IMPACT OF HEAT AND NUTRITIONAL STRESS ON RUMEN FERMENTATION CHARACTERISTICS AND METABOLIC ACTIVITY IN BUCKS

by

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(2010-20-108)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Impact of Heat and Nutritional Stress on Rumen Fermentation Characteristics and Metabolic Activity in Bucks"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"Impact of Heat and Nutritional Stress on Rumen Fermentation Characteristics and Metabolic Activity in Bucks"** is a record of research work done independently by Ms. Chaidanya K., (2010-20-108) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Dedicated to

My Beloved Family

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SYMBOLS AND ABBREVIATIONS

ACP	- Acid phosphatase
ALP	- Alkaline phosphatase
ALT	- Alanine aminotransferase
APADH	- Acetylpyridine Adenine Dinucleotide (Reduced)
APADP	- Acetylpyridine-Adenine Dinucleotide Phosphate
APADPH	- Acetyl Pyridine Dinucleotide Phosphate (Reduced)
AST	- Aspartate aminotransferase
BLAST	- Basic Local Alignment Search Tool
BW	- body weight
C	- Control
CAT	- Catalase
CCl_4	-Carbon tetrachloride
cDNA	- Complementary DNA
CH_4	. Methane
CMCase	- Carboxy methyl cellulase
CO_2	. Carbondioxide
CS	- Combined stress
$CuSO_4$	- Copper sulphate
DNA	- Deoxyribo nucleic acid
DNS	- Dintro salicylic acid
EC	- Extracellular
ELISA	– Enzyme-linked immunosorbent assay.
TE buffer	– Tris- Ethylenediaminetetraacetic acid buffer
FAO	– Food and agriculture organisation
FAOSTAT	– Food and Agriculture Organization Corporate Statistical Database
GAPDH	- Glyceraldehyde-3-phosphate dehydrogenase
GC	- Gas chromatography
GDP	- Gross domestic product
GH	- Growth hormone

GHG - Greenhouse gas

GLM - General linear model

GnRH - Gonadotropin releasing hormone

GSH-Px - Glutathione peroxidase

Hr - Hour

H & E –Haematoxylin and Eosin

H_2SO_4 - Sulphuric acid

HCO_3 - Bicarbonate

$HgCl_2$ - Saturated mercuric chloride

HPA - Hypothalamus pituitary axis

HRP - Horseradish peroxidase

HS - Heat stress

HSP–70 - Heat shock protein

IN - Intracellular

IFCC - International Federation of Clinical Chemistry

IGF-I - Insulin like growth factor – I

INCCA - Indian Network for Climate Change Assessment

IPCC – Intergovernmental panel on climate change

K_2SO4 - Potassium sulphate

LDH – Lactate Dehydrogenase

LH - Luteinizing hormone

LN_2 - Liquid nitrogen

MDH - Malate dehydrogenase

MoEF – Ministry of environment and forests

mRNA – messenger Ribo nucleic acid

$(NH_4)_2SO_4$ - Ammonia sulphate

Na_2SO_4 - Sodium Sulfate

NaOH -Sodium Hydroxide

NCBI - National Center for Biotechnology Information

NDDB – National dairy development board.

NDF - Neutral Detergent Fiber

NFC	- Non Fibrous Carbohydrate
NH_3	- Ammonia
NH_3-N	- Ammonia nitrogen
ns	– Non significant
NS	- Nutritional stress
NSC	- Non-structural carbohydrate
OD	- Optical density
PBMCs	- Peripheral blood mono nuclear cells
PBS	– Phosphate buffered saline
Pmol/litre	- Picomole/lite
PO_4	- Phosphate buffer
PUFA	- Polyunsaturated fatty acids
RNA	-Ribonucleic acid
RH	- Relative humidity
ROS	- Reactive oxygen species
RR	- Respiration rate
RT	- Rectal temperature
RT	-qPCR- Real time quantitative polymerase chain reaction
SEM	– Standard Error Mean
SOD	- Super-oxide dismutase
SPSS	– Statistical package for the social sciences
SRL	- Strained rumen liquor
T_3	- Triiodothyronine
T_4	- Thyroxine
TA	- Total activity
TCA	-Trichloro acetic acid
THI	-Temperature humidity index
TMB	- Tetra methyl benzidine
TNZ	– Thermo neutral zone
TRH	- Thyrotropin releasing hormone
TSH	- Thyroid stimulating hormone

TVFA	- Total volatile fatty acids
VFAs	- Volatile fatty acids
°	- Degree
E	– East
N	– North
°C	– Degree centigrade
m	– Meter
mm	– millimetre
Kg	– Kilogram
hrs	– Hours
w/v	- weight/volume
rpm	– Revolution per minute
min	– Minute
ml	– Milliliter
mmol/L	- millimole per liter
gm	– Gram
N	– Normal
mg	– Milligram
µg	– Microgram
nm	– Nanometer
µL	– Microlitre
IU	– International Unit
Abs	– Absorbance
µM	– Micrometer
g	– Gravity
ng	- nanogram/millilitre
Fig.	– Figure
H_2O	– Water
pNPP	- ρ-Nitrophenyl phosphate

CHAPTER 1

INTRODUCTION

Globally, agriculture provides livelihood for more people than the industries (Upton, 2004). Millions of rural people still keep livestock in traditional production systems, where they support livelihoods and household food security. Domesticated mammals and birds contribute directly to the livelihoods of hundreds of millions of people, including an estimated 70 per cent of the world's rural poor (FAO, 2015). They provide a wide range of products and services including food, transport, fibers, fuel and fertilizer. Livestock contribute 40 per cent of the global value of agricultural output and support the livelihoods and food security of almost a 1.3 billion people. (Herrero *et al*., 2013). The livestock sector is one of the fastest growing sectors of the agricultural economy. The growth and transformation of the sector provide opportunities for agricultural development, poverty reduction and food security gains. Majority of the extremely poor people of the world are known to be mainly dependent on agriculture and its allied sectors.

Worldwide the demand for animal based food is increasing and the developing countries are the major exporters. Apart from these food products the non-food products are also exported by the developing nations. In many developing countries livestock keeping is a multifunctional activity. Beyond their direct role in generating food and income in many countries livestock is a valuable asset, serving as a store of wealth, collateral for credit, an essential safety net during times of crisis. In addition, they also provide a cushioning effect against crop failures (FAOSTAT, 2014).

According to Porsche *et al*. (2011), India being susceptible to extreme climate events, India ranks second in the 2011 Climate Change Vulnerability Index. The impact of climate change on the ecosystem is worsened due to rapid urbanization, industrialization and economic development. They added that this is further aggravated by the occurrence of floods, droughts, and decreased water

availability from glacial melt and decreasing rainfall. From the climate data of the last century it has been observed that there has been an increase of 0.5 °C in the mean annual temperature. Pande and Akermann (2011) reported that after 1990s the average annual temperature has been on increasing trend. Six out of the ten most severe drought disasters globally, occurred in India in the last century affecting up to 300 million people, and adversely impacting its agricultural sector. According to World Bank (2013), a warming trend has begun to emerge over south Asia, particularly in India, which appears to be due to human induced climate change. Over the same period a decline in total rainfall amounts during the monsoon period likely due to the effects of anthropogenic activities has been observed. Furthermore, there are observed increases in the frequency of the most extreme precipitation events, as well as increases in the frequency of short drought periods. Models used in a study by the Indian Network for Climate Change Assessment (INCCA) show a projected increase in annual mean temperatures of up to 1.5°C and 2.0°C by the 2030s (MoEF, 2010). In addition, projections for 2020, 2050 and 2080 also show an all-round warming due to increase in Greenhouse gas (GHG) concentrations (MoEF, 2012).

Animal husbandry forms an integral component of Indian economy as bulk of the population depends on agriculture for their sustenance. More than half of the population lives in rural areas and is highly dependent on climate-sensitive sectors such as agriculture, livestock, fisheries and forestry (Sterrett, 2011). The majority of the farmers are small and marginal and they rely upon small ruminants like goat and sheep for their livelihood securities.

Increased environmental temperature leads to variations in the basic rumen physiology. Heat stress (HS) reduces the total production of volatile fatty acids (VFAs) with individual variation. Passage rate and retention time of digesta is also influenced by rise in ambient temperature and thus affects digestibility. The high ambient temperature also causes various changes in the characteristics and pattern of rumen fermentation. This results in changes in the rumen pH, changes of the microbial population inside the rumen and production of VFAs. The change

in microbiota due to HS may change the fermentation pattern in the rumen resulting in variation in digestibility, VFA production and also Methane (CH_4) emission (Yadav *et al*., 2013). The long-term changes induced by a low plane of nutrition is known to decrease gut mass and reduce O_2 consumption by visceral tissue, and reduce VFA absorption (Doreau *et al*., 1997). In the tropics, there occurs seasonal fluctuations of feed resources as the availability depends upon the amount of rainfall received, which is considered as another major constraint during the summer. The alteration in VFA profiles and shifts in rumen pH due to dietary shifts are well known phenomena and their impact on animal function are widely accepted. Microbes alter their growth depending upon the substrate or the type of feed available, pH and rate of passage from the rumen and finally causing changes in the end products of fermentation like VFAs. CH_4 comprises between 20 and 30 per cent of total gases produced in the rumen and can represent a significant feed energy loss (Shibata, 2010).

Livestock are often considered to be one of the most important means of food and economic security for poor and marginal farmers (Funston *et al*., 2010; Sejian *et al*., 2014a). Climate change and global warming cause great threat to entire livestock population across the world. Climatic factors or seasonal changes greatly influence the behavior of animals due to neuroendocrine response to climatic elements, consequently affecting production and health of animals (Sejian *et al*., 2013). Heat is produced in animals by the oxidation of absorbed nutrients in the cells. Kumar (1998) reported lower metabolic rate in Marwari, Sirohi, Jamunapari and Barbari bucks in hot period than that in moderate or in cool period. Resting heat production was significantly higher in Barbari breed than that in Jamunapari, Marwari and Sirohi breeds. The overall metabolic rate of Marwari and Sirohi bucks were comparatively lower than that of Jamunapari bucks and significantly lower than that of Barbari bucks. A lower metabolic rate is advantageous to life in arid region where water and food are at a premium, since this would reduce water loss and food requirement (Yousef, 1984). No further

reduction in the resting heat production as a result of exposure to solar radiation in hot period was observed in the bucks.

Thyroid gland is one of the most sensitive organs to the ambient heat variation (Rasooli *et al*., 2004). Appropriate thyroid gland function and activity of thyroid hormones are considered crucial to sustain the productive performance in domestic animals (Todini, 2007). When the animals start to suffer due to heat, food ingestion is reduced and metabolism slows down causing a hypo-function of the thyroid gland (McManus *et al*., 2009). One of the principal functions of glucocorticoid under thermal stress is to control hepatic gluconeogenesis and insulin concentration in such conditions directly depicts the energy status of animal to sustain production under such extreme environmental conditions (Sejian *et al*., 2008). Hence measuring the metabolic hormones such as thyroid hormones and insulin will give a clue about how an animal adapts itself to the changing environments by altering their metabolic activity.

As a result of climate change, livestock reared in tropical countries like India are vulnerable to various environmental stresses like HS, nutritional stress (NS) and walking stress (Sejian *et al*., 2013). These environmental stresses affect the growth and production potential of the animals as these animals tries to adapt to these environmental challenges by bringing about various physiological, behavioral and hormonal mechanisms. One of the primary way by which the animals tries to adapt to hot environment is by reducing their metabolic activity to prevent the additional metabolic heat load during summer. These effects are brought about by altering the endocrine activity of hypothalamo-pituitary-thyroid activity. Thyroid function declines as an acclimation response to alleviate the additional heat load by declining both Triiodothyronine (T_3) and Thyroxine (T_4) levels during short and long term exposure to solar radiation in goats. This reduction may be due to effect of high ambient temperature on hypothalamus and pituitary glands to decrease the thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH) which enable animal to reduce their basal metabolism (Gupta *et al*., 2013). Appropriate thyroid gland function and thyroid hormone

activity are considered crucial to sustain the productive performance in animals (Todini, 2007). The animals try to reduce their metabolic activities in order to cope up to the stress and maintain homeostasis. In addition, the plasma thyroid hormone concentrations are correlated with feed intake in several ruminant species (Kafi *et al*., 2012). The Changes of blood thyroid hormone concentrations are an indirect measure of the changes in thyroid gland activity and circulating thyroid hormones can be considered as indicators of the metabolic and nutritional status of the animals (Sejian *et al*., 2012a).

Animals reared under hot environment are generally subjected to more than one stress at a time. This seriously hampers the animal production and reproduction under such environmental conditions (Naqvi and Sejian, 2010). In tropical countries these environmental challenges occur simultaneously rather than in isolation and the impact of these are more severe when they occur cumulatively than they occur individually. These affect the production and survival of the livestock vulnerable in these environments. All these ultimately affect the livelihood and survival of thousands of poor people who rely upon animal agriculture for the existence, causing a huge financial burden to the farmers. But studies involving effect of stress on farm animal productivity have generally considered one stress at a time. Meagre reports are available in literature regarding studies involving the effects of more than one stress in farm animals. However Moberg (2000) described a hypothetical scheme indicating how two stressors can summate together and influence normal functions in mammalian species. The effects of combined stresses on the animals are more severe than that of the individual stress (Sejian *et al*., 2012b; Sejian *et al*., 2013). However, there are only few studies available on the effects of combined and multiple stresses on livestock. The adverse effects of these cumulative stresses are to be identified for developing suitable adaptation strategies to sustain livestock production in the changing climate scenario especially in the tropical regions. Also elucidating the rumen fermentation pattern of the livestock under the influence of multiple climatic factors are important for developing suitable mitigation strategies for

reducing the contribution of livestock towards climate change. Hence an attempt has been made in this study to establish the effect of more than one stress simultaneously in goat.

With these background, the study was conducted to establish the impact of HS and NS simultaneously on the rumen fermentation pattern and metabolic activities in buck. Such information would be very valuable as under the changing climatic condition, HS and NS stress are the major stresses occurring and it is very vital to study these stresses influence on rumen fermentation pattern and metabolic activity. These attempts are first of its kind in any livestock species and the outcome of this study in goat may be useful in understanding the combined stress effect on rumen fermentation pattern and metabolic adaptation in other livestock species. This may add value to the existing knowledge on adaptation of livestock to changing climatic scenario and these information may be very precious if one attempts improving goat production under the changing climatic condition.

The objectives of the present study are

1. To determine the impact of HS and NS on rumen fermentation pattern and volatile fatty acids production in bucks
2. To observe the impact of HS and NS on the metabolic hormone and enzyme profile in bucks
3. To study the impact of HS and NS on the Heat shock protein (HSP) expression in visceral organs of bucks

CHAPTER 2

REVIEW OF LITERATURE

2.1 Importance of livestock to Indian economy

Worldwide, agriculture provides a livelihood for more people than any industry (Moran, 2009). Growth in agricultural production and productivity is required to elevate rural incomes, and to meet the food and raw material needs of the more rapidly growing urban populations. Agriculture has crucial role in reducing poverty as major portion of the world's poor population survive in rural areas and they depend largely on agriculture and its allied sectors for their livelihood security (FAO, 2012). Livestock provide over half of the value of global agricultural output and one third in developing countries (Upton, 2004). The majority of the world's estimated 1.3 billion poor people live in developing countries depending directly or indirectly on livestock for their survival and livelihood (World Bank, 2008; Moyo and Swanepoel, 2010). Ali (2007) reported that at the global level, it is estimated that 70 per cent of the rural poor's livelihoods are supported by the animal sector, representing approximately 20 per cent or about one fifth of animal products traded worldwide. Globally, livestock contributes about 40 per cent to the agricultural gross domestic product (GDP) and constitutes about 30 per cent of the agricultural GDP in the developing world (World Bank, 2009; Moyo and Swanepoel, 2010). These estimates highlight the essential contribution of livestock to sustainable agricultural development. The livelihood of the rural poor in developing countries depends majorly on local natural resource-based activities such as crop and livestock production, hunting, fuel wood, fishing, and minor forest product collection (Yusuf, 2014). Livestock acts as insurance policies and bank accounts in many parts of the developing world (Pell *et al*., 2010). Across the developing world, livestock contribute, on average, 33 per cent of household income in mixed crop-livestock systems and 55

per cent of pastoral incomes and according to livestock accounts for 68 per cent of the household income in the developing world (Herrero *et al*., 2009).

India's livestock sector is one of the largest in the world, accounting for 57 per cent of world's buffalo population and 16 per cent of world's cattle and ranking first in respect of cattle and buffalo population, third in sheep and second in goat population in the world (Kumar *et al*., 2012). Livestock is an integral component of the economy as the Indian economy is agrarian based. Animal husbandry contributes to the national and socio- economic development of the country. Apart from its contribution to national and agricultural economy it also provides employment generation opportunities, asset creation and it acts as a best source of insurance for farmers against the vagaries of the natural calamities like drought, ensuring social and financial security. It also ensures nutrition and food security for the people as livestock is the main source of animal protein for the population. About 70 million of the rural population owns one or the other livestock species. In India, 15-20 per cent of the rural families are landless and 80 per cent of the land owners are marginal and small farmers, they rely on livestock for their livelihood. Women contribute to the 69 per cent of the workforce engaged in livestock sector. Occurrence of natural hazards like drought is not a bizarre in Indian sub-continent. Livestock forms a source of means of reducing the risks associated with crop failure and a diversification strategy for resource poor small scale farmers and their communities in the marginal areas with harsh environments (Thornton *et al*., 2007; Vandamme *et al*., 2010; Moyo and Swanepoel, 2010). Livestock also contributes to the crop production by providing draught animal power and manure (Herrero *et al*., 2013).

2.2 Significance of rearing small ruminants

Small ruminants (goats and sheep) form a significant economic and ecological niche in agricultural systems throughout the developing countries. Small ruminants also play a complementary role to other livestock in the utilization of available feed resources and provide one of the practical means of

using vast areas of natural grassland in regions where crop production is least possible (Baker and Rege, 1994). The largest number of goats is observed in Asia, followed by Africa, representing about 59.7 per cent and 33.8 per cent, summing up to 93.5 per cent out of the total number of the world, respectively. The total number of goats in the world increased by 146 per cent of the total number (590.1 million) encountered in 1990. Number of goats in the world has been increasing since 1990 by about 1per cent to 4per cent each year and during the same period, cattle number increased by 5 per cent, while that of sheep decreased by 10 per cent, reflecting the emergence of goats as a major livestock species (Aziz, 2010).

In developing countries rearing of the small ruminants plays a major role in providing livelihoods for the majority of the population. They form the most readily and immediately available source of income to meet financial or social burden of the population. Small ruminants make a valuable contribution to the rural poor people, these contributions range from precious animal proteins (meat and milk) to fiber and skins, draught power in the highlands, food security and stable households. The feed requirement of small ruminants such as goat and sheep are comparatively less than that of the large ruminants because of their small body size. Most poor and marginal farmers rely heavily on small ruminant's production primarily due to their very low initial investments required. Their relative smaller size and early maturity or higher reproductive efficiency makes them suitable to meet the existing needs of milk and meat. Also they utilize marginal lands effectively without compromising on production (Devendra, 2005). In developing countries like India rearing of small ruminants provides substantial proportion of self-employment opportunities, especially for women as they play a greater role in day to day management of small ruminants especially goat.

2.3 Importance of goat rearing

Small ruminants especially goats provide livelihood to millions of rural poor in most of the developing countries of the Asia and Africa, where 95 per cent

of the world's goat population is concentrated and also where bulk of the world's poor population live (Nyathi, 2007). Small ruminants are well integrated in the farming systems of the marginal farmers who find goats as having a wide potential for their socio-economic progress. Goats offer a strong opportunity for the upliftment of agencies suitable for interventions including micro credit, extension, technical and marketing support especially to women, landless and small farmers. They further added that the major motivational factor for rearing goats among the small and marginal farmers was their feeding behavior, stall feeding is not essential they can be reared even by feeding only kitchen waste and allowing to graze them in harvested fields where milch cattle do not thrive. Morand-Fehr *et al*. in 2004 reported that due the wide adaptability of goats to harsh environments and limited feeding requirements during the last 20 years, the number of goats around the world increased by about 58 per cent when the increases of cattle and sheep stocks were 10 and 22 per cent only, respectively. In contrast with cattle (45per cent) and sheep (40per cent), goat farming is specific to low income countries particularly in tropical areas of Asia and Africa where about 80 per cent of the goats around the world are reared.

Goats are 2.5 times more economical than sheep on free range grazing under semi-arid conditions. They are considered as poor man's cow as they have immense contribution towards rural and national economy. The initial investments, housing and management requirements are less for goat rearing. They can survive in adverse environments and are capable of adapting in various agro climatic conditions with the available shrubs and trees where the land is less fertile and no crops can be grown. Goats can continue in very severe environments because of several adaptation mechanisms, such as the ability of walking distances, adaptability to drought the behavior to select the most nutritive plant fractions, the efficient digestion of the less nutritive portions rich in fiber due to a very intensive urea cycle etc. (Haenlein 2001; Iniguez, 2004; Morand-Fehr *et al*., 2004). Goats are known for their better drought tolerance on comparing to the other species of livestock which makes them important

especially in tropical countries and due to their wide adaptability. Morand-Fehr *et al*. (2004) reported that during the last 20 years, the number of goats around the world increased (by about 60 per cent) not only in the countries with low income (75 per cent) but also in those with high (20 per cent) or intermediate (25 per cent) income. Goats are used to modify the vegetation cover in many African and Asian countries, in order to clear areas rich in bushes, shrubs, thorny vegetation or under woods, so that later on other species such as sheep and cattle can graze on a more nutritive vegetation and more open areas, however at the same time by the removal of the undergrowth the spread of forest fires can be reduced or avoided (Hart, 2001).

2.4 Factors influencing goat rumen fermentation

Ruminants have the ability to convert cellulose and hemicellulose to energy with the help of microorganisms. Microorganisms present in the rumen ferment the fibers and produce VFAs, which are then used by the host animal as source of energy. The rumen fermentation results in the production of VFAs, microbes, gases like Ammonia (NH_3), CH_4 and Carbondioxide (CO_2). The microbial population comprises of bacteria, fungi and protozoa. The VFAs produced are; Acetate, Propionate, Butyrate, Valarate, Iso-Valarate, Iso-Butyrate. The formations of these acids require or produce hydrogen and the hydrogen along with CO_2 forms CH_4. The formation of propionic acid requires hydrogen and acetic and butyric acid produces hydrogen (Allard, 2009). Also the Ruminant livestock can produce 250 to 500 L of CH_4 per day (Iqbal and Hashim, 2014). There are many factors that influence the rumen fermentation pattern in the animals. These include age, breed, sex, type of diet fed, environmental and seasonal variations etc.

2.4.1 Species difference

It has been reported that species difference existed in rumen fermentation between cattle, sheep and goat (Alcaide *et al*., 2000). However, they further reported that between sheep and goat not much difference was obtained for rumen

fermentation when they fed medium quality diet with energy being at maintenance level. Further, they reported that no clear trend in concentration of both total as well as individual VFAs was observed between sheep and goat with the exception of an increased proportion of valeric acid in the rumen liquor of sheep. However, Garcia *et al*. (1995) observed that in a grazing condition there existed difference in rumen fermentation between sheep and goat with higher fermentation recorded being in goat. They attributed this difference to the higher passage rate as well higher degradation rate in the rumen of goat as compared to sheep.

2.4.2 Age differences

The age of the animal is an important factor influencing rumen fermentation. In 8 and 10 week old lambs, most of the fermentation occurs in the rumen. The concentration of VFAs in the rumen in calves has been found to reach adult levels at 6 weeks of age in animals fed a high-roughage diet and at 7-8 weeks for a high concentrate diet (Qadis *et al*., 2014). Similarly in 8 and 10 week old lambs, most of the fermentation occurs in the rumen. The concentration of VFAs in the rumen in calves has been found to reach adult levels at 6 weeks of age in animals fed a high-roughage diet and at 7-8 weeks for a high concentrate diet (Allard, 2009). The digestibility of feeds and efficiency of feed utilization increased with age in growing lambs (Allard, 2009). Allard, (2009) reported that when Lambs at 15, 24 and 40 weeks of age fed with dried clover, the digestibility of crude protein increased by 10 per cent from the age of 15 weeks to 40 weeks but the digestibility of organic matter and cell wall constituents decreased by 1.8 per cent and further he stated that no change in the rate of production of VFAs per unit of feed intake or rate of absorption of VFAs were seen (Allard, 2009).

2.4.3 Rumen pH

Meng-zhi *et al.* (2008) reported that in goats the pH of rumen liquor to be a colligation index of the level and condition of rumen fermentation. According to them, its change has a strong effect on the vigor of rumen microbes as rumen

microbial organisms only thrive within a narrow pH value range (5.5 to 7.5) suitable for microbial growth. The decline in ruminal pH will increase ruminal total volatile fatty acids (TVFA), which was mainly attributed to a significant increase in ruminal butyrate, rather than the accumulation of ruminal lactic acid. The acidity of the rumen fluid or the rumen pH influences the fermentation in two ways: 1. When the pH values are lower by 6.5 reduces the activity of fibrolytic micro-organisms degrading cell walls (Dijkstra *et al*., 1992). Hence pH determines the cell wall degradability and its contribution to microbial growth and towards VFA production. 2. pH determines the profile of VFA produced. Increased intake of concentrate meals leads to a more acidic rumen fluid and increased rate of substrate fermentation leading to increased rates of VFA production.

2.4.5 Type of diet

The composition of diet fed to the livestock is another important factor which influences rumen fermentation in dairy steers (Anantasook *et al*., 2013). The amounts of digestible nutrients especially the carbohydrate fraction also influences the fermentation pattern in livestock (Beauchemin and McGinn, 2006). Furthermore, a diet rich in fat also influenced the fermentation characteristics in the rumen of Buffaloes (Jentsch *et al*., 2007). Further Sejian *et al*. (2011); Soto-Navarro *et al*. (2014) reported that the type of forage consumed also influenced rumen fermentation in cattle and sheep respectively. Sejian *et al*. (2011) observed that the level of propionic acid was more in sheep fed high quality roughage of corn silage and alfalfa hay in cattle. Similarly, Saro *et al*. (2014) reported that sheep fed the lower quality forage showed higher abundance of some cellulolytic bacteria and fungi, which could be interpreted as an adaptation to digest a more fibrous and complex forage. They also observed that concentration of Ammonia nitrogen (NH_3-N) and TVFA, Carboxy methyl cellulase (CMCase) and α-amylase activities were greater in alfalfa fed sheep as compared to grass hay fed sheep. Further, there are also reports that high level of crude protein in the diet also

increased rumen fermentation characteristics and also influenced the type of microbial population in rumen (Chanthakhoun *et al*., 2010).

The ruminants are usually fed with poor quality forages, cereals grain straw, grain diets. The types of feedstuffs consumed by the ruminants influence the microbial population and feedstuff retention time in the rumen (Hernandez-Sanabria *et al*., 2010). Nutritional balance of the diet consumed by the ruminants will influence the microbe's ability to digest feedstuffs and overall ability of the microbe to live and grow. Almost all feedstuffs are deficit in certain nutrients crucial for optimum microbial growth. The daily feed intake and rumen fill were found to be some of the major factors influencing the fermentation pattern in cattle (Carberry *et al*., 2012). Further, the proportion of concentrates in dietary dry matter and the composition, rate and extent of degradation of individual feed fractions (the types of carbohydrate and protein) in dietary dry matter also affects the fermentation pattern (Bannink, 2006). Changes in dry matter intake not only affect the amount of substrate available for microbial degradation, but it also changes fermentation conditions and the size of the microbial population (Mekuriaw *et al*., 2013).

