UNIVERSITY OF NAIROBI

Analysis of Metabolite Compounds in Ruminants and their Contribution to Greenhouse Gas Emission

BY

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A Research thesis submitted in partial fulfillment of the requirements for the Award of the Degree of Master of Science in Analytical Chemistry of the University of Nairobi

DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work, or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

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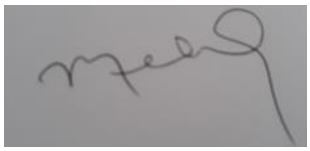

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DEDICATION

To my parents Godfrey and Irene Omondi, brothers Charles, Javan, sisters Lydia, Lavender and Pamela, for your endurance, sacrifice and support throughout this journey.

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ABSTRACT

Ruminants such as cattle, goats, sheep, and camels host highly diverse microbial communities in their rumens that ferment fibrous plant materials to produce energy. However, this fermentation process also generates significant amounts of volatile organic compounds and greenhouse gases, such as carbon dioxide and methane, which are released into the environment through various excretion routes. The accumulation of these gases in the atmosphere contributes to global warming and climate change. To address this issue, it is important to understand the differences in rumen chemistry and microbial communities among various ruminant species and their impact on greenhouse gas production. In a recent study, rumen contents from cattle, sheep, goats, and camels were analyzed using Solid Phase Microextraction followed by Gas Chromatography Mass Spectrometry (SPME-GC/MS) and High Performance Liquid Chromatography (HPLC) to determine their metabolite composition, including their greenhouse gas emission contribution. The results revealed significant differences in rumen metabolites between ruminant species, both qualitatively and quantitatively. While certain compounds, such as p-cresol, camphor, skatole, α-pinene, and carbon dioxide, were found in all ruminants, others were unique to individual livestock species. Monoterpenes, sesquiterpenes, and hydrocarbons, contributed to the dissimilarity in metabolite compounds' composition across all the species. Compared to grazers like cattle and sheep, browsers like goats and camels had similar volatile organic compound profiles, microbial populations, and greenhouse gas emission footprints. Cattle and sheep were found to have a relatively high carbon dioxide emission footprint compared to goats and camels. Additionally, Camels had relatively little carbon dioxide emission footprint which was ascribed to their low ruminal pH (6.5), therefore suggestive of CO_2 emission inhibition by acids. The revealed variation of volatile metabolite compounds in livestock rumen plays an important role in GHG production through enteric fermentation. By understanding the metabolic pathways and microbial populations involved in rumen fermentation, it may be possible to design more sustainable management practices that reduce GHG emissions in livestock. This is particularly important given the growing global demand for livestock products and the contribution of enteric fermentation to overall agricultural GHG emissions.

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LIST OF ABBREVIATIONS

GC-MS	Gas Chromatography-Mass Spectrometry
GHG	Greenhouse Gases
GI	Gastrointestinal tract
HPLC	High Performance Liquid Chromatography
NVOCs	Non -Volatile Organic Compounds
NMDS	Non-metric Multidimensional Scaling
VOCs	Volatile Organic Compounds
HS-SPME	Headspace-Solid Phase Microextraction
PCR	Polymerase Chain Reaction
HS-SPME	Headspace-Solid Phase Microextraction
SCFAS	Short Chained Fatty Acids
VFAs	Volatile fatty acids

CHAPTER ONE: INTRODUCTION

1.1 Background

Livestock farming is estimated to contribute close to 40% of the global agricultural GDP thereby creating employment opportunities to nearly 1.3 billion people worldwide (Graham *et al.,* 2022). The rising global population, estimated to reach 10 billion by 2050 (UN, 2019), is exerting pressure on livestock products, which must be produced proportionately. At the same time, the global climate change continues to threaten the survival and sustainability of the global ecosystems, which also includes sustainable livestock production.

The continuing rise in global temperatures brought on by the release of greenhouse gases like methane, carbon dioxide, and nitrous oxide exacerbates global climate change (Wallace *et al.,* 2015, Monteiro *et al.,* 2018). As the atmospheric concentration of greenhouse gases rises, the earth's temperature keeps rising. This is due to the possibility that greenhouse gases (CO_2, CH_4, NO_2, and water vapor) could trap radiation in the atmosphere, leading to a phenomenon known as global warming that blankets the earth's lithosphere (Ramanathan & Feng, 2009). Additionally, over time, continuing emissions of greenhouse gases reduce the amount of hydroxyl radicals in the atmosphere. These gases have a longer lifespan since there are less OH^o reactions to worry about (Mellouki *et al.,* 2015), hence their continued accumulation in the atmosphere.

Given the buildup of greenhouse gases in the atmosphere, global weather patterns are changing. As a result, widespread severe environmental disasters such powerful heat waves, frequent droughts, storms, an increase in human and animal diseases, and a decline in the availability of food are taking place (Grossi *et al.,* 2019). Higher heat and humidity have an impact on crop and livestock output because they make people more susceptible to disease. For instance, Rose and colleagues (2014) stated that tropical and temperate climates provide evidence that climatic variations may have a major impact on the seasonal transmission of gastro-intestinal nematodes in livestock. Furthermore, there have been significant setbacks to crop production, which have led to lower yields and inferior quality (Research Institute (IFPRI), 2009). More floods, fatalities, and the eviction of both people and animals from their natural habitat have been reported as a result of the melting of glaciers brought on by global warming and the rise in precipitation. The rising frequency and hardship that greenhouse gases cause to the global population has heightened the desire to carefully analyze how diverse industries contribute to them. Agriculture now receives special attention as a result of this. For instance, recent research

shows that, in terms of greenhouse gas emissions, agricultural emissions are a significant factor in the global climate change (Poore & Nemecek, 2018).

1.2 Agriculture as a source of Greenhouse Gas Emission

Human activities, which have increased global warming rates over the past 2000 years, are one of the main drivers of the dramatic rise in the tropospheric concentrations of greenhouse gases (Figure 1), (Ghussain, 2019, IPCC 2021 Summary for Policy Makers, 2021). Agriculture is among the top anthropogenic activities attributed to greenhouse gas emission. Recent estimates indicate that 24% of all global greenhouse gas emissions are caused by the agricultural activities in relation to other sectors (Figure 2A; Intergovernmental Panel on Climate Change & Edenhofer, 2014; Kuyah *et al.* 2014).

Agriculture encompasses both crop cultivation and livestock production, and both have a significant impact on greenhouse gas emissions. However, animal farming accounts for over 14.5% of the total agricultural greenhouse gas emissions (Grossi *et al,* 2019). This is largely due to enteric fermentation, which refers to the production of gases in the digestive system of animals. Among the primary greenhouse gases, methane, mainly produced by ruminants via enteric fermentation accounts for 16% of the total global greenhouse gases emitted by different sectors (figure 2B).

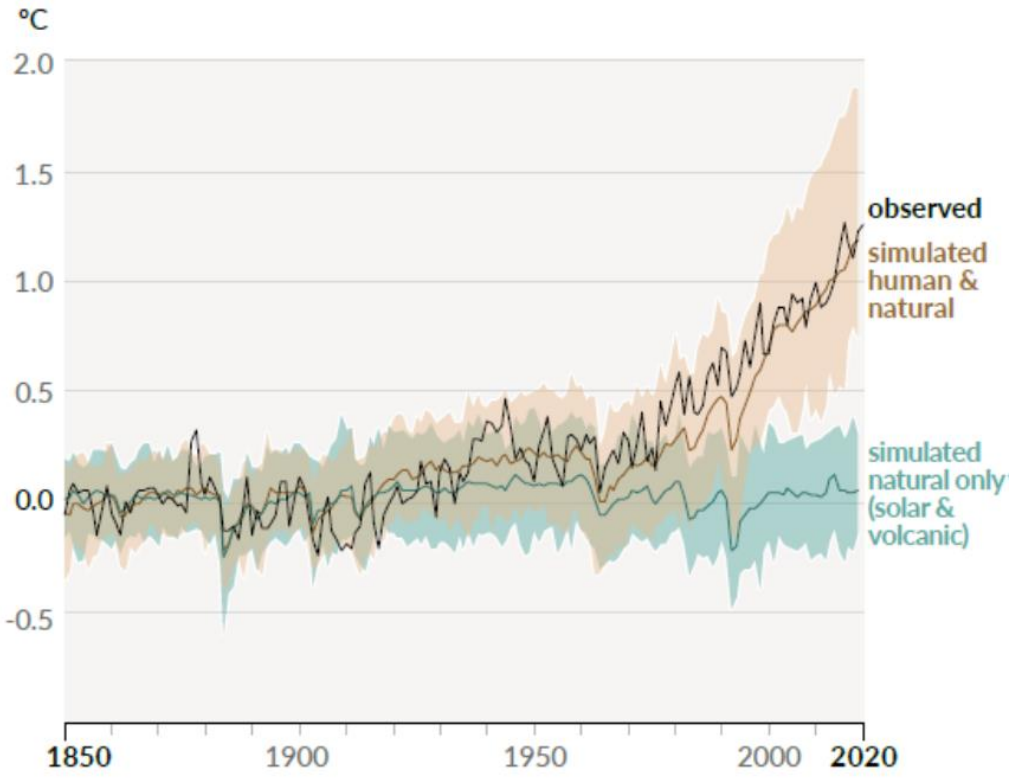

Figure 1: Change in global surface temperature (annual average) as observed and simulated using human and natural and natural only factor (both 1850-2020) source (IPCC 2021 Summary for Policy Makers.,2021).

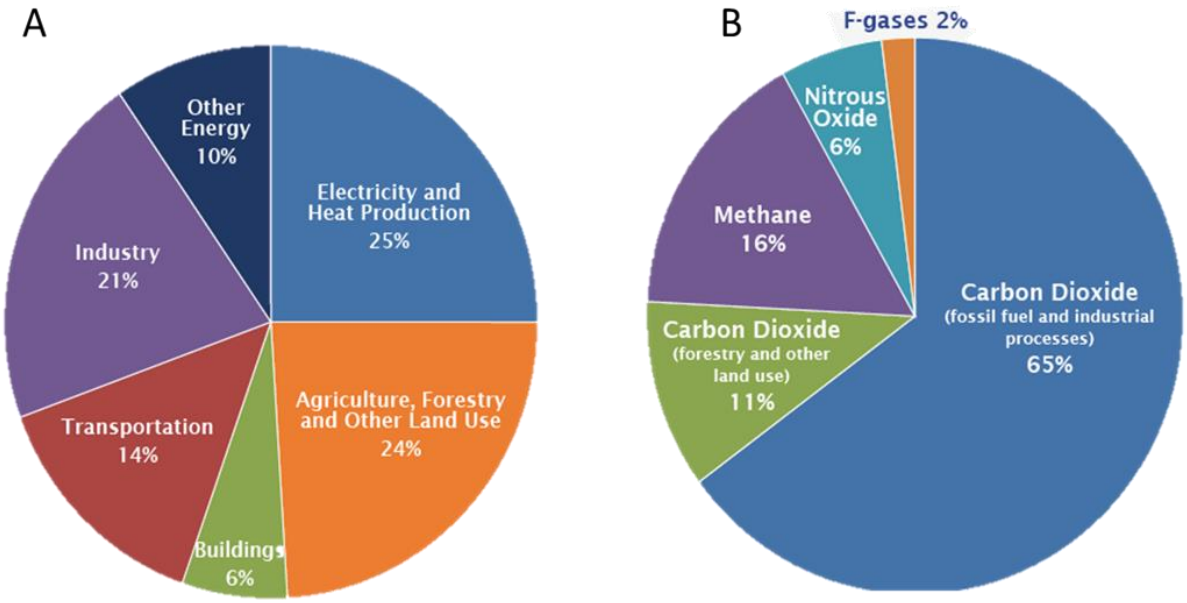

Figure 2: (A) global greenhouse gas emission by sector, (B) global greenhouse gas emission by gas type *source* (Intergovernmental Panel on Climate Change & Edenhofer, 2014)

By 2050 (UN, 2017), the world's population is predicted to reach 10 billion people. This will result to a sharp increase in demand for livestock products. Therefore, increased greenhouse gas emissions, which will hasten the pace of climate change. In order to help develop mitigation techniques for reducing livestock emission, more research is therefore needed. Consequently, decrease the environmental impact while making sure there is enough food for a growing global population.

1.3 Statement of the problem

Climate change continue to ravage, causing disruptions which have drastically affected the ecological life balance thus threating the existence of the global flora and fauna. This circumstance is aggravated by continued emission of greenhouse gases among other volatile organic compounds. Studies have demonstrated that livestock, specifically ruminants, are key players to this effect. Consequently, the rising population, estimated to reach 10 billion by 2050 (UN, 2017), is exerting pressure on livestock products, which must be produced proportionately. Growing livestock emissions have drawn the attention of the global

community and scientists in the recent years. Evidence points out that these emissions are a threat to livestock production (Sundström *et al.*, 2014), as well as mankind. Ruminants possess a special gastrointestinal tract endowed with a collection of complex microorganisms, which collectively govern the metabolic activities and arbitrate the production of metabolites including greenhouse gases. However the overall composition of rumen metabolites including greenhouse gases has been overlooked, also their variation across cow, goat, sheep and camel. Additionally, rumen chemodiversity including greenhouse gas emission variations across cow, sheep, goat and camel are yet to be reported. We hypothesize that various livestock vary in their greenhouse emission footprint. This could form a basis for focused metabolic manipulations or engineered metabolisms that ensure reduced greenhouse emissions which could be part of the solution to global climate change.

1.4 Objectives

1.4. 1 General Objective

To characterize metabolite compounds' composition, rumen microbiota and diet composition in rumens of camels, cattle, sheep and goats, in relation to greenhouse gas production

1.4.2 Specific Objectives

1. To identify volatile and non-volatile-organic compounds in cattle, goats, camels, and sheep.
2. To determine the effectiveness of HS-SPME and Porapak-Q adsorbent for rumen metabolite analysis.
3. To determine the composition of rumen microbial communities in cattle, camels, goats, and sheep.
4. To establish the diet composition in rumens of cattle, sheep, goats and camels

1.5 Justification and significance

Scientific research has shifted attention to the progressive climatic shifts due to continued global warming. Livestock emissions are among the major contributors of concern to researchers, in the context of greenhouse effect. For this reason, the rumen has been the center of focus due to presence of complex microbial communities whose significant role in ruminal fermentation enhance production of greenhouse gases. However, little is known in regards the ruminal chemical composition including their association with the microbial population and their synergistic effect on greenhouse gas emissions. The present study aims at establishing the

general chemical outlook of cattle, goats, sheep and camels rumens including greenhouse gases and rumen microbes. Additionally, the study seeks to establish the variation of these metabolites across individual livestock such as camels, cattle, goats and sheep and their association to greenhouse emissions. The project finding offers insights into the rumen chemical diversity including greenhouse gas and their variation between various livestock, and the associated ruminal macrobiota. This information is of significance in designing mitigation strategies for metabolite manipulation using rumen microbes as a potential way to mitigate greenhouse gas emission Vis-a-Vis global warming from ruminants. This will have practical scientific applications for manipulating greenhouse gases emission to reduce global warming and consequently make livestock green without compromising the health of the animals. This is in line with the global sustainable development goal (SDG) number thirteen on climate action and also the need to address food security.

1.6 Research questions

The present study aimed at answering the following research questions;

1. Do ruminants vary in their volatile organic compounds composition and distribution?
2. Do livestock vary in their rumen microbial domain populations?
3. Do livestock exhibit dissimilarity in greenhouse emission footprint?
4. Is there a correlation between rumen metabolite and relative greenhouse emission footprint in livestock?
5. Does diet preference influence metabolite and microbiota composition in livestock?

1.8 Scope of study

The present study was geared towards establishing how the composition of rumen metabolite generally relates to greenhouse gas emission in cattle, camels, goats and sheep as well as to better understand how rumen microbial population, including dietary composition, may influence rumen metabolite composition and greenhouse emission in livestock.

CHAPTER TWO: LITERATURE REVIEW

2.1 Atmospheric chemistry of Greenhouse Gases and Volatile organic compounds

Greenhouse gases (GHGs) and volatile organic compounds (VOCs) are major atmospheric pollutants that significantly impact climate change and air quality. GHGs, such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), trap heat in the atmosphere, leading to global warming. On the other hand, VOCs contribute to the formation of ozone (O3) and other secondary pollutants that pose a risk to human health and ecosystems (Koppmann, 2020). In recent times, there has been a growing concern over the role of VOCs in air pollution and climate change. A study has revealed that VOCs may contribute to global warming at the same level as CO2, and reducing VOC emissions could be an effective measure to combat climate change (Koppmann, 2020).

The chemistry of atmospheric pollutants is complex and is affected by several factors, including atmospheric conditions, sources of emissions, and atmospheric transport (Mellouki *et al.,* 2015). Moreover, the interaction of VOCs in the atmosphere can vary significantly based on the region and the type of emission source, underlining the importance of region-specific air quality policies (Fiore *et al.,* 2015). The majority of radiatively active compounds in the Earth's atmosphere are chemically reactive, implying that atmospheric chemistry plays a crucial role in regulating their lifetime and overload. Myhre and colleagues (2013) suggest that chemically active gaseous compounds in the atmosphere can be influenced by their interaction with other species, including aerosols and water, in their immediate surroundings, or by interacting with solar radiation (photolysis).

The sun's continual flow of ultraviolet, visible, and near infrared radiations, as well as emissions caused by both natural and human activity, determine what makes up the atmosphere (Isaksen *et al*., 2009). Furthermore, numerous interactions and patterns of temporal or geographical variability that result in large nonlinearities and a wide variety of pertinent time frames are used to characterize the chemistry of the atmosphere (Kleinman *et al.,* 2001, Isaksen *et al.,* 2009). Thus, the underlying design of the Earth-atmosphere climate system, and consequently the fundamental mechanisms operating within it, lead to the combination of global climate change and atmospheric composition. As a result, climatic systems are largely dependent on atmospheric composition and chemistry which, according to Isaksen *et al.,* (2009), influence climatic shifts through regulating the sun’s radiation budget as well as terrestrial radiation and thermal structure (Vardavas & Taylor, 2007). Therefore, the

atmospheric buildup of chemically active gases like CO_2, CH_4 including water vapour is dependent on their chemical interactions within the atmosphere (Ehhalt *et al*., 2000).

To indirectly influence the abundances and lifetime of direct greenhouse gases, carbon monoxide (CO), Nitrous oxides (NOx), and Sulphur dioxide (SO2) undergo chemical interactions with these gases in the atmosphere (Myhre *et al.,* 2013). These compounds also act as precursors for secondary organic particles and undergo photo-oxidation and photo-reduction reactions, driven by radiation energies from the sun, resulting in the production of the hydroxyl radical OH° (Isaksen *et al.,* 2009, 2014). This radical catalyzes various chemical reactions that impact ozone and other climate-relevant chemicals. Additionally, atmospheric photochemistry can transform volatile organic compounds into multifunctional oxygenated organic compounds and contribute to the formation of tropospheric ozone (Mellouki *et al.,* 2015; Yuan *et al.,* 2017). Tropospheric ozone's infrared absorptions contribute to approximately 10% of the natural greenhouse effect caused by radiatively active species such as water, carbon dioxide, nitrous oxide, and methane (Mellouki *et al.,* 2015; Yuan *et al.,* 2017).

2.1.1 Methane

Methane, is one of the chemically active climate gases whose atmospheric load and effect is heavily dependent on the tropospheric chemistry and can cause global warming up to 100-years (Ehhalt *et al*., 2000). It is mostly produced from different sources including ruminant's digestive tract due to enteric fermentation in the rumen (Monteiro *et al.,* 2018). The atmospheric presence and abundance of methane is regulated its reaction with hydroxyl radical (OH°) supplied through the photo dissociation of ozone and subsequently its reaction with water vapour (Myhre *et al*., 2013). Therefore, methane's photochemistry is of climatic significance since it affects the concentrations of both stratospheric water vapour, molecular hydrogen and carbon monoxide (CO) (Vardavas & Taylor, 2007).

The hydroxyl radical OH° initiates several chemical reactions within the atmosphere which subsequently affect chemicals of significant impact on the climate, like ozone, methane, and secondary particles (Myhre *et al.,* 2013, Isaksen *et al.,* 2014). Methane reacts with hydroxyl radical OH° present in the atmosphere through a solar initiated photo dissociation. This happens via a series of chemical reactions, yielding formaldehyde CH_2O which undergoes further photolysis to produce carbon monoxide (CO) and molecular hydrogen. One of the reaction paths is as outlined below.

$$CH_4 + OH^\circ \rightarrow CH_3 + H_2O \quad (1)$$

$$CH_3 + O_2 + M \rightarrow CH_3O_2 + M \quad (2)$$

(M can be any molecule either NO_X or O_2)

$$CH_3OOH + OH^\circ \rightarrow CH_2O + OH + H_2O \quad (3)$$

$$CH_2O + hv \rightarrow CO + H_2 \quad (4)$$

Similar to methane (CH_4) and carbon monoxide (CO), molecular hydrogen is also oxidized by the atmospheric hydroxyl radical OH°. Consequently, the effectiveness of OH° radical oxidation of both methane and carbon monoxide affects the atmospheric mixing ratio (Vardavas & Taylor, 2007, Isaksen *et al.*, 2009).

$$H_2 + OH^\circ \rightarrow H_2O + H \quad (5)$$

The chemical interactions of methane and hydroxyl radical OH° within the atmosphere is consequential in methane emission impacts. Therefore, as methane emissions continue to rise, the available hydroxyl radical OH° present to oxidize it diminishes. This results to an irregular increase in methane surface mixing ratio (Myhre *et al.*, 2013), and subsequently methane lifetime and burden.

2.1.2 Carbon dioxide and Water vapour

Carbon dioxide (CO2) is a minor constituent of the Earth's atmosphere that has a critical function in controlling the planet's temperature. The atmospheric CO_2 concentration has risen considerably in recent times, predominantly due to human actions such as the combustion of fossil fuels and farming. This rise in atmospheric CO_2 is termed as atmospheric carbon dioxide enrichment, or CO_2 enrichment, and is believed to be a key contributor to global warming (IPCC, 2013).This is due to its capacity to trap infrared radiation, as a result of its vibration frequencies matching those of the atmosphere and earth's surface (Ramanathan & Feng, 2009). These radiations are then re-emitted back to the Earth's surface, contributing to the maintenance of heat within the surface. The continued CO_2 absorption and emission, creates a blanket that protects the earth from the cold of outer space, hence increasing the surface temperatures (Kweku *et al.*, 2018). Increased CO_2 levels also lead to an increase in the amount of water vapor in the atmosphere, thus leading to high reaction rates between oxygen atoms in the excited state and raises the water vapour concentrations in the atmosphere (Isaksen *et al.* 2014).

Water vapor (H_2O) is another potent greenhouse gas in the atmosphere, with concentrations ranging from as low as a few parts per million (ppm) to as high as 5% by volume. Unlike CO_2, which is well-mixed in the atmosphere, the distribution of water vapor is more variable and depends on factors such as temperature, pressure, and humidity (Koppmann, 2020).Moreover, water vapor facilitates production of hydroxyl radical OH° in the troposphere by reacting with excited oxygen atoms generated via ozone photolysis (Li *et al.*, 2018). The OH° so produced enhances the oxidizing capacity of the troposphere, thereby resulting in a drop of CH_4, CO, and H_2 levels due to an increase in the oxidation of these three gases by OH in the troposphere (Li *et al.*, 2018; Loyd *et al.*, 2016). As a primary oxidant in the atmosphere, the hydroxyl radical (OH) determines the fate of most atmospheric pollutants, including carbon monoxide. This reactions therefore, contribute to the overall carbon cycle, and helps to regulate the concentrations of greenhouse gases in the atmosphere (Myhre *et al.*, 2013).

$$O_3 \rightarrow O^* + O_2 \ (O^* \ excited \ state \ oxygen \ atom < 320nm \qquad (6)$$

$$O^* + H_2O \rightarrow 2OH^{\circ} \qquad (7)$$

Water vapour reaction with excited state oxygen atoms (Equation 7 & 8)

$$CO_2 + hv \rightarrow CO + O \qquad (8)$$

$$H_2O + hv \rightarrow 2H + O \qquad (9)$$

$$H_2O + hv \rightarrow H + OH^{\circ} \qquad (10)$$

Additionally, water vapor acts as a positive feedback mechanism in the climate system, amplifying the warming effect of other greenhouse gases such as CO_2. This is because as the Earth's temperature rises, more water evaporates into the atmosphere, leading to an increase in water vapor and further warming (IPCC & Edenhofer, 2014).

The chemical reaction between CO_2 and H_2O in the atmosphere is referred to as the carbon dioxide-water vapor cycle. This cycle plays a crucial role in regulating the Earth's temperature by controlling the amount of energy absorbed and re-emitted by the atmosphere. The reaction between CO_2 and H_2O leads to the formation of carbonic acid (H_2CO_3), which can further react with atmospheric minerals to form bicarbonates and carbonates. These chemical reactions play an important role in regulating the Earth's carbon cycle and in maintaining the planet's temperature stability (Yumol et al., 2020). Equation for the reactions are represented below.

$$CO_2 + H_2O \rightarrow H_2CO_3 \qquad (11)$$

$$H_2CO_3 + \text{M (atmospheric mineral)} \rightarrow MCO_3^- + H^+ \text{(bicarbonate)}$$

$$2H^+ + MCO_3 \text{ (carbonate)} \qquad (12)$$

Where M represents any atmospheric mineral and MCO3^- and MCO3 represent bicarbonate and carbonate forms of the atmospheric mineral, respectively.

2.1.3 Volatile organic compounds

Volatile organic compounds find their way into the atmosphere from various sources, both natural and human induced emissions (Koppmann, 2010). Majorly these compounds constitute saturated hydrocarbon (alkanes), unsaturated hydrocarbons (alkenes and aromatic compounds) and oxygenated compounds (alcohols, aldehydes ketones and esters) (Ehhalt *et al.,* 2000, Koppmann, 2010). Other sources of VOCs include biogenic sources, mostly plants, which release isoprene, monoterpenes, and sesquiterpenes, among other unsaturated chemicals (Laothawornkitkul *et al.,* 2009, Koopmans, 2010, Mellouki *et al.,* 2015). Although there are many substances that are classified as VOCs, benzene and some of its organic derivatives, such as toluene, ethyl benzene, and xylene, are the most prevalent in the environment. As a result, they are used as a benchmark when determining the environmental levels of VOC exposure (Li *et al.,* 2021).

