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Untargeted Urine Metabolomics Profiling during Different Phases of Estrous Cycle in Surti Goats

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ABSTRACT

Background: Estrus detection is crucial for reproductive performance of goats which requires accurate, economical and easy techniques. Present study was undertaken to delineate urine metabolomics profile to identify biomarkers for different phases of estrous cycle in Surti goats.

Methods: Ten non-pregnant Surti goats were synchronized for estrous using intra-vaginal progesterone sponges (impregnated with 60 mg Medroxyprogesterone acetate) for 11 days followed by intramuscular injection of 125 µg Cloprostenol ($PGF_2\alpha$ analogue). Estrus was confirmed based on physiological and behavioral signs. Urine samples collected during proestrus, estrus and diestrus were analyzed using gas chromatography-mass spectrometry. Web-based interface MetaboAnalyst 5.0 was used for data preprocessing, normalization, statistical analysis and data visualization. Statistical analysis comprised data normalization, one-way ANOVA and post-hoc Fisher LSD test, hierarchical clustering, principal component analysis and partial least squares discriminant analysis.

Result: Total 56 metabolites were found to vary during different phases of estrous cycle. The study was successful in generating untargeted urine metabolomics profile of Surti goats that can be used for identifying specific biomarkers for different phases of estrous. Two metabolites 4-amino-2-hydroxybenzoic acid (2-dieth-4-A-2) and 4-Methylphenol (p-Cresol) emerged as potential biomarkers for detecting estrus. These results can serve as base line reference paving way for future studies in larger group that may further validate these findings.

Key words: Estrous cycle, GC-MS, Surti goats, Urine metabolites.

INTRODUCTION

Accurate and timely detection of estrus in goats is key for improving reproductive and production performance but compared to smaller herd size it is difficult especially in large herd owing to labor cost, requirement of advanced facilities further marred by ambiguous physiological and behavioral signs. Estrus synchronization and fixed-time insemination serves well to enhance reproductive efficiency of small ruminants like goats (Rahman *et al.,* 2008). To bolster the ease and precision of estrus detection studies can focus on non-invasively collected biological sample like urine that may contain biomolecules specific to different phases of estrous cycle. These metabolites can be explored through metabolomic studies to delineate underlying biochemical pathways related to health and disease, including pregnancy and pregnancy-associated dysfunctions (Phillips *et al.,*2018; Velho *et al.,* 2018). Gas chromatography coupled to mass spectrometry (GC/MS) has been shown to be a robust method for metabolomics studies (Want *et al.,* 2007). Several studies have utilized the technique of GC/MS for wider nature of studies (Sunitha *et al.,* 2023). Non-targeted GC/MS analysis from urine, serum and tissue extracts has been successful in metabolomics analysis and this approach has been used to identify metabolites that are associated with different disease states and to gain insights into metabolic pathways of different physiological or pathological conditions (Jiye *et al.,* 2005).

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Even though urine has been explored as good biomarker for general health status in goats (Khushboo *et al.,* 2021), 4-Methylphenol (p-Cresol) has been reported in urine during estrus by (Sudhan *et al.,* 2017) in certain farm animals, Muniasamy *et al.* (2017) in Murrah buffaloes as well as in high concentration by Rajanarayanan and Archunan (2011) in female buffalo. This pheromone has also been established in deer (Whittle *et al.,* 2000) and mare (Buda *et al.,* 2012) during period of sexual receptivity. Some reports also suggest the its not only in estrus but during diestrus in dog urine (Divya, 2012). However, in

buffalo estrus phase different compounds such as hydracyrlic acid, 3-bromo-1-propanol and benzyl serine were found to be upregulated in urine (Doshi *et al*., 2024). Though some studies have been done in other species that report different compounds, very few or but almost none studies have been done in goats. The region of present study *i.e*. western coastal belt of South Gujarat is home tract of Surti breed of goat (Deshpande and Sabapara, 2010).

Considering importance of goats in livestock industry worldwide, necessity of accurate and timely detection of estrus, existing gap in the knowledge of phase specific metabolites for estrous cycle of goats, non-invasive nature of urine sample collection and nearby abundance of Surti breed of goat, we conducted the present study with the objective to delineate comprehensive urine metabolomics profile of Surti goats during different phases of the estrous cycle. During this study we aimed to identify metabolites that could pave way to serve as biomarkers for different phases of estrous cycle in goats.

