SERUM METABOLITES AND MINERALS PROFILE DURING BUFFALO ESTROUS CYCLE

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE
IN
ANIMAL BIOCHEMISTRY

BY

PUSHPANJALI SINGH

(B.Sc. Life Sciences)

ANIMAL BIOCHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
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Approved by

16/10/2021
EXTERNAL EXAMINER

Varij Nayan 16.10.21
DR. VARIJ NAYAN
MAJOR ADVISOR
(GUIDE)

MEMBERS, ADVISORY COMMITTEE

1. **Dr. P.S. YADAV**
   Principal Scientist
   APR Division
   P. Singh 16.10.21
2. **Dr. NAVNEET SAXENA**
   Principal Scientist
   AN&FTDivision
   N Saxena 16/10/2021
3. **Dr. N.L. SELOKAR**
   Scientist
   APR Division
   (Presently, ABTC, NDRI)
   16/10/21
4. **Dr. SANJAY KUMAR**
   Senior Scientist
   AGB Division
   (Director's Nominee)
   16.10.2021

भा. कृ. अनु. प. - केंद्रीय भैंस अनुसंधान संस्थान
हिसार-125001 (हरियाणा), भारत
ICAR-CENTRAL INSTITUTE FOR RESEARCH ON BUFFALOES
HISAR-125001 (HARYANA), INDIA
www.cirb.res.in

Dr. VARIJ NAYAN
Senior Scientist,
Animal Biochemistry, APR Division
Email: Varij.Nayan@icar.gov.in

CERTIFICATE

This is to certify that the thesis entitled, **"SERUM METABOLITES AND MINERALS PROFILE DURING BUFFALO ESTROUS CYCLE"** submitted by **Ms. PUSHPANJALI SINGH** towards the partial fulfilment of the requirement for the award of the degree of **MASTER OF SCIENCE** in **ANIMAL BIOCHEMISTRY** of the **National Dairy Research Institute (Deemed University),** Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Date: 16th **September, 2021**

Varij Nayan
16.10.21
(Dr. VARIJ NAYAN)
MAJOR ADVISOR
(GUIDE)

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PUSHPANJALI SINGH

ABSTRACT

Estrus identification in buffaloes has been a major problem for many years. The lack of reliable methods and the incorrect timing of insemination affects effective reproductive management in buffaloes due to their poor estrus expression. The recent advancement of molecular techniques in animal research, such as metabolomics, has given new ideas for biological fluids-based estrus metabolites biomarker. Serum is a primary carrier of small molecules in the body, contains a massive amount of information and making it perfect for biochemical and minerals analysis. A significant amount of knowledge has been gained in recent years on different biological fluids for estrus identification, however, no single metabolite was identified in serum. Even though metabolomics is one of the most powerful strategies in molecular mechanisms, little research has been conducted on the serum metabolome analysis of estrus and diestrus buffalo. Therefore, this study aimed to explore serum metabolome differences between estrus and diestrus buffaloes, as well as their biochemical and minerals profile during estrus and diestrus. Serum samples from six buffaloes in estrus and diestrus were analyzed in UPLC-Q-Orbitrap HRMS to generate metabolomic profiles. The findings revealed that serum metabolites were widely distributed in lipid metabolism and those metabolites were significantly upregulated during estrus. A total of 789 metabolites was identified in both positive and negative electrospray ionization ion mode with 71 significantly different metabolites narrowed down between two groups of buffalo. Further pathway analysis revealed that the 15 significantly different metabolites were primarily enriched in fatty acid lipid metabolism-related pathways such as primary bile acid synthesis, arachidonic acid metabolism, glycerophospholipid metabolism, and unsaturated fatty acid biosynthesis. Serum samples from 19 buffaloes were analyzed for biochemical parameters and minerals profile. The current study found that only aspartate aminotransferase, bilirubin in total, and bilirubin indirect level show a significant difference ($P<0.05$) in estrus in comparison to the diestrus group. In minerals analysis, iron and magnesium show a significant difference($P<0.05$) in estrus and diestrus. Thus, these findings provide valuable information towards the identification of potential metabolites biomarker for the investigation of physiological conditions like estrus in buffalo.

भैंसों के एस्ट्रस के दौरान सीरम मेटाबोलाइट और खनिज रूपरेखा

सारांश

भैंसों में एस्ट्रस की पहचान कई वर्षों से एक बड़ी समस्या रही है, विश्वसनीय तरीकों की कमी और गर्भाधान का गलत समय भैंसों में प्रभावी प्रजनन प्रबंधन को प्रभावित करता है। पशु अनुसंधान में आणविक तकनीकों की हालिया प्रगति, जैसे कि उपापचयी, ने जैविक तरल पदार्थ पर आधारित एस्ट्रस चक्र के दौरान मेटाबोलाइट बायोमार्कर के लिए नए विचार दिए हैं। सीरम शरीर में छोटे अणुओं का एक प्राथमिक वाहक है, इसमें भारी मात्रा में जानकारी होती है। जो इसे जैव रासायनिक और खनिज विश्लेषण के लिए एकदम सही बनाता है। हाल के वर्षों में एस्ट्रस की पहचान के लिए विभिन्न जैविक तरल पदार्थों पर एक महत्वपूर्ण मात्रा में ज्ञान प्राप्त किया गया है, हालांकि सीरम में एक भी मेटाबोलाइट की पहचान नहीं की गई थी। भले ही मेटाबॉलिकम आणविक तंत्र में सबसे शक्तिशाली नीतियों में से एक है, लेकिन एस्ट्रस और डायस्ट्रस भैंस के सीरम उपापचयी विश्लेषण पर बहुत कम शोध किया गया है। इसलिए इस अध्ययन का उद्देश्य एस्ट्रस और डायस्ट्रस भैंसों के बीच उपापचयी के अंतर के साथ-साथ एस्ट्रस और डायस्ट्रस के दौरान उनके जैव रासायनिक और खनिज प्रोफाइल का पता लगाना है। मेटाबॉलिक प्रोफाइल बनाने के लिए यूपीएलसी-क्यू-ऑर्बिट्रैप एचआरएमएस में एस्ट्रस और डायस्ट्रस में छह भैंसों से सीरम के नमूनों का विश्लेषण किया गया था। निष्कर्षों से पता चला कि सीरम मेटाबोलाइट्स फैटी एसिड चयापचय में व्यापक रूप से वितरित किए गए थे और एस्ट्रस के दौरान मेटाबोलाइट्स को काफी हद तक अपग्रेड किया गया था। सकारात्मक और नकारात्मक दोनों प्रकार के इलेक्ट्रोस्प्रे आयनीकरण आयन मोड में कुल 789 मेटाबोलाइट्स की पहचान की गई थी, जिसमें 71 अलग-अलग मेटाबोलाइट्स भैंस के दो समूहों के बीच संकुचित हो गए थे। आगे के मार्ग विश्लेषण से पता चला कि 15 अलग-अलग मेटाबोलाइट्स मुख्य रूप से फैटी एसिड लिपिड चयापचय से संबंधित मार्गों जैसे प्राथमिक पित्त एसिड संश्लेषण, एराकिडोनिक एसिड चयापचय, ग्लिसरोफॉस्फोलिपिड चयापचय, और असंतृप्त फैटी एसिड बायोसिंथेसिस में समृद्ध थे। जैव रासायनिक मापदंडों और खनिज प्रोफाइल के लिए 19 भैंसों के सीरम नमूनों का विश्लेषण किया गया। वर्तमान अध्ययन में पाया गया कि डायस्ट्रस समूह की तुलना में केवल एस्पार्टेट एमिनोट्रांस्फरेज, कुल बिलीरुबिन और बिलीरुबिन अप्रत्यक्ष स्तर एस्ट्रस में एक महत्वपूर्ण अंतर ($P<0.05$) दिखाते हैं। खनिज विश्लेषण में, आयरन और मैग्नीशियम एस्ट्रस और डायस्ट्रस में एक महत्वपूर्ण अंतर ($P<0.05$) दिखाते हैं। इस प्रकार ये निष्कर्ष भैंस में एस्ट्रस जैसी शारीरिक स्थितियों की जांच के लिए संभावित मेटाबोलाइट बायोमार्कर की पहचान की दिशा में बहुमूल्य जानकारी प्रदान करते हैं

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ABBREVIATIONS

ACRONYMS	**MEANING**
%	Percentage
µg	Microgram
µl	Microliter
µM	Micromolar
°C	Degree celsius
AA	Arachidonic acid
AI	Artificial insemination
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BID	Bilirubin indirect
BIT	Bilirubin in total
BPC	Base peak chromatogram
CCTV	Closed-circuit television
dl	Deciliter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FAB	Fast atom bombardment
FAO	Food and agriculture organization
FC	Fold change
FSH	Follicle stimulation hormone
GC-MS	Gas chromatography-mass spectrometry
GnRH	Gonadotropin-releasing hormone
HRMS	High-resolution mass spectrometry
HSP-70	Heat shock protein-70
IgG	Immunoglobulin G
IVF	In-vitro fertilization

IVP	In-vitro embryo production
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography-mass spectrometry
LH	Luteinization hormone
m/z	Mass-to-charge ratio
mg	Milligram
ml	Milliliter
MS	Mass spectrometry
ng	Nanogram
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCA	Principle component analysis
PG	Prostaglandin
PGE2	Prostaglandin E_2
PLA2	phospholipase A2
PLS-DA	Partial Least Squares-Discriminant analysis
ppm	Parts per million
PUFAs	Polyunsaturated fatty acids
RBC	Red blood cell
RNA	Ribonucleic acid
RT	Retention time
SEM	Standard error of the mean
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
TIC	Total ion chromatogram
TLR-4	Toll like receptor
TOF	Time of flight

CHAPTER-1

Introduction

INTRODUCTION

Livestock production is a key element of the agricultural economy of developing countries, and it is intertwined with the social and cultural lives of millions of farmers. Because of the growing demand for milk and milk products, livestock provide numerous opportunities for employment and income. Indian livestock population records 535.78 million which has shown a rise of about 4.6% since 2012 of which 109.85 million are buffaloes showing an increase of about 1.0% over the previous census (livestock census provisional data for 2019, BAHS-2019). The economic well-being of a country, especially a developing one, is highly reliant on productive livestock, and the productive life of an animal is determined by its reproductive efficiency.

Domestic water buffalo (*Bubalus bubalis*) is a very important livestock resource in Asia, particularly in the southeast, which includes India. Throughout Indian history, buffaloes have played an important role in milk production, meat industries, and other agricultural activities such as draught animals. Buffalo has gained popularity due to their high milk yield and fat percentage, and they are regarded as a mainstay of the Indian dairy industry (Mondal *et al.,* 2009). At present, Indian buffaloes have a vital influence in dairy industry development and contribute about 49% share in total milk and 18.85% of meat production of India (BAHS-2019, DADF). Despite buffaloes' great potential in the dairy industry, a true understanding of their production potential has yet to be achieved, and their reproductive efficacy has been hindered by a variety of factors including heat detection, late puberty, seasonality of calving, longer postpartum anestrus, and calving interval. Estrus detection in buffalo is difficult, resulting in a significant economic loss due to decreased reproductive efficiency. These are also known as shy breeders or "silent estrus animals" because the typical signs of estrus in buffalo are relatively weak or silent, and most signs appear late at night and often go unnoticed, making them difficult to detect. (Suthar and Dhami, 2010). Estrus detection is a major issue due to poor expression of estrus signs in buffaloes, and inefficient heat detection reduces the herd's fertility status; each missed heat is equivalent to a

21-day loss in production (Kumar *et al.,* 2013). Heat detection is labor-intensive, and its success entirely depends on a dairy farm's abilities, skill, approach, and attitude. Lacunae in the heat detection system will eventually result in a loss to a dairy farm. It has been reported that 50% of estrus events go undetected, and more than 20% of inseminations occur at inappropriate times due to incorrect estrus identification, primarily during the summer season (Sharma *et al.,* 2008). which leads to huge economic loss to buffalo farmers. Currently, estrus detection in buffaloes is primarily done through behavioral and physiological signs such as bellowing, mounting, increased activity, licking, circling, swollen vulva, clear discharge mucus, and frequent urination; however, these signs are not as noticeable as they are in cows. Therefore, the need for a simple field applicable estrus identification method would be much useful to buffalo farmers. Although the cervicovaginal fluid fern patterns confirm the estrus stage for a breeding decision, the fluid discharge is absent during the silent-heat condition. In such conditions, alternative and reliable heat detection methods are required in buffaloes.

In that case, methods that use non-invasive or minimally invasive materials will be extremely beneficial to farmers in the field. Because non-invasive fluids such as saliva, urine, milk, vaginal discharges, and minimally invasive fluids such as a drop of blood are simple to collect by a non-expert person. Furthermore, these biological samples are excellent sources of biomarkers representing animal pathophysiology and can be used to better understand the biological process, Biomarkers are any biomolecules that are a measurable indicator of a biological state or have physicochemical properties that are related to a specific physiological condition. The Analysis of low-molecular-weight metabolites in biological samples has become a valuable tool in research. After all, it provides a powerful approach to discover metabolite biomarkers in biological systems because it is a dynamic biomolecule that changes continuously with the physiological condition of the body. And analysis of metabolite has the potential to generate novel non-invasive diagnostic tests as well as provides a unique insight into established novel metabolic pathways, which are simple and cost-effective yet retain high sensitivity and specificity characteristics. For example, estrus-associated volatile compounds and other metabolites were identified in urine, saliva, and vaginal fluid in buffaloes. Plasma or serum biochemical profiles were used for clinical and metabolic analysis of individual animals, groups, or herds, as well as to assess physiological changes. Because serum contains numerous substances such as enzymes, proteins, hormones, lipids, and so on. When processed and correctly interpreted, this substance testing provides necessary information on the animal's clinical condition, health status, nutritional state, and metabolic state. As a result of changes in various biochemical constituents responsible for reproductive failure (Prabha *et al.,* 2000) and lower-level concentration of circulatory minerals result in impaired reproductive function leading to the cessation of cyclic activity (Mortson *et al.,* 1972) because minerals play a major role in the regulation of hormone and enzyme for initiation of estrus (Dhoble and Gupta, 1986). And the use of blood metabolites for assessing nutritional status and pathophysiological conditions of animals is becoming popular because several factors like nutrition, physiological status of an animal, feeding, breed, age, and season affect the level of blood metabolites. Therefore, blood metabolites can be used to benefit the buffalo population. Several studies on metabolomics have been conducted in various biological samples, for the identification of metabolites related to various diseases. But the serum metabolome and serum biochemical and minerals for estrus and diestrus specific have yet to be explored. With this background, the current study has been designed with the following **objectives:**

1. **To profile the serum metabolome during buffalo estrus and diestrus.**
2. **To evaluate the serum biochemical and mineral profile during buffalo estrus and diestrus.**

CHAPTER-2

Review of Literature

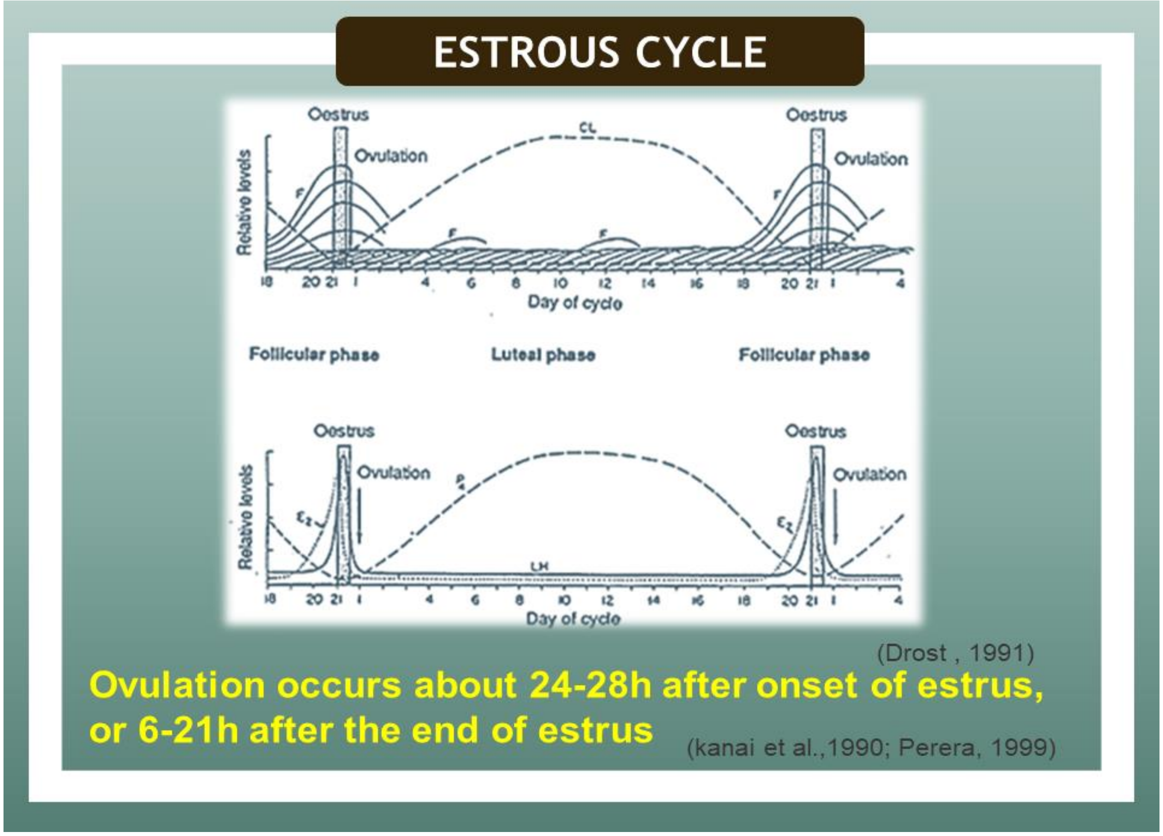
REVIEW OF LITERATURE

Buffaloes are the most important livestock species in the dairy industries worldwide, and they play an important role in the agricultural economies of many developing Asian countries, accounting for nearly 97% of the world's buffalo population (Mondal *et al.,* 2006). Buffalo is an important animal in many farming systems around the world because it adapts easily to changing ecological and harsh environmental conditions. They are efficient animals when it comes to utilizing low-quality roughages, and resisting some bovine tropical diseases. Buffaloes have played a key role in overall social development in terms of meat production, milk production, and agricultural activities (Nanda and Nako, 2003). Successful breeding and milk production are dependent on the reproductive efficiency of female animals. Animal reproduction is an essential component of sustainable livestock production. Improving reproductive efficiencies requires great attention and strategies. Despite its all merits, the reproductive efficiency of this shy breeder is hampered by several factors, including delayed puberty, the poor manifestation of estrus signs regarded as “silent heat” (Suthar and Dhami, 2010). long calving intervals, longer postpartum anestrus period, and a low conception rate (Madan *et al.,* 1996), which results in population decline and a significant reduction in farmers' financial returns. Silent heat is a major impediment to buffalo reproduction efficiency and failure of heat detection is a major factor contributing to low fertility. Which ultimately leads to huge economic loss to our country. The behavioral signs of estrus are less obvious in buffalo than in cattle which makes it difficult to predict estrus and time of ovulation thus reduces breeding efficiency (Selvam *et al.,* 2017). There are several behavior signs associated with estrus like micturition, bellowing, vulvar swelling, mucus discharge, restlessness, stand to be mounted, and inappetence. But these signs are relatively weak in buffalo, therefore, require continuous monitoring and experienced handling of the animal to effectively predict the correct time of estrus, and timely detection of heat in female buffalo is a pre-requisite for successful artificial insemination as it would help inappropriate time of ovulation (Kumar *et al.,* 2013) in turns which will increase conception rate. Understanding the regular mechanisms involved in the estrous cycle is required to improve buffalo reproductive efficiency.

2.1. Estrous cycle

Buffaloes are polyestrous animals. The mean length of the cycle is 21 days and it is divided into four phases: proestrus, estrus, metestrus, and diestrus. Estrus is a period when a female is sexually receptive (in heat). The duration of estrus generally ranges from about 5 to 27 hours and ovulation occurs about 24-28 hours after onset of estrus or 6 to 21 hours after the end of estrus (Kanai *et al.,* 1990). Estrus generally last a shorter time in hot climates, and signs of estrus may only occur late at night or early in the morning. The signs of estrus in buffalo are substantially less pronounced than in cattle. The cycle is been regulated by gonadotropin hormones. Buffalo's estrus cycle follows a similar pattern to cattle in terms of hormone changes as shown in (figure 2.1). During the follicular phase, the dominant follicle becomes extremely receptive to LH and continues to grow, while the diameter of the follicle rises and it begins to release estrogen and inhibin. The inhibin triggers a negative feedback loop in the adenohypophysis, reducing FSH levels and making them insufficient for the development of the other subordinate follicles.

The increasing estrogen secretion by the preovulatory dominant follicle associated with decreased progesterone serum concentration leads to regression of the corpus luteum. The pre-ovulatory peak of LH is induced by GnRH (De Abreu *et al.,* 2017). Following ovulation, the luteal phase is marked by an increase in serum progesterone levels released by the corpus luteum, because progesterone determines limiting the peak of LH required for ovulation to occur. The corpus luteal’s primary function is to produce progesterone for pregnancy maintenance. Furthermore, progesterone reduces gonadotropin secretion throughout pregnancy, preventing the occurrence of estrus. If a pregnancy is not recognized by the mother, the corpus luteum shrinks due to the action of prostaglandins generated by the uterus, which lowers progesterone levels in the blood, and the new estrous cycle begins.



ESTROUS CYCLE

(Drost , 1991)

Ovulation occurs about 24-28h after onset of estrus, or 6-21h after the end of estrus (kanai et al.,1990; Perera, 1999)

Figure 2.1. Schematic representation of the pattern of secretion of LH, estrogen and progesterone in estrous cycle.

