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Plant extracts as an alternative for antibiotics in saanen goats' semen extender

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Abstract

The widespread use of antibiotics contributes to the development of antibiotic resistance. Therefore, exploring suitable alternatives to antibiotics is crucial. This study aimed to determine the antibacterial effect of mulberry, roselle and aloe vera extracts on bacteria isolated from buck semen and their effectiveness as an alternative to antibiotics for semen extender. The antibacterial activity of plant extract against identified bacteria was determined by using disc diffusion method. Semen was added to the extender with mulberry, roselle or aloe vera extract and incubated at 37 °C for 3 h and cryopreserved for 24 h before the assessment of bacterial load and semen quality were investigated. Results shows that mulberry extract was the only plant extract that significantly inhibited both gram-negative and gram-positive bacteria, with greater inhibitory zone on gram positive bacteria (22.33 ± 1.12 mm) compare to Roselle (8.1 ± 1.08 mm) and Aloe vera (9.6 ± 1.05 mm) resulting in the lowest bacterial load (763 ± 4.57 CFU/ml) and improved sperm quality during the 3-hour incubation period. Whereas, aloe vera and roselle extracts were highly effective for decreasing bacterial contamination and protecting the sperm quality of post-thawed semen. The study concluded that, mulberry extract was a suitable alternative to commercial antibiotics for fresh semen extenders, while aloe vera and roselle were more recommended for cryopreservation purposes.

Keywords: *antibiotics, antibacterial activity, goat, plant extract, semen*

Introduction

Every semen sample contains bacteria, which may enter due to local infections in the genital tract, systemic infections caused by bacteremia, or inflammation of the genitourinary tract. The bacterial contamination of ejaculate during collection can also be caused by external sources like faecal bacteria, environment, and other compounds of animal origin contained in the diluents and buffers or equipment (Gangwar et al. 2020). Numerous bacteria, including *Staphylococcus aureus*, are found in semen and they are known to cause a number of illnesses in humans and animals (McMillan et al. 2016). *Staphylococcus aureus*, for example, is connected to mastitis in goats and cows, one of the most expensive illnesses in the dairy industry. Mastitis is an infectious condition that incurs significant financial losses for dairy and food farmers worldwide (Xing et al. 2016). These iseases, in one way or another, may harm inseminated females or cause sperm quality to rapidly deteriorate (Al-Kass et al. 2019).

The removal of bacteria from semen or their growth inhibition has been accomplished by using a variety of techniques, including the inclusion of antibiotics to semen extenders, traditional centrifugation and mechanical removal of bacteria by using single-layer centrifugation (Morrell and Wallgren 2014; Santos and Silva 2020). The use of semen extenders containing antibiotics was the most popular strategy to prevent bacterial growth. However, the inclusion of antibiotics can cause bacterial resistance, hence, the exploration of traditional antibiotic to combat this issue was in rising mode.

Consequently, the interest in herbal plants was increased due to the notion that plant-based medications are less likely to cause adverse effects than synthetic ones. This makes the quest for a natural alternative

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an appealing choice, especially when combined with the lower cost of plant preparations. It is evident from literature that mulberry, roselle and aloe vera have a lot of promises in terms of their anti-inflammatory, anti-cancer, anti-hypertensive capabilities and many other significant biological functions (Gupta et al. 2020; Miroddi et al. 2015; Patel 2014; Yang and Lee 2012).

Despite the extensive history and popular acceptance of antibacterial properties of these plant extracts, only a few articles had discussed their ability as an alternative to antibiotics in semen extender. Previous study mostly focuses on the antioxidant effect of mulberry, roselle and aloe vera on freezing and chilling semen. Mulberry demonstrated protective effects for bovine spermatozoa against cryoinjuries and the post-thawed oxidative stress (Suleman et al. 2022). Moreover, it improved sperm traits when added to semen dilution in Awassi ram (Abdul et al. 2022). The effects of roselle on the sperm functions as well as oxidative stress in the sperm and testes in high fat diet-induced obese rats was shown previously (Puchai et al. 2019). It was also found that the aqueous extract of roselle calyx could reduce ROS production in sperm freezing and thawing process in bovine sperm (Jamaludin et al. 2012). Aloe vera has been reported as an efficient cryoprotectant on several species, such as domestic cat (Barbosa et al. 2020), collared peccary (Souza et al. 2016), fish (Garcia et al. 2016; Yong et al. 2017), bovine (Singh et al. 2020) and ovine (Brito et al. 2014). A recent study on goat by (Zareie et al. 2021) focused on determining the use of aloe vera extract to reduce oxidative stress of sperm during cryopreservation but limited studies focus to determine the antibacterial activity of aloe vera on goat semen.

To the best of our knowledge, no study has been done to directly compare the antibacterial ability of mulberry, roselle and aloe vera extracts and its effect on semen of Saanen buck. Therefore, this study was conducted to determine the antibacterial ability of mulberry, roselle and aloe vera extract and the possibility of these plants extract as an alternative substance to replace antibiotics in semen extenders of Saanen goats.

Materials and method

Preparations of plant extracts

The mulberry (*Morus nigra*), roselle (*Hibiscus sabdariffa* L.) and aloe vera (*Aloe barbadensis mille*) plant were purchased from a local plant nursery. The leaves part of mulberry, calyx of roselle fruits and leaves of the aloe vera plant were collected and washed with distilled water. Aloe vera leaves were cut open , and the fresh pulp was collected. The samples were then oven dried at 40 °C for 48 h. The dried plants were ground, kept in a sealed container and stored in a refrigerator (4 °C) prior to extraction. Ethanolic extracts of mulberries, roselle and aloe vera were prepared by dissolving 200g of plant powder with 95% ethanol at 30 °C for 48 h. The extracts were then filtered by using Whatman no. 1 filter paper. The filtrate was freeze-dried by lyophilisation after being concentrated under vacuum at 45 °C by using a rotary evaporator. Solvents (ethanol) were eliminated from the concentrates by placing them in a recirculating air incubator. The total extract of mulberries, roselle and aloe vera were each dissolved in water at a concentration of 50 mg/ml and stored at −20 °C for further use.

Two experiments were conducted to study bacterial species present in the buck semen and the antibacterial activity of plant extract against the bacteria present in vitro (Experiment 1). The effectiveness of plant extract in fresh and post-thawed semen on bacterial control and quality, including in vivo fertility of spermatozoa, was determined in Experiment 2.