MATERIALS AND METHODS

The study was carried out in Department of Veterinary Physiology and Biochemistry, Veterinary College, Kamdhenu University, Navsari in collaboration with Food Quality Testing Laboratory (FQTL), NAU, Navsari and Livestock Research Station, Kamdhenu University, Navsari. The study was done following ethical guidelines and was approved by Institutional animal ethics committee (IAEC vide 092-VCN-VPY-2020). The schematic of urine metabolomics profiling during different phases of estrous cycle in Surti goats is depicted in Fig 1 as described below:

Animal selection and management

Ten non-pregnant sexually mature Surti goats (aged >2 Years) irrespective of parity were randomly selected from LRS, KU, Navsari (Gujarat, India). They were maintained under standard practices of housing, feeding and management. Green fodders (Hybrid Napier or Jowar and tree leaves) were provided in the open paddock. In addition, they were provided concentrate mixtures @ 0.25 kg/head/day inside the pen. The animals had free access to fresh and clean drinking water all the time inside the pens. Total duration of study period was of 6 months spanning rainy season (July to September) to early winter season (October to December) characteristic of south costal region of Gujarat in Navsari district.

Estrous synchronization

Selected goats were synchronized for estrous with intra-vaginal progesterone sponges impregnated with 60 mg Medroxyprogesterone acetate (MAP) kept for 11 days followed by intramuscular injection of 125 µg Cloprostenol ($PGF_2\alpha$ analogue) at sponge removal.

Determination of different phases and urine sample collection

The period between sponge removal and onset of estrus was considered proestrus. The period after estrus *i.e*., 6 days as in present study was considered diestrus. Estrus was successfully confirmed by observing behavioral signs such as restlessness, frequent micturition, frequent bleating, clustering around buck, mounting on other animals, wagging of tail, standing to be mounted and physiological signs like vulvar hyperemia, vulvar edema, opening of cervical-os and cervico-vaginal mucus discharge. Urine samples were collected in sterile and clean grease free airtight glass bottle separately from all the goats during proestrus, estrus and diestrus (6 days following estrus) aseptically by passing the catheter in urinary bladder. Collected urine samples were analyzed on the same day for metabolomic studies.

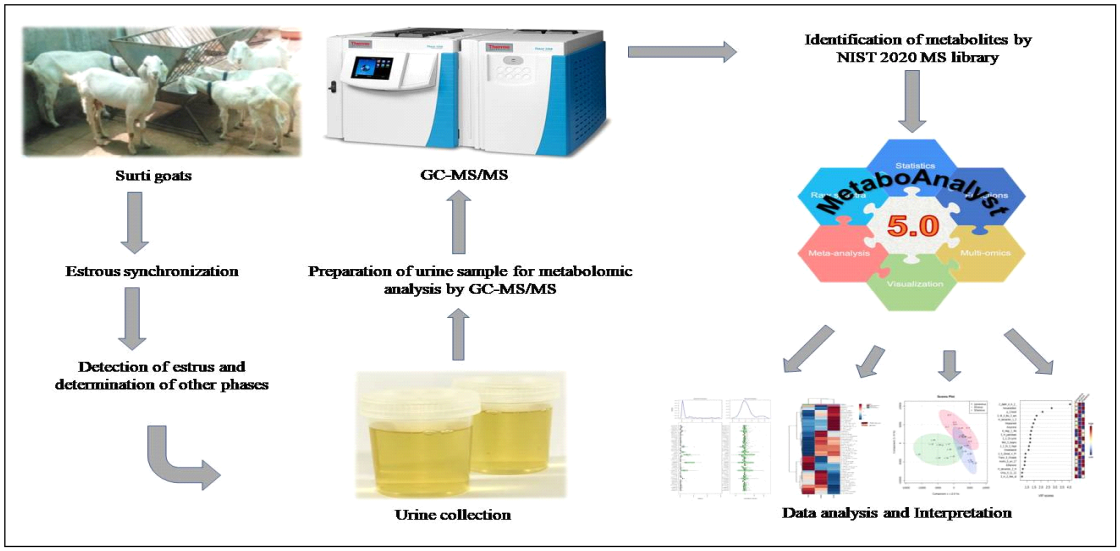

Fig 1: Schematic of urine metabolomics profiling during different phases of estrous cycle in Surti goats.

