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Isolation and identification of common bacterial contaminants in Malabari buck semen

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Abstract
The present study was undertaken to isolate and characterise the predominant bacterial contaminants in semen from Malabari bucks maintained at the Artificial Insemination Centre, College of Veterinary and Animal Sciences, Pookode, Wayanad. Semen was collected using a sterilised artificial vagina and evaluated for macroscopic characteristics. Microbiological analysis involved enrichment in selective broths, sub-culturing on eosin methylene blue agar (EMB) and Baird-Parker agar (BP), followed by colony morphology, Gram's staining and biochemical identification. The average ejaculate volume was 0.88 ± 0.02 mL, with colour ranging from creamy to creamy yellow and consistently high sperm density (DDDD). Cultural characterisation revealed two predominant colony types, which after Gram's staining appeared as Gram-positive cocci in clusters and Gram-negative rods. Biochemical profiles confirmed the isolates as *Staphylococcus aureus* (catalase positive, oxidase negative) and *Escherichia coli* (IMViC pattern: + + - -).

Keywords: Semen, Malabari Bucks, Bacteria, *S. aureus*, *E. coli*

1. Introduction
Artificial Insemination (AI) using cryopreserved semen is an advanced reproductive technique that plays a vital role in accelerating genetic progress and improving livestock breeds. By enabling the distribution of superior sire genetics over long distances, AI supports precision breeding, reproductive cycle management, and controlled mating strategies, ultimately enhancing herd productivity and efficiency (Leboeuf *et al.*, 2000; Gangwar *et al.*, 2016) [1, 2]. Despite its advantages, semen quality can be compromised during collection, dilution, freezing and thawing due to exposure to physical, chemical and microbial stressors. Among these, bacterial contamination is a significant concern, as it is nearly impossible to eliminate completely, even under strict hygienic conditions (Yániz *et al.*, 2010) [3].
Sources of contamination include the animal's genital tract, the environment, collection instruments, and handlers (Ahmed *et al.*, 2017) [4]. Common bacteria that are frequently isolated from semen are *Escherichia coli*, *Staphylococcus spp.*, *Pseudomonas spp.*, *Bacillus spp.*, and *Klebsiella spp.* (Ahmed *et al.*, 2017; Yániz *et al.*, 2010; Andrabi *et al.*, 2016) [4, 3, 5]. They negatively influence sperm by secreting toxins and enzymes that damage membranes, disrupt mitochondrial activity and induce premature acrosome reactions. They also produce metabolic byproducts such as ammonia and lactic acid, altering semen pH, while generating Reactive Oxygen Species (ROS) that cause lipid peroxidation, DNA fragmentation and protein damage, ultimately triggering sperm apoptosis and reducing fertilising capacity. These harmful effects are further intensified during cryopreservation, when sperm are already vulnerable to oxidative stress (Villegas *et al.*, 2005; Yániz *et al.*, 2010; Schulz *et al.*, 2010; Santos and Silva, 2020) [6, 3, 8, 9]. The present study was undertaken to isolate and characterise the predominant bacterial contaminants present in the semen of Malabari bucks.

2. Materials and Methods
The study was conducted from October 2024 to June 2025 utilising the Malabari bucks maintained at the Artificial Insemination under the Department of Animal Reproduction,

Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Pookode, Wayanad.

2.1 Semen collection
Semen was collected using a sterilised Danish-type artificial vagina maintained at 42 to 45 °C with sufficient pressure to facilitate proper ejaculation. Prior to each collection, the teaser buck was permitted two false mounts. The ejaculates were collected into tubes and immediately placed in a 37 °C water bath, after which they were taken to the semen evaluation laboratory for detailed analysis.

2.2 Macroscopic evaluation of semen samples
Semen samples were evaluated following the method of Bhai *et al.* (2015) [12]. Ejaculate volume was measured directly from the collection vial, while colour was visually graded based on creaminess. Density was assessed based on the opacity of a semen drop under visual inspection and graded from D to DDDD.