Experiment 1: Determination of the antibacterial activity of plant extract

Animals and semen collection

Semen was collected from five mature and healthy Saanen bucks maintained at Ladang 4, MARDI Kluang. Semen was collected by using an artificial vagina at weekly intervals (triplicates).

Bacterial load

The bacterial load of aseptically obtained semen samples was assessed using the standard plate count (SPC) method. The SPC was conducted by spread plate method by using the serial dilution method. Fresh semen samples were transferred to 99 ml dilution blanks (44 – 45 °C) which contained 2% sodium citrate solution. Shaking was carried out several times until emulsification (first dilution). Then, 1 ml of samples were transferred from the first dilution to 99 ml dilution blanks which contained 2% sodium citrate solution (second). Steps were repeated and were then poured into each well of inoculated plates aseptically. The petri dishes were then incubated at 37 °C for 48 h and the number of colonies that arose was counted with a colony counter. The bacterial load in semen was expressed as a number of colony-forming units (CFU) per ml of the semen. This was determined by using the following formula:

Number of colonies × dilution factor of the plate/ml of the diluted semen

Identification of bacteria

Samples from fresh semen were cultivated for aerobic bacteria on blood agar (Gibco) by using normal microbiological procedures. The colonies that grew after being incubated for 24 h at 37 °C were chosen based on their morphological traits, and they were then cultivated once more on blood agar to get a pure culture. Isolates were typed by Gram staining.

Antibacterial activity of plant extract

Each semen sample was plated on nutrient agar and incubated at 37 °C in 5% of CO_2 for 24 h. The antibacterial activity of roselle fruits, mulberry leaves and aloe vera leaves powder extract against identified gram-positive and gram-negative bacteria were tested by disc diffusion method. A 6 mm diameter paper disc was soaked in 100 µl of the extracts and then placed on the surface of media that contained inoculated bacteria. Sterile distilled water which was also added to a paper disc was designated as a control. The plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested bacteria. All tests were performed in triplicate.

Experiment 2: Comparing herbal extracts and commercial antibiotics on sperm survivability and quality of fresh and frozen thawed semen

Extender preparation and dilution

A Tris-based extender that contained 3.63 g of tris, 0.50 g of fructose, 14 ml of egg yolk and 1.99 g of citric acid was used as the base extender. Then, extender was divided into five aliquots for the addition of herbal extract. The first aliquot (T1) of the extender served as a positive control which contained synthetic antibiotic streptomycin and penicillin (1000 IU ml). In the second part (T2) of the extender, mulberry extract was added. Roselle extract was added to the third aliquot (T3) while aloe vera extract was added to the fourth aliquot (T4). Five parts (T5) of the extender did not contain antibiotics and served as controls. All diluted semen in T1, T2, T3, T4, and T5 were incubated at 37 °C for 3 h and cryopreserved for 24 h before the assessment of bacterial load and semen quality were investigated.

Semen cryopreservation

Each semen sample was diluted at 37 °C in a single step with one of the five aliquots of experimental extender in order to contain approximately 100 x 10^6 spermatozoa/ml. The semen was diluted, then allowed to cool to 4 °C for 2 h and equilibrated for another 2 h at 4 °C. Semen was then filled in 0.25 ml straws and then plunged into liquid nitrogen. Semen straws were frozen for 24 h, then thawed for at least 30 s at 37 °C to evaluate the quality and bacterial count of the semen.

Sperm quality evaluation

Sperm motility

Percentage of motility was determined by counting the motile and non-motile sperm cells. Diluted semen of each group was divided into five microliters (µl), put on a pre-warmed slide, covered with a cover slip and examined under a compound light microscope. Approximately 200 spermatozoa were observed in four fields to provide an accurate assessment of the percentage motility.

Sperm viability and normality

A small drop of the diluted semen was mixed in 2 – 3 drops of eosin-nigrosin stain and after 1 min a smear was prepared on a clean slide. Dead spermatozoa are either partly or completely stained, whereas unstained spermatozoa are living spermatozoa that did not take on any colour. The percentage of normal sperm was also determined from the stained sperm smears by observing the head, midpiece and tail morphology of the sperm, as described in Mohammed et al. (2022). A total of 200 spermatozoa were counted at various fields of the slide.

Sperm membrane integrity

The sperm membrane integrity was assessed by using hypo-osmotic swelling test (HOST) technique, as previously reported by Ramu and Jeyendran (2013). Briefly, 1 ml of hypo-osmotic solution (HOST, 13.5 g/l of fructose, 7.35 g/l of sodium citrate, in 1 l of distilled water, 94:02 ± 1:11 mos/kg) was mixed with 200 µl of semen sample. Then, the mixture was incubated at 37 °C for 30 min. A drop (10 µl) of the mixture was put onto a coverslip-covered microscopic slide and the number of spermatozoa with swollen tails were counted under phase-contrast microscope at 400 × magnification.

Statistical analysis

The data are presented as the means ± SEM, and statistical analysis was done by using SPSS computer software package (IBM SPSS, Version 26). Two-way analysis of variance (ANOVA) was done to compare differences in bacterial count amongst treatment groups and times for each semen parameter. Duncan post hoc multiple comparisons were used to compare the within-class means when the main effects were determined to be statistically significant, whereby $p < 0.05$ was regarded as significant. The study employed linear regression and Pearson's correlation coefficient to examine the degree of linear association between semen parameters and microbial load.

Results and discussion

Isolation and identification of bacteria from buck semen

In the present study, bacterial load ranged from 0 to 1650 CFU/ml in Saanen goats. The average bacterial load in fresh semen was 747 ± 15.06 CFU/ml which was a bit higher than reported in the semen of Jamunapari, Barbari and Jakhrana bucks found at 540.50 ± 55.88 CFU/ml, 391.81 ± 46.33 CFU/ml and 388.93 ± 44.71 CFU/ml, respectively (Gangwar et al., 2021). This difference

might be due to the difference in environment, seasonal and breed that may influence the presence of bacteria. Despite higher bacterial load, the semen quality of fresh ejaculate was within an acceptable good percentage with sperm motility at above 85%, viability at 80%, normality at 75%, and membrane integrity at 80%.