Despite their low concentrations and short life time in the atmosphere, climate active VOCs significantly influence the global tropospheric chemistry (Koppmann, 2010), which directly relate to global warming (Mellouki *et al.*, 2015). Additionally, several human health complications such as asthma, atopic dermatitis and neurologic problems are attributed to atmospheric residence of these compounds (Montero-Montoya *et al.*, 2018) .

The diverse chemical habits exhibited by volatile organic compounds in the atmosphere have gained prominence in recent times due to their contribution in enhancing greenhouse effect which subsequently affects the global climate. This phenomenon is contributed by their greenhouse effect capability, and involvement in secondary organic aerosols production, tropospheric ozone and small micro sized particles once in the atmosphere (Li *et al.,* 2021; Montero-Montoya *et al.,* 2018)

Similar to other greenhouse gases, the atmospheric dissociation of VOCs is ignited by the reaction of hydroxyl radical OH° produced by the reaction of water vapour and an excited

oxygen atom. Additionally, ozone and nitrate radicals also initiate VOC degeneration, specifically unsaturated compounds. An overview of the various oxidation pathways for VOCs is demonstrated in figure 3 below.

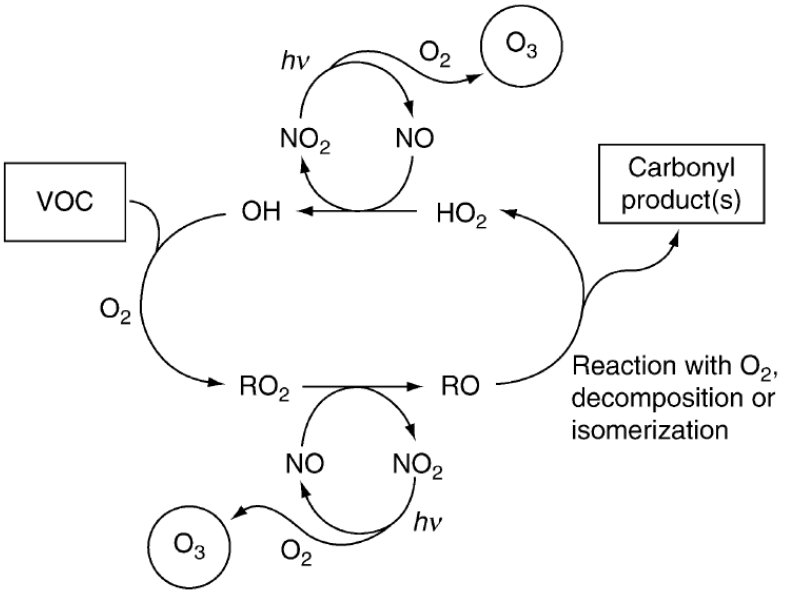

Figure 3: Oxidation pathways of atmospheric VOCs into the first stable carbonyl products in the presence of NOx, and the formation of ozone (Figure adopted from R. Koppman (2010) Chemistry of Volatile Organic compounds in the atmosphere. pp. 274)

With the rising global concern on progressive global warming and climate change occasioned by the increased concentrations of greenhouse gases among other VOCs, studies point out anthropogenic emissions as the leading source of these compounds with livestock emissions taking the center stage in this category.

2.2 Greenhouse gas emissions from livestock

Despite the socio-economic importance of livestock farming to the global community, environmental impacts associated with livestock cannot be overlooked. Due to enteric fermentation, livestock like cattle, goats, sheep, and camels produce considerable amounts of greenhouse gas emissions (Grossi *et al.,* 2019). Studies have indicated that more than 35% of global anthropogenic methane and 9% of global anthropogenic CO_2 emission arise from the livestock sector (Graham *et al.,* 2022)

Livestock have a four-chambered digestive system; the largest chamber, the rumen, contains complex microbial communities and digestive enzymes (Gupta et al., 2014;Wallace et al.,

2015;Medjekal & Ghadbane, 2021).These microbes are pivotal in rumen metabolic activities, precisely digestion, hence determine the generation and release of various volatile organic compounds including greenhouse gases. A summary of rumen digestion process is illustrated in figure 4.

These microbiota colonies readily degrade ingested fibrous plant materials (MacLeod *et al.*, 2012, Monteiro *et al*., 2018) through various metabolic pathways like fermentation and glycolysis and transform them into short chained fatty acids, vitamins and ammonia (Place et al., 2011, Zhang *et al*., 2021). Acetate, propionate, and butyrate are the three main volatile fatty acids created during this process, and the animal uses them as a source of energy (Figure 4). Moreover, gases like methane (CH_4), and carbon dioxide (CO_2) also form part of the byproducts, which are eliminated from the ruminal ecosystem through eructation (Martin *et al.*, 2010, Monteiro *et al.*, 2018).

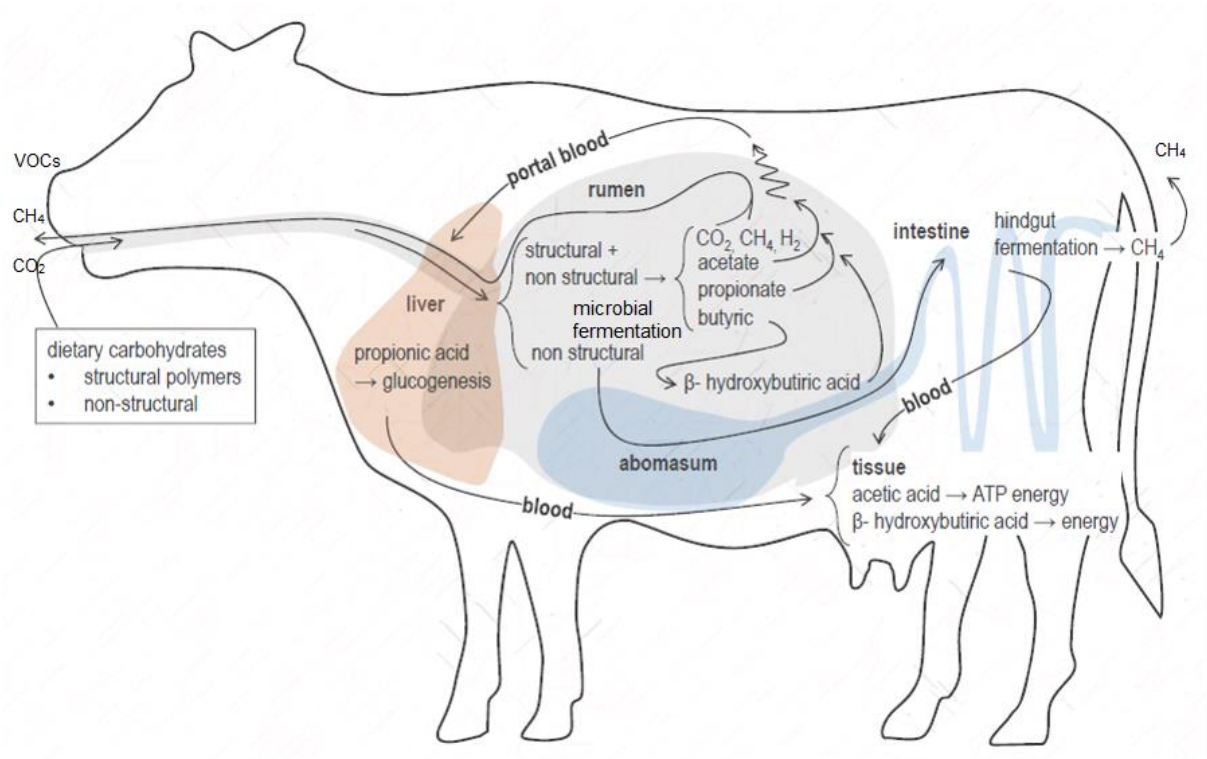

Figure 4: Summary of rumen fermentation and production of greenhouse gases modified from García-Yuste, (2020)

2.3 Anatomy of ruminants' digestive System

Ruminants such as cattle, sheep, and goats, are characterized by their unique, multi-chambered stomach (Figure 5), which allows them to efficiently extract nutrients from tough and fibrous

plant materials (García-Yuste, 2020). This feature makes ruminants to be at an advantaged position over the monogastric animals as they can procure energy and nutrients from plant biomass which can't be utilized directly by other animals (Medjekal & Ghadbane, 2021).

The ruminant stomach is divided into four main parts: rumen and reticulum, omasum, and abomasum. The rumen and reticulum are the largest parts and are considered the "first stomach", also referred to as reticulo-rumen. This is due to the absence of a clear boundaries separating the two regions (García-Yuste, 2020). They hold majority of the fermented plant material, and is are lined with microbes, such as bacteria and protozoa, which break down the plant fibers and release nutrients for the animal to digest (Wallace *et al.*, 2015). Additionally, are endowed with reticulated mucosa containing absorbent papillae which are highly keratinized, therefore able to maintain the circulation of food particles in and out of the rumen (García-Yuste, 2020, Medjekal & Ghadbane, 2021). Located on the stomach's right side, is the omasum, which is attached to both the reticulum and abomasum through the reticulo-omasal and omaso-abomasal orifices, respectively. The omasum contains plate-like folds or laminae conjoined to the greater curvature of the omasum and to its ends, furthermore, presence of highly keratinized papillae ensures the ease of thrusting of food in the omasum towards the abomasum (Harfoot, 1978). Finally, the abomasum is the last chamber of the GI tract. It is a tubular organ that connects the small intestine to the omasum. The abomasum serves as the only secretory basin because it is lined with glandular mucosa that contains secretory cells that release pepsin, hydrochloric acid, and mucus (Medjekal & Ghadbane, 2021).

Overall, the anatomy of the ruminant digestive system is specifically adapted to their herbivorous diet and allows them to extract the maximum amount of nutrients from the tough plant material that they consume. The presence of the rumen and reticulum, which are filled with microbes that break down the plant materials, is a key characteristic that sets ruminants apart from other mammals.

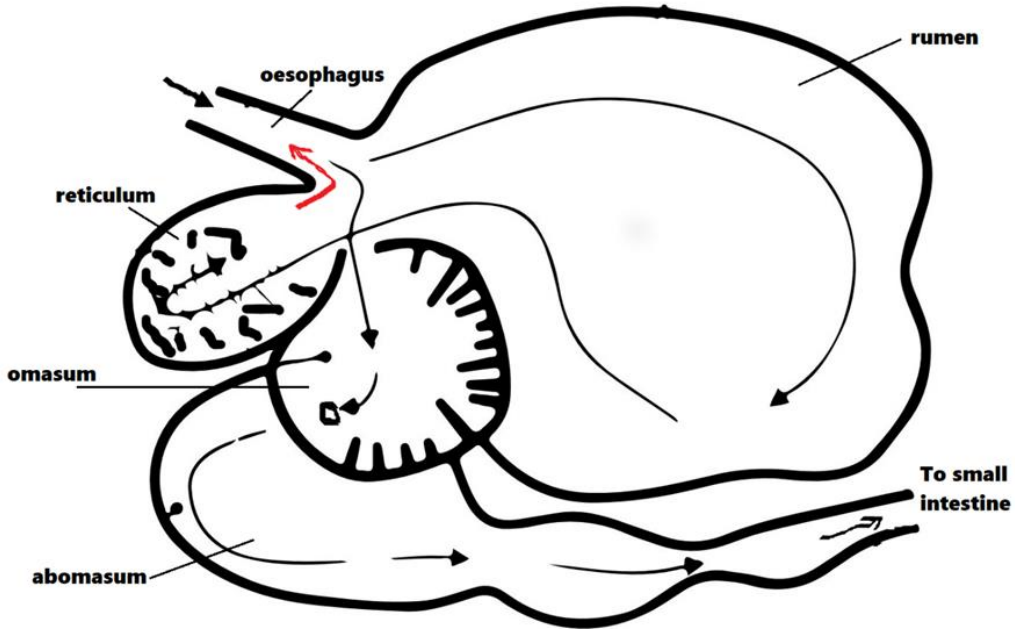

Figure 5: Simplified structure of the ruminants' stomach the arrow shows the movement of food to various sections. Red arrow indicates the regurgitation of food for rumination. Modified from García-Yuste, (2020)

2.4 Physico-chemical characteristics in the rumen

The rumen is contemplated to be akin to a reactor continuously working with anaerobic micro flora (Medjekal & Ghadbane, 2021). The constant anaerobic environment in the rumen facilitate fermentation. It exhibits an averagely constant internal temperatures of 39˚C which sometimes fluctuates between 39˚C and 40˚C or may go up to 41˚C in scenarios of acute fermentation (Medjekal & Ghadbane, 2021). The pH is also maintained at a fairly acidic to neutral state of pH 5.5 to 7.5 by the large quantities of saliva which acts as a buffer due to the high levels of bicarbonate which helps in maintaining the pH levels, however the pH may sometimes go down during intense fermentation (García-Yuste, 2020)

The rumen ecosystem is defined by 3 phase stratification model based on the gravity of the residing components i.e. gases, solids and liquids (García-Yuste, 2020). The gas phase region constitutes of CH_4, CO_2, and H_2 which are produced in large volumes during the fermentation process.(Harfoot, 1981). The gas pool typically contains between 60 and 65 percent CO_2, 25 to 30 percent CH_4, 6 to 9 percent N_2, 0.3 to 0.6 percent O_2, 0.1 to 0.3 percent H_2, and 0.001 percent H_2S (Medjekal & Ghadbane, 2021). These gases are usually removed from the ruminal ecosystem via eructation. The liquid/ fluid-like phase house a host of complex symbiotic microorganisms (Morgavi, 2015, Zhang *et al.*, 2021). Besides,

the dense and finely ground plant material is found in the bottom phase along with liquids; on top of this layer, the coarser, less ground material floats like a raft, and gases ascend to the surface (Garca-Yuste, 2020). Moreover, monocarboxylic acids, which are primarily organic compounds generated because of the metabolic activities, including cations such as sodium may also be present in the rumen.

2.5 Rumen micro-flora

The rumen microflora is a complex and dynamic community, with the microbial population and composition constantly changing in response to various environmental factors, such as diet and the host animal's physiological state (Liu et al., 2017). This diversity is important for maintaining the health and productivity of the host animal, as different microorganisms perform different functions in the rumen fermentation process (Zhang et al., 2021). For example, some bacteria specialize in breaking down complex carbohydrates, while others break down proteins and lipids (Zeng, 2017) .

Majority of the microbiome that live in the rumen are anaerobes and facultative bacteria, which can easily thrive and metabolize in the described environmental circumstances. There are more than 350 families of bacteria, fungus, and ciliated protozoa among the primary microbial species found in the rumen (Grossi *et al.,* 2019). The most prevalent type of bacteria in these macrobiota colonies, which come in a variety of forms such cellulolytic and hemi cellulolytic (Medjekal & Ghadbane, 2021). Although bacteriodetes are the most numerous and actively involved in the digestion process, other groups of microorganisms like ciliated protozoa and fungi are also helpful in the success of the process even though their role may not be as pronounced as in the case of bacteria (Myer *et al.,* 2017). Rumen microflora therefore plays a crucial role in the digestive processes of ruminant animals thus making the rumen to be functionally flexible and highly adapted to distinct types of diets. The balance of rumen microflora is essential for the health and productivity of the host animal.

2.6 Ruminal fermentation

Ruminal fermentation refers to the metabolic process by which microbes in the rumen of ruminant animals, such as cattle, goats and sheep, break down complex carbohydrates (e.g. cellulose and hemicellulose) and proteins in the ingested feed into simpler, more readily usable compounds (e.g. short-chain fatty acids, amino acids, and glucose) (Morgavi, 2015). It is strictly an anaerobic process, and heavily relies on the cellulose degrading bacteria, digestive

enzymes, fungi, protozoa and yeast in the rumen (Gupta *et al.*, 2014, Morgavi, 2015,Wang *et al.*, 2016, Monteiro *et al.*, 2018). This process, which occurs in the rumen, is crucial to humankind because it turns fibrous, unappealing vegetation into milk and meat, which are essential sources of protein and fat for humans.

Ruminal fermentation is a complex, dynamic process that is influenced by a number of factors, including the type and quantity of feed, the presence of other microbes in the rumen, and the presence of antibiotics and other chemicals (Owens & Basalan, 2016). The microbial population in the rumen ferments carbohydrates to produce heat, energy, gases (CH_4, CO_2, H_2), and organic acids (Medjekal & Ghadbane, 2021).These gases (CH_4, CO_2) are later eructated from the animal system through the mouth or into the environment. Presence of cofactors such as NADH within the ruminal environment is significant for the anaerobic fermentation to aid various redox reactions; for instance; carbon dioxide is reduced to methane (Equation 12), sulphates to sulphides, nitrates to ammonia whereas unsaturated fatty acids reduced to their saturated forms (Beauchemin et al., 2020, García-Yuste, 2020).

$$CO_2 + 8H \rightarrow CH_4 + 2H_2O \qquad (33)$$

Polysaccharides, primarily cellulose, hemicellulose, and starch, are digested in the rumen to produce glucose, hexoses, and pentoses, which are then metabolized to produce volatile fatty acids (VFAs) and carbon dioxide CO_2 (Figure 6), Beauchemin *et al.*, 2020). These include acetate, propionate and butyrate which primarily cater for the energy demands of ruminants while other by products; CH_4, CO_2, ammonia–nitrogen are eliminated from the animal ecosystem into the atmosphere (Morgavi, 2015, Grossi *et al.*, 2019; Owens & Basalan, 2016). The synthesis of acetate and butyrate via glycolysis yields metabolic hydrogen (H) (Morgavi, 2015, Grossi *et al.*, 2019, Beauchemin *et al.*, 2020). This proceeds through the dehydrogenation reaction involving hydrogenase activity which yields molecular hydrogen (H_2). The molecular hydrogen so produced is readily taken up by the archea bacteria to reduce carbon dioxide through methanogenesis process, hence producing methane (Monteiro *et al.*, 2018). Thus archea bacteria is able to maintain the ruminal biochemical balance within the ruminal ecosystem (Morgavi, 2015, Wallace *et al.*, 2015, Monteiro *et al.*, 2018). Figure 6 below highlights various fermentation reactions and associate routes and table 1 shows different chemical components arising from different food types.

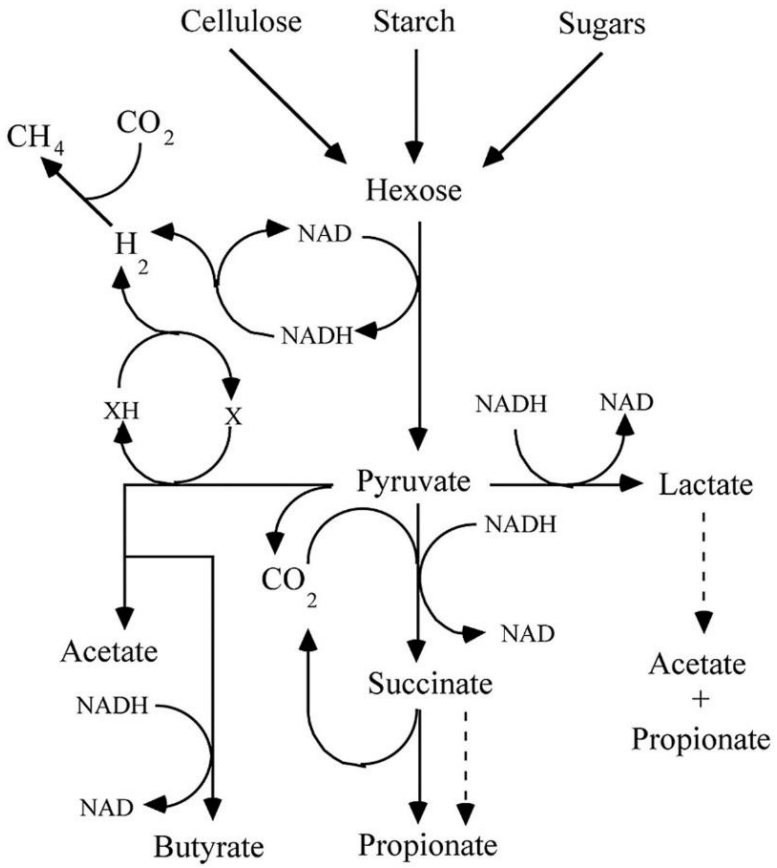

Figure 6: Rumen fermentation routes including co-factors: generation of greenhouse gases: X represents alternative electron carrier e.g. ferredoxin (Russell, 2001)

Table 1: Rumen metabolic conversion of main food components into final products of digestion. Modified from (García-Yuste, 2020)

Food Component	Polymer Chemical component	Monomer Chemical component	Digestion output
Crude fibre	Long- chain carbohydrate (pentosan)	Pentoses	Acetate, propionate, butyrate
Nitrogen-free extract	Long- chain carbohydrate (hexosan)	Glucose and other hexoses	Volatile fatty acids (acetate, propionate, butyrate)
Crude fat	Triglycerides, galactosides	Fatty acids & glycerol	Propionate, saturated fatty acids
Crude protein	Protein, non-protein	Amino acids	Acetate, propionate, butyrate, isobutyrate, isovalerate, ammonia
Ash	Minerals	Elements	Reduced elements, microbial cells, CO_2 , CH_4

2.7 Selected analytical methods for extraction of volatile organic compounds

Volatile organic compounds exhibit a broad spectrum of varied chemical characteristics in addition to probable matrix effects, which impact on their availability and concentration within the sample (Balasubramanian & Panigrahi, 2011, Boyacı *et al.,* 2018). Therefore, the choice of sample extraction and analysis of is of significance handling such compounds.

Recent developments and advancement of various analytical technologies continue to profit the ever growing field of metabolomic studies. The introduction and improvement of existing analytical instrumentation such as gas chromatography-mass spectrometry (GC-MS) and complementary sample preparations method like solid phase microextraction (SPME) has enhanced a progressive growth of bioanalytical studies (Aristizabal-Henao *et al.*, 2021). This has made it possible to determine a variety of metabolic byproducts from biological samples like urine, serum, and feces, that demand advanced analytical procedures (Getahun *et al*., 2020,Aristizabal-Henao *et al.*, 2021). Additionally, bioanalytical studies employ relatively smaller sample volumes. Therefore, need rapid, non-depletive, and high throughput sample extraction methods which can easily be interphased with existing analytical instruments (Boyacı *et al.*, 2018 , Aristizabal-Henao *et al.*, 2021).

2.7.1 Headspace-Solid Phase microextraction (HS-SPME)

SPME is a widely used sampling technique for trapping VOCs in various samples matrices. This technique is based on the partition of the analytes between gaseous mobile phase and a stationary liquid phase immobilized on an inert solid surface, hence is best suited for highly volatile and thermally stable compounds (Cai *et al* 2021). It achieves a greater sensitivity, high resolution and allows alienation of structurally identical molecules (Domínguez *et al.*, 2019). Additionally, current research by Song and colleagues (2019) shows that HS-SPME is a quick and non-destructive method for extracting volatile organic compounds from the matrices of biological material.

2.7.2 Porapak Q- Adsorbent (Super Q)

Porapak Q-Adsorbent, also known as Super Q, is a popular adsorbent used for trapping VOCs in air samples. Recent studies have evaluated the potential of Super Q in comparison with other adsorbents for VOC trapping, as well as for specific applications such as indoor air quality assessment, breath analysis, and environmental monitoring. Even and colleagues (2021) compared the effectiveness of Super Q with other adsorbents for trapping VOCs in indoor air samples. The results showed that Super Q was more effective than other adsorbents for a wide range of VOCs, and it was particularly effective for capturing volatile organic compounds of higher molecular masses. In addition, recent studies by Getahun and teammates ,have also demonstrated the effectiveness of super Q in trapping volatile organic compounds in urine, breath, dung including plants (Getahun, 2020; Getahun et al., 2022; Tawich *et al.*, 2021). Overall, these studies suggest that Super Q is an effective adsorbent for trapping VOCs in a variety of sample matrices, including but not limited to biological samples such as ruminal fluid.

2.8. Analytical instrumentation

Recent technological advancement in various analytical instruments have enabled the ease and cost of carrying out research. Different analytical instruments such as gas chromatography coupled with mass spectrometry (GC/MS) and high performance liquid chromatograph, have widely been employed in analyzing various sample matrices

2.8.1 Gas Chromatography/Mass spectrometry (GC/MS)

Gas Chromatography/Mass spectrometry (GC/MS) is a powerful analytical technique used to separate, identify and quantify volatile compounds in a sample. The technique involves

separating the sample into its individual components, and then ionizing those components to create charged particles that can be analyzed by mass spectrometry (Wei *et al.,* 2023).

GC/MS is a two-step process that involves gas chromatography and mass spectrometry. In gas chromatography, the sample is introduced into a column containing a stationary phase that separates the individual components based on their physical and chemical properties (Jain *et al.,* 2016). The separated components are then passed through a detector, which measures the concentration of each component as it elutes from the column. In mass spectrometry, the separated components are ionized and fragmented into charged particles, which are then separated based on their mass-to-charge ratio. The resulting mass spectrum provides information on the molecular weight, structure, and identity of each component *(*Bukhaiti *et al.,* 2017; S. Liu *et al.,* 2020)

The GC/MS approach therefore find applications in a number of analytical disciplines, including forensic science, pharmaceuticals, food and flavor analysis, and environmental analysis. For instance, it is used in environmental analysis to detect and quantify contaminants in the air, water, and soil. In forensic analysis, it is used to analyze drugs, explosives, and other materials at crime scenes (Biniarz *et al*., 2017; Bukhaiti *et al.,* 2017).. Hence the technique can be used to analyze metabolite compounds in rumen contents.

2.8.2 High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is another analytical technique that is for separating, identifying, and quantifying non-volatile components of a sample. It is technique is based on the interaction between the sample components and a stationary phase, which is packed in a column, and a mobile phase, which is passed through the column at high pressure (McDonald *et al.,* 2016).