The fermentation end products in the rumen, especially the VFA, is influenced by the proportion of Neutral Detergent Fiber (NDF) versus Non Fibrous Carbohydrate (NFC) in the ration (Bannink *et al*., 2006). However, starch-based diets favor propionate production, while forage-based diets high in cellulose, hemicellulose, and lignin favor the production of acetate and butyrate (Knapp *et al*., 2014). Likewise soluble sugars tend to enhance greater proportions of butyrate and also fiber digestibility (Hindrichsen *et al*., 2005). Passage rate influences the fermentation pattern as well as microbial growth rates and extent of digestion in ruminants. The faster passage rate of feed materials indicates a lower extent of rumen fermentation. Greater passage rates also increases microbial energy requirements as the cells have to divide more frequently to maintain rumen populations (Knapp *et al*., 2014). Rumen fermentation is also affected by decreasing retention time (Christophersen, 2008).

Digestion of food in the rumen occurs by a combination of microbial fermentation and physical breakdown during rumination. The most important factor that influences rumen fermentation is the type and number of microbes present in the rumen (Anantasook *et al*., 2013). Microbial attack is carried out by a mixed population of bacteria and ciliate protozoa, together with a smaller, but possibly metabolically important, population of anaerobic fungi (Mardalena *et al.*, 2014). VFA content is one of the key indicators for rumen fermentation, and is mainly affected by dietary composition. Total ruminal VFA concentration depends on type of diet, level of feed intake, frequency of feeding and feed additives (Araba *et al*., 2002). Propionic acid production is favored by feeding diets which are high in concentrates (Da-cheng *et al*., 2013). They further indicated that rumen fermentation patterns changed significantly with increased NFC: NDF ratio. Ammonia is another important component which influences microbial protein synthesis (Arelovich *et al*., 2014). Ammonia is the nitrogen source of main microbes in rumen (Bandle and Gupta, 1997). NH_3-N concentration is also a colligation indicator of degradation and utilization of nitrogen source by rumen microbes.

2.4.6 Microbial populations

The rumen ecology includes a complex association of microorganisms including bacteria (10^{11}–10^{12} cells/ml), fungi (10^3 –10^6 cells/ml), archaea (10^7 – 10^9 cells/ml), and protozoa (10^4 –10^6cells/ ml), these interact with the feed, their host and each other. This ecosystem is relatively poorly understood, particularly inter-species interactions and interactions with the host (Wright and Klieve, 2011). The rumen has an anaerobic environment where the degradation of plant materials happens in a very short time. The type of microbes which are most efficient at deriving nutrients from feedstuffs will be the dominant population in the rumen. During rumen fermentation, rumen bacteria play a primary role in the conversion of feed components to peptides, NH_3, and VFAs. These end products provide the host with essential nutrients and VFAs provide metabolic energy, thereby affecting its performance or productivity and health.

Microbial degradation of substrates in the rumen depends on intrinsic characteristics that determine the susceptibility of the substrate to be degraded and utilized by micro-organisms. These characteristics differ between types of substrate and between types of feedstuffs. Intrinsic characteristics are important determinants of substrate degradation and utilization by microorganisms, VFA production (Knapp *et al*., 2014). Different types of fermented carbohydrate give different profiles of VFA production (Bannink *et al*., 2006). According to Hernandez-Sanabria *et al*. (2012), the balance between microbial production and host epithelial transport and absorption represents the concentration of VFAs in the rumen. They also added that the concentration of VFA can be interpreted as one of the indicators of microbe- microbe interactions as well as host- microbe interactions.

2.5 Impact of Heat Stress on rumen fermentation

HS affects the rumen health due to variety of biological and management reasons (Bernabucci *et al*., 2010). According to Bernabucci *et al*. (2010), the heat stressed cows consume less feeds and as a result ruminate less and as a consequence the buffering agents entering the rumen are reduced. In addition to that the total VFA content increases and the pH decreases. As enhanced blood flow to the periphery occurs during HS so as to increase the heat dissipation and a decreased circulation into the gastro intestinal tract the digestion end products are absorbed less efficiently. They further added that the increased respiration rates or panting during summer leads to acidosis as more CO_2 will be exhaled at the time of panting. The body needs to maintain a 20: 1 HCO_3 (bicarbonate) to CO_2 ratio for an effective blood pH buffering system, because of the hyperventilation-induced decrease in blood CO_2, the kidney secretes HCO_3 to maintain this ratio. This reduces the amount of HCO_3 that can be used through saliva to buffer and maintain a healthy rumen pH (Choubey and Kumar, 2012). Further, the panting cows frequently drool reducing the quantity of saliva available for the rumen. The drop in saliva HCO_3 quantity and the reduced amount of saliva entering the rumen

make the heat-stressed cow much more prone to acute rumen acidosis (Kadzere *et al*., 2002).

Increased environmental temperature reduces the rumination time and lowers appetite by having a direct negative effect on the appetite center of the hypothalamus (Soriani *et al*., 2013). The rumination, blood flow to the rumen epithelium and reticular motility is reduced during HS and dehydration whereas, the volume of digesta in the rumen of beef cows and Bedouin goats (Silanikove, 1985), riverine buffalo (Korde *et al*., 2007) and Egyptian buffalo (Marai and Habeeb, 2010) increased. HS reduced the VFA production (Tajima *et al*., 2007 and Nonaka *et al*., 2008). Nonaka *et al*. (2008) reported that in Holstein heifers the ratio of acetate to propionate decreased during HS and the molar concentration of acetate decreased whereas propionate and butyrate concentration increased non significantly. As compared with the normal temperature high ruminal temperature decreased the total VFA concentration in male calves (Salles *et al*., 2010). However, ruminal temperature did not affect the molar proportion of VFA (Salles *et al*., 2010 and King *et al*., 2011). The variations in fermentation pattern are due to the variations in the microbial population (Uyeno *et al*., 2010). High ruminal temperature increased the ruminal pH from 5.82 to 6.03 in lactating dairy cattle (Hall, 2009 and Yadav *et al*., 2013).

Further, an increase in diet digestibility in cattle when exposed to high temperature was reported by Nonaka *et al*. (2008) Holstein Heifers. The reasons for the high diet digestibility due to high environmental temperature could be attributed to the reduction in passage rate (Dikmen *et al*., 2012; Soriani *et al*., 2013) and due to the changes in feed composition or due to the reduction in dry matter intake (Smith *et al*., 2013; Yadav *et al*., 2013). In contrast to this in small ruminants (Silanikove 1985; Yadav *et al*., 2013) and cattle (Christopherson, 1985; Mathers *et al*., 1989 and Yadav *et al*., 2013) no or negative relationships between high ambient temperature and feed digestibility has been reported. Christopherson (1985) stated that when cattle were fed with forage based diets the dry matter digestibility in hot environments increased. Similarly, in a study conducted by

Mathers *et al*. (1989) in Ayrshire cattle the dry mater digestibility was found to be significantly higher at 33 °C than at 20 °C with moderate diet quality but was similar at 33°C and at 20°C with a high quality diet. Yadav *et al*. (2012) reported that the digestibility at 25 and 30°C has no change and at 35°C it increases and again at 40°C it decreases and the reason for this decrease is attributed to the change in rumen pH, temperature, motility and flora and fauna as a result of higher intensity of thermal stress. In addition, lower ruminal pH and higher concentration of lactic acid was found in heat stressed cattle and this may imply that a high lactic acid concentration and lower ruminal pH might be involved in inhibiting the ruminal motility during HS (Yadav *et al*., 2012).

2.6 Impact of Heat Stress on metabolic activity

Optimized livestock production is depended upon many factors like environmental stresses, climatic factors, health status, nutrient availability, and genetic potential. Animals require a diet which is balanced for the specific requirements of the animal and it must contain the correct proportions of energy, protein, fiber, vitamins, minerals and water in order to perform its biological functions and to maintain its performances. All the body functions are hindered when the animal is exposed to stress. Moberg (2000) defined stress as a multi-factorial and growing concern in animal production, which influences both short- and long-term performances. It is the biological response elicited when an individual perceives a threat to its homeostasis. The threat was the stressor. When stress response truly threatens the animal's well-being then the animal experience strain. The stress in animal can be of abiotic and biotic origin. Abiotic stress is the negative impact of non-living factors on living organisms in a specific environment, adversely affecting the performance or individual physiology of the animal. Biotic stresses include living disturbances such as pathogenic microorganisms. The most important of all abiotic stresses is HS followed by cold stress (Soren, 2012). In the tropics and sub tropics the key constraint faced by the animals is the HS due to the elevated temperature. Animal productivity is maximized in a narrow thermal range as energy, and nutrients are diverted away

from growth, milk, reproduction toward maintaining homeostasis when environmental conditions are not ideal. This change in nutrient partitioning priority decreases animal performance and is therefore a huge economic burden to the farmers (O'Brien *et al*., 2010).

Climate change has both direct and indirect stresses on livestock production. The direct stresses result from atmospheric parameters like temperature, humidity, solar radiation, wind velocity and rainfall, whereas the indirect stresses comprises of like pests and pathogens, occurrence of diseases, reduced nutrient quality in fodder crops, low availability of pasture lands and less availability of feed and water (IPCC, 2007). Livestock when exposed to stress undergoes reduction in productive and reproductive efficiency and the animal's natural defenses to a significant extent as during stress period major part of the energy generated will be utilized for thermogenesis or thermolysis or rather than for body metabolic activities (Babinszky *et al*., 2011). The animal productivity is severely affected above the thermo neutral zone. Constant exposure of livestock to high temperature causes a rise in its body temperature, a decline in feed intake, increase in water intake, a decrease in production of milk, changes in milk composition, reduction in growth and even a loss in body weight (BW). The extent of reduction in feed intake is proportional to the thermal stress. VFAs are reduced in summer, especially acetic acid (Prasad *et al*., 2012). Feed efficiency is considerably reduced (Soren, 2012).

According to Aggarwal and Upadhayay (2013), the HS alters the rumen fermentation pattern and the metabolic activity of the livestock. During HS the activation of the hypothalamic–pituitary–adrenal axis and the consequent increase in plasma glucocorticoid concentrations are two of the most important responses that the animals show. The short- and long-term environmental heat affects the endocrine glands and in turn leads to release of hormones, namely, thyroxin, cortisol, growth hormone and catecholamine. The thyroid hormones are important for the animal's adaptation. The thyroid gland secretes the hormones T_3 and T_4 which are the major mechanism for acclimation and are also known as the HS

markers in livestock (Horowitz, 2001). Both the T_3 and T_4 are associated with the metabolic homeostasis and are found to alter with the climatic changes (Sejian *et al*., 2013). It is a well-established fact that the endogenous level of thyroid hormone decreases when there is heat acclimation (Horowitz, 2001). The Thyroid hormones acts on many different target tissues, stimulating oxygen utilization and heat production in every cell of the body (Todini, 2007). These hormones are primary determinants of basal metabolic rate and have positive correlation with tissue production (Aggarwal and Upadhayay, 2013). Both T_3 and T_4 are biologically active form but T_3 is several times more active when compared with T_4. They play an important function in the expression of endogenous seasonal rhythms of neuroendocrine reproductive activity in sheep (Lehman *et al*., 2010).Horowitz (2001) stated that in cow the plasma (T_4) and (T_3) levels have been observed to decline under HS as compared to thermo-neutral conditions. In young and old buffalo calves the level of thyroid hormones tends to decline when exposed to acute heat (Nessim, 2004). When the ambient temperature rose from 24°c to 38°c inside the climate chamber in male Freisien calves the level of T_3 declined. (Habeeb *et al*., 2001). Yousef *et al.* (1997) found that in Friesian calves plasma T_3 level reduced from 151 to 126 ng/dl when exposed to direct solar radiation. In lactating buffaloes with the increase of ambient temperature from 17.5 to 37.1 the level of plasma T_3 varied significantly ($p< 0.01$) (Habeeb *et al*., 2000). Perez *et al*. (1997) stated that the level of blood T_3 and T_4 has a negative correlation with Temperature humidity index (THI), Relative humidity (RH), rectal temperature (RT) and respiration rate (RR) in Friesian heifers during the hot humid and dry winter season. A decline in the plasma concentrations of T_3 from 2.2 to 1.16ng/ml has been reported in cattle (Farooq *et al*., 2010) whereas a reduced thyroid activity in thermal acclimated cattle has been reported by Kumar *et al.*, (2011).The decline in thyroid hormones along with decreased plasma growth hormone (GH) level has a synergistic effect to reduce heat production. Kumar *et al*. (2011) reported that during HS there were significant reductions in concentrations of T_3 and T_4 in plasma and in milk of lactating cows. However, a significant increase in T_3 but not in T_4 level was observed during HS in cross bred

cattle (IVRI, 1984). Rasooli *et al*. (2004) stated that the thyroid hormones changes during the HS may be some of the attempts to reduce the metabolic heat production besides other endocrine and metabolic changes brought about by the animals. An inverse relation between blood thyroid hormones and increased ambient temperature in goats was found out (Todini, 2007) and in sheep (Webster *et al*., 1991; Starling *et al*., 2005; Todini, 2007). Sivakumar *et al.* (2010) reported in heat stressed goats T_3 and T_4 level decreased from 4.55 and 21.27 to 3.21 and 16.70pmol/L. There is decline in T_3 and T_4 levels during short and long term exposure to solar radiation in goats (Helal *et al*., 2010; Gupta *et al*., 2013). Thyroid function decreases as an acclimation response to alleviate HS. According to Smith and Vale (2006), this reduction may be due to effect of heat on hypothalamus pituitary axis (HPA) to decrease in TRH which enable animal to reduce basal metabolism. However, in contrast a study conducted by McGuire *et al*. (1991) the T_4 concentrations tended to increase in the thermal stress treatment compared with the thermal comfort restricted intake treatment. They further concluded that the effects of nutrition and thermal stress did not noticeably alter concentrations of metabolic hormones in lactating dairy cows. Similarly, Sejian *et al.* (2008) reported significant increase in T_4 level in heat stressed goats and found no difference between control and HS groups for T_3 level.

2.7 Impact of Heat Stress on metabolic enzyme activities

The Acid phosphatase (ACP) and Alkaline phosphatase (ALP) are generally considered as the metabolic enzymes. In a study on sheep, Sejian *et al*. (2010a) concluded that both ACP and ALP decreased significantly in HS and combined stress i.e., HS and NS stress groups as compared to the control (TNZ). However, NS alone did not significantly influence the level of these enzymes in their study. Similar result of lower ALP in heat stressed goat was reported by other authors (Helal *et al*., 2010 and Gupta *et al*., 2013).All these authors attributed this reduced metabolic enzymes in heat exposed sheep to the reduction in both thyroid activity as well as circulating thyroid hormone concentration in their study. Banerjee *et al*.(2015) reported significant increase Aspartate

aminotransferase (AST) in goats. They also observed that the level of AST was significantly higher for cold stress (Gaddi and Chegu breeds) as compared to HS (Sirohi and Barbari breeds) in their study and they attributed this difference to breed variation in adaptation to different climatic condition. The increased activity in some enzymes with rising temperature may be due to the accelerated reactions at higher temperature. Further, Gupta *et al*. (2013) reported significantly higher concentration of both Alanine aminotransferase (ALT) and AST in goat and concluded that both AST and ALT are helpful in diagnosis of welfare of animals. In addition, both Naqvi *et al*. (1991) and Nazifi *et al*. (2003) also reported similar HS induced significantly higher concentration of both ALT and AST in sheep. They attributed this increase to reflect higher adaptation capability in these animals. However, Sharma and Kataria (2011) reported that HS only significantly increased ALT but observed no effect on AST concentration in goat. Similarly HS induced non-significant effect on AST in goat was reported by Ocak *et al*., (2009).

2.8 Impact of nutrition on rumen functions

Forages are perennial and annual crops grown for use as pasture, green chop, hay, silage. They contain energy, significant levels of protein, fiber, and vitamins A and E and on sun curing they may contain significant levels of vitamin D (NDDB, 2012). Adequate amount of forage and fiber is essential to maintain normal rumen function and metabolism (Dschaak, 2012). Extremes in either direction can adversely affect animal performance and health.

In order to meet the animal's energy needs, for adequate microbial protein synthesis and for maintaining normal fiber digestion as well as other rumen functions sufficient propionic acid production is essential and this could be met by proper intake of non-structural carbohydrate (NSC). Inadequate NSC may depress the energy available from propionic and lactic acid production, reduce microbial protein synthesis, and decrease fiber digestion. Excessive NSC may lead to reduced fiber digestibility, acetic acid production, as well as cause abnormalities in rumen tissue, which may lead to ulcers and liver abscesses.

Rumen microorganisms cannot tolerate high levels of fat. Unsaturated fats are more likely to interfere with the ruminal fermentation than saturated fatty acids. However, Saturated fats are believed to be relatively inert in the rumen because of their high melting point and low solubility in rumen fluid whole oilseeds are less likely to interfere with ruminal fermentation compared to free oils (Chalupa*et al*., 1984). Whole oilseeds are slowly digested, allowing for a slow release of the oil in the rumen and more extensive microbial hydrogenation (Chen *et al*., 2008). Further, appropriate mineral mixture concentrations are required for maintaining normal rumen function (Garg *et al*., 2007). For example, magnesium is essential for rumen digestion, phosphorus is essential for normal rumen metabolism, sulfur is essential for synthesis of amino acids by rumen microbes while calcium can have pronounced effect on rumen metabolism.

2.9 Impact of Nutritional Stress on metabolic activity in livestock

Rangelands are the major systems in which livestock are reared in the majority of the world. The climatic changes have led to the decline in pasture quantity and quality. As, a consequence of this the animals are threatened by the reduced availability of feed. The NS is the major environmental threat the animals are exposed to especially in the tropics. In the tropics especially during the summer or dry period only the 30 per cent of the fodder availability of that of the spring is available. Under-nutrition in livestock can occur in late spring and summer due to increased energy output for thermoregulation and concurrent reduction in energy intake. Impact of seasonal fluctuations and climatic extremes on herbage quality and quantity are considered as an important source of influence on the well-being of livestock in extensive Production systems. This can lead to the impairing of reproductive and productive efficiency of grazing animals (Ali and Hayder, 2008). The extreme heat condition during the summer months have negative impacts on the grazing animals, and are capable of inducing nutritional imbalances in them (Sejian *et al*., 2014a). In semi-arid tropical regions low quality and inadequate feed is a chief factor in under-production of animals (Funston *et al*., 2010).

Nutrition state is closely related to endocrine and neuro endocrinal cues (Sejian *et al.*, 2014b).They further reported that the more the feed is restricted, the lower the thyroid hormone concentrations in sheep. Kong *et al*. (2004) energy balance can also play a major role in affecting the decrease in plasma thyroid hormone levels in small ruminants, thus indicating the importance of optimum nutrition for maintaining appropriate thyroid hormone levels in sheep (Todini, 2007). Similarly, Kong *et al*. (2004) and Kafi *et al*. (2012) stated that the plasma thyroid hormone concentrations are correlated with feed intake in several ruminant species. The quantity and quality of food eaten is a major factor determining plasma concentrations of Thyroid hormone (Todini, 2007). Thyroid hormones could directly depict the nutritional status of the animals (Sejian *et al*., 2014b). Further, they documented that the circulating thyroid hormone concentration in blood can be a good indicator for NS in sheep. Similarly, Caldeira *et al*. (2007) stated that the Circulating Thyroid hormone concentrations seem better correlated with feed intake than adiposity status.

The level of plasma free thyroid hormone and T_3 were found to be reduced in the feed deprived adult sheep. While, on increasing the nutrition supplementation led to an in increase the level of T_3 in the adult sheep. The level of plasma T_4 increased on supplementing concentrate to the lactating ewes (Todini, 2007) and plasma T_3 levels were higher in rams with high amounts of ingested energy and protein (Zhang *et al*., 2004).

In the Sahel desert, plasma T_3 and T_4 levels did not change significantly from the beginning of the cool season (December) until the end of the dry warm season (May), but a highly significant rise of both hormones was observed at the onset of the humid warm season (June) (Assane and Sere, 1990). The reason for this is that an enhanced thyroid activity during the humid warm season in such environments is functional for the animals facing the increased availability of both quality and quantity of food, following the seasons characterized by food shortage.

Leptin is a hormone produced by the ob gene in adipose tissue and often called as obese gene (Zhang *et al*., 1994). It is a polypeptide hormone of 167 amino acids and secreted by adipose tissue in direct proportion to fat tissue content, nutritional status and tissue location (Chilliard *et al*., 2005). Leptin may be found in the circulation in the free form or complexed with leptin-binding proteins, and this characteristic appears to be species-specific and dependent upon physiological status (Garcia *et al*., 2002). According to Zieba *et al*. (2005), the expression and secretion of leptin are associated with body fat mass and are affected by alterations in feed intake. The leptin gene expression in Peripheral blood mono nuclear cells (PBMCs) was regarded as an indirect measure of physiologically sustainable concentrations of leptin and the messenger Ribo nucleic acid (mRNA) expression of leptin in lymphocytes is an indirect indicator of nutritional status of animal (Hyder, 2012). Food deprivation results in a decline in the circulating leptin, and if untreated, results in a cessation of reproduction, a survival mechanism that is achieved relatively quickly in monogastrics and less quickly in ruminants (Schreihofer *et al*., 1993). Leptin stimulates the hypothalamic–adenohypophyseal axis especially in nutritionally stressed animals (Zieba *et al*., 2005). The stimulation of the hypothalamic–gonadotropic axis by leptin in cattle and sheep is observed predominantly in animals and tissues pre-exposed to profound negative energy balance. Leptin increases the release of Gonadotropin releasing hormone (GnRH) by the hypothalamus (Amstalden, 2003). It was found that the nutrition stress reduces the leptin concentrations and when the animals are re-fed, the hormone levels gets re-estabilished in beef heifers (León *et al*., 2004). Similar findings have been reported in heifers (Amstalden *et al*., 2000) and in cows (Amstalden, 2003) where fasting decreases concentrations of leptin, insulin and (Insulin like growth factor – I) IGF-I, and Luteinizing hormone (LH) pulsing frequency and the infusion of recombinant leptin regulates the insulin concentrations and thereby resulting in hyper stimulation of LH secretion. The leptin gene is highly preserved across species and was found to be specifically located on chromosome 4q32 in the bovine (Stone *et al*., 1996). Hyder (2012) reported that there was a significant reduction

in serum leptin levels in the nutritionally stressed goats only on 10th and 30th day of study. The animals have characteristic physiological ability to survive the NS by altering the leptin levels which are required for maintaining the physiological normalcy.

2.10 Concept of multiple stresses

Optimized livestock production is governed by so many factors such as genetic potentiality of the animals, environmental and climatic factors, health status, nutrient availability, and other factors (Soren, 2012). In the present scenario, one of the major challenges affecting the livestock production is the changing climate. Its negative impacts are felt more severely by poor people in developing and developed countries, who rely heavily on the natural resource for their survival. Rural poor communities depend entirely on agriculture and livestock for their livelihood (Sejian *et al*., 2014b). The climate change has brought about various changes like increase in the environmental temperature, changes in pattern and distribution of rainfall, elevated levels of CO_2 in the atmosphere etc. all these factors are the root cause for decline in pasture availability, reduced nutrient quality of the available pastures and water scarcity, these all factors pose a great threat to the livestock production and survival. In the tropics HS is the most important environmental stress affecting the livestock production. Apart from the HS also the NS form another constraint, as the pasture availability decreases during the summer. Rangelands are the largest land use system on earth. These pastoral systems are those in which people depend predominantly for rearing livestock and livelihoods (Sejian *et al*., 2013). Reports indicate that only 30 per cent of the fodder available during that of the spring is available during the dry period in the tropics. Another major threat faced in arid and semi-arid tropical environments are their low biomass productivity, high climatic variability, and scarcity of water (Maurya *et al*., 2010; Sejian *et al*., 2010a; Sejian *et al*., 2013). Marai *et al*. (2010) stated that factors such as water

deprivation, nutritional imbalance and nutritional deficiency may exacerbate the impact of HS. Apart from all these environmental challenges the productive potentials of animals are also affected by walking stress. Walking stress is another important stress which hampers livestock production especially in extensive system of rearing (Sejian *et al*., 2012b). During extreme summer months as a result of reduction in pasture availability the animals have to walk a long distance in search of pasture and these act as additional stress to these animals. Sejian *et al*. (2013) reported that these stresses lead to alteration in the process of homeostasis and metabolism of the animals. Sejian *et al.* (2010a) and Soren, (2012) reported that the HS occurs coupled with NS and the effect of these cumulative stresses have more severe negative impact on the productive and reproductive functions of the animals than when they occurred individually. Thus the animals in the tropic are vulnerable to these environmental stresses, leading to hampering the sustainability and productivity of the livestock in these regions and ultimately causing a huge financial burden for the poor and marginal farmers. In a study conducted on Malpura ewes by Sejian *et al*. (2010b) the level of T_3 and T_4 were found significantly lower in the combined stress (HS and NS) group. Further, Sejian *et al*. (2014b) documented significant reduction in the T_3 and T_4 levels when ewes are subjected to multiple stresses (heat, nutritional and walking stress). They attributed the reason for this to be due to the reduced metabolic activity of these ewes to suppress heat production. They further added that the level of ACP and ALP also declined in the combined stress (HS and NS) group on comparison with the control group (*ad libitum* and TNZ), even though the nutritionally deprived group did not showed any significant variation in the levels of these metabolic enzymes. However, there are no reports on influence multiple stresses on the impact of metabolic activity in goats.

2.11 Impact of Heat Stress and nutritional status on antioxidant status in livestock

It has been reported that HS was associated to bring oxidative stress in livestock (Bernabucci *et al*., 2002; Mujahid *et al*., 2005; Sivakumar *et al*., 2010)

possibly leading to mitochondrial oxidative damage, as the mitochondria are generally accepted as the largest source of reactive oxygen species (ROS). ROS, the major culprits for causing oxidative stress, are constantly generated *in vivo* as an integral part of metabolism. They trigger progressive destruction of polyunsaturated fatty acids (PUFA), ultimately leading to membrane destruction (Kumar *et al*., 2011). The oxidative stress is characterised by increased plasma levels of super-oxide dismutase (SOD) and glutathione peroxidase (GPx). Animal body employs antioxidants to quench these free radicals. The enzymatic antioxidants like SOD, catalase (CAT) and GPx act by scavenging both intracellular and extracellular superoxide radical and preventing lipid peroxidation of plasma membrane. AST is positively correlated with and show the importance of liver function for the maintenance of oxidative status (Tanaka *et al*., 2011). Estimation of ROS and the biological antioxidants in plasma reflects the oxidative status of the animals. Severe negative energy balance is reported to be highly associated with high oxidative stress in cows (Pedernera *et al*., 2010). Further, it has been reported that the total antioxidant capacity of goats were affected by HS (Sivakumar *et al*., 2010). In small ruminants such as goats, the HS has pronounced effects on the oxidative status of animals than nutritional factors. However, oxidative stress in dairy goats can also be affected by plane of nutrition (Celi *et al*., 2010).