Preparation of urine sample for metabolomic analysis by GC-MS/MS

Urine (25 ml) was mixed with an equal volume of Dichloromethane (DCM), stirred, filtered and separated. Thereafter it was dried in nitrogen gas, reconstituted with DCM, filtered using a 0.22 µm filter and collected in GC vial out of which 2 µl extract was injected into GC-MS/MS (TRACE 1310 GC; TSQ 9000, Thermo Scientific) under following conditions - injector temperature 245°C, gas ûow rate 1 ml/min through fused silica capillary column of 0.25-ìm Rxi-5 MS column (30 m×0.25 mm). The initial temperature program was as follows: oven temperature started from 40°C that was held for 4 min, ramped with 3°C/min to 300°C and held for 10 min. The ion source temperature was 250°C. Ions were generated at 70 eV with electron ionization and recorded at 500 amu/sec over the mass range *m/z* 35 to 560. The total run time was 110 minutes. Finally, the identification of unknown compounds was made by probability base matching using the NIST 2020 MS library.

Statistical analysis

Statistical analysis was done using MetaboAnalyst 5.0 (web-based interface) that included data preprocessing, normalization, statistical analysis, pathway analysis and data visualization. Univariate analysis *i.e.*, ANOVA, post hoc Fisher LSD test and multivariate analysis like Principal Component Analysis (PCA) for data reduction, hierarchical clustering with Heatmap for classification and Partial Least Squares-Discriminate Analysis (PLS-DA) to identify the variables that best discriminate between groups or classes in a data set, were used in the current study. In PLS-DA regression model,VIP (Variable Importance in the Projection) plot was used to rank metabolites based on their importance in discriminating between different estrous phases (VIP scores:>1-significant;>2-highly significant).

RESULTS AND DISCUSSION

In this study, gas chromatography-mass spectrometry (GC-MS/MS) based urine analysis of Surti goats was done for estrus specific metabolites during estrous cycle. As there are not much studies that have explored urine metabolomics profiling of estrous cycle in goats, we reviewed whatever scientific literature was available to possibly align them with findings of the present study.

During urine metabolomic studies using GC-MS/MS analysis, standardization of metabolomics workflow is necessary for generating data that is precise and has higher reproducibility. Gas chromatography-mass spectrometry (GC-MS) based metabolomics is ideal for identifying and quantitating small molecular metabolites (<650 Daltons), including small acids, alcohols, hydroxyl acids, amino acids, sugars, fatty acids, sterols, catecholamines, drugs and toxins. The technique often uses chemical derivatization to make these compounds volatile enough for gas chromatography. GC-MS-based metabolomics is a versatile technique that can be used for both targeted and untargeted analysis of metabolites in biological samples. This approach may help to discover novel compounds and metabolic pathways associated with specific biological processes or diseased states. Volatile compounds on GC-MS based metabolomics easily allow integrating targeted assays for absolute quantification of specific metabolites with untargeted metabolomics to discover novel compounds. In the present study, GC-MS analysis revealed a total of 56 compounds which were selected after filtering of data by Interquartile range (IQR). Before subjecting for clustering or multivariate technique, data was preprocessed to handle missing values, normalization and appropriate data transformations (*e.g.*, log transformation, Pareto scaling) to ensure the data's suitability for clustering analysis. Data normalization was done by Quantile normalization (Fig 2) followed by Pareto scaling in MetaboAnalyst 5.0. Pareto scaling basically is a preprocessing technique that makes data more amenable to analysis, as variables with both small and large variances contribute more equitably to overall variation in the dataset. MetaboAnalyst provides various visualization options to explore and interpret the clustering results, such as heatmap plots or principal component analysis (PCA) plots that helps to visualize the clustering patterns and identify sample clusters associated with different experimental conditions.

One-way analysis of variance

Eleven distinct metabolites were found significant using one-way ANOVA by MetaboAnalyst 5.0, suggesting their important contribution in goats across stages of the estrous cycle (Table 1).

Hierarchical clustering

Using MetaboAnalyst 5.0, hierarchical clustering was applied to metabolomics data to explore patterns, relationships and similarities among metabolites based on their metabolic profiles. It may be helpful in revealing underlying structure and similarities in the data, which useful for identifying potential biomarkers or gaining insights into the metabolic changes associated with different experimental conditions. Heatmap of discovered urine metabolites in estrous synchronized Surti goats during proestrus, estrus and diestrus phases are shown in Fig 3. Upregulation and downregulation of metabolites are mentioned in Fig 4.