The stationary phase is typically a solid support coated with a layer of a specific material, such as silica gel or a polymer, which interacts with the sample components based on their physical and chemical properties. The mobile phase is typically a solvent or mixture of solvents that is chosen based on the sample and stationary phase properties (Bhati *et al.,* 2022). As the sample components pass through the column, they interact with the stationary phase and are separated based on their chemical and physical properties (Nie & Nie, 2019). The availability of numerous stationary phases for HPLC, the most common being silica particles modified with C18 groups, also complimented by the availability of different mobile phases presents several options for researchers (McDonald *et al.*, 2016; Nie & Nie, 2019).

Complex mixtures can be separated into individual components, which can then be detected and measured using the suitable detectors and data management techniques, making this technique one of the most sought-after analytical tool. This is because of its reliability and high sensitivity in analyzing non-volatile organic compounds in various sample matrices (Bhati *et al.*, 2022; McDonald *et al.*, 2016).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Reagents and equipment

Pure analytical grade dichloromethane (DCM), hexane, methanol, LC-MS grade water, acetonitrile, and metaphosphoric acid purchased from Sigma Aldrich and Kobian Kenya limited were used for various extraction and sample preparation procedures.

Volatile organic compounds (VOCs) in the ruminal fluid were extracted by a ,65μm PDMS/DVB, stableflex 24Ga, manual holder SPME fibers (Supelco, Bellefonte, PA, USA) and Type Q Porapak™ porous polymer adsorbent assembled with a portable volatile collection pump (Sigma Scientific, USA). On the other hand, liquid-liquid extraction was employed in extracting non-volatile compounds from the rumen fluid. The extracted compounds, both volatile and non- volatiles, were later analyzed using Gas Chromatography (GC-(HP-7890A, Agilent technologies, USA, interfaced with a Mass Spectrometer (MS-597C, Agilent technologies, USA) and High performance Liquid Chromatography (HPLC) respectively. Whereas the UV-Vis spectrophotometer (BioSpec Inc, USA) was used for ammonia determination, ruminal fluid pH were determined by 3310, (Xylem Analytics, Germany) pH meter. Genomic DNA extraction from the rumen fluid for microbiome and diet identification was carried using Isolate II genomic DNA extraction kit (Bioline meridian biosciences), while a Qiagen gel extraction Kit (Hilden Germany) was used for DNA extraction from excised gels. A 96-well Proflex thermal cycler PCR system (Applied Biosystems) and Quant Studio3 (Applied Biosystems) were used for convectional and quantitative polymerase chain reactions. Respectively. Additionally, 5417R centrifuge (Eppendorf, Germany), falcon tubes (50 mL), vials (1.5 mL), pipettes (1000, 100 and 10 microliter (Eppendoff), 1 L airtight glass odor collection jars Sigma Scientific, USA),centrifuge tubes (1.5 and 2 mL, (Eppendorf) were also used to carry out the this study.

3.2 Rumen content sample collection

Rumen contents (500 mL) were collected from 5 of each randomly selected, freshly slaughtered boran cattle (*Bos indicus),* goats (*Capra aegagrus hircus*), and sheep (*Ovis aries*) at choice meats abattoir in Kahawa West, Nairobi County while those from camels (*Camelus dromedaries*) were collected at Athi River camel abattoir in Machakos County. The samples were collated into 1L sterile airtight freeze-resistant glass jars from and the pH of each

determined using a portable pH meter (3310, Xylem Analytics, Germany). The collected sample were then kept in kept in a cooler box. These were later used for analysis of volatile and non-volatile compounds. On the other hand, 50 mL of rumen fluid, were also taken from each of the ruminants that were sampled, snap frozen in liquid nitrogen, and kept in a cooler box. These were then used for genomic DNA extraction. The samples were then transported to the chemistry and molecular biology research laboratories at the chemical ecology unit and animal health theme respectively, both based at the International Center of Insect Physiology and Ecology (*icipe*), Duduville campus, Kasarani, Nairobi county.

3.3 Extraction of Volatile Organic Compounds from Rumen Contents

To determine the most suitable method for extraction volatile compounds from the ruminal content, two odor collection techniques, headspace Solid Phase Microextraction and PoraPak Q adsorbent (figure 7D-E), were first employed to trap volatile organic metabolites from cattle rumen sample at varying time frames (5minutes to 1 hour) . The results obtained by the two techniques were later compared to determine at the most suitable odor collection technique for volatile rumen metabolite extraction adopted.

3.3.1 Volatile organic compounds' trapping by Solid Phase Microextraction (SPME)

Volatile rumen metabolites were trapped from 500 mL freshly obtained ruminal fluid samples in airtight glass odor collection jars (Sigma Scientific,USA) using the headspace-solid phase microextraction (HS-SPME) technique according to Omondi and team, (2022). Prior to volatile trapping, the SPME fiber (figure 7A) was preconditioned at 250°C (injector port temperature) for half an hour on a Gas chromatography system (GC-(HP-7890B, Agilent technologies, USA) as per the manufacturer's guideline. The sample and headspace were both kept at the same temperature (37°C) for 15 minutes allow for equilibration. After that, conditioned manual holder SPME fiber (65µm, PDMS/DVB (polydimethyl siloxane/divinylbezene), stableflex 24Ga, Supelco, Bellefonte, PA, USA) was introduced into the sample headspace for the adsorption of volatile organic compounds (figure 7A-B). The SPME adsorbed volatiles were then analyzed by GC-MS.

3.3.2 Volatile organic compounds' trapping by Porapak-Q adsorbent

PoraPak^{TM}-Q 50-80 mesh 30 mg (Figure 7C, Sigma Scientific, USA) adsorbents were cleaned and condition with 1 mL of GC-MS-grade hexane prior to extraction. Then, the same quantity of dichloromethane was added (DCM). Using a portable volatile collection pump and

PoraPak^{TM}-Q adsorbent, volatiles were extracted from a 200 mL newly obtained ruminal fluid sample using a dynamic headspace volatile extraction technique (figure 7E, Sigma Scientific, USA).The PoraPak-Q adsorbent was attached to a Teflon tube affixed to a portable odor collection pump then introduced onto the sample headspace. A pull set at 2 L/min was used to draw volatiles through to the adsorbent for adsorption, and clean air was pumped onto the sample at a rate of 2.5 L/min to enable volatile equilibration at the headspace. After 300 µl of GC/MS-grade hexane was passed through the PoraPak-Q adsorbent to elute the adsorbed volatiles, the eluate was collected into a 1.5 mL vial and subsequently analyzed by GC-MS.

Figure 7: Volatile organic compounds' extraction materials and methods used on this study: (A) SPME fibers, (B) SPME holder, (C) PoraPak Q adsorbent (D), Headspace-SPME rumen odor collection setup, (E) Dynamic head space with PoraPak-Q adsorbent set up

3.4 Extraction of volatile fatty acids (VFA) and ruminal ammonia-N

A 10 mL aliquot of the ruminal fluid from each livestock species was put in 15-ml sterilized centrifuge tube (UltraCruz^{TM}) and centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant was then transferred into a clean separate tube and a solution of 25% metaphosphoric acid added in volume ratio of 1:5. The mixture was then chilled for 5 minutes

in ice then slightly agitated using a vortex mixer (Scientific industries Inc, USA) to homogenize, and later centrifuged at 14000 rpm for 15 minutes. 5 mL of the supernatant was used for volatile fatty acids trapping using SPME.

3.4. 1 Determination of ammonia concentration in livestock rumen

Ruminal ammonia was determined and quantified by colorimetric method through a catalyzed indophenol reaction as highlighted by Utomo and team (2022). The reaction involves the oxidation of ammonia by hypochlorite in the presence of phenol and a catalyst, such as sodium nitroprusside. The resulting indophenol blue complex is then measured spectrophotometrically to determine the concentration of ammonia in the sample. The reaction proceeds as follows:

$$NH_3 + 2H_2O + 2NaOCl + C_6H_5OH + Na_2[Fe(CN)5N \rightarrow (C_6H_4O)2NCl + Na_2CO_3 + 2NaCl + H_2O + Na_2[Fe(CN)5NO) \quad (14)$$

3.4.1.1 Preparation of ammonia standards

A 100 gL^{-1} ammonium stock solution was prepared by dissolving 0.04 g of dried ammonium sulfate in a 100 mL of deionized water. For the working standards, 5, 10, 15, 20, 25 and 30 mL of the stock solution was pipetted into separate 100 mL volumetric flasks and topped up with deionized water. These were represented as, 5, 10, 15, 20, 25, and 30 gL^{-1} ammonium solution respectively. A blank was included to represent standard 0.

3.4.1.2 Determination of wavelength for maximum absorbance

First, the wavelength for maximum absorbance by ammonia was determined by measuring the highest standards (similarly prepared as the samples) at different wavelengths starting from 350 nm to 800 at intervals of 50 nm. Absorbance values were recorded at each wavelength interval and used to plot an absorbance curve from which the wavelength at which the maximum absorbance by ammonia was noted and used for ruminal ammonia analysis.

3.4.1.3 Sample preparation for ruminal ammonia determination

1 mL aliquot of the supernatant previously obtained and standards were pipetted into 5 mL of reagent A (solution mixture of phenol and sodium nitroprusside). This was followed by addition of 5 mL of reagent B (mixture of sodium hypochlorite & sodium hydroxide). The solution was then left for 1 hour for the color to stabilize and later the absorbance was measured in a UV/VIS spectrophotometer (Biospec-mini, (BioSpec Inc, USA) at a wavelength (640nm) determined during absorbance experiments in the previous section. Ammonia concentration

was then calculated from the standard calibration curve developed. The analysis was done in triplicates with both the samples and standards.

3.5 Extraction of sugars and non-volatile metabolites

Ruminal content aliquots of 1 mL were pipetted into 15 mL falcon tubes. 5 mL of LC-MS grade water–acetonitrile (25:75) solutions was added to ruminal content and the mixture vortexed thoroughly. The mixture was then sonicated for 20 minutes then centrifuged at 14 000 rpm as detailed in Cheseto *et al*., (2020). The supernatant was later transferred into clean 15 mL falcon tubes and evaporated using a vacuum concentrator at 60^{o}C for 1 hour. The concentrates were then diluted with 1mL of water–acetonitrile (25:75) solution (LC-MS grade) and transferred into 1.5 mL vials and analyzed by HPLC.

3.5.1 Preparation of sugar standards and mobile phase

A stock solution containing 2% of simple sugars (fructose, glucose and sucrose) was prepared by weighing 2g of the individual sugars, mixed together and dissolved in double distilled-deionized water to form a blend. The blend was used as standards to determine the presence of these sugars in rumen content. 1L LC-MS grade Acetonitrile-water (75:25) was then sonicated for 30 minutes and used as HPLC mobile phase solvents for analysis of sugars.

3.5.2 Spectrophotometric method for total sugar determination in rumen fluid from livestock

Total sugars in rumen content was determined by UV-VIS spectrophotometry the amount following the modified reported by Trejo and co-workers (2022). 100 µl samples and standards solutions, were pipetted into different clean glass test tubes. 5mL of 2.5 mL hydrochloric acid was then added and the mixtures and placed into a boiling water bath for 1 hour. Distilled water, 10 mL, was later added to the digest. The mixture was then centrifuged at 4200 rpm for 5 minutes. The supernatant was later transferred to a clean test tube. Supernatant (1mL) was pipetted into a test tube and 1ml 50% phenol solution and 5 mL of concentrated sulphuric acid was added respectively. 500 µl of the mixture was then diluted with 1.5 mL of deionized water and left to stand for 1 hour for colour development and stabilization. Absorbance was then measured using a UV-VIS spectrophotometer (BioSpec Inc, USA) at 490nm wavelength.

3.6 Chemical Analysis

Chemical analysis of all the ruminal content samples was carried out in the laboratory using the following instruments: UV-VIS spectrophotometer for ruminal ammonia determination, gas chromatography coupled with mass spectrometry (GC-MS) for volatile rumen metabolites and volatile fatty acids identification, and high performance liquid chromatography (HPLC) for analysis of sugars.

3.6.1 GC-MS analysis of volatile rumen metabolites and volatile fatty acids

The volatile metabolites and volatile fatty acids extracted by solid phase microextraction were analyzed by Gas chromatography (GC-(HP-7890A, Agilent technologies, USA) coupled with Mass spectrometry (MS-5975C, Agilent technologies, USA). SPME fiber was inserted into the GC-MS injection port and temperature program initiated for desorption, chromatographic separation and identification of individual volatiles. Volatile desorption was conducted at the injection port at 250 ºC for 2 minutes in splitless mode (Dursun *et al.*, 2017). The HP-5MSI, 30 m X 0.25 mm i.d, 0.25 μm m thick capillary column (J & W Scientific, USA), immobilized with 5% (phenyl methyl silicone) as the stationary phase, was used to separate the volatiles through chromatography. At a flow rate of 1.2 mL per minute, 99.99% pure helium gas transported the volatile metabolites. The oven's initial temperature was set to 30 °C, where it was maintained for five minutes, before gradually rising at a rate of 10 °C/min to 280 °C, where it was maintained at an isothermal state for ten and a half minutes. The mass spectrometry (MS) detector was operating in the scan mode within a mass range of 8 to 550 m z−1 at 1 scan s−1, with electron energy of 70 eV. The MS interface line was preheated to 230°C. In total, the study took 35 minutes.

Based on their separate mass spectral data and retention times, individual compounds were identified using computer-aided comparison against the MSD library (NIST, 2005, NIST 05a, AND Adams MS HP, USA). The percentage composition of individual compounds were determined by integration of their peak areas.

3.6.2 HPLC Analysis

High Pressure Liquid Chromatography was used to analyze sugars in ruminal fluid (glucose, fructose, and sucrose). Water- acetonitrile mixture 75:25 (v/v) was used as the mobile phase, and the flow rate was maintained at 1 mL per minute. A 250 mm × 4.6 mm, 5 μm LC-NH_2 column, was used to accomplish chromatographic separation (Supelco in Bellefonte,

Pennsylvania). Finally, the data was then acquired using the LC solution program (Agilent technologies, USA).

3.7 Rumen microbiome DNA isolation and characterization

Genomic DNA for rumen microbiome was isolated and characterized using bacterial and archaeal 16S rRNA, protozoal 18S rRNA genes and fungal ITS1 gene amplicons sequencing. From the collected bovine rumen fluid samples according to Wallace and team (2019).

3.7.1 Genomic DNA extraction from rumen content

A 1 mL aliquot of the bovine rumen fluid was mixed with an equivalent volume of lysis buffer in a 2 mL centrifuge tube. 10% of 2 mm zirconia beads (BioSpec Inc., USA) was then added to the mixture. Afterwards, the mixture was shaken three times on mini tissue lyser machine (Qiagen, Hilden, Germany), at a cadence of 30Hz for 30 seconds per cycle. This made it possible for the microorganism's cell membranes to rupture and discharge nucleic acid materials. The discharged nucleic acid material was then isolated using the Isolate II genomic DNA extraction tool (Bioline Meridian Biosciences) based on the manufacturers procedures. The quality and quantity of the DNA extracts were then checked by a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, United States) as described by Tawich and colleagues (2021).

3.7.2 PCR amplification, sequencing and microbiota identification

Rumen microbe specific PCR-based mechanisms that targeted the 16S rRNA genes of bacteria, archaea, protozoa, and fungi as well as the 18S rRNA genes was conducted for rumen microbiota identification. Universal primers previously used by Whitehead & Cotta, (2001) , Rooke and team (2014), and Wallace and colleagues (2019), were used for PCR amplifications.. 1 µl of the DNA extracts and was mixed with a 2 µl of 5 × HOT FIREPol Blend Master mix (Solis BioDyne, Estonia), 6 µl nuclease-free water and 0.5 µl of each primer (10 µM), to make up a 10-µl reaction volume. These were then placed on a 96-well proflex thermal cycler (Applied Biosystems) for gene amplification. An PCR ultrapure nuclease-free water was used as negative (-ve) control .The obtained PCR products, were then resolved on a 2% agarose gel electrophoresis, and later visualized a UV transluminator attached to digital camera (Gel Logic 200 Imaging System, Kodak, Japan). A 100-bp gene ruler (Solis BioDyne, Estonia) was used to deduce the obtained band sizes. The bands were then excised, and the DNA re-extracted from them using QIA quick gel extraction Kit (Qiagen, Hilden Germany)

according to the manufacturer's procedures. The re-extracted DNA were then sent for sequencing at Macrogen Inc (Netherlands). Using Geneious software, obtained sequences were cleaned, edited, and aligned, resulting in a congruent sequence made up of contigs from both the forward and reverse sequences. The specific microbiota taxa were later identified by aligning the processed sequences against the GenBank database using the NCBI BLAST1 search engine.

3.7.3 Rumen microbiome diversity richness

The identified rumen microbiome species were quantified to establish the abundance of individual species across the rumens of catte, goats, sheep and camels. This was achieved by real time quantitative-PCR (RT-q-PCR) technique. The already obtained DNA extracts, were subjected to quantitative-PCR amplification using ArchF (CCTGCTCCTTGCACACAC) and ArchR (CCTACGGCTACCTTGTTAC) primers for archea, whereas BactF (GGATTAGATACCCTGGTAGT),and BactR (CACGACACGAGCTGACG) for respectively. Similarly, prozoa were amplified using CiliF (CGATGGTAGTGTATTGGAC) and CiliR (GGAGCTGGAATTACCGC) primers. 10-µl reaction volumes containing 0.5 µl of each primer (10 µM), 1 µl DNA extract, 2 µl of 5 × HOT FIREPol evergreen (Solis BioDyne, Estonia), and 6 µl nuclease-free water, were placed on 96-well, Quant studio3 thermal cycler (Applied Biosystems) for amplification. Bacterial, Archeal and Protozoal abundance were then calculated from triplicate C_t values using the universal bacterial calibration equation, already determined from standard calibration curves.

3.8 Livestock plant diet identification

Plant diet identification for cow, camel, goat and sheep were established using a PCR-based technique targeting an aggregate of two gene chloroplast comprising of coding (rbcL gene) and non-coding gene spacer region (trnH-psbA) primers (Tawich et al., 2021). Chloroplast DNA was extracted from bovine rumen fluid using Isolate II genomic DNA extraction mini kit (Bioline meridian biosciences) according to the manufacturer's guidelines and thereafter amplified and sequenced for positive identification.

3.8.1 Chloroplast DNA amplification and plant identification

The Proflex 96-well thermal cycler (Applied Biosystems) was used for the PCR amplifications, which were carried out in 10-µl reaction volumes with 0.5 µl of each primer (10 µM), 1 µl of DNA template, 2 µl of 5x HOT FIREPol Blend Master mix (Solis BioDyne, Estonia), and 6 µl

nuclease-free water. Ultrapure nuclease-free water used as the DNA template for the PCR negative (-ve) control and positive (+ve) control (extracted maize plant DNA). Obtained amplicons were run through a gel electrophoresis to determine the individual amplicons via a 2% agarose gel dyed with 0.5µL ethidium bromide. Later, the gel images were viewed under a UV transluminator attached to digital camera (Gel Logic 200 Imaging System, Kodak, Japan) and their respective band sizes deduced using 100-bp DNA ruler (Solis BioDyne, Estonia). The bands were later cut, and the DNA re-extracted from them using QIAquick gel extraction Kit (Qiagen, Hilden Germany) according to the manufacturer's procedures and sent for sequencing at Macrogen Inc (Netherlands). The realized sequences were clarified, edited, and adjusted using Geneious software, with contigs from both the forward and reverse sequences forming a congruent sequence. Individual plant species were singled out by comparing obtained sequences with the GenBank database by the use of NCBI BLAST1 search engine.

3.9 Data Analysis

Choosing the appropriate sample size especially when conducting livestock related studies may be quite a challenge sometimes due to herd variability (Wallace *et al.,* 2019);Baleba *et al.,* 2019). In this studywh, a minimum of 5 animals randomly selected from cattle, sheep, camel and goat herds were used.

Beforehand, calculations to determine the minimum sample size suitable for metabolite composition determination were performed according to Getahun and team (2022). The results showed that a minimum of 4 animals per species were required to identify distinctive rumen metabolite, hence in the current investigation, 5 animals from each species' herd were employed. Due to the justification difficulties that may arise when using an even number of samples, the present study therefore chose to work with 5.

Subsequent statistical analysis was conducted based on the nature of the obtained data using R statistical analysis software version 1.4.1106 as described by Albaladejo and team (2015), and also PAST version software Version 4.02, and GraphPad Prism version 9. Multivariate analysis of identified metabolites using non-metric multidimensional scaling plot (NMDS) and a combined color coded matrix plot with a cluster dendrogram was used show how livestock cluster based on their metabolite profiles. Pearson's correlation model was used to highlight metabolite correlation among individual animals, metabolite functional groups and carbon footprint. Simper analysis and one-way ANOSIM was employed to show which compounds greatly contributed to metabolite variation in livestock. Chord similarity index and

Fruchterman-Reingold algorithm was used in metabolite network correlation analysis. One way ANOVA and independent pairwise t-test was used to establish variations among livestock based on the identified metabolites and later compared by Bray-Curtis dissimilarity index for signature rumen metabolites identification.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Comparative determination of volatile organic compounds by headspace SPME-GC/MS and dynamic headspace-Porapak Q- GC/MS methods

The study aimed to compare two analytical methods, headspace-SPME and dynamic headspace Porapak-Q adsorbent, for the extraction and analysis of volatile organic compounds from cattle rumen fluid. Gas chromatography-mass spectrometry was used to analyze the samples, at different extraction times, 5 minutes, 15 minutes, 30 minutes and 1 hour, for both methods.

The results showed that the number and diversity of compounds extracted varied significantly between the two techniques and with different extraction times (Figure 8; A-D). More compounds were extracted with longer extraction times (1 hour), and SPME extracted more compounds within a shorter extraction period (30 minutes) than Porapak-Q adsorbent (Figure 8E-F). This was in agreement with past studies which have demonstrated SPME followed by GC/MS analysis as a rapid, sensitive and non-destructive technique for determination of metabolite compounds in diverse matrices (Boyacı *et al.,* 2018; Domínguez *et al.,* 2019; Dursun *et al.,* 2017). Additionally, most bioanalytical research such as metabolomic studies, which prefer analytical procedures that use relatively low sample volumes, and high throughput like HS-SPME-GC/MS methods (Boyacı *et al*., 2018, Aristizabal-Henao *et al*., 2021). The SPME-GC/MS method as presented simplicity and efficiency in quick metabolite extraction and analysis against Porapak-Q adsorbent method (Omondi *et al.,* 2022).These findings, thus provide valuable information for researchers seeking to analyze volatile organic compounds in bovine rumen content. Therefore, SPME-GC/MS method was adopted for subsequent determination of volatile organic compounds in bovine rumen contents from camels, sheep, goats and cattle.

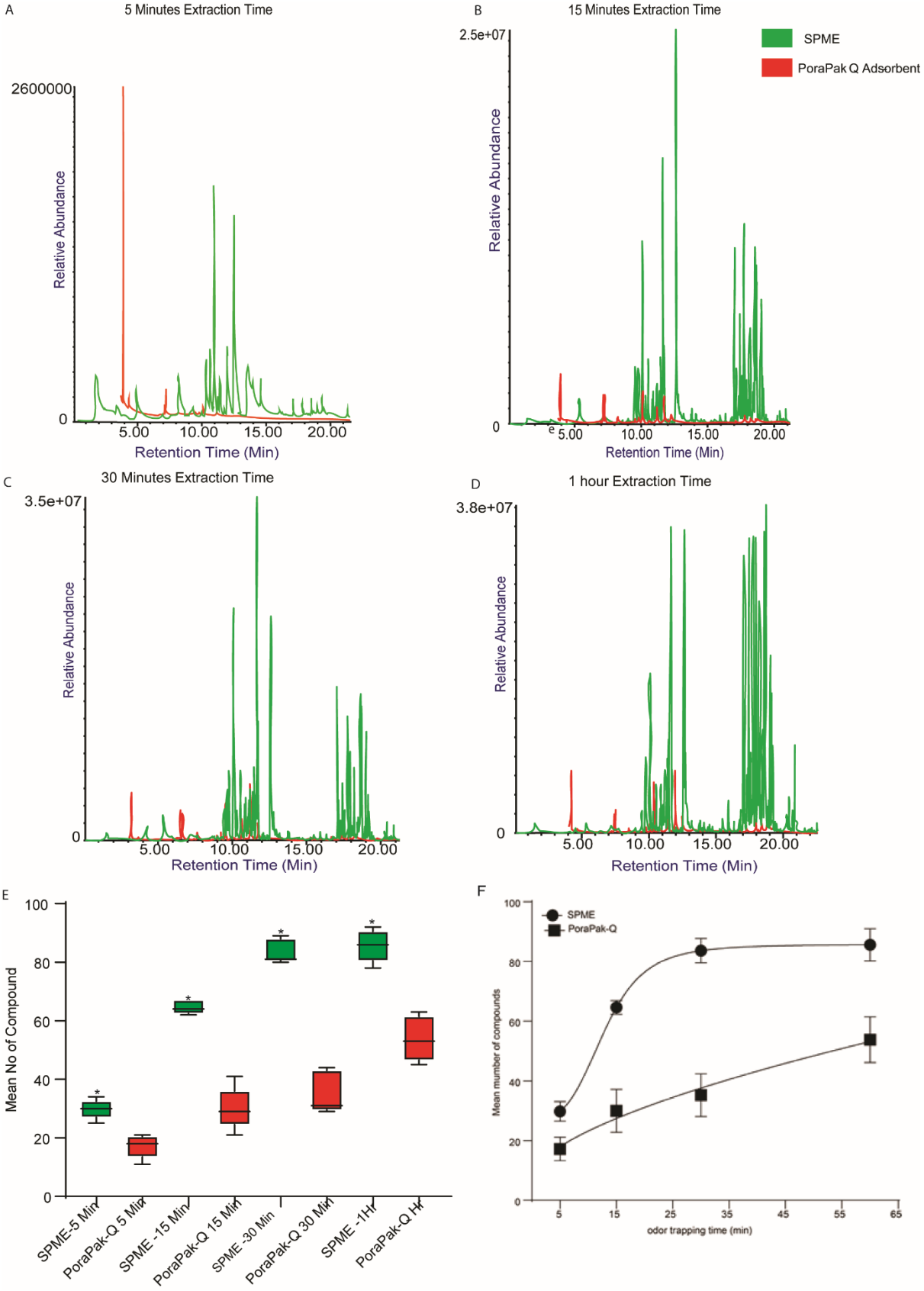

Figure 8: Representative GC-MS chromatogram of cattle rumen metabolite profile trapped using HS-SPME and Porapak-Q from rumen at different extraction times (A-D, (E) Box plot representation of mean number of extracted compounds by the two methods (F) A nonlinear

regression curve of the number of metabolites trapped as a function of odor collection time for the two methods, n =5.*, P < 0.001.