2.12 Heat Shock Protein expression in digestive tract

The body responds to a variety of insults that damage intracellular proteins by producing HSPs. These 'molecular chaperones' stabilize proteins, protecting them against denaturation, and re–fold denatured proteins, restoring their functionality (Kregel, 2002). Exposure to heat results in redistribution of blood to the periphery and compensatory reduction in the blood supply to the gut, which damages cells lining the gut, permitting endotoxin to enter the body (Cronje, 2005). Exposure to heat induces production of HSP–70 in the gut, liver and other tissues (Hotchkiss *et al*., 1993; Flanagan *et al*., 1995). The beneficial effects of HSP are so numerous that it is not surprising that the productive life of Holstein

cattle is associated with promoter variants in the gene that encodes HSP–70 expression (Schwerin *et al*., 2003). The finding that the greatest increase in HSP concentrations following exposure to heat occurs in the tissue of the liver and gut (Flanagan *et al*., 1995) provides additional support for the contention that damage to the cells of the gut is central to the adverse effects of heat load (Cronje, 2005). The possibility that the integrity of the gut lining is critical to resilience against hyperthermia has great significance for livestock species because energy–dense production diets are known to damage the gut lining (Cronje, 2005). Although it seems probable that gut permeability in hyperthermic ruminants would be compromised to a similar extent as in monogastric species, it is possible that the consequences of this would be more severe since the rumen is populated with bacteria that are predominantly of the endotoxin–producing gram–negative type (Cronje, 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Location

The experiment was carried out at the National Institute of Animal Nutrition and Physiology experimental livestock farm, Bengaluru, India which is located in southern Deccan plateau of the country at longitude 77º 38´E and the latitude of 12º 58´N and at altitude of 920 m above mean sea level. The average annual maximum and minimum ambient temperature ranges between 15 to 36ºC. The mean annual relative humidity ranges between 20 and 85%. The annual rainfall in this area ranges from 200 to 970 mm with an erratic distribution throughout the year. The average annual minimum and maximum temperature ranges between 15-22 and 27-34ºC respectively. The average annual RH ranges between 40-85 %. The experiment was carried out during April-May. The temperature and RH variations during the study period (April-May) ranged between 24-38 and 30-38 % respectively under hot semi-arid environment.

The THI values were calculated as per method described by McDowell (1972). Accordingly the formula used was $THI = 0.72(T_{db} + T_{wb}) + 40.6$ where, T_{db} = Dry bulb temperature in ºC; T_{wb} = Wet bulb temperature in ºC.

3.2 Animals

Osmanabadi is a dual purpose (meat and milk) hardy goat breed, which originated in the semi-arid areas of central tropical India. The Osmanabadi breed derives its name from its habitat and distributed in Ahmednagar, Solapur and Osmanabad districts in Maharashtra (Motghare *et al.,* 2005, Deokar *et al.,* 2006). It has spread over a wide range of agro-climatic conditions in Maharashtra and adjoining parts of Karnataka and Andhra Pradesh. The goats are large in size. Coat color varies, but mostly it is black (73%) and the rest are white, brown or spotted. The average BW of adult male and female animals are 34 kg and 30 kg respectively. The breed is considered useful both for meat and milk. Average

daily yield varies from 0.5 to 1.5 kg for a lactation length of about 4 months. In favorable conditions the does will breed regularly twice a year and twinning is common in this breed.

The study was conducted in 24 (one year old) Osmanabadi bucks weighing between 15 to 20 kg. The animals were housed in well-ventilated sheds made up of asbestos roofing at the height 2.4 m and open from side and maintained under proper hygienic conditions. Prophylactic measures against goat diseases like goat pox, peste des petits ruminants, enterotoxaemia; endo and ectoparasitic infestations were carried out as prescribed by the health calendar of the institute to ensure that the animals were in healthy condition throughout the study.

3.3 Technical program

The study was conducted for a period of 45 days. Twenty four adult bucks were used in the study. They were randomly allocated into four groups of six animals each viz., C (n=6; control), HS (n=6; heat stress), NS (n=6; nutritional stress) and CS (n=6; combined stress). The animals were stall fed with a diet consisting of 60% roughage (Hybrid Napier) and 40 % concentrate (maize 36 kg, wheat bran 37 kg, soya bean meal 25 kg, mineral mixture 1.5 kg and common salt 0.5kg/100 kg of feed). C and NS bucks were maintained in the shed in thermo-neutral zone (TNZ) while HS and CS bucks were exposed outside to summer heat stress between 10:00 h to 16:00 h. C and HS bucks were provided with *ad libitum* feeding while NS and CS bucks were provided with restricted feed (30% of intake of *ad libitum*) to induce nutritional stress. All four group animals were fed and watered individually throughout the study period. All cardinal weather parameters were recorded both inside and outside the shed. Rumen liquor and blood samples were collected at fortnightly interval. The study was conducted after obtaining approval from the institute ethical committee for subjecting the animal to both heat and nutritional stresses and for slaughtering the animals for collection of organs for histopathological section and gene expression.

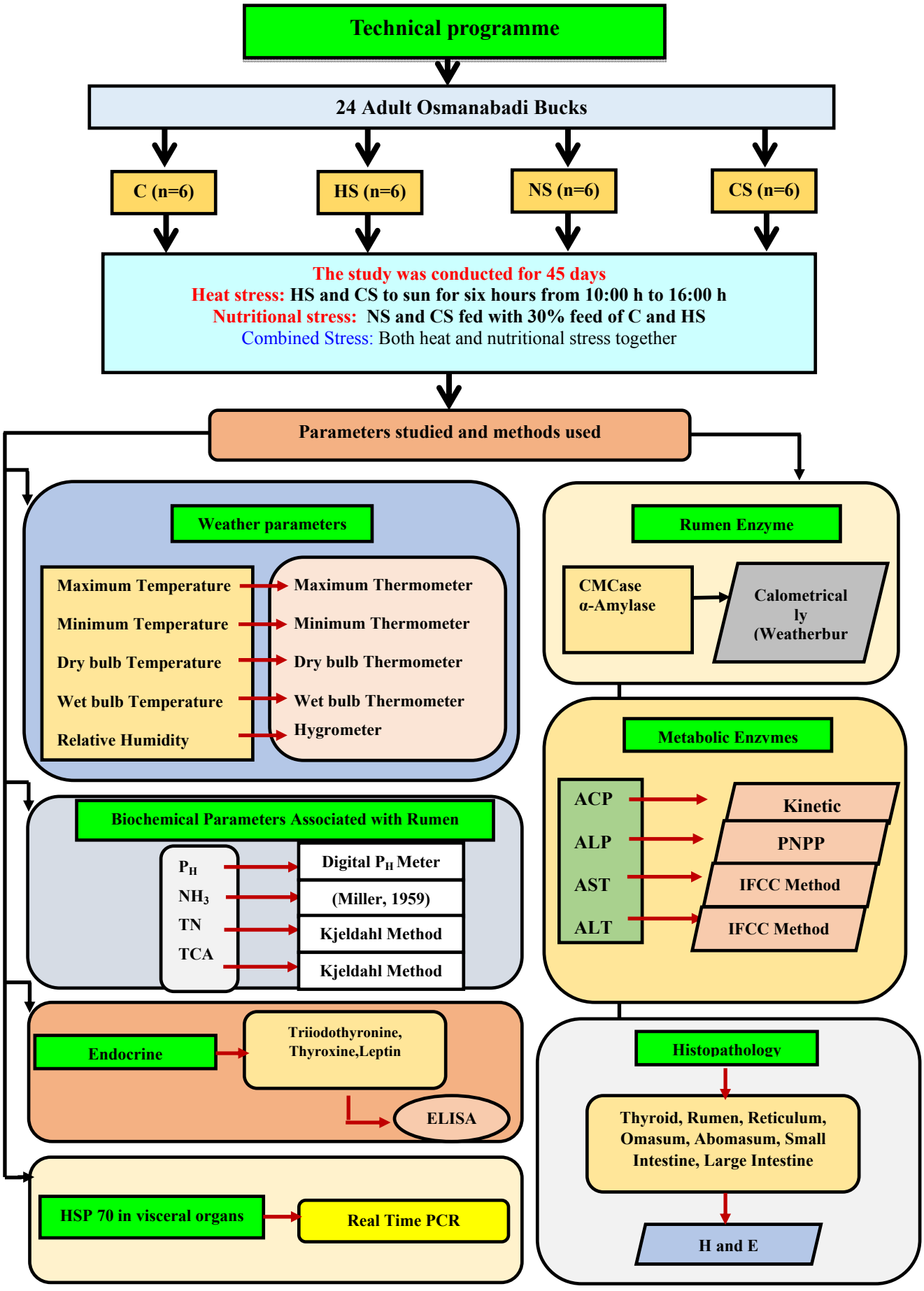


Fig 1: Represents overall technical programme for the entire study.

ACP- Acid phosphatase, ALP- Alklaline phosphatase, AST- Aspartate Aminotransferase, ALT- Alanine Aminotransferase, C-Control, CMCase-Carboxymethyl cellulase, CS-Combined stress, ELISA-Enzyme Linked Immunosorbent Assay,H and E-Haematoxylin and Eosin,HS-Heat stress, HSP-Heat Shock Protein,NS-Nutritional stress, PCR- Polymerized Chain Reaction, TCA- Tricholoro acetic acid, TN- Total nitrogen.

The study was conducted after obtaining approval from the institute ethical committee for subjecting the animal to both heat and nutritional stresses. Fig.1 depicts the technical programme of the current study.

3.4 Weather parameters

The weather parameters were recorded twice daily (8:00 hr and 14:00 hr) for the entire study period. The maximum, minimum, wet bulb and dry bulb temperatures were recorded using maximum thermometer, minimum thermometer, wet bulb thermometer and dry bulb thermometer respectively. The humidity was recorded using hygrometer.

3.5 Rumen liquor collection Apparatus

- Hand held vacuum pump
- Conical flask with an inlet
- Perforated silicon tubes
- Hard plastic pipe (8 inches)

Procedure

1. The plastic pipe was inserted into the mouth of the animal. The perforated silicon tube was then introduced through the plastic pipe into the stomach (rumen).
2. Due care was taken so that the silicon tube does not enter the trachea (animal will not cough)
3. Once the tube has reached the rumen the other end of the tube was connected to the collecting flask which was attached to the suction pump. Suction was created by means of the hand held vacuum pump.
4. Rumen liquor was drawn into the collecting flask.
5. After collection it was immediately transferred in a plastic bottle and stoppered and then placed in an ice box and brought to the laboratory.

Table 1: Ingredients and chemical composition of concentrate mixture and hybrid napier hay fed to goats

Attribute	Concentrate mixture (kg/100 kg)	Napier hay (*Pennisetum purpureum*)
Ingredients		
Maize	36	-
Wheat bran	37	-
Soybean meal	25	-
Mineral mixture	1.5	-
Salt	0.5	-
Chemical composition (%)		
Dry matter	92.9±0.079	94.0±0.289
Organic matter	95.9±0.190	95.4±0.298
Crude protein	19.6±0.176	6.21±0.098
Ether extract	1.82±0.183	1.49±0.026
Total ash	4.10±0.190	4.64±0.298
Fibre fractions (%)		
Neutral detergent fibre	40.4±1.400	82. 9±0.881
Acid detergent fibre	11.1±0.239	64.6±1.950
Acid detergent lignin	2.14±0.029	12.3±0.651
Nutritive value		
Total digestible nutrients %*	72.2	55.0
Digestible energy (kJ/kg) *	13.3	10.1
Metabolizable energy (kJ/kg) *	10.9	8.28

*Calculated values

3.5.1 Rumen associated parameters

3.5.1.1 pH determination

The pH of the rumen liquor was determined using a digital pH meter.

Procedure

The pH meter was switched-on at least 2 hrs prior to recording of the pH of the rumen liquor. The pH meter was calibrated with buffer (pH 4 and pH 7). The electrode of the pH meter was placed into the bottle containing the rumen liquor and pH was recorded.

3.5.1.2 Strained rumen liquor (SRL)

The rumen liquor was filtered through four layers of cheese/ muslin cloth to obtain strained rumen liquor.

3.5.2 Sampling of rumen liquor

3.5.2.1 Estimation of VFAs through gas chromatography (GC)

For VFA estimation 0.2 ml of metaphosphoric acid (25%; w/v) was added to 0.8 ml of strained rumen liquor (SRL) in micro centrifuge tube and samples were centrifuged at 5000 rpm for twenty min. The supernatant was stored at -20°C and later analyzed for VFA concentrations by GC (Agilent; Model 7890A GC System,Shanghai, China) using Flame Ionization Detector, programmable temperature vaporizer injector and capillary column (Agilent J&W DB-WAX GC Column 40 m × 0.18 mm × 0.18 μm).The analytical conditions for fractionation of VFA were injection port temperature 250^{o}C, column temperature step up from 60 to 200^{o}C in 7 min with a hold time of 10 min and the detector temperature was maintained at 300^{o}C with GC grade air, hydrogen and nitrogen was used as carrier gas with flow rate of 1.0 ml/min. The injector was equipped with a glass liner of glass wool to separate particles of dirt from the sample. The Rumen liquor

samples were injected by an automatic injector at an injection volume of 1 µL using the split method and a 30:1 splitting ratio. Standard solutions of appropriate concentration (mmol/L) were prepared from the individual substances namely acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid of analytical purity (Sigma-Aldrich) and the individual VFAs in the rumen liquor sample were identified based on the retention time of the standard. The concentrations of the individual VFA in the rumen liquor samples were determined by recording the area of both the VFA mixed standards and as well as the sample and expressed as mmol/L.

3.5.3 Sampling of rumen liquor for other biochemical parameters

20 ml of SRL was sampled for biochemical analysis. About 2-3 drops of saturated mercuric chloride ($HgCl_2$) was added in the SRL sample and stored at -20^{o}C till analyzed.

3.5.3.1 Total nitrogen

Total nitrogen was estimated by Kjeldahl method using total nitrogen analyzer complete with digestion and auto distillation unit and accessories (MODEL NO. KB 20S and VAP 45 S,Gerhardt, Cologne, Germany).

Principle

When sample containing nitrogen is digested with Sulphuric acid (H_2SO_4), the total nitrogen is converted into ammonia sulphate $(NH_4)_2SO_4$. In the presence of alkali, ammonia is released from the ammonium sulphate and released ammonia is distilled and trapped in a known volume of standard acid, which is then back titrated with the standard alkali. The process is completed in three steps. The reactions during estimations are as follows:

Digestion

Organic nitrogen + conc. $H_2SO_4 = (NH_4)_2SO_4$

Plate 1: Collection of rumen liquor

Plate 2: Preparation of reagents for estimation of rumen associated parameters

Distillation

$(NH_4)_2SO_4 + 2NaOH = Na_2SO_4 + 2NH_3 + 2H_2O$

Trapping

$2NH_3 + H_2SO_4 = (NH_4)_2SO_4$

Apparatus

- Electronic / chemical balance
- Micro Kjeldahl distillation assembly
- Digestion bench
- Kjeldahl flasks (100 ml capacity)
- Pipette
- Conical flask
- Beaker
- Burette
- Volumetric flask (100 ml capacity)

Reagents

- Digestion mixture ($K_2SO4 + CuSO_4$ in the ratio of 9:1): 90gm potassium sulphate and 10gm copper sulphate mixed together.
- Concentrated H_2SO_4
- 40% Sodium hydroxide (NaOH) solution: weigh 40gm NaOH pellets and add water to 100 ml
- 0.01N NaOH solution
- 0.01N H_2SO_4
- Methyl red indicator: dissolve 0.1gm methyl red indicator in 60 ml ethanol and add distilled water to make volume upto 100 ml.

Procedure

Digestion

1. 2.0 ml of strained rumen liquor was taken in a Kjeldahl flask.

2. 10 ml of concentrated $H_2SO_4$was added to it.
3. 2-3 gm digestion mixture was added.
4. The flask was kept on digestion bench and allowed to boil gently. Care was taken to avoid the bumping of the contents.
5. When the solution became clear blue, the flask was removed from the digestion bench and allowed to cool.
6. 5 to 10 ml of distilled water was added to the Kjeldahl flask. The whole material was transferred in to a 100 ml volumetric flask with repeated washings of distilled water. The volume was made up to 100 ml with distilled water.

Distillation

1. The Kjedahl distillation assembly was set
2. 10 ml 0.001$(NH_4)_2SO_4$ (A) was taken in a conical flask and 2-3 drops of indicator was added into it.
3. The flask was kept under the condenser in such a way that the tip of the condenser was dipped in acid, so that to avoid ammonia loss during distillation.
4. 10 ml aliquot of digested sample was taken and transferred into the Kjeldahl assembly.

   15- 20 ml 40% NaOH was added to make the aliquot contents alkaline and the stopper was put immediately and allowed for distillation for 15 min.

Titration

1. The flask was removed after washing the tip of the condenser with distilled water.
2. The contents of the flask was titrated with standard till pink colour developed (end point).
3. The volume of alkali (B) used for titration was recorded.
4. A blank was run using all reagents but no sample following the similar procedure to estimate the nitrogen content of the reagents, if any.

Calculation

1 ml 0.01N H_2SO_4 = 0.00014 gm nitrogen

$$\text{Nitrogen / 100 ml rumen liquor} = \frac{v \times 0.00014 * D}{V \times A} \times 100$$

Where, v = a-b

D = Dilution (volume made in volumetric flask)

V= Initial volume of rumen liquor taken for the digestion

A= Aliquot taken (10 ml)

a=Titre volume of sample (ml)

b= Titre volume of blank (ml)

Nitrogen of sample = nitrogen of sample – nitrogen of blank

3.5.3.2 Trichloro acetic acid (TCA)

TCA was estimated by Kjeldahl method usingtotal nitrogen analyzer complete with digestion and auto distillation unit and accessories (MODEL NO. KB 20S and VAP 45 S,Gerhardt, Cologne, Germany).

Principle

TCA perceptible nitrogen is the fraction which represents true protein. True proteins are precipitated by exposing it to low pH by adding trichloroacetic acid. These precipitated proteins are analysed further for nitrogen content by micro- Kjeldahl method as described for total nitrogen.

Procedure

1. 2 ml of rumen liquor was taken in a centrifuge tube.
2. 2 ml of 20 % TCA solution was added into the tube.
3. The tubes were left undisturbed overnight.
4. The tubes were centrifuged at 5000 rpm for 10 min.
5. The precipitate was transferred with repeated washings of distilled water into Kjeldahl flask.
6. Digestion, distillation and titration was carried out as described for total nitrogen.

3.5.3.2 NH_3-N

NH_3-N in rumen liquor was estimated by calorimetric method as described by Weatherburn,(1967) using spectrophotometer.

Reagents

Solution A: 1 gm phenol and 5 mg sodium nitroprusside was dissolved in 100 ml of distilled water.

Solution B: 0.5 gm NaOH and 0.84 ml sodium hypochlorite was dissolved in 100 ml distilled water. Solution A and B were stored in amber colour bottles in refrigerator.

Stock standard solution of $(NH_4)_2SO_4$: 0.048 gm$(NH_4)_2SO_4$was dissolved in 100 ml distilled water to get a final concentration of 10 mg of NH_3-N per 100 ml of solution.

Working standard solution of $(NH_4)_2SO_4$: 10 ml solution was taken and made up to 100 ml (The solution contains 0.01 mg $(NH_4)_2SO_4$per ml).

Procedure

Prepare tubes as follows

Tube No.	1	2	3	4	5	6	7	8	9
Dist. Water (ml)	1.00	0.95	0.90	0.80	0.60	0.40	0.20	0.00	0.90
Standard Sol. (ml)	-	0.05	0.10	0.20	0.40	0.60	0.80	1.00	-
Sample	-	-	-	-	-	-	-	-	0.10
NH_3-N (µg) in tubes	0.00	0.5	1.0	2.0	4.0	6.0	8.0	10.0	?

1. 5 ml of solution A was added in all tubes.
2. Immediately after adding solution A, solution B was added in all the tubes and the contents were mixed vigorously.

3. All the tubes were incubated for 15 min at 39°C for colour development
4. The absorbance "A" was recorded at 625 nm against blank.
5. The calibration curve was prepared by plotting "A" against standard NH_3-N concentration.
6. The concentration of NH_3-N in the sample was calculated by reading "A" on the calibration curve.
7. (U-2900) UV- VIS Spectrophotometer double beam with inbuilt LCD Monitor, Hitachi, Kyoto, Japan was used to record the absorbance.

3.6 Estimation of Rumen enzymes

The rumen enzymes were estimated on both 0^{th}day and 45^{th} day. 2 ml of rumen liquor sample was transferred and centrifuged at 4°C for 20 min at 14,000 rpm. The supernatant was collected and was deep frozen and analysed for the extracellular enzyme activity. To the pellets 0.1 molar of Phosphate buffer (PO_4), 400µL of carbon tetrachloride (CCl_4) and 400 µL Lysozyme (0.4%) were added and incubated at 39°C for 3 hrs. Centrifuge at 4°C after 3 hrs for 20 min at 14,000 rpm. The supernatant was collected and was deep frozen for analysing intracellular enzyme activity.

Principle

3.6.1 CMCase (EC.3.2.1.4: endo – 1, 4 β – glucanase):

The enzyme catalyses the hydrolysis of cellulose, releasing glucose by breaking the β – 1, 4- linkages. The enzyme is of microbial origin. The amount of glucose released is measured calorimetrically to estimate the enzyme activity.

3.6.2 α–Amylase (EC 3.2.1.1:1, 4-α-D-Glucanohydrolase):

The enzyme attacks on α -1, 4 – glucan linkages of starch and glycogen releasing maltose, isomaltose, larger oligo- saccharides and glucose. The α–Amylase, activity is determined by measuring the rate of release of reducing sugars during the incubation of enzyme with the substrate.

Reagents

PO_4Buffer (0.1 molar, $_P$H 6.8):

Solution A: 28.392 gm of Disodium hydrogen phosphate was weighed and the volume was made up to 1000ml with distilled water.

Solution B: 31.206 gm of sodium dihydrogen phosphate was weighed and the volume was made up to 1000ml with distilled water.

24.5 ml of Solution A and 25.5 ml of Solution B was added and the volume was made upto100 ml with distilled water and was checked for$_P$H6.8.

0.4% Lysozyme: 0.4gm of Lysozyme was added and the volume was made up to 100 ml with PO_4 buffer.

CMCase: 1gm of CMC was added in 50 ml of distilled water and the content was heated. The volume was made up to 100 ml after cooling, with distilled water.

Dintro salicylic acid (DNS): 10gm of sodium hydroxide, 10 gm of DNS and 2gm of phenol were added the volume was made up to 1000 ml (Just before use 0.05% of sodium sulphite is to be added per 100 ml of DNS).

Rochelle salt (40%): 40 gm of potassium sodium tartarate was weighed and the volume was made up to 100 ml.

Standard Glucose solution (0.1%): 0.1 gm Glucose was weighed and the volume was made up to 100 ml with distilled water.

Starch solution (1%):1gm starch was added to 50 ml distilled water and the content was heated and the volume was made up to 100 ml after cooling, with distilled water

Procedure:

The tubes were prepare as follows for calculating the standard curve:

Tube No.	1	2	3	4	5	6	7	8	9
Dist. Water (µL)	1000	875	750	625	500	375	250	125	0
Glucose Standard (µL)	0	125	250	375	500	625	750	875	1000

DNS (µL)	1500	1500	1500	1500	1500	1500	1500	1500	1500
Rochelle salt (µL)	500	500	500	500	500	500	500	500	500
Dist. Water (µL)	7250	7250	7250	7250	7250	7250	7250	7250	7250

1. The contents were mixed by vortexing and kept in boiling water bath for 10 min and 500 µL of Rochelle salt and 7250 µL of distilled water were added into each tube.
2. The absorbance"A" was measured at 575 nm.
3. The calibration curve was prepared by plotting "A" against standard Glucose concentration.
4. (U-2900) UV- VIS Spectrophotometer double beam with inbuilt LCD Monitor, Hitachi, Kyoto, Japan was used to record the absorbance.

Protocol for CMCase:

The CMCase enzyme activity was estimated calorimetrically using Modified DNS method for reducing sugars described by Miller, (1959) using spectrophotometer.

Tube No.	Test	Control	Blank
Dist. Water (µL)	-	-	1000
PO_4(µL)	500	500	-
CMCase (µL)	250	250	-
Sample (µL)	250	250	
DNS (µL)	-	1500	1500

The contents of the tubes were mixed and incubated at 39°Cfor 60 min.

1. DNS was added to the test and was kept in boiling water bath for 10 min.
2. 500 µL of Rochelle salt and 7250 µL of distilled water were added to every test tube and was mixed well by vortexing.
3. The absorbance was read at 575 nm against Blank.
4. The enzyme activity was expressed asµmolof glucose/ml/hr.
5. (U-2900) UV- VIS Spectrophotometer double beam with inbuilt LCD Monitor, Hitachi, Kyoto, Japan was used to record the absorbance.

Protocol for α-Amylase:

The α-Amylase enzyme activity was estimated calorimetrically using Modified DNS method for reducing sugars described by Miller, (1959) using spectrophotometer.

Tube No.	Test	Control	Blank
Dist. Water (µL)	-	-	500
PO_4(µL)	250	250	-
Starch(µL)	125	125	-
Sample (µL)	125	125	-
DNS (µL)	-	1500	1500

1. The contents of the tubes were mixed and incubated at 39°Cfor 60 min.
2. DNS was added to the test and was kept in boiling water bath for 10 min.
3. 500 µL of Rochelle salt and 7250 µL of distilled water were added to every test tube and was mixed well by vortexing.
4. The absorbance was read at 575 nm against Blank.
5. The enzyme activity was expressed as µmolof glucose/ml/hr.
6. (U-2900) UV- VIS Spectrophotometer double beam with inbuilt LCD Monitor, Hitachi, Kyoto, Japan was used to record the absorbance.

3.7 Blood collection

Five ml of blood samples were collected at fortnightly interval from all four groups simultaneously at 11:00 h using 20 gauge sterilized needles and plastic syringe from external jugular vein in tubes. Heparin (Sisco Research Laboratories pvt. Ltd, Bombay) was used as the anticoagulant at the rate of 10 IU per ml of blood.

3.7.1 Plasma separation

Plasma was separated from blood by centrifugation at 3500 rpm at room temperature for 20 min. The plasma was then divided into aliquots in micro centrifuge tubes, and kept frozen at -20°C till further analysis. Plasma samples were used to estimate enzymes and endocrine parameters.

3.7.2 Estimation of metabolic enzymes

3.7.2.1 AST

The plasma AST was estimated using International Federation of Clinical Chemistry (IFCC) method using spectrophotometer (Proton, Bangalore, India).

Principle

$$\text{L-Aspartate + 2-Oxoglutarate} \xleftrightarrow{\text{AST}} \text{L-Glutamate + oxaloacetate}$$

$$\text{Oxaloacetate + APADH (NADH Analogue) + } H^{+} \xleftrightarrow{\text{MDH}} \text{L-Malate + APAD (Oxidised NADH Analogue)}$$

Where,

AST -Aspartate aminotransferase

MDH -*Malate dehydrogenase*

APAD -Adenine-dinucleotide

Plate 3: processing of rumen liquor for estimation of rumen enzymes

Plate 4: Collection of blood from the animals

Procedure

1. 0.8 ml of R1, 0.2 ml of R2 and 100µL of plasma were added and mixed well in a test tube by vortexing.
2. The content was incubated for 60 sec and after incubation, the change in optical density per 60 sec during 180 sec against distilled water at 340 nm as was recorded as follows:
3. A0- exactly after 60 sec
4. A1, A2, A3- exactly after every 60 sec for 180 sec
5. The absorbance was recorded using Bio spectrophotometer Basic, Eppendorf, Hamburg, Germany.