2.3 Isolation and identification of bacteria from semen samples
The collected semen samples were diluted ten-fold in sterile phosphate-buffered saline (PBS), transported to the laboratory within one hour, and incubated at 37 °C until further processing for inoculation.

2.4 Preparation of microbial media
For microbiological evaluation, the required culture media were prepared by dissolving the appropriate quantity of dehydrated media powder in 100 mL of triple-distilled water and heating until complete dissolution. The media was then sterilised in an autoclave at 121 °C under 15 psi pressure for 15 min. Following sterilisation, the media were dispensed into sterile Petri dishes under aseptic conditions in a laminar airflow cabinet. Once solidified, the plates were incubated at 37 °C for 24 h to verify sterility, after which they were sealed in polyethylene bags and stored at 4 °C until further use. The different media which were prepared according to the manufacturer's instructions for the study include buffered peptone water broth, brain heart infusion broth, Eosin Methylene Blue (EMB) agar and Baird-Parker (BP) agar.

2.5 Bacterial identification
The samples were incubated in BPW broth and BHI broth for 18 to 24 h to enrich bacterial growth. Following incubation, 100 µL of broth culture was inoculated by spread plate technique on EMB agar and BP agar plates and incubated at 37 °C for 24 h. If no bacterial growth was observed, the plates were further incubated for an additional 24 h (in total 48 h) at 37 °C before being re-assessed. Samples were considered bacteriologically sterile only if growth was not detected after the extended incubation period.

Bacterial isolation and identification were performed based on colony morphology by visual observation, cultural characteristics by subjecting to Gram's staining and biochemical characterisation including catalase test, oxidase test and IMViC (Indole production, Methyl red) tests were performed following the methodology outlined by Quinn *et al.* (2011) [10].

A loopful of bacterial growth from isolated colonies was transferred onto a clean, dry glass slide for catalase testing. A drop of three per cent hydrogen peroxide was added, and the immediate appearance of oxygen bubbles within 5 to 10 s was taken as a positive reaction. For the oxidase test, tetramethyl-p-phenylenediamine-impregnated oxidase discs were used. Bacterial colonies were applied directly to the disc surface. Development of a deep purple colour within 10 s indicated a strong positive reaction, colour development between 10 and 60 s indicated a weak positive, and no colour change after 60 s was interpreted as negative.

Indole production was determined by inoculating bacterial cultures into tryptone water, followed by incubation at 37 °C for 48 h. After incubation, 0.50 mL of Kovac's reagent was added along the test tube wall. The formation of a cherry-red layer indicated positive indole production. The methyl red test assessed glucose fermentation to stable acidic end products. Bacteria were inoculated into glucose phosphate peptone water and incubated at 37 °C for 48 h. Two drops of methyl red indicator were added post-incubation, with the development of a red colour signifying a positive result. For the Voges-Proskauer test, bacteria were inoculated into glucose phosphate peptone broth and incubated at 35 °C for 48 h. After incubation, one millilitre broth aliquots were mixed with 0.60 mL of five per cent α-naphthol and 0.20 mL of 40 per cent potassium hydroxide, shaken vigorously, and kept in a slanting position for 15-30 minutes. A pink colour indicated a positive acetoin production. Citrate utilization was tested using Simmons citrate agar slants inoculated with bacterial cultures and incubated aerobically at 37 °C for up to seven days. Positive utilization was indicated by growth and a colour change of bromothymol blue from green to blue due to alkalinization.