4.2 Rumen metabolite composition

In the current research, a total of 218 rumen metabolite compounds of diverse chemical classification, including greenhouse gases were determined in the rumen bovine samples obtained from four livestock species, cattle, goats, sheep and camel. Metabolite chromatogram profiles obtained (Figure 9) highlighted significant dissimilarity in chemodiversity displayed by different livestock species.

The identified metabolite compounds included greenhouses gases such as CO_2, volatile fatty acids (acetic, butyric & propanoic), aromatic compounds (toluene), aldehydydes (butanal), ketones (4-Octanone, nonanone & undecanone), monoterpenes & sesquiterpenes (limonene, camphene, α-pinene, & caryophyllene), hydrocarbons: long and short chain (tridecane, 4-octene, 3 heptyne, tricosane & eicosane), alcohols (decanol, 3-hexyn-1-ol) among other chemical compounds. These compounds provide a general overview of various chemical metabolic byproducts resulting from microbial induced fermentation which is characteristic of the rumen digestion process (Martin *et al.*, 2010). Moreover, fecal predictive indolic and phenolic compounds such as p-cresol, and indole including 3-propyl phenol, skatole and benzylaldehyde also associated with livestock dung (Mansourian *et al*., 2016, Getahun *et al*, 2020) were also detected in the ruminal bovine samples of all the livestock studied. The presence of plant related chemical groups such monoterpenes, sequiterpenes, was suggestive of the diet-microbe interaction aiding digestion in livestock rumens. Contrary to known greenhouse gases like carbon dioxide and methane (Ramanathan & Feng, 2009), biogenic compounds containing one or more isoprene groups such monoterpenes (Laothawornkitkul *et al.*, 2009) and oxygenated volatile organic compounds for instance aldehydes and ketones (Mellouki *et al.*, 2015) has also been highlighted as key drivers of atmospheric chemistry which potentially contribute to greenhouse effect. Therefore the displayed interspecific variation in rumen metabolite profiles of cattle, camel, goat and sheep, outlines the critical role of enteric fermentation in regulating livestock chemodiversity and potentially the enteric greenhouse emissions.

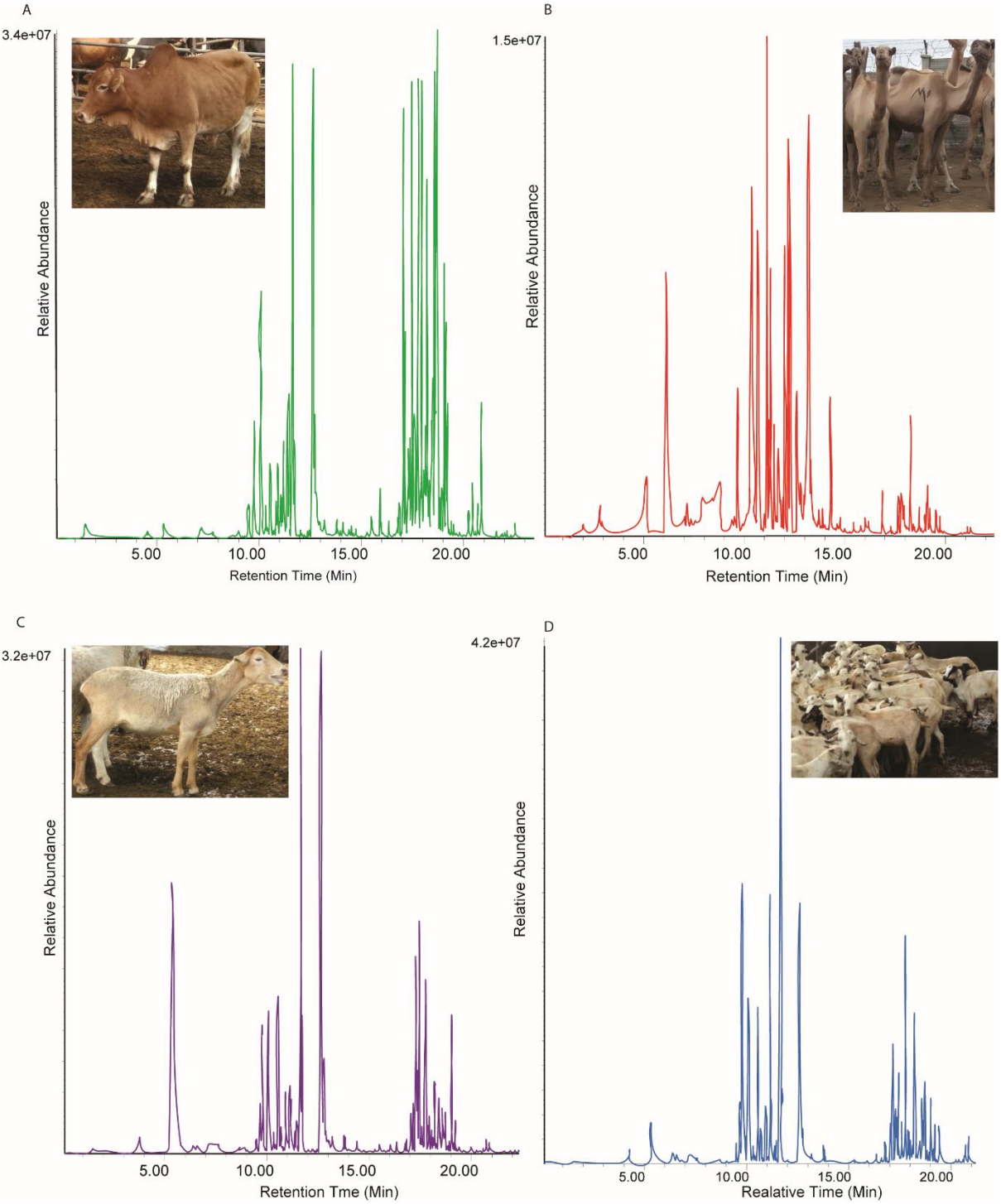

Figure 9: GC-MS chromatogram profiles (A-D) of rumen metabolites for various livestock species

A Pearson's correlation analysis on the identified compounds among individual species population, to establish whether herd population dynamics or host genetic make-up may influence the overall metabolite composition. Although, minimal variability in metabolite composition among individual species was revealed, majority of the metabolite compounds

were conserved within herd populations (Figure 10). The findings imply that livestock of similar species, have a conserved and comparable metabolite composition, irrespective of gender, age, locality or rumen microbiome as also highlighted by Wallace and team (2019),and including Zhang and co-workers (2021b).

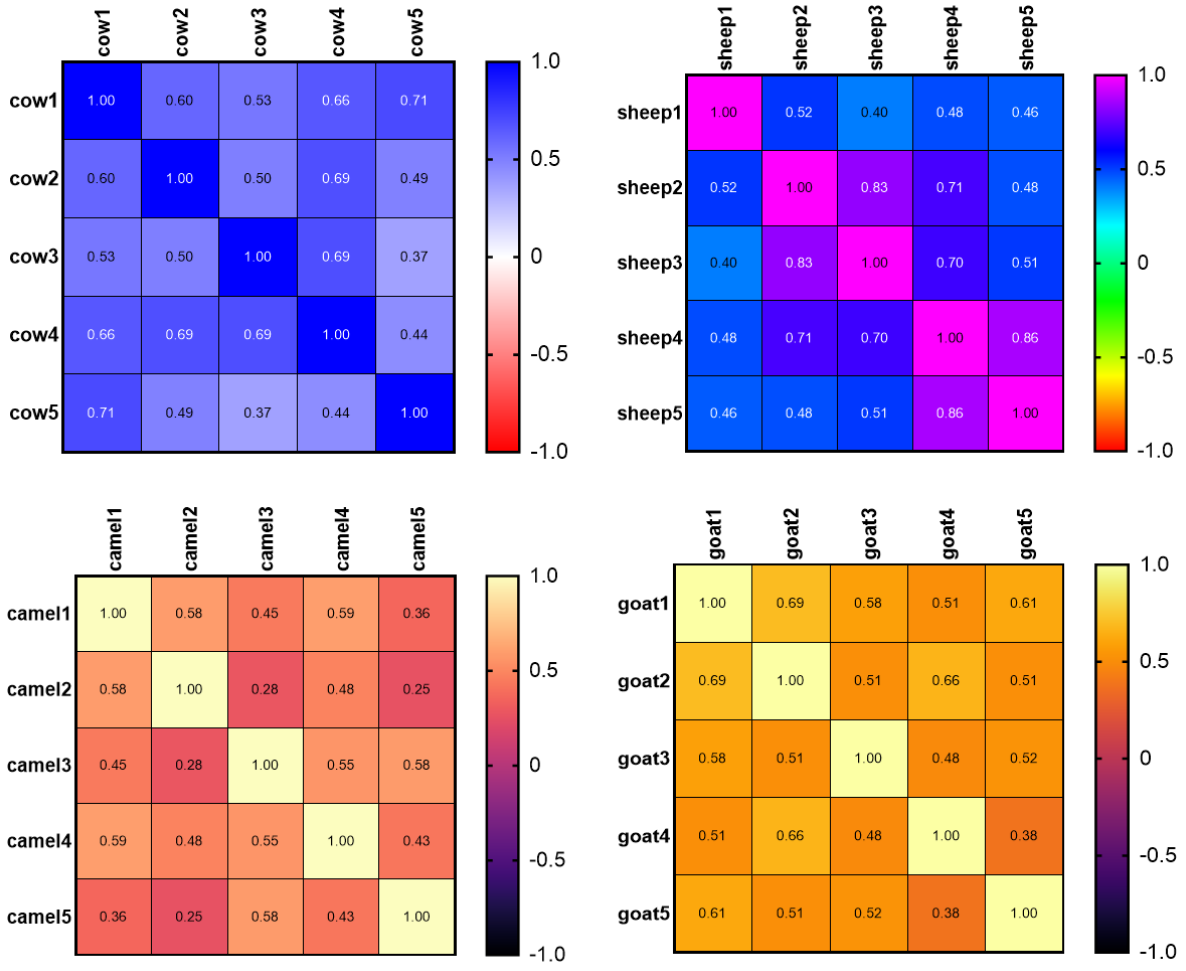

Figure 100: Color coded Pearson's correlation plots for identified rumen metabolite among individual animal in their respective species

A further examination of the identified metabolites compounds showed that each respective species had its own pool of unique chemical compounds (Figure 11). For example, 2- nonene and α- selinene were uniquely identified in cattle, urea and hydrocinnamic acid in camel, butyl butanoate and 2-methyl-1-heptanol in sheep, and 1, 3 cyclohexadiene and phenyl ethyl alcohol in goat. However there was similarity in the general metabolite composition among individual populations (Figure 10). Volatile fatty acid such as acetate, butyric and propionic acids which are the key rumen fermentation drivers including ammonia resulting from broken down proteins, were identified in all livestock hence an indication of a similar metabolic pathway

despite the difference in livestock species(Monteiro *et al.*, 2018; Owens and Basalan, 2016). Hence the displayed interspecific variation in rumen metabolite profiles of cattle, camels, goats and sheep, outlines the critical role of enteric fermentation in regulating livestock chemodiversity and potentially the enteric greenhouse emissions.

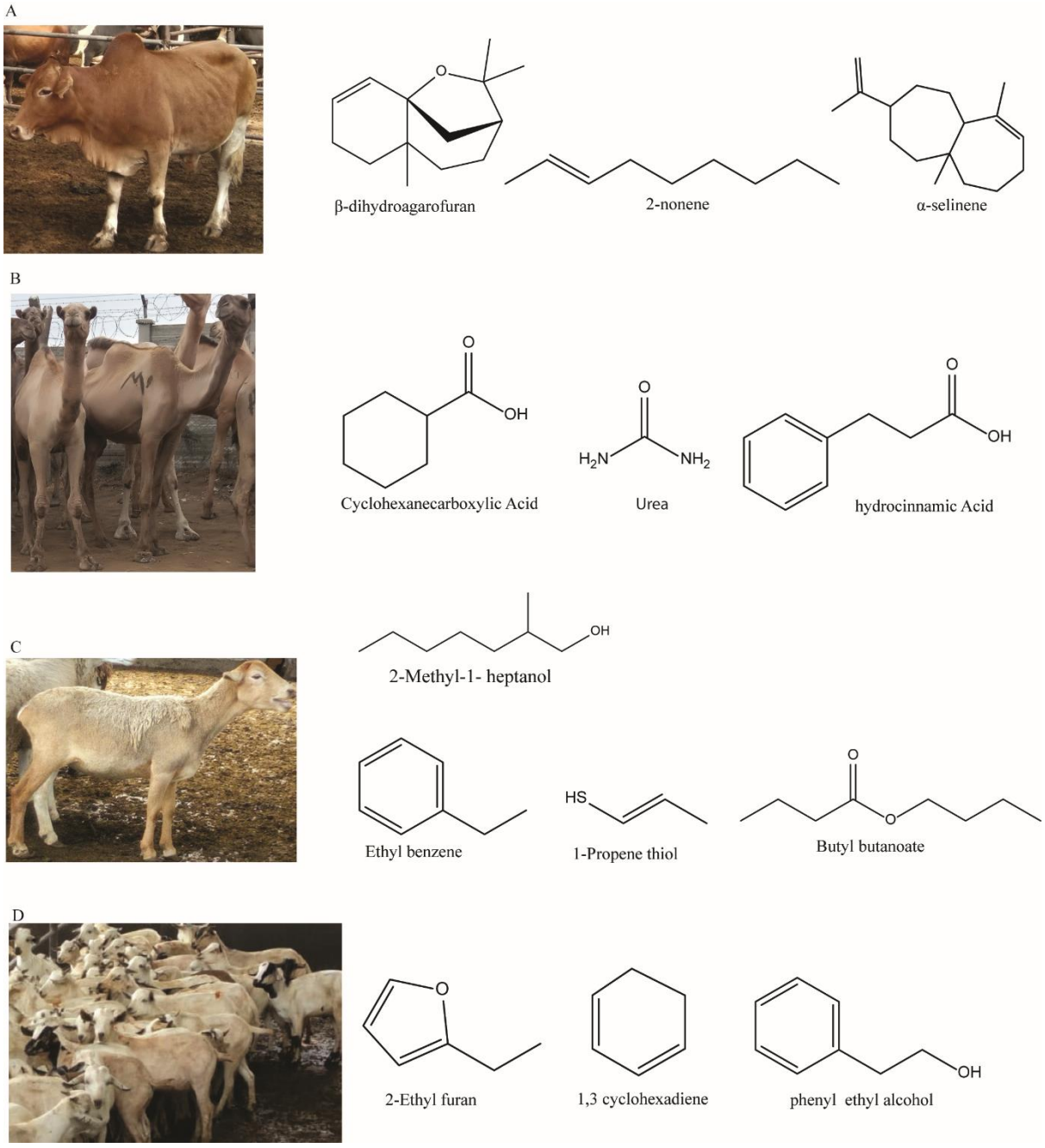

Figure 111: Identified signature compounds (A-D) from the total analyzed metabolites in four livestock species

4. 3 Variation of metabolite compounds in livestock rumen

The metabolite compounds detected in the rumen of cattle, sheep, goat and camels were analyzed using a multivariate analysis employing non-metric multidimensional scaling (NMDS) and cluster analysis (Figure 12). The comparative investigation of rumen fermentation by products among various livestock species (cow, sheep, goat & camel) highlighted significant interspecific chemodiversity of rumen metabolites between livestock (Figure 9 & Figure 12). This study demonstrates that different livestock groups exhibit a diverse chemical composition resulting from rumen fermentation as revealed by their distinct metabolite chromatogram profiles both in relative abundances and identities (Figure 9). Additionally, percent contribution of individual compounds to the total identified metabolites varied among the four livestock species (Figure 12A). Cattle and sheep are clustered together and similarly Goats and camels (Figure 12A-B). The distinct clustering of livestock demonstrated the dissimilarity in metabolite composition among individual groups of animals. Browsers like goats and camels which mostly feed on non-grasses including leaves, shrubs and herbaceous dicots appear to exhibit similar metabolite profiles compared to grazers like cattle and sheep which feed purely on grass (Figure 12A-B).

The observed dissimilarity in rumen metabolic profiles further points out that diet choice may potentially influence the general rumen metabolic processes and consequently metabolite and greenhouse gas production in ruminants. For instance, recent study by Garcia and colleagues (2017), reported that diet composition and source influence rumen fermentation due to the difference in digestibility of various feed components. Furthermore, dry matter intake has often influenced measures of ruminal digestion and CH_4 production (Garcia *et al.,* 2017). For example, diets that are high in fiber and low in starch may escalate the production of propionate, which may then lower methane production (Jalč *et al.,* 2013; Pecka-Kiełb *et al.,* 2021). In contrast, diets that are high in starch and low in fiber may increase the production of acetate and butyrate, which are VFAs that can increase the production of methane(Min *et al.,* 2022). Additionally, the existence of specific microbial populations or their lack in the rumen, such as methanogens and protozoa, can also affect the amount of methane produced during enteric fermentation (Palangi & Lackner, 2022). Therefore, it is crucial to take into account the function of metabolite compounds in livestock rumens in GHG production when designing livestock diets and management strategies. By manipulating the types and amounts of nutrients provided to livestock, it is potentially feasible to decrease the quantity of methane generated during enteric fermentation. Research studies have indicated that feed additives like plant

extracts and ionophores can modify rumen fermentation patterns and reduce methane production (Palangi & Lackner, 2022). Likewise, strategies such as improving forage quality and incorporating legumes into grazing systems can also reduce methane emissions by promoting the production of propionate over acetate and butyrate (Chen *et al*., 2020). Hence the variation of metabolite compounds in livestock rumen highlighted in the present research, could play an important role in GHG production through enteric fermentation. Hence assist in management of greenhouse gas emission in livestock. This is particularly important given the growing global demand for livestock products and the contribution of enteric fermentation to overall agricultural GHG emissions.

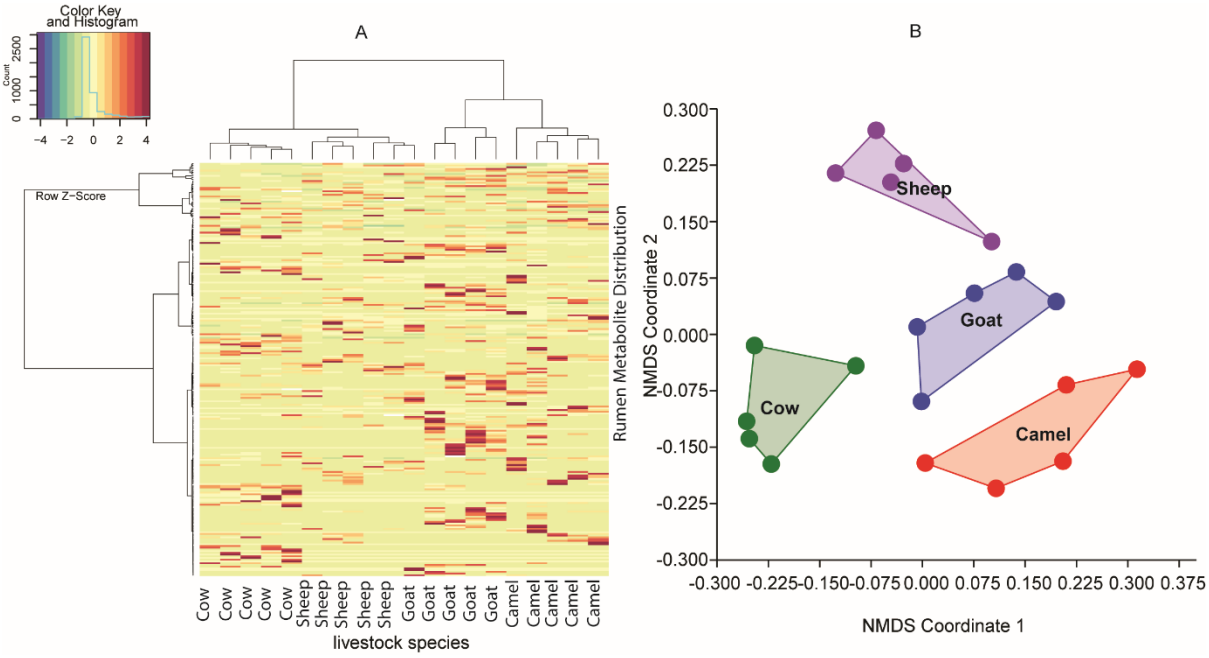

Figure 122: A-Heatmap coded matrix showing relative percent contribution of individual metabolite to the total composition of each livestock species, B-Multivariate analysis of livestock metabolites identified by non-metric multidimensional scaling (NMDS)

4.4 Metabolite variation by chemical functional groups

The present work further examined the chemical functional group variety of the identified metabolite compounds in livestock rumen, in order to comprehend the displayed livestock chemodiversity based on the hypothesis that rumen metabolite diversity corresponds with greenhouse gas emission in livestock. Based on their functional groups, the identified metabolite compounds from the four livestock species were classified into their appropriate chemical families. Aromatic hydrocarbons, acids, hydrocarbons (alkane, alkene, and alkynes),

phenols, alcohols, indoles, amines, monoterpenes, sesquiterpenes, ketones, esters, ethers, and thiols were among the chemical families found in the rumen metabolic products.

The variation of each chemical species among the four livestock groups was then established using one way ANOVA and an independent t-test. The relative amounts of aromatic hydrocarbons did not differ significantly ($p > 0.005$; Figure 13B) across the four cattle species. Alcohols, hydrocarbons, monoterpenes, acids, and sesquiterpenes, however demonstrated significant variations in relative concentration (Figure 13A, C–F; P 0.005). Compared to goats, which showed high abundance in alcohols and sesquiterpenes, cattle, sheep, and camels all displayed similar relative concentrations of these chemical groups. Likewise, camels had significantly high acids concentrations than cattle, sheep and goats (ANOVA, $P = 0.004$; Figure 13C). Whereas goats displayed notably high hydrocarbon concentrations (independent t-test, $P = 0.001$), monoterpenes were more dominant in sheep than the other ruminants (independent t-test, $P > 0.05$). Both camels and sheep showed similarity in concentrations of monoterpenes and sesquiterpenes while cattle and goats exhibited high sesquiterpenes concentrations (Figure 13E-F). Whereas phenols were equally concentrated in cattle, sheep and camel than in goats (Figure 14A, ANOVA, $P = 0.002$), ketones concentrations varied among camels and cattle and similarly between goats and sheep (ANOVA, $P = 0.001$). Similarly, indoles concentrations in sheep and goats were comparable than in cattle and camel, and likewise to thiols (Figure 14E). Other chemical families like ethers and amine also varied among the four ruminants investigated (Figure 14F, 15A). Analysis of similarities (ANOSIM) and similarity percentage (SIMPER) analysis was employed to highlight chemical functional groups that significantly contributed to the metabolite compound dissimilarity among the four livestock ($P = 0.0001$, $R = 0.7427$). Hydrocarbons, monoterpenes, sesquiterpenes and phenols had the highest variation contribution among the 4 livestock species compared to thiols and esters which had the least variation contributions (Figure 15B-C).