Calculations

1. The average change in absorbance per min (Abs/min) was calculated.
2. Activity of AST in IU/L
3. At 340 nm in IU/L = Abs/min *1975

3.7.2.2 ALT

The plasma ALT was estimated using IFCC method using spectrophotometer (Proton, Bangalore, India).

Principle

$$\text{L-Alanine + 2-Oxoglutarate} \xleftrightarrow{\text{ALT}} \text{L-Glutamate + Pyruvate}$$

$$\text{Pyruvate + APADPH (NADH Analogue) + } H^{+} \xleftrightarrow{\text{LDH}} \text{D-Lactate + APADP (Oxidised NADH Analogue)}$$

ALT-Alanine aminotransferase

LDH-*Lactate Dehydrogenase*

APADPH-Acetyl Pyridine Dinucleotide Phosphate

APADP-Acetylpyridine-Adenine Dinucleotide Phosphate

Procedure

1. 0.8 ml of R1, 0.2 ml of R2 and 100 µL Plasma were added in to a test tube and mixed well by vortexing.
2. The content was incubated for 10 sec and after incubation, the change in optical density per 60 sec was measured during 240 sec against distilled water at 340 nm as follows.
3. Ao- exactly after 10 sec
4. AI, A2, A3, A4- Exactly after every 60 sec for 240 sec.
5. The absorbance was recorded using Bio spectrophotometer Basic, Eppendorf, Hamburg, Germany.

Calculations

1. The average change in Abs/ min was recorded.
2. Activity of ALT in IU/L
3. At 340 nm in IU/L =Abs/min* 2225

3.7.2.3 ALP

The plasma ALP was estimated using ρ-Nitrophenyl phosphate (pNPP) Kinetic method using Spectrophotometer (Coral, Goa, India).

Principle

p-Nitrophenyl phosphate ⟶ p- Nitrophenol + phoshpate

Procedure

1. 1ml of working reagent and 0.02 ml of sample were added in a test tube and incubated at the assay temperature (37°C/ 30°C/25°C) for 1 min.
2. The contents were mixed well and the initial absorbance A_0 was measured after 1 min.

3. The absorbance reading was repeated after every 1, 2 and 3 min. The mean absorbance change per min was calculated.
4. The absorbance was recorded using Bio spectrophotometer Basic, Eppendorf, Hamburg, Germany.

Calculation

ALP activity (IU/L) =change of Abs/ min * 2754

3.7.2.4 ACP

The plasma ACP was estimated using kinetic method using Spectrophotometer (ACCUREX, Mumbai, India).

Principle:

In acidic PH of buffer system, acid phosphatase hydrolyses α-naphthylphosphate to α- naphthol and phosphate. The α – naphthol is then coupled with Diazotised Fast red TR to form a Diazo Dye which has a strong absorbance at 405 nm. The increase in absorbance is directly proportional to the level of acid phosphatase in plasma.

$$\alpha\text{- naphthylphosphate} + H_2O \xrightarrow{ACP} \alpha - \text{naphthol} + \text{phosphate}$$

$$\alpha\text{- naphthol} + \text{Fast Red TR} \longleftrightarrow \text{Diazonium Dye}$$

Procedure

1. The required amount of working solution was pre warmed at 37°C before use.
2. 0.01 ml of specimen was added to 1 ml of total ACP working solution.
6. The contents were mixed thoroughly and the assay mixture was immediately transferred to the thermo stated cuvette. The stop watch was started simultaneously, the first reading was recorded at 300^{th} sec and subsequently 3 more readings with 60 sec interval at 405 nm. The absorbance was recorded using Bio spectrophotometer Basic, Eppendorf, Hamburg, Germany.

Calculation

Concentration in IU/L = 743 * change in Abs/min.

3.7.3 Estimation of endocrine parameters

Endocrine parameters estimated in the study were T_3, T_4 and Leptin. The parameters T_3 (LDN, Nordhorn, Germany), T_4 (CALBIOTECH, *Austin Drive Spring Valley*, USA), Leptin (LDN, Nordhorn, Germany) were estimated by Enzyme -linked immunosorbent assay (ELISA).

3.7.3.1 T_3

Principle

The principle of the enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, controls and samples) and an enzyme – labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the color formed is inversely proportional to the concentration of T_3 in the sample. A set of standards is used to plot a standard curve from which the amount of T_3 in patient samples and controls can be directly read.

Procedure

1. It was assured that all reagents reached room temperature before use. Calibrators, controls and specimen samples were assayed in duplicate. Once the procedure was started, all steps were completed without interruption.
2. The working solution of the T_3- Horseradish peroxidase (HRP) conjugate and wash buffer were prepared.

3. 50µL of each calibrator, control and specimen sample were pipetted into correspondingly labelled wells in duplicate.
4. 100µL of the conjugate working solution was pipetted into each well using multichannel pipette.
5. The plate was incubated on a plate shaker (approximately 200 rpm) for 1 h at room temperature.
6. The wells were washed 3 times with prepared wash buffer (300µL/ well for each wash) and the plate was taped firmly against absorbent paper to ensure that it is dry.
7. 150µL of tetra methyl benzidine (TMB) substrate was pipetted into each well at timed intervals.
8. The plate was incubated on a plate shaker at room temperature for 10-15 min (or until calibrator A attained a dark blue colour for desired OD)
9. 50µL of stopping solution was added into each well at the same timed intervals as done for pipetting TMB substrate.
10. The plate was read on a microwell plate reader (Thermo Scientific, MULTISCAN GO, Finland) at 450 nm within 20 min of addition of the stopping solution.

Calculations

1. The mean optical density of each calibrator duplicate was calculated.
2. A calibrator curve was drawn on semi- log paper with the mean optical densities on the Y- axis and the calibrator concentrations on the X-axis.
3. The mean optical density of each unknown duplicate was calculated.
4. Value of the unknowns were read directly of each unknown duplicate.

3.7.3.2 T_4

Principle of the test

The principle of the enzyme immunoassay test follows the typical competitive binding scenario. The samples, working T_4- HRP conjugate and Anti-T_4-Biotin solution are added to the wells coated with streptavidin. T_4 in the

patient's plasma competes with a T_4 enzyme (HRP) conjugate for binding sites. Unbound T_4 and T_4 enzyme conjugate is washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of T_4 in the samples. A standard curve is prepared relating color intensity to the concentration of the T_4.

Assay Procedure

1. It was assured that all reagents reached room temperature before use. Calibrators, controls and specimen samples were assayed in duplicate. Once the procedure was started, all steps were completed without interruption.
2. The wash buffer was prepared.
3. 25µL of each calibrator, control and specimen sample were pipetted into correspondingly labelled wells.
4. 50µL of the working T_4 enzyme conjugate solution was pipetted into each well using multichannel pipette.
5. 50µL of T_4-Antibody-Biotin solution was added to all wells.
6. The microplate was swirled gently for 20-30 sec in order to mix the reagents.
7. The plate was covered and incubated for 60 min at room temperature.
8. The liquid from all wells were removed and the plate was washed 3 times with 300µL of 1X wash buffer. The plate was blotted on absorbent paper towel.
9. 100µL of TMB substrate solution was added to all wells.
10. The plate was covered and incubated at room temperature for 15 min.
11. 50µL of stopping solution was added into each well and mixed gently for 15- 20 sec (300 µL per well).
12. The plate was read on a microwell plate reader (Thermo Scientific, MULTISCAN GO, Finland) at 450 nm within 15minof addition of the stopping solution.

Calculations

1. A graph was plotted with the absorbance for T_4 standards (vertical axis) versus T_4 standard concentrations (horizontal axis) on a linear graph paper. Best curve through the points were drawn and the standard curve was constructed.
2. The absorbance for controls and each unknown sample from the curve was read. The value for each control or unknown sample was recorded.

3.7.3.3 Leptin

Principle of the test

The principle of the following enzyme immunoassay test follows a typical two step capture or sandwich type assay. The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for a different epitope of leptin is conjugated to biotin. During the first step, leptin present in the samples and standards is bound to the immobilised antibody and to the biotinylated antibody, thus forming a sandwich complex. Excess and unbound biotinylated antibody is removed by a washing step. In the second step, streptavidin- HRP is added, which binds specially to any bound biotinylated antibody. Again, unbound streptavidin- HRP is removed by a washing step. Next, the enzyme substrate is added (TMB), forming a blue coloured product that is directly proportional to the amount of leptin present. The enzymatic reaction is terminated by the addition of the stopping solution, converting the blue colour to a yellow colour. The absorbance is measured on a microtiter plate reader at 450 nm. A set of standards is used to plot a standard curve from which the amount of leptin in patient samples and controls can be directly read.

Assay procedure:

1. The working solutions of the streptavidin- HRP- conjugate and wash buffer were prepared.
2. 20µL of each standard, control and specimen samples were pipetted into the corresponding wells in duplicate.

3. 80µL of the monoclonal anti- leptin- biotin conjugate was pipetted into each well.
4. The plate was incubated on a plate shaker (approximately 200 rpm) for 1 h at room temperature.
5. The wells were washed 3 times with prepared wash buffer (300µL/ well for each wash) and the plate was taped firmly against absorbent paper to ensure that it is dry.
6. 100µL of prepared streptavidin- HRP-conjugate was pipetted into each well.
7. The plate was incubated on a plate shaker (approximately 200 rpm) for 30 min at room temperature,
8. The wells were washed 3 times with prepared wash buffer (300µL/well for each wash) and the plate was taped firmly against absorbent paper to ensure that it is dry.
9. 100µL of TMB substrate was pipetted into each well at timed intervals.
10. The plate was incubated on a plate shaker for 10-15 min at room temperature.
11. 50µL of stopping solution was pipetted into each well at the same timed interval as in the above step.
12. The plate was read on a microwell plate reader (Thermo Scientific, MULTISCAN GO, Finland) at 450 nm within 20 min after addition of the stopping solution.

Calculation

1. The mean optical density of each calibrator duplicate was calculated.
2. A calibrator curve was drawn on a semi- log paper with the mean optical densities on the Y- axis and the calibrator concentrations on the X-axis.
3. The mean optical density of each unknown duplicate was calculated.
4. The values of the unknowns wereread directly off the standard curve.

3.8 Histopathology

Histopathological observations in different groups of animals subjected to different kinds of stress: The tissues were collected immediately after sacrifice from same site in all the animals from C (n=6), HS (n=6), NS (n=6) and CS (n=6)

in buffered 10% formalin. The fixed tissues were processed routinely to get Haematoxylin and Eosin (H and E) stained sections (Luna, 1968). The organs collected for the study includes thyroid, rumen, Omasum, abomasum, reticulum, small intestine and large intestine. The slides were interpreted in comparison with that of C group animals and representative lesions were photographed.

3.9 Gene expression

Principle

Samples are lysed and homogenized in lysis buffer, which contains guanidine thiocyanate, a chaotropic salt capable of protecting RNA from endogeneous RNases. The lysate is then mixed with ethanol and loaded on a purification column. The chaotropic salt and ethanol cause RNA to bind to the silica membrane while the lysate is spun through the column. Subsequently, impurities are effectively removed from the membrane by washing the column with wash buffers. Pure RNA is then eluted under low ionic strength conditions with nuclease-free water.

3.9.1 Sample collection and storage

At the end of the study Rumen and Intestine were collected from each animal of all the groups immediately after slaughter. The samples were cut into small pieces, washed in Phosphate buffered saline (PBS) and immersed in RNA shield (Zymo Research, USA) and stored at -80°C till future use.

3.9.2 Sample preparation for RNA isolation

After thawing, the tissues were removed from RNA shield (Zymo Research, USA) and immediately processed for RNA isolation using GeneJET RNA Purification Kit (Thermo Scientific, Lithuania) and the procedure was followed as per manufacturer's protocol with slight modifications:

About 30 mg of the frozen tissue was weighed and placed into liquid nitrogen (LN_2) (-196) and was ground thoroughly with RNAase ZAP (Ambion,

USA) treated mortar and pestle. The powdered tissue was transferred immediately into a 1.5 ml microcentrifuge tube containing 300 µL of lysis buffer supplemented with β-mercaptoethanol and vortexed for 10 sec in order to mix thoroughly. 10 µL of proteinase K was added in 590 µL of (Tris –Ethylenediaminetetraaceticacid) TE buffer was added to 300 µL of lysate vortexed and incubated at room temperature for 10 min and centrifuged for 5 min at 12000 g. The supernatant was transferred into a new RNAase-free 1.5 µL microcentrifuge tube. 450 µL of ethanol (96-100%) was added and mixed by pipetting. Upto 700 µL of lysate was transferred to GeneJET RNA purification column inserted in a collection tube and was centrifuged for 1 min at 12000 g. The flow through solution was discarded and the purification column was placed back into the collection tube. The step was repeated until all the lysate was transferred into the column and centrifuged. The collection tube containing the flowthrough was discarded and the purification column was placed into a new 2 ml collection tube. About 700 µL of wash buffer1 was added to the purification column and centrifuged for 1 min at 12000 g. The flow through solution was discarded and the column was placed back into the collection tube. About 600 µL of wash buffer 2 was added to the purification column and was centrifuged for 2 min at 12000 g. The collection tube containing the flowthrough solution was discarded and the purification column was transferred to a sterile 1.5 ml RNase-free microcentrifuge tube. About 40µL of nuclease free water was added to the centre of the GeneJET RNA purification column membrane and centrifuged for 3min at 12000 g inorder to elute RNA. The purified RNA was stored at -80°C until complementary DNA (cDNA) synthesis.

3.9.3 DNase treatment

Total RNA isolated from the tissues was treated with DNase (TURBO DNA-free, Ambion, USA) in order to eliminate the genomic Deoxy ribo nucleic acid (DNA) contamination in total RNA. During and after DNase treatment, 1µL of RNase inhibitor (20U/µL, Invitrogen, USA) was added. After DNase treatment quality and quantity was analyzed using Spectrophotometer (ND-1000, Thermoscientific, USA).

3.9.4 cDNA Synthesis

The total RNA was reverse transcribed into cDNA using Maxima first strand cDNA synthesis kit for Real time quantitative polymerase chain reaction (RT-qPCR) (Thermo Scientific, Lithuania). After thawing, the components of the kit were mixed and briefly centrifuged and stored on ice.4 µL of 5X Reaction Mix, 2 µL Maxima Enzyme Mix, 1 µg of Template RNA and Water, nuclease free was added to make up the total volume to 20 µL into a sterile, RNAase- free tube, and were mixed gently and centrifuged and subjected to reverse transcribing PCR (incubated for 10 min at 25°C followed by 30min at 50°C and the reaction was terminated by heating at 85°C for 5 min).The converted cDNA was diluted to a final concentration of 25ng/µL using nuclease free water and 2 µL of diluted cDNA (50 µg) was used for qPCR experiments.

3.9.5 Primer design and synthesis

Gene specific primers were designed using online National Centre for Biotechnology Information(NCBI) primer design software (Primer3, http://bioinfo.ut.ee/primer3/) and specificity was checked using Primer3 and Basic Local Alignment Search Tool(BLAST) (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/). The preferences were given to the primers binding to the exon-exon junction. The primers were titrated with different concentrations (10, 5 2.5 and 1 µM) for selecting optimum concentration to be used for qPCR experiments.

3.9.6 Quantitative RT-PCR analysis

The relative expression of selected genes was studied using SYBR green chemistry (Maxima SYBR green qPCR master mix, Fermentas, USA). A 20 µL reaction was carried out in duplicates using 50ng of template and 1 µM primer concentration. The RT qPCR reaction conditions were as follows: enzyme activation at 95°C for 10 min and amplification cycle (40 cycles; initial denaturation at 95°C for 15 sec, annealing at 60°C for 30sec and extension at 72°C for 30 sec).The melt curve analysis was performed to check the non-specific

amplification. The GAPDH gene was used as an internal control and the relative expression was analyzed using the formula, $2^{-\Delta\Delta CT}$(Tarif *et al*., 2012). The results were expressed in fold change as compared to C group (C =1fold).

3.10 Statistical analysis

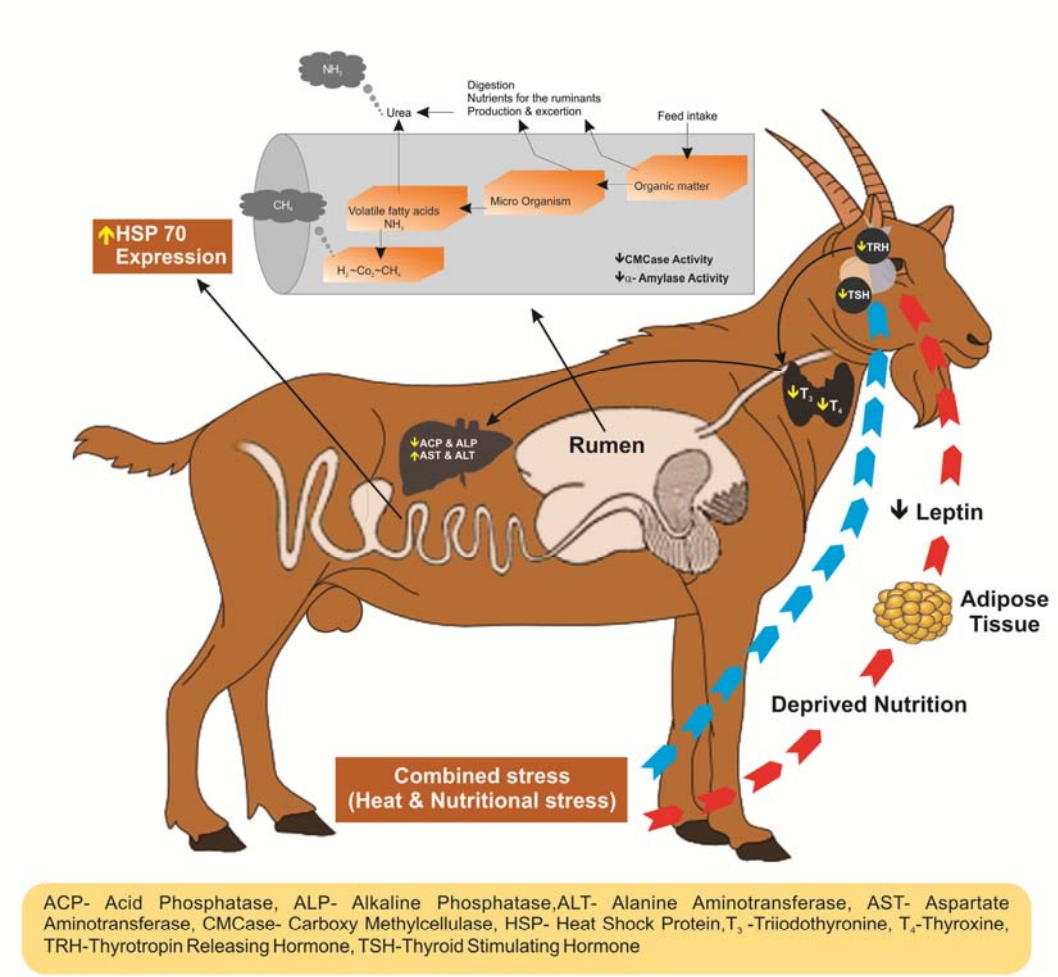
The data was analyzed by general linear model (GLM) repeated measurement analysis of variance (Statistical package for the social sciences (SPSS) 16.0). Effect of fixed factors namely group (control, heat, nutritional and combined stresses) was taken as between subject factor and days (longitudinal time over which experiment was carried out (Day0, Day 15, Day 30 and Day 45) were taken as within subject factor and also interaction between group and days was analyzed on the various parameters studied. Comparison of means of the different subgroups were made by Duncan's multiple range tests as described by Kramer (1957).

Fig. 2 depicts the concept of the study conducted. It shows in how an animal's metabolic activities and rumen fermentation may get affected when exposed to combined stress or what changes are expected from an animal out of the study.

Table 2: Primers used for HSP70 expression. GAPDH used as reference gene to normalize the gene expression of target genes

Gene ID	Primers	Primer sequence (5"- 3")	Primer Length (bp)	Product Size (bp)	Accession No
HSP70	F	TGGCTTTCACCGATACCGAG	20	167	NM_001285703.1
	R	GTCGTTGATCACGCGGAAAG	20		
GAPDH	F	GGTGATGCTGGTGCTGAGTA	20	265	AF030943
	R	TCATAAGTCCCTCCACGATG	20		

HSP70 -Heat Shock Protein 70; GAPDH - Glyceraldehyde 3-phosphate dehydrogenase



ACP- Acid Phosphatase, ALP- Alkaline Phosphatase,ALT- Alanine Aminotransferase, AST- Aspartate Aminotransferase, CMCase- Carboxy Methylcellulase, HSP- Heat Shock Protein,T_3 -Triiodothyronine, T_4-Thyroxine, TRH-Thyrotropin Releasing Hormone, TSH-Thyroid Stimulating Hormone

Fig. 2: Concept figure of the present study

CHAPTER 4

RESULTS

4.1 Weather parameters

The maximum, minimum, wet and dry bulb temperatures and RH were recorded and the THI was calculated on an average at fortnightly interval. The obtained THI inside during both morning and afternoon are described in table 3. The THI values inside the shed shows that the animals were in comfort zone both during morning and afternoon.

The THI outside the shed during both morning and afternoon are described in table 4. The THI values outside the shed shows that during the morning hours the animals were in comfort zone while during afternoon the animals were under extreme distress. The THI values were calculated as per method described by McDowell (1972). Accordingly the formula used was THI = $0.72(T_{db} + T_{wb}) + 40.6$ where, T_{db} = Dry bulb temperature in ºC; T_{wb} = Wet bulb temperature in ºC. The THI values 72 and less are considered comfortable; THI values between 75 and 78 are considered stressful and THI above 78 considered Extreme distress.

Table 3: Mean and SEM of weather data during the experimental period inside the shed

Time of Recording	Minimum temperature (ºC)	Maximum temperature (ºC)	Dry bulb temperature (ºC)	Wet bulb temperature (ºC)	RH (%)	THI
Morning (8:00 hrs)	21.50 ±0.60	32.37 ±0.20	23.80 ±0.21	20.10 ±0.17	78.67 ±4.67	72.21 ±0.25
Afternoon (14:00 hrs)	24.4 ±0.56	35.60 ±0.72	26.47 ±0.28	20.93 ±0.09	45.67 ±7.21	74.73 ±0.22

SEM-Standard Error Mean; RH- Relative Humidity; THI- Temperature Humidity Index

Table 4: Mean and SEM of weather data during the experimental period in the outside environment

Time of Recording	Minimum temperature (ºC)	Maximum temperature (ºC)	Dry bulb temperature (ºC)	Wet bulb temperature (ºC)	RH (%)	THI
Morning (8:00 hrs)	26.93 ±2.87	34.03 ±0.52	23.13 ±0.20	22.97 ±0.66	61.00 ±7.77	73.92 ±0.57
Afternoon (14:00 hrs)	27.23 ±3.46	38.33 ±0.52	29.57 ±0.38	26.53 ±0.71	37.00 ±4.16	80.99 ±0.25

SEM-Standard Error Mean; RH- Relative Humidity; THI- Temperature Humidity Index

4.2 VFA profile of rumen liquor in bucks

The effects of HS, NS and CS on rumen liquor VFA profile are described in table 5. Rumen liquor acetic acid level did not differ between the groups for the treatments. However, the experimental days significantly ($P<0.01$) influenced rumen liquor acetic acid level. Further, interaction between groups and experimental days also did not significantly influence rumen liquor acetic acid level. Rumen liquor propionic acid level showed significant ($P<0.01$) changes for the treatment. There were significant ($P<0.01$) differences in propionic acid level between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P<0.01$) influenced rumen liquor propionic acid level in the study. In addition, interaction between groups and experimental days also significantly ($P<0.05$) influenced the rumen liquor propionic acid level in the study. Rumen liquor butyric acid level showed significant ($P<0.01$) changes for the treatment. There were significant ($P<0.01$) differences in butyric acid level between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P<0.01$) influenced rumen liquor butyric acid level in the study. In addition, interaction between groups and experimental days also significantly ($P<0.01$) influenced the rumen liquor butyric acid level in the study. Rumen liquor valeric

acid level showed significant ($P<0.01$) changes for the treatment. There were significant ($P<0.01$) differences in valeric acid level between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P<0.01$) influenced rumen liquor valeric acid level in the study. In addition, interaction between groups and experimental days also significantly ($P<0.05$) influenced the rumen liquor valeric acid level in the study. Rumen liquor iso-valeric acid level did not show significant changes for the treatment. However, both experimental days and interaction between groups and experimental days significantly ($P<0.01$) influenced the rumen liquor isovaleric acid level in the study. Rumen liquor TVFA level did not show significant changes for the treatment. However, experimental days significantly ($P<0.01$) influenced the rumen liquor TVFA level in the study. Further, interaction between groups and experimental days also significantly ($P<0.01$) influenced the rumen liquor TVFA level in the study. Rumen liquor acetate: propionate ratio showed significant ($P<0.01$) changes for the treatment. There were significant ($P<0.01$) differences in propionic acid level between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P<0.01$) influenced rumen liquor acetate: propionate ratio in the study. However, interaction between groups and experimental days did not significantly influence the rumen liquor acetate: propionate ratio in the study.