3. Results

Table 1: Macroscopic characteristics of fresh semen ejaculates from Malabari buck (N=32)

Semen characteristics	Mean ± SE
Volume (mL)	0.88±0.02
Colour	Creamy to creamy yellow
Density (D to DDDD)	DDDD

3.1 Bacterial isolation and identification
3.1.1 Cultural characterisation
The development of distinctive colonies was noted on the BP agar and EMB agar culture plates after incubation. The distinctive colonies on BP agar, which were round, smooth, convex, jet-black, glossy, and ranging in diameter from 1 to 2 mm with distinct and well-defined edges, were indicative of *S. aureus* (Plate 1). The colonies having a diameter of around 2 to 3 mm on EMB agar looked round, smooth, and moist. Additionally, they had darker centres and a distinctive green metallic sheen when exposed to reflected light indicative of *E. coli* (Plate 2).

3.1.2 Cultural and morphological characterisation
Following Gram's staining, the colonies from the BP agar plate displayed purple, spherical cocci grouped in asymmetrical clusters resembling grapes, which is indicative of Gram-positive *S. aureus* (Plate 3) and the colonies from the EMB agar plate showed up as pink to red, rod-shaped cells that were usually grouped singly or in short chains, which is indicative of Gram-negative *E. coli* (Plate 4).

3.1.3 Biochemical identification of *S. aureus*
The catalase test was performed with a small portion of the bacterial colony from the BP plate, and the instantaneous formation of effervescence confirmed a positive catalase reaction (Plate 5). Another small portion was colony was put

through the oxidase test and within 60 s, the disc showed no change in colour, suggesting a negative oxidase reaction (Plate 6). Oxidase-negative and catalase-positive data together led to the bacterium's identification as *S. aureus.*

3.1.4 Biochemical identification of *E. coli*

The colony from EMB agar was tested for indole production, with the addition of Kovac's reagent resulting in the immediate appearance of a pink to cherry-red ring at the medium surface, indicating a positive indole reaction (Plate 7A). For the methyl red test, bacteria cultured in MR-VP media developed a distinct red coloration upon addition of methyl red indicator, confirming a positive result (Plate 7B). Conversely, the Voges-Proskauer test was negative, as no pink to red colour formed within 15 min after adding Barritt's reagents A and B to the MR-VP culture, with the medium remaining yellow brown (Plate 7C). When inoculated onto Simmons citrate agar, the colony showed no visible growth and the medium retained its green colour after incubation, indicating a negative citrate utilisation test (Plate 7D). The organism which exhibited the patterns of indole positive, methyl red positive, Voges-Proskauer negative and citrate negative (+ + - -) was determined to be *E. coli* based on the results of the IMViC test.

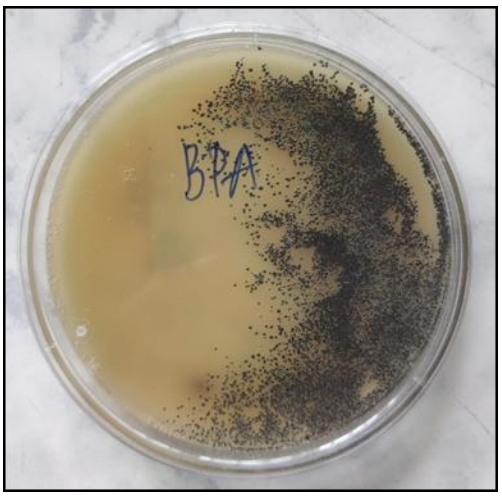

Fig 1: Baird-Parker agar plate with *S. aureus* colonies

Fig 2: Eosin Methylene Blue agar plate with *E. coli* colonies

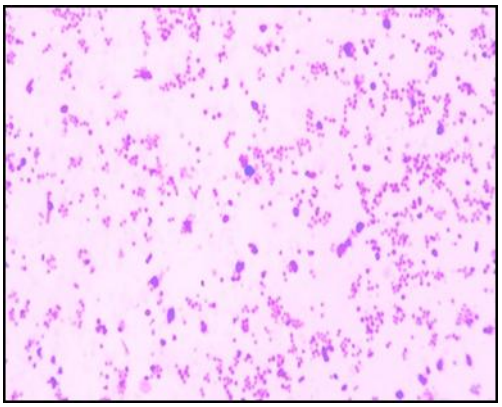

Fig 3: Gram-positive cocci (*S. aureus*)