2.2. Need for estrus detection

Silent estrus is one of the most common problems in buffaloes. Buffaloes are seasonally polyestrous, and heat expression is limited to only eight months of the year. From March to June, buffaloes are generally sexually dormant, with only 3% of them showing signs of heat. Estrus signs, such as swollen vulva, vaginal mucus discharge, frequent urination, mounting, bellowing, restlessness, and sniffing other individual's vagina, appear in buffalo at a very low intensity and are difficult to detect. Whereas in cattle they all are a prominent indicator of estrus. The most acceptable strategy for increasing the buffalo population is artificial insemination (AI), but pregnancy success in buffalo is limited due to misinterpretation of the time of estrus, and AI success is dependent on the optimal time of insemination, which is 10-24 hours after the onset of the estrus phase. As a result, detecting estrus at the right time is critical for reproductive management. Buffaloe's estrus detection is primarily based on behavioral signals (Table 2.1).

Table 2.1. Estrus detection method based on behavioral signs (Choudhary *et al.,* 2018)

Estrus determination methods	Efficiency (%)
Mucous discharge	62.07
Frequent micturition	55.17
Swollen vulva	41.38
Chin resting	27.59
Restless	27.59
Sniffing vulva of a herd mate	20.69
Mounting on another buffalo	10.34
Standing heat	3.45
Bellowing	37.93
Flehmen reaction	24.14

Furthermore, numerous heat detection methods for buffalo and cows have been developed. Methods such as using a teaser animal or pedometer, analyzing the ovary by ultrasonography, using CCTV & Video Camera, and monitoring hormone levels in blood require skill and experience, and all of these methods are not much user-friendly and stressful in the case of buffaloes due to their behavior and habitat. Hence, a non-invasive or minimally invasive approach for identifying estrus in buffalo utilising biofluids is required.

2.3. Current estrus detection methods by using different biofluids

Biofluids have a broader dynamic range than tissue, they are regarded as useful resources for the finding of biomarkers. A biomarker is a biological marker discovered in biofluid that can be used to determine if a procedure is normal or abnormal, as well as the presence of disease or illness, and to comprehend the animal's physiological and pathological parameters. So far many research has been conducted employing biofluids such as urine, saliva, blood/serum, cervicovaginal fluid, and milk to identify estrus-specific biomarkers.

2.3.1. Salivary Evaluations

In cases of silent heat, the salivary ferning pattern approach is also utilized to detect estrus. Saliva is a non-invasive fluid that may be obtained simply and represents the pathophysiology of animals. During estrus, the concentration of salts in saliva fluctuates rapidly, forming a fern-like crystallization pattern. (Ravinder *et al.,* 2016). Under a microscope, the fern pattern indicates that the animal is in estrus. The right period for buffalo insemination is indicated by a rise in branching in the fern pattern. According to onteru *et al.,* (2016) the direct saliva transcript analysis in buffaloes, saliva demonstrated considerably higher expression of the Heat shock protein 70 (HSP70) and Toll-like receptor 4 (TLR4) during estrus than during diestrus.

2.3.2. Cervicovaginal mucus

The cervicovaginal mucus fern pattern in buffaloes is an extremely useful method to determine estrus, and the crystallization appears as a fern when studied under a microscope because of variations in electrolytes in cervical secretions triggered by changes in circulating ovarian hormones. Buffaloes with a typical fern pattern are in estrus and should be inseminated. A recent study discovered that the expression of heat shock protein-70 was higher during the estrus, suggesting that it might be used as a biomarker for estrus detection in buffaloes (Muthukumar *et al.,* 2014).

2.3.3. Milk and plasma

Somatic cell counts or progesterone concentrations can be used to detect estrus in buffaloes using milk. Buffaloes' somatic cell counts and milk proteins both increased considerably during estrus. Progesterone concentrations in milk and plasma in buffaloes decrease dramatically during estrus and hence can be used to identify estrus. In the experiment plasma progesterone concentrations in Murrah buffaloes declined from 0.30-0.23 ng/mL two days before estrus to 0.09-0.03 ng/mL on the day of estrus, then progressively increased to 0.27-0.03 ng/mL two days following estrus (Gupta *et al.,* 2001). The problem with progesterone assays for estrus detection is that they are only effective during particular times of the period.

2.3.4. Chemical cues in urine and feces

To determine estrus, an LH bovine kit was used to quantify urine LH. The findings reveal a significant rise in urine LH during estrus, but neither before or after estrus phase (Selvam *et al.,* 2017). Usage of Nanoparticle peptide-based biosensor in the detection of estrus described the use of a peptide-based biosensor for sensing luteinizing hormone, which works as an ovulation predictor (Nayan, 2020). Fecal material are readily available sample. During estrus, feces include a variety of chemical substances, including volatile chemicals present in urine or vaginal mucus that act as pheromones. Buffalo feces included twenty-seven volatile chemicals, with 4-methyl phenol and trans-verbenol identified solely in the feces of buffaloes in estrus (Karthikeyan *et al.,* 2013). Buffalo bulls developed flehmen after being given a combination of the two drugs. As a result, these two substances might be used to determine buffalo estrus. The urine of estrus synchronized buffaloes included P-cresol and oleic acid (9-octadecenoic acid), which was identical to buffaloes in natural estrus (Muniasamy *et al.,* 2017).

So, concerning all the above studies, many more compounds or metabolites can be identified by using the serum as a minimally invasive sample which will be easily available in comparison to urine which becomes a tedious process sometimes, and because of a large quantity of urine samples given by animal is diluted for metabolite identification compare to serum sample, a technique such as high-resolution mass spectrometry (HRMS) can be used for lower metabolite identification in serum sample which may be an estrus specific and could be a potential metabolite biomarker for estrus detection.

2.4. Metabolites

Metabolism is a series of chemical reactions that occur in living organisms. It is necessary for the biological system's regular function as well as the organism's ability to grow and reproduce, retain its structure, and adapt to a continuously changing environment. Metabolite refers to the substrate, intermediate, and product of metabolism reactions. Unlike genes and proteins, which are affected by epigenetic regulation and post-translational modifications, (figure 2.2). Metabolites are the final products of protein and enzyme activity in metabolic pathways, and their levels can be regarded as the biological system's final responses to genetic or environmental alterations and can be used to assess cellular health (Manchester *et al.,* 2016).

Metabolites are dynamic molecules that change every second in the body and are important indicators of pathophysiological condition and can provide information for the identification of early differential disease markers and help to understand their occurrence and progression. The information flow through different omics levels is described as the central dogma of molecular biology and the whole set of Metabolites represents the metabolome. Metabolome composition is influenced not only by the type of sample but also by internal and external factors, so the metabolome can be viewed as an interface between genetically determined processes and environmental processes. Hence, metabolite is thought to be the biological system's final response to genetic, environmental, or nutritional modification (Whitfield *et al.,* 2004). So, metabolome analysis can be an effective method for identifying diagnostic markers and investigating unidentified pathological conditions.

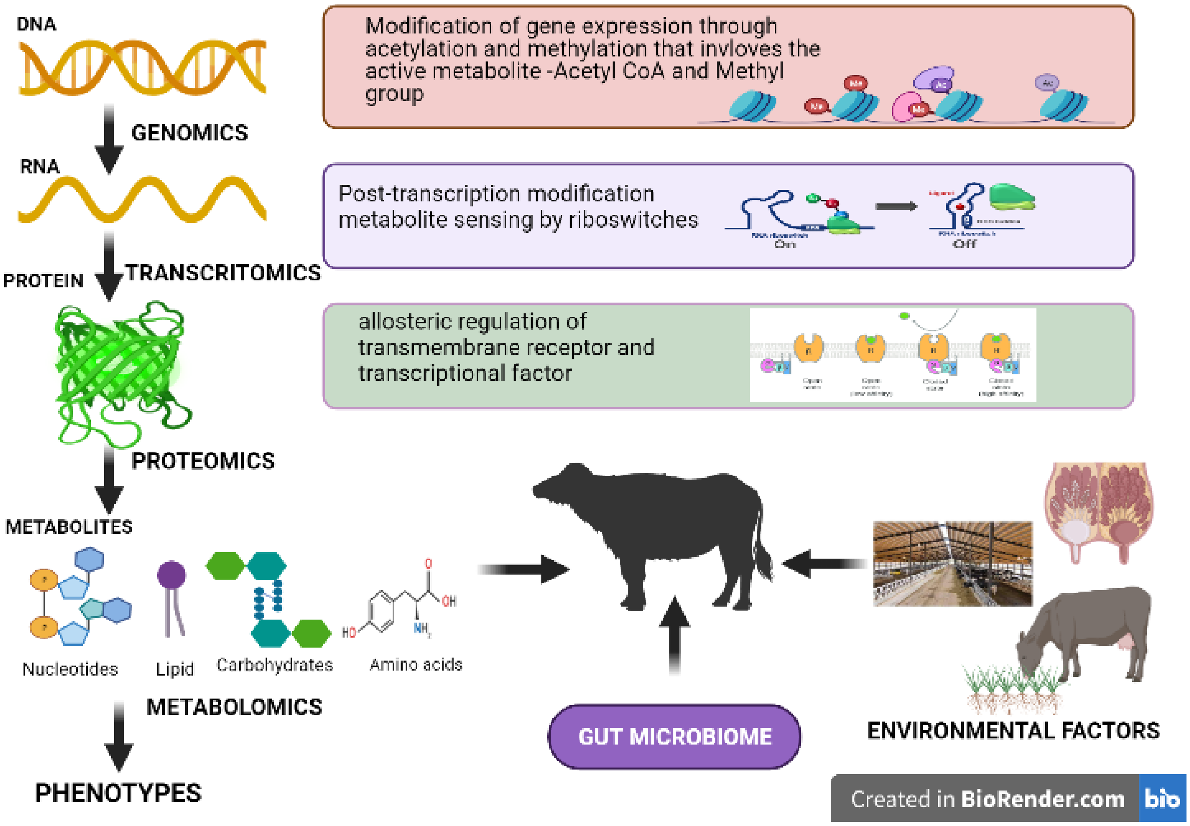


Figure 2.2. A diagrammatic representation of different levels of omics cascade

2.5. Metabolomics- A new entry to omics cascade

Using high-resolution analytics platforms and statistical methodologies, metabolomics is the process of detecting and measuring all low molecular weight final products (metabolites) of metabolism in a biological sample (Cambiaghi *et al.,* 2017). It provides a functional view of an organism as determined by the sum of its genes, RNA, protein, and environmental factors, and thus a measure of a biological system's phenotype. Metabolomics represents not only the downstream output of the genome, but also the upstream input from the environment, and is thus placed at the bottom of the "omics" cascade (Wishart, 2016; Zaitsu *et al.,* 2016) because genes control what may happen whereas metabolites determined what has happened (Dunn *et al.,* 2011). It gives a comprehensive picture of an organism's metabolic condition, which can reveal relevant info about disease pathophysiology (Xi *et al.,* 2014). Metabolomics is a relatively new member of the omics family, which includes genomes, transcriptomics, and proteomics. Metabolomics is a technique that can be used to explain the etiology of disease, generate disease biomarkers, and analyze and monitor complicated disorders (Martin *et al.,* 2012). Metabolomics has been used in a variety of studies, including disease diagnosis (Zhang *et al.,* 2012), nutrition, environmental science, food science drug development, and monitoring of drug metabolism, crop characterization, and toxicology (Cuykx *et al.,* 2018).

2.6. Targeted and untargeted metabolomics

Targeted and untargeted metabolomics are two types of metabolomics methods. Untargeted metabolomics includes both metabolite measurement and identification and is used to identify as many metabolites as feasible. It uses relative quantification (fold change) and comparison of the sample to detect the existence of all potential metabolites in a sample (Cambiaghi *et al.,* 2017). The aim is to determine the whole metabolome in an unbiased manner, usually to develop novel ideas that will be evaluated later in the research phase. On the other hand, untargeted metabolomics frequently results in the generation of huge amounts of complicated data that pose challenges in terms of optimal and unbiased strategies for data acquisition processing and statistical analysis of complex data, and Despite having substantial metabolomics spectra libraries (Dunn *et al.,* 2013), identification of unknown compounds is a tough element of untargeted metabolomics, hence an advanced bioinformatics tool is required to interpret the results (Gorrochategui *et al.,* 2016). On the other side, the metabolites chosen for quantification in target metabolomics are already known and represent specific pathways or classes of molecules. The key benefit of this method is increased analytical depth as a result of increased sensitivity and accuracy. One limitation of the targeted approach is that the compounds of interest must be known ahead of time and be analyzed. A new hypothesis may be constrained by the fact that the targeted metabolomics encompasses a small number of compounds.

2.7. Analytical platforms for metabolomics

Analytical platforms used in metabolomics research include Nuclear magnetic resonance (NMR) and Mass spectrometry (MS). Generally, Mass spectrometry combined with various separation techniques such as gas chromatography and liquid chromatography, and NMR is performed with direct analysis.

2.7.1. Nuclear magnetic resonance

NMR is based on the resonance frequency, which represents the spin behavior of atomic nuclei in a magnetic field. NMR is a non-destructive, non-invasive, non-equilibrium approach for evaluating micromolar metabolites that require little or no sample preparation (Reo, 2002; Beckonert *et al.,* 2007). NMR has been frequently employed it is a non-invasive method to diagnose disease (Nagana *et al.,* 2008; Capati *et al.,* 2017). NMR has many benefits in metabolomics analysis. It has highly reproducible and quantitative properties (Pan and Raftery, 2007), and it is a robust, non-destructive technique for measuring various types of small-molecule metabolites (Creek *et al.,* 2012). The drawback of NMR in the metabolomics analysis is its low sensitivity and spectral resolution, as well as its ability to detect only the most abundant compound in a given sample.

2.7.2. Mass spectrometry

MS is the most widely used technique because it allows for accurate metabolite identification, particularly when combined with chromatographic separation methods to improve mass-resolving capabilities. Every mass spectrometer has three interconnected parts: an ionization source that adds charge to the molecules to be analyzed, a mass analyzer that separates molecules based on their mass/charge ratio (m/z), and a detector. Mass spectrometry is a well-established method for measuring the mass to charge ratio of charged particles in metabolomics studies (Baran *et al.,* 2009). The main disadvantages of the MS technique are the need to separate or purify the sample before directing it into the mass analyzer, as well as the instrument's high cost. Direct injection is a quick technique that is frequently used in metabolomics analysis. The main advantage of MS is that there is no loss of metabolites when samples are directly injected into MS. The main limitation is the low and inconsistent ionization efficiencies in complex biological samples (Zhang *et al.,* 2012). Combining MS with chromatography can reduce ion suppression by separating complex samples before entering the MS, and the two most popular systems are Gas chromatography-MS (GC-MS) and Liquid chromatography-MS (LC-MS).

2.7.3. Gas chromatography-mass spectrometry

In metabolomics analysis, GC-MS can separate and detect naturally occurring volatile metabolites (alcohols, aldehydes, esters, etc.) as well as metabolites that become volatile after derivatization (Zhang *et al.,* 2012). The core principle of GC separation is based on the partitioning of specific molecules between gas and liquid phases at a given temperature, as well as the effect of the portioning on their movement through the GC column. Molecules in the gas phase pass through the capillary column of the GC and are then detected by Mass Spectrometry. Because molecules have different volatility and partition differently between the gas and liquid phases depending on temperature, using an increasing temperature gradient on the GC column allows for molecule separation in time. The main benefits of GC-M are its increased sensitivity, highly reproducible and robust technique, low cost, excellent separation, detection of more metabolites, and the availability of extensive and good software and databases for identification (Schauer *et al.,* 2005). The disadvantage of GC-MS is that it is only appropriate for volatile, thermally stable compounds or compounds that can be produced through chemical derivatization, which significantly increases processing and analysis time (Segers *et al.,* 2019).

2.7.4. Liquid chromatography-mass spectrometry

In LC-MS, Before MS detection, liquid samples are directly injected into the LC column, and metabolites are separated based on their interaction with a stationary phase. The stationary phase is typically represented by spherical beads with specific functional groups that define the nature of the separation on the surface. When compared to gas chromatography-mass spectrometry (GC-MS), LC-MS can analyze non-volatile metabolites without derivatization, thus provides good coverage of the metabolome. By avoiding multiple, time-consuming sample preparation processes, overall measurement variances can be reduced, resulting in more consistent and comparable metabolomics results in LC-MS. Once the sample is injected into the LC-MS instrument, it is subjected to liquid chromatography. The sample is dissolved into a liquid fluid known as the "mobile phase". The sample-carrying solution is then forced under high pressure through a column packed with the “stationary phase,” which is made up of small particles, a porous monolithic layer, or a porous layer. At different times, different compounds in the sample solution elute from the column. The time at which a compound elutes from the column is referred to as its retention time. However, because only a small number of metabolites in an experiment share the same or similar retention time, liquid chromatography reduces sample complexity and reduces background noise in mass spectrometry detection. Compounds are injected into the mass spectrometer after eluting from the liquid chromatography. An ion source, a mass analyzer, and a mass spectrometer, and a detector are the three essential components of a mass spectrometer. The ion source converts the electrically compounds into charged molecular ions. Various techniques, such as electrospray ionization (ESI), atmospheric pressure chemical ionization, atmospheric pressure photoionization, fast atom bombardment (FAB), are used to achieve this conversion. Among these, ESI is by far the most commonly used method in most LC-MS-based metabolomics studies. It can ionize a wide range of metabolites with varying molecular weights and compound polarities. Because metabolites have such diverse chemical properties, it is often necessary to analyze the biological sample in both positive and negative ionization modes to maximize metabolome coverage (Theodoridis *et al.,* 2011). Mass analyzers in mass spectrometry are used to separate ions of analytes by their mass/charge ratio in the gas phase under a high vacuum using electric and magnetic potentials. Commonly used mass analyzers include quadrupole, ion trap, time-of-flight (TOF), and Orbitrap. The detector converts the abundances of the ions from the mass analyzer into electrical signals by recording the charge induced or current produced when an ion hits or passes through the detector. A general outline of LC-MS is shown in (figure 2.3).

2.8. Importance of metabolomics in livestock

The livestock population is beneficial in every way. They produce high-quality food items such as eggs, milk, and other dairy products. Despite being a potential source of all necessities, their use is restricted due to some common prevalent disease conditions in livestock. In most cases of the disease, the metabolic pathway has been either activated or deactivated, so a specific metabolite is an important player in the biological system and acts as a biomarker for the disease condition. With the most recent advances in analytical chemistry, specific metabolites can be analyzed with metabolic tools and technologies. Another advantage of metabolomics analysis for livestock is that the sample is always obtained non-invasively (saliva, urine, feces) or minimally invasively (serum and plasma).

In recent years, several papers on livestock research have been published that may assist farmers, veterinarians, and livestock researchers. These include animal products, animal nutrition, milk quality, animal fertility, animal reproduction, animal genetics and breeding, processing of animal products, animal health and disease tendency, animal physiology, and reproduction. According to the current status of livestock metabolomics, nearly half of all metabolomics research has been conducted in bovine. Porcine and ovine metabolomics research came in second and third with 25% and 13%, respectively, followed by equine and caprine metabolomics studies with 5% and 4%, respectively (figure 2.4).

2.9. Metabolomics in livestock

2.9.1. Metabolomics in Reproduction

Reproduction is a process that involves sexual differentiation, sexual maturation, gametogenesis, fertilization, embryo development, and gamete fertility. Massive molecular analysis techniques and tools are now available to evaluate the metabolome, which deals with all of the complex reproduction processes. Several studies have been conducted on the metabolome of the female reproductive system, which includes ovarian follicular fluid, ovarian follicles, embryo culture, and endometrial fluid.

2.9.1.1. Follicular fluid

Follicular fluid is important for determining fertility because it protects the oocyte from proteolysis and provides the microenvironment for embryonic development, follicular fluids are important in the quality of embryos and oocyte production and maturation. High-resolution mass spectrometry was used to look for markers that could be connected to metabolic pathways that contribute to higher or poorer reproductive performance in follicular fluid samples from two groups with high and low fertility. (Guerreiro *et al.,* 2018). Studies have found a link between the levels of non-esterified fatty acid metabolites in serum and Follicular Fluid in early postpartum cows with negative energy balance, which may lead to changes in the quality of oocyte cells, and a negative energy balance may impede fertility of high-yielding dairy cows by affecting oocyte quality via increased NEFA concentrations in FF (Leory *et al.,* 2005). The metabolic characterization of FF in lactating cow and heifer follicles using gas chromatography (GC-MS) based mass spectrometry indicated higher saturated fatty acid contents in cow follicles than in heifer follicles (Bender *et al.,* 2010). Another study looked at the changes in follicular fluid metabolites in dairy cows with inactive ovaries and discovered that eight compounds were considerably elevated and six were significantly decreased (Bai *et al.,* 2020).