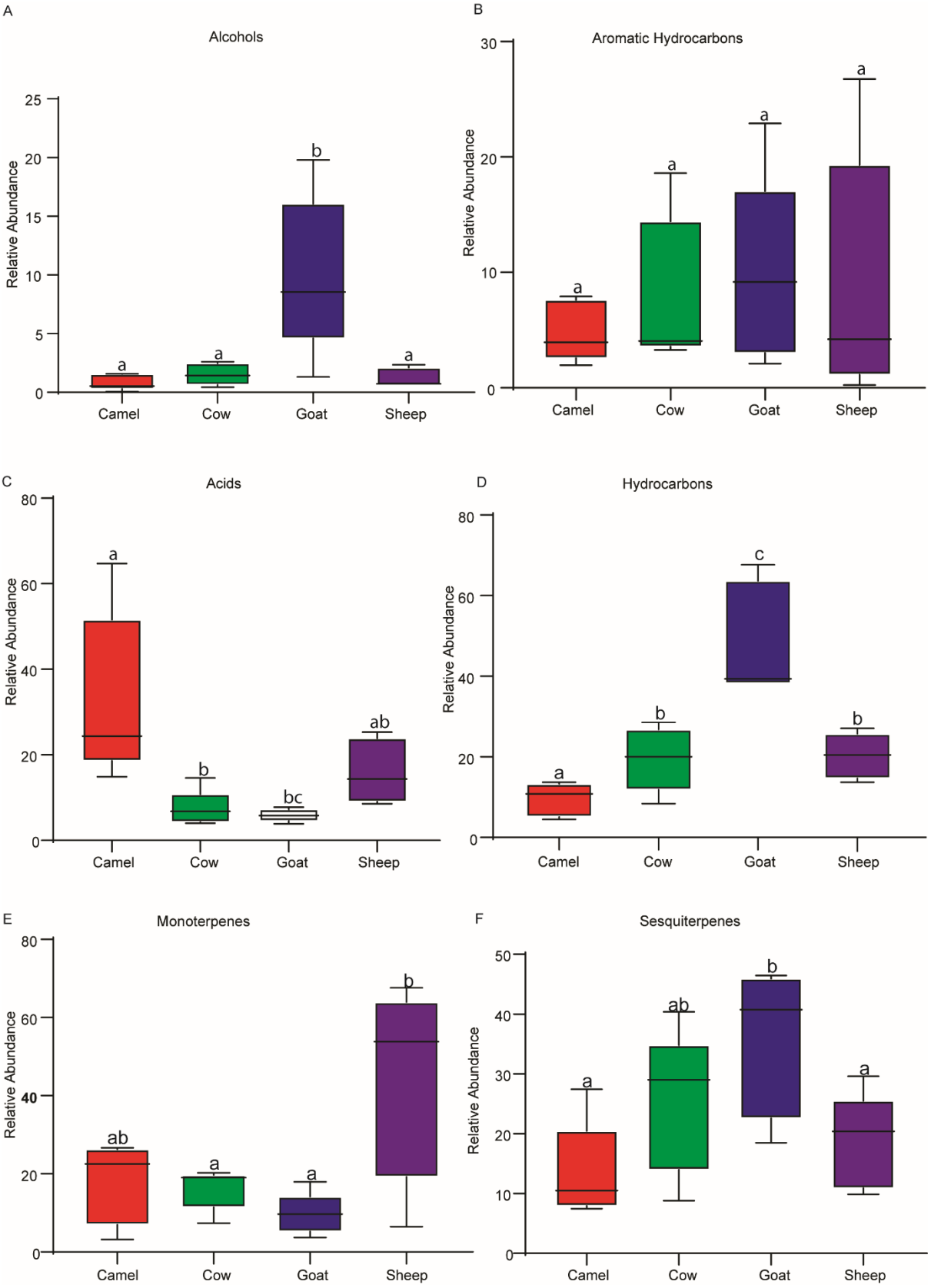

Figure 13: Variation of chemical families (A-F) identified in rumen metabolites in different livestock species, the different letters on bars show statistical significance ($P < 0.05$)

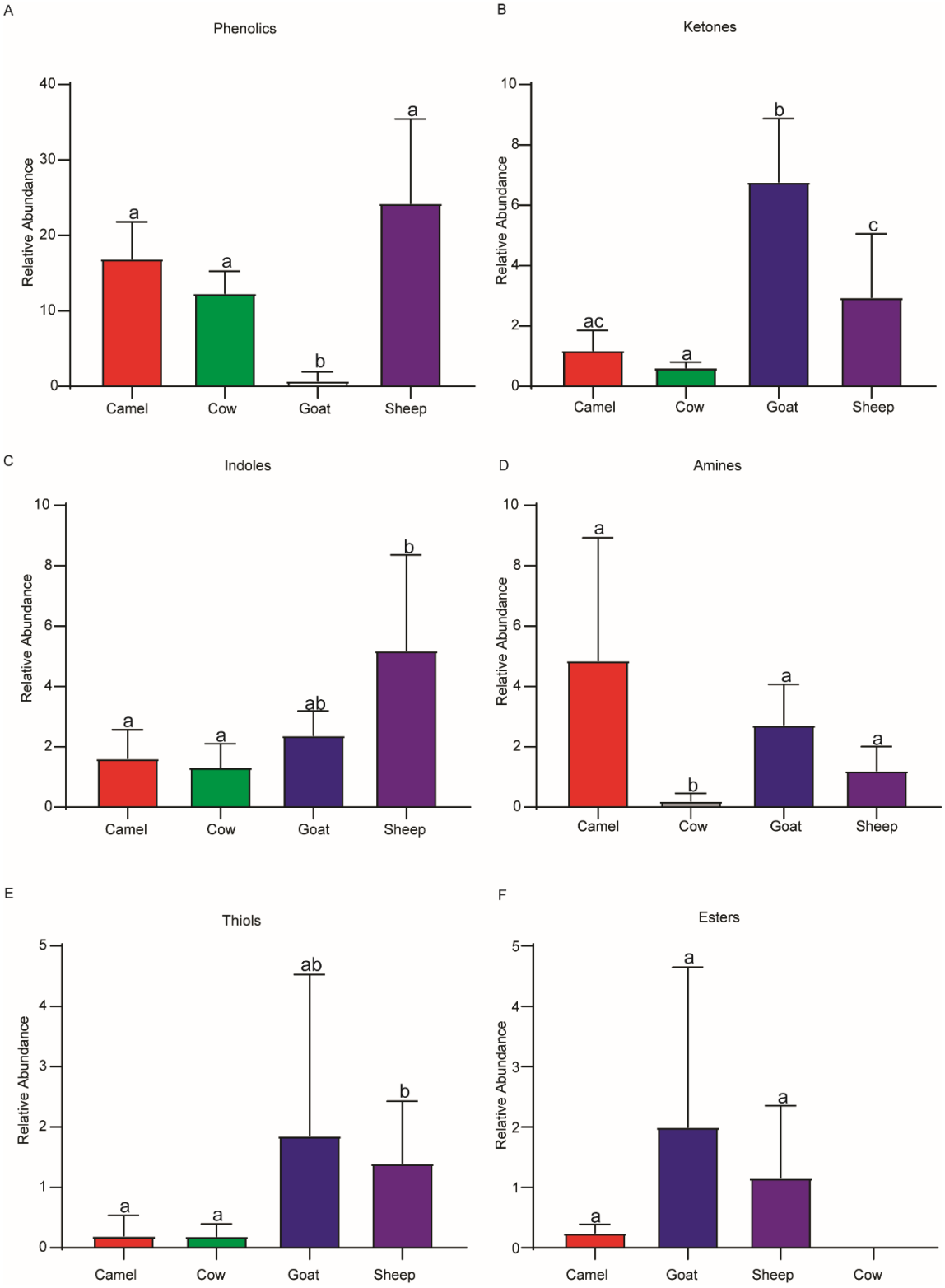

Figure 14: Variation of chemical families (A-F) identified in rumen metabolites in different livestock species, the different letters on bars show statistical significance ($P < 0.05$)

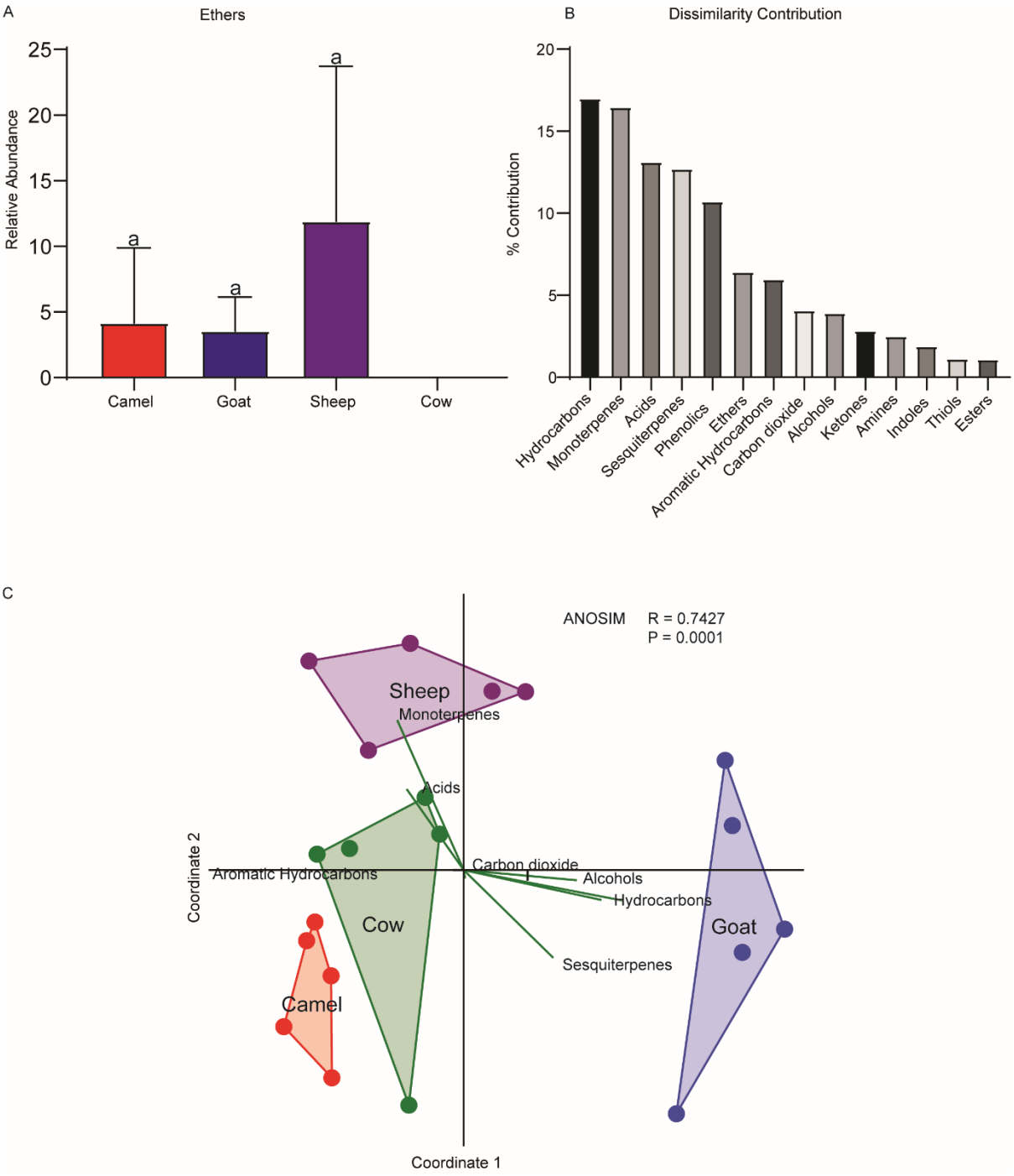

Figure 15: Variation of chemical families (A) identified in rumen metabolites in different livestock species, (B) dissimilarity contribution of individual chemical groups of rumen metabolite identified by non-metric multi-dimensional scaling plot (NMDS)

The variation in metabolite compounds classes highlighted among cattle, camels, sheep and goats, features relevant aspects of rumen metabolite composition, and further points to the chemodiversity exhibited by ruminant livestock. Such a variation may influence greenhouse gas production in the rumen, as certain functional groups can promote or inhibit the production of methane during rumen fermentation (Black *et al.*, 2021).Metabolite compound classes such

as terpenes, alcohols, phenols, acids, indoles, aromatic hydrocarbons, and hydrocarbons are present in livestock feed and can influence GHG production through their interactions with the microorganisms in the rumen (Owens & Basalan, 2016). Chemical compound classes like terpenes (monoterpene and sesquiterpenes), which are generally constituted in livestock feed, including plants and plant-based by-products, inhibit methanogenic bacteria in the rumen This, in turn, results in a decline in the production of methane, as observed by Vera and team (2020). For instance, essential oils containing terpenes such as limonene, eucalyptol, and caryophyllene, decreased methane production in vitro and in vivo (Patra & Saxena, 2009). Similarly, alcohols which this study demonstrates to be highly abundant in goats (Figure 13A), has been shown to decrease methane production in the rumen, likely through a similar mechanism involving the inhibition of methanogenic (Patra & Saxena, 2010).

Phenols and indolic compounds, mainly derived from microbial fermentation of aromatic amino acids, and was present in cattle, camels and sheep rumen, have been highlighted to reduced greenhouse gas production in livestock rumen by inhibiting methanogenic bacteria(Min *et al.,* 2022). A study by Gutierrez and co-workers (2021), also highlighted that phenolic compounds like tannins reduced methane production in the rumen by forming complexes with proteins and other metabolites, thereby inhibiting methanogenic bacteria (Gutierrez *et al.,* 2021). Similarly, Ungerfeld, (2015) demonstrated that indoles, produced through the degradation of tryptophan, inhibited methanogenic bacteria, thereby lowering methane production (Ungerfeld, 2015).

Contrary to phenols and indoles, acids, particularly short-chain fatty acids (SCFAs), which are a major product of rumen fermentation. In the present study, it is evident that acid concentrations varied among the four ruminants (Figure 13C), generally this may be ascribed to the various microbial populations or feed composition. Acids have been shown to enhance greenhouse gas production, probably through the generation of hydrogen gas, which is a substrate for methanogenic bacteria in livestock rumen (Chiri *et al.*, 2021; Rooke *et al.*, 2014). In addition, hydrocarbons, another class of compounds that was found in livestock rumen (Figure 14D), are largely constituted in plant carbohydrates like glucose and sucrose, the main energy sources for livestock (Khan *et al.,* 2020). The presence of hydrocarbons, mainly unsaturated fatty acids and alkanes stimulate the production of hydrogen gas in the rumen, therefore enhancing GHG production during enteric fermentation (Olijhoek *et al.,* 2018).

The highlighted variation in metabolite compound classes in livestock rumen, can significantly influence GHG production during enteric fermentation. Chemical compound classes such as terpenes, alcohols, and phenols can inhibit methanogenic bacteria, leading to a reduction in methane production, while others, such as SCFAs and unsaturated fatty acids, can stimulate methane production. The effects of these chemical compound on GHG production are complex and depend on factors such as their concentration, the microorganisms present, and the feed composition. Hence, additional research is necessary to gain a complete understanding of the contribution of different metabolite compound classes to greenhouse gas production in the rumen of livestock.

4.5 Ruminal ammonia and total sugars

Ruminal ammonia concentration was significantly high in cattle, and camel compared to goat and sheep (Figure 16-B, ANOVA, $P < 0.05$). There was no significant variation realized in the amount of sugars among the livestock (Figure 16-A, ANOVA, $P > 0.05$). Despite the statistical similarity of sugar concentrations in the 4 ruminants, individual concentrations between animals was different. For instance, cattle had more sugars than camels (Figure 16).

This study depicts no significant variation in sugar concentrations among the four ruminant species (figure 16-A), however camel had the lowest concentrations of sugars than cattle, sheep and goats. Simple sugars such as glucose, fructose and sucrose are key energy sources from plants hence utilized by ruminants' to provide instant energy for facilitating different metabolic processes in the animal's body system (Khan *et al.*, 2020, Mokaya *et al.*, 2022). Besides, notable differences in ruminal ammonia concentrations were realized between large ruminants (cattle & camels) and small ruminants (goat & sheep, Figure 16-B).

In the rumen, ammonia and amino acids are usually produced through bacterial aided fermentation of water soluble proteins such as albumens and globulins (Owens & Basalan, 2016). The produced ammonia helps in maintaining the ruminal bacterial growth therefore promotes more extensive ruminal fermentation of carbohydrates. Additionally, ammonia neutralizes ruminal acids and elevates urinary output, enhancing water absorption and ruminal fluid turnover (Owens & Basalan, 2016). Since larger ruminants like cattle and camels tend to drink a lot of water, they exhibited higher concentrations of ruminal ammonia than small ruminants like goats and sheep (figure 16-B).

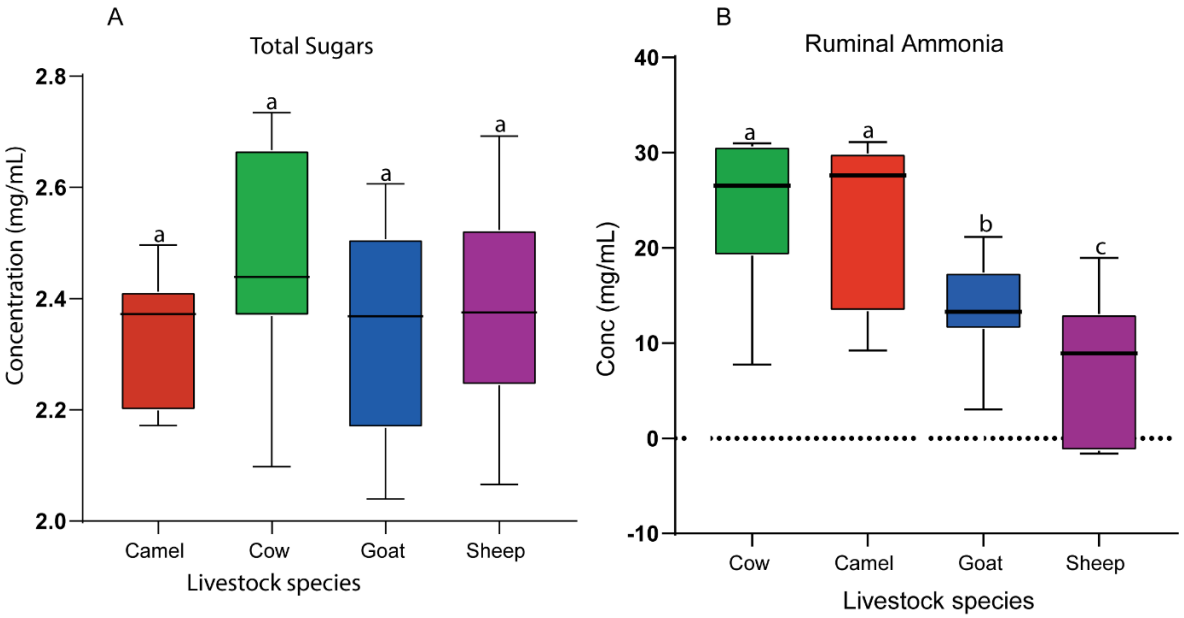

Figure 16: A-total sugar concentration (ANOVA, P > 0.05), and B-ruminal ammonia concentration in ruminants (ANOVA, P < 0.05), the different letters on bars show statistical significance (P < 0.05)

4.6 Ruminal pH

In biological processes, such as rumen fermentation, pH is important (Garca-Yuste, 2020). As a result, the pH of ruminal fluid from cattle, goats, sheep, and camels was analyzed in the current study. Cattle, sheep and goats did not exhibit any significant differences in ruminal fluid pH (ANOVA, P > 0.05), however, this were significantly different from that of camels (ANOVA, P < 0.05), as seen in Figure 17. Camel ruminal pH ranged from 6.3 to 6.5, with an average of 6.3, while that of cattle, sheep, and goats was between 7.0 and 7.4, with an average of 7.2. (Figure 17). The low pH in camel also matched the high acid concentrations earlier realized in camel rumen (Figure 13C). Literature also highlights that high energy diets like browses which are primarily fed on by camels produce a lot of acids during their fermentation (Shi *et al.*, 2020). Ruminal pH affects how fast bacteria ferment in ruminants; for example, ruminal pH decreases during vigorous fermentation (Villot *et al.*, 2018,Garca-Yuste, 2020). Additionally, according to Owens and colleagues (2016), ruminal pH plays a substantial role in determining the content and quantity of fermentation byproducts. The current study also shows that observed variations in pH may be responsible for differences in the concentration of volatile organic compounds and the carbon footprint of ruminants.

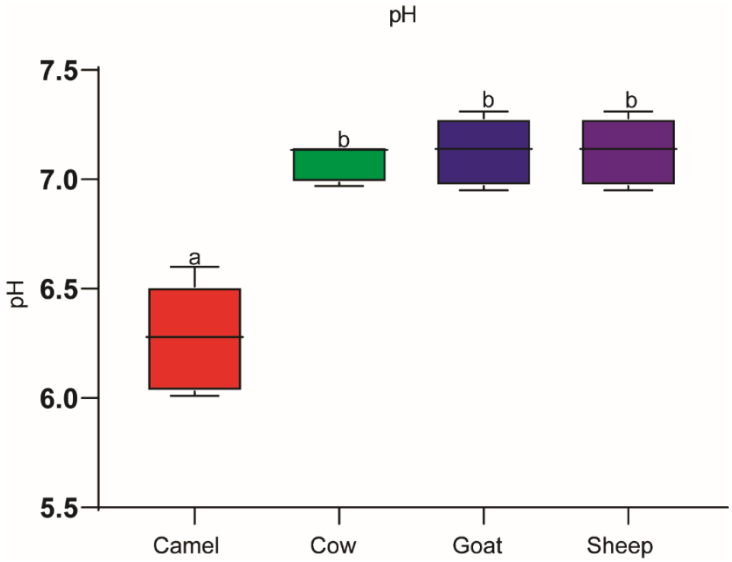

Figure 17: Box plots showing ruminal pH measured in different ruminants, the different letters on bars show statistical significance ($P < 0.05$)

4.7 Variation in greenhouse gas footprint

A significant variation in livestock carbon footprint (Figure 18, ANOVA, $P < 0.05$) which could be suggestive of greenhouse gas emission dissimilarity in livestock was realized in the present study. Camel had the least relative CO_2 relative abundance while cattle registered the highest abundance. On the other hand, camel and goat showed similarity in carbon dioxide foot print (independent t-test, $P > 0.05$). The same was also realized in cattle and sheep which also displayed similarity in relative carbon dioxide abundance (independent t-test, $P > 0.05$).

Other than methane, carbon dioxide, a byproduct of ruminal fermentation is one of the primary greenhouse gases that have been attributed to facilitate global warming therefore climate change (Lynch *et al.*, 2021). The current study reveals the differences in carbon footprint among ruminants which could translate to relative emission capacities. According to prior research, cattle constitute the main source of enteric GHG gases (Grossi *et al.*, 2019), this study also reflects this where cattle showed the highest relative carbon dioxide abundance. Furthermore, browsers (camel and goat) displayed the lowest CO_2 abundance compared to grazers (cattle & sheep, Figure 18). Notably, camel showed the least abundance of carbon dioxide, based on the previous findings reported in this study, camel exhibited the highest acid abundance, which conforms to the low pH realized (Figure 17). Hence, the study assumes that acidic environment reduces greenhouse gas emission footprint in livestock.

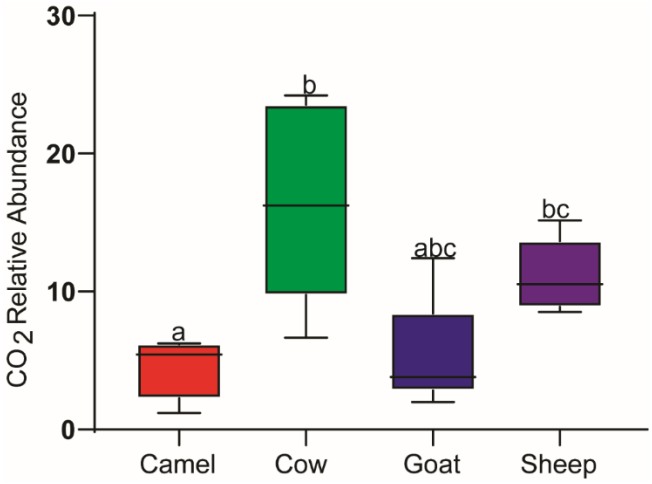

Figure 18: Relative CO_2 emission in various livestock species (ANOVA, $P < 0.05$), different letters on bars show statistical significance ($P < 0.05$)

4.8 Rumen metabolite interaction with carbon dioxide

Having established that cattle, camel, sheep and goat varied in their rumen metabolite composition including relative carbon dioxide emission, the study further investigated how various chemical identities of the identified metabolites associated with carbon dioxide abundance in the rumen. For this reason, correlation analysis was conducted in order to get insights of which metabolites inhibited or absorbed CO_2 in livestock rumen. The findings demonstrated that relative carbon dioxide emission negatively interacted with acids, phenolic, indolic, amines, ketoses and ethers in general (Figure 19). On the other hand, metabolites from other chemical identities such as hydrocarbons, monoterpenes, sesquiterpenes, alcohols, aromatic hydrocarbons and thiols registered a positive interaction with CO_2 (Figure 19).

The relative CO_2 emission tends to be reduced by acids as shown in figure 19, this tends to agree with findings in the previous sections in this study that which showed that camels exhibited less relative carbon dioxide concentrations (Figure 18). Although it had been highlighted that higher acid concentrations may increase methane production during rumen fermentation, the effect of acids on carbon dioxide and other metabolite compounds need to be explored. However, Khan and team (2020) reported that high abundance of some organic acids are associated with specific plant metabolites which enhance drought tolerance in some plant species. A similar case may be possible as demonstrated in the current study with interaction of carbon dioxide with various rumen metabolites from different chemical families. Moreover, literature also suggests that ratios of specific organic acids generated stoichiometrically governs the amounts and composition of the gases produced during rumen fermentation

(Owens & Basalan, 2016). These findings therefore prompted further investigations into establishing how individual acid rumen metabolites could be associated with relative carbon dioxide abundance in the rumen.