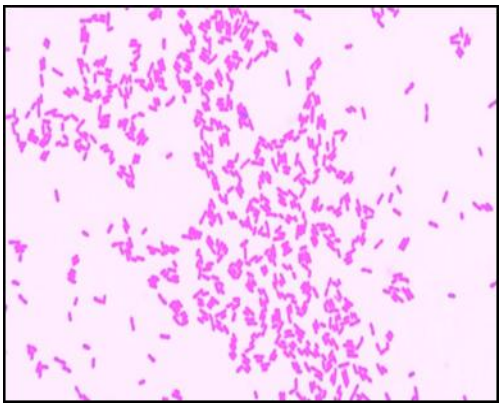

Fig 4: Gram-negative bacilli (*E. coli*)

Fig 5: Positive catalase test

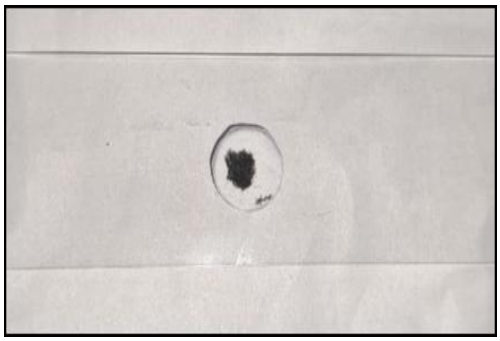

Fig 6: Negative oxidase test

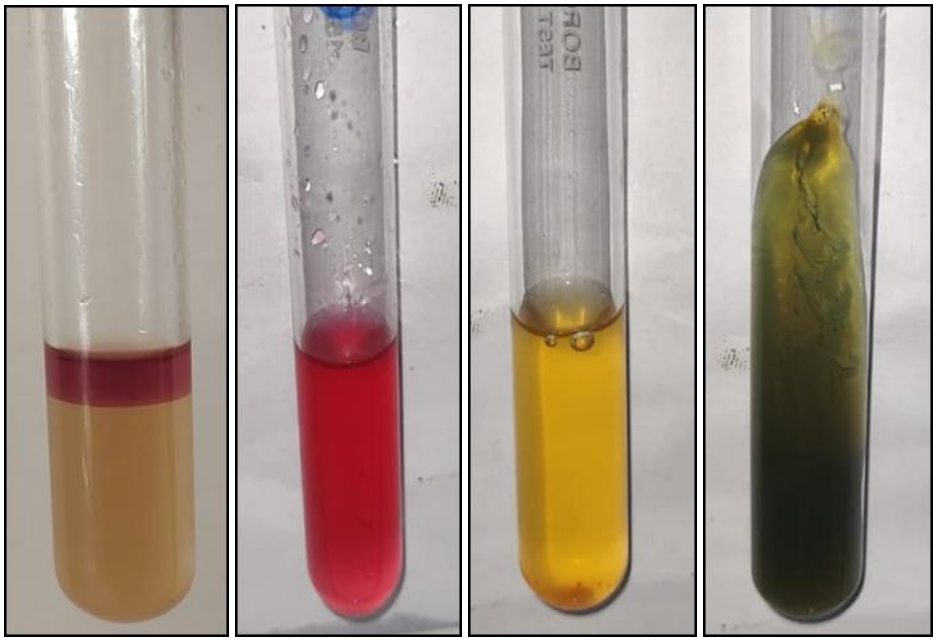

Fig 7(A): Positive Indole test

Fig 7(B): Positive Methyl red test

Fig 7(C): Negative Voges-Proskauer test

Fig 7(D): Negative Citrate test

4. Discussion

4.1 Macroscopic characteristics of fresh semen ejaculates from Malabari buck

The ejaculate volume of Malabari bucks in the present study ranged from 0.70 to 1.10 mL, with a mean of 0.88 ± 0.02 mL. These values are in close agreement with reports by Bhai *et al.* (2015) [12] and Behera *et al.* (2015) [11], but slightly higher than those of Pawshe *et al.* (2017) [14], Akhila *et al.* (2016) [15], and Kanimozhi *et al.* (2023) [16], who documented means between 0.63 and 0.78 mL. Lower averages (0.50-0.65 mL) were noted by Shiny (2011) [17] and Krishnan (2017) [18], whereas Urmila (2022) [19] and Vigneshwaran *et al*. (2024) [20] reported slightly higher values (0.97-1.17 mL). Variations among studies may reflect differences in buck age, nutrition, season, and semen collection practices (Arrebola *et al.*, 2016) [21]. Semen colour varied from creamy white to creamy yellow, consistent with earlier descriptions in Malabari bucks (Shiny, 2011; Bhai *et al.*, 2015; Kanimozhi *et al.*, 2023) [17, 12, 16]. Similar creamy shades have been reported in related studies (Prasanth and Mathai, 1996; Ranjini, 1998; Rajan, 2010; Behera *et al.*, 2015; Krishnan, 2017) [22, 11, 23, 24, 18]. Ejaculatory colour differences likely result from sperm density and accessory gland secretions, and crossbreeding with Alpine goats has also been associated with subtle variations in semen appearance (Kutty and Mathew, 2000) [25]. All ejaculates in this study showed the highest density grade (DDDD), reflecting the presence of concentrated spermatozoa. Comparable results were reported by Shiny (2011) [17], Behera *et al.* (2015) [11], Bhai *et al.* (2015) [12], Krishnan (2017) [18], and Kanimozhi *et al.* (2023) [16]. Similar findings in crossbred Malabari bucks (Prasanth and Mathai, 1996; Ranjini, 1998; Rajan, 2010) [22, 23, 24] suggest that this trait is conserved. Seasonal influences have also been highlighted, with summer ejaculates exhibiting higher density compared to winter collections, possibly due to photoperiod and nutrition-driven variations in testicular activity (Kutty and Mathew, 2000; Ritar and Salamon, 1991; Arrebola *et al.*, 2016) [25, 26, 21].