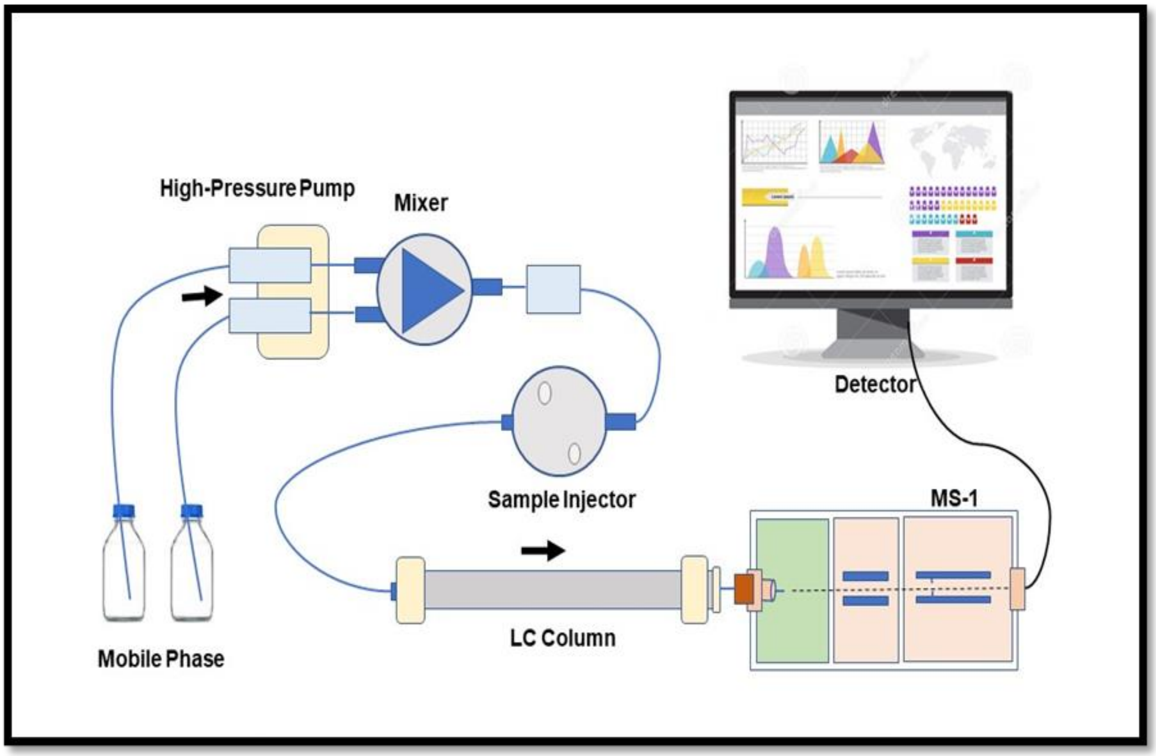


Figure 2.3. Representing the general outline of LC-MS

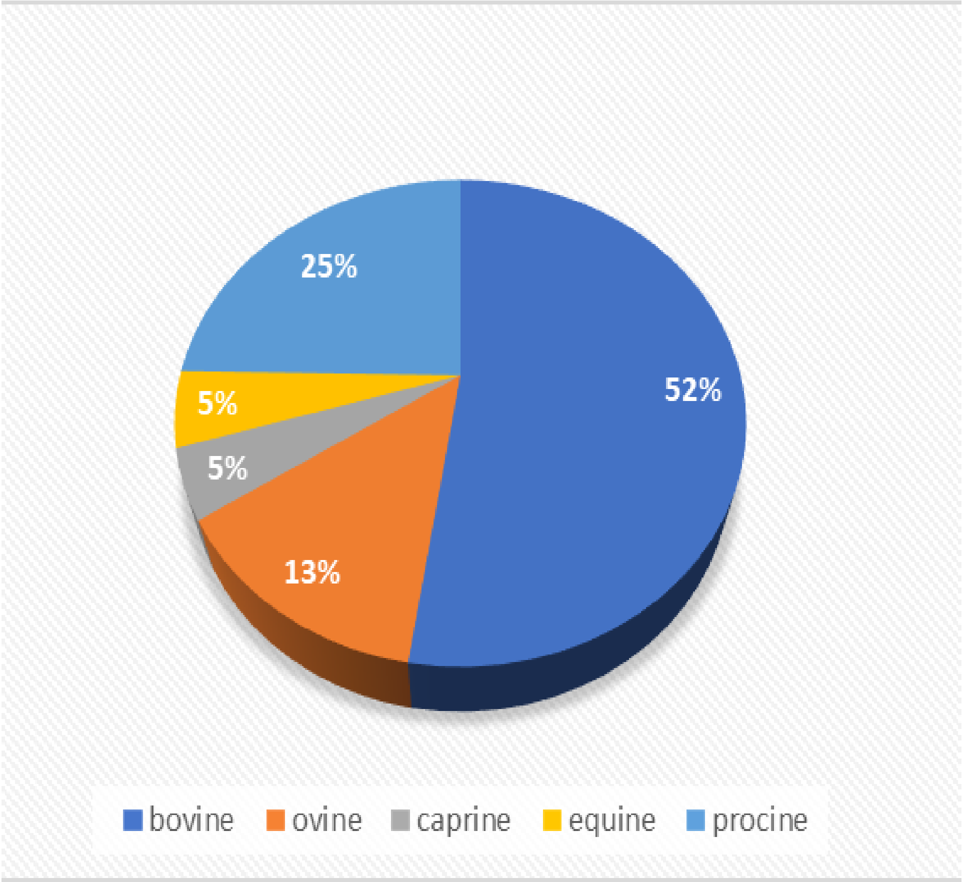


Figure 2.4. Percentage share of metabolomics among various species of livestock (Adapted from goldansaz *et al.,* 2017).

2.9.1.2. Metabolomics in embryo culture media

The versatility of metabolomics applications has allowed us to non-invasively monitor the metabolic activity of embryos in a complex culture environment, allowing us to find a non-invasive alternative to searching for a marker related to embryo viability by analyzing the cultured media surrounding the embryo. Many of the chemicals detected in the culture medium, including glucose, lactate, and pyruvate, oxygen, amino acids, and triglycerides, are used in embryo metabolic activities and may be linked to embryo viability. Oxygen consumption has provided accurate evidence of the pregnancy rates of bovine IVP embryos (Muñoz *et al.,* 2014). Another non-invasive method for embryo selection was based on the depletion and appearance of amino acids in culture media (Singh *et al.,* 2007). The spent culture medium could reveal vital information about a bovine's physiological state. In a recent study, bovine embryos (2 cells to blastocyst stage) were divided into two groups: fast-divided embryos and slow-divided embryos. The study found that fast-cleaved embryos consumed more culture media metabolites such as methionine, lysine, threonine, histidine, acetate, phenylalanine, arginine, tyrosine, tryptophan (Perkel *et al.,* 2017). Ultimately, metabolomics data can provide in-depth information on the biochemical pathways used by embryos under various conditions, allowing for the modification of in vitro metabolism to promote increased embryo growth and higher conception rates.

2.9.1.3. Metabolomics in endometrial uterine fluid

Many metabolites found in the uterine fluid have signalling capabilities, and some of them affect embryonic preimplantation development. The metabolome of the bovine uterus changes during the early stages of the estrous cycle and is likely to participate in the regulation of embryonic preimplantation development (Tribulo *et al.,* 2019). On day 7 of the estrous cycle, plasma and uterine luminal fluid samples were taken from two distinct groups of repeat breeder and healthy cows. Metabolomics analysis identified 17 plasma metabolites observed at concentrations that distinguished between the two groups, but no metabolites in the uterine luminal fluids for repeat breeding problems in cattle (Funeshima *et al.,* 2021).

2.9.1.4. Metabolomics in seminal plasma

The use of a metabolomics tool to look for biomarkers in seminal plasma has proven successful, as has the use of a metabolomics tool to identify potential biomarkers of male fertility and infertility (Kovac *et al.,* 2013). After NMR analysis, high and low fertility biomarkers were revealed in seminal plasma and the serum of bulls (Kumar *et al.,* 2015). Infertile bull's seminal plasma contains larger levels of citrate, lactate, glycerylphosphorylcholine, and glyceryl phosphorylethanolamine than fertile bull's seminal plasma (Deepinder *et al.,* 2007).

2.10. Metabolomics in nutrition and rumen metabolome

Nutri-metabolomics studies have reframed molecular nutrition, digestion, and metabolism, ultimately presenting a new metabolite into an animal system that serves as a new biomarker, and this new metabolite is influenced directly or indirectly by nutrients. Cattle feed influences the ruminal microbial metabolome, which in turn influences the systemic metabolome (Saleem *et al.,* 2013). Acetate, glucose and propionate levels in ruminal fluid differed between Holstein dairy cows fed corn stover or a blend of alfalfa hay and corn silage (Zhao *et al.,* 2014). In a second study, Holstein dairy cows on low-concentration and high-concentrate diets had different ruminal amino acid profiles, including alanine, leucine, and glycine (Zhang *et al.,* 2017). In lactating Holstein cows, high grain diets increased ruminal levels of harmful and inflammatory chemicals (Ametaj *et al.,* 2010).

2.11. Metabolomics in disease diagnosis

The absence of relevant symptoms in many diseases is frequently discovered at a later stage. The more advanced stage of disease necessitates more invasive diagnostic and treatment procedures. The importance of early molecular diagnosis in increasing survival rates cannot be overstated. By identifying the biochemical changes that occur during disease, metabolomics tools can be used to predict disease at an early point. Milk fever is a metabolic disorder that usually develops during and after parturition. To detect milk fever, metabolites such as Amyloid A protein in serum, calcitonin gene, IgG heavy chain C-region, and albumin are utilized (Collard *et al.,* 2000). In plasma metabolomics research, the ratio of alanine to a branched amino acid determines the duration of hypoxia (Solberg *et al.,* 2010). Dervishi and co-workers used the systemic metabolome to identify holstein dairy cows at risk of retained placenta and metritis (Dervishi *et al.,* 2018).

2.12. Serum biochemical parameters

An individual's blood biochemical profile enables clinical investigation of the presence of various metabolites and other constituents in the body, which estimate the severity of diseases and the health status of animals (Piccione *et al.,* 2010). Further biochemical parameters are important in determining an animal's physiological, nutritional, and pathological status. Biochemical indicator information can aid in the diagnosis of metabolic disorders that can impair livestock reproductive performance (Amle *et al.,* 2014; Kaminski *et al.,* 2014). Because blood indices change during different physiological states, it is imperative to analyze biochemical constituents during different physiological states. Various studies reference values may not be completely applicable to our climate conditions. As a result, a biochemical profile in buffaloes in our climate at various physiological states is required.

Alanine aminotransferase was previously known as serum glutamate-pyruvate transaminase (SGPT). The transition of an amino group from L-alanine to alpha-ketoglutarate is catalyzed by ALT. Alanine aminotransferase (ALT) is generally the most useful enzyme for identifying the presence of hepatocellular damage. It is found in many tissues but its greatest activity is in the liver (Boyd *et al.,* 1983). When the liver is damaged or inflamed, it can release ALT into the bloodstream and this leads to serum ALT level rises. Aspartate transaminase (AST), also known as serum glutamic-oxaloacetic transaminase (SGOT), is an enzyme that play important role in amino acid metabolism by transferring alpha-amino acid from aspartate to glutamate. The liver, heart, skeletal muscle, kidneys, brain, and red blood cells all contain AST. Because AST is found in many parts of the body other than the liver, it should not be used solely as an indicator of liver damage unless other supporting enzymes are measured (Giffen *et al.,* 2002). An increase in transaminase enzyme activity has been found after heat stress or during the summer season (Marai *et al.,* 1995). Serum ALT, AST, and their ratio (AST/ALT ratio) are used as indicators for liver health. The end-product of heme catabolism produces bilirubin. Bilirubin is a yellow chemical formed during the normal breakdown of haemoglobin. As red blood cells degrade, the iron-containing portion of haemoglobin is transformed to bilirubin. Total bilirubin is the sum of direct and indirect bilirubin in the body; a small increase in bilirubin concentration does not indicate abnormality; however, a large increase in bilirubin concentration indicates the presence of a medical condition or underlying disease. It is primarily increased during liver damage or bile diseases because bilirubin is formed in the liver and stored in the bile. Hyperbilirubinemia causes early sexual maturation and late ovulation in animals (Saiduddin and Davis, 1993). When the liver degrades protein, it creates urea as a waste product. Urea is a helpful indicator for kidney and liver health since it represents urea breakdown and urea removal by the kidney. It provides a general overview of animal metabolic and overall health. The levels of urea in cyclic and non-cyclic cows did not differ significantly (Ahmad *et al.,* 2004).

Serum total protein in the blood is a complex mixture of amino acids that play an important role in body metabolism. Albumins and globulins are two types of proteins found in serum. The main purpose of serum protein is to maintain blood volume constant and to transport diverse compounds through the bloodstream by forming complexes. Albumin makes up more than half of serum protein and is produced mostly in the liver. It is involved in fatty acid metabolism, the movement of hormones, vitamins, and metals, and the maintenance of colloidal osmotic pressure. When compared to the control cycle group, the mean values of serum total protein, albumin, and globulin were considerably ($p<0.05$) lower in the smooth inactive ovaries group (El-Razek *et al.,* 2019). Glycerol and three fatty acids are combined to form a triglyceride. The principal carrier of dietary fat through the bloodstream is triglycerides. They are essential for the production of other lipids like cholesterol. Cholesterol helps to maintain cell membrane integrity and is involved in cell signalling. Lipoproteins transfer cholesterol from the liver to the rest of the body in ruminants. Cholesterol is the precursor of steroid hormones released by the ovary; hence it plays an important part in its physiology. Furthermore, past researchers found that mean cholesterol levels in cyclic buffaloes were higher than those seen in this study of surti buffaloes with inactive ovaries (Jayachandran *et al.,* 2013).

2.13 Minerals profile

Domestic animal's nutritional status has a significant impact on their reproductive behavior. Mineral deficiency is frequent in cattle, and the severity of the deficiency depends on the varieties of feed utilized, the physiological status of the animals, and the weather condition. Mineral deficiency in one ore multiple numbers or imbalance can induce infertility, poor conception, anestrous, and other issues of reproduction. (Hidiroglou, 1979). Several trace elements act as cofactors in the synthesis of the hormone, influencing biochemical functions related to reproduction (Valley,1976). Because of the role of minerals in the endocrine system and tissue integrity, minerals have a significant role to play in the follicular growth and fertility in dairy cows. Minerals that affect ruminant reproduction are usually found in the trace elements category (Cu, Co, Zn, Fe, and Mn). Many types of trace minerals have been linked to various reproductive problems (Jain, 1993). Repeat breeding in cattle can be caused by an excess or lack of nutrients (Das *et al.,* 2002). A strong association between copper and zinc has been shown with reproductive hormones (Prasad *et al.,* 1989). Sex hormone synthesis is decreased by deficiency of manganese and perhaps other steroids by inhibiting the synthesis of cholesterol and its precursors (Doisey, 1973). Copper has involvement in FSH, LH, and estrogen activities, and therefore plays a major role in maintaining optimal fertility (Desai *et al.,* 1982). Low iron levels in serum have been noticed to affect normal gonadal activity, possibly by producing inadequate hormonal output and degradation of general physiological function (Sharma *et al.,* 1988). Based on the previous literature, it is possible to postulate that a lack of certain micro and trace minerals in the blood causes reproductive failure in animals.

Materials and Methods

MATERIALS AND METHODS

The present study was performed to assess the Serum metabolites and minerals and biochemical profile in healthy buffalo heifers at buffalo farm, central Institute for research on buffaloes. (CIRB), Hisar, Haryana.

3.1. Experimental animals

All animals were housed in a shed having a concrete floor with asbestos roofing with proper feeding arrangement. The animal was fed an adequate quantity of green (berseem, mustard, maize, sorghum, etc.) and dry fodder (wheat straw), and the concentrate is supplemented with the mineral mixture and common salt prepared by the Institute. 6 healthy heifers were selected for metabolomics analysis and 19 buffalos' heifers were selected for biochemical and minerals analysis. And blood was collected from this animal during the estrous cycle at day zero (estrus) and day 10 (diestrus). Experimental details are shown in the flow diagram (figure 3.1).

3.2. Experimental design

3.2.1. Visual observation

Healthy heifers with normal vital signs such as temperature, respiratory rate, pulse rate, and free from genital disease or infections were selected from the shed. They were observed for estrus specific behavioral signs like bellowing, clear mucous discharge, frequent micturition, red and swollen vulva, mounting, allowing to be mounted by other buffaloes, nervousness, etc. And Animals showing prominent estrus signs were separated and thereafter estrus was confirmed by ultrasonography. Heifers with follicle sizes of more than 12mm were confirmed as estrus animals.

3.2.2. Blood sample collection

After confirmation of estrus the blood sample was collected from zero-day estrus heifers and 10th day diestrus heifers via jugular vein puncture with a vacutainer needle in the serum clot activated vacutainer serum tube. The blood sample was collected during this period in the early morning chilled on an ice pack then transported to the laboratory and kept in a slanting position for 45min followed by centrifuge at 3000rpm for 15min at 4°C and serum sample was separated within 12h of collection. After that serum was harvested and stored in multiple 2ml eppendorf tubes in -20°C until further metabolomics, biochemical and minerals serum analysis to prevent loss of bioactivity and contamination and were thawed on an ice bath for approximately 2h before use. Biochemical analysis was performed on the same day of blood collection.

3.3. Metabolomics analysis

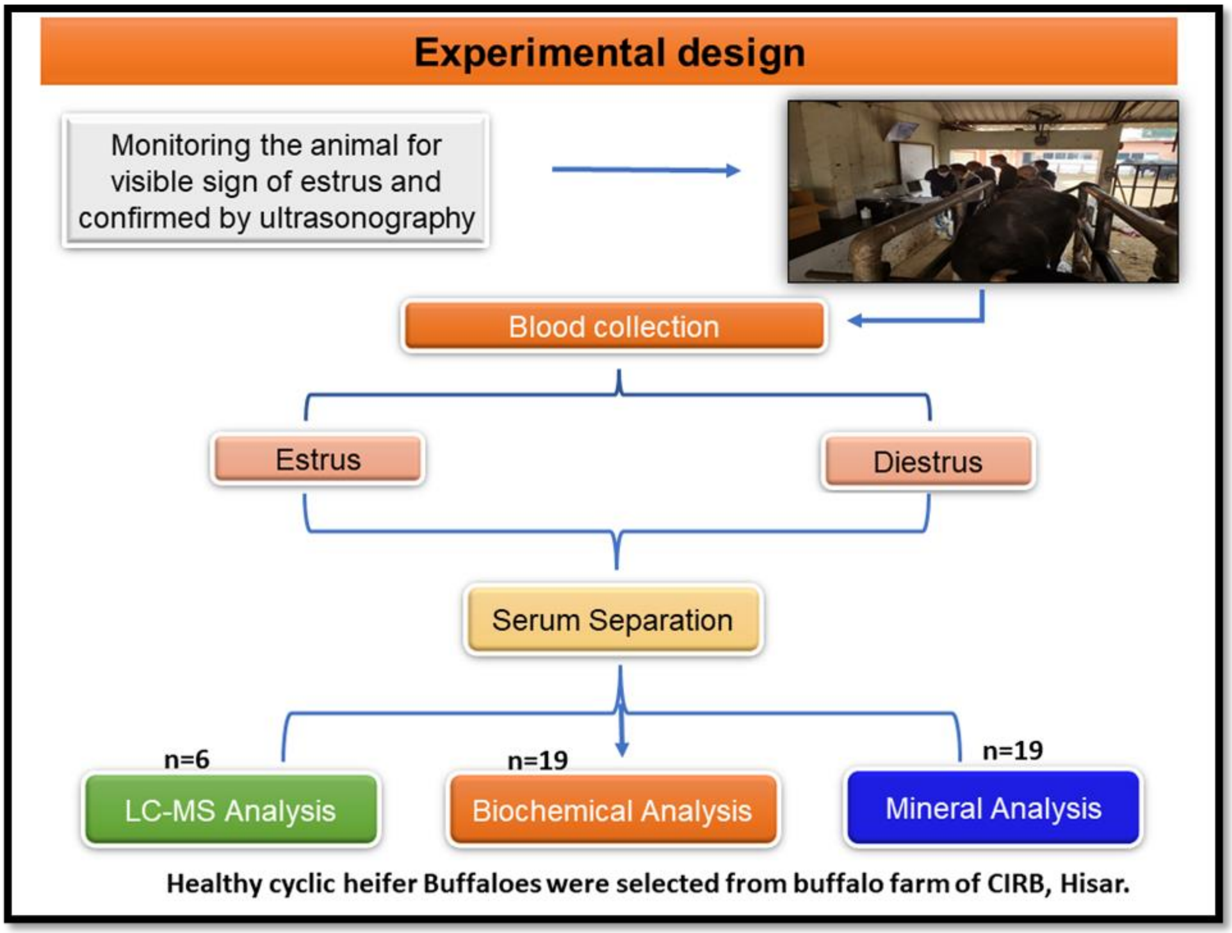
A typical metabolomics study consists of several different parts which can be grouped into four main steps Sample preparation, Data acquisition, Data processing, and Data interpretation (figure 3.2).

3.3.1. Sample preparation and metabolite extraction

The first step in the metabolomics workflow is the preparation of the biological sample (serum). Samples were taken out from -20° C and thawed slowly at 4°C and mix thoroughly. For metabolite extraction to each sample, pre-chilled methanol/chloroform was added to precipitate proteins. The mixture was subjected to a vortex for 3 min, then stood for 10 min to 60 min to promote the precipitation. Then the mixture was centrifuged at 16,000g for 15min at 4˚C, and the supernatant was collected and the supernatant was transferred to a new centrifuge tube and dried in a vacuum centrifugal concentrator. The dry residue was reconstituted in ultrapure water or methanol and used for metabolomics analysis. To validate the stability of sequence analysis, a quality control (QC) sample was prepared by pooling the same volume from each serum sample and then extracted and analyzed in the same way as the samples. The pooled QC sample and blank (pure acetonitrile) sample were injected after every 10 samples during the analytical run for evaluating the repeatability.

3.3.2. Data acquisition

Once the sample is ready, different techniques can be used to separate and characterize chemically diverse groups of metabolites present in the serum sample. In this study, LC-HRMS techniques were used. LC-HRMS analysis was performed on an



Healthy cyclic heifer Buffaloes were selected from buffalo farm of CIRB, Hisar.

Figure 3.1. Flow chart representing the experimental design.