Microbiological analyses of the semen showed presence of gram-positive and gram-negative bacteria. *Figure 1* shows the morphology of bacteria collected from the buck semen. Gram-positive bacteria isolated from the buck semen appeared spherical and formed grape-like clusters. They were identified under genus Staphylococcus. Whereas, gram-negative bacteria were observed as bacillus or rod-shaped and were arranged in chains and loosely tangled clumps. They were identified under the genus Enterobacter. The presence of *Staphylococcus spp*. was previously reported in other goat breeds such as Jamunapari, Barbari, and Jakhrana (Gangwar et al. 2021). The species identified in these three breeds were *Staphylococcus aureus, Staphylococcus sciuri, Staphylococcus haemolyticus, Staphylococcus chromogenes, Staphylococcus epidermidis*, and *Staphylococcus simulans*. Similarly, a previous study in ram semen ejaculates recorded the presence of *Staphylococcus aureus* and *Staphylococcus epidermidis* and other Gram-negative bacteria belonging to enterobacteriaceae were found in a higher proportion of semen samples (Ahmed et al. 2018; Yániz et al. 2010). Similar studies were conducted on bull and boar, which also reported Staphylococcus (Reda et al. 2020) and Enterobacteriaceae species (Costinar et al. 2021) in the semen.

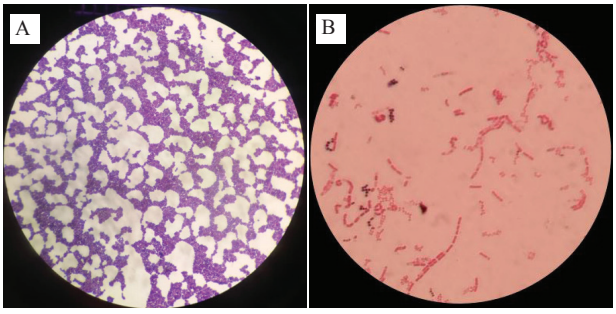

Figure 1. Gram-positive bacteria (A) and Gram-negative bacteria (B) identified under light microscope (1000X magnification)

In vitro antibacterial activity of plant extracts

Figure 2 shows that gram-positive and gram-negative bacteria exhibited different susceptibility responses against mulberry, roselle and aloe vera extracts. The results showed that the three plant extracts significantly inhibited the growth of gram-positive bacteria but only mulberry extract showed a significant inhibitory effect on gram-negative bacteria. Mulberry extract showed significant antibacterial activity (p <0.05) with the largest inhibitory zone of 22.3 mm and 10.8 mm for both gram-positive and gram-negative bacteria, respectively (*Table* 1).

Although aloe vera and roselle extracts exhibited antibacterial activities against gram-positive bacteria; however, no significant inhibitory effect was observed on the gram-negative bacteria. Thus this study revealed that impact of a plant extract can differ, depending on whether gram-positive or gram-negative microbes are present. Plant extract showed stronger antibacterial action against gram-positive bacteria than gram-negative bacteria. Similar outcomes were observed in a prior investigation on foodborne bacteria which used plant extracts. As compared to gram-negative bacteria (*E. coli*), Krasaekoopt et al. (2005) found that the plant extract used in the study showed the largest zones of inhibition on *Staphylococcus aureus*, a gram-positive bacterium. This may be explained as gram-positive bacteria have a single layer of cell wall, but gram-negative bacteria have many layers and a more complicated structure (Mai-Prochnow et al. 2016).

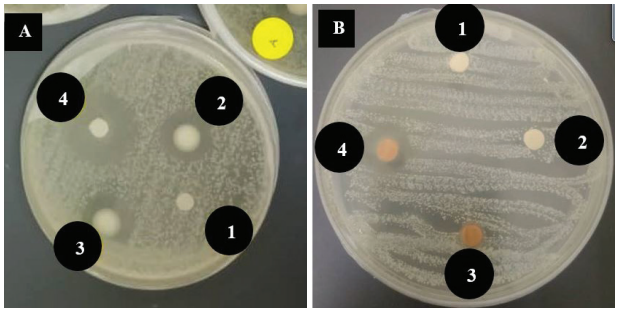

Figure 2. Antibacterial activity of plant extract on gram-positive bacteria (A) and gram-negative bacteria (B). (1) control; (2) roselle extract; (3) aloe vera extract; (4) mulberry extract

Correlation between semen quality and total bacterial load

Correlation and regression analysis revealed that the percentage of sperm motility, viability, plasma membrane integrity and normality were negatively affected by the rise in bacterial load of semen (*Tables* 2, *Figure* 3). The strongest correlation was observed between bacterial load and motility ($r = –0.947$, p <0.05) and viability ($r = –0.922$, p <0.05). Multiple regression analysis also revealed that with the increase of bacterial load in the buck semen, the semen quality was compromised.

Observations from this study affirmed that there was a correlation between bacterial load and semen quality, as an increase in bacterial numbers led to the deterioration of sperm quality in terms of motility and viability and caused morphological alterations and membrane integrity in the sperm cells of goats. These findings were in agreement with other studies on goat (Gangwar et al. 2021), cow (Reda et al. 2020), boar (Luther et al. 2023), and human (Eini et al. 2021). Higher microbial counts in the semen of bulls were shown to be negatively correlated with sperm motility and vitality.

Table 1. Inhibition zone of Mulberry, Roselle and Aloe vera extracts on gram-positive and gram-negative bacteria (Mean ± SEM). Difference superscripts in the same column indicate significant difference (p <0.05)

Label	Plant extract	Inhibition diameter (mm)	
		Gram-positive bacteria	Gram-negative bacteria
1	Control (distilled water)	6.0 ± 0.40 [a]	6.0 ± 0.22 [a]
2	Roselle	8.1 ± 1.08 [b]	6.2 ± 1.09 [a]
3	Aloe vera	9.6 ± 1.05 [b]	6.1 ± 1.11[a]
4	Mulberry	22.3 ± 1.12[c]	10.8 ± 1.20 [b]

Table 2. Correlation among semen traits and bacterial load in Saanen bucks

Semen traits	Motility	Membrane integrity	Viability	Normality	Bacterial load
Motility	1	0.534*	0.899**	0.788**	-0.947**
Membrane integrity		1	0.649*	0.729**	-0.666*
Viability			1	0.770**	-0.922**
Normality				1	-0.724*
Bacterial load					1

*. Correlation is significant at the 0.05 level (2-tailed)
**. Correlation is significant at the 0.01 level (2-tailed)