Table 5: Effect of heat stress, nutritional stress and combined stresses (heat and nutritional stress) on volatile fatty acids production in goats

FACTOR	C2 (mmol/L)	C3 (mmol/L)	C4 (mmol/L)	C5 (mmol/L)	C5i (mmol/L)	TVFA (mmol/L)	C:P (mmol/L)
GROUP	ns	**	**	**	ns	ns	**
C	57.85^{a}±2.01	21.33^{a}±0.80	09.9^{a}±0.50	1.06^{a}±0.07	0.73^{a} ±0.10	108.80^{a}±9.03	2.76^{b}±0.12
HS	56.03^{a}±2.17	20.21^{a}±1.14	9.93^{a}±0.54	0.91^{a}±0.08	0.81^{a}±0.08	109.11^{a}±9.56	2.89^{b}±0.15
NS	52.75^{a}±3.12	15.46^{b}±1.61	6.15^{b}±0.86	0.69^{b}±0.08	0.87^{a}±0.10	90.89^{a}±08.72	3.70^{a}±0.18
CS	50.84^{a}±3.17	13.65^{b}±1.13	5.76^{b}±0.78	0.67^{b}±0.09	0.86^{a}±0.12	71.69^{b}±07.12	3.85^{a}±0.20
DAYS	**	**	**	**	**	**	**
0	60.76±1.73	22.99±1.03	10.7±0.52	1.26±0.05	1.32±0.11	112.9±10.7	2.72±0.11
15	55.47±3.21	16.43±0.94	7.26±0.36	0.79±0.03	0.75±0.08	93.14±8.48	3.65±0.19
30	52.43±2.60	16.82±0.88	6.50±0.55	0.57±0.06	0.45±0.04	89.59±8.75	3.28±0.12
45	48.81±1.58	14.41±0.76	7.28±0.45	0.80±0.06	0.75±0.06	84.90±9.13	3.55±0.13
GROUP* DAYS	ns	*	**	**	**	**	ns
C0	63.84±3.12	23.70±1.87	10.32±0.95	1.29±0.09	1.13±0.21	120.0±19.3	2.71±0.21
C15	53.47±5.80	19.74±1.69	09.32±0.65	0.97±0.06	0.55±0.16	100.8±15.3	2.75±0.34
C30	56.80±4.69	21.75±1.58	08.94±0.99	0.77±0.10	0.33±0.09	106.0±15.8	2.66±0.22
C45	57.29±2.85	20.11±1.37	11.01±0.81	1.22±0.14	0.92±0.12	108.5±16.5	2.91±0.24
HS0	59.94±3.49	23.50±2.09	09.70±1.06	1.21±0.10	1.06±0.21	117.7±21.6	2.60±0.23
HS15	55.19±6.48	21.46±1.89	10.54±0.73	0.94±0.07	0.68±0.16	109.9±17.1	2.72±0.38
HS30	50.89±5.25	19.20±1.77	08.75±1.11	0.69±0.12	0.45±0.09	99.66±17.6	2.64±0.24
HS45	58.09±3.19	16.70±1.54	10.72±0.91	1.16±0.15	1.05±0.12	109.2±18.4	3.59±0.27
NS0	67.61±3.12	26.29±1.87	12.33±0.95	1.23±0.09	1.50±0.18	130.5±19.3	2.61±0.21
NS15	44.96±5.80	10.79±1.69	04.06±0.65	0.61±0.06	0.89±0.14	73.44±15.3	4.15±0.34
NS30	54.03±4.70	13.73±1.58	04.26±0.99	0.47±0.10	0.48±0.07	87.37±15.8	4.04±0.22
NS45	44.38±2.85	11.01±1.37	03.94±0.81	0.43±0.14	0.63±0.11	72.31±16.5	4.02±0.24
CS0	51.64±4.03	18.46±2.41	10.41±1.22	1.31±0.12	1.64±0.26	83.37±24.9	2.96±0.27
CS15	68.23±7.48	13.72±2.18	05.13±0.84	0.63±0.08	0.87±0.19	88.42±19.8	4.96±0.44
CS30	48.01±6.06	12.62±2.04	04.04±1.28	0.36±0.13	0.53±0.10	65.38±20.4	3.79±0.28
CS45	35.47±3.68	09.82±1.77	03.44±1.05	0.38±0.17	0.42±0.15	49.59±21.3	3.67±0.31

C2- Acetic acid; C3- Propionic acid; C4- Butyric acid; C5- Valeric acid; C5i- Iso Valeric acid; TVFA- Total volatile fatty acid; C:P- Acetate : Propionate ratio; C-Control; HS-Heat Stress; NS-Nutritional Stress; CS-Combined Stress

**Indicates statistical significance at $P < 0.01$; * Indicates statistical significance at $P < 0.05$; ns - Indicates non-significant; Values bearing different superscripts within a column differ significantly with each other

Figure 3: Effect of heat stress, nutritional stress and combined stresses (heat and nutrition stress) on the volatile fatty acid production in goats

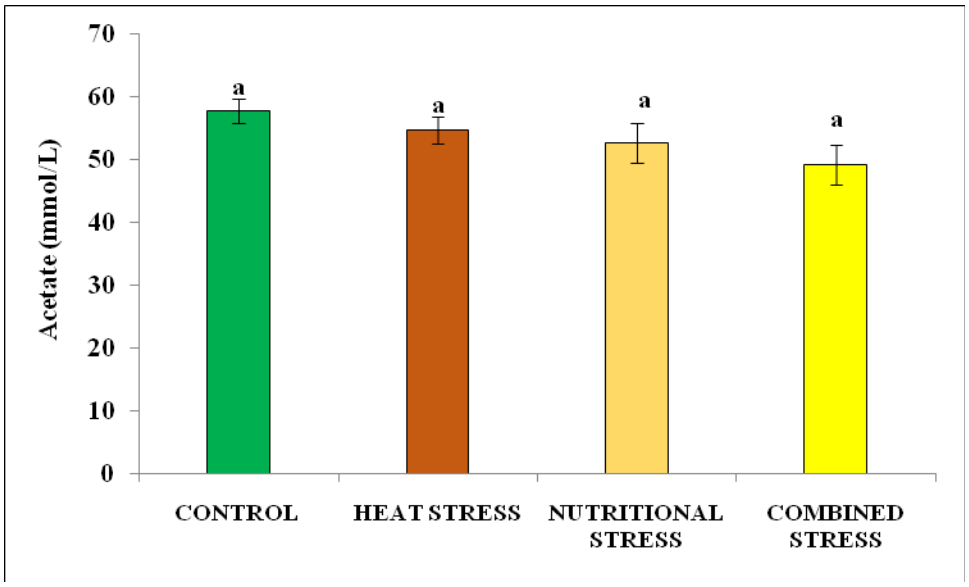


Fig. 3(a):Acetate

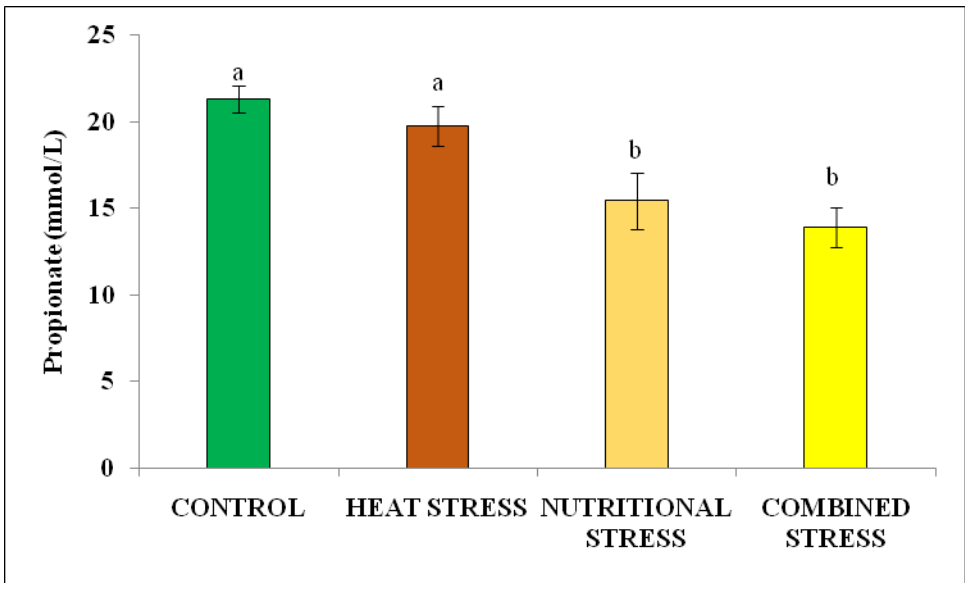


Fig. 3(b):Propionate

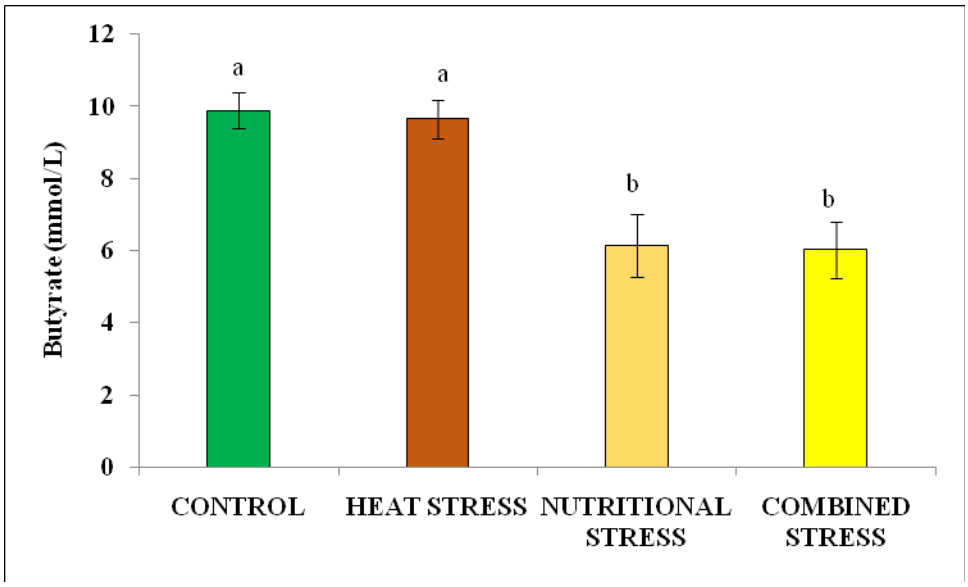


Fig. 3(c):Butyrate

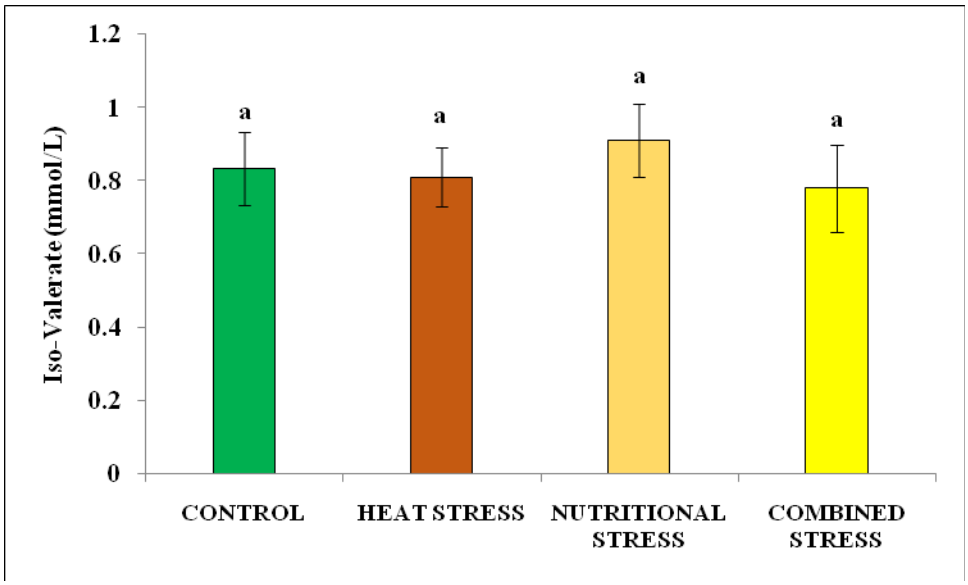


Fig. 3(d):Iso-Valerate

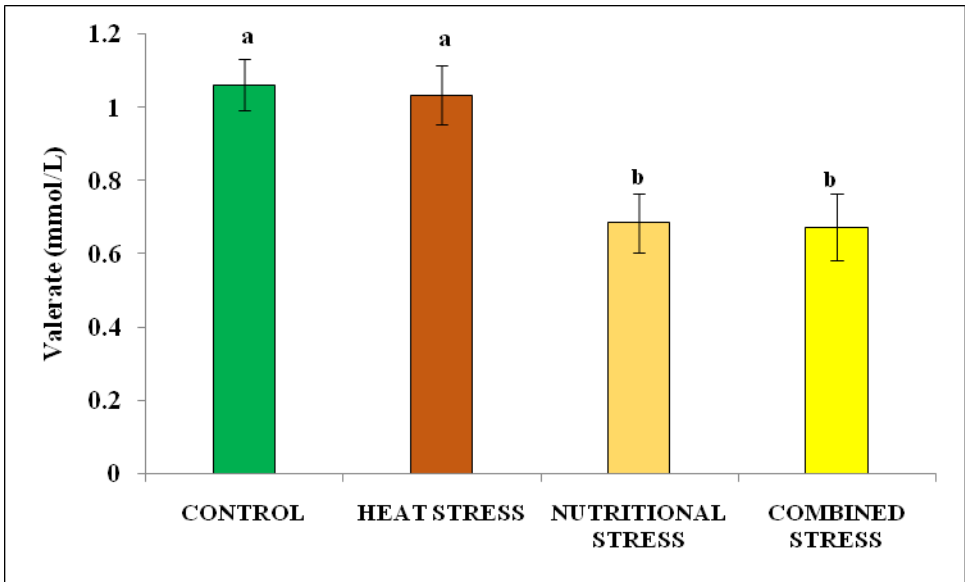


Fig. 3(e):Valerate

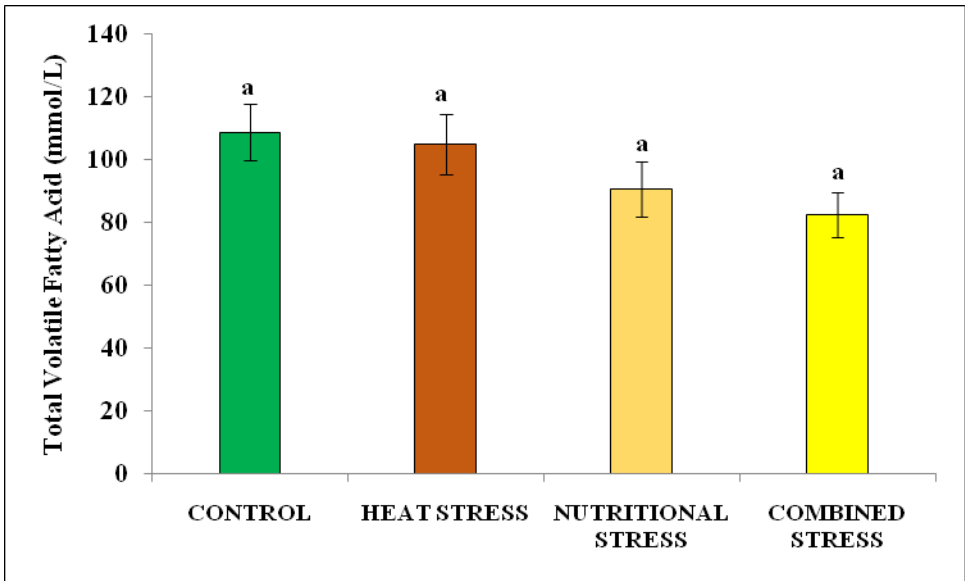


Fig. 3(f):Total Volatile Fatty Acid

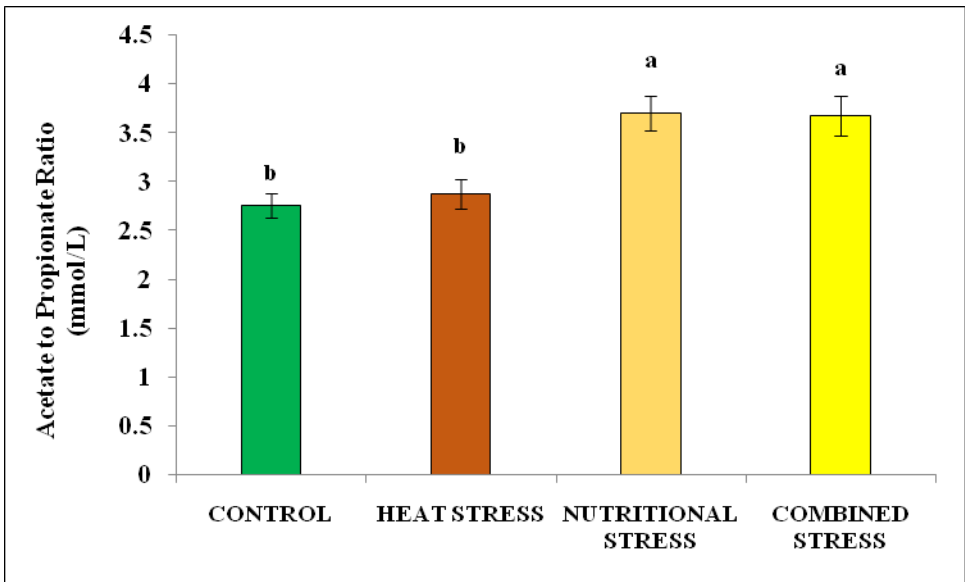


Fig. 3(g):Acetate to Propionate Ratio

4.3 Biochemical parameter of rumen liquor in bucks

The effects of HS, NS and CS on biochemical parameters of rumen liquor are described in table 6. NH_3-N in rumen liquor showed significant ($P<0.05$) changes for the treatment. The highest concentration of NH_3-N was recorded in C while the lowest in CS group. These effects were significantly ($P<0.01$) different between the C and CS groups. NH_3-N in HS and NS groups did not differ with each other as well as with C and CS groups. Further, experimental days significantly ($P<0.05$) influenced NH_3-N throughout the study period. In addition, there was significant ($P<0.01$) interaction between groups and experimental days for the rumen liquor NH_3-N in the study. The treatment, experimental days and interaction between treatment and experimental days did not influence rumen liquor pH in the study. TCA of rumen liquor also showed significant ($P<0.01$) changes for the treatment. There were significant ($P < 0.01$) differences in TCA between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P < 0.01$) influenced TCA in the study. In addition, interaction between groups and experimental days also differed significantly ($P < 0.01$) for the TCA in the study. Total nitrogen of rumen liquor also showed significant ($P<0.01$) changes for the treatment. There were significant ($P<0.01$) differences in Total nitrogen between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P<0.01$) influenced Total nitrogen in the study. In addition, interaction between groups and experimental days also significantly ($P<0.01$) influenced the rumen liquor Total nitrogen in the study.

Table 6: Effect of heat stress, nutritional stress and combined stresses (heat and nutritional stress) on the biochemical parameter of rumen liquor in goats

FACTOR	NH_3 N (mg/100ml)	pH	TCA (mg/100ml)	TOTAL NITROGEN (mg/100ml)
GROUP	*	ns	**	**
C	32.09 [a] ± 1.47	6.57 [a] ± 0.03	158.79 [a] ± 8.44	233.77[a] ± 9.93
HS	28.77[ab]± 1.72	6.58 [a] ± 0.11	140.11 [a] ± 11.4	212.32[a] ± 9.99
NS	27.13[ab] ± 1.10	6.66 [a] ± 0.05	100.17 [b] ± 10.4	163.34[b] ± 10.9
CS	22.37 [b] ± 1.67	6.74 [a] ± 0.07	97.89 [b] ± 12.44	161.20[b] ± 13.1
DAYS	**	ns	**	**
0	31.29 ± 1.32	6.59 ± 0.04	171.80 ± 8.0	242.37 ± 8.59
15	26.21 ± 1.61	6.45 ± 0.06	124.03 ± 6.7	180.22 ± 6.32
30	27.99 ± 1.40	6.77 ± 0.11	93.88 ± 8.98	165.37 ± 8.29
45	24.88 ± 1.61	6.74 ± 0.07	107.25 ± 8.1	182.67 ± 8.42
GROUP * DAYS	*	ns	**	**
C0	31.41 ± 2.56	6.64 ± 0.08	158.26 ± 15.7	234.86 ± 16.66
C15	31.91 ± 3.12	6.48 ± 0.12	175.17 ± 13.0	241.25 ± 12.26
C30	32.33 ± 2.71	6.60 ± 0.20	134.18 ± 17.4	202.04 ± 16.09
C45	32.73 ± 3.13	6.57 ± 0.14	167.55 ± 15.7	256.93 ± 16.34
HS0	30.66 ± 2.56	6.65 ± 0.08	180.52 ± 15.7	246.40 ± 16.66
HS15	27.61 ± 3.12	6.35 ± 0.12	139.10 ± 13.0	194.54 ± 12.26
HS30	28.23 ± 2.71	6.70 ± 0.20	105.84 ± 17.4	190.18 ± 16.09
HS45	28.60 ± 3.12	6.62 ± 0.14	134.96 ± 15.7	218.18 ± 16.34
NS0	31.73 ± 2.56	6.54 ± 0.09	164.64 ± 17.6	231.98 ± 18.62
NS15	26.18 ±3.12	6.49 ± 0.13	98.84 ± 14.58	155.96 ± 13.71
NS30	28.96 ± 2.71	6.76 ± 0.22	67.06 ± 19.48	135.52 ± 17.99
NS45	21.65 ± 3.12	6.86 ± 0.15	70.14 ± 17.53	129.92 ± 18.27
CS0	31.37 ± 2.56	6.53 ± 0.09	183.79 ± 15.7	256.26 ± 16.66
CS15	19.12 ± 3.12	6.47 ± 0.13	82.99 ± 13.04	129.14 ± 12.26
CS30	22.45 ± 2.71	7.02 ± 0.22	68.43 ± 17.42	133.73 ± 16.09
CS45	16.55 ± 3.12	6.92 ± 0.15	56.34 ± 15.68	125.66 ± 16.34

NH_3 N- Ammonia Nitrogen;$_P$H- Rumen liquor $_P$H; TCA- Tricarboxylic acid; C-Control; HS-Heat Stress; NS-Nutritional Stress; CS-Combined Stress

**Indicates statistical significance at $P < 0.01$; * Indicates statistical significance at $P < 0.05$; ns - Indicates non-significant; Values bearing different superscripts within a column differ significantly with each other

Figure 4: Effect of Effect of Heat stress, Nutritional stress and combined stresses (Heat and nutritional) on the biochemical parameters of rumen liquor (a) TCA (b) Total nitrogen (c) $_{P}H$ (d) NH_3 –N in goats

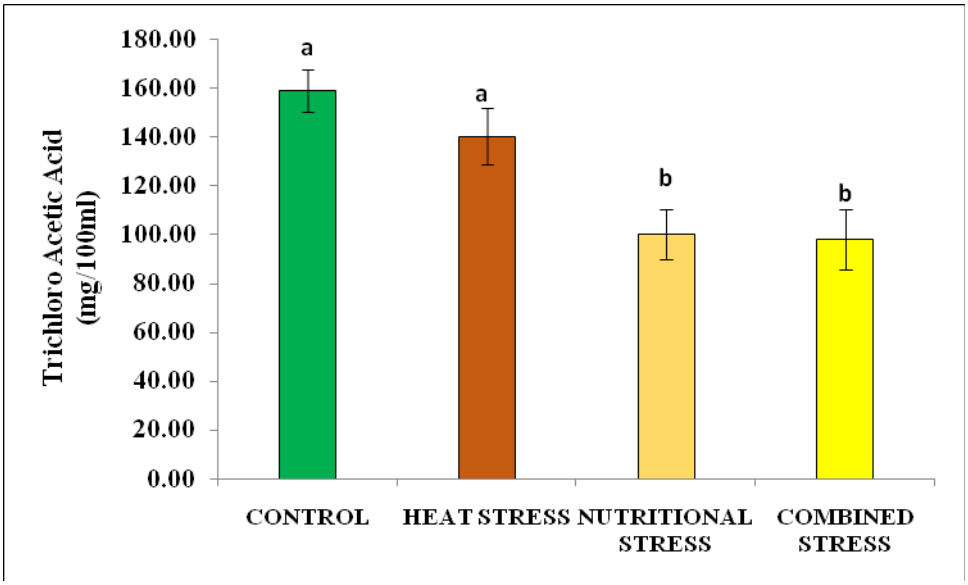


Fig. 4(a): Trichloro Acetic Acid

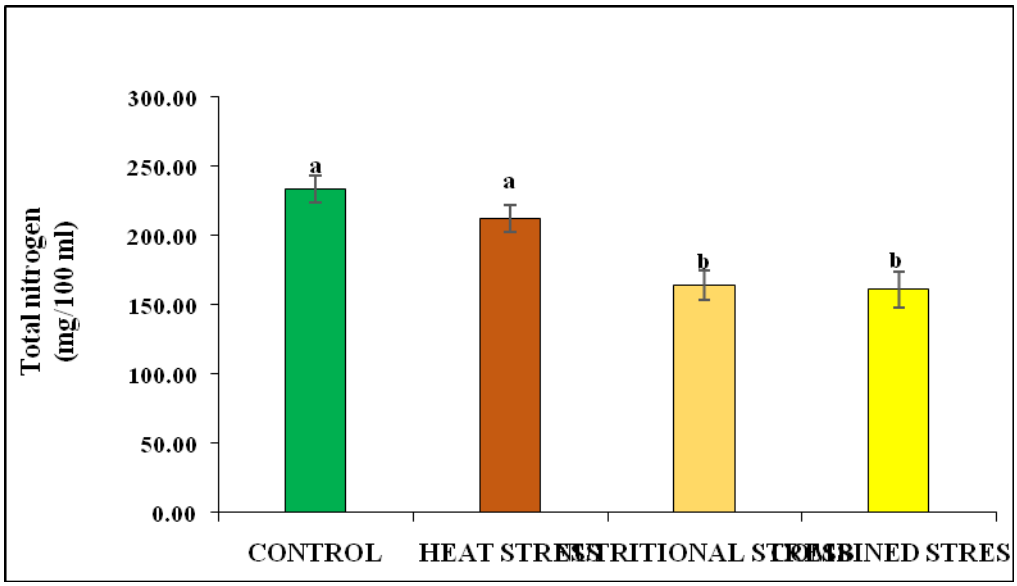


Fig. 4(b): Total nitrogen

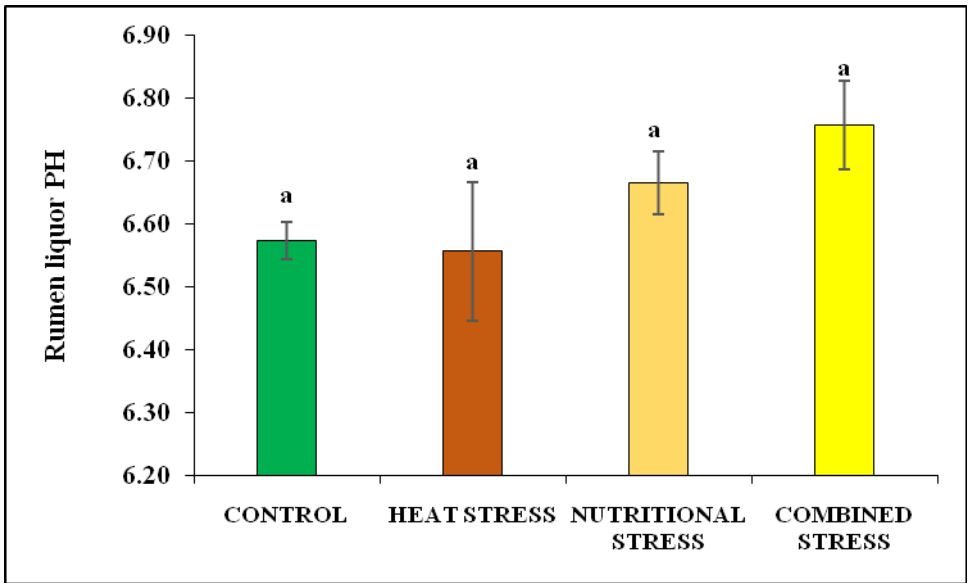


Fig. 4(c): pH

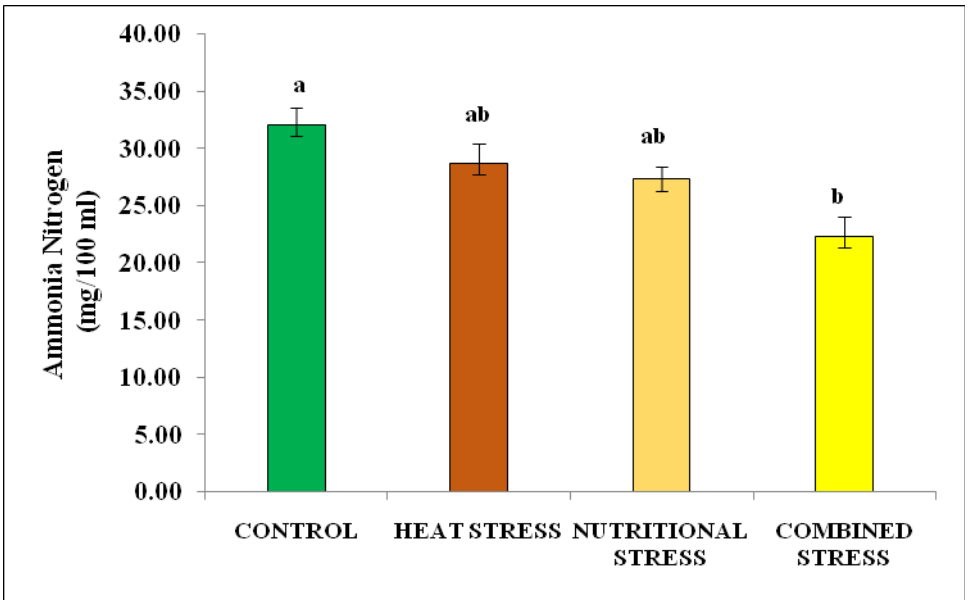


Fig. 4(d): Ammonia Nitrogen

4.4 α-Amylase enzyme activity of rumen liquor in bucks

The effect of HS, NS and CS on α-Amylase enzyme activity of rumen liquor is described in table 7. The α-Amylase EC, α-Amylase IC and α-Amylase TA of rumen liquor did not differ between the groups. However, the experimental days influenced all these parameters in the study. The experimental days significantly influenced α-Amylase EC ($P < 0.05$), α-Amylase IC ($P < 0.05$) and α-Amylase TA ($P < 0.01$) in rumen liquor. In addition, there was significant ($P<0.01$) interaction between groups and experimental days only for α-Amylase IC activity in the study.