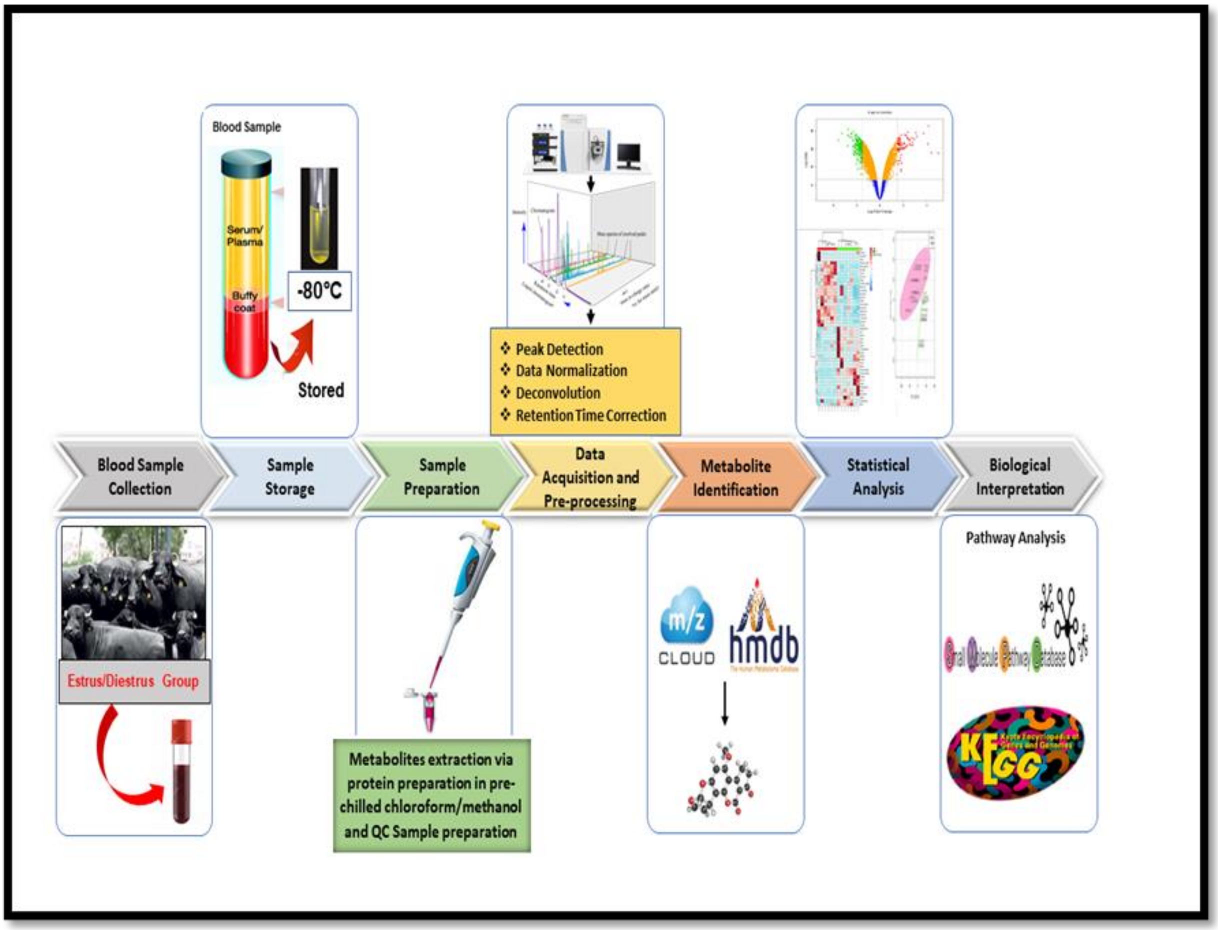


Figure 3.2. A schematic illustration of methods and procedure of untargeted metabolomics work plan for objective 1.

HPLC Ultimate 3000 system (Dionex), coupled with a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Inc.).

3.3.2.1. Chromatographic analysis condition

Chromatographic separation was performed on a Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm) and the column temperature was maintained at 50°C with binary gradient elution at a flow rate of 0.3mL/min, the injection volume was 2µl, mixing mobile phase A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). The linear elution gradient program was used as follows:

Gradient:

Sl. No.	Time	Flow (ml/min)	B%	Curve
1	0:00	0.500	7	5
2	16:00	0.500	95	5
3	20:00	0.500	95	5
4	25:00	0.500	7	5

3.3.2.2. Mass spectrometry condition

Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source. And was operated in positive or negative polarity mode to detect as many compounds as possible, both positive and negative ion modes were performed for each sample with a heated electrospray interface with spray voltage 1.9kV and capillary temperature of 300°C. Each sample was injected twice, with one injection in positive ion mode and another one in negative ion mode. The instrument operated at a 70,000 resolution with a full-scan acquisition ranging from 70 to 1,500m/z with automatic gain control (AGC) target of 3×10^{-6} charges and a maximum injection time (IT) of 100ms.

3.3.3. Data pre-processing and multivariate statistical analysis

Generally, this pre-processing involves noise reduction, retention time correction, peak detection, and integration, and chromatogram alignment. Compound Discoverer 3.1 (CD 3.1) software (Thermo Fisher Scientific) was applied to process and analyze the mass spectrum data, for instance, selection of spectra, peak picking and retention times alignment, molecular formula assignment, and candidate comparison with the Mz-Cloud database. The p values were also obtained by CD 3.1 software with Student's t-test. The resulting data matrix containing variables, sample code, and peak area were exported as CSV files and imported into compound discover to conduct multivariate statistical analysis for PCA, PLS-DA, volcano plot box plot, and heat map. The student's t-test was used to assess the significant difference between two groups.

3.3.4. Data interpretation

In this last step, the selected metabolites are linked to the biological context under study. And to further characterize the metabolic changes and the metabolic pathways involved, the differentiated metabolites were first annotated with the Kyoto Encyclopedia of Genes and Genomes and Human Metabolome Database. Data were then processed and analyzed using MetaboAnalyst 5.0 by R software. Two modules of MetaboAnalyst were used, namely pathway analysis and enrichment analysis, which are based on the KEGG database and Small Molecule Pathway Database, respectively.

3.3.5. Serum biochemical and minerals profile

The study was conducted on 19 heifers' buffaloes for the study of blood biochemical profiles in 19 buffaloes were subjected to blood collection during the estrus and diestrus phase of the estrous cycle. Serum was separated and stored at -20°C and analyzed for biochemical parameters using a biochemical auto-analyzer to determine the concentration of different biochemical parameters (figure 3.3) For mineral analysis Wet digestion of samples was done using the double acid mixture. The serum minerals were determined using atomic absorption spectrophotometer (figure 3.4).

3.3.6. Statistical Analysis

The data were collected in the form of a report sheet in biochemical and mineral analysis and presented for statistical analysis in Graph Prism software. The data were represented as mean± standard deviation; student paired t-test was used to assess the significance of the difference between groups and p-value less than 0.05 ($p<0.05$) was considered as statically significant.

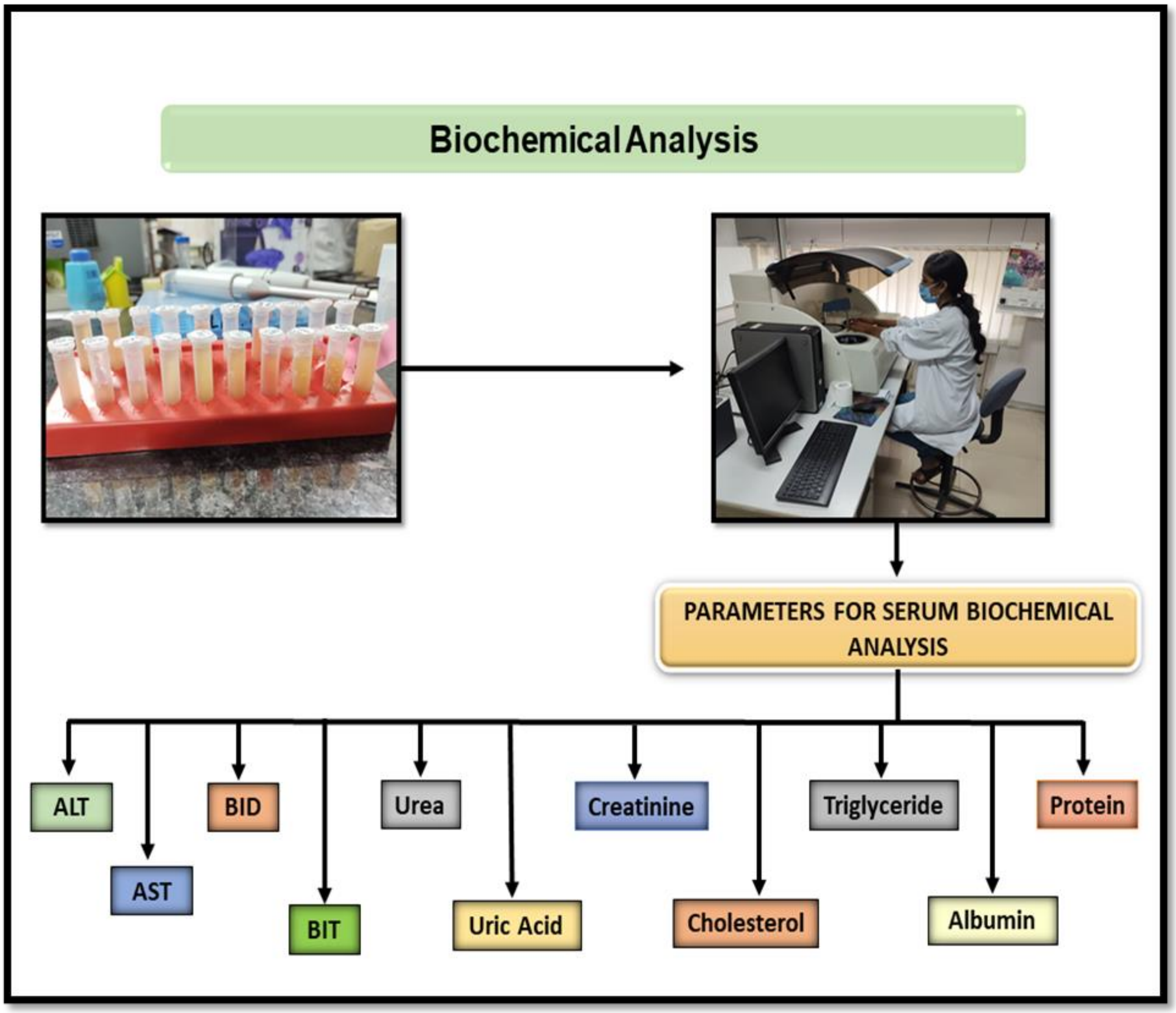


Figure 3.3. A brief outline of the work plan for activity 1 in objective 2.

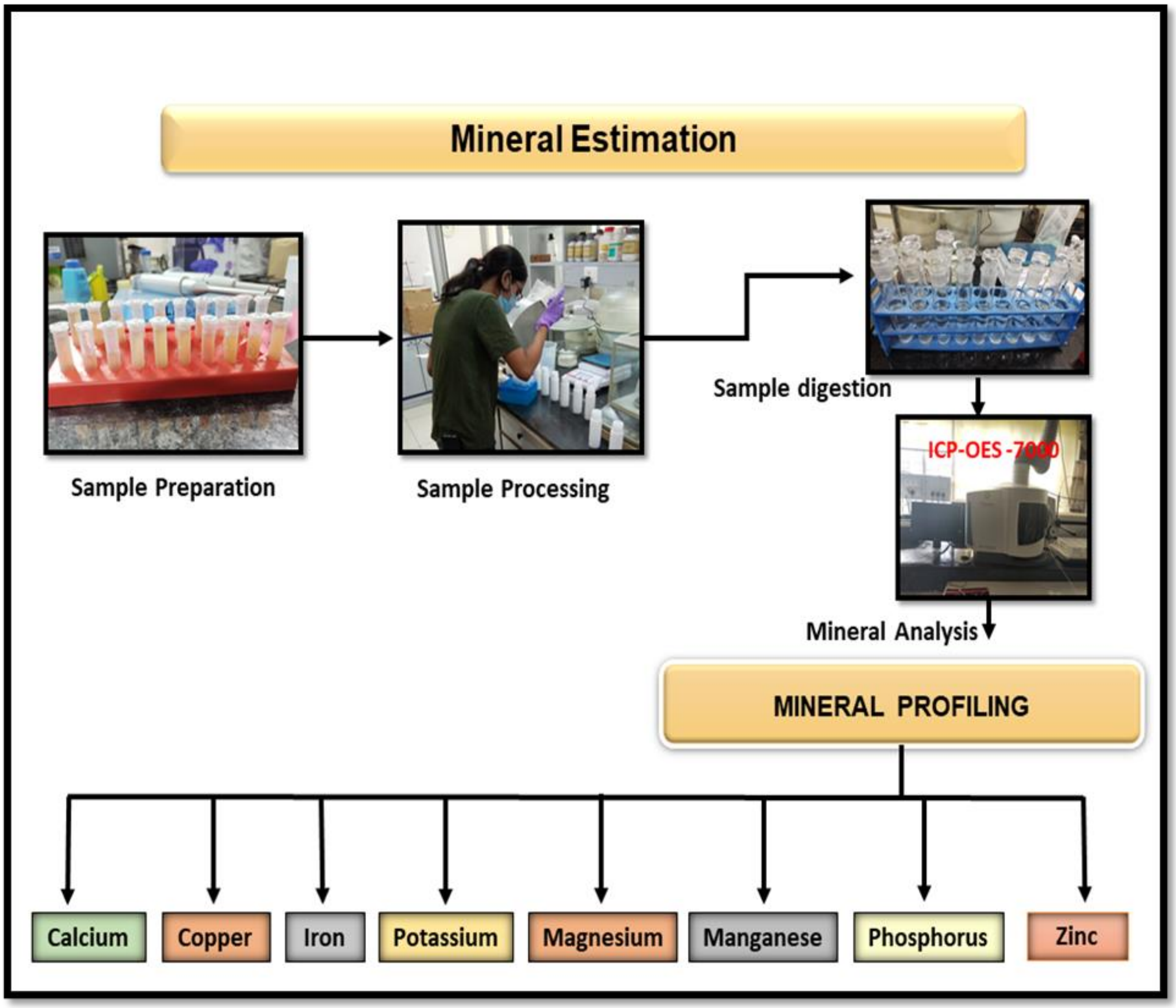


Figure 3.4. A brief outline of the work plan for activity 2 in objective 2.

CHAPTER-4

Results and Discussion

4.1. Serum metabolome profile

The current study was focused on determining the comparative metabolomics profile of serum and identifying the differential expression of metabolites during the estrus and diestrus phases of the buffalo estrous cycle. LC-MS orbitrap was used for high throughput profiling for non-targeted serum metabolites. Upon LC-MS analysis, for both positive and negative ion mode output is given as spreadsheet of mass-to-charge ratio peaks for each sample and with m/z values providing molecular formula by comparison to metabolite database, the identified metabolites were analyzed by compound discoverer software, the data was presented based on the individual metabolites found according to their m/z values, retention times, numbers of detected peaks, molecular names and their abundances in each of serum samples. Based on the MS acquisition, the data were divided into positive and negative ionization modes. A total of 789 metabolites were detected in the serum samples out of which 243 were detected in negative ion mode and 546 metabolites in positive ion mode. For negative ion detection, 52 metabolites were identified after matching on the MS database and for positive ion mode 143 were identified with their chemical name and chemical formula (figure 4.1) and 6 metabolites were common in both negative and positive ion mode (table 4.1). The total ion chromatogram depicts the abundance of ions produced during analysis by adding the intensities of all mass spectral peaks, with each peak representing a unique mass to charge ratio and retention time. This mass is then compared to a database to identify the metabolite. This representative spectrum was obtained using total ion chromatograms (TIC) in positive or negative ion mode, and it includes retention times (RT), peak intensity, and resolution. The chromatogram peak shows good reproducibility, indicating good repeatability and stability. As shown in (Figure 4.2) adjacent peaks were well separated from each other. This indicated that chromatographic and mass spectrometric conditions were appropriate for sample identification.

4.2. Statistical analysis

To uncover significant differences between metabolites and biological states in metabolomics study univariate and multivariate statistical analyses are used, visualization tools in statistical analysis of metabolomics data are an integral part of this process. Before analysis data were subjected to log-transformed and normalized using autoscaling and median centering (figure 4.3). Normalization is a pre-processing step in omics data analysis to compare different samples to make valid interference and to remove technical artifacts.

4.2.1. Univariate method

Univariate is the most common approach which analyzes each variable separately and includes fold change, t-test, box whisker plot, and volcano plot. Univariate analyses are frequently used to obtain an overview of important features before performing multivariate analyses.

4.2.1.1. Fold change analysis

The goal of fold change (FC) analysis is to compare the absolute value of change between two group means. The significant features are those features whose FCs are beyond the given FC threshold (either up or down). Fold change was calculated by diestrus concentration/estrus concentration, and threshold Fold change for this study was taken 1.

4.2.1.2. T-test

To screen out differential metabolites from the LC-MS result the p-value was chosen at $p \leq 0.05$ for statistical significance, with help of the fold change threshold and Student's t-test, significant difference metabolites between the two groups were further narrow down. For both positive and negative modes, the metabolites were sorted to identify significant metabolites between two groups a total of 71 metabolites were screen down in negative and positive ion mode of LC-MS acquisition, which was significantly different metabolites across estrus and diestrus groups 22 were in negative mode and 49 in positive mode (table 4.2 and 4.3).

Table 4.1. Representing the metabolites number in positive and negative polarity and the common number of metabolites in both modes.

	Negative ion mode	Positive ion mode	Total numbers of metabolites
Numbers of metabolites	243	546	789
Numbers of identified metabolites	52	143	195
Numbers of unidentified metabolites	191	403	594
Numbers of common metabolites	-	-	6

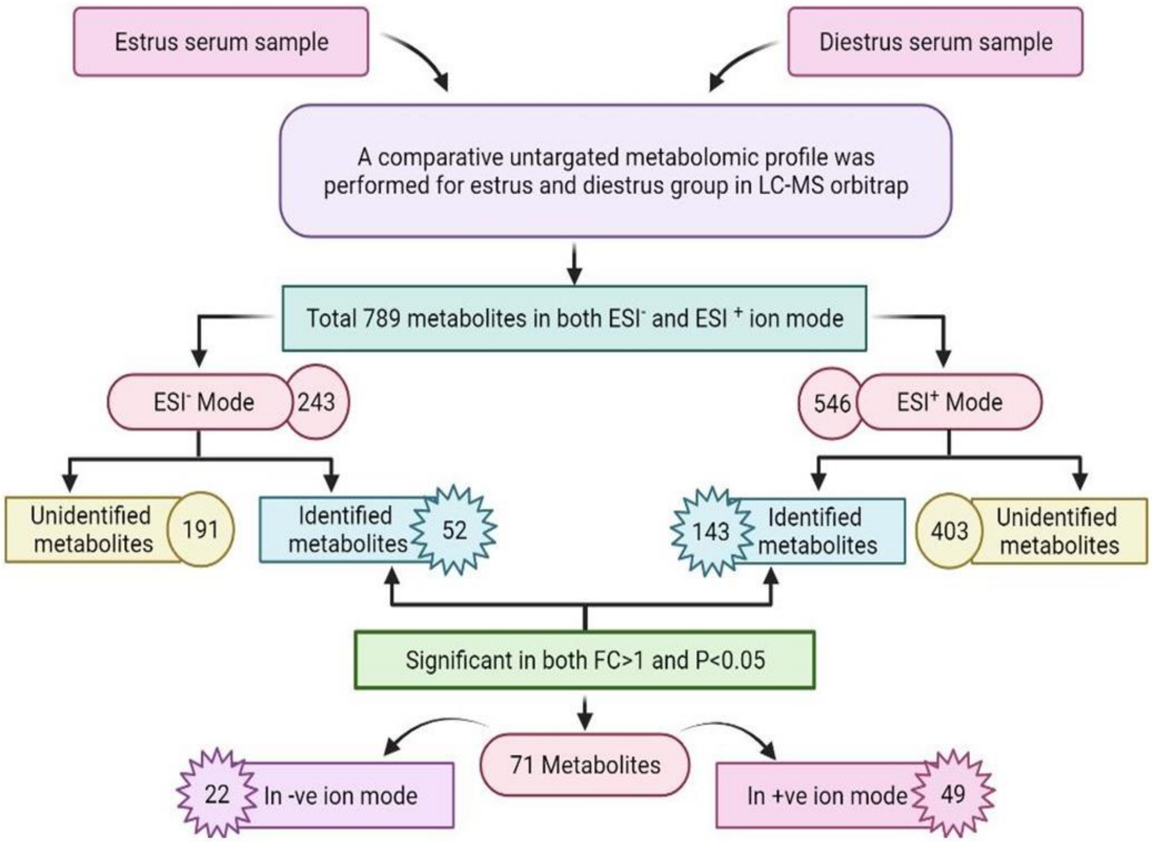


Figure 4.1. Flow chart depicting the strategy used to identify significant metabolites of estrus and diestrus.

Table 4.2. The list of significantly different metabolites between Estrus and Diestrus in negative ion mode.