Principal component analysis (PCA)

PCA was done to identify underlying patterns, grouping of samples and reveal important features (*e.g.*, metabolites) driving the observed differences between samples. The scree plot shows that the first 5 principal components explain 59.9% of the total variability in the data (Fig 5). This suggests that these components are most important for describing the underlying patterns in the data.

Score plots (Fig 6) shows samples in the reduced-dimensional space defined by the principal components.

Samples that are close together in the score plot are more similar in their metabolic profiles. Score plot obtained indicates that there is a clear separation among the phases of estrous cycle (proestrus, estrus and diestrus) based on the metabolomic data. However, there is also significant overlap among the phases, indicating some similarities in the metabolic profiles between different phases.

Partial least squares discriminant analysis (PLS-DA)

Multivariate statistical technique PLS-DA elucidated the understanding how the model discriminated and classified samples into predefined phases of the estrous cycle based on their metabolic profiles of different metabolomes. It generated a two-dimensional score plot describing the clear separation between proestrus, estrus and diestrus phases of the estrus cycle (Fig 7). The first, second, third, fourth and fifth components of PLS-DA analysis explained 13.5%, 9%, 12.4%, 11.3% and 6.3% variations of all 56 metabolites respectively. Further, the variable importance in the projection (VIP) score in the estrous cycle indicatesthe importance of each metabolite in the PLS-DA model. VIP score on a scale of 1.0-3.0 (Fig 8) is used to identify the most discriminant features (*e.g.*, metabolites). These features may serve as potential biomarkers or important variables associated with the studied conditions. Metabolites with the highest VIP values are the most powerful group discriminators. Typically, VIP values >1 are significant and VIP values >2 are highly significant. These metabolites could be further investigated to gain insights into the biological processes that underlie the observed differences between the different stages.