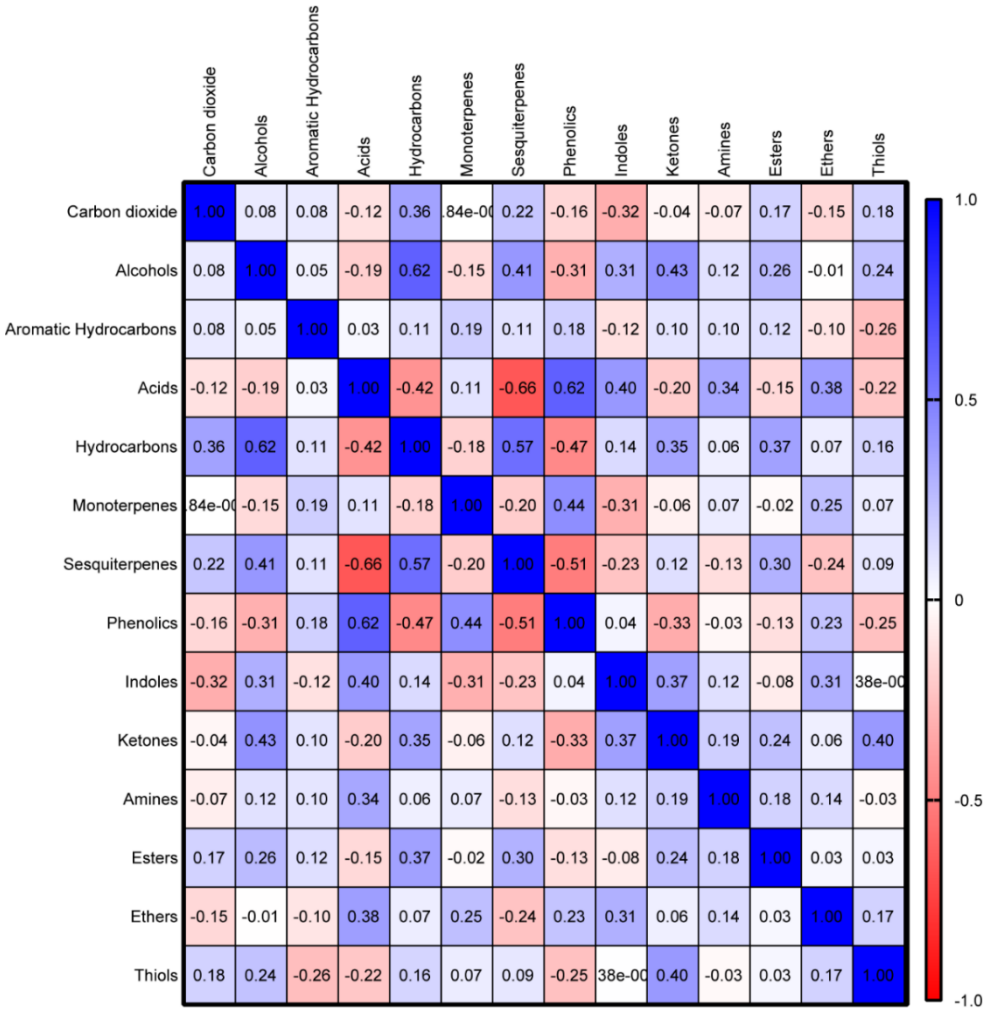

Figure 19: Color coded matrix Pearson's correlation plot for carbon dioxide interaction with identified rumen metabolite chemical families from all the 4 livestock species

A Pearson's correlation analysis was employed to highlight how individual acid compounds associated with the relative carbon dioxide concentrations. The results showed that propanoic acid, hexanoic acid, 2-methyl butanoic acid and carbamic acid tend to downregulate carbon dioxide concentrations compared to other acids (Figure 20). Contrary to acetic and butanoic acid, propanoic acid demonstrated interaction with carbon dioxide. Although it is one of the primary sources of energy generated during rumen fermentations, the fermentation pathway of propanoic acid differs from that of acetic and butanoic acid in that it does not produce hydrogen (Chen *et al.*, 2020, Zhang *et al.*, 2021). Furthermore, propionate is a gluconeo-genic volatile fatty acid hence can facilitate availability of more energy to livestock. The demonstrated

interaction between propanoic acid (propionate) and carbon dioxide (Figure 20), is consistent with a recent study by Chen and team (2020) which showed that propionate acid bacteria reduced methane production in vitro. Therefore, stimulating the propionate fermentation pathway could decrease methane emission. Likewise, the current research suggests that altering the biosynthetic pathways of major acids like propanoic acid produced during rumen fermentation could potentially reduce the overall greenhouse gas footprints in livestock.

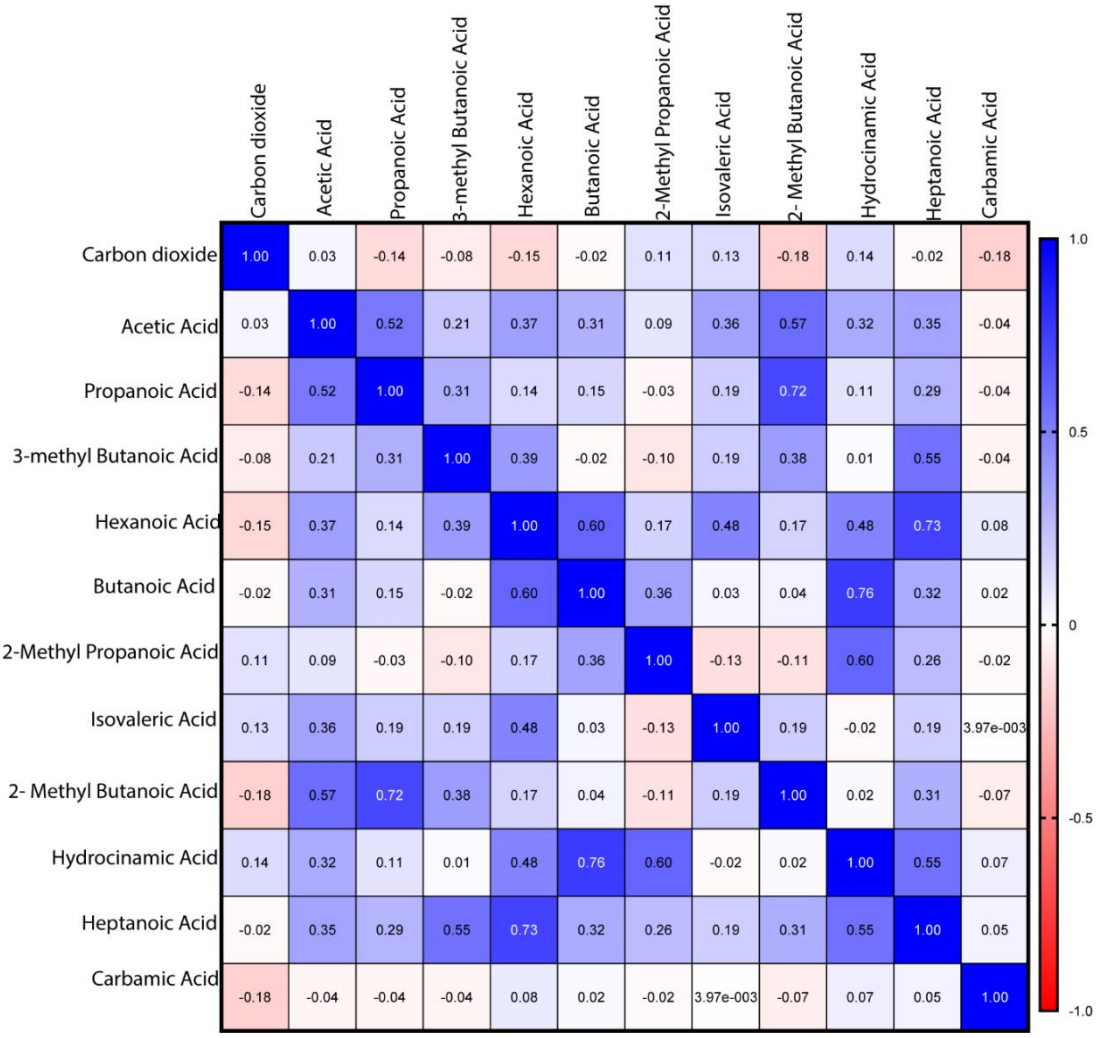

Figure 20: Color coded matrix Pearson's correlation plot showing carbon dioxide interaction with acids identified in the 4 livestock species

4.9 Rumen metabolite network correlation analysis

In this study various biosynthetic pathways that characterize metabolite compound synthesis were evaluated. A correlation network analysis on the identified metabolite compounds was conducted to establish if there were any correlation in their biosynthetic pathways among the

four ruminants studied. The relative concentrations of these compounds were subjected to a correlation network analysis using Fruchterman-Reingold algorithm (Fig 21-22). Recent metabolomic studies have used network correlation cluster analysis with different prediction models to suggest similarity in metabolite biosynthesis pathways (Toubiana *et al.*, 2020, Colantonio *et al.*, 2022).

These metabolic routes provide a hint of the overall relationship between several compounds that share biosynthesis route, thereby creating room for potential manipulation. In the present research, the four ruminants displayed variation in their metabolite correlation networks (Fig 21-22). However, despite the difference, compounds of similar chemical identities were interconnected, hence suggesting a similarity in their synthesis routes. For instance, terpenes such as valencene, β-caryophyllene, camphene and pinene, displayed a strong correlation (thick connecting lines) among them, and similarly, volatile fatty acids (Figure 21-22). Additionally, fecal predictive compounds like p-cresol and 6-methyl, indole were all interconnected with carbon dioxide, hence highlighting a correlation in their biosynthetic pathway. A similar correlation was also demonstrated by Colantonio and colleagues (2022), who highlighted the correlation in biochemical pathway of apocarotenoid volatiles and fatty acid derived volatiles (Colantonio *et al.*, 2022).

The shown association in biosynthetic pathways , therefore presents a window for mitigating greenhouse gas production in ruminants by manipulating the rumen fermentation pathways, however more studies is still needed.

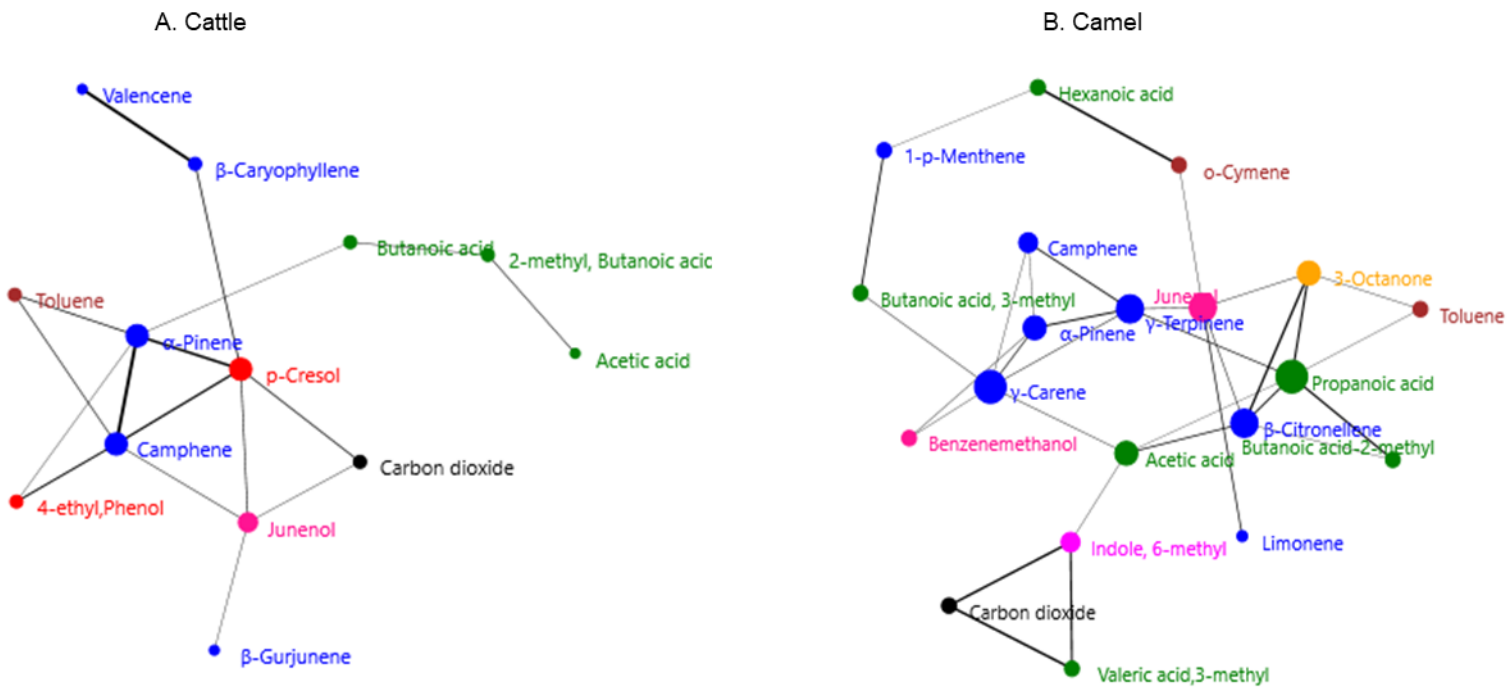

Figure 211: Metabolite correlation network plots. Clusters show metabolite biosynthesis pathways. The size of each metabolite node represents metabolite relative concentrations. The thickness of the lines connecting metabolites is scaled relative to the correlation between the compounds.

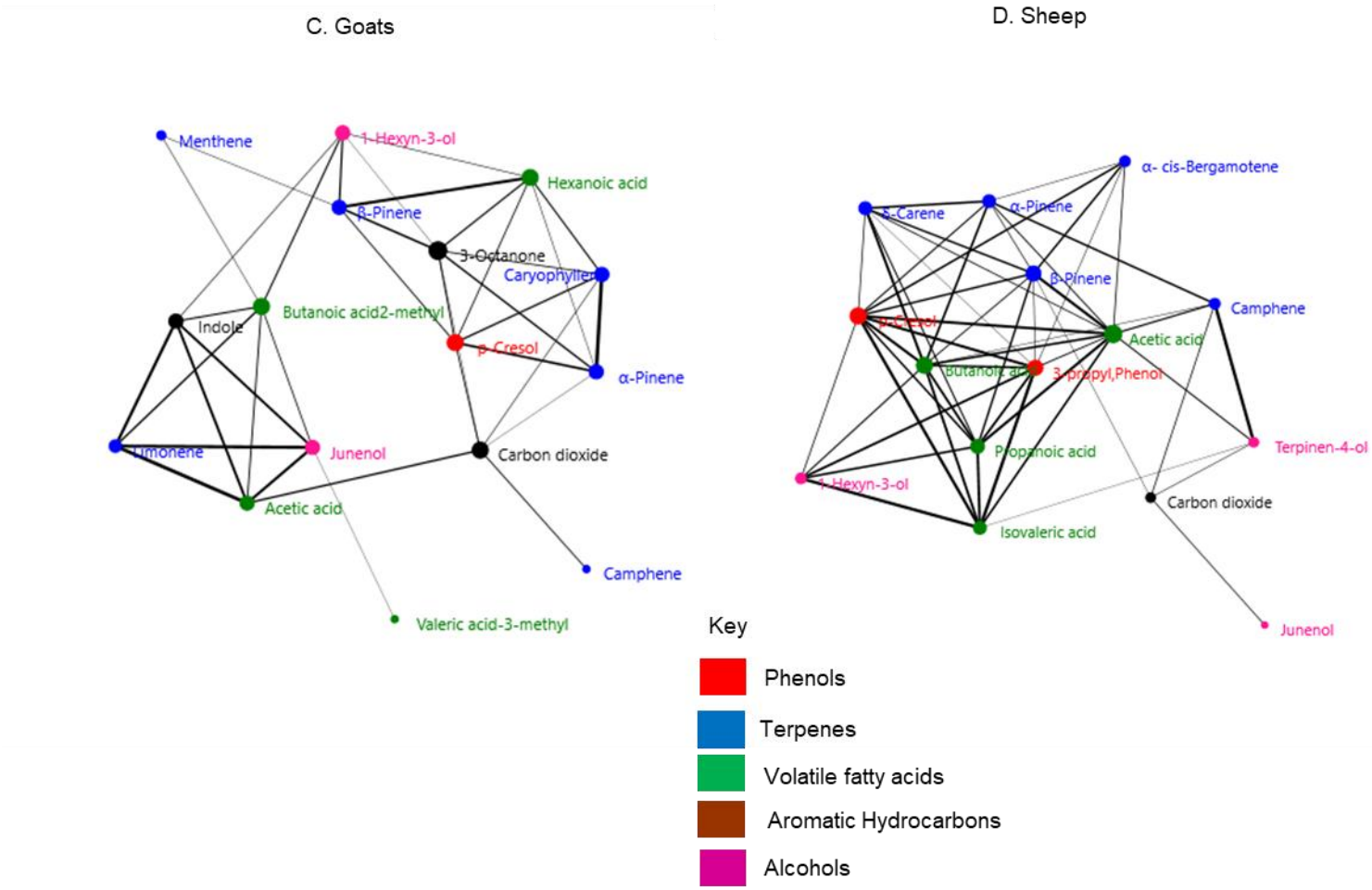

Figure 222: Metabolite correlation network plots (goats and sheep). Clusters show metabolite biosynthesis pathways. The size of each metabolite node represents metabolite relative concentrations. The thickness of the lines connecting metabolites is scaled relative to the correlation between the compounds.

5.0 Rumen microbiota diversity

Rumen microbiota communities contribute significantly to rumen fermentation by assisting in the breakdown of plant fibers and cellulose to produce energy used by the animal as well as the production of metabolites, which include greenhouse gases like carbon dioxide and methane (Morgavi, 2015; Grossi *et al.,* 2019, Zhang *et al.,* 2021;. Rooke *et al.,* 2014, Wang *et al.,* 2016).The present research investigated rumen microbiota distribution in cattle, camel, goat and sheep to understand if revealed metabolite chemodiversity and relative greenhouse gas emission could be associated with microbial diversity in livestock.

The results showed that livestock have a remarkable rumen microbiota composition and biodiversity which vary between cattle, sheep, goat and camel (Figure 25A). Among the established microbial domains present in livestock rumen included bacteria, archea, protozoa and fungi (Figure 23 and 24) therefore consistent with most studies which have previously demonstrated presence of these microbiota domains in livestock (Zeng, 2017, He *et al.,* 2018, Wallace *et al.,* 2019). While some microbial communities were maintained throughout the four species of livestock, others were specific to certain livestock groups (Figure 23).Cattle and sheep harbor similar rumen microbes, some of which are absent in camel and goat. Likewise, goat and camel have specific microbiota that are missing in cattle and sheep (Figure 25: A-B) for instance uncultured *neocallimastigales,* uncultured, *Prevotellaceae bacterium*, *Methanomicrobium mobile strain*, *Methanobrevibacter millerae* were uniquely present in camel and goat. Similar uncultured *Methanobrevibacter sp* was distinctly present in cattle and sheep, therefore supporting earlier studies (Zeng, 2017, He *et al.,* 2018, Wallace *et al.,* 2019). Moreover, uncultured *rumen methanogen M6 gene,* and *Nigrospora sphaerica isolate* were present in cattle, *Ostracodinium sp* were distinctly present in sheep. Similarly, uncultured *Bacteroidetes bacterium*, and *Ophyroscolex purkynjei* were identified in goat whereas *Entodinium caudatum* were only in camels (Figure 23A).

Additionally, there was consistency in shared rumen microbes such as uncultured *Archaeon clone, uncultured Rumen protozoa*, and uncultured *anaerobic bacterium* between the studied livestock species that corresponded with previous studies (Morgavi, 2015, Zeng, 2017,C. Liu *et al.,* 2017, He *et al.,* 2018, Zhang *et al.,* 2021). Generally, *euryarchaeota, bacteroidetes,* and ciliate protozoans were the most dominant phyla, a similar observation had also been presented in recent studies by He *et al.,* (2018), Wallace *et al.,* (2019) , and Zhang *et al.,* (2021) which

identified these phyla as the prevalent among the existing microbial populations in the gastrointestinal tracts of most ruminants.

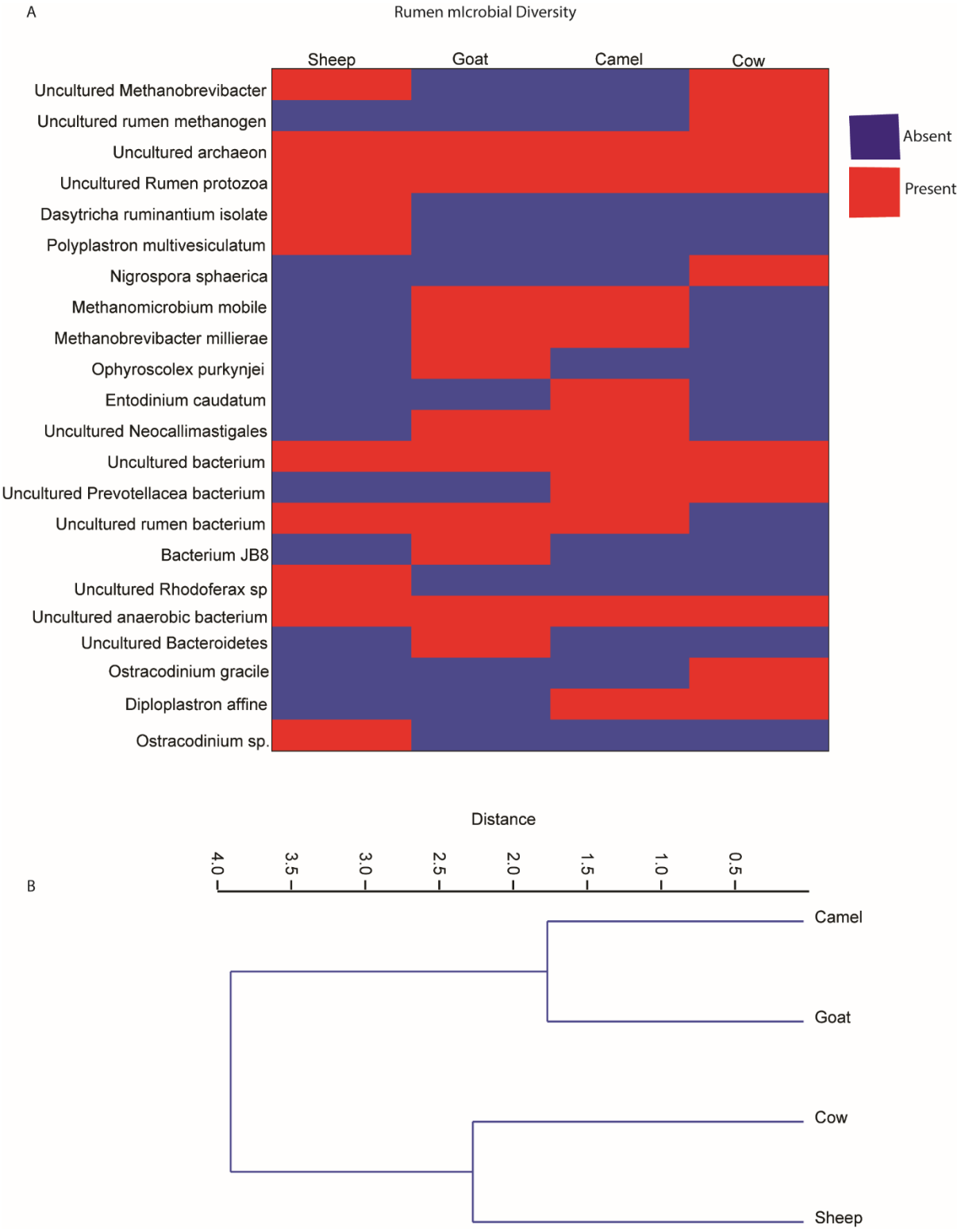

Figure 23: A-Heatmap coded matrix showing identified rumen microbial distribution in livestock species, B- Multivariate livestock cluster of identified rumen microbes using classical cluster analysis by ward's method

5.1 Rumen Microbiota Richness

The microbial biodiversity revealed in the present study prompted further investigation to access the rumen microbiota richness in cattle, camel, goat and sheep. Real Time-quantitative-PCR (RT-q-PCR) technique was employed using universal primers to determine the total copy number of the protozoal 18S rRNA gene, bacterial 16S rRNA gene, and the archaeal 16S rRNA gene in order assess the rumen microbial domain richness in the four examined livestock groups.

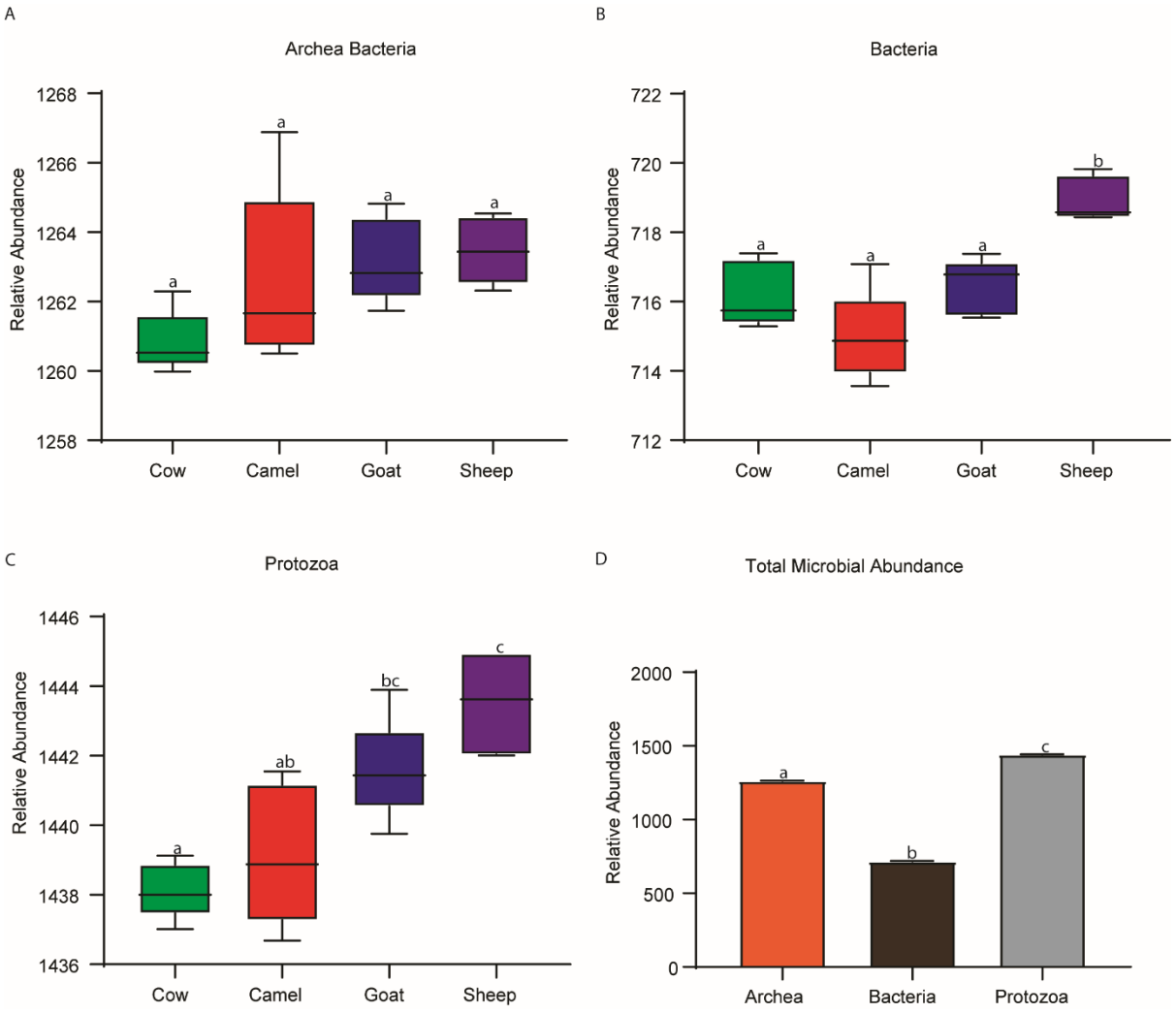

Figure 234: Rumen microbiota diversity population in different livestock species (A-C), and (D) total abundance of different rumen microbiota domains, bars followed by different letters are statistical significance (ANOVA, $P < 0.05$)

The microbiota population richness realized in the present study was consistent with previous observations (Whitehead & Cotta, 2001, Myer *et al.*, 2017, He *et al.*, 2018) in that archea bacteria and protozoa were the most abundant compared to the general bacteria across all the livestock species (Figure 24). Protozoa and general rumen bacteria exhibit a predator-prey kind

of association (Russell, 2001), therefore implies that predominantly bacteria which are mostly Gram-negative are susceptible to protozoal predation are suppressed by protozoa (Wallace *et al.*, 2019), thus allowing the crop up of other microbial communities to fill the niche for instance archea bacteria. The 3 rumen microbial domains, archea, bacteria and protozoa positively correlated with relative carbon dioxide abundance in livestock. Archea and bacteria had the highest correlation factor, 0.548 & 0.546 respectively whereas protozoa had the lowest correlation factor of 0.274 (Figure 25).