The bacterial contamination of semen caused detrimental effects on sperm quality, either directly on the nutrients source for sperms, which was represented in semen diluents, or indirectly by the formation of endotoxins and metabolic toxic byproducts that could harm sperms (Fraczek and Kurpisz 2015; Luther et al. 2023). Study on *S. aureus* released alpha-toxin, which could directly affect membrane permeability by the formation of small pores that enabled an uncontrolled flux of Na+ into the cell and subsequent apoptosis and necrosis (Villegas et al. 2005). According to Kaur and Prabha (2013), the decreased sperm viability occurred due to bacteria which produced soluble spermatotoxic substances. They also discovered that sperm immobilisation or decreased motility was caused by bacteria-producing sperm inhibitory factor (SIF). SIF lowered mitochondrial ATPase activity, which in turn inhibited sperm motility and viability. Loss of sperm motility and normal sperm morphology after bacterial semen infection might be the result of adhesion phenomena and sperm agglutination. The surface of sperm is rich in glycoproteins, it can interact with bacteria through receptor-ligand interactions. Previous studies also showed a markedly increased incidence of ASA (antibodies against sperm antigen) in seminal plasma from urinary tract infection (UTI) patients (Fraczek and Kurpisz 2015). The reason for this might be interactions between bacteria and spermatozoa at the receptor-ligand level, as the sperm surface is rich in glycoproteins, and thus susceptible to bacterial harm. Additionally, an increased presence of bacterial load triggers the immune response, causes oxidative stress, and thereby contributes to sperm structure alterations (Ďuračka et al. 2021). According to earlier research, spermatozoa quality and ROS levels were adversely correlated (Wang et al. 2021). Polyunsaturated fatty acids make up the majority of lipids in membrane-bound sperm and are especially vulnerable to oxidative stressors. A higher proportion of spermatozoa with damaged membranes may result from increased ROS production brought on by an increase in the bacterial load.

Impact of plant extracts on bacterial load and sperm traits

In the current study, bacterial load varied (p <0.05) according to time. The results displayed an increase in viable bacterial counts in all groups of diluted semen, stored from 0 to 3 h at 37 °C (*Table* 3). The bacterial count between groups at 0 h was non-significant, but after 3 h of incubation, the bacterial load increased significantly but reduced in number in the post-thawed semen. As compared to the negative control (no antibiotic), all treatment groups recorded lower bacterial counts (p <0.05) during 3 h of incubation time and after post-thawed. Semen added with Mulberry extract (763 ± 4.57 CFU/ml) showed the lowest bacterial count amongst groups after 3 h of incubation, whereas roselle extracts (650 ± 2.10 CFU/ml) and aloe vera extract (587 ± 3.47 CFU/ml) showed lower bacterial count and a higher effect on bacterial contamination control in post-thawed semen.

Table 4 shows the quality of fresh (0 h) sperm which was not significantly different between groups (p >0.05). The average percentage of sperm motility was 87.58 ± 15.66 %, membrane integrity was 80.69 ± 21.85 %, viability was 85.44 ± 18.36 % and normality was 79.25 ± 16.80 % for Saanen buck. After 3 h of incubation at 37 °C, the sperm quality was most affected when no antibiotics were added to the extender (*Figure* 4). The percentage of sperm motility, viability, normality and plasma membrane integrity showed the lowest with no antibiotics as compared to when plant extracts were added. Mulberry extract provided more efficient preservation (p <0.05) of

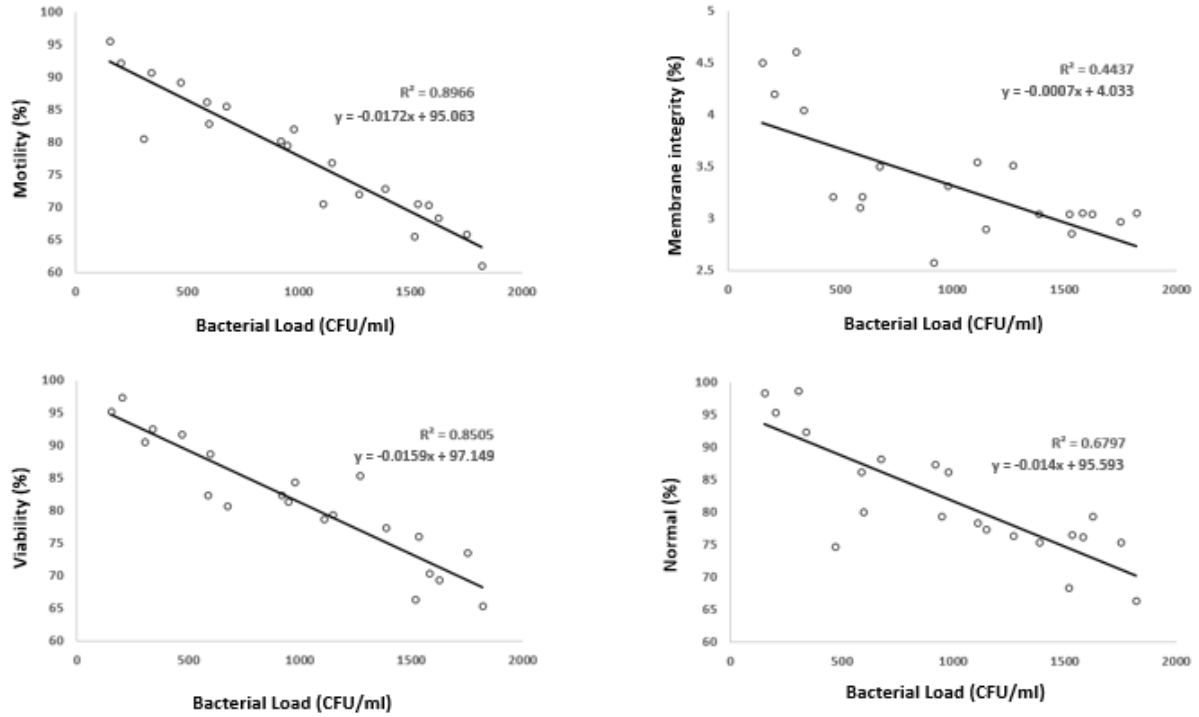

Figure 3. Scatter plot distribution of individual observations for bacterial load with different semen parameters in Saanen bucks

the sperm motility and viability as compared to all the groups during 3h of incubation. However, the percentage of plasma membrane integrity was not significantly different as compared to commercial antibiotics. Sperm motility and viability for semen extender with commercial antibiotics, mulberry and aloe vera were not significantly different between groups (p >0.05) but significantly higher as compared to a control group with no antibiotic (p <0.05). The percentage of normality was similar in all treatment groups but significantly higher as compared to control.