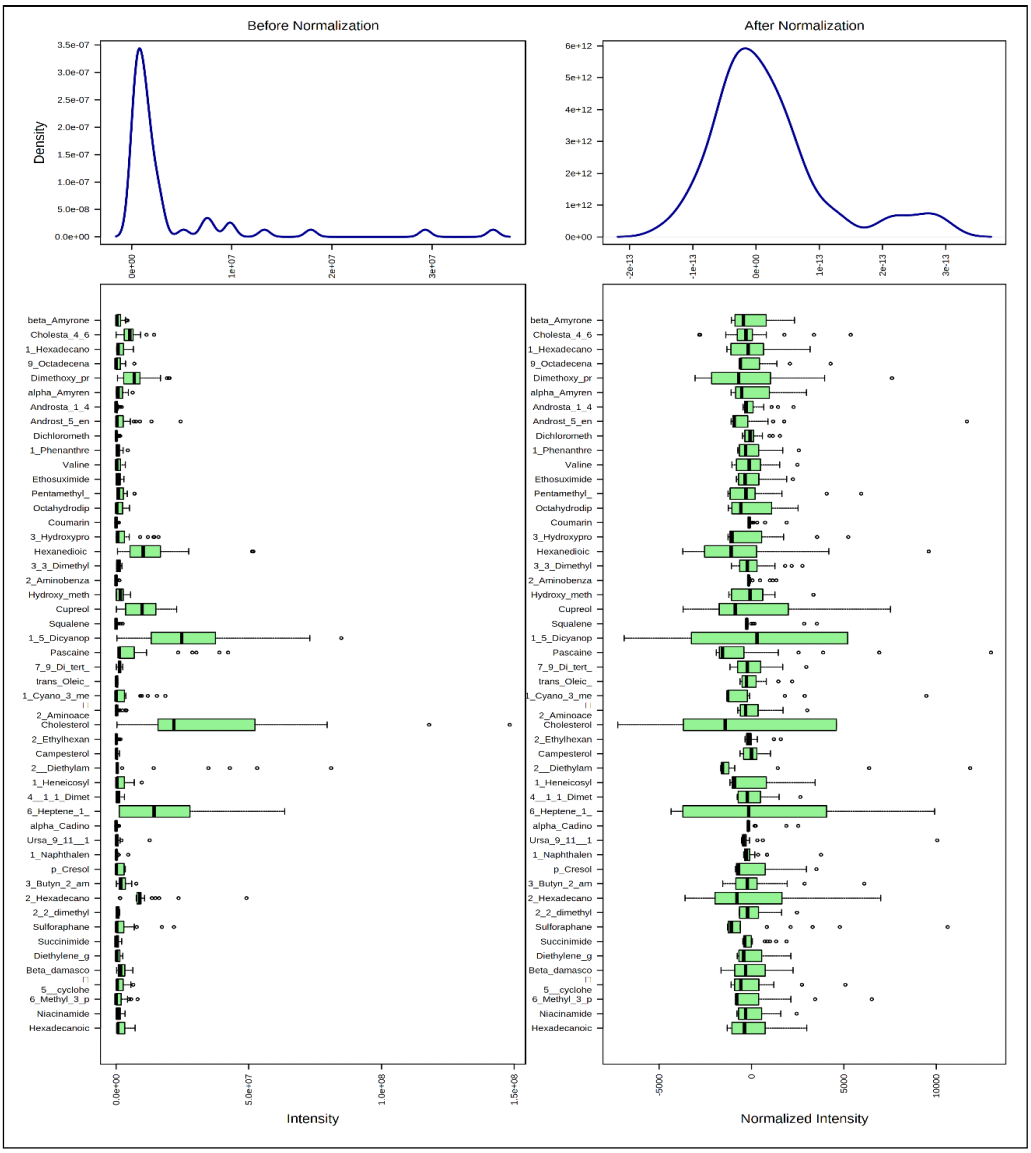

Fig 2: Data normalization using quantile normalization.

Metabolites with VIP score of more than 1 included three metabolites *viz.* methylbut-3-yn-2-amine (2-M-3-Bu-2-am), 6-methyl-3-propan-2-yloxan-2-one (Met-3-propan) and (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylheptan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a] phenanthren-3-ol (cholesterol) in proestrus phase whereas four metabolites *viz.* (4*aR*,6*aR*,6*bS*,8*aR*,12*aR*,14*aR*,14*bR*)-4,4,6*a*,6*b*,8*a*,11,

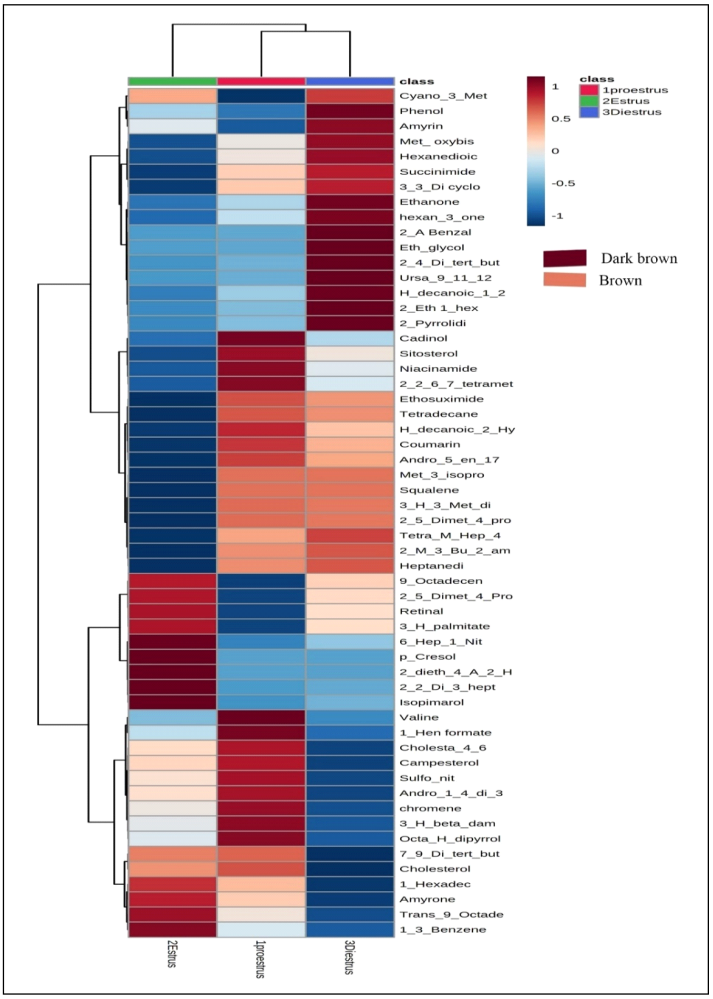

Fig 3: Unsupervised hierarchical clustering heat map of metabolites from proestrus, estrus and diestrus phases of Surti goats. (Different rows for Urine metabolites and different columns for phases of estrous).