The bacteriological analysis of Malabari buck semen in this study predominantly identified *E. coli* and *S. aureus* as the main bacterial contaminants. These findings align with Ronald and Prabhakar (2001) [27], who reported *E. coli, Staphylococcus spp., Bacillus spp., and Proteus spp.* as principal bacterial species in bull semen. Similarly, Andrabi *et al.* (2016) [5] reported *S. aureus, E. coli, and Pseudomonas aeruginosa* as predominant bacteria in buffalo bull ejaculates. Consistent with the summary by Yániz *et al.* (2010) [3], *E. coli* is frequently isolated across various species, including humans, cattle, horses, dogs, pigs, goats, and sheep. Likewise, *S. aureus* is commonly found in the semen of animals such as dogs, stallions, bulls, boars, and bucks, often because it exists as a commensal organism on skin and mucous membranes.

The predominance of *E. coli* and *S. aureus* in this study concurs with earlier reports identifying these bacteria as primary semen contaminants in domestic animals, including rams and bulls, reflecting their ubiquity in the environment and their ease of access to semen during collection and handling (Yousef *et al.*, 2021) [28].

5. Conclusion

The study identified *E. coli* and *S. aureus* as the predominant bacterial contaminants in Malabari buck semen. These organisms impair sperm function through toxin production, oxidative stress, and pH alterations, with their effects being aggravated during cryopreservation. The findings reinforce the necessity of stringent hygienic handling, regular bacteriological assessment, and the exploration of effective antimicrobial alternatives to maintain semen quality and improve the success of artificial insemination programmes in goats.

6. Acknowledgements

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7. Conflict of Interest

The authors declare that they have no conflict of interest.

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