Sl. No.	Name of Metabolite	Fold Change	P-Value
1.	Arachidonic acid	-1.72	0.000721558
2.	b-D-Glucopyranosiduronic acid, (3a,5b)-24-[(carboxymethyl)amino]-24-oxocholan-3-yl	-3.95	0.004740027
3.	Cholic acid	-1.45	0.001262191
4.	Glycocholic acid	-2.2	0.00171355
5.	Glycodeoxycholic acid	-2.92	0.004371303
6.	Glycoursodeoxycholic acid	-5.78	2.44905E-05
7.	L-alpha-lysophosphatidylcholine	-1.37	0.002281206
8.	Methyl N, O-dioctanoyltyrosinate	-4.61	0.000700953
9.	N'-[3-{[4-(Dimethylamino) phenyl] amino}-2-({[4-(dimethylamino) phenyl] amino} methyl)-2-nitropropyl]-N, N-dimethyl-1,4-benzenediamine	-1.56	0.001747653
10.	NP-016385	-2.78	0.003918501
11.	Prostaglandin E2	-1.91	0.005716731
12.	Taurochenodeoxycholic acid	-6.38	0.001536247
13.	Triphenyl[bis(2,4,6-trichlorophenoxy)] stiborane	1.15	0.001066205
14.	(1aS,2S,2aS,5R,5aS,6S,7aR)-2-Acetoxy-5-hydroxy-5-isopropyl-2a,7a-dimethyldecahydroazuleno[5,6-b] oxiren-6-yl (2E)-2-methyl-2-butenoate	-1.42	0.000986722
15.	(2R)-3-{[(2-Aminoethoxy) (hydroxy) phosphoryl] oxy}-2-hydroxypropyl (11Z)-11-icosenoate	-1.6	0.001211924
16.	11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid	-2.2	0.000642506
17.	17-AAG	-6.81	0.002285501
18.	1-icosanoyl-sn-glycero-3-phosphoethanolamine	-1.62	0.004530178
19.	1-stearoyl-sn-glycero-3-phosphoethanolamine	-1.84	0.001202313
20.	2-[(11Z,14Z)-icosadienoyl]-sn-glycero-3-	-2.09	0.011737311

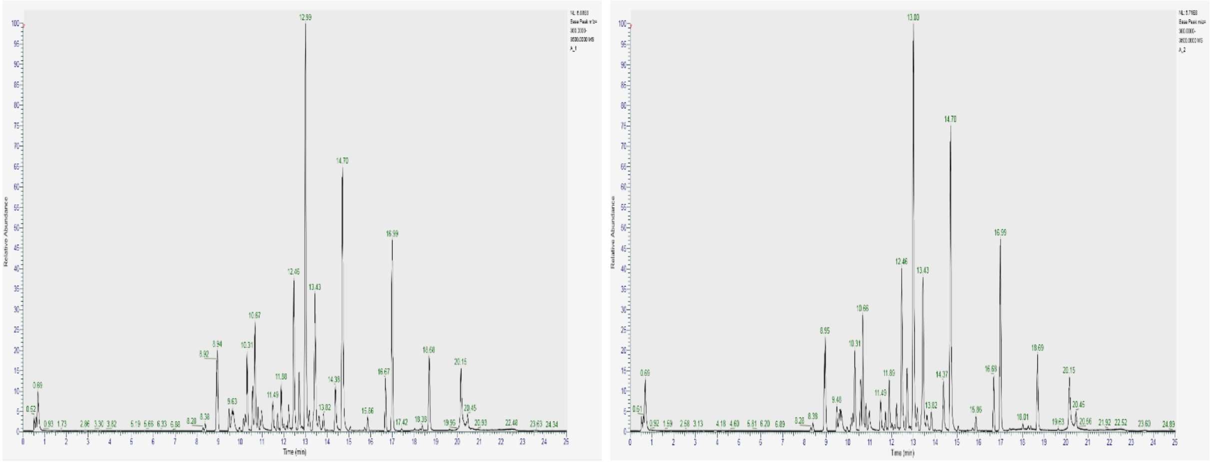
	phosphoethanolamine		
21.	6:2 Fluorinated telomer sulfonate	-1.73	0.000882567
22.	7-ketodeoxycholic acid	-1.48	0.002149206

Table 4.3. The list of significantly different metabolites between Estrus and Diestrus in positive ion mode.

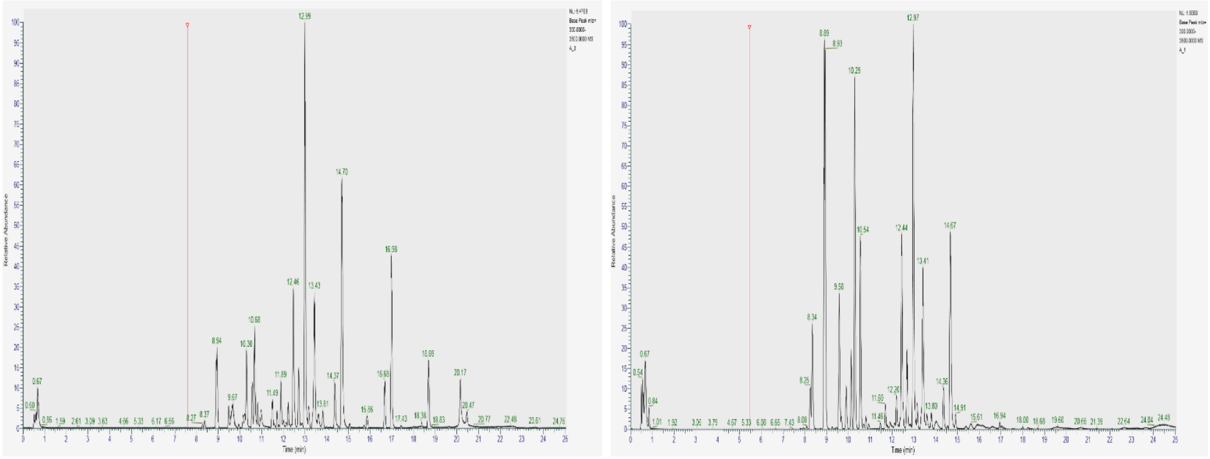
Sl. No.	Name of Metabolite	Fold Change	P-Value
1.	Benzyl S-benzyl-N-[6-(benzyloxy)-5-{[(benzyloxy) carbonyl] amino}-6-oxohexanoyl] cysteinylvalinate	-2.15	0.00877954
2.	Bis(4-ethylbenzylidene) sorbitol	-1.33	0.000348526
3.	Ethyl 3-(abieta-8,11,13-trien-18-ylamino)-2-methyl-3-oxopropanoate	-5.42	2.15238E-05
4.	Glycodeoxycholic acid	-2.81	0.001942421
5.	Heptyl N-[(allyloxy)carbonyl]-N-methylglycyl-N-methyl glycinate	-1.33	0.003189647
6.	Lacto-N-fucopentaose-2	-2.17	0.018223495
7.	LysoPC(18:3(9Z,12Z,15Z))	-1.39	0.001985991
8.	LysoPC(20:5(5Z,8Z,11Z,14Z,17Z))	-1.44	0.000170539
9.	LysoPC(22:5(7Z,10Z,13Z,16Z,19Z))	-1.63	0.000777162
10.	Methyl N, O-dioctanoyltyrosinate	-4.52	0.000587519
11.	N-(3,12-Dihydroxy-24-oxocholan-24-yl) glycine	-5.14	0.00015295
12.	N-[(3alpha,7alpha,12alpha,20R)-3,7,12-Trihydroxy-24-oxocholan-24-yl] glycine	-2.64	6.53774E-05
13.	N-docosanoylsphinganine	-2.25	0.006022954
14.	O-[Hydroxy(octadecyloxy) phosphoryl]-L-serine	-1.46	0.000563993
15.	stearoylcarnitine	-1.34	0.001756427
16.	Taurochenodeoxycholic Acid (sodium salt)	-2.5	0.00032562
17.	Taurodeoxycholic Acid	-2.51	0.000306715
18.	Agigenin	-1.08	0.00151531
19.	L-alpha-lysophosphatidylcholine	-1.12	0.001112547

20.	LysoPC(22:4(7Z,10Z,13Z,16Z))	-1.18	0.000210473
21.	LysoPC(P-18:0)	-1.19	0.000348526
22.	N-[(2S,3R,4E)-1-{[4-O-(beta-D-Galactopyranosyl)-beta-D-glucopyranosyl] oxy}-3-hydroxy-4-octadecen-2-yl] dodecanamide	1.55	0.315680747
23.	1-[(9Z)-hexadecenoyl]-sn-glycero-3-phosphocholine	-1.46	0.000868335
24.	1-arachidonoyl-sn-glycero-3-phosphocholine	-1.09	0.000539758
25.	1-heptadecanoyl-sn-glycero-3-phosphocholine	-1.27	0.028536168
26.	1-hexadecyl-2-acetyl-sn-glycero-3-phosphoethanolamine	-1.29	0.000170539
27.	1-Linoleoyl-sn-glycero-3-phosphocholine	-1.09	0.000621611
28.	1-oleoyl-sn-glycero-3-phosphoethanolamine	-1.05	0.000978563
29.	1-O-OCTADECYL-SN-GLYCERO-3-PHOSPHOCHOLINE	-1.03	0.003941109
30.	1-Palmitoyl-2-hydroxy-sn-glycero-3-PE	-1.16	0.000707276
31.	1-stearoyl-sn-glycero-3-phosphoethanolamine	-1.2	0.001267187
32.	(2R)-1-{[(2-Aminoethoxy) (hydroxy)phosphoryl] oxy}-3-hydroxy-2-propanyl (11Z)-11-icosenoate	-1.1	0.001335065
33.	(2R)-1-{[(2-Aminoethoxy) (hydroxy) phosphoryl] oxy}-3-hydroxy-2-propanyl docosanoate	-1.07	0.003833623
34.	(2S,8S,11S,14S,17S,23S,26R)-8-Benzyl-14,23-di-sec-butyl-11-{[(2-methyl-3-buten-2-yl) oxy] methyl}-28-thia6,9,12,15,21,24,29 heptaazatetracyclo[24.2.1.0~2,6~.0~17,21~] nonacos-1(29)-ene-7,10,13,16,22,25-hexone	-1.18	0.052404569
35.	(6E)-6-(Methoxyimino) pregnane-3,20-diyl diacetate	-2.16	6.53774E-05
36.	1-(4-Biphenylyl)-1-dodecanone	-2.27	9.96477E-05
37.	1,2,2a,3,4,4a,5,6,6a,7,8,8a,9,10,10a,11,12,12a-Octadecahydrocoronene	-2.27	0.000210473
38.	1,2,3,7,8-Pentabromooxanthrene	-1.69	0.001004104

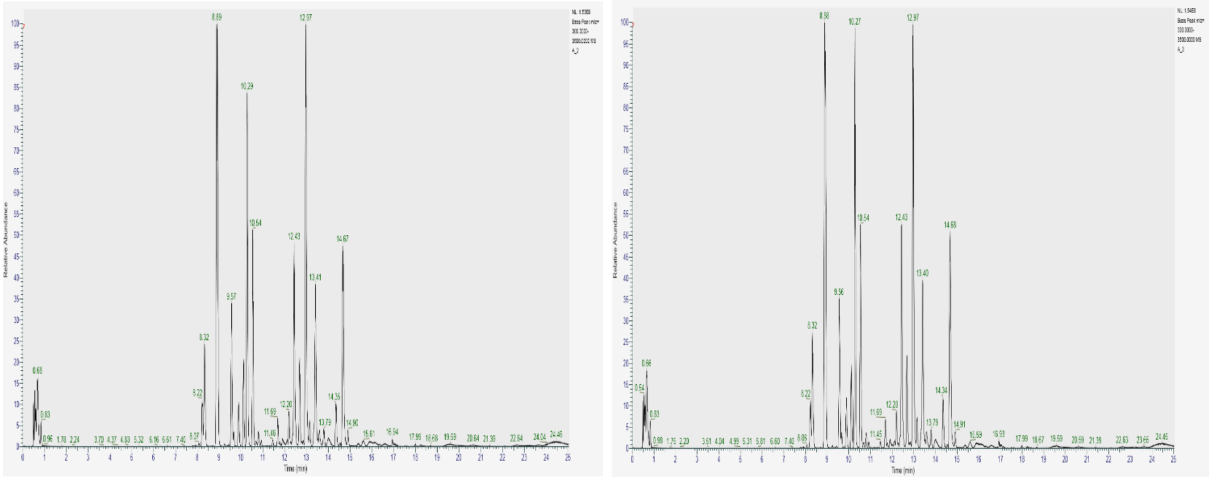
39.	1-[(1Z,9Z)-octadecadienyl]-sn-glycero-3-phosphocholine	-1.29	0.000571402
40.	1,2,3-Tris(2,4-dimethylphenyl) guanidine	-1.9	0.000833709
41.	1-[(8Z,11Z,14Z)-icosatrienoyl]-sn-glycro-3-phosphocholine	-1.3	0.001826817
42.	1-[(9Z)-hexadecenoyl]-sn-glycero-3-phosphocholine	-1.36	0.003448078
43.	11-Hydroxy-3,16-bis[(trimethylsilyl)oxy] androst-5-en-17-one	-2.53	0.00032562
44.	16(R)-HETE	-1.47	0.000348526
45.	1-oleoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine	-1.73	0.00823362
46.	2,6-Di(bicyclo[2.2.1]hept-2-yl)-4-(2-methyl-2-propanyl)phenol	-2.16	0.001079579
47.	2-[(2E,5E,7E,11E)-10-Hydroxy-3,7,9,11-tetramethyl-2,5,7,11-tetradecatetraen-1-yl]-5,6-dimethoxy-3-methyl-4-pyridinol	-2.17	2.24134E-05
48.	24-Amino-24-oxochol-5-en-3-yl acetate	-3.88	0.000426068
49.	3-{[(2-Aminoethoxy) (hydroxy) phosphoryl] oxy} propyl stearate	-1.71	0.001079579



Positive A1 **Positive A2**



Positive A3 **Negative A1**



Negative A2 **Negative A3**

Figure 4.2. Representation of total ion chromatogram of a serum sample from UPLC Orbitrap MS in ESI+ and ESI-

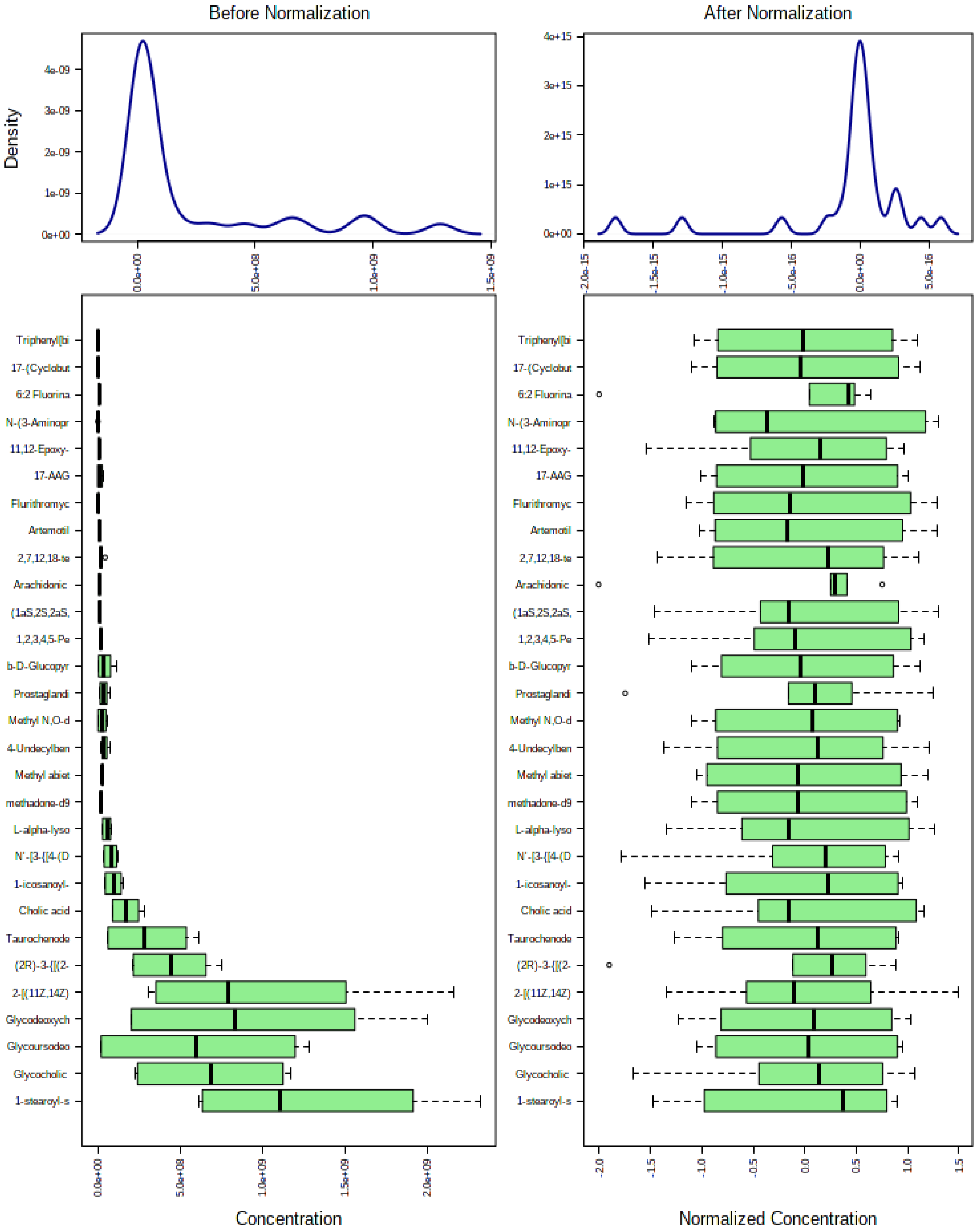


Figure 4.3. Data normalization by metabolites before using mass spectrometry spectra (Selected methods- Data transformation is log transformation and Data Scaling is auto-scaling).

4.2.1.3. Box and whisker plot

Differences between estrus and diestrus groups were visualized using a box plot, which is a method for depicting data by quartiles. It compares the estrus and diestrus groups based on their median intensity, which is the midpoint of the data set, with half of the values above and half of the values below. The median of the estrus group is higher than that of the diestrus group, revealing a clear difference between the two groups. It also revealed that there is no difference within groups because the median within the group lies in the same plane and maintains the uniformity of the two samples. And dotted point both sides of the box are outliers of the data (figure 4.4).

4.2.1.4. Volcano plot

A volcano plot is an easy way to visualize the metabolite that is statistically as well as biologically significant among estrus and diestrus groups and each point representing the metabolites that are very much statistically significant in terms of negative log of the p-value on the Y-axis and log of the fold change on X-axis. As shown in the figure green dots are downregulated significant metabolites and having less than lower fold change value (-1) whereas red dots are upregulated significant metabolites and having greater than upper fold change value (1) between the two groups. And grey color metabolites are those which represent no significant difference between the estrus and diestrus groups (figure 4.5).

4.2.2. Multivariate method

Untargeted metabolites obtained from estrus and diestrus samples were compared for multivariate analysis. It implies the observation and analysis of more than two statistical variables at the same time and can identify relationship patterns between two groups. PCA and PLS-DA are two multivariate analysis methods.

4.2.2.1. Principle component analysis

PCA is a statistical method for converting a set of correlated variables into a set of linearly uncorrelated variables known as principal components. It helps to visualize the large set of data into two-dimensional form PC1 and PC2 components. The PCA analysis was plotted as a 2D score plot between component 1 on X-axis and component 2 on Y-axis with 82.5% and 7.4% variation respectively in negative ion mode for the estrus and diestrus group and positive ion mode PC1 and PC2 variation is 78.0% and 11.4% respectively. PCA revealed a well-differentiated and cluster pattern in the score plot and also indicate a significant metabolome difference between the estrus and diestrus groups (figure 4.6).

4.2.2.2. Partial least squares-discriminant analysis (PLS-DA)

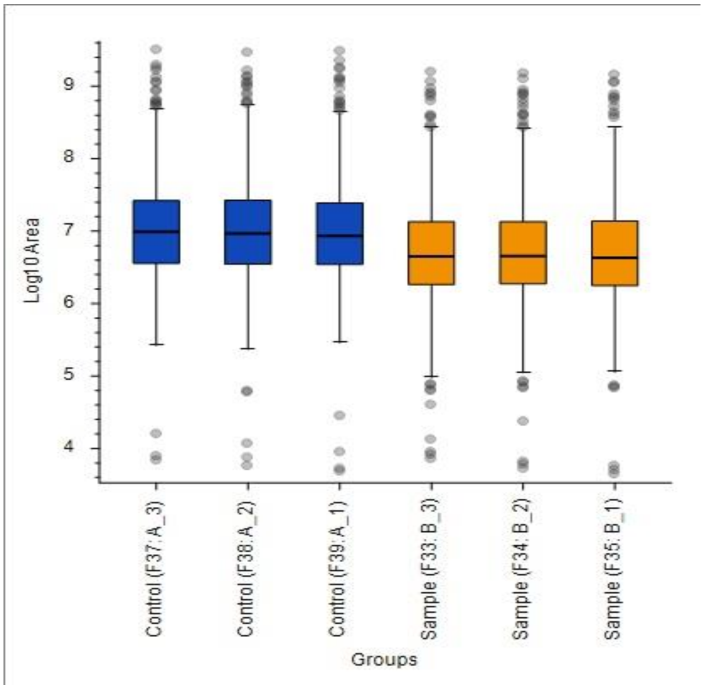
PCA is insufficient for determining the difference between the two groups. As a result, a multivariate analysis with partial least squares discriminates analysis (PLS-DA) was performed to identify a set of compounds that can be used to discriminate between groups, and in this study, distinct separation among two groups was found in both positive and negative ion modes and Score plot revealed more clear differences between the metabolites in the two kinds of the sample group and indicating a significant difference in metabolite components between estrus and diestrus animals (figure 4.7).

