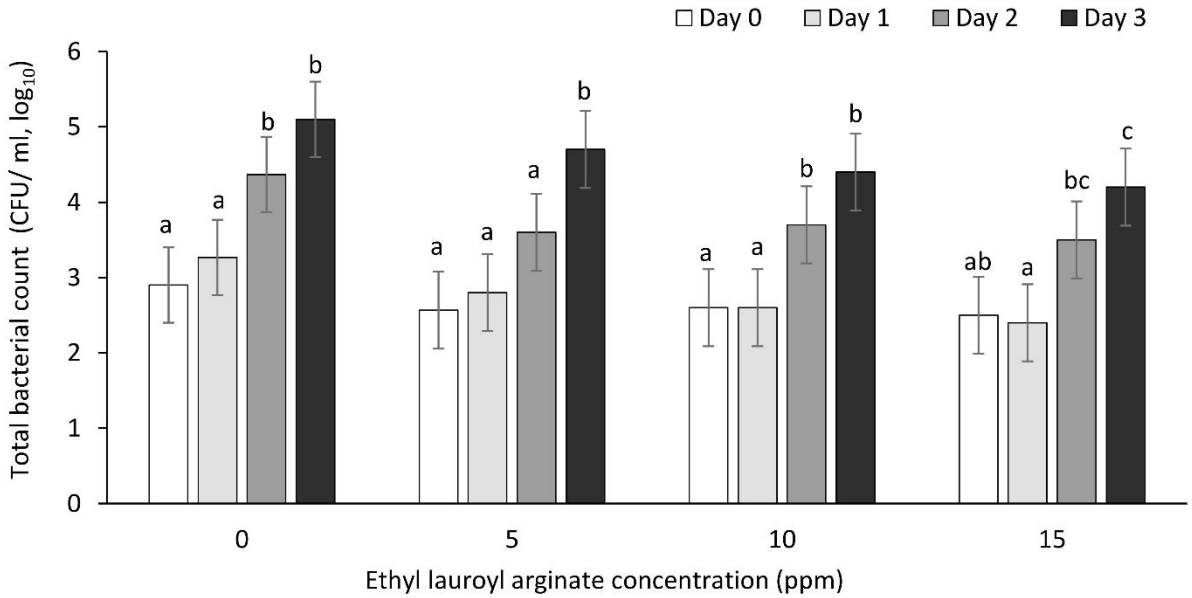

Figure 3 Total bacterial count over 3 days of storage with four different concentrations of Ethyl lauroyl arginate (ELA) (n = 3 per subgroup). [a,b,c] Different letters within each ELA concentration indicate significant differences ($P < 0.05$).

ELA is an amino acid-based cationic surfactant synthesized from L-arginine, lauric acid, and ethanol (Ma *et al*., 2023). This cationic surfactant is well-structured to bind to the negatively charged surfaces of bacteria, helping control bacterial growth, mainly through membrane damage and oxidative stress (Ma *et al*., 2023). Most bacterial cells carry a net negative charge due to the presence of peptidoglycan, which is rich in carboxyl and amino groups (Martínez *et al*., 2020). In Gram-negative bacteria, the high negative charge primarily arises from outer phospholipids and lipopolysaccharides. In Gram-positive bacteria, the negative charge is due to teichoic acids, which are rich in phosphate groups, contributing further to the overall negative charge of their cell walls (Brown *et al*., 2013). In the case of sperm cells, Magdanz *et al*. (2019) found that the charge distribution on the sperm membrane is not uniform. While the overall net charge of the sperm cell is negative, there are positively charged regions, particularly on the sperm heads. It can be inferred that the potential toxicity of ELA at high concentrations to sperm quality may be related to the differences in charge distribution. This charge disparity could lead to ELA attaching to sperm cells, causing alterations in their structure and function. Interestingly, no differences were observed in sperm acrosome integrity among the four groups, nor were there any changes in acrosome integrity from day 0 to day 3 of storage. This could be due to the positive charge on the sperm heads matching the positive charge of ELA, preventing its binding to the acrosome region and thus avoiding harmful effects. However, further research is strongly recommended to better understand the molecular mechanisms by which ELA interacts with sperm cells.