Table 7: Effect of heat stress, nutritional stress and combined stresses (heat and nutrition stress) on the α -amylase enzyme activity in goats

FACTOR		α-Amylase EC (μ mol of glucose/ml/hr)	α-Amylase IC (μ mol of glucose/ml/hr)	α-Amylase TA (μ mol of glucose/ml/hr)
GROUP		ns	ns	ns
	C	$77.17^{a} \pm 11.49$	$50.28^{a} \pm 9.23$	$127.45^{a} \pm 22.15$
	HS	$85.32^{a} \pm 18.93$	$28.10^{a} \pm 3.02$	$113.42^{a} \pm 20.60$
	NS	$60.71^{a} \pm 09.66$	$58.09^{a} \pm 14.0$	$118.80^{a} \pm 27.43$
	CS	$55.14^{a} \pm 17.62$	$52.31^{a} \pm 10.3$	$107.46^{a} \pm 29.46$
DAYS		**	**	**
	0	089 ± 12.17	62.03 ± 10.86	151.04 ± 19
	45	50.16 ± 5.60	32.36 ± 04.55	82.53 ± 9.5
GROUP * DAYS		ns	**	*
	C0	75.82 ± 24.35	34.49 ± 21.7	110.31 ± 38.0
	C45	78.52 ± 11.22	66.07 ± 9.11	144.59 ± 19.1
	HS0	123.5 ± 24.35	34.66 ± 21.7	158.16 ± 38.0
	HS45	47.14 ± 11.22	21.54 ± 9.11	68.68 ± 19.09
	NS0	81.81 ± 24.35	97.17 ± 21.7	178.99 ± 38.0
	NS45	39.61 ± 11.22	18.99 ± 9.11	58.61 ± 19.09
	CS0	74.90 ± 24.35	81.79 ± 21.7	156.7 ± 38.01
	CS45	35.38 ± 11.22	22.85 ± 9.11	58.22 ± 19.09

EC-Extracellular activity; IC- Intracellular activity; TA- Total activity; C-Control; HS-Heat Stress; NS-Nutritional Stress; CS-Combined Stress

**Indicates statistical significance at $P < 0.01$; * Indicates statistical significance at $P < 0.05$; ns - Indicates non-significant; Values bearing different superscripts within a column differ significantly with each other

Figure 5: Effect of Heat stress, Nutritional stress and combined stresses (Heat and nutritional) on the (a) Extracellular activity of α-Amylase enzyme (b) Intracellular activity of α-Amylase enzyme (c) Total activity of α-Amylase enzyme in goats.

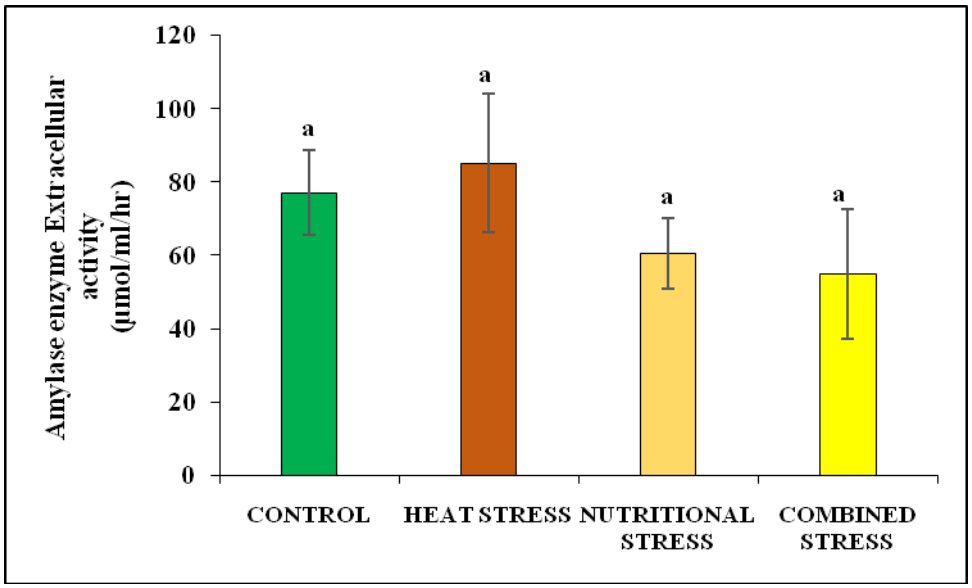


Fig. 5(a): Extracellular activity of α-Amylase enzyme

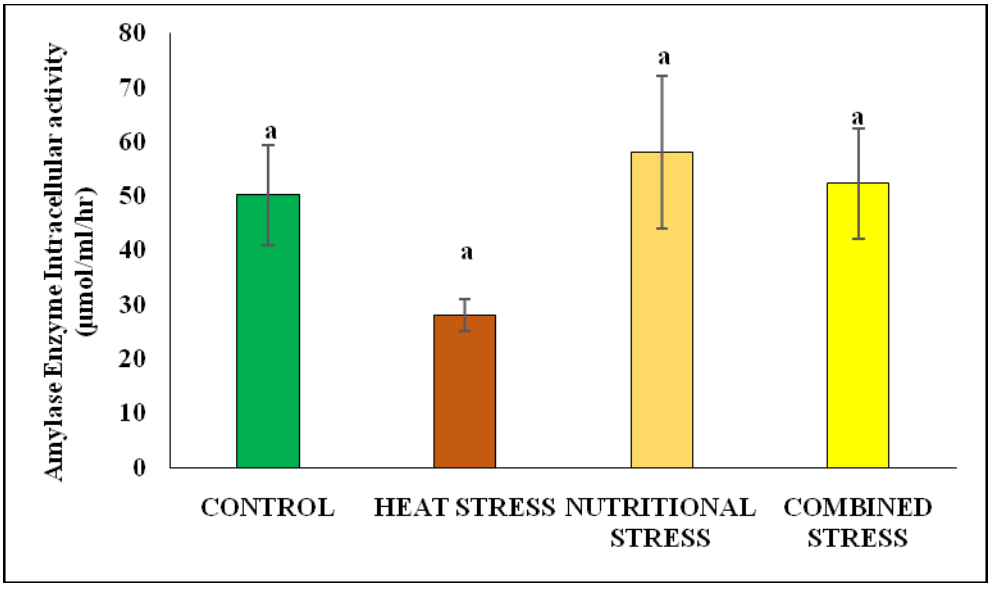


Fig. 5(b): Intracellular activity of α-Amylase enzyme

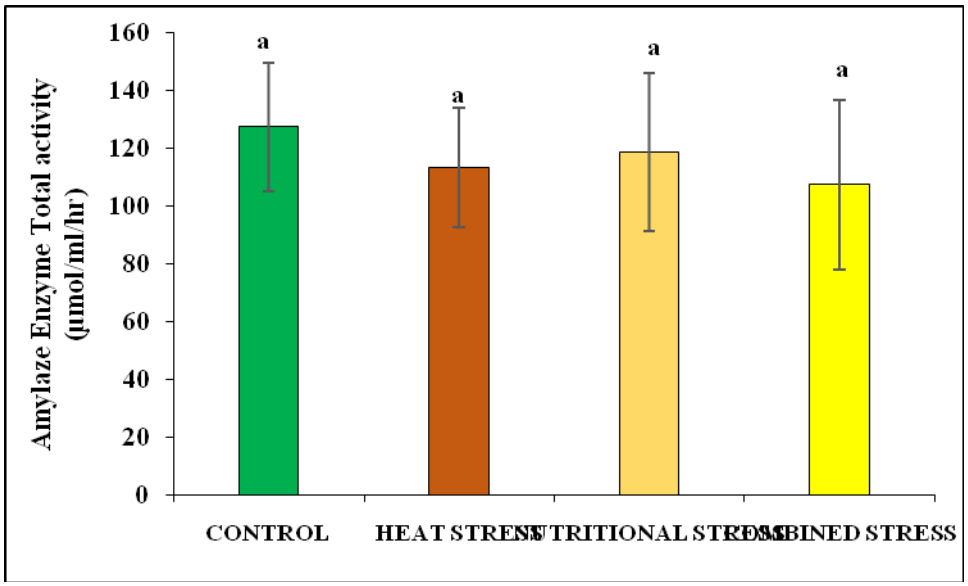


Fig. 5(b): Total activity of α-Amylase enzyme

4.5 CMCase activity of rumen liquor in bucks

The effect of HS, NS and CS on CMCase enzyme activity of rumen liquor is described in table 8. The carboxy methyl cellulase EC, CMCase IC and CMCase TA of rumen liquor differed significantly (P<0.01) between the groups. Further, the experimental days significantly influenced CMCase EC, and CMCase TA of the rumen liquor in the study. In addition, there was significant (P<0.01) interaction between groups and experimental days only for CMCase EC activity of the rumen liquor in the study.

Table 8: Effect of Heat stress, Nutritional stress and combined stresses (Heat stress and Nutritional stress) on the Carboxy methyl cellulase activity in goats

FACTOR	Carboxy methyl cellulase EC (μ mol of glucose/ml/hr)	Carboxy methyl cellulase IC (μ mol of glucose/ml/hr)	Carboxy methyl cellulase TA (μ mol of glucose/ml/hr)
GROUP	**	**	**
C	$4.46^{a} \pm 0.35$	$5.11^{a} \pm 0.31$	$9.57^{a} \pm 0.50$
HS	$3.99^{b} \pm 0.20$	$5.23^{a} \pm 0.43$	$9.23^{a} \pm 0.58$
NS	$3.44^{c} \pm 0.08$	$4.13^{b} \pm 0.29$	$7.58^{b} \pm 0.34$
CS	$3.66^{c} \pm 0.10$	$3.59^{b} \pm 0.14$	$7.25^{b} \pm 0.18$
DAYS	**	ns	**
0	4.36 ± 0.11	4.83 ± 0.22	9.20 ± 0.27
45	3.41± 0.11	4.20 ± 0.16	7.62 ± 0.18
GROUP * DAYS	**	ns	Ns
C0	5.37 ± 0.21	4.96 ± 0.44	10.33 ± 0.54
C45	3.55 ± 0.06	5.26 ± 0.33	08.81 ± 0.35
HS0	4.51 ± 0.21	6.14 ± 0.44	10.67 ± 0.54
HS45	3.47 ± 0.06	4.33 ± 0.33	07.79 ± 0.35
NS0	3.64 ± 0.21	4.56 ± 0.44	08.21 ± 0.54
NS45	3.25 ± 0.06	3.70 ± 0.33	06.95 ± 0.35
CS0	3.93 ± 0.21	3.64 ± 0.44	07.57 ± 0.54
CS45	3.39 ± 0.06	3.54 ± 0.33	06.94 ± 0.35

EC-Extracellular activity; IC- Intracellular activity; TA- Total activity; C-Control; HS-HeatStress; NS-Nutritional Stress; CS-Combined Stress

**Indicates statistical significance at $P < 0.01$; * Indicates statistical significance at $P < 0.05$;ns -Indicates non-significant; Values bearing different superscripts within a columndiffer significantly with each other

Figure 6: Effect of Heat stress, Nutritional stress and combined stresses (Heat and nutritional) on the (a) Extracellular activity of CMCase enzyme (b) Intracellular CMCase activity of enzyme (c) Total activity of CMCase enzyme in goats

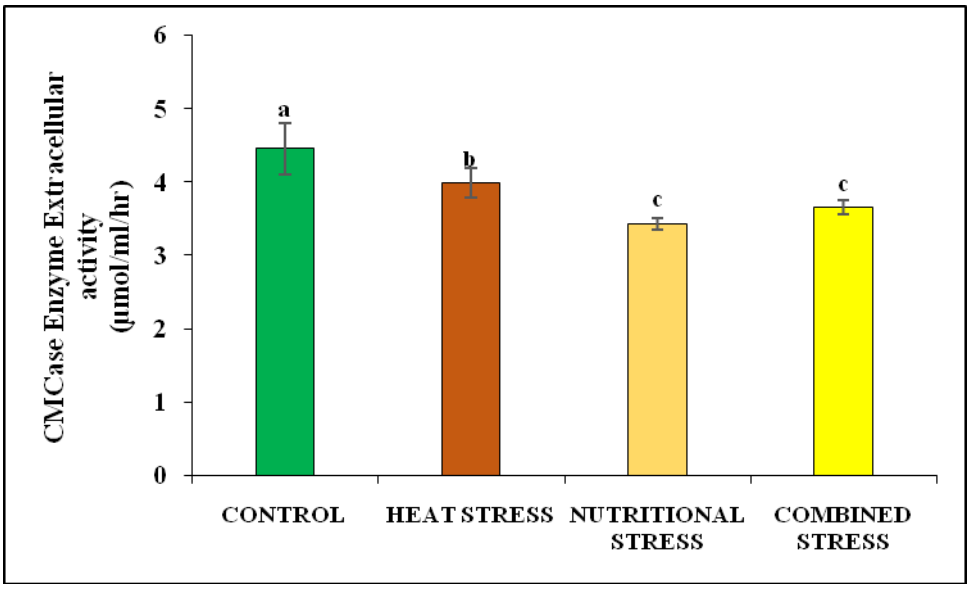


Fig. 6(a): Extracellular activity of CMCase enzyme

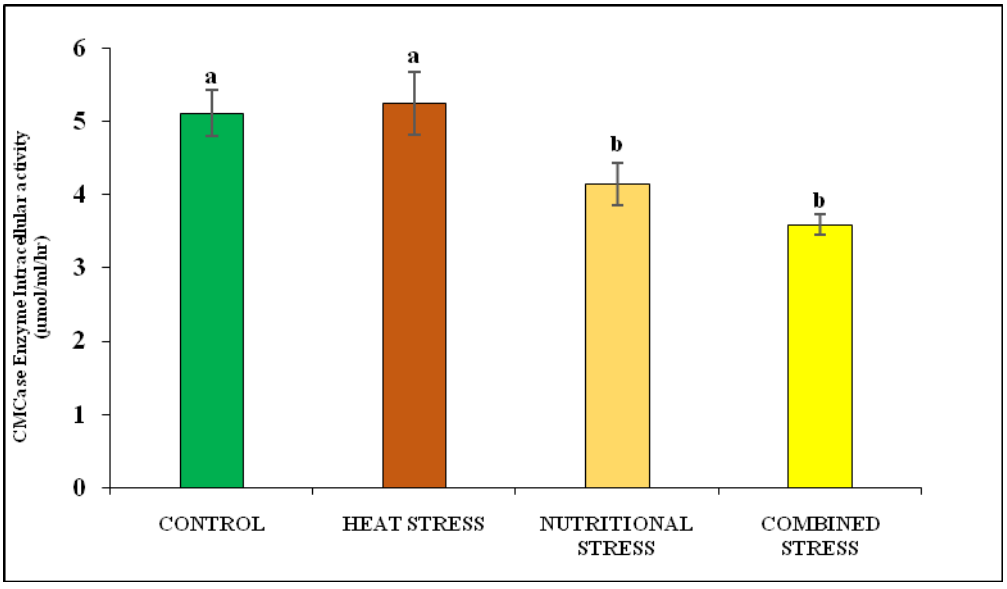


Fig. 6(b): Intracellular CMCase activity of enzyme

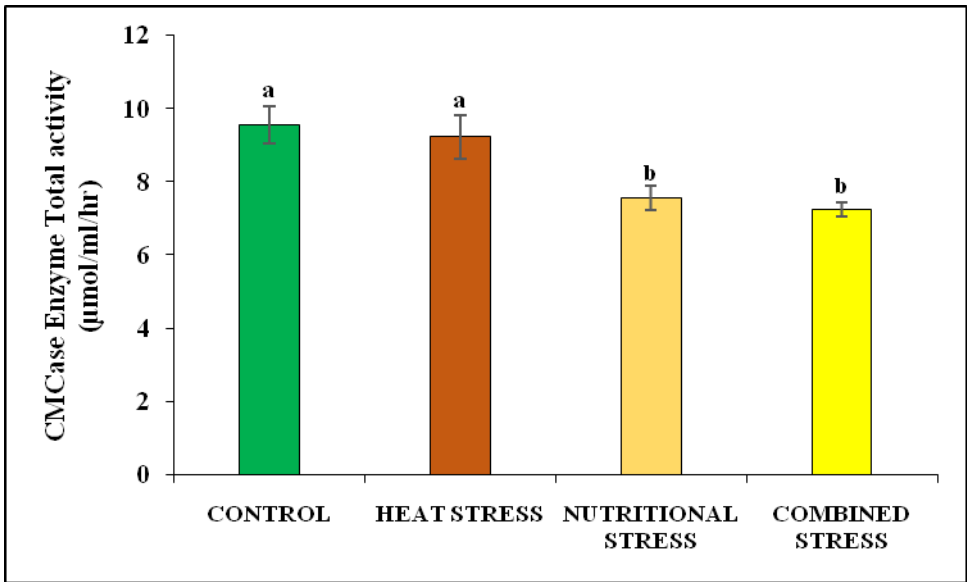


Fig. 6(c): Total activity of CMCase enzyme

4.6 Blood metabolic enzyme activities in bucks

The effects of HS, NS and CS on blood metabolic enzyme profile are described in table 9. Plasma ACP level did not differ between the groups for the treatment. However, the experimental days highly significantly ($P<0.01$) influenced plasma ACP level. Further, interaction between groups and experimental days also did not significantly influence plasma ACP level. Plasma ALP level showed significant ($P<0.01$) changes for the treatment. There were significant ($P<0.01$) differences in ALP between the *ad libitum* (C and HS) and restricted feeding (NS and CS) groups. Further, experimental days also significantly ($P<0.01$) influenced ALP in the study. In addition, interaction between groups and experimental days also significantly ($P<0.05$) influenced the plasma ALP in the study. Plasma ALT level showed significant ($P<0.05$) difference for the treatment. The highest plasma ALT level was recorded in CS group and this level was significantly different with C and HS groups. However plasma ALT did not differ between both *ad libitum* fed groups (C and HS) and restricted feeding groups (NS and CS). Further, experimental days also significantly ($P<0.05$) influenced plasma ALT level in the study. However, interaction between groups and experimental days did not significantly influence the plasma ALT level. Plasma AST level showed significant ($P<0.05$) difference for the treatment. The highest plasma AST level was recorded in CS group and this level was significantly different with C and HS groups. However plasma AST did not differ between both *ad libitum* fed groups (C and HS) and restricted feeding groups (NS and CS). Further, experimental days also highly significantly ($P<0.01$) influenced plasma AST level in the study. However, interaction between groups and experimental days did not influence significantly the plasma AST level.

Table 9: Effect of heat stress, nutritional stress and combined stresses (heat and nutritional stress) on the metabolic enzymes in goats

FACTOR	ACP (IU/L)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)
GROUP	ns	**	*	*
C	$2.41^{a} \pm 0.18$	$327.01^{a} \pm 30.8$	$15.76^{b} \pm 0.73$	$63.61^{b} \pm 4.62$
HS	$2.20^{a} \pm 0.20$	$310.41^{a} \pm 23.4$	$17.94^{b} \pm 1.05$	$69.21^{b} \pm 4.72$
NS	$1.95^{a} \pm 0.27$	$133.38^{b} \pm 23.83$	$20.85^{ab} \pm 2.3$	$86.3^{ab} \pm 10.4$
CS	$1.94^{a} \pm 0.19$	$123.25^{b} \pm 20.75$	$25.61^{a} \pm 2.54$	$107.6^{a} \pm 15.3$
DAYS	**	**	*	**
0	2.63 ± 0.18	281.72 ± 18.99	16.736 ± 1.02	58.87 ± 3.68
15	2.48 ± 0.28	215.75 ± 18.34	19.767 ± 1.95	80.52 ± 6.41
30	1.73 ± 0.16	218.19 ± 23.35	21.729 ± 2.04	76.16 ± 5.79
45	1.65 ± 0.16	178.40 ± 27.49	21.924 ± 2.06	111.9 ± 15.1
GROUP * DAYS	ns	*	ns	ns
C0	2.65 ± 0.36	273.41 ± 37.9	16.968 ± 2.05	63.64 ± 07.35
C15	2.52 ± 0.57	350.18 ± 36.7	15.754 ± 3.90	63.86 ± 12.81
C30	2.11 ± 0.31	378.63 ± 46.7	15.575 ± 4.08	73.95 ± 11.57
C45	2.35 ± 0.32	305.83 ± 54.9	14.739 ± 4.12	52.10 ± 30.09
HS0	2.56 ± 0.36	297.79 ± 37.9	14.648 ± 2.05	52.36 ± 07.35
HS15	2.78 ± 0.57	345.53 ± 36.7	16.502 ± 3.90	66.05 ± 12.81
HS30	1.61 ± 0.31	317.01 ± 46.7	19.469 ± 4.08	66.27 ± 11.57
HS45	1.86 ± 0.32	281.21 ± 54.9	21.133 ± 4.12	92.17 ± 30.09
NS0	2.72 ± 0.36	285.45 ± 37.9	18.733 ± 2.05	54.75 ± 07.35
NS15	2.35 ± 0.57	82.52 ± 36.68	21.230 ± 3.90	85.63 ± 12.81
NS30	1.43 ± 0.31	97.77 ± 46.69	21.648 ± 4.08	76.51 ± 11.57
NS45	1.28 ± 0.32	67.78 ± 54.98	21.786 ± 4.12	128.17 ± 30.1
CS0	2.60 ± 0.36	270.22 ± 37.9	16.595 ± 2.05	64.74 ± 07.35
CS15	2.27 ± 0.57	84.76 ± 36.68	25.583 ± 3.90	106.54 ± 12.8
CS30	1.78 ± 0.31	79.25 ± 46.69	30.223 ± 4.08	87.89 ± 11.57
CS45	1.12 ± 0.32	58.76 ± 54.98	30.038 ± 4.12	171.39 ± 30.1

ACP- Acid phosphatase;ALP- Alkaline phosphatase; ALT- Alanine aminotransferase; AST-Aspartate aminotransferase; C-Control; HS-Heat Stress; NS-Nutritional Stress; CS-Combined Stress

**Indicates statistical significance at $P < 0.01$; * Indicates statistical significance at $P < 0.05$;ns-Indicates non-significant; Values bearing different superscripts within a column differ significantly with each other

Figure 7: Effect of Effect of Heat stress, Nutritional stress and combined stresses (Heat and nutritional) on the metabolic enzymes in goats (a) ACP (b) ALP (c) ALT (d) AST in goats