In vitro study showed mulberry extract demonstrated the highest antibacterial activity on both gram-positive and negative-bacteria isolated from fresh buck semen. Therefore, it is indicated as the most effective plant extract as compared to roselle and aloe vera extract. The results are in accordance with the in vivo study which showed after 3 h of incubation with an extender that was added with mulberry extracts, the buck semen showed the lowest bacterial loads and better overall sperm quality. Previously, a study on Awassi ram semen proved that by adding mulberry leaf extract to a low concentration (Tris) diluent had an effective role in improving the studied semen characteristics of the sperm (Abdul et al. 2022). Herbal extracts of the root and leaves of mulberry were found to inhibit gram-positive and gram-negative bacteria (Sharifee et al. 2022). The activities were principally attributed to soluble protein and phenolic compounds, including mulberrin (Kuwanon C), albanol A (Mulberrofuran G), and albanol B (Zafar et al. 2013). Some previous studies also found that mulberry root and leaves contain quercetin, and fascinatingly, it was the leaves that contained the most active ingredient, quercetin substance (Enkhmaa et al. 2005), which was known to be effective against gram-positive and gram-negative bacteria (Grajek et al. 2015; Salem et al. 2013). Quercetin can destroy membranes of bacteria, resulting in the inhibition of their growth (Shengnan Wang et al. 2018).

A decrease in sperm quality was observed after cryopreservation in all groups (*Figure* 5). However, all treatment groups, either added with commercial antibiotic or plant extracts, provided higher sperm quality as compared to the control group (p <0.05). Overall results indicated that semen diluted in an extender added with roselle or aloe vera extract showed better post-thawed quality. Semen added with roselle or aloe vera extract showed significantly higher sperm viability than other groups, while the percentage of motility and plasma membrane integrity for roselle or aloe vera groups was comparable with commercial antibiotics. The mulberry extract group showed lower sperm motility and membrane integrity as compared to other treatment groups, but was comparable to the commercial antibiotic group for viability and normality percentage. Interestingly, the mulberry extracts did not show the same protective effect on post-thawed semen. Although from an in vitro study, roselle and aloe vera extracts were unable to show strong antibacterial activity on bacteria isolated from fresh buck semen, but in post-thawed semen both extracts proved to be more effective in decreasing bacterial contamination and preserving the semen quality of the buck.

Aloe vera and roselle extracts are known for their antibacterial and antioxidant properties (Gupta et al. 2020; Khan et al. 2023; Singh et al. 2020). The protective effect on aloe vera extracts on cryopreserve semen has been demonstrated in cattle (Singh et al. 2020). The study

Table 3. Effect of plant extracts on bacterial count (Mean ± SEM) of 0 Hour, 3 h incubation (37 °C) and post-thawed semen. Difference superscript in the same column indicate significant difference (p <0.05)

Group	Bacterial count (CFU/ml)		
	0 Hour	3 hours	Post thawed
Commercial antibiotic (Positive control)	696 ± 5.03[a]	941 ± 12.02 [b]	719 ± 3.11[b]
No antibiotic (Negative control)	723 ± 9.40 [a]	1605 ± 9.45 [c]	1290 ± 2.05 [c]
Mulberry	750 ± 4.70 [a]	763 ± 4.57 [a]	726 ± 2.41 [b]
Roselle	718 ± 3.00 [a]	1082 ± 8.21[b]	650 ± 2.10 [a]
Aloe vera	688 ± 9.51 [a]	937 ± 6.31[b]	587 ± 3.47 [a]

Table 4. Effect of plant extracts on semen quality of fresh semen. Difference superscripts in the same column indicate significant differences (p <0.05)

Group	Sperm quality (Fresh, 0 Hour)			
	Motility (%)	Membrane integrity (%)	Viability (%)	Normal (%)
Commercial antibiotic (Positive control)	86.52 ± 12.35 [a]	80.20 ± 11.20 [a]	82.11 ± 14.21 [a]	83.41 ± 6.20 [a]
No antibiotic (Negative control)	90.55 ± 9.55 [a]	82.16 ±16.32 [a]	80.76 ± 12.11 [a]	75.30 ± 14.20 [a]
Mulberry	88.67 ± 3.65 [a]	83.52 ± 10.21 [a]	80.44 ± 17.19 [a]	82.24 ± 21.45 [a]
Roselle	91.14 ± 19.32 [a]	80.26 ± 13.02 [a]	88.38 ± 21.25 [a]	83.36 ± 16.10 [a]
Aloe vera	89.21 ± 20.20 [a]	82.66 ± 9.25 [a]	89.12 ± 18.21 [a]	82.09 ± 8.22 [a]

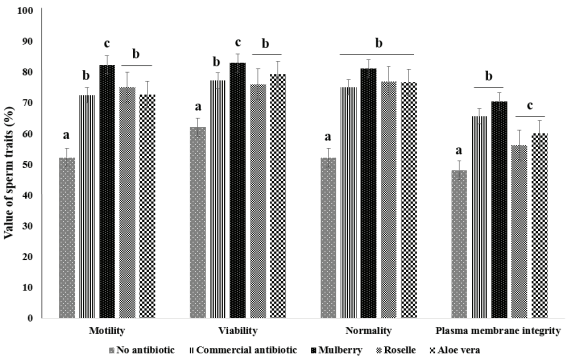

Figure 4. Effect of plant extract compared to commercial and no antibiotic added to semen extender on buck sperm traits at 3 hours of incubation at 37 °C. Bar with different letters differed significantly (p <0.05)