4.3 Hierarchical clustering

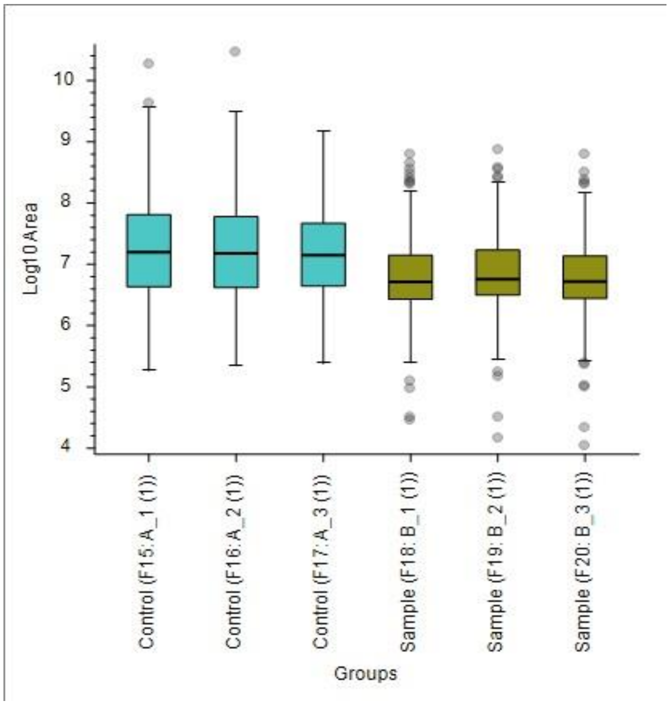
Cluster analysis is used to identify groups of metabolites that share similar properties or that are part of the identical biological pathway. Dendrograms and heatmaps are the two modules used in hierarchical clustering which show the correlation between metabolites and selected samples in a two-dimensional color-coded rectangles map (heat map), with each rectangle, reflects the relative amount of a specific metabolite in a specific sample. In hierarchical clustering analysis, the estrus and diestrus samples tended to cluster well with clear clades separation in the dendrogram. The heat map provides an overview of all metabolites and samples at a glance. All identified metabolites of the Estrus and diestrus group of animals show metabolic change based on the relative quantities of each metabolite concentration. Color profile in the heat map shows the intensity of a particular compound from dark red to dark green showing the intensity of gradient from higher to lower, and color code difference between estrus and diestrus revealed most of the metabolites differed significantly between the two

Control= Estrus

Sample= Diestrus



Positive ion mode

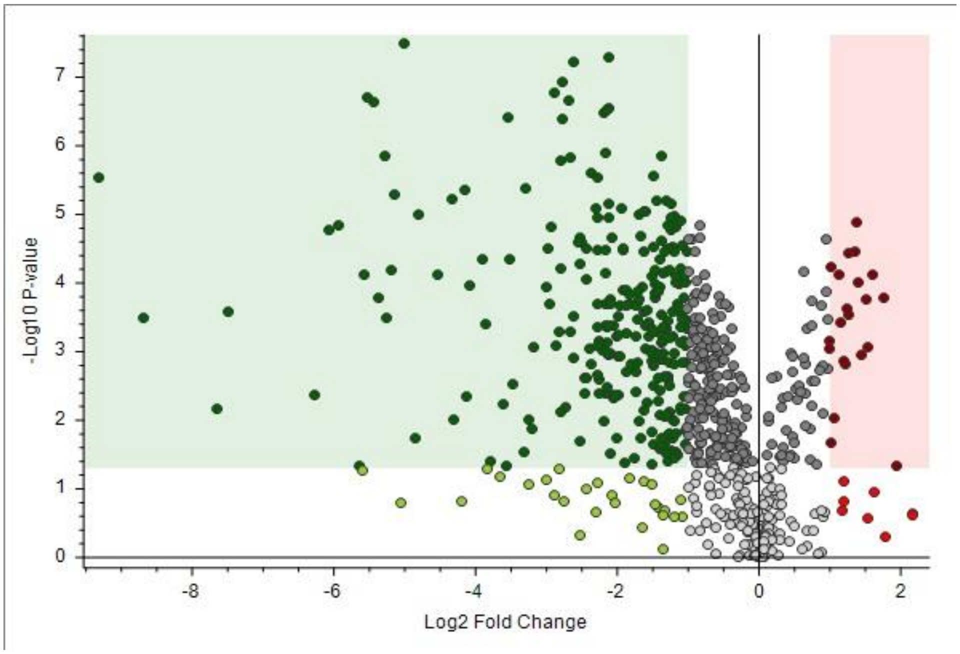


Negative ion mode

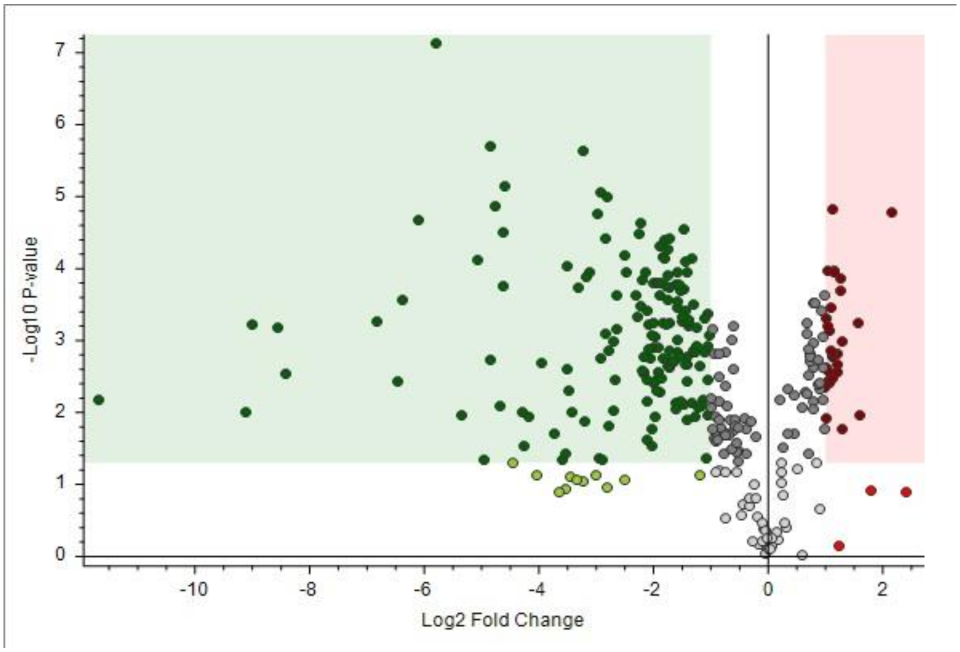
Figure 4.4. Box- whisker plot derived from estrus and diestrus groups of serum samples

🔴 Significant and greater than upper FC value

🟢 Significant and less than lower FC value

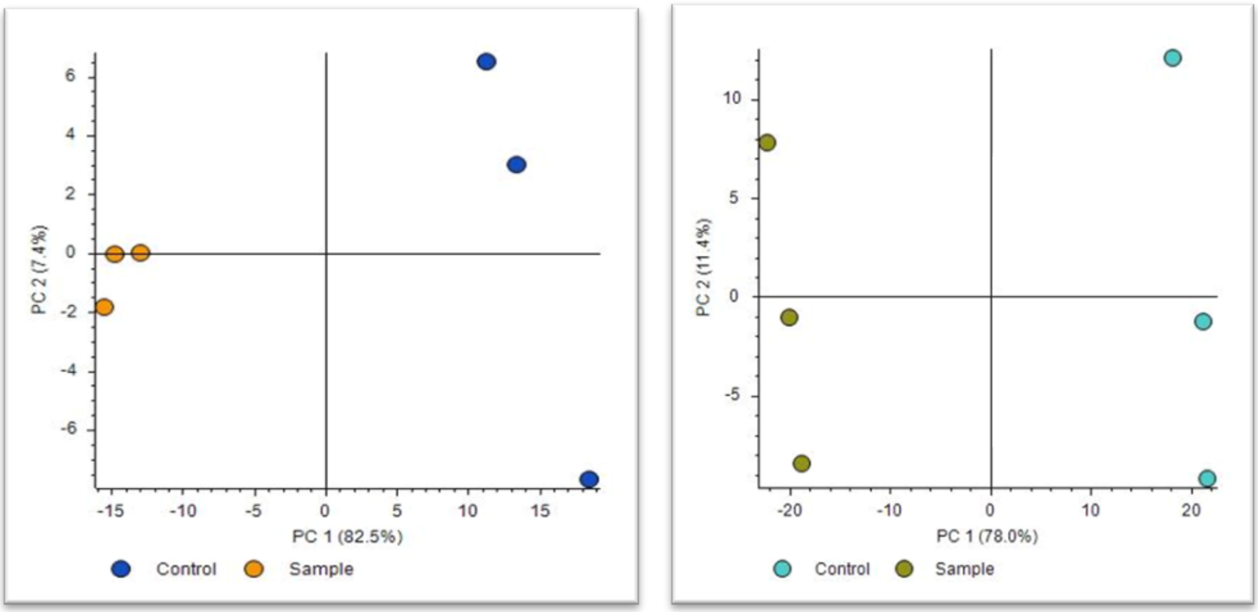


Positive ion mode



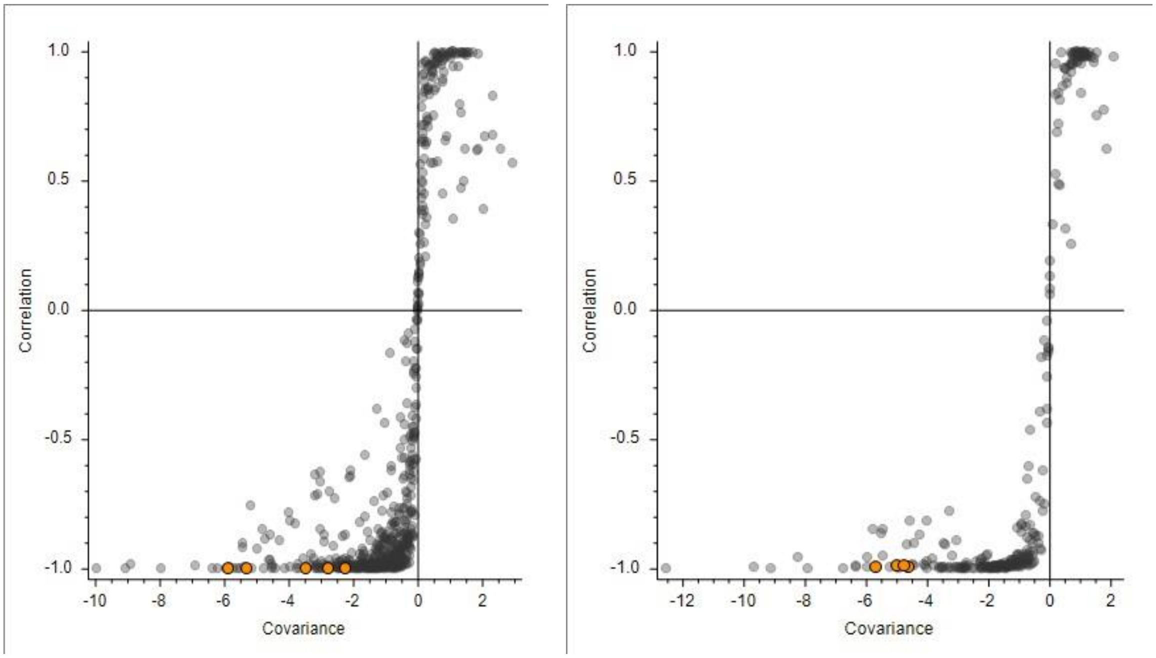
Negative ion mode

Figure 4.5. Volcano plot of differential expressed metabolite of estrus and diestrus with at least 1-fold change at 0.05 p-value. Each point in the volcanic plot represents a metabolite, The expression of red metabolite was upregulated, that of green metabolite was down-regulated, and the black metabolite was not significantly different.



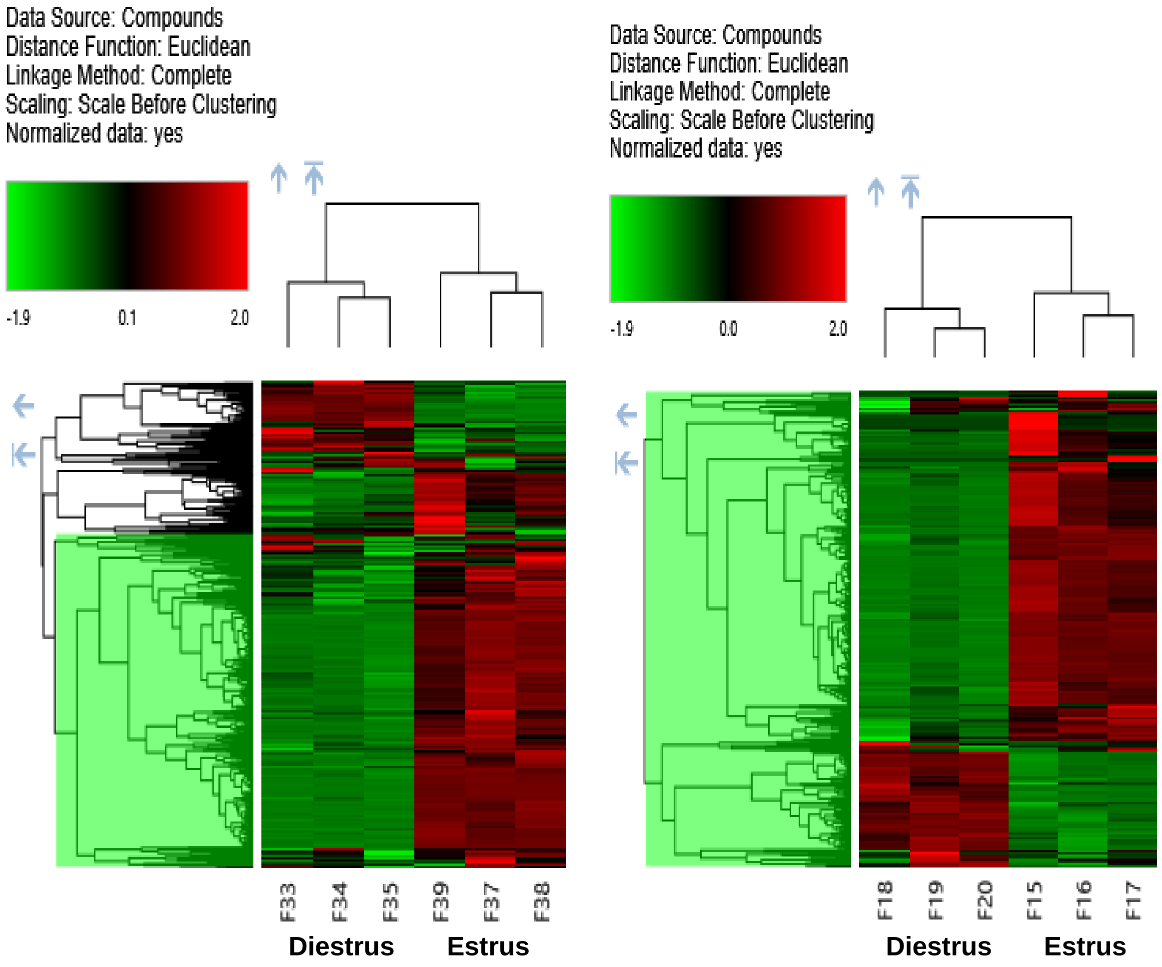
Positive ion mode **Negative ion mode**

Figure 4.6. Principal component analysis 2D score plot for estrus and diestrus serum samples analyzed in the positive ion and negative ion mode.



Positive ion mode **Negative ion mode**

Figure 4.7. Partial least square discrimination analysis (PLS-DA) score plot of estrus and diestrus serum samples in positive and negative ion mode.



Positive ion mode

Negative ion mode

Figure 4.8. Hierarchical clustering heat map of all identified serum metabolites for visualizing the metabolite variation across estrus and diestrus. With row representing the metabolite and column representing the samples.

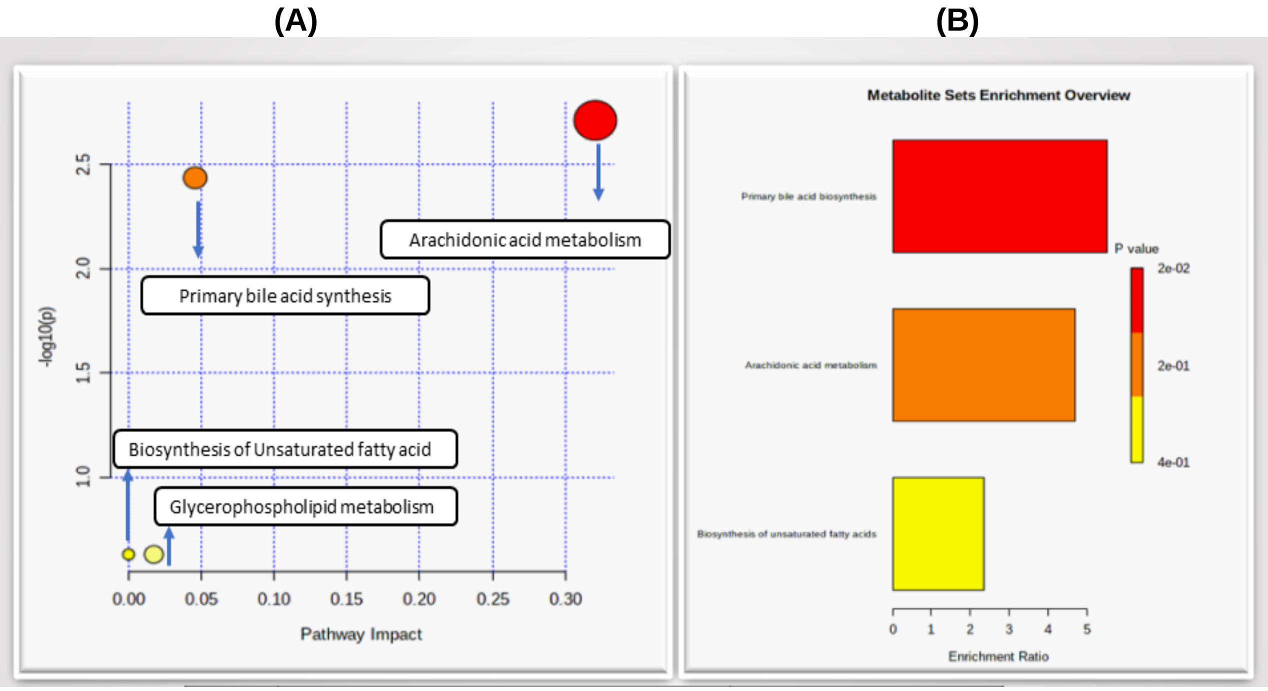
groups are highly expressed or upregulated in estrus while decreased or down regulated in diestrus (figure 4.8).

4.4. Metabolic pathway analysis

The output of the statistical analysis is typically a long list of significant metabolites, and the final step of untargeted metabolomics data analysis is linking these significantly expressed metabolites to the biological context, which is accomplished through enrichment analysis and pathway analysis. These are the two main modules of Metaboanalyst 5.0 for metabolic pathway analysis. To investigate the function of 71 differential metabolites, they are imported to metaboanalyst 5.0 and among 71 metabolites only 15 metabolites were matched with the KEGG pathway database and result showed that the differential metabolites were mainly involved in fatty acid metabolism pathways such as primary bile acid synthesis, arachidonic acid metabolism, biosynthesis of unsaturated fatty acid and glycerophospholipid metabolism. The p-value determines the colour of each circle. The darker colour indicates the pathway's most significant change metabolite, while the size of the circle reflects the pathway impact score and pathway analysis revealed that arachidonic acid metabolism is the darkest colour with the highest impact score indicating the most significant change metabolites, followed by primary bile acid synthesis, glycerophospholipid metabolism, and unsaturated fatty acid biosynthesis (figure 4.9). 15 significant metabolites are listed, along with the name of the relevant metabolic pathway (table 4.4).

4.5. Serum biochemical and minerals profile

A total of 19 healthy heifers were selected for serum biochemical and minerals analysis depicts the Mean ± SEM values of serum minerals and biochemical parameters at significant value ($p \leq 0.05$). In biochemical analysis, no significant difference was observed in most of the parameters namely ALT, Urea, Creatinine, Triglyceride, Cholesterol, Uric acid, albumin, and protein between two groups of animals but estrus and diestrus animals differ significantly in AST, BIT, and BID (figure 4.10) and the mean serum value for biochemical analysis in the present study was recorded 156.31±4.92 to 144.10±4.43 IU/L, 0.16±0.017 to 0.12±0.008 mg/dl, and 0.12±0.011 to 0.09±0.0041 mg/dl from estrus to diestrus respectively (table 4.5). In minerals analysis, the level of minerals including calcium, copper, iron, potassium, magnesium, manganese, phosphorus, and zinc was measured in serum samples then compared between estrus and diestrus groups. The present study did not show a significant level of difference in calcium, copper, manganese, phosphorus, and zinc. Both groups of animals differ significantly in iron and magnesium (figure 4.11) and the mean serum value for minerals analysis in the present study was recorded 10.12±0.968 to 7.88±0.365 ppm, and 3.24±0.069 to 2.96±0.061 mg/dl from estrus to diestrus respectively (table 4.6).



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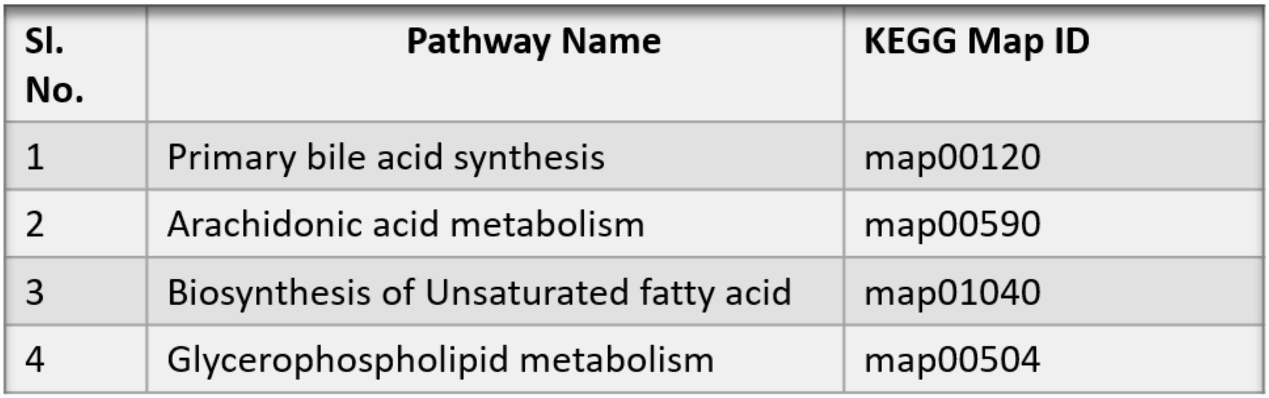


Sl. No.	Pathway Name	KEGG Map ID
1	Primary bile acid synthesis	map00120
2	Arachidonic acid metabolism	map00590
3	Biosynthesis of Unsaturated fatty acid	map01040
4	Glycerophospholipid metabolism	map00504

4.9. Metabolomics pathway analysis (A) plot show pathway and (B) enrichment analysis generated from metal analyst software by using the KEGG database. (C) Metabolic pathways of differential metabolites of estrus and diestrus with KEGG Map ID.