In our previous study, we proposed that when ejaculates are collected from boars with high sperm quality, antibiotics could be excluded from semen extenders, allowing the use of semen doses for up to two days, provided that strict hygienic protocols are followed during semen collection and processing (Ngo *et al*., 2023). This suggestion was based on the observation that significant bacterial growth was only detected after the first day of storage in semen without antibiotics (Ngo *et al*., 2023). The current study aligns with our earlier findings (Ngo *et al*., 2023), as the bacterial load in the 0 ppm group only began to increase after the first day of storage. Furthermore, the present study suggests that ELA at a concentration of 5 ppm could serve as an alternative to antibiotics for preserving semen doses in BTS extenders for up to two storage days, extending preservation by one day compared to the 0 ppm group. This is due to the bacterial load being maintained at around 3 $\log_{10}$ and sperm viability at 71.3%. This approach is practical, as many breeding organizations require that sperm motility on the expiration day be between 45–70%, and bacterial contamination should not exceed 1,000 CFU/ml (~2–3 $\log_{10}$) when semen doses are sold on the global market (Waberski *et al*., 2019). Moreover, mesophilic aerobic bacteria only negatively impact sperm quality or fertility at levels above 10^7 CFU/ml (Bussalleu *et al*., 2011; Ubeda *et al*., 2013).

Our findings present an alternative antimicrobial agent aimed at reducing antibiotic use in the boar artificial insemination industry. ELA, encapsulated in organic nanoparticles at concentrations of 10 to 15 ppm, effectively controlled bacterial growth during short-term storage, reducing it to 3.2 to 3.3 $\log_{10}$ CFU/ml without the need for antibiotics. However, at these concentrations, certain sperm quality parameters, such as progressive motility and membrane integrity, were compromised. Therefore, the use of 5 ppm of ELA encapsulated in organic nanoparticles is recommended, as it successfully reduced the bacterial load from 3.9 to 3.4 $\log_{10}$ CFU/ml without negatively affecting sperm characteristics during short-term storage, making it a viable option for semen extenders without antibiotics.

Acknowledgment

This research was funded by the National Research Council of Thailand (NRCT) (N42A660892). CongBang Ngo is a grantee of the C2F PhD Scholarship at Chulalongkorn University. We would like to thank Dr. Preechaphon Taechamaeteekul for assisting with the preparation of the sperm evaluation figure, which enhanced the quality of our manuscript.

References

Arana L, Gallego L and Alkorta I 2021. Incorporation of antibiotics into solid lipid nanoparticles: a promising approach to reduce antibiotic resistance emergence. Nanomaterials (Basel) 11(5):1251.

Brown S, Santa Maria JPJr and Walker S 2013. Wall teichoic acids of gram-positive bacteria. Annu Rev Microbiol. 67:313–336.

Bussalleu E, Yeste M, Sepúlveda L, Torner E, Pinart E and Bonet S 2011. Effects of different concentrations of enterotoxigenic and verotoxigenic *E. coli* on boar sperm quality. Anim Reprod Sci. 127:176–182.

Demircan B and Özdestan Ocak Ö 2021. The effects of ethyl lauroyl arginate and lemon essential oil added edible chitosan film coating on biogenic amines formation during storage in mackerel fillets. J Food Process Preserv. 45:e15454.

EFSA 2019. Scientific opinion on the safety of ethyl lauroyl arginate (E243) as a food additive in the light of the new information provided and the proposed extension of use. EFSA J. 17:5621.

Gączarzewicz D, Udała J, Piasecka M, Błaszczyk Ba and Stankiewicz T 2016. Bacterial contamination of boar semen and its relationship to sperm quality preserved in commercial extender containing gentamicin sulfate. Pol J Vet Sci. 19:451–459.