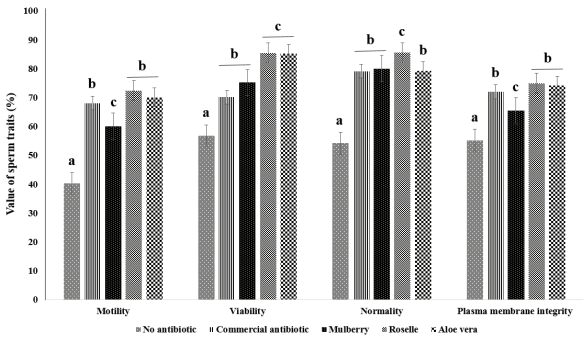

Figure 5. Effect of plant extract compared to commercial and no antibiotic added to semen extender on buck sperm traits after post-thawed. Bar with different letters differed significantly (p <0.05)

showed a significant increased progressive motility, live spermatozoa, acrosomal integrity and HOST positive spermatozoa, while a significantly decreased sperm abnormalities in post-thawed semen that was similar to this current study. In oxidative stress evaluation, the MDA level was also decreased by aloe vera extracts. The aloe vera metabolites associated with antibacterial activity are anthraquinones, glucomannan and acemannan (Maan et al. 2018). Roselle calyx extracts have previously shown to effectively and dose-dependently inhibit the growth of numerous bacteria such as *E. coli, S. aureus, Str. Mutans* and *P. aeruginosa* (Al-Hashimi and Al-Hashimi 2012). The antibacterial activity of the calyx extracts of roselle can be attributed to the action of the phytochemical compounds it contains (Babayi et al. 2004). These bioactive compounds are known to act by different mechanisms and exert antimicrobial action. Flavonoids are hydroxylated phenolic substances known to be synthesised by plants in response to microbial infection and it should not be surprising that they were found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cowan 1999). Moreover, the polyphenolic nature of the flavonoid gossyypetin in roselle extract could also

be attributed to the antibacterial activity against bacteria in buck semen (Mounnissamy et al. 2002). In addition, roselle extract contains high antioxidant properties. These plant extracts not only inhibit the growth of bacteria, but also helps to provide antioxidants and reduce oxidative stress in semen during freezing. A previous study on Boer goat to determine the ability of roselle extract added to an extender to improve buck semen was unsuccessful due to factors, such as poor extraction method and/or degradation of anthocyanin pigment during the extraction and storage process (Mohammed et al. 2022). However, this current study was able to demonstrate a good effect on the post-thawed sperm quality of Saanen goats.

Roselle and aloe vera extracts demonstrated better post-thaw sperm quality may due to their strong antioxidant and membrane-stabilizing effects, which counteracted cryopreservation-induced damage (Costa et al. 2021; Zareie et al. 2021). Their effectiveness in decreasing bacterial contamination post-thaw further highlights their potential as natural alternatives to commercial antibiotics in semen extenders. Although these extracts did not exhibit strong antibacterial activity in vitro against bacteria isolated from fresh semen, their inclusion in the extender likely reduced bacterial contamination in post-thawed semen. This could be due to the ability of some compounds in the extracts to inhibit bacterial growth indirectly or enhance the semen extender's overall efficacy in controlling contamination. In contrast, mulberry extract showed limited cryoprotective effects, may due to weaker or less targeted biochemical properties for semen preservation (Kutluyer et al. 2014). The mulberry extract may have lacked sufficient antioxidant capacity or contained compounds less effective at stabilizing the sperm membrane during cryopreservation. Additionally, some bioactive compounds in mulberry could have interacted negatively with sperm, contributing to reduced motility and membrane integrity. This possibility requires further investigation in future studies to identify and evaluate the specific components of mulberry extract and their impact on sperm preservation. Despite its limitations, mulberry extract still preserved viability and normality comparably to commercial antibiotics, suggesting it provided some level of protection, although weaker than roselle or aloe vera.

Conclusion

Overall, this study observed that semen extenders without any addition of antibiotic or antibacterial plant extract were more prone to bacterial contamination reflected by the highest bacterial load number, and thus amongst other groups, it showed the lowest sperm quality after 3 h incubation at 37 °C and 24 h freezing. All three tested plant extracts showed good antibacterial activities in the present study and comparable inhibitory effects with commercial antibiotics on bacterial growth as well as being able to protect the quality of buck sperm. Their inclusion in the composition of buck semen extender had proven to work as a natural and reliable substitute to commercial antibiotics since it was found to have antibacterial properties against the bacteria isolated from buck semen and effectively inhibited bacterial growth in semen extender and resulted in higher sperm quality. Mulberry extract is a more suitable alternative to commercial antibiotics for fresh semen extenders while aloe vera and roselle are more recommended for cryopreservation purposes.

References

Abdul, N., Abdul, H., Mahmood, F. A., & Mahmood, R. M. (2022). The effect of white mulberry , morus alba l leaf extract on some the effect of white mulberry , morus alba l leaf extract on some characteristics of the cooled semen of awassi rams. January.

Ahmed, E., Islam, M., Alam, M., Jha, P., Ghosh, S., Naher, N., & Bari, F. (2018). Bacterial contamination of ram semen used for artificial insemination in indigenous ewes. *Bangladesh Veterinarian*, *34*(1), 20–26. https://doi.org/10.3329/bvet.v34i1.38709

Al-Hashimi, A., & Al-Hashimi, A. G. (2012). Antioxidant and antibacterial activities of Hibiscus sabdariffa L. extracts. *African Journal of Food Science*, *6*(21), 506–511. https://doi.org/10.5897/AJFS12.099

Al-Kass, Z., Eriksson, E., Bagge, E., Wallgren, M., & Morrell, J. M. (2019). Bacteria detected in the genital tract, semen or pre-ejaculatory fluid of Swedish stallions from 2007 to 2017. *Acta Veterinaria Scandinavica*, *61*(1). https://doi.org/10.1186/S13028-019-0459-Z

Babayi, H., Kolo, I., Okogun, J., & Ijah, U. (2004). The antimicrobial activities of methanolic extracts of Eucalyptus camaldulensis and Terminalia catappa against some pathogenic microorganisms. *Biokemistri*, *16*(2). https://doi.org/10.4314/BIOKEM.V16I2.32578

Barbosa, B. de S., dos Santos, F. A., do Macêdo, L. B., Izzo, R. G., Fernandes, D. P., Praxedes, É. A., Silva, A. R., & Bezerra, M. B. (2020). Effect of supplementation of *Aloe vera* extracts in cold storage media and cryopreservation of domestic cat epididymal spermatozoa. *Animal Reproduction*, *17*(1), e20190067. https://doi.org/10.21451/1984-3143-AR2019-0067

Costa, F., Vasconcelos, E., Azevedo, V., Paulino, L., Soares, M., Silva, J., Silva, A., & Souza, A. (2021). Aloe vera increases mRNA expression of antioxidant enzymes in cryopreserved bovine ovarian tissue and promotes follicular growth and survival after in vitro culture. *Cryobiology*. https://doi.org/10.1016/j.cryobiol.2021.07.005.