Table 4.4 Significantly different metabolites and their matched pathway in KEGG database

Metabolites	**Metabolic pathway name**
Cholic acid	Primary bile acid synthesis
Glycocholic acid	Primary bile acid synthesis
Glycodeoxycholic acid	Primary bile acid synthesis
Taurochenodeoxycholic acid	Primary bile acid synthesis
Taurodeoxycholic acid	Primary bile acid synthesis
Chenodeoxycholic acid	Primary bile acid synthesis
LysoPC (18:3 (9Z,12Z,15Z))	Glycerophospholipid metabolism
LysoPC (20:5 (8Z,11Z,14Z,17Z))	Glycerophospholipid metabolism
LysoPC (22:5 (7Z,10Z,13Z,16z,19Z))	Glycerophospholipid metabolism
LysoPC (22:4(7Z,10Z,13Z 16Z))	Glycerophospholipid metabolism
LysoPC (18:0)	Glycerophospholipid metabolism
Arachidonic acid	Arachidonic acid metabolism
16(R)HETE	Arachidonic acid metabolism
Prostaglandin E2	Arachidonic acid metabolism
Arachidonic acid	Biosynthesis of unsaturated fatty acid

Table 4.5. Biochemical Analysis of Serum sample of Estrus and Diestrus.

Values are expressed as Mean ± S.E.M. (N=19) and significantly different at $P<0.05$.

Parameters	Estrus	Diestrus
ALT (IU/L)	65.26±2.142	62.36±1.302
AST (IU/L)	156.31±4.929	144.10±4.432
BID (mg/dl)	0.12±0.011	0.09±0.004
BIT (mg/dl)	0.16±0.017	0.12±0.008
UREA (mg/dl)	25.35±1.089	26.84±1.145
CREATININE (mg/dl)	1.78±0.046	1.76±0.038
TRIGLYCERIDES (mg/dl)	12.94±1.135	13.63±0.765
CHOLESTEROL (mg/dl)	86.68±2.576	87.26±2.653
URIC ACID (mg/dl)	0.36±0.052	0.35±0.057
ALBUMIN (g/dl)	2.59±0.060	2.53±0.050
PROTEIN (g/dl)	6.93±0.151	6.90±0.102

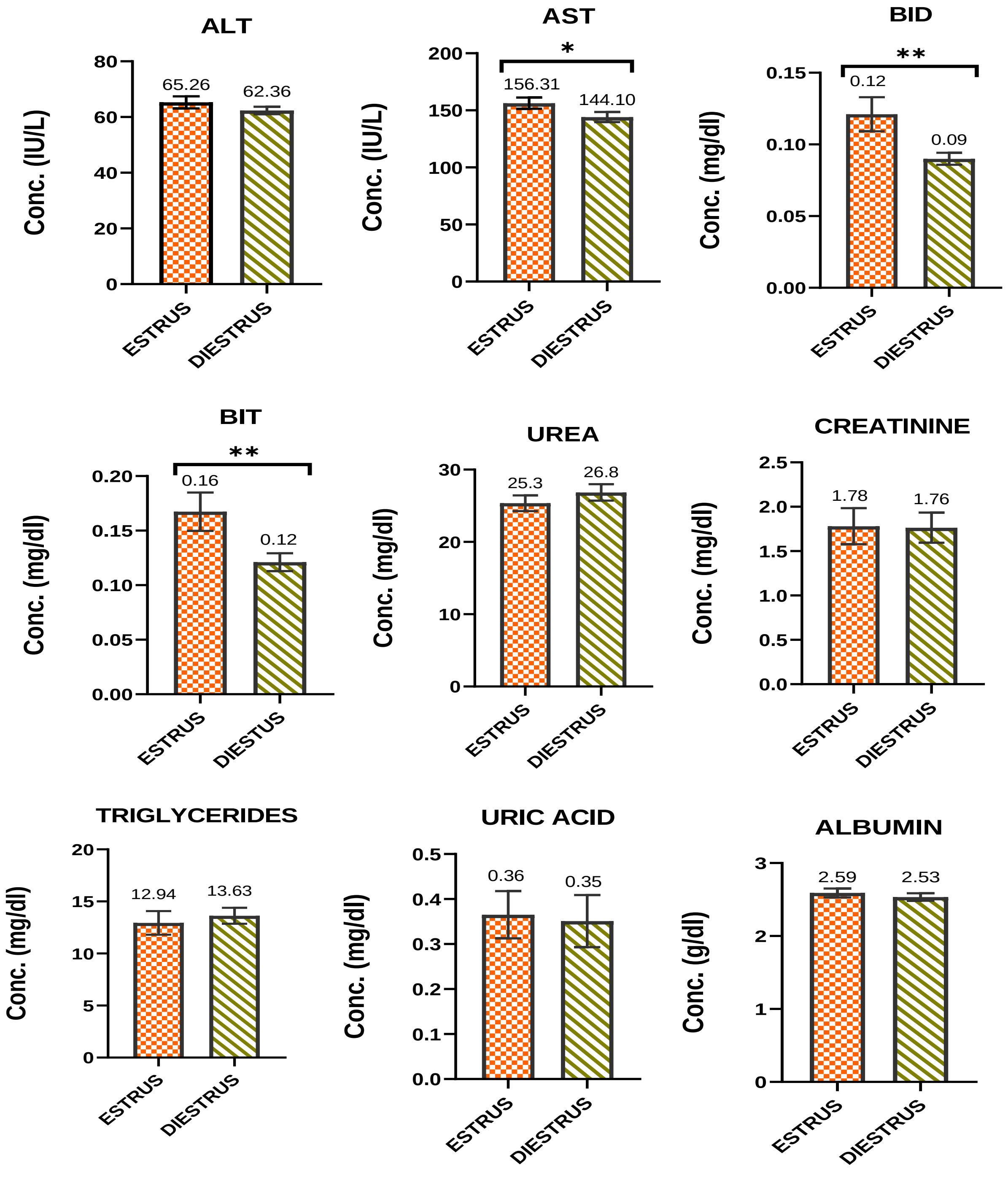
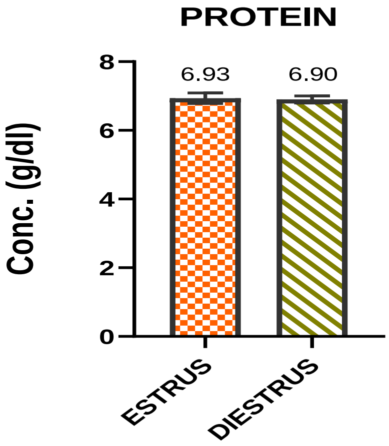


Figure 4.10. Biochemical Analysis of Serum sample of estrus and diestrus. Results are expressed as Mean ± S.E.M. (N=19) and bars with * and ** are significantly different at $P<0.05$

In Cont…



Biochemical Analysis of Serum sample of estrus and diestrus.
Results are expressed as Mean ± S.E.M. (N=19) and bars with * and ** are significantly different at $P<0.05$.

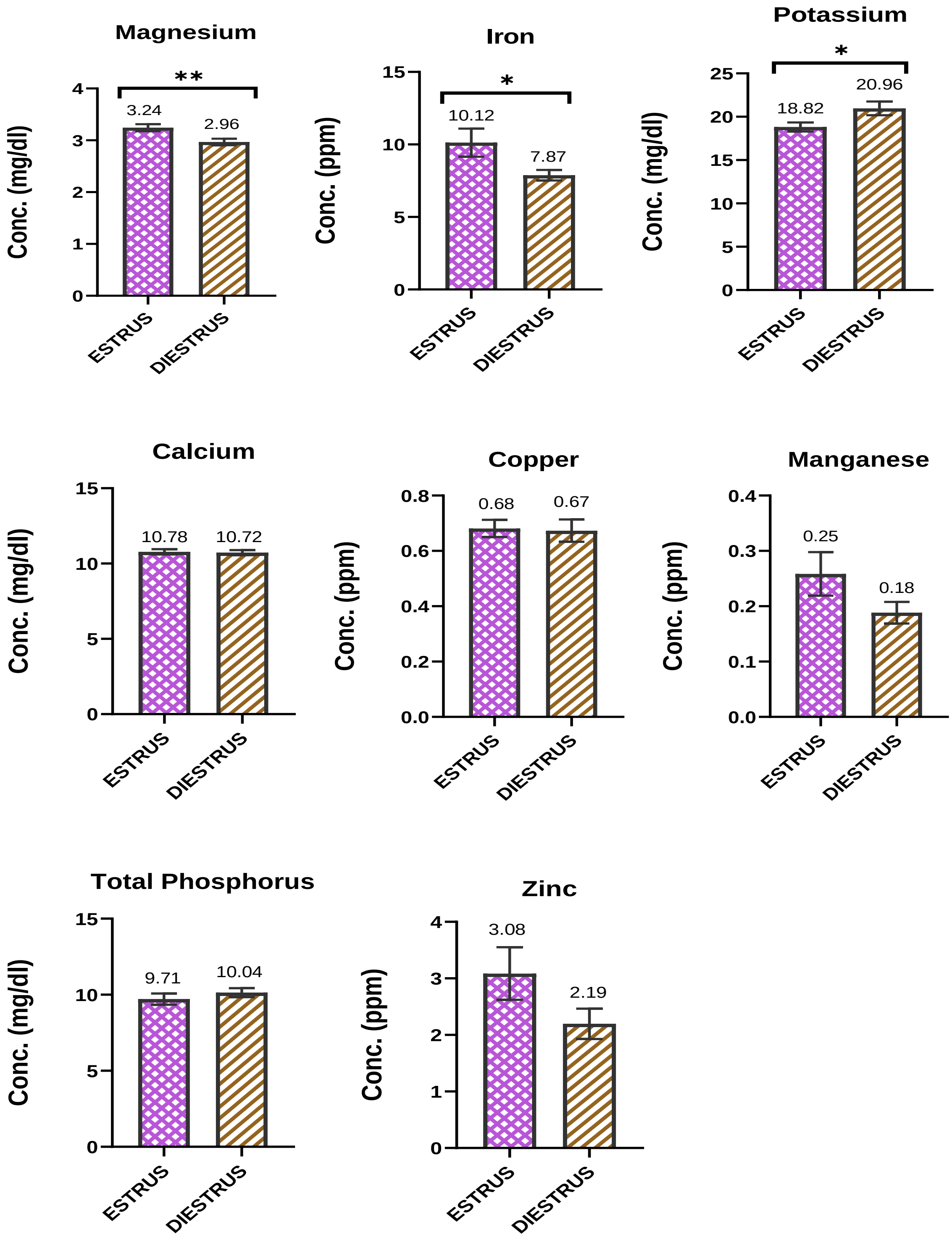


Figure 4.11. Mineral Estimation of Serum sample of estrus and diestrus
Values are expressed as Mean ± S.E.M. (N=19) and bars with * and ** are significantly different at $P < 0.05$.

Table 4.6. Minerals Profile of Serum sample of Estrus and Diestrus.

Values are expressed as Mean ± S.E.M. (N=19) and significantly different at $P<0.05$.

Parameters	**Estrus**	**Diestrus**
Calcium (mg/dl)	10.78±0.176	10.72±0.168
Copper (ppm)	0.68±0.031	0.673±0.040
Iron (ppm)	10.12±0.968	7.88±0.365
Potassium (mg/dl)	18.82±0.523	20.96±0.797
Magnesium (mg/dl)	3.24±0.069	2.96±0.061
Manganese (ppm)	0.25±0.039	0.18±0.0196
Total Phosphorus (mg/dl)	9.71±0.369	10.14±0.295
Zinc (ppm)	3.08±0.464	2.19±0.268

4.6. Discussion

Metabolomics has been used in many fields in recent years, and it has been discovered that the metabolome of buffalo serum may provide additional insight into the buffalo pathophysiological condition. In this study, serum samples from the estrus and diestrus groups were analyzed using an LC-MS-based untargeted metabolomics method to reveal the significant metabolite difference between the estrus and diestrus groups. It was discovered that the two groups have a significant serum metabolome difference. The study's major findings are summarized below.

A total of 15 key metabolites were identified in serum samples from two groups. and these potential metabolites were involved in a pathway of arachidonic acid metabolism, primary bile acid synthesis, glycerophospholipid metabolism, and biosynthesis of unsaturated fatty acid. It was found that the differential metabolites between the two groups were mainly enriched in fatty acids metabolism and were mostly upregulated in estrus compare to diestrus. Estrus is defined as the sexual receptivity phase of the estrous cycle, when the concentration of estrogen from the maturing follicle reaches a peak, produces behavioral signs and changes in the genital organs. Ruminant reproduction is closely linked to the availability of energy. Fats are an important source of energy and also serve as the precursor for steroid hormones and the growth of oocytes and follicles. In this study Arachidonic acid and PGE2 were the most important metabolites involved in the arachidonic acid metabolism pathway. Arachidonic acid, a member of the omega-6 polyunsaturated fatty acid (PUFA) family, is one of the most important essential fatty acids for oocyte biology, with optimal levels required for normal oocyte growth and fertility (Lapa *et al.,* 2011). Mostly polyunsaturated fatty acids primarily serve as an energy reservoir for oocyte maturation, the precursors of steroidogenesis, membrane biogenesis, and fertility in mammals (Marei *et al.,* 2009). AA improves oocyte maturation by modulating second messengers during oocyte maturation (Khajeh *et al.,* 2017) and by regulating the intracellular signal pathway (Zhang *et al.,* 2019). Furthermore, the arachidonic acid metabolism pathway produces its derivatives through various metabolic pathways (COX), resulting in the synthesis of different eicosanoids, which are precursors for key signalling molecules such as prostaglandins (PGs), which play a key role in many reproductive processes. Prostaglandin E2 is a pro-inflammatory eicosanoid that contributes to the inflammation reaction associated with ovulation. PGE2 is directly related to ovulation because it promotes the release of LHRH from the hypothalamus. Its levels rise in response to an increase in LH, enhancing the inflammatory response and activating collagenases and other proteolytic enzymes to degrade follicle connective tissue and lead to ovulation (Boots and Jungheim, 2015). The stimulus that induces arachidonic acid to be released or metabolized in the ovary during ovulation is unknown. However, PLA2 activity is increased during the preovulatory LH surge, hence the COX pathway of AA concluded that LH is the primary regulator of ovarian PLA2 activity. LysoPCs were found to be significantly expressed across estrus and diestrus in the current study. LysoPCs are byproducts of PC catabolism, which is controlled by the phospholipases A1, A2, and D. The expression of Lysophosphatidylcholine family molecules were upregulated in estrus, implying that glycerolphospholipid metabolism in the estrus group was enhanced. Lyso-pc, like PGE2, is an inflammatory product that can stimulate macrophage activity and has important physiological functions such as promoting fat metabolism and eliminating cholesterol from serum. Arachidonic acid is a key metabolite involved in the metabolism of unsaturated fatty acids. Arachidonic acid is an essential unsaturated fatty acid that aids reproduction by acting as energy production, a precursor for prostaglandin, and steroid hormone synthesis. The differences in the levels of all identified bile metabolites between the two groups were mainly from primary bile acid synthesis, which was downregulated in the diestrus group. cholesterol is a major precursor for bile acid synthesis in the liver and is released into the intestine to aid in the absorption of lipid molecules. Primary bile acids are produced in the liver and converted to secondary bile acids in the gut by intestinal flora. In the current study, however, no difference in cholesterol levels was observed between the two groups of animals. This could be because in diestrus animals' primary bile acid production is reduced due to liver abnormalities. There is little information available on the connection between bile acid and female reproduction, including bile acid found in cattle follicular fluid (Sanchez *et al.,* 2016). However, the precise role of bile acid in reproduction and its function is unknown.

In an analysis of serum concentration of biochemical parameters, no significant difference was observed in most of the parameters namely ALT, Urea, Creatinine, Triglyceride, Cholesterol, Uric acid, albumin, and protein between two groups of animals. Three parameters AST, BIT, and BID has been found significantly different among estrus and diestrus group. AST concentration increases during estrus example of an anabolic condition that has been reported elsewhere (Yaqub *et al.,* 2013). This enzyme has been linked to muscle activity because it catalyzes the formation of oxaloacetate during gluconeogenesis (Doornenbal *et al.,* 1988). Increased AST concentrations in heifers during estrus may be related to increased physical activity. This could be because ovarian steroids increase hepatocellular membrane permeability to this enzyme.

Furthermore, because AST is a muscle enzyme, high AST activity observed during the estrus phase in this study could be attributed to the heightened neuromuscular excitability associated with this stage of the estrous cycle. Increased bilirubin concentrations in estrus indicate a change in the liver physiological condition when compared to diestrus, and a significant difference was found between estrus and diestrus in bilirubin concentration indicate some liver problems. An adequate concentration of haemoglobin in the blood is required during estrus for proper oxygen and nutrient transport to the organs, as well as gonadal cell metabolic activities. Iron levels were found to be significantly higher in the estrus group. Although iron is not as important in reproduction as other minerals, it does play an important role as a cofactor in enzymatic reactions such as steroid hormone synthesis, oxygen binding, and energy metabolism during the estrous cycle. Serum iron deficiency has been linked to changes in gonadal activity, such as reduced follicular growth and fertility. (Ceylan *et al.,* 2008), and may result in improper oxygenation during estrus. Thus, in this study, the high concentration of iron in serum during estrus supports the physiological change during estrus. The magnesium and iron levels in the estrus group were found to be significantly higher. Though magnesium does not play a direct role in reproduction, it is involved in a variety of enzymatic reactions catalyzed by ATP-linked enzymes. Furthermore, magnesium influences calcium and phosphorus absorption (Sharma *et al.,* 2004). As a result, magnesium deficiency can have a secondary effect on reproduction. Stressful events, such as estrus, require a significant amount of magnesium ion as a cofactor in the energy-producing ATP reactions.

CHAPTER-5

Summary and Conclusion

SUMMARY AND CONCLUSION

Metabolomics profile between estrus and diestrus

- LC-MS orbitrap based approach was employed to carry out the metabolomics profile between estrus and diestrus heifers
- The LC-MS analysis revealed a total 789 metabolites in both negative and positive ion modes.
- Based on fold change and P-value metabolites were further narrow down and 71 metabolites were extracted which were significantly expressed during the comparative analysis of the estrus and diestrus group.
- All significantly different metabolites were imported to the metaboanalyst (version 5.0), the relevant metabolic pathway involves the most significant metabolites under this study were Primary bile acid synthesis, arachidonic acid metabolism, glycerophospholipid metabolism, and biosynthesis of unsaturated fatty acid. KEGG was used for enrichment analysis.
- Fifteen significant metabolites which were identified in relevant metabolic pathways after matching on the KEGG database in this study are Cholic acid, Glycocholic acid, Glycodeoxycholic acid, Taurochenodeoxycholic acid, Taurodeoxycholic acid, and chenodeoxycholic acid in primary bile acid synthesis. LysoPCs in glycerophospholipid metabolism and arachidonic acid, Prostaglandin E2 in arachidonic acid metabolism.
- The role of bile acid and its major functions in reproduction remains unknown. The most significant metabolite with greater (Taurochenodeoxycholic acid) fold chain can be further explored for its impact on reproductive function.
- Most of the significantly identified metabolites were upregulated (fold change >1) in the estrus phase as compared to the diestrus phase of the cycle.

Biochemical and minerals profile between Estrus and diestrus

- AST levels were significantly ($P \leq 0.05$) higher in estrus than diestrus.
- BID and BIT levels were significantly ($P \leq 0.05$) higher in the estrus group.

- Serum Iron and Magnesium concentrations were significantly ($P \leq 0.05$) higher in the estrus group
- Serum Potassium levels were lower in estrus than diestrus.

Conclusion

In this study, we looked at the differences in metabolomics profiles, biochemical profiles, and mineral profiles between estrus and diestrus groups of healthy heifers. The variation in fold change, statistically significant p-value, and expression level of metabolites in their respective groups reflect changes in the metabolome between the estrus and diestrus stages of the cycle. 15 significantly different metabolites were identified among the serum of estrus and diestrus by using LC-MS Orbitrap metabolomics with the different multivariate and univariate methods. These metabolites were found to be enriched primarily in the fatty acid metabolism pathway, which may explain the important role of lipid metabolites in steroidogenesis, oocyte maturation, follicular development, and the inflammatory effect on ovulation and energy production during estrus. The increase in AST activity explains the physical activity during the estrus phase. During estrus, metabolic transitions occur in combination with the preparation of the reproductive tract, which explains physiological changes with an increase in magnesium and iron concentrations required as cofactors in ATP-linked enzymatic reactions, energy metabolism, and oxygen binding for proper blood flow during estrus. To summarise the present study, the metabolic pathway involves significant metabolites and significant biochemicals and minerals parameters provide further understanding of the physiology of the estrus and can be used as a practical information for the development and identification of the potential biomarker.

Bibliography

Bibliography

Ahmad, I., Lodhi, L.A., Qureshi, Z.I. and Younis, M., 2004. Studies on blood glucose, total proteins, urea and cholesterol levels in cyclic, non-cyclic and endometritic crossbred cows. *Pakistan Veterinary Journal*, *24*(2), pp.92-94.

Ametaj, B.N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M.J., Dunn, S.M., Xia, J. and Wishart, D.S., 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics*, *6*(4), pp.583-594.

Amle, M., Patodkar, V., Shelar, R. and Birade, H., 2014. Serum biochemical levels of repeat breeder cross bred cows under rural condition of Satara District of Maharashtra. *Int. J. Adv. Vet. Sci. Technol*, *3*(1), pp.109-113.