Table 1: Metabolites identified by One-way ANOVA in MetaboAnalyst 5.0.

Metabolites	p value	Fisher's LSD
p-Cresol	8.75 x 10-8	2Estrus - 1proestrus; 2Estrus - 3Diestrus
Hexadecanoic acid- 1-(hydroxymethyl) -1-2-ethanediyl ester2Estrus -	5.52 x 10-6	3Diestrus - 1proestrus; 3Diestrus - 2Estrus
2-2-Dimethyl-3-heptanone	5.38 x 10-5	2Estrus - 1proestrus; 2Estrus - 3Diestrus
β-Amyrone	3.56 x 10-4	1proestrus - 3Diestrus; 2Estrus - 3Diestrus
trans-9-Octadecenoic acid- pentyl ester	8.29 x 10-4	1proestrus - 3Diestrus; 2Estrus - 3Diestrus
2,5,5,6,8a-Pentamethyl-trans-4a-5,6,7,8,8a -hexahydro-gamma-chromene	1.06 x 10-3	1proestrus - 2Estrus; 1proestrus - 3Diestrus; 2Estrus-3Diestrus
Cyclopentanecarboxylic acid- 3-3-dimethyl	1.84 x 10-3	1proestrus - 2Estrus; 3Diestrus - 2Estrus
Benzaldehyde-2-amino	2.73 x 10-3	3Diestrus - 1proestrus; 3Diestrus - 2Estrus
2-(Diethylamino)ethyl 4-amino-2-hydroxybenzoate	2.81 x 10-3	2Estrus - 1proestrus; 2Estrus - 3Diestrus
1-Heneicosyl formate	3.65 x 10-3	1proestrus - 2Estrus; 1proestrus - 3Diestrus
Ethanone- 1-(2-aminophenyl)	8.89 x 10-3	3Diestrus - 1proestrus; 3Diestrus - 2Estrus

11,14*b*-octamethyl-2,4*a*,5,6,7,8,9,10,12,12*a*,14,14*a*-dodecahydro-1*H*-picen-3-one (Amyrone), hept-6-enenitrile (6-Hep-1-Nit), 3-hydroxypropyl hexadecanoate (3-H-palmitate) and 2-2-dimethylheptan-3-one (2-2-Di-3-hept) in estrus phase and three metabolites namely [(2S)-2-hexadecanoyloxy-3-hydroxypropyl] hexadecanoate (2-Hexadecanoyl), Heptanedinitrile (Heptanedi) and 3-3-dimethylcyclopentane-1-carboxylic acid (3-3-Di cyclo) in diestrus phase of Surti goats.

Highly significant metabolites (VIP value>2) included two metabolites such as 4-amino-2-hydroxybenzoic acid (2-dieth-4-A-2) and 4-Methylphenol (p-Cresol) that were

Fig 4: Upregulated and downregulated metabolites during different phases of estrous of Surti goats.

detected in estrus phase whereas one metabolite *i.e.* Bis(2-ethylhexyl) hexanedioate (Hexanedioate) that was identified in diestrus phase of Surti goats.

Following data normalization, one-way ANOVA and post hoc Fisher LSD test of urine metabolites was performed with data obtained for proestrus, estrus and diestrus to identify metabolites that varied significantly in different phases of estrous cycle. Fisher LSD test demonstrated that during estrus p-cresol, 2-2-Dimethyl-3-heptanone and 2-(Diethylamino)ethyl-4-amino-2-hydroxybenzoate were significantly higher than proestrus and diestrus. As compared to proestrus and estrus, hexadecanoic acid-1-(hydroxymethyl)-1-2-ethanediyl ester, benzaldehyde-2-amino and ethanone-1-(2-aminophenyl) were significantly