Costinar, L., Herman, V., Pitoiu, E., Iancu, I., Degi, J., Hulea, A., & Pascu, C. (2021). Boar Semen Contamination: Identification of Gram-Negative Bacteria and Antimicrobial Resistance Profile. *Animals, 12*(1), 43. https://doi.org/10.3390/ANI12010043

Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews*, *12*(4), 564. https://doi.org/10.1128/CMR.12.4.564

Ďuračka, M., Belić, L., Tokárová, K., Žiarovská, J., Kačániová, M., Lukáč, N. & Tvrdá, E. (2021). Bacterial communities in bovine ejaculates and their impact on the semen quality. *Systems Biology in Reproductive Medicine*, *67*(6), 438–449. https://doi.org/10.1080/19396368.2021.1958028

Eini, F., Kutenaei, M. A., Zareei, F., Dastjerdi, Z. S., Shirzeyli, M. H. & Salehi, E. (2021). Effect of bacterial infection on sperm quality and DNA fragmentation in subfertile men with Leukocytospermia. *BMC Molecular and Cell Biology*, *22*(1), 1–10. https://doi.org/10.1186/S12860-021-00380-8/FIGURES/3

Enkhmaa, B., Shiwaku, K., Katsube, T., Kitajima, K., Anuurad, E., Yamasaki, M. & Yamane, Y. (2005). Mulberry (Morus alba L.) Leaves and Their Major Flavonol Quercetin 3-(6-Malonylglucoside) Attenuate Atherosclerotic Lesion Development in LDL Receptor-Deficient Mice. *The Journal of Nutrition*, *135*(4), 729–734. https://doi.org/10.1093/JN/135.4.729

Fraczek, M. & Kurpisz, M. (2015). Mechanisms of the harmful effects of bacterial semen infection on ejaculated human spermatozoa: potential inflammatory markers in semen. *Folia Histochemica et Cytobiologica*, *53*(3), 201–217. https://doi.org/10.5603/FHC.A2015.0019

Gangwar, C., Kumaresan, G., Mishra, A. K., Kumar, A., Pachoori, A., Saraswat, S., Singh, N. P. & Kharche, S. D. (2020). Molecular detection of important abortion-causing microorganisms in preputial swab of breeding bucks using PCR-based assays. *Reproduction in Domestic Animals*, *55*(11), 1520–1525. https://doi.org/10.1111/RDA.13801

Gangwar, C., Mishra, A. K., Gururaj, K., Kumar, A., Kharche, S. D., Saraswat, S., Kumar, R. & Ramachandran, N. (2021). Semen quality and total microbial load: An association study in important Indian Goat breeds during different seasons. *Andrologia*, *53*(4), 1–13. https://doi.org/10.1111/and.13995

Garcia, R. R. F., Streit, D. P., Cabrita, E., C.Godoy, L., Garcia, R. R. F., Streit, D. P., Cabrita, E. & C.Godoy, L. (2016). Semen Cryopreservation in Brazilian Freshwater Fish: Advances and Main Challenges. *Cryopreservation in Eukaryotes*. https://doi.org/10.5772/65037

Grajek, K., Wawro, A. & Kokocha, D. (2015). Bioactivity of Morus Alba L. Extracts-an Overview. *International Journal of Pharmaceutical Sciences and Research*, *6*(8), 3110. https://doi.org/10.13040/IJPSR.0975-8232.6(8).3110-22

Gupta, V. K., Kumar, A., Pereira, M. de L., Siddiqi, N. J. & Sharma, B. (2020). Anti-Inflammatory and Antioxidative Potential of Aloe vera on the Cartap and Malathion Mediated Toxicity in Wistar Rats. *International Journal of Environmental Research and Public Health 2020, Vol. 17, Page 5177*, *17*(14), 5177. https://doi.org/10.3390/IJERPH17145177

Jamaludin, M., Hanis, M., Balkis, S. & Mohamed, J. (2012). *Effects of Aqueous Extract of Hibiscus sabdariffa L. (Roselle) Calyx on Bovine Sperm Membrane*.

Kaur, K., & Prabha, V. (2013). Sperm impairment by sperm agglutinating factor isolated from Escherichia coli: Receptor specific interactions. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/548497

Khan, R. U., Naz, S., De Marzo, D., Dimuccio, M. M., Bozzo, G., Tufarelli, V., Losacco, C. & Ragni, M. (2023). Aloe vera: A Sustainable Green Alternative to Exclude Antibiotics in Modern Poultry Production. In *Antibiotics 12*(1). https://doi.org/10.3390/antibiotics12010044

Kutluyer, F., Kayim, M., Öğretmen, F., Büyükleblebici, S. & Tuncer, P. (2014). Cryopreservation of rainbow trout Oncorhynchus mykiss spermatozoa: effects of extender supplemented with different antioxidants on sperm motility, velocity and fertility. *Cryobiology*, 69 3, 462-6. https://doi.org/10.1016/j.cryobiol.2014.10.005.

Luther, A. M., Nguyen, T. Q., Verspohl, J. & Waberski, D. (2023). Storage of boar semen at 17 °C without conventional antibiotics in an extender containing an organic bactericidal substance. *Frontiers in Veterinary Science*, *10*, 1294903. https://doi.org/10.3389/FVETS.2023.1294903/BIBTEX

Maan, A. A., Nazir, A., Khan, M. K. I., Ahmad, T., Zia, R., Murid, M. & Abrar, M. (2018). The therapeutic properties and applications of Aloe vera: A review. *Journal of Herbal Medicine*, *12*, 1–10. https://doi.org/10.1016/J.HERMED.2018.01.002

Mai-Prochnow, A., Clauson, M., Hong, J. & Murphy, A. B. (2016). Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Scientific Reports, 6*(1), 1–11. https://doi.org/10.1038/srep38610

McMillan, K., Moore, S. C., McAuley, C. M., Fegan, N. & Fox, E. M. (2016). Characterization of Staphylococcus aureus isolates from raw milk sources in Victoria, Australia. *BMC Microbiology*, *16*(1), 1–12. https://doi.org/10.1186/S12866-016-0789-1/FIGURES/5

Miroddi, M., Navarra, M., Calapai, F., Mancari, F., Giofrè, S. V., Gangemi, S. & Calapai, G. (2015). Review of Clinical Pharmacology of Aloe vera L. in the Treatment of Psoriasis. *Wiley Online LibraryM Miroddi, M Navarra, F Calapai, F Mancari, SV Giofrè, S Gangemi, G CalapaiPhytotherapy Research, 2015•Wiley Online Library*, *29*(5), 648–655. https://doi.org/10.1002/ptr.5316