Archunan, G., 2013. Gas chromatographic mass spectrometric analysis of estrus specific volatile compounds in buffalo vaginal mucus after initial sexual foreplay. *Journal of Buffalo Science*, *2*(1), pp.1-7.

Bai, Y., Zhang, F., Zhang, H., Xu, C., Wu, L. and Xia, C., 2020. Follicular Fluid Metabolite Changes in Dairy Cows with Inactive Ovary Identified Using Untargeted Metabolomics. *BioMed Research International*, *2020*.

Baran, R., Reindl, W. and Northen, T.R., 2009. Mass spectrometry-based metabolomics and enzymatic assays for functional genomics. *Current opinion in microbiology*, *12*(5), pp.547-552.

Beckonert, O., Keun, H.C., Ebbels, T.M., Bundy, J., Holmes, E., Lindon, J.C. and Nicholson, J.K., 2007. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature protocols*, *2*(11), p.2692.

Bender, K., Walsh, S., Evans, A.C.O., Fair, T. and Brennan, L., 2010. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. *Reproduction*, *139*(6), pp.1047-1055.

Boots, C.E. and Jungheim, E.S., 2015, July. Inflammation and human ovarian follicular dynamics. In *Seminars in reproductive medicine* (Vol. 33, No. 04, pp. 270-275). Thieme Medical Publishers.

Boyd, J.W., 1983. The mechanisms relating to increases in plasma enzymes and isoenzymes in diseases of animals. *Veterinary Clinical Pathology*, *12*(2), pp.9-24.

Cambiaghi, A., Ferrario, M. and Masseroli, M., 2017. Analysis of metabolomic data: tools, current strategies and future challenges for omics data integration. *Briefings in bioinformatics*, *18*(3), pp.498-510.

Capati, A., Ijare, O.B. and Bezabeh, T., 2017. Diagnostic applications of nuclear magnetic resonance–based urinary metabolomics. *Magnetic resonance insights*, *10*, p.1178623X17694346.

Ceylan, A., Serin, I., Aksit, H. and Seyrek, K.A.M.İ.L., 2008. Concentrations of some elements in dairy cows with reproductive disorders. *Bull Vet Inst Pulawy*, *52*, pp.109-112.

Choudhary, K.K., Bharadwaj, A., Sharma, R.K., Jerome, A., Khanna, S. and Kavya, K.M., 2018. Efficacy of different methods for detecting the onset of ovarian cyclicity in post-partum Murrah buffaloes. *Tropical animal health and production*, *50*(7), pp.1559-1564.

Collard, B.L., Boettcher, P.J., Dekkers, J.C., Petitclerc, D. and Schaeffer, L.R., 2000. Relationships between energy balance and health traits of dairy cattle in early lactation. *Journal of dairy science*, *83*(11), pp.2683-2690.

Creek, D.J., Jankevics, A., Burgess, K.E., Breitling, R. and Barrett, M.P., 2012. IDEOM: an Excel interface for analysis of LC–MS-based metabolomics data. *Bioinformatics*, *28*(7), pp.1048-1049.

Cuykx, M., Claes, L., Rodrigues, R.M., Vanhaecke, T. and Covaci, A., 2018. Metabolomics profiling of steatosis progression in HepaRG® cells using sodium valproate. *Toxicology letters*, *286*, pp.22-30.

Das, S., Bandopadhya, S.K., Basu, S., Ghosh, B.B. and Dattagupta, R., 2002. Blood mineral profile of normal cyclic and repeat breeder crossbred cows under rural condition. *Indian J. Anim. Reprod*, *23*(2), pp.167-169.

De Abreu, A.C.M.R., Busato, E.M., Bergstein-Galan, T.G., Bertol, M.A.F. and Weiss, R.R., 2017. Bovine Reproductive Physiology and *Reproduction Biotechnology in Farm Animals*, p.37.

Deepinder, F., Chowdary, H.T. and Agarwal, A., 2007. Role of metabolomic analysis of biomarkers in the management of male infertility. *Expert Review of Molecular Diagnostics*, *7*(4), pp.351-358.

Dervishi, E., Zhang, G., Mandal, R., Wishart, D.S. and Ametaj, B.N., 2018. Targeted metabolomics: new insights into pathobiology of retained placenta in dairy cows and potential risk biomarkers. *animal*, *12*(5), pp.1050-1059.

Desai, M.C., Thakkar, T.P., Dharshana, R.A. and Janakiraman, K., 1982. Note on serum copper and iron in Surti buffalo in relation to reproduction and gonadotropins. *Indian journal of animal sciences*.

Dhoble, R.L. and Gupta, S.K., 1986. Serum calcium and inorganic phosphorous during postpartum anoestrous in buffaloes. *Indian J. Anim. Hlth*, *25*, pp.123-126.

Doisy, E.A., 1973. Micronutrient controls on biosynthesis of clotting proteins and cholesterol. *Trace substances in environmental health*, *6*, p.193.

Doornenbal, H., Tong, A.K. and Murray, N.L., 1988. Reference values of blood parameters in beef cattle of different ages and stages of lactation. *Canadian Journal of Veterinary Research*, *52*(1), p.99.

Dunn, W.B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., Brown, M., Knowles, J.D., Halsall, A., Haselden, J.N. and Nicholls, A.W., 2011. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nature protocols*, *6*(7), pp.1060-1083.

Dunn, W.B., Erban, A., Weber, R.J., Creek, D.J., Brown, M., Breitling, R., Hankemeier, T., Goodacre, R., Neumann, S., Kopka, J. and Viant, M.R., 2013. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics*, *9*(1), pp.44-66.

El-Razek, A., Emad, M.M. and Allam, T.S., 2019. Some Biochemical Parameters and Hematological Picture in Cases of Smooth Inactive Ovaries in Buffalo-Cows. *Alexandria Journal for Veterinary Sciences*, *61*(1).

Funeshima, N., Miura, R., Katoh, T., Yaginuma, H., Kitou, T., Yoshimura, I., Konda, K., Hamano, S. and Shirasuna, K., 2021. Metabolomic profiles of plasma and uterine luminal fluids from healthy and repeat breeder Holstein cows. *BMC Veterinary Research*, *17*(1), pp.1-10.

Giffen, P.S., Pick, C.R., Price, M.A., Williams, A. and York, M.J., 2002. Alpha-glutathione S-transferase in the assessment of hepatotoxicity—its diagnostic utility in comparison with other recognized markers in the Wistar Han rat. *Toxicologic pathology*, *30*(3), pp.365-372.

Goldansaz, S.A., Guo, A.C., Sajed, T., Steele, M.A., Plastow, G.S. and Wishart, D.S., 2017. Livestock metabolomics and the livestock metabolome: A systematic review. *PloS one*, *12*(5), p.e0177675.

Gorrochategui, E., Jaumot, J., Lacorte, S. and Tauler, R., 2016. Data analysis strategies for targeted and untargeted LC-MS metabolomic studies: Overview and workflow. *TrAC Trends in Analytical Chemistry*, *82*, pp.425-442.

Guerreiro, T.M., Gonçalves, R.F., Melo, C.F.O.R., de Oliveira, D.N., Lima, E.O., Visintin, J.A., de Achilles, M.A. and Catharino, R.R., 2018. A metabolomic overview of follicular fluid in cows. *Front. Vet. Sci*. 5: 10.

Gupta, K.A. and Purohit, G.N., 2001. Use of vaginal electrical resistance (VER) to predict estrus and ovarian activity, its relationship with plasma progesterone and its use for insemination in buffaloes. *Theriogenology*, *56*(2), pp.235-245.

Hidiroglou, M., 1979. Trace element deficiencies and fertility in ruminants: a review. *Journal of Dairy Science*, *62*(8), pp.1195-1206.

https://epashuhaat.gov.in/documents/ProvisionalKeyResultsof20thLivestockCensus, 2019.

Jain, N.C., 1993. *Essentials of veterinary hematology*.

Jayachandran, S., Nanjappan, K., Muralidharan, J., Selvaraj, P. and Manoharan, A., 2013. Blood biochemical and mineral status in cyclic and postpartum anestrus buffaloes. *Int. J. Food Agric. Vet. Sci*, *3*(1), pp.99-97.

Kaminski, P., Jerzak, L., Sparks, T.H., Johnston, A., Bochenski, M., Kasprzak, M., Wiśniewska, E., Mroczkowski, S. and Tryjanowski, P., 2014. Sex and other sources of variation in the haematological parameters of White Stork Ciconia ciconia chicks. *Journal of Ornithology*, *155*(1), pp.307-314.

Karthikeyan, K., Muniasamy, S., SankarGanesh, D., Achiraman, S., Saravanakumar, V.R. and Archunan, G., 2013. Faecal chemical cues in water buffalo that facilitate estrus detection. *Animal reproduction science*, *138*(3-4), pp.163-167.

Kanai, Y., Latief, T.A., Ishikawa, N. and Shimizu, H., 1990. Behavioural and hormonal aspects of the oestrous cycle in swamp buffaloes reared under temperate conditions. In *Domestic buffalo production in Asia,* pp. 113-120.

Khajeh, M., Rahbarghazi, R., Nouri, M. and Darabi, M., 2017. Potential role of polyunsaturated fatty acids, with particular regard to the signaling pathways of arachidonic acid and its derivatives in the process of maturation of the oocytes: Contemporary review. *Biomedicine & Pharmacotherapy*, *94*, pp.458-467.

Kovac, J.R., Pastuszak, A.W. and Lamb, D.J., 2013. The use of genomics, proteomics, and metabolomics in identifying biomarkers of male infertility. *Fertility and sterility*, *99*(4), pp.998-1007.

Kumar, A., Kroetsch, T., Blondin, P. and Anzar, M., 2015. Fertility-associated metabolites in bull seminal plasma and blood serum: 1H nuclear magnetic resonance analysis. *Molecular reproduction and development*, *82*(2), pp.123-131.

Kumar, P., Shankar Rao, T.K., Kumar, N., Chaurasia, S. and Patel, N.B., 2013. Heat detection techniques in cattle and buffalo. *Veterinary World*, *6*(6).

Lapa, M., Marques, C.C., Alves, S.P., Vasques, M.I., Baptista, M.C., Carvalhais, I., Silva Pereira, M., Horta, A.E.M., Bessa, R.J.B. and Pereira, R.M., 2011. Effect of trans-10 cis-12 conjugated linoleic acid on Bovine Oocyte Competence and Fatty Acid Composition. *Reproduction in domestic animals*, *46*(5), pp.904-910.

Leroy, J.L.M.R., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., Genicot, G. and Van Soom, A., 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction*, *130*(4), pp.485-495.

Madan, M.L., Das, S.K. and Palta, P., 1996. Application of reproductive technology to buffaloes. *Animal Reproduction Science*, *42*(1-4), pp.299-306.

Manchester, M. and Anand, A., 2017. Metabolomics: strategies to define the role of metabolism in virus infection and pathogenesis. *Advances in virus research*, *98*, pp.57-81.

Marai, I.F.M., Habeeb, A.A., Daader, A.H. and Yousef, H.M., 1995. Effects of Egyptian subtropical summer conditions and the heat-stress alleviation technique of water spray and a diaphoretic on the growth and physiological functions of Friesian calves. *Journal of Arid Environments*, *30*(2), pp.219-225.

Marei, W.F., Wathes, D.C. and Fouladi-Nashta, A.A., 2009. The effect of linolenic acid on bovine oocyte maturation and development. *Biology of Reproduction*, *81*(6), pp.1064-1072.

Martin, F.P., Collino, S., Rezzi, S. and Kochhar, S., 2012. Metabolomic applications to decipher gut microbial metabolic influence in health and disease. *Frontiers in physiology*, *3*, p.113.

Mondal, S., Prakash, B.S. and Palta, P., 2006. Endocrine aspects of oestrous cycle in buffaloes (Bubalus bubalis): an overview. *Asian-australasian journal of animal sciences*, *20*(1), pp.124-131.

Mondal, S., Suresh, K.P. and Nandi, S., 2009. Endocrine profiles of oestrous cycle in buffalo: a meta-analysis. *Asian-Australasian Journal of Animal Sciences*, *23*(2), pp.169-174.

Mortson, H.R., Allen, J.H. and Smith R.M. (1972). Role of minerals in reproduction. British J. Nutri., 27:127

Muniasamy, S., Selvam, R.M., Rajanarayanan, S., Saravanakumar, V.R. and Archunan, G., 2017. p-cresol and oleic acid as reliable biomarkers of estrus: evidence from synchronized Murrah buffaloes. *Iranian journal of veterinary research*, *18*(2), p.124.

Muñoz, M., Uyar, A., Correia, E., Ponsart, C., Guyader-Joly, C., Martínez-Bello, D., Guienne, M.L., Fernandez-Gonzalez, A., Díez, C., Caamaño, J.N. and Trigal, B., 2014. Metabolomic prediction of pregnancy viability in superovulated cattle embryos and recipients with Fourier transform infrared spectroscopy. *BioMed research international*, *2014*.

Muthukumar, S., Rajkumar, R., Karthikeyan, K., Liao, C.C., Singh, D., Akbarsha, M.A. and Archunan, G., 2014. Buffalo cervico-vaginal fluid proteomics with special reference to estrous cycle: heat shock protein (HSP)-70 appears to be an estrus indicator. *Biology of reproduction*, *90*(5), pp.97-1.

Nagana Gowda, G.A., Zhang, S., Gu, H., Asiago, V., Shanaiah, N. and Raftery, D., 2008. Metabolomics-based methods for early disease diagnostics: A review. *Expert review of molecular diagnostics*, *8*(5), pp.617-633.

Nanda, A.S. and Nakao, T., 2003. Role of buffalo in the socioeconomic development of rural Asia: Current status and future prospectus. *Animal Science Journal*, *74*(6), pp.443-455.

Nayan, V., Sinha, E.S., Onteru, S.K. and Singh, D., 2020. A proof-of-concept of lateral flow based luteinizing hormone detection in urine for ovulation prediction in buffaloes. *Analytical Methods*, *12*(26), pp.3411-3424.

Onteru, S.K., Baddela, V.S., Ravinder, R., Kaipa, O., Nayan, V., Singh, P., Baithalu, R.K. and Singh, D., 2016. Direct saliva transcript analysis as a novel non-invasive method for oestrus marker detection in buffaloes. *Biomarkers*, *21*(2), pp.99-101.

Pan, Z. and Raftery, D., 2007. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and bioanalytical chemistry*, *387*(2), pp.525-527.

Perkel, K.J. and Madan, P., 2017. Spent culture medium analysis from individually cultured bovine embryos demonstrates metabolomic differences. *Zygote*, *25*(6), pp.662-674.

Piccione, G., Casella, S., Lutri, L., Vazzana, I., Ferrantelli, V. and Caola, G., 2010. Reference values for some haematological, haematochemical, and electrophoretic parameters in the Girgentana goat. *Turkish Journal of Veterinary and Animal Sciences*, *34*(2), pp.197-204.

Prabha, B., Singh, C., Murtuza, M. and Pandey, R.P., 2000. Total serum calcium and inorganic phosphorus concentration in crossbred (Friesian× Hariana) pregnant cows and calves. *Indian Journal of Animal Sciences*, *70*(1), pp.50-51.

Prasad, C.S., Sharma, P.V., Obireddy, A. and Chinnaiya, G.P., 1989. Trace elements and ovarian hormonal levels during different reproductive conditions in crossbred cattle. *Indian J. Dairy Sci*, *42*(3), pp.489-492.

Ravinder, R., Kaipa, O., Baddela, V.S., Sinha, E.S., Singh, P., Nayan, V., Velagala, C.S.N., Baithalu, R.K., Onteru, S.K. and Singh, D., 2016. Saliva ferning, an unorthodox estrus detection method in water buffaloes (Bubalus bubalis). *Theriogenology*, *86*(5), pp.1147-1155.

Reo, N.V., 2002. NMR-based metabolomics. *Drug and chemical toxicology*, *25*(4), pp.375-382.

Saiduddin, S. and Davis, D.R., 1993. Reproductive abnormalities in the prepubertal jaundiced gunn rat. *Neonatology*, *63*(3), pp.177-182.

Saleem, F., Bouatra, S., Guo, A.C., Psychogios, N., Mandal, R., Dunn, S.M., Ametaj, B.N. and Wishart, D.S., 2013. The bovine ruminal fluid metabolome. *Metabolomics*, *9*(2), pp.360-378.

Sanchez-Guijo, A., Blaschka, C., Hartmann, M.F., Wrenzycki, C. and Wudy, S.A., 2016. Profiling of bile acids in bovine follicular fluid by fused-core-LC–MS/MS. *The Journal of steroid biochemistry and molecular biology*, *162*, pp.117-125

Samuthirapandi, M., Shanmugam, V. and Archunan, G., Gas Chromatographic Monitoring and Quantification of the Estrus-Specific Pheromone Compounds in Female Murrah Buffalo Urine.

Schauer, N., Steinhauser, D., Strelkov, S., Schomburg, D., Allison, G., Moritz, T., Lundgren, K., Roessner-Tunali, U., Forbes, M.G., Willmitzer, L. and Fernie, A.R., 2005. GC–MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS letters*, *579*(6), pp.1332-1337.

Segers, K., Declerck, S., Mangelings, D., Heyden, Y.V. and Eeckhaut, A.V., 2019. Analytical techniques for metabolomic studies: a review. *Bioanalysis*, *11*(24), pp.2297-2318.

Selvam, R.M., Onteru, S.K., Nayan, V., Sivakumar, M., Singh, D. and Archunan, G., 2017. Exploration of Luteinizing hormone in murrah buffalo (Bubalus bubalis) urine: Extended surge window opens door for estrus prediction. *General and comparative endocrinology*, *251*, pp.121-126.

Sharma, H.C., Dhami, A.J., Sharma, S.K., Sarvaiya, N.P. and Kavani, F.S., 2008. Assessment of estrus detection and insemination efficiency of AI workers in buffaloes through plasma progesterone profile under field conditions. *The Indian Journal of Animal Sciences*, *78*(7).

Sharma, M.C., Joshi, C., Saxena, N. and Das, G., 2004. Role of minerals in reproductive performance of livestock. *Livest. Intl*, *8*, pp.5-10.

Sharma, V.K., Vadodaria, V.P., Kavani, F.S. and Siddiquee, G.M., 1988. Studies on circulatory levels of trace minerals in normal cyclic and primary infertile kankerej heifers. *Indian Veterinary Journal*, *65*(4), pp.332-335.

Singh, R. and Sinclair, K.D., 2007. Metabolomics: approaches to assessing oocyte and embryo quality. *Theriogenology*, *68*, pp. S56-S62.

Solberg, R., Enot, D., Deigner, H.P., Koal, T., Scholl-Bürgi, S., Saugstad, O.D. and Keller, M., 2010. Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs. *PloS one*, *5*(3), p.e9606.

Suthar, V.S. and Dhami, A.J., 2010. Estrus Detection Methods in Buffalo. *Veterinary World*, *3*(2).

Theodoridis, G., Gika, H.G. and Wilson, I.D., 2011. Mass spectrometry-based holistic analytical approaches for metabolite profiling in systems biology studies. *Mass spectrometry reviews*, *30*(5), pp.884-906.

Tríbulo, P., Balzano-Nogueira, L., Conesa, A., Siqueira, L.G. and Hansen, P.J., 2019. Changes in the uterine metabolome of the cow during the first 7 days after estrus. *Molecular reproduction and development*, *86*(1), pp.75-87.

Vallee, B.L., 1976. Zinc biochemistry: a perspective.

Whitfield, P.D., German, A.J. and Noble, P.J.M., 2004. Metabolomics: an emerging post-genomic tool for nutrition. *British Journal of Nutrition*, *92*(4), pp.549-555.

Wishart, D.S., 2016. Emerging applications of metabolomics in drug discovery and precision medicine. *Nature reviews Drug discovery*, *15*(7), pp.473-484.

Xi, B., Gu, H., Baniasadi, H. and Raftery, D., 2014. Statistical analysis and modeling of mass spectrometry-based metabolomics data. In *Mass spectrometry in metabolomics* (pp. 333-353). Humana Press, New York, NY.

Yaqub, L.S., Kawu, M.U., Ayo, J.O., Ambali, S.F. and Habibu, B., 2013. Effect of oestrous cycle on serum electrolytes and liver enzymes in Red Sokoto goats. *Afr. J. Biochem. Res*, *7*(9), pp.174-178.

Zaitsu, K., Hayashi, Y., Kusano, M., Tsuchihashi, H. and Ishii, A., 2016. Application of metabolomics to toxicology of drugs of abuse: A mini review of metabolomics approach to acute and chronic toxicity studies. *Drug metabolism and pharmacokinetics*, *31*(1), pp.21-26.

Zhang, A., Sun, H., Wang, P., Han, Y. and Wang, X., 2012. Modern analytical techniques in metabolomics analysis. *Analyst*, *137*(2), pp.293-300.

Zhang, G., Deng, Q., Mandal, R., Wishart, D.S. and Ametaj, B.N., 2017. DI/LC-MS/MS-based metabolic profiling for identification of early predictive serum biomarkers of metritis in transition dairy cows. *Journal of agricultural and food chemistry*, *65*(38), pp.8510-8521.

Zhang, N., Wang, L., Luo, G., Tang, X., Ma, L., Zheng, Y., Liu, S., A Price, C. and Jiang, Z., 2019. Arachidonic acid regulation of intracellular signaling pathways and target gene expression in bovine ovarian granulosa cells. *Animals*, *9*(6), p.374.

Zhang, R., Zhu, W., Jiang, L. and Mao, S., 2017. Comparative metabolome analysis of ruminal changes in Holstein dairy cows fed low-or high-concentrate diets. *Metabolomics*, *13*(6), pp.1-15.

Zhao, S., Zhao, J., Bu, D., Sun, P., Wang, J. and Dong, Z., 2014. Metabolomics analysis reveals large effect of roughage types on rumen microbial metabolic profile in dairy cows. *Letters in applied microbiology*, *59*(1), pp.79-85.