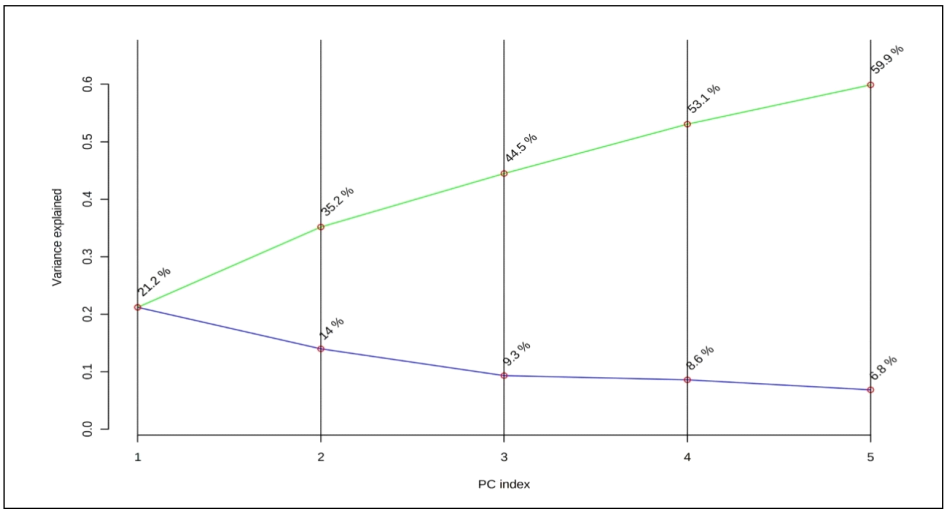

Fig 5: Scree plot shows the variance explained by PCs.

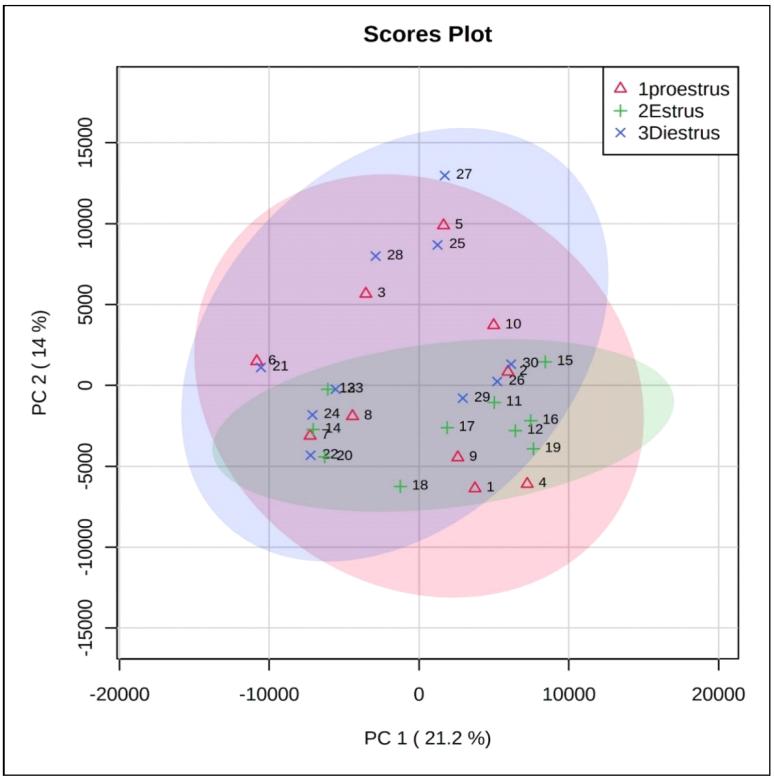

Fig 6: Principal component analysis (PCA) score plots of PC1 versus PC2 from metabolite profiles of urinary samples derived from three phases of the estrous cycle.

Fig 7: Partial least square-discriminant analysis (PLS-DA) score plot.