Mohammed, N. H., ShikhMaidin, M. & Yien, C. Y. S. (2022). Roselle Calyx Extract as an Antioxidant Supplement on Tris-Base Extender for Goat Semen. *Jurnal Ternak*, *13*(1), 23–29. https://doi.org/10.30736/jt.v1i1.85

Morrell, J. M. & Wallgren, M. (2014). Alternatives to Antibiotics in Semen Extenders: A Review. *Pathogens 2014, Vol. 3, Pages 934-946*, *3*(4), 934–946. https://doi.org/10.3390/PATHOGENS3040934

Mounnissamy, V., Gunasegaran, R. & Gopal, V. (2002). *Diuretic activity of gossypetin isolated from Hibiscus sabdariffa in rats*. https://www.cabidigitallibrary.org/doi/full/10.5555/20043009214

Patel, S. (2014). Hibiscus sabdariffa: An ideal yet under-exploited candidate for nutraceutical applications. *Biomedicine and Preventive Nutrition*, *4*(1), 23–27. https://doi.org/10.1016/j.bionut.2013.10.004

Puchai, C., Kokmas, W., Kruevaisayawan, H., Sakara, T. & Onrawee, K. (2019). Effect of Hibiscus sabdariffa on sperm quality and testicular oxidative stress in rats fed with high fat diet. *Journal of Physiological and Biomedical Sciences Available*, *32*(2), 36–41.

Ramu, S. & Jeyendran, R. S. (2013). The hypo-osmotic swelling test for evaluation of sperm membrane integrity. *Methods in Molecular Biology*, *927*, 21–25. https://doi.org/10.1007/978-1-62703-38-0_3

Reda, A. A., Almaw, G., Abreha, S., Tadeg, W. & Tadesse, B. (2020). Bacteriospermia and Sperm Quality of Cryopreserved Bull Semen Used in Artificial Insemination of Cows in South Wollo Zone, Ethiopia. *Veterinary Medicine International*, *2020*. https://doi.org/10.1155/2020/2098315

Salem, M. Z. M., Aly, H., Gohar, Y. & El-Sayed, A.-W. (2013). Biological Activity of Extracts from Morus alba L., Albizzia lebbeck (L.) Benth. and Casuarina glauca Sieber Against the Growth of some Pathogenic Bacteria. *International Journal of Agricultural and Food Research*, *2*(1). https://doi.org/10.24102/IJAFR.V2I1.122

Santos, C. S. & Silva, A. R. (2020). Current and alternative trends in antibacterial agents used in mammalian semen technology. In *Animal Reproduction* (Vol. 17, Issue 1, pp. 1–13). Colegio Brasileiro de Reproducao Animal. https://doi.org/10.21451/1984-3143-AR2019-0111

Singh, P., Agarwal, S., Singh, H., Verma, P. K., Pandey, A. K. & Kumar, S. (2020). Antioxidant Effects of Aloe vera as Semen Additive in Cryopreservation of Cattle Bull Semen. *International Journal of Current Microbiology and Applied Sciences*, *9*(9), 1625–1635. https://doi.org/10.20546/ijcmas.2020.909.202

Souza, A. L. P., Lima, G. L., Peixoto, G. C. X., Silva, A. M., Oliveira, M. F. & Silva, A. R. (2016). Use of Aloe vera–based extender for chilling and freezing collared peccary (Pecari tajacu) semen. *Theriogenology*, *85*(8), 1432–1438. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.01.007

Suleman, S., Kanwal, M., & Ali, R.. (2023). Morus nigra Fruit Extract Safeguards Frozen Thawed Bovine Sperm Parameters. *Ijas.Rasht.Iau.Ir*. Retrieved February 21, 2024, from https://ijas.rasht.iau.ir/article_702543_5e38bcc31fd54d2c4e31ef6e90721e32.pdf

Wang, Shengnan, Yao, J., Zhou, B., Yang, J., Chaudry, M. T., Wang, M., Xiao, F., Li, Y. & Yin, W. (2018). Bacteriostatic Effect of Quercetin as an Antibiotic Alternative In Vivo and Its Antibacterial Mechanism In Vitro. *Journal of Food Protection*, *81*(1), 68–78. https://doi.org/10.4315/0362-028X.JFP-17-214

Wang, Sutian, Zhang, K., Yao, Y., Li, J. & Deng, S. (2021). Bacterial Infections Affect Male Fertility: A Focus on the Oxidative Stress-Autophagy Axis. *Frontiers in Cell and Developmental Biology*, *9*, 727812. https://doi.org/10.3389/FCELL.2021.727812

Xing, X., Zhang, Y., Wu, Q., Wang, X., Ge, W. & Wu, C. (2016). Prevalence and characterization of Staphylococcus aureus isolated from goat milk powder processing plants. *Food Control*, *59*, 644–650. https://doi.org/10.1016/J.FOODCONT.2015.06.042

Yang, J. Y. & Lee, H. S. (2012). Evaluation of antioxidant and antibacterial activities of morin isolated from mulberry fruits (*Morus alba* L.). *Journal of the Korean Society for Applied Biological Chemistry*, *55*(4), 485–489. https://doi.org/10.1007/s13765-012-2110-9

Yániz, J. L., Marco-Aguado, M. A., Mateos, J. A. & Santolaria, P. (2010). Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15 °C. *Animal Reproduction Science*, *122*(1–2), 142–149. https://doi.org/10.1016/J.ANIREPROSCI.2010.08.006

Yong, S. Y., Nguang, S. I. & Kari, A. (2017). Comparison between the effect of egg yolk-based extender and Aloe vera (*Aloe barbadensis*) based extender on red tilapia (*Oreochromis niloticus*) sperm quality. *Journal of Fundamental and Applied Sciences*, *9*(2S), 813–820. https://doi.org/10.4314/JFAS.V9I2S.50

Zafar, M. S., Muhammad, F., Javed, I., Akhtar, M., Khaliq, T., Aslam, B., Waheed, A., Yasmin, R. & Zafar, H. (2013). White mulberry (*Morus alba*): A brief phytochemical and pharmacological evaluations account. *International Journal of Agriculture and Biology*, *15*(3), 612–620.

Zareie, K., Farshad, A., Rostamzadeh, J., Azimi, G. & Ariyan, F. (2021). Freezability of Goat Epididymal Sperm using Aloe vera Extract and Trehalose in Diluents. *Austin Journal of Veterinary Science & Animal Husbandry*, *8*(2). https://doi.org/10.26420/austinjvetscianimhusb.2021.1078