Keeratikunakorn K, Kaewchomphunuch T, Kaeoket K and Ngamwongsatit N 2023. Antimicrobial activity of cell free supernatants from probiotics inhibits against pathogenic bacteria isolated from fresh boar semen. Sci Rep. 13:5995.

Kim TS, Ham SY, Park BB, Byun Y and Park HD 2017. Lauroyl arginate ethyl blocks the iron signals necessary for *Pseudomonas aeruginosa* biofilm development. Front Microbiol. 8:970.

Ma QM, Davidson PM and Zhong QX 2020. Properties and potential food applications of lauric arginate as a cationic antimicrobial. Int J Food Microbiol. 315:108417.

Ma Y, Ma Y, Chi L, Wang S, Zhang D and Xiang Q 2023. Lauric arginate ethyl ester: An update on the antimicrobial potential and application in the food systems. Front Microbiol. 14:1125808.

Martínez B, Rodríguez A, Kulakauskas S and Chapot-Chartier MP 2020. Cell wall homeostasis in lactic acid bacteria: threats and defences. FEMS Microbiol Rev. 44(5):538–564.

Magdanz V, Gebauer J, Sharan P, Eltoukhy S, Voigt D and Simmchen J 2019. Sperm-particle interactions and their prospects for charge mapping. Adv Biosyst. 3(9):e1900061.

Morrell JM, Cojkic A, Malaluang P, Ntallaris T, Lindahl J and Hansson I 2024. Antibiotics in semen extenders - a multiplicity of paradoxes. Reprod Fertil Dev. 36:RD23218.

Ngo CB, Suriya S; Nutthee A and Tummaruk P 2022. Semen production capacity among boar breeds and effect of organic antioxidant supplementation on sperm characteristics during the hot season in Thailand. Thai J Vet Med. 52(3):441–449.

Ngo CB, Suwimonteerabutr J, Prapasarakul N, Morrell JM and Tummaruk P 2023. Bacteriospermia and its antimicrobial resistance in relation to boar sperm quality during short-term storage with or without antibiotics in a tropical environment. Porc Health Manag. 9:21.

Ngo CB, Suwimonteerabutr J, Morrell JM and Tummaruk P 2024. Sow reproductive performance following artificial insemination with semen doses processed using Single Layer Centrifugation without antibiotics in the tropics. Theriogenology 226:194–201.

Ruckman SA, Rocabayera X, Borzelleca JF and Sandusky CB 2004. Toxicological and metabolic investigations of the safety of Nα-Lauroyl-L-arginine ethyl ester monohydrochloride (LAE). Food Chem Toxicol. 42: 245–259.

Rungruangsak J, Sangkaphet S, Buranaamnuay K, Pongpeng P and Tummaruk P 2021a. Boar sperm production in a tropical environment. Thai J Vet Med. 51(2):213–220.

Rungruangsak J, Suwimonteerabutr J, Buranaamnuay K, Chankrachang A and Tummaruk P 2021b. A comparative study of two methods to determine acrosome integrity of frozen-thawed boar sperm: FITC-PNA/EthD-1 versus Coomassie blue staining. Veterinarska Stanica 52 (6):629–638.

Schulze M, Dathe M, Waberski D and Müller K 2016. Liquid storage of boar semen: Current and future perspectives on the use of cationic antimicrobial peptides to replace antibiotics in semen extenders. Theriogenology 85:39–46.

Suwimonteerabutr J, Chumsri S, Tummaruk P and Nuntapaitoon M 2020. Butaphosphan and Cyanocobalamin supplementation in semen extender on chilled boar sperm quality and life span. Front Vet Sci. 7:592162.

Ubeda JL, Ausejo R, Dahmani Y, Falceto MV, Usan A, Malo C and Perez-Martinez CF 2013. Adverse effects of members of the Enterobacteriaceae family on boar sperm quality. Theriogenology 80:565–570.

Waberski D, Riesenbeck A, Schulze M, Weitze KF and Johnson L 2019. Application of preserved boar semen for artificial insemination: Past, present and future challenges. Theriogenology 137: 2–7.