Rumen microbiota domain richness positively correlated with relative carbon dioxide emission in all the livestock (Figure 25). Despite protozoal richness being the highest (Figure 23D), it had a weak correlation compared to archaeal and bacterial populations which had a strong correlation with relative carbon dioxide emission in livestock. This type of association might be expected since most of the rumen archea and some bacterial species are largely methanogenic (Wallace *et al.*, 2019).

Recent studies show that various factors other than microbiota diversity, such as heritable traits, diet and host genotype metabolic phenotypes and microbial metagenome to significantly influence greenhouse emission in livestock (Morgavi, 2015;Rooke *et al.*, 2014; Wallace *et al.*, 2019; Wallace *et al.*, 2015). Also, a prediction study by Zhang and co-workers (2021), demonstrated that rumen microbiota functions on ruminal metabolic pathway was largely associated with carbohydrate breakdown, therefore regulates carbon footprint, sugar synthesis including greenhouse gas production. Similarly, the present study highlights that rumen metabolite-microbiota interaction may be another important factor to predict and explain greenhouse emission in livestock though this needs to be explored further.

The revealed microbiota variation highlighted in the current study, was suggestive that diet preferences by different livestock groups contributed to the exhibited microbial biodiversity (Rooke *et al.*, 2014), for instance browsers like camel and goat displayed similarity in microbiota community, in relation to grazers like cattle and sheep (Figure 23B). This therefore prompted further investigation to establish the diet composition in cattle, camel, goat and sheep

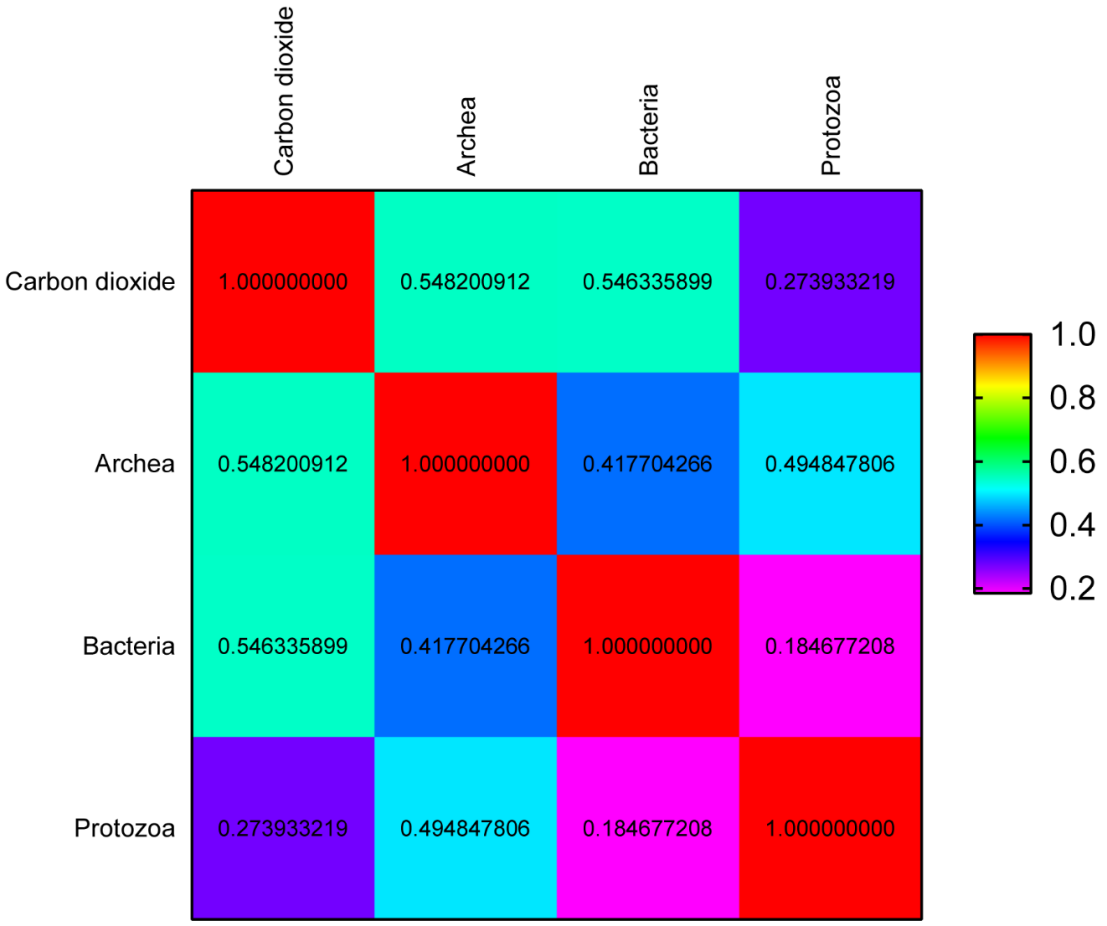

Figure 245: Pearson's color coded correlation matrix plot showing carbon dioxide interaction with rumen microbial domain population

5.2 Plant diet identification

To get an insight of diet composition in the cattle, camel, goat and sheep, plant diet assessment in digester samples obtained from the four livestock species were established using a PCR-based method using the rbcL gene and the trnH-psbA gene spacer region to target a pair of genes that make up the chloroplast marker.

Camel and goat were found to have consumed plants species such *Acacia concinna, Searsia tripartite, Vachellia nilotica, Sonchus webbii*. These plant families are predominantly associated with dry and semi-dry environments (Li *et al.*, 2008) which is characteristic of most habitats hosting camel and goat therefore providing nutritional needs to these animals. Camel and goat are generally classified as browsers hence are specially adapted to feed on shrubs and woody plants which mainly grow in dry and semi- dry climates (Ward *et al.*, 2020). Additionally, these group of livestock have a diverse pool of plant species to feed on as demonstrated in the current study (Table 2). On the other hand cattle and sheep which are

primarily grazers (Ward *et al.*, 2020) had consumed *Cenchrus ciliaris*, a common species from the grass family, including other low lying vegetation such as *Cenchrus americanus* and *Rhus transvaalensis.* Generally grazers (cattle and sheep) tend to be more selective on their diet preference (Cuchillo-Hilario *et al.*, 2018), thus had limited diet choices compared to browsers (Table 2). The dietary variation that was found was also mirrored in the corresponding microbiota communities, which jointly affect the host's nutrition and energy balance, including fermentation byproducts (Myer *et al.*, 2017). Diet quality and nutritional composition influences metabolite composition, which means that nutritionally rich foods have diverse metabolite composition (Cuchillo *et al.*, 2018). For instance, cattle and sheep consume grass, which has lower nutritional value, showed less metabolite diversity than camels and goats which consume woody plants and bushes with its high fiber content (Clauss *et al.*, 2008, Ward *et al.*, 2020).

Table 2: List of plant species that are consumed by different livestock groups

Plant Species	Family	Animal
Eleusine coracana	*Poacea*	Camel
Searsia tripartita	*Sersia*	Camel
Lysiloma watsonii	*Fabaceae*	Camel
Rhus gueinzii(Searsia gueinzii)	*Sersia*	Camel
Vachellia nilotica	*Fabaceae*	Camel
Malvastrum coromandelianum	*Malvaceae*	Camel/Goat
Acacia concinna	*Fabaceae*	Camel/Goat
Laurophyllus capensis	*Anacardiaceae*	Camel/Goat
Senegalia lasiophylla	*Leguminosae*	Camel/Goat
Rhus transvaalensis	*Anacardiaceae*	Cow
Loranthaceae sp	*Loranthaceae*	Cow/sheep
Asplenium obtusatum	*Aspleniaceae*	Cow/sheep
Cenchrus ciliaris	*Poacea*	Cow/sheep
Cenchrus americanus	*Poaceae*	Cow/sheep
Boechera divaricarpa	*Brassicaceae*	Goat
Cissus quadrangularis	*Vitaceae*	Goat
Paraprenanthes sororia	*Asteraceae*	Goat
Spondias purpurea	*Anacardiaceae*	Goat
Sonchus webbii	*Asteraceae*	Goat/Camel
Beilschmiedia roxburghiana	*Lauracaceaae*	Sheep
Styrax ferrugineus	*styracaceaae*	Sheep

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

The characterization and comparative analysis of metabolite compounds' composition and their relative greenhouse emission contribution in cattle, camels, goats and sheep, reveals relevant interspecific diversity in metabolite composition, metabolite chemical classes in livestock which may consequently, influence the greenhouse gas emission capacities in various livestock as highlighted in this study.

The SPME-GC/MS technique has shown promise in the rapid extraction of volatile organic compounds, including carbon dioxide, from ruminal content, with an extraction time of 30 minutes as compared to 1 hour for Porapak-Q adsorbent. Its success in bioanalytical studies suggests that it could be useful in expanding the scope of biochemical research questions that can be addressed. However, it is worth noting that the SPME fiber may become saturated more quickly depending on the concentration of analytes competing for adsorption sites, unlike Porapak-Q adsorbent.

Although the established metabolite variation in the current study could be ascribed to the difference in livestock species, the presence of diverse microbial domain populations including diet composition, revealed in cattle, camels, goats and sheep is suggestive of the existing difference in livestock rumen microbiome and diet preference, thus may contribute to greenhouse gas emission variation. This is because the interactions between microbial communities and various diets, influence the ruminal metabolic processes thus regulate metabolite compound and greenhouse production in ruminants.

The composition of metabolite compounds in the rumen of livestock is a significant factor in the production of greenhouse gases through enteric fermentation. Knowledge of the microbial populations and metabolic pathways involved in rumen fermentation can aid in the development of sustainable livestock diets and management practices aimed at reducing GHG emissions. This is especially critical in light of the increasing global demand for livestock products and the significant contribution of enteric fermentation to overall agricultural greenhouse gas emissions.

5.2 Recommendations

Based on the observed findings, the present study recommends the following.

1. The shown variation in metabolite compound composition, including their contributions to the production of greenhouse gases in cattle, sheep, camels, and goats, can be used to reduce enteric fermentation-related greenhouse gas emissions from livestock.
2. A longitudinal research focused on individual livestock species should be carried out to understand the relationship between the highlighted parameters such as rumen metabolite, microbiota and greenhouse gases emission.
3. More investigations on rumen metabolite diversity in relation to greenhouse gas emission a more restricted set up, for instance, restricted diet, age, livestock gender, and weight is hereby recommended. Additionally, investigations into the role of individual metabolite compound in greenhouse gas production in livestock should be considered.
4. The use of headspace-solid phase microextraction techniques followed gas chromatographic analysis (HS-SPME), is hereby recommended for bioanalytical studies which demand rapid and high throughput analytical techniques.
5. Real time greenhouse emission measurements using larger livestock populations should be carried out to establish a clear picture of the emission variations between livestock in different seasons and geographies.
6. Observed finding in camels were very interesting, thus further research should be conducted on camels in relation to greenhouse gases emission
7. Whole genome sequencing followed by detailed bioinformatics on rumen microbiome distribution in cattle, goat, sheep and camel, should be explored to establish the unique microbiota population and their role in rumen fermentation and greenhouse gases emission.

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APPENDICES

Appendix 1

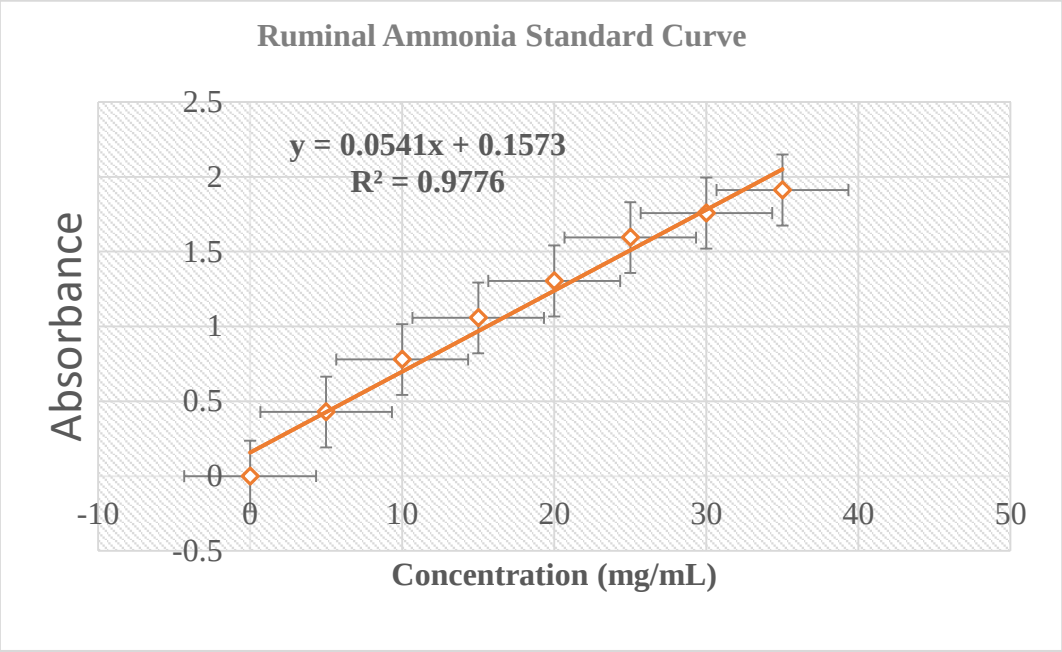


Table 1: Rumen Ammonia Measurements

Sample Id	Absobance	Concentration(mg/mL)	Dillution Factor	Final Conc
C1	1.535	25.4658	1	25.4658
C2	1.816	30.65989	1	30.6599
C3	1.773	29.86506	1	29.8651
C4	1.834	30.99261	1	30.9926
C5	1.65	27.5915	1	27.5915
Ca1	1.77	29.80961	1	29.8096
Ca2	1.794	30.25323	1	30.2532
Ca3	1.643	27.46211	1	27.4621
Ca4	1.535	25.4658	1	25.4658
Ca5	1.66	27.77634	1	27.7763
GT1	0.882	13.39556	1	13.3956
GT2	0.87	13.17375	1	13.1738
GT3	0.851	12.82255	1	12.8226
GT4	1.052	16.53789	1	16.5379
GT5	1.084	17.12939	1	17.1294
S1	0.859	12.97043	1	12.9704
S2	0.601	8.201479	1	8.2015
S3	1.182	18.94085	1	18.9409
S4	0.88	13.3586	1	13.3586
S5	0.773	11.38078	1	11.3808

Key: Ca= Camel, S=sheep, C=Cow, G=Goat

Negative concentration= below detectable limit

Appendix 2

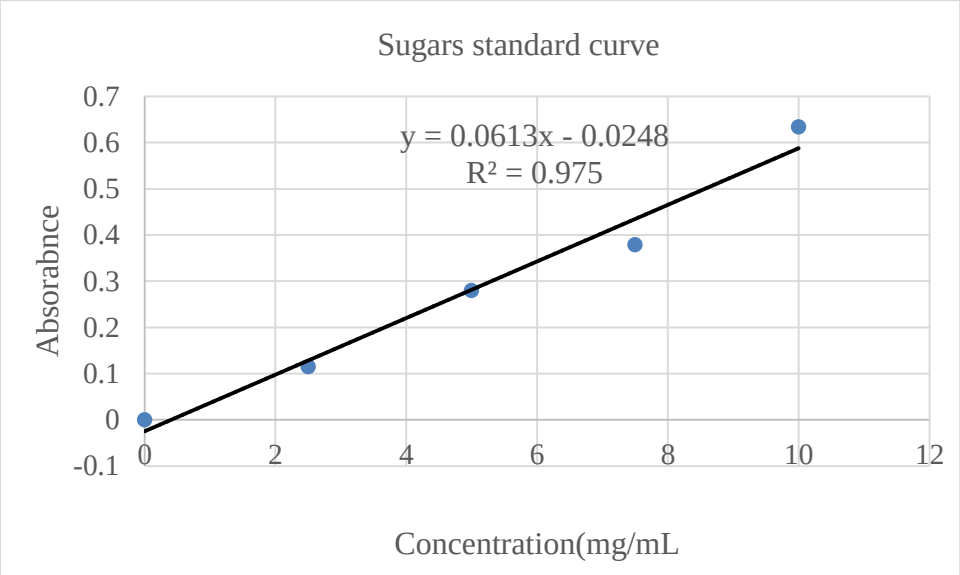


Table 2: Total ruminal Sugars Concentration Measurements

Sample Id	Absorbancce	Conc (mg/mL)	Dillution Factor	Final conc
Ca1	0.248	1.248	2	2.496
Ca2	0.19	1.19	2	2.38
Ca3	0.228	1.228	2	2.456
Ca4	0.088	1.088	2	2.176
Ca5	0.103	1.103	2	2.206
C1	0.049	1.049	2	2.098
C2	0.189	1.189	2	2.378
C3	0.318	1.318	2	2.636
C4	0.333	1.333	2	2.666
C5	0.335	1.335	2	2.67
G1	0.191	1.191	2	2.382
G2	0.242	1.242	2	2.484
G3	0.303	1.303	2	2.606
G4	0.041	1.041	2	2.082
G5	0.098	1.098	2	2.196
S1	0.033	1.033	2	2.066
S2	0.077	1.077	2	2.154
S3	0.137	1.137	2	2.274
S4	0.346	1.346	2	2.692
S5	0.182	1.182	2	2.364

Appendix 3

Table 3: Livestock Rumen Microbiota

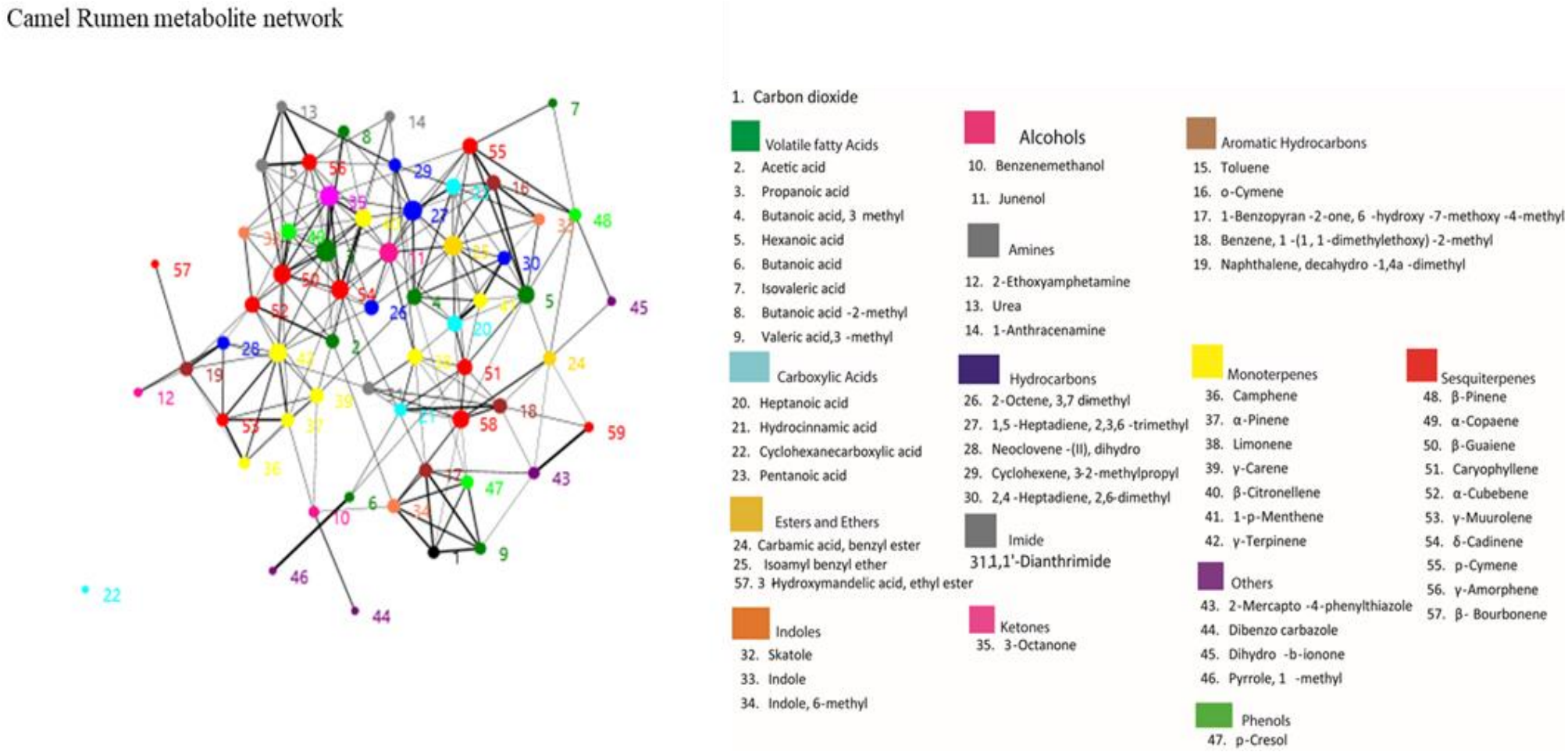
Methanogen	Animal
Uncultured Methanobrevibacter sp.	Cattle
Uncultured rumen methanogen M6 gene	Cattle
Uncultured archaeon clone	Cattle
Methanomicrobium mobile strain	Camel
Uncultured archaeon	Camel
Methanobrevibacter millerae	Camel
Methanomicrobium mobile strain	Goat
Uncultured archaeon	Goat
Methanobrevibacter millerae	Goat
Uncultured Methanobrevibacter sp.	Sheep
Uncultured archaeon clone	Sheep

Protozoa	Animal
Uncultured Rumen protozoa	Cattle
Ostracodinium gracile	Cattle
Diploplastron affine	Camel
Uncultured rumen protozoa	Camel
Entodinium caudatum	Camel
Polyplastron multivesiculatum	Camel
Diploplastron affine	Camel
Ophyroscolex purkynjei	Goat
Uncultured rumen protozoa	Goat
Uncultured Rumen protozoa	Sheep
Dasytricha ruminantium isolate	Sheep
Polyplastron multivesiculatum isolate	Sheep
Ostracodinium sp.	Sheep

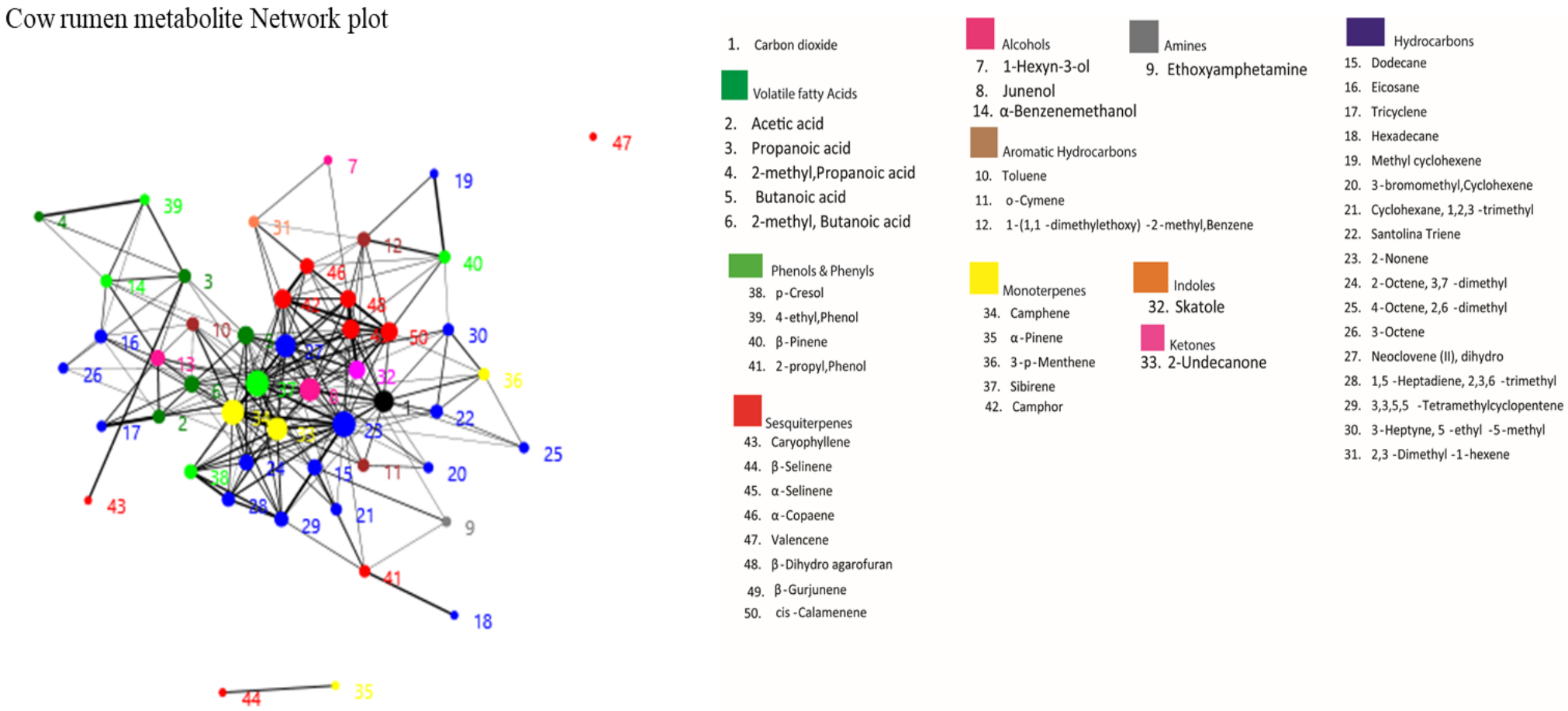
Rumen Bacteria	Animal
Uncultured bacterium	Cattle
Uncultured bacterium clone	Cattle
Uncultured Prevotellaceae	Camel
Uncultured bacterium	Camel
Uncultured rumen bacterium	Camel
Uncultured rumen bacterium	Goat
Uncultured rumen bacterium	Goat
Uncultured Bacteriodetes	Goat
Uncultured anaerobic bacterium	Goat
Bacterium JB8	Goat
Uncultured bacterium clone	Goat
Uncultured bacterium	Sheep
Uncultured anaerobic bacterium	Sheep
uncultured rumen bacterium	Sheep
Uncultured Rhodoferax sp	Sheep