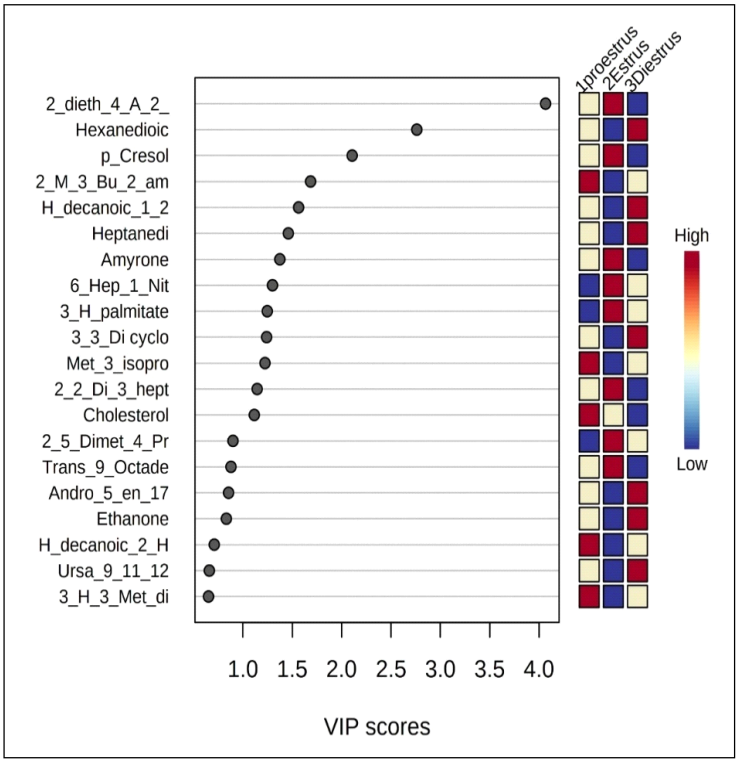

Fig 8: VIP score of metabolites of proestrus, estrus and diestrus phases of estrous cycle of Surti goats based on PLS-DA model.

higher in diestrus phase. During proestrus phase,1-heneicosyl formate was significantly higher compared to other two phases. β-Amyrone as well as the trans-9-Octadecenoic acid-pentyl ester were significantly higher during the proestrus and estrus phase as compared to diestrus. 2,5,5,6,8a-Pentamethyl-trans-4a-5,6,7,8,8a-hexahydro-gamma-chromene levels were significantly higher in proestrus than in estrus and significantly lower in diestrus. Among the metabolites identified to be higher in estrus, p-cresol has been reported in mammals with almost similar results. Across mammals, microbial activity is responsible for producing cresols along with aromatic compounds that are metabolized and excreted in urine (Fiege, 2000; De Preter *et al.,* 2004) in free form as well as conjugated form. p-cresol endogenously is produced from tyrosine by intestinal anaerobic bacteria (Bone *et al.,* 1976). Even though cresols naturally are present in mammalian urine (Spiehs and Varel, 2009), there is dearth of studies that link its presence with ovarian activity.

A recent report by Doshi *et al*. (2024) suggests upregulation of hydracyrlic acid, 3-bromo-1-propanol and benzyl serine in buffalo urine during estrus phase however most the earlier studies concur the dominating presence of 4-Methylphenol (p-Cresol) in urine during estrus. Volatile phenolic compound pheromone 4-Methylphenol (p-Cresol) in urine considered as pheromone has been reported in animals when they are sexually receptive *i.e*., during estrus (Sudhan *et al.,* 2017). During estrus phase of Murrah buffaloes in both synchronized as well as naturally cyclic, 4-methylphenol (p-Cresol) was present in urine (Muniasamy *et al.,* 2017). Its high concentration in female buffalo urine during estrus period has been reported by Rajanarayanan and Archunan (2011). Presence of this pheromone has also been established in deer (Whittle *et al.,* 2000) and mare (Buda *et al.,* 2012) during period of sexual receptivity. Its role as an indicator of estrus is bolstered by reports of its presence in other biological samples such as saliva (Karthikeyan *et al.,* 2014) and faeces (Karthikeyan *et al.,* 2013). Beyond acting as an attractant, it is also suggested to stimulate neuroendocrine pathways for enhanced libido as well as increase in sperm count in male buffaloes (Archunan and Rajanarayanan, 2010) and influence penile erection in stallion (Buda *et al.,* 2012). Its increased presence could also be reasoned by its antimicrobial activity in genital region (Morris *et al*. 1979) during sexual activity. Some reports also suggest the presence of 4-methylphenol (p-Cresol) not only in estrus but during diestrus in dog urine (Divya, 2012).

CONCLUSION

This study was successful in generating comprehensive untargeted metabolomics profiling from urine sample of Surti goats during different phases of estrous cycle. Two urinary metabolites, namely 4-amino-2-hydroxybenzoic acid (2-dieth-4-A-2) and 4-Methylphenol (p-Cresol) can serve as potential biomarkers for detecting the estrus phase in Surti goats. Although the findings of this study are intriguing, it is important to acknowledge that they are preliminary in nature and various other metabolites that were delineated along with their upregulation and downregulation for proestrus, estrus and diestrus can facilitate the identification of more urinary biomarkers specific for different estrous phases in goats. Future studies in larger group may further validate these findings.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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