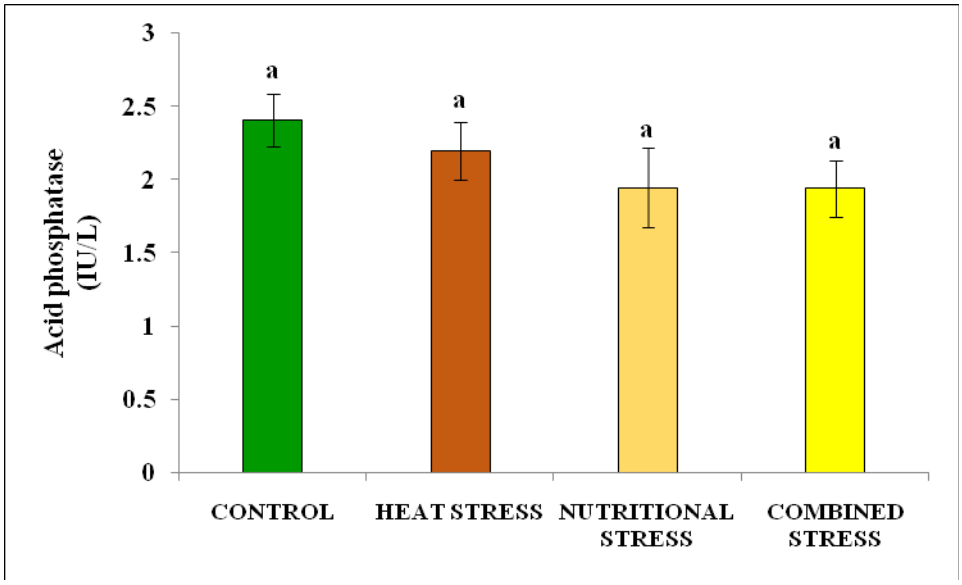


Fig. 7(a):Acid phosphatase

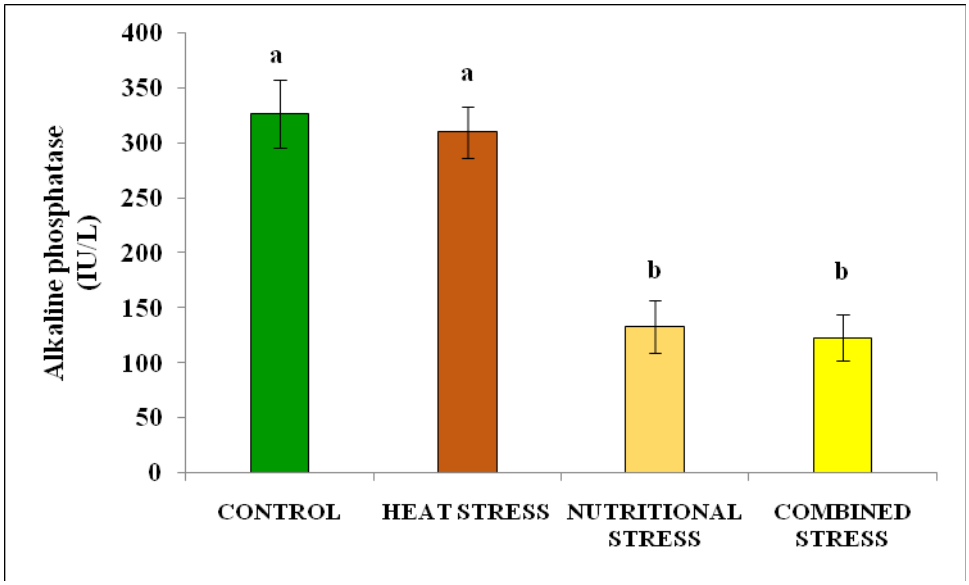


Fig. 7(b):Alkaline phosphatase

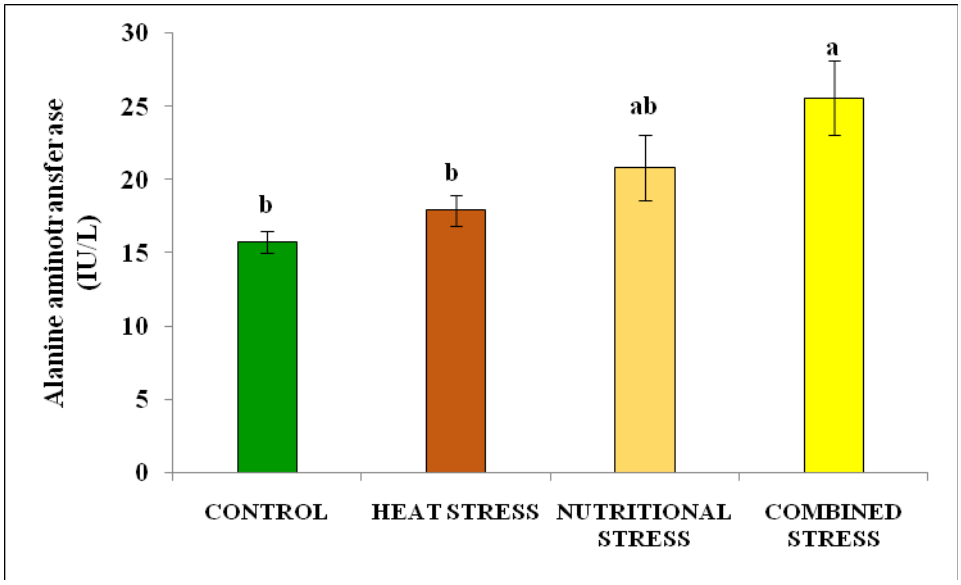


Fig. 7(c):Alanine aminotransferase

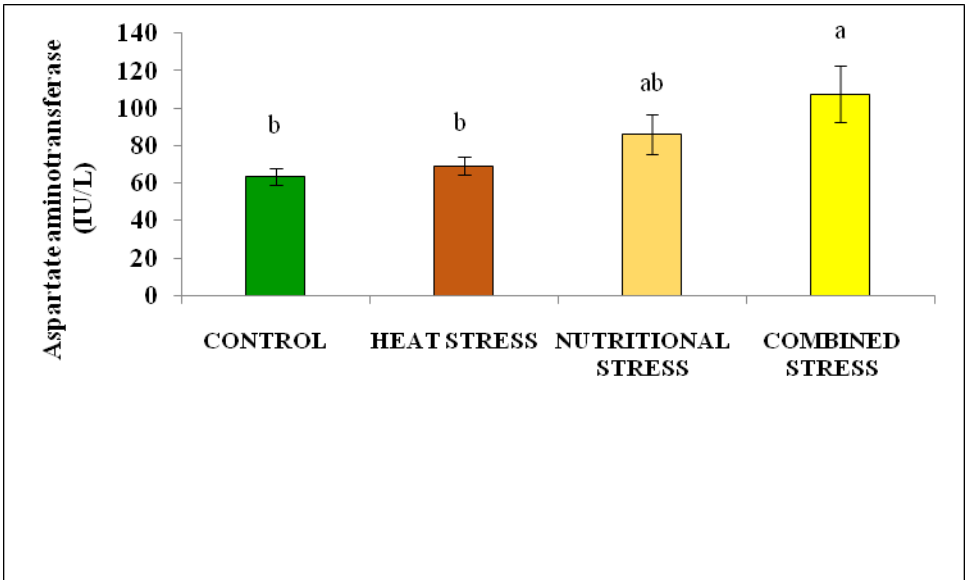


Fig. 7(c):Aspartate aminotransferase

4.7 Endocrine parameters

The effects of HS, NS and CS on plasma endocrine profile are described in table 10. Plasma T_3 level showed significant ($P<0.01$) variation between the groups. The highest plasma T_3 was recorded in C group and the lowest in all stress groups (HS, NS and CS). However, plasma T_3 level did not differed between the stress groups. Further, the experimental days and interaction between treatment and experimental days did not influence plasma T_3 level. Plasma T_4 level also showed significant ($P<0.01$) variation between the groups. The highest plasma T_4 was recorded in C group and the lowest in CS group bucks. The level of plasma T_4 differed significantly between HS and CS but did not differ between CS and NS. Further, plasma T_4 level between HS and NS also did not differed with each other. However, plasma T_4 in all stress groups was significantly lower as compared to C group. Further, experimental days also significantly ($P<0.05$) influenced plasma T_4 level. However, interaction between treatment and experimental days did not influence plasma T_4 level. Plasma leptin level also showed significant ($P<0.01$) variation between the groups. The highest plasma leptin was recorded in C group and the lowest in CS group bucks. The level of plasma leptin differed significantly among all the stress groups HS, NS and CS. However, plasma leptin level did not differ between C and HS group. Further, experimental days also significantly ($P<0.05$) influenced plasma leptin concentration. However, interaction between treatment and experimental days did not influence plasma leptin level.

Table 10: Effect of heat stress, nutritional stress and combined stresses (heat and nutrition stress) on the endocrine parameters in goats

FACTOR	T_3 (ng/ml)	T_4 (µg/dL)	Leptin (ng/ml)
GROUP	**	**	**
C	$2.37^{a} \pm 0.16$	$17.76^{a} \pm 0.98$	$2.53^{a} \pm 0.10$
HS	$1.48^{b} \pm 0.20$	$13.42^{b} \pm 1.02$	$2.37^{a} \pm 0.16$
NS	$1.53^{b} \pm 0.25$	$12.4^{bc} \pm 1.16$	$1.85^{b} \pm 0.15$
CS	$1.08^{b} \pm 0.23$	$10.27^{c} \pm 1.15$	$1.38^{c} \pm 0.20$
DAYS	ns	*	*
0	2.19 ± 0.24	16.92 ± 1.67	2.43 ± 0.17
15	1.50 ± 0.12	12.08 ± 0.74	1.89 ± 0.18
30	1.39 ± 0.19	12.85 ± 1.03	1.99 ± 0.10
45	1.38 ± 0.31	12.04 ± 0.83	1.82 ± 0.13
GROUP * DAYS	ns	ns	ns
C0	2.13 ± 0.49	16.92 ± 3.34	2.59 ± 0.34
C15	2.40 ± 0.24	19.65 ± 1.49	2.43 ± 0.36
C30	2.45 ± 0.38	17.79 ± 2.07	2.63 ± 0.21
C45	2.49 ± 0.61	16.68 ± 1.66	2.45 ± 0.26
HS0	2.53 ± 0.49	18.12 ± 3.34	2.33 ± 0.34
HS15	1.61 ± 0.24	08.71 ± 1.49	2.23 ± 0.36
HS30	1.10 ± 0.38	14.29 ± 2.07	2.59 ± 0.21
HS45	0.68 ± 0.61	12.55 ± 1.66	2.34 ± 0.26
NS0	2.13 ± 0.49	16.61 ± 3.34	2.41 ± 0.34
NS15	1.19 ± 0.24	11.82 ± 1.49	1.71 ± 0.36
NS30	1.12 ± 0.38	10.95 ± 2.07	1.74 ± 0.21
NS45	1.69 ± 0.61	10.38 ± 1.66	1.54 ± 0.26
CS0	2.00 ± 0.49	16.01 ± 3.34	2.39 ± 0.34
CS15	0.81 ± 0.24	08.15 ± 1.49	1.19 ± 0.36
CS30	0.86 ± 0.38	08.38 ± 2.07	0.99 ± 0.21
CS45	0.66 ± 0.61	08.54 ± 1.66	0.95 ± 0.26

T_3- Triiodothyronine;T_4- Thyroxine; C-Control; HS-Heat Stress; NS-Nutritional Stress; CS-Combined Stress

**Indicates statistical significance at $P < 0.01$; * Indicates statistical significance at $P < 0.05$; ns-Indicates non-significant; Values bearing different superscripts within a column differ significantly with each other

Figure 8: Effect of heat stress, nutritional stress and combined stresses (heat and nutritional stress) on the endocrine parameters in goats.

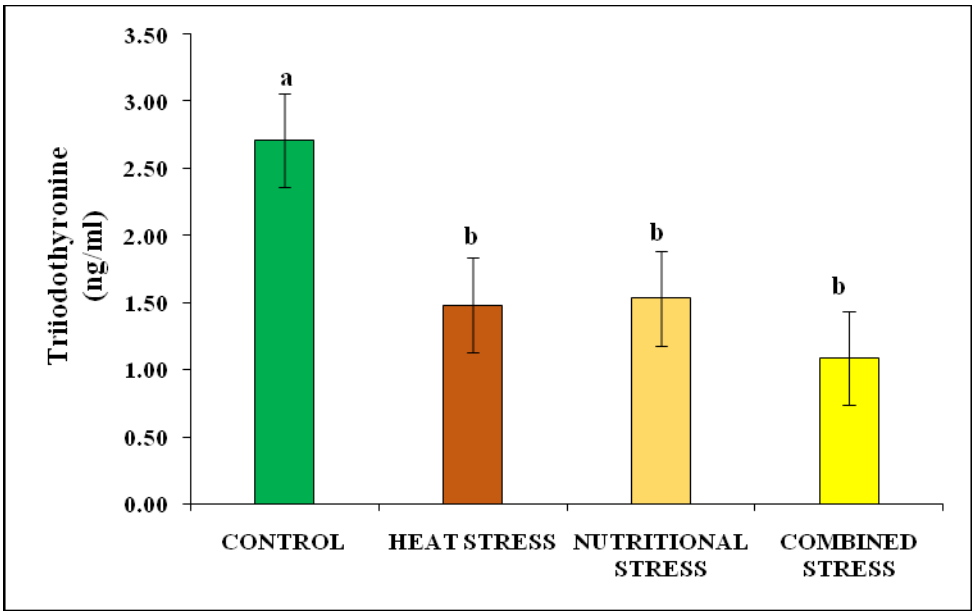


Fig. 8(a): Triiodothyronine

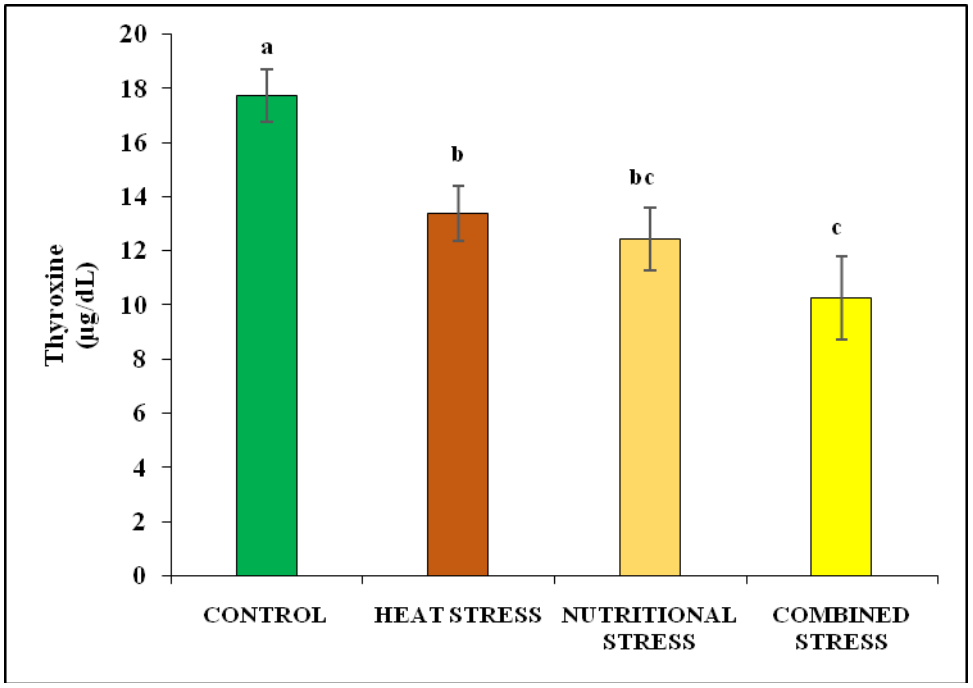


Fig. 8(b): Thyroxine

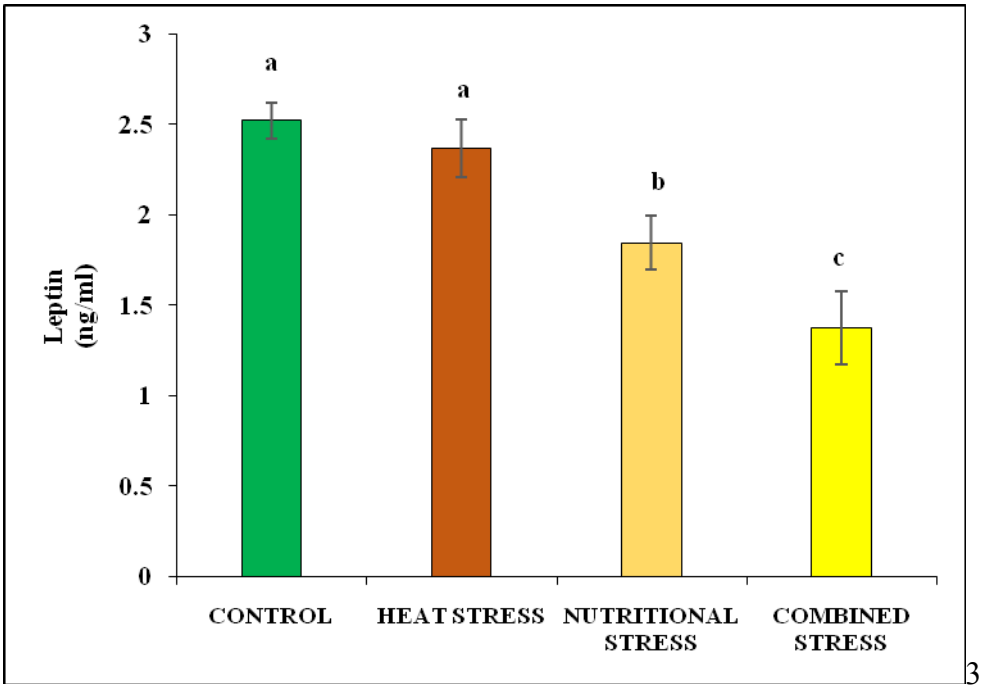


Fig. 8(c):Leptin

4.8 Gene Expression

4.8.1 Rumen HSP70 expression

Rumen HSP70 mRNA transcript expression between the C, HS, NS and CS groups of goats are described in Fig.10. The results revealed that rumen HSP70 mRNA expression was evident in C (1 fold), HS (1.23 fold), NS (0.34 fold) and CS (1.29 fold). On comparative basis, the higher expression of rumen HSP70 mRNA was recorded in CS goats (Fig. 10). Within the stress groups, the highest rumen HSP70 mRNA expression was recorded in CS group followed by HS and NS groups.

4.8.2 Intestinal HSP70 Expression

Intestinal HSP70 mRNA transcript expression between the C, HS, NS and CS groups of goats are described in Fig. 11. The results revealed that intestinal HSP70 mRNA expression was evident in C (1 fold), HS (1.62 fold), NS (0.87 fold) and CS (0.93 fold). On comparative basis, the higher expression of intestinal HSP70 mRNA was recorded in HS goats (Fig. 11). Within the stress groups, the highest intestinal HSP70 mRNA expression was recorded in HS group followed by CS and NS groups.

4.9 Histopathology of Rumen and Thyroid

The H and E staining results of different organs studied revealed that only thyroid and rumen section showed significant changes between the groups. However, reticulum, Omasum, abomasum, small intestine and large intestine did not showed significant changes for different treatments in the study. The rumen H and E section showed significant changes for different stresses. The length of rumen villi and thickness was reduced more in CS followed by HS, NS compared to C, whereas keratinization was observed highest in CS group followed by HS and NS groups compared to C group. Similarly, the thyroid section also showed significant changes for different stresses. These changes in different stress groups (HS, NS and CS) were compared with C group. The thyroid alveoli showed significantly lower thyroglobulin activity in HS group followed by CS and NS groups as compared to C group.

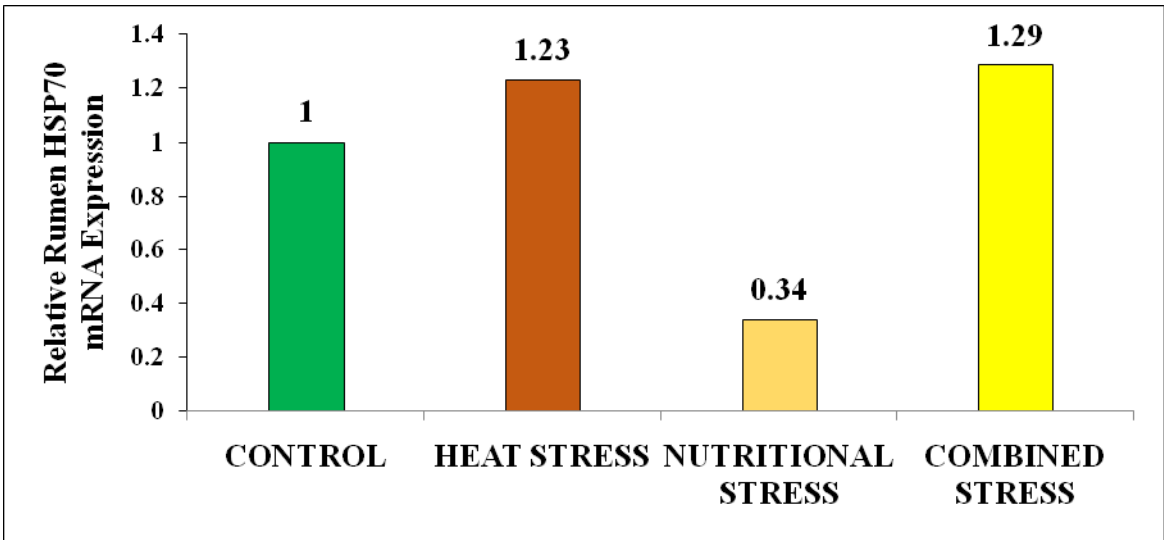


Fig. 9: Rumen HSP70 mRNA transcript expression between the control, heat stress, nutritional stress, combined stresses (heat & nutritional) groups of goats

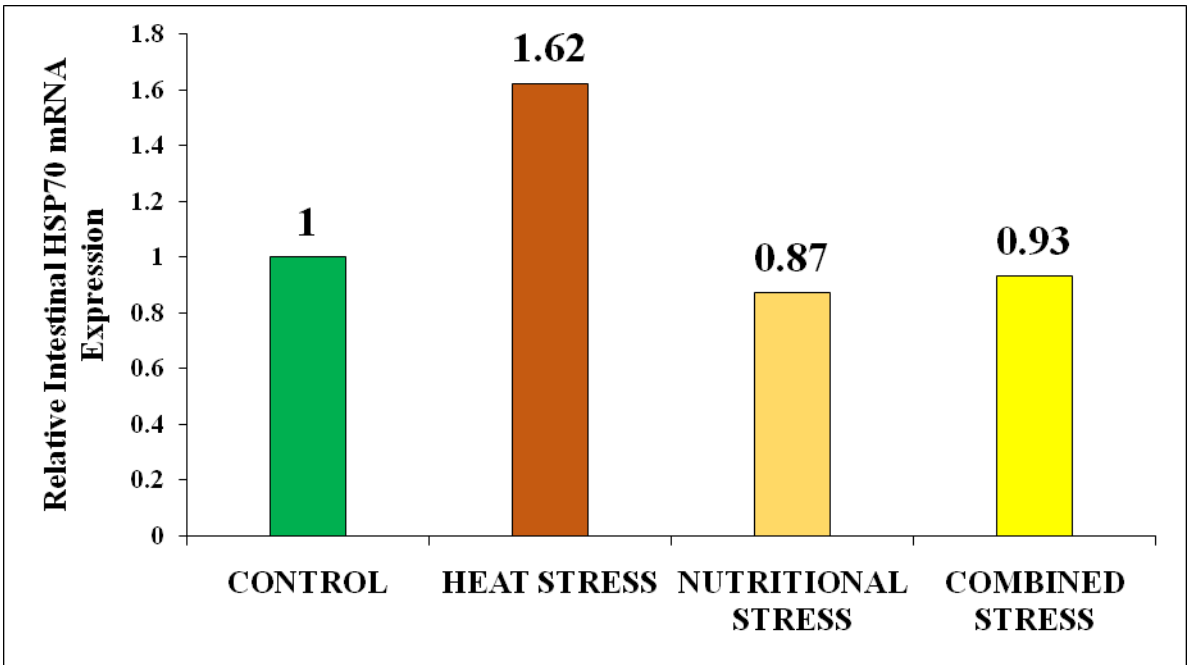


Fig. 10: Intestinal HSP70 mRNA transcript expression between the control, heat stress, nutritional stress, combined stresses (heat & nutritional) groups of goats

the study.

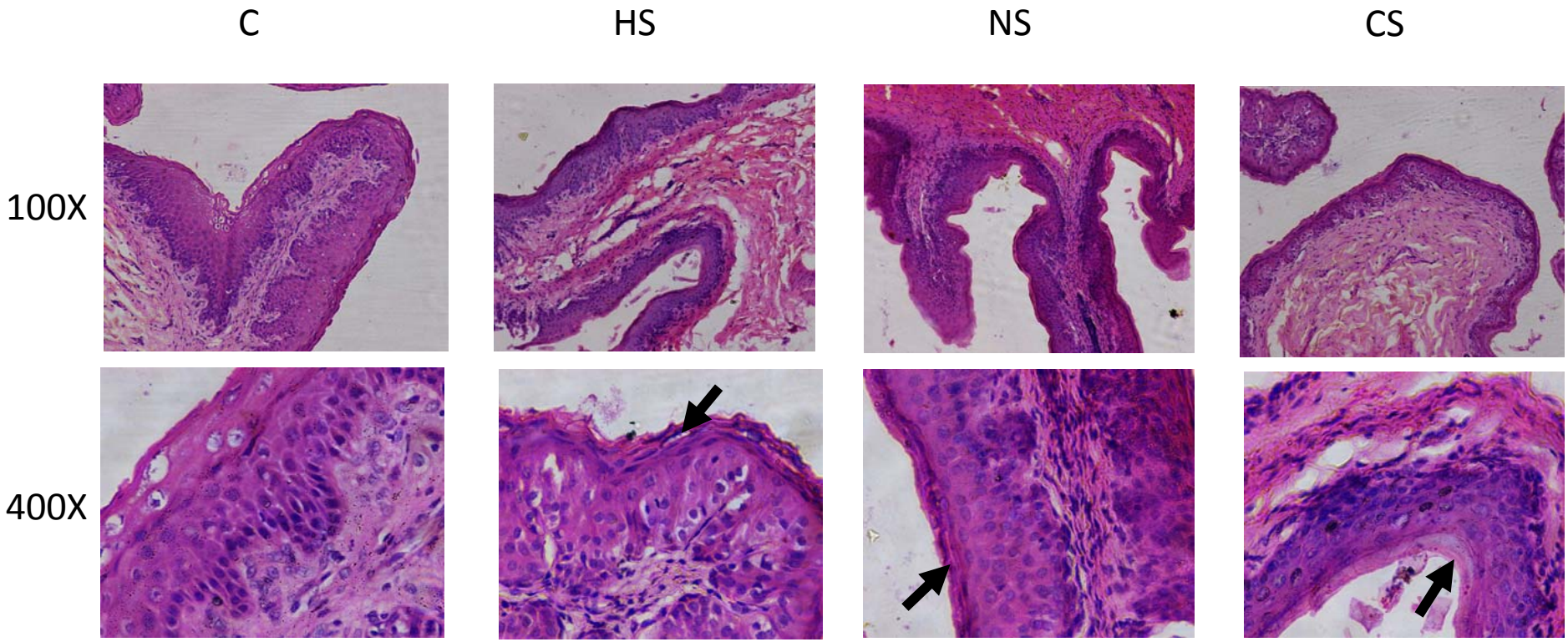


Plate 5: Histopathological changes in H&E stained sections from rumen of C (Control; n=6), HS (Heat Stress; n=6), NS (Nutritional Stress; n=6) and CS (Combined stress; n=6) animals subjected to different kinds of stress. The length of rumen villi and thickness was reduced more in CS followed by HS, NS compared to C, whereas keratinization was observed highest in CS group followed by HS and NS groups compared to C group.

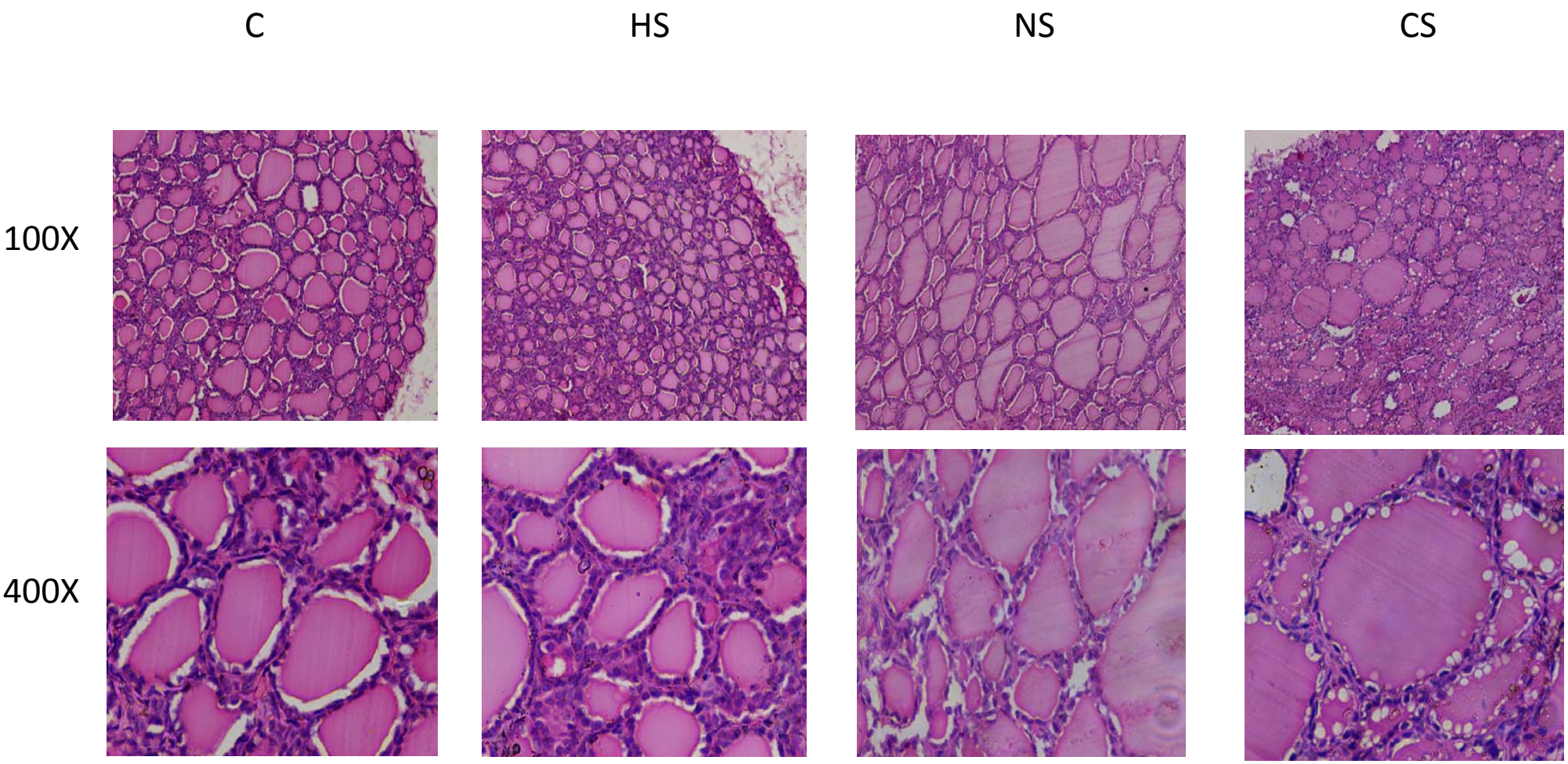


Plate 6: Histopathological changes in H&E stained sections from thyroid glands of C (Control; n=6), HS (Heat Stress; n=6), NS (Nutritional Stress; n=6) and CS (Combined stress; n=6) animals subjected to different kinds of stress. The HS group thyroid alveoli showed less amount of thyroglobulin activity followed by CS and NS groups compared to C group.

CHAPTER 5
DISCUSSION

Research pertaining to environmental stress influence on rumen fermentation pattern and metabolic profiles in livestock are very meagre. In the changing climate scenario, these environmental stresses do not occur individually but rather they occur simultaneously. Hence research efforts are needed to study the cumulative impact of environmental stresses on rumen fermentation pattern and metabolic activity. In that line, this is the first experiment which attempted to establish the cumulative impact of both HS and NS simultaneously on goat rumen fermentation pattern and metabolic activities. In the changing climatic condition, goat is considered to be an important species for the livelihood security of poor and marginal farmers. Hence the outcome of this research can add lot of value to the very limited existing knowledge available in this field. This in turn may pave way for development of suitable mitigation strategies to improve goat production in the changing climatic condition.

5.1 VFA production

Kadzere *et al.* (2002) observed that HS reduced the concentration of VFA in the rumen of cattle, a response which would not be expected to cause the inhibition of reticulo-rumen motility associated with HS. The treatments in the study significantly influenced production of propionic acid, butyric acid and valeric acid. However, HS alone did not significantly influence the different VFA production. However, both NS and CS groups showed significant variation in the types of VFAs production. This shows the sensitivity of nutritional status to influence the VFAs production. HS in animals results in dramatic reduction in roughage intake, gut motility and rumination which in turn contribute to decreased VFA production and may contribute to alteration in acetate: propionate ratio (Kadzere *et al*., 2002). The TVFAs production was significantly lower only in CS group as compared to other group. This could be attributed to both HS and NS in

CS group goats. There are reports suggesting HS reduced VFA production (Tajima *et al*., 2007; Nonaka *et al*., 2008). Similarly Salles *et al*. (2010) reported that the higher ruminal temperature significantly decreased the TVFAS concentration in cattle. However, there are also reports indicating that ruminal temperature did not affect the proportion of VFA in cattle (Salles *et al*., 2010; King *et al*., 2011; Yadav *et al*., 2013). This difference in TVFA concentration between the groups could be attributed to the type of microbial population residing in rumen and HS brings about reduction by reducing the activity of particular microbes (Uyeno *et al*., 2010). Further, there are also reports that HS brings about reduction in TVFA production by increasing the pH of rumen liquor (Hall, 2009; Yadav *et al*., 2013). Few authors have reported that changes in rumen fermentation pattern also may be brought about by changes in DMI (Smith *et al*., 2013; Yadav *et al*., 2013). The experimental days significantly influenced the production of various VFAs. Further, the interaction between treatment and experimental days significantly influenced all VFAs production except acetic acid. This showed that the relationship between the groups for different VFAs production changed over time indicating that the animals are trying to adapt to the existing conditions. The acetate: propionate ratio also differed significantly between the groups. The highest ratio was obtained in both NS and CS groups as compared to C and HS groups again indicating the role of nutrition in inducing changes in rumen fermentation pattern. However, Nonaka *et al*. (2008) contradicted our finding and reported that the ratio of acetate to propionate decreased during HS. The highest acetate to propionate ratio in NS and CS groups indicate that the level of methane production could be more in these groups as generally acetate production results in releasing of four hydrogen molecules in the rumen which will be acted upon by the methane producing bacteria to produce methane. The non-significant influence of interaction between treatment and experimental groups on acetate: propionate ratio indicates that the treatment effect persisted indicating both NS and HS can lead to more methane production in goat.

5.2 Biochemical parameter of rumen liquor

Beede and Collier (1986) concluded that rumen function during HS may be altered by pituitary effects on basal metabolism, such as via reduced somatotropin and thyroid hormones. The rumen liquor NH_3-N of CS group significantly differed with C group. However, both HS and NS independently did not influence rumen liquor NH_3-N. But the cumulative effect significantly influenced. This shows the significance of this study to establish the cumulative impact of both HS and NS in goat in the changing climate. The significantly lower NH_3-N in CS group could be attributed to the joint impact of HS and NS. However, there are reports suggesting influence of nutrition alone on the rumen ammonia concentration (Chanjula *et al*., 2014). They reported that energy deficient diet significantly decreased rumen liquor NH_3-N concentration. Rumen liquor pH has strong correlation with rumen microbial activity and rumen TVFAs production (Dijkstra *et al*. 1992; Meng-zhi *et al*., 2008). However, in the present experiment the treatment both HS and NS individually or in combination did not influence the rumen liquor pH. But there are reports suggesting lower rumen liquor pH during HS in cattle (Collier *et al*., 1982; Kadzere *et al*., 2002; Yadav *et al*., 2012). Further, the non-significant influence of experimental days and interaction between treatment and experimental days suggests that the non-significant influence of these stresses on rumen liquor pH persisted over the time in goat. Both TCA and total nitrogen showed similar trend to the treatments with significantly lower values being in CS and NS groups as compared to both C and HS groups. Similarly, the experimental days and interaction between treatment and experimental days also significantly influenced NH_3-N, TCA and total nitrogen content of rumen liquor. This shows that these parameters varied over the period indicating that at some point their concentration was significantly different while at some other time the values were non-significantly different.

5.3 Enzyme parameter of rumen liquor

The activities of α-Amylase showed no definite trend related to both HS and NS in the study. The treatment did not influence both extracellular and intracellular α-Amylase activities. Further the TA of α-Amylase of rumen liquor

also did not differed between the groups. This shows that neither HS nor NS was able to influence α-Amylase activities. Further the non-significant interaction between treatment and experimental days for EC α-Amylase activity shows that the non-significant treatment effects on EC α-Amylase activity persisted over the entire experimental schedule. However, the significant interaction between treatment and experimental days for IC and TA ofα-Amylase shows that the enzyme activity varied over the period indicating that at some point the enzyme activity was significantly different while at some other time the values were non-significantly different. CMCase activity extracellular showed higher variation for the treatment with all stress groups (HS, NS and CS) significantly lower than C group. This shows the severity of HS and NS individually as well as cumulatively influencing the rumen liquor EC CMCase activity. The significantly lower EC CMCase activity in CS groups as compared to HS group may be attributed to the additional NS in CS group. Saro *et al*. (2014) also reported the significant influence of quality of nutrition on CMCase activity in sheep. They reported that the animal with low quality feed had significantly lower CMCase activity in sheep as compared to high quality feed in sheep. The significant influence of experimental days and interaction between treatment and experimental days suggests that the animals were trying to adapt to the existing stressful condition to alter the EC CMCase activity in goat. Further, both intracellular and TA of CMCase of rumen liquor was significantly lower in NS and CS groups as compared to *ad libitum* fed groups (C and HS). This shows the sensitivity of CMCase activities for the environmental stresses. Both HS and NS could have reduced the number of microorganisms residing in the rumen. The reduced CMCase activities could be due to lower protozoa concentration as a result of these stresses. There are reports suggesting lower CMCase activities in defaunated animals (Santra and Karim, 2002; Eugene *et al*., 2004; Wina *et al*., 2006). The non-significant effect of interaction between treatment and experimental days suggests that the effect of treatment on IC and TA of CMCase persisted over the time. This shows that IC and TA of CMCase may be reliable indicator for both HS and NS in goats.

5.4 Blood metabolic enzymes

The treatment did not influence ACP but significantly influenced ALP. The significantly lower ALP was recorded in both NS and CS groups as compared to C and HS groups. This shows that HS alone was not severe to induce reduction in ALP level. However, when HS and NS combined it significantly reduced plasma ALP level. This difference could be attributed to the different levels of feed intake between the groups. In a similar study on sheep, Sejian *et al*. (2010b) concluded that both ACP and ALP decreased significantly in HS and combined stress groups as compared to the C (TNZ). As a general adaptive mechanism the animals try to reduce their metabolic activity to reduce the additional heat load to the animals. There are reports suggesting lower ALP in heat stressed goat (Helal *et al*., 2010; Gupta *et al*., 2013). All these authors attributed this reduced metabolic enzymes in heat exposed goat to the reduction in both thyroid activity as well as circulating thyroid hormone concentration in their study. Similarly in the current study also both T_4 and T_3concentrations were significantly lower in both HS and CS groups. This proves the adaptive capability of goat to both HS and CS by lowering their metabolic activity to prevent the additional heat load to these animals. The significant interaction between treatment and experimental days for plasma ALP shows that the relationship between the groups changed over time indicating that the animals are trying to adapt to the existing condition.

ALT and AST also showed significant difference between the groups. Both ALT and AST was significantly higher in CS group as compared to C and HS group. The increased activity in some enzymes with rising temperature may be due to the accelerated reactions at higher temperature. Similarly Gupta *et al*. (2013) reported significantly higher concentration of both ALT and AST in goat during HS and concluded that both AST and ALT are helpful in diagnosis of welfare of animals. In addition, both Naqvi et al. (1991) and Nazifi et al. (2003) also reported similar HS induced significantly higher concentration of both ALT and AST in sheep. They attributed this increase to reflect higher adaptation capability in these animals. Further, Banerjee et al. (2015) reported significantly higher level of AST in heat stressed goats. However, in our study the level of AST

was significantly higher only when HS was coupled with NS. This shows the severity of cumulative effect of combined stress in the study. However, Sharma and Kataria (2011) reported that HS only significantly increased ALT but observed no effect on AST concentration in goat. Similarly HS induced non-significant effect on AST in goat was reported by Ocak et al. (2009). The non-significant difference in interaction between treatment and experimental groups shows that the effect of different stresses on both plasma AST and ALT persisted throughout the experiment indicating their significance for adaption in goat.

5.5 Endocrine parameters

Plasma thyroid hormone concentrations differed significantly between the groups. Both T_4 and T_3 was significantly lower in stress groups. Both the T_3 and T_4 are associated with the metabolic homeostasis and are found to alter with the climatic changes (Sejian *et al*., 2013). Appropriate thyroid gland function and activity of T_3 and T_4 are also considered crucial to sustain the productive performance in domestic animals (Todini, 2007).Both T_4 and T_3 significantly reduced in both HS and NS groups in goat. In a similar study in Malpura ewes, Sejian *et al*. (2014b) reported significantly lower T_3 and T_4 concentration in NS group. They observed that, the more the feed is restricted, the lower the thyroid hormone concentrations in sheep. During summer, the exposure of animals to high ambient temperature was associated with depression of thyroid activity thereby causing a relatively lower concentration of thyroid hormones (Rasooli *et al*., 2004; Stockman *et al*., 2011). It is an established fact that depression of thyroid function during HS was part of the process of metabolic adaptation, by which the heat production may consequently be maintained at low level (Sejian *et al*. 2010b). There are evidences suggesting that thermal stress acts directly on the HPA axis causing a reduction in TSH secretion (Rasooli *et al*. 2004; Sejian and Srivastava, 2010). In addition, blood thyroid hormones are considered to be good indicators of the nutritional status of an animal (Riis and Madsen 1985; Rhoads *et al*. 2009). Further, energy balance also can play a major role in affecting the decrease in plasma Thyroid hormone levels in small ruminants (Kong *et al*., 2004), thus signifying the importance of optimum nutrition for maintaining

appropriate thyroid hormone levels in sheep (Todini, 2007). Consistent with this notion, plasma thyroid hormone concentrations are correlated with feed intake in several ruminant species (Kong *et al*., 2004; Kafi *et al*., 2012). Further, thyroid hormones decide the metabolic activity and metabolic activity intern gets drastically affected due to NS. This shows that thyroid hormones could directly depict the nutritional status of the animals. Based on the above findings, it could be inferred that circulating thyroid hormone concentration can be a good indicator for NS in goat. The non-significant difference in interaction between treatment and experimental groups shows that the effect of stresses on both plasma T_3 and T_4 persisted throughout the experiment indicating their significance for adaption in goat.

Changes in nutritional status are accompanied by changes in blood metabolites and hormones such as IGF-1 and leptin in ruminant species (Chilliard *et al*., 2001). Blood leptin levels respond to changes in nutritional status in livestock (Amstalden, 2003, Lee *et al*., 2005). Leptin has multiple physiological effects and plays a pivotal role in control of body growth and adaptation. The level of plasma leptin was found to be significantly lower in NS as compared to C goats. Amstalden (2003) also reported similar finding of low level of circulating leptin in cow subjected to short-term feed restriction and attributed this to the diminished synthesis of leptin in adipose tissue. These results were consistent with previous studies revealing that the level of leptin decreased with food deprivation or under nutrition (Adam *et al*., 2000; Lee *et al*., 2005]. The reduction in circulating leptin during feed restriction may be attributed to the reduction in plasma insulin levels as there are reports suggesting reduction in both leptin and insulin in parallel with the reduction in BW (Havel *et a*l., 1996; Recabarren *et al*., 2004). The reduced leptin concentration in feed restricted goats could be to stimulate the appetite center in the hypothalamus to stimulate feed intake and initiate adaptive mechanisms by stimulating glucocorticoid production to favor hepatic gluconeogenesis and to decrease thyroid activity to maintain homeostasis in animals (Friedman and Halaas, 1998; Lee *et al*., 2005; Hyder *et al*., 2013). The non-significant difference in interaction between treatment and experimental

groups shows that the effect of stresses persisted on plasma leptin concentration persisted throughout the experiment indicating their significance for adaption in goat.

5.6 Rumen and intestinal HSP70 gene expression

HSP70 is one of the most abundant and best characterized HSP family that consists of highly conserved stress proteins, expressed in response to stress, and plays crucial roles in environmental stress tolerance and adaptation in goat (Gupta *et al*., 2013; Banergee *et al*., 2014; Mohanarao *et al*., 2014).On comparative basis, the higher expression of rumen HSP70 messenger Ribonucleic acid (mRNA) was reported in CS goats followed by HS group. However, in NS group the expression of rumen HSP70 mRNA is down regulated. There are reports suggesting expression of HSP in rumen (Cronje, 2005). Cronje (2005) attributed this HSP expression in rumen to redistribution of blood to the periphery and compensatory reduction in the blood supply to the gut, which damages cells lining the gut, permitting endotoxin to enter the body. These consequences are more severe in CS group since the rumen is populated with bacteria that are predominantly of the endotoxin–producing gram–negative type and since two stresses are impacting simultaneously the CS group, the endotoxin production would be more in this group leading to more HSP70 expression (Cronje, 2005). The down regulation of HSP70 in NS group signifies the importance of optimum nutrition to induce more functional HSP70 expression in goat. Further, the different folds of HSP70 expression in HS, NS and CS indicates the different role of HSP70 in adaptation of goats to HS, NS and CS. However, in the intestine HSP70 expression was significantly higher in HS group as compared to NS and CS groups. This again signifies the importance of optimum nutrition for HSP70 expression. The lower expression of HSP70 in CS as compared to HS group could be attributed to the restricted feeding in CS group. There are reports indicating expression of HSP70 in gut during HS (Hotchkiss *et al*., 1993; Flanagan *et al*., 1995). Exposure to heat results in redistribution of blood to the periphery and compensatory reduction in the blood supply to the gut, which damages cells lining the gut leading to endotoxin production which ultimately culminates in increased HSP70 expression

in intestine (Cronje, 2005). The finding that the greatest increase in HSP concentrations following exposure to heat occurs in intestine (Flanagan *et al*., 1995) provides additional support for the contention that damage to the cells of the gut is central to the adverse effects of heat load (Cronje, 2005). The possibility that the integrity of the gut lining is critical to resilience against hyperthermia has great significance for livestock species because energy–dense production diets are known to damage the gut lining (Cronje, 2005). The results clearly indicate that HSP70 expression in both rumen and intestine could be attributed to the level of endotoxin produced in these tissues.

5.7 Histopathology

The H and E staining results of different organs studied revealed that only rumen and thyroid sections showed significant changes between the groups but did not influence reticulum, Omasum, abomasum, small intestine and large intestine. This shows that in goat environmental stresses influence only rumen to bring about changes in rumen fermentation pattern and further influence thyroid gland to reduce the metabolic activity. The reduced rumen villi length and thickness and further the higher keratinization in CS group rumen indicate the hyper function of rumen in these bucks. In addition, the higher expression of rumen HSP70 mRNA also supports this notion in CS goats. The thyroid alveoli showed significantly lower thyroglobulin activity in HS group followed by CS and NS groups as compared to C group. This indicates the lower functioning ability of thyroid gland in all stress groups (HS, NS and CS) as compared to C group bucks. The lower level of both T_3 and T_4 in all stress groups in the current study proves the lower activity of thyroid gland in stress groups. This could be the adaptive mechanisms of stress group (HS, NS and CS) bucks to produce less metabolic heat. The reduced thyroglobulin activity in CS and NS groups as compared to HS group could be attributed to the feed restriction in

CHAPTER 6

SUMMARY AND CONCLUSION

A study was designed to establish the impact of Heat Stress and Nutritional Stress simultaneously on the rumen fermentation pattern and metabolic activities in buck. Such information would be very valuable as under the changing climatic condition, Heat Stress and Nutritional Stress are the major stresses occurring and it is very vital to study these stresses influence on rumen fermentation pattern and metabolic activity. These attempts are first of its kind in any livestock species and the outcome of this study in goat may be useful in understanding the combined stress effect on rumen fermentation pattern and metabolic adaptation in other livestock species.

Twenty four adult Osmanabadi bucks (average Body Weight 16.0 kg) were used in the present study. The bucks were divided into four groups viz., C (n=6; control), HS (n=6; heat stress), NS (n=6; nutritional stress) and CS (n=6; combined stress). The study was conducted for a period of 45 days. The animals were stall fed with a diet consisting of 60% roughage (Hybrid Napier) and 40 % concentrate (maize 36 kg, wheat bran 37 kg, soya bean meal 25 kg, mineral mixture 1.5 kg and common salt 0.5kg/100 kg of feed). C and HS bucks had *ad libitum* access to their feed while NS and CS bucks were under restricted feed (30% intake of C bucks) to induce NS. The HS and CS bucks were exposed to solar radiation for six hours a day between 10:00 h to 16:00 h to induce HS. Rumen metabolic enzymes, types and Total Volatile Fatty Acid produced, blood metabolic enzymes and endocrine responses were recorded at fortnightly interval. After slaughter different organs were collected for Heat Shock Protein 70 gene expression and for histopathological observations. The data were analyzed using repeated measures analysis of variance.

The activities of α-Amylase showed no definite trend related to both Heat Stress and Nutritional Stress in the study. It showed that neither Heat Stress nor Nutritional Stress was able to influence α-Amylase activities. Further the non-

significant interaction between treatment and experimental days for EC α-Amylase activity showed that the non-significant treatment effects on EC α-Amylase activity persisted over the entire experimental schedule. EC CMCase showed higher variation for the treatment with all stress groups (HS, NS and CS) showing significantly lower EC CMCase activity than C group. IC and TA CMCase activity of rumen liquor was significantly lower in NS and CS groups as compared to *ad libitum* fed groups (C and HS).The significantly lower EC CMCase activity in CS groups as compared to HS group may be attributed to the additional NS in CS group. The non-significant effect of interaction between treatment and experimental days suggests that the effect of treatment on IC and total CMCase activities persisted over the time. This shows that IC and total CMCase activities may be reliable indicator for both HS and NS in goats.

The highest concentration of NH_3-N ($P<0.05$) was high in C while the lowest in CS group. The rumen liquor NH_3-N of CS group significantly differed with C group. The significantly lower NH_3-N in CS group could be attributed to the joint impact of HS and NS. Both TCA and total nitrogen showed similar trend to the treatments with significantly lower values being in CS and NS groups as compared to both C and Hs groups. Similarly, the experimental days and interaction between treatment and experimental days also significantly influenced NH_3-N, TCA and total nitrogen content of rumen liquor. This indicates that the animals were trying to adapt to the existing conditions.

There were significantly ($P<0.01$) higher levels of propionic acid, butyric acid, and valeric acid in *ad libitum* (C and HS) fed groups as compared to restricted fed (NS and CS) groups. The TVFA production was significantly lower only in CS group as compared to other group. This could be attributed to both HS and NS in CS group goats. Further, the interaction between treatment and experimental days significantly influenced all VFAs production except acetic acid. This showed that the relationship between the groups for different VFAs production changed over time indicating that the animals are trying to adapt to the existing conditions. Further, the acetate and propionate ratio was significantly ($P<0.01$) higher in CS

and NS groups as compared to C and HS groups. The non-significant influence of interaction between treatment and experimental groups on acetate: propionate ratio indicates that the treatment effect persisted indicating both NS and HS can lead to more CH_4 production in goat.

The treatment did not influence ACP but significantly influenced ALP. There were significantly ($P<0.01$) higher levels of ALP in *ad libitum* (C and HS) fed groups as compared to restricted fed (NS and CS) groups. This shows that HS alone was not severe to induce reduction in ALP level. However, when HS and NS combined it significantly reduced plasma ALP level. The significant interaction between treatment and experimental days for plasma ALP shows that the relationship between the groups changed over time indicating that the animals are trying to adapt to the existing condition. However, plasma AST and ALT showed reverse trend ($P<0.05$) to plasma ALP. The non-significant difference in interaction between treatment and experimental groups shows that the effect of different stresses on both plasma AST and ALT persisted throughout the experiment indicating their significance for adaption in goat.

The highest ($P<0.01$) plasma T_3 and T_4 was recorded in C group as compared to stress groups (HS, NS and CS). The non-significant difference in interaction between treatment and experimental groups shows that the effect of stresses on both plasma T_3 and T_4 persisted throughout the experiment indicating their significance for adaption in goat. Further, the highest leptin concentration was recorded in C group while the lowest level in CS group. The non-significant difference in interaction between treatment and experimental groups shows that the effect of stresses persisted on plasma leptin concentration persisted throughout the experiment indicating their significance for adaption in goat.

The higher expression of rumen HSP70 mRNA was reported in CS goats. Within the stress groups, the highest rumen HSP70 mRNA expression was reported in CS group followed by HS and NS groups. Further, the higher expression of intestinal HSP70 mRNA was reported in HS goats. Within the stress

groups, the highest intestinal HSP70 mRNA expression was reported in HS group followed by CS and NS groups. The H and E staining results of different organs studied revealed that only thyroid and rumen section showed significant changes between the groups. The thyroid alveoli showed significantly lower thyroglobulin activity in HS group followed by CS and NS groups as compared to C group. The length of rumen villi and thickness was reduced more in CS followed by HS, NS compared to C, whereas keratinization was observed highest in CS group followed by HS and NS groups compared to C group.

The present study revealed that bucks subjected to Heat Stress and Nutritional Stress separately had less detrimental effects on buck's rumen fermentation pattern and metabolic activities. Further, when compared to NS, HS had less significant effect on the rumen fermentation pattern and metabolic activities in the bucks. This indicates that when nutrition is not a limiting factor then bucks were able to better cope up with HS. However, when both these stresses were coupled, it had serious consequences on rumen fermentation pattern and metabolic activities studied in these bucks. In addition, the study indicated that Osmanabadi bucks possessed the capability to adapt to the detrimental effects of environmental stresses which is evident from the significant interaction of treatment and experimental days on α-Amylase IC, α-Amylase TA, EC CMCase, NH_3-N, TCA, total nitrogen, Propionic acid, Butyric acid, Valeric acid, Iso-Valeric acid, TVFA and ALP. In addition, the study indicated that plasma T_3, T_4, leptin concentration and rumen HSP70gene may act as ideal biological marker for assessing the impact of combined stress on rumen fermentation pattern and metabolic activities in bucks. Further studies are required to have a clear understanding of these associations at a mechanistic level if we are to fully exploit the potential of nutritionally manipulated rumen fermentation and metabolic activities in bucks under HS condition.

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ABSTRACT

A study was conducted to assess the combined effect of heat stress and nutritional restriction on rumen fermentation characteristics and metabolic activities in Osmanabadi Bucks. Twenty four adult Osmanabadi bucks (average body weight (BW) 16.0 kg) were used in the present study. The bucks were divided into four groups viz., C (n=6; control), HS (n=6; heat stress), NS (n=6; nutritional stress) and CS (n=6; combined stress). The study was conducted for a period of 45 days. C and HS bucks had *ad libitum* access to their feed while NS and CS bucks were under restricted feed (30% intake of C bucks) to induce nutritional stress. The HS and CS bucks were exposed to solar radiation for six hours a day between 10:00 h and 16:00 h to induce heat stress. The data were analyzed using repeated measures analysis of variance. The carboxy methyl cellulase (CMCase) Extracellular activity (EC), CMCase Intracellular activity (IC) and CMCase total activity (TA) of rumen liquor differed significantly ($P<0.01$) between the groups. The highest concentration of ammonia nitrogen ($P<0.05$) was recorded in C while the lowest in CS group. There were significantly ($P<0.01$) higher levels of trichloroacetic acid (TCA) in *ad libitum* (C and HS) fed groups as compared to restricted fed (NS and CS) groups. There were significantly ($P<0.01$) higher levels of total nitrogen in *ad libitum* (C and HS) fed groups as compared to restricted fed (NS and CS) groups. There were significantly ($P<0.01$) higher levels of propionic acid, butyric acid, and valeric acid in *ad libitum* (C and HS) fed groups as compared to restricted fed (NS and CS) groups. Further, the acetate and propionate ratio was significantly ($P<0.01$) higher in CS and NS groups as compared to C and HS groups. There were significantly ($P<0.01$) higher levels of plasma alkaline phosphatise (ALP) in *ad libitum* (C and HS) fed groups as compared to restricted fed (NS and CS) groups. However, plasma Aspartate amino transferase (AST) and alanine amino transferase (ALT) showed reverse trend ($P<0.05$) to plasma ALP. The highest ($P<0.01$) plasma Triiodothyronine(T_3) and Thyroxine(T_4) was recorded in C

group as compared to stress groups (HS, NS and CS). Further, the highest leptin concentration was recorded in C group while the lowest level in CS group. The higher expression of rumen heat shock protein 70(HSP70) messenger Ribonucleic acid(mRNA) was reported in CS goats. Further, the higher expression of intestinal HSP70 mRNA was reported in HS goats. The thyroid alveoli showed significantly lower thyroglobulin activity in HS group. The length of rumen villi and thickness was reduced more in CS, whereas rumen keratinization was observed highest in CS group. It can be concluded from this study that when two stressors occur simultaneously, they may have severe impact on rumen fermentation characteristics and metabolic activities of bucks as compared to that would occur individually. Further, the study established the adaptive capability of Osmanabadi bucks to the detrimental effects of environmental stresses to alter their fermentation and metabolic activities. This is evident from the significant influences of interaction between treatment and experimental days on majority of the parameters studied.

IMPACT OF HEAT AND NUTRITIONAL STRESS ON RUMEN FERMENTATION CHARACTERISTICS AND METABOLIC ACTIVITY IN BUCKS

by

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ABSTRACT OF THE THESIS

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ABSTRACT

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