Fungi	
Nigrospora sp	Cattle
Uncultured Neocallimastigales	Camel
Nigrospora sp	Sheep
Nigrospora sphaerica isolate	Sheep

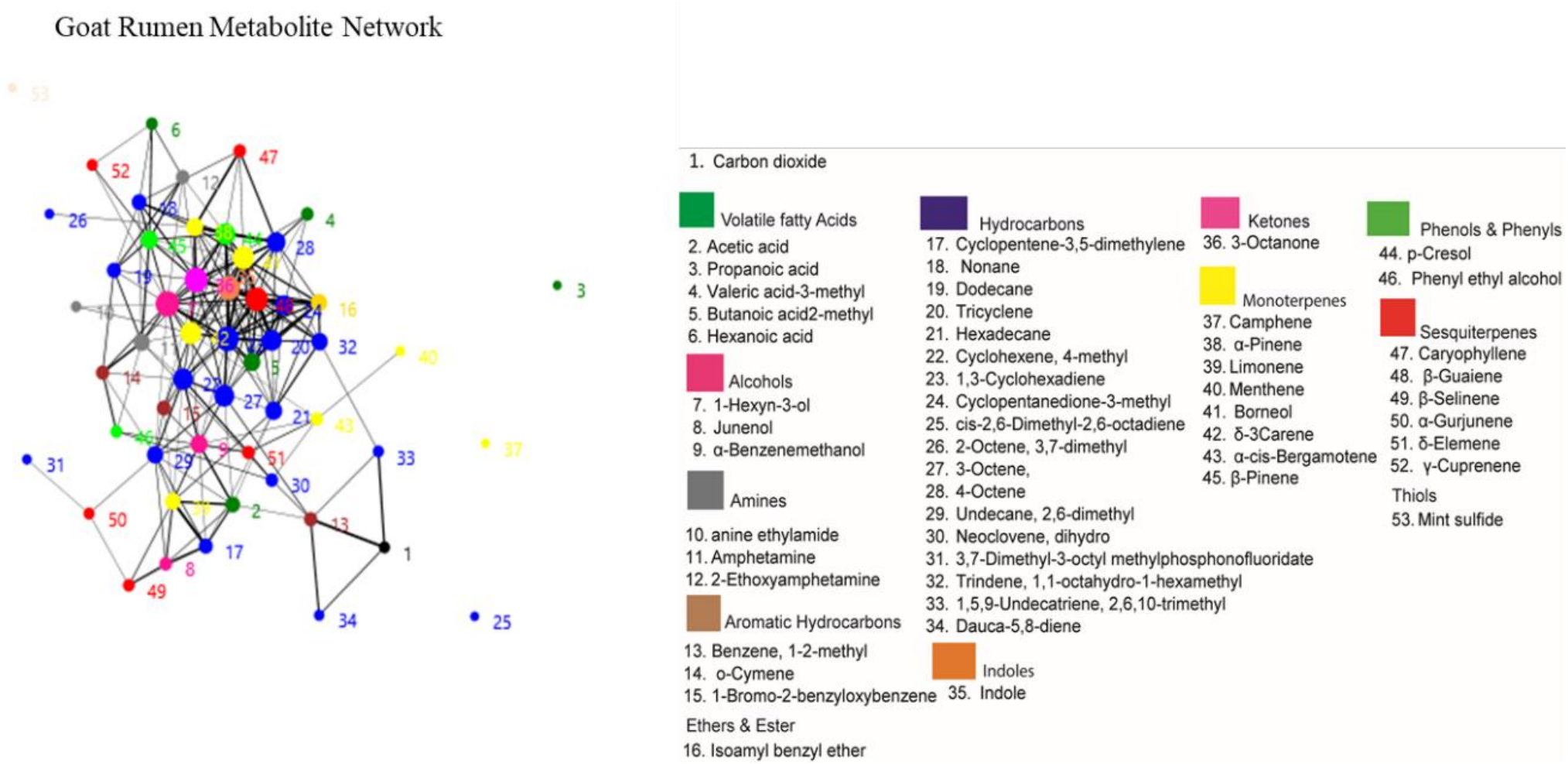
Appendix 4: Correlation network plots



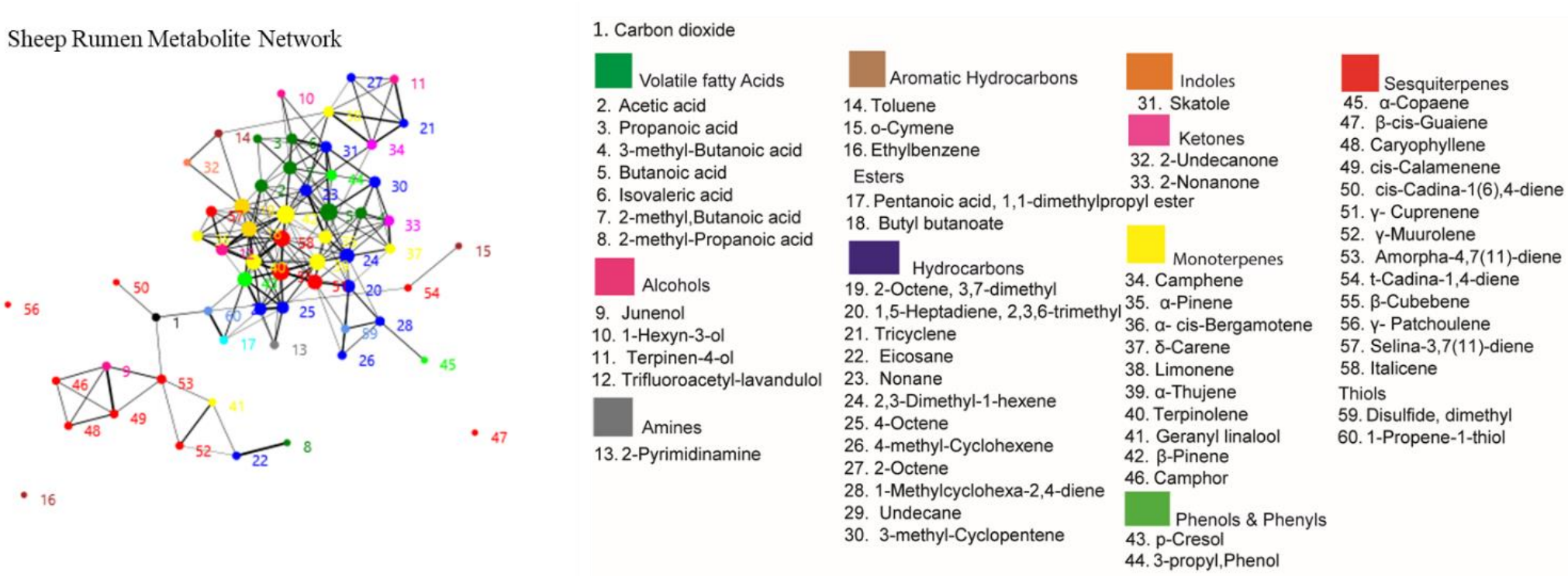
Correlation network plot of camel rumen metabolites and their assigned clusters based on their known chemical classification. The size of each metabolite node represents metabolite relative abundance. The thickness of the lines connecting metabolites is scaled relative to the correlation between the metabolites. The identity of each metabolite is denoted by number in the legend



Correlation network plot of Cattle rumen metabolites and their assigned clusters based on their known chemical classification. The size of each metabolite node represents metabolite relative abundance. The thickness of the lines connecting metabolites is scaled relative to the correlation between the metabolites. The identity of each metabolite is denoted by number in the legend



Correlation network plot of goat rumen metabolites and their assigned clusters based on their known chemical classification. The size of each metabolite node represents metabolite relative abundance. The thickness of the lines connecting metabolites is scaled relative to the correlation between the metabolites. The identity of each metabolite is denoted by number in the legend



Correlation network plot of Sheep rumen metabolites and their assigned clusters based on their known chemical classification. The size of each metabolite node represents metabolite relative abundance. The thickness of the lines connecting metabolites is scaled relative to the correlation between the metabolites. The identity of each metabolite is denoted by number in the legend

Appendix 5

Table 4: Cattle Rumen Metabolite

Compound Name	CAS	Retention Time	Chemical family
1. 1-Hexyn-3-ol	000105-31-7		Alcohol
2. Junenol	000472-07-1.	20.2449	Alcohol
3. α-Benzenemethanol	048115-38-4	2.3272	Alcohol
4. Ethoxyamphetamine	135014-84-5	3.7607	Amine
5. Toluene	000108-88-3	5.3507	Aromatic Hydrocarbon
6. o-Cymene	000527-84-4	12.2268	Aromatic Hydrocarbon
7. 1-(1,1-dimethylethoxy)-2-methyl,Benzene	015359-98-5	2.6837	Aromatic Hydrocarbon
8. Octadecanoic acid	000057-11-4	25.889	Carboxylic acid
9. Carbon dioxide	000124-38-9	1.5657	Greenhouse gas
10. Dodecane	000112-40-3	14.5336	Hydrocarbon
11. Eicosane	000112-95-8	22.6865	Hydrocarbon
12. Tricyclene	000508-32-7	9.4942	Hydrocarbon
13. Hexadecane	000544-76-3	19.8193	Hydrocarbon
14. Methyl cyclohexene	000591-47-9	12.4507	Hydrocarbon
15. Cyclohexane, 1,2,3-trimethyl	001678-97-3	9.203	Hydrocarbon
16. 2-Nonene	006434-78-2	9.0239	Hydrocarbon
17. 2-Octene, 3,7-dimethyl	006874-32-4	11.1295	Hydrocarbon
18. 4-Octene, 2,6-dimethyl	006874-32-4	10.9279	Hydrocarbon
19. 3-Octene	007642-04-8	6.4034	Hydrocarbon
20. Neoclovene (II), dihydro	030824-81-8	19.2373	Hydrocarbon
21. 1,5-Heptadiene, 2,3,6-trimethyl-	033501-88-1	11.5102	Hydrocarbon
22. 3,3,5,5-Tetramethylcyclopentene	038667-10-6	12.0254	hydrocarbon
23. 3-Heptyne, 5-ethyl-5-methyl	061228-10-2	11.3756	Hydrocarbon
24. 2,6-Dimethyl-1,3,6-heptatriene	000928-67-6	14.108	hydrocarbon

25. 2,6-dimethyl-Undecane	017301-23-4	14.7351	hydrocarbon
26. Skatole	000083-34-1	17.3108	Indole
27. 3-methyl-Indole	000083-34-1	17.4453	Indole
28. 2-Undecanone	000112-12-9	16.4824	Ketone
29. Camphene	000079-92-5	10.6816	Monoterpene
30. α-Pinene	000080-56-8	10.3904	Monoterpene
31. 3-p-Menthene	000500-00-5	10.8381	Monoterpene
32. β-Pinene	000127-91-3	11.2863	Monoterpene
33. Camphor	000076-22-2	13.7721	Monoterpene
34. Limonene	000138-86-3	12.2718	Monoterpene
35. α-Cubebene	017699-14-8	17.3111	Monoterpene
36. p-Cresol	000106-44-5	13.1677	Phenolic
37. 4-ethyl,Phenol	000123-07-9	14.7578	Phenolic
38. 2-propyl,Phenol	000644-35-9	16.5669	Phenolic
39. Phenol, 3-propyl-	000621-27-2	16.5942	phenollic
40. Sibirene	014029-18-6	19.73	Sesquiterpene
41. Caryophyllene	000087-44-5	18.0949	Sesquiterpene
42. β-Selinene	000473-13-2	21.6111	Sesquiterpene
43. α-Selinene	000473-13-2	19.5282	Sesquiterpene
44. α-Copaene	003856-25-5	17.6695	sesquiterpene
45. Valencene	004630-07-3	18.6771	Sesquiterpene
46. β-Dihydro agarofuran	005956-09-2	19.1715	Sesquiterpene
47. β-Gurjunene	017334-55-3	17.5797	sesquiterpene
48. cis-Calamenene	072937-55-4	19.5508	Sesquiterpene
49. cis- β-Guaiene	000088-84-6	18.1395	sesquiterpene
50. Acetic acid	000064-19-7	4.0967	Volatile fatty acid
51. Propanoic acid	000079-09-4	6.3147	Volatile fatty acid
52. 2-methyl,Propanoic acid	000079-31-2	7.2382	Volatile fatty acid
53. Butanoic acid	000107-92-6	7.68	Volatile fatty acid
54. 2-methyl, Butanoic acid	000116-53-0	9.5839	Volatile fatty acid

Appendix 6

Table 5: Camel Rumen Metabolite

Compound	CAS	Retention Time	Chemical family
1. Junenol	000472-07-1.	20.8051	Alcohol
2. Terpinen-4-ol	000562-74-3	14.8027	Alcohol
3. 3-methyl,1-Hexanol	013231-81-7	8.0365	Alcohol
4. Urea	000057-13-6	3.2582	Amine
5. 1-Anthracenamine	000610-49-1	11.0398	Amine
6. Toluene	000108-88-3	6.1572	Aromatic Hydrocarbon
7. o-Cymene	000527-84-4	12.2045	Aromatic Hydrocarbon
8. 1-Benzopyran-2-one, 6-hydroxy-7-methoxy-4-methyl	006345-62-6	21.2978	Aromatic Hydrocarbon
9. Benzene, 1-(1,1-dimethylethoxy)-2-methyl	015359-98-5	12.7867	Aromatic Hydrocarbon
10. Naphthalene, decahydro-1,4a-dimethyl	001008-18-0	19.1027	Aromatic Hydrocarbon
11. p-Cymene	000099-87-6	12.227	Aromatic Hydrocarbon
12. Heptanoic acid	000111-14-8	16.7061	carboxylic acid
13. Hydrocinnamic acid	000501-52-0	15.3623	carboxylic acid
14. Cyclohexanecarboxylic acid	000098-89-5	17.5572	Carboxyllic acid
15. Pentanoic acid	000097-61-0	9.5168	Carboxyllic acid
16. Carbamic acid, benzyl ester	1000314-73-3	20.581	Ester
17. 3-Hydroxymandelic acid, ethyl ester	017066-67-0	14.2424	Ester
18. Dodecanoic acid, 2-hexen-1-yl ester	1000159-97-0	12.5626	Ester
19. Isoamyl benzyl ether	000122-73-6	6.0899	Ether
20. Ethyl allophanate	000626-36-8	1.9688	Ether

21. 1-Methyl-cyclohexyl propionate	091328-37-9	12.9882	Ether
22. Carbon dioxide	000124-38-9	1.5658	Greenhouse gas
23. 2-Octene, 3,7-dimethyl	006874-32-4	11.1295	Hydrocarbon
24. Neoclovene-(II), dihydro	030824-81-8	19.2373	Hydrocarbon
25. 2,4-Heptadiene, 2,6-dimethyl	004634-87-1	7.7476	Hydrocarbon
26. 4-Octene, 2,6-dimethyl	062960-77-4	9.9425	Hydrocarbon
27. 3,6-dimethyl-Decane	017312-53-7	12.7868	Hydrocarbon
28. 2- Octene	013389-42-9	7.3441	Hydrocarbon
29. Nonane	000111-84-2	9.6734	Hydrocarbon
30. 1,1'-Dianthrimide	000082-22-4	16.863	Imide
31. Skatole	000083-34-1	17.9382	Indole
32. Indole	000120-72-9	16.7511	Indole
33. Indole, 6-methyl	003420-02-8	18.2741	Indole
34. 1H-Indole, 3-methyl-	000083-34-1	17.8708	indole
35. 3-Octanone	000106-68-3	11.5326	Ketone
36. 6-methyl-5-Hepten-2-one	000110-93-0	11.5098	Ketone
37. Camphene	000079-92-5	10.7038	Monoterpene
38. α-Pinene	000080-56-8	10.3902	Monoterpene
39. Limonene	000138-86-3	12.8148	Monoterpene
40. γ-Carene	013466-78-9	12.4766	Monoterpene
41. β-Citronellene	10281-55-7	11.2381	Monoterpene
42. 1-p-Menthene	000500-00-5	11.7115	Monoterpene
43. γ-Terpinene	005392-40-5	12.7866	Monoterpene
44. β-Pinene	000127-91-3	11.2861	Monoterpene
45. 2-Mercapto-4-phenylthiazole	002103-88-0	11.0398	Others
46. Dibenzo carbazole	000207-84-1	13.7275	Others
47. Pyrrole, 1-methyl	000096-54-8	11.3082	Others
48. p-Cresol	000106-44-5	13.0779	Phenolic
49. Phenol, 3-methyl-	000108-39-4	15.4743	Phenollic
50. α-Copaene	003856-25-5	17.6693	Sesquiterpene
51. β-Guaiene	000088-84-6	19.7524	Sesquiterpene
52. Caryophyllene	000087-44-5	18.274	Sesquiterpene

53. α-Cubebene	017699-14-8	17.2888	Sesquiterpene
54. γ-Muurolene	024268-39-1	18.9684	Sesquiterpene
55. δ-Cadinene	000483-76-1	19.4613	Sesquiterpene
56. γ-Amorphene	006980-46-7	19.5282	Sesquiterpene
57. Cis-Calamenene	072937-55-4	19.5731	sesquiterpene
58. Cyclic octaatomic sulfur	010544-50-0	25.4188	Thiol
59. Dimethyl sulfide	000075-18-3	2.0134	thiol
60. Acetic acid	000064-19-7	5.3733	Volatile fatty acid
61. Propanoic acid	000079-09-4	7.5907	Volatile fatty acid
62. Butanoic acid, 3-methyl	000503-74-2	9.5168	Volatile fatty acid
63. Hexanoic acid	000142-62-1	10.1214	Volatile fatty acid
64. Butanoic acid	000107-92-6	8.9569	Volatile fatty acid
65. Isovaleric acid	000503-74-2	9.36	Volatile fatty acid
66. Butanoic acid-2-methyl	000116-53-0	9.1586	Volatile fatty acid

Appendix 7

Table 6: Goat Rumen Metabolite

Compound	**CAS**	**Retention Time**	**Chemical Family**
1. Junenol	000472-07-1.	20.7826	Alcohol
2. α-Benzenemethanol	006589-55-5	2.7528	Alcohol
3. Phenyl ethyl alcohol	000060-12-8	14.4856	Alcohol
4. anine ethylamide	001999-43-5	20.4015	Amide
5. Amphetamine	000300-62-9	18.2966	Amine
6. 2-Ethoxyamphetamine	135014-84-5	1.9687	Amine

7. Benzene, 1-2-methyl	000611-14-3	21.2975	Aromatic Hydrocarbon
8. o-Cymene	000527-84-4	12.2045	Aromatic Hydrocarbon
9. 1-Bromo-2-benzyloxybenzene	031575-75-4	12.2266	Aromatic Hydrocarbon
10. Toluene	000108-88-3	6.1964	Aromatic Hydrocarbon
11. Glutaric acid, dodecyl tetrahydrofurfuryl ester	1000359-66-8	12.988	Ester
12. Sulfurous acid, 2-ethylhexyl hexyl ester	1000309-20-2	13.0776	Ester
13. 3-Hydroxymandelic acid, ethyl ester	1000071-88-9	13.1896	Ester
14. Carbon dioxide	000124-38-9	1.5211	Greenhouse gas
15. Cyclopentene-3,5-dimethylene	000000-00-0	11.6667	Hydrocarbon
16. Nonane	000111-84-2	9.696	Hydrocarbon
17. Dodecane	000112-40-3	15.049	Hydrocarbon
18. Tricyclene	000508-32-7	9.5166	Hydrocarbon
19. Hexadecane	000544-76-3	19.7969	Hydrocarbon
20. Cyclohexene, 4-methyl	000591-47-9	11.7118	Hydrocarbon
21. 1,3-Cyclohexadiene	000592-57-4	11.6446	Hydrocarbon
22. Cyclopentanedione-3-methyl	000765-70-8	11.6666	Hydrocarbon

23. cis-2,6-Dimethyl-2,6-octadiene	002492-22-0	10.9276	Hydrocarbon
24. 2-Octene, 3,7-dimethyl	006874-32-4	11.1449	Hydrocarbon
25. 3-Octene,	007642-04-8	6.8513	Hydrocarbon
26. 4-Octene	014850-23-8	6.4033	Hydrocarbon
27. Undecane, 2,6-dimethyl	017301-23-4	6.4033	Hydrocarbon
28. Neoclovene, dihydro	030824-81-8	19.2372	Hydrocarbon
29. 3,7-Dimethyl-3-octyl methylphosphonofluoridate	0345260-82-4	22.8205	Hydrocarbon
30. Methyl cyclohexene	000591-47-9	12.4506	Hydrocarbon
31. 1,5,9-Undecatriene, 2,6,10-trimethyl	062951-96-6	17.49	Hydrocarbon
32. Dauca-5,8-diene	142928-08-3	19.5733	Hydrocarbon
33. Indole	000120-72-9	16.0117	Indole
34. Indole, 1-methyl-2-phenyl	003558-24-5	13.1003	indole
35. Skatole	000083-34-1	18.6324	indole
36. 3-Octanone	000106-68-3	11.5326	Ketone
37. Cyclohexanone	092368-82-6	19.9291	Ketone
38. Camphene	000079-92-5	10.0764	Monoterpene

39. α-Pinene	000080-56-8	10.3903	Monoterpene
40. Limonene	000138-86-3	11.7339	Monoterpene
41. Menthene	000500-00-5	11.1516	Monoterpene
42. δ-3Carene	000554-61-0	12.7417	Monoterpene
43. α-cis-Bergamotene	018252-46-5	17.8931	Monoterpene
44. β-Pinene	000127-91-3	10.7036	Monoterpene
45. Terpinene	000099-85-4	10.1268	Monoterpene
46. Citronellene	002436-90-0	10.6714	Monoterpene
47. p-Cresol	000106-44-5	13.7579	Phenolic
48. Phenol, 2-propyl-	000644-35-9	16.2136	Phenollic
49. Phenol, 3-propyl-	000621-27-2	16.348	Phenollic
50. Phenol, 2,4-dimethyl-	000105-67-9	15.17	phenollic
51. 2-propyl-Phenol	000644-35-9	15.4966	phenollic
52. Caryophyllene	000087-44-5	18.2966	Sesquiterpene
53. β-Guaiene	000088-84-6	18.3187	Sesquiterpene
54. β-Selinene	000473-13-2	19.5281	Sesquiterpene

55. α-Gurjunene	017334-55-3	18.453	Sesquiterpene
56. δ-Elemene	020307-84-0	17.1095	Sesquiterpene
57. γ-Cuprenene	029621-78-1	21.3199	Sesquiterpene
58. Cyclic octaatomic sulfur	010544-50-0	24.7242	Thiol
59. Mint sulfide	072445-42-2	21.611	Thiol
60. Acetic acid	000064-19-7	4.903	Volatile fatty acid
61. Propanoic acid	000079-09-4	6.7172	Volatile fatty acid
62. Valeric acid-3-methyl	000105-43-1	9.2929	Volatile fatty acid
63. Butanoic acid2-methyl	000116-53-0	9.472	Volatile fatty acid
64. Hexanoic acid	000142-62-1	9.3149	Volatile fatty acid

Appendix 8

Table 7: Sheep Rumen Metabolite

Compound	**CAS**	**Retention Time**	**Chemical family**
1. Carbon dioxide	000124-38-9	1.5435	Greenhouse gas
2. Acetic acid	000064-19-7	4.903	Volatile fatty acid
3. Propanoic acid	000079-09-4	6.986	Volatile fatty acid
4. 3-methyl-Butanoic acid	000503-74-2	8.7325	Volatile fatty acid
5. Butanoic acid	000107-92-6	8.621	Volatile fatty acid
6. Isovaleric acid	000503-74-2	9.4497	Volatile fatty acid

7. 2-methyl,Butanoic acid	000116-53-0	9.6512	Volatile fatty acid
8. 2-methyl-Propanoic acid	000079-31-2	7.7699	Volatile fatty acid
9. Junenol	000472-07-1.	20.2447	Alcohol
10. 1-Hexyn-3-ol	000105-31-7	11.9803	Alcohol
11. Terpinen-4-ol	000100-49-2	20.2671	Alcohol
12. Trifluoroacetyl-lavandulol	58461-27-1	20.8942	Alcohol
13. 2-Pyrimidinamine	000109-12-6	11.0175	Amine
14. Toluene	000108-88-3	6.1797	Aromatic Hydrocarbon
15. o-Cymene	000527-84-4	12.2045	Aromatic Hydrocarbon
16. Ethylbenzene	000100-41-4	15.0261	Aromatic Hydrocarbon
17. Pentanoic acid, 1,1-dimethylpropyl ester	023361-78-6	19.9983	Ester
18. Butyl butanoate	000109-21-7	17.0643	Ester
19. 2-Octene, 3,7-dimethyl	006874-32-4	11.1294	Hydrocarbon
20. 1,5-Heptadiene, 2,3,6-trimethyl	033501-88-1	11.1964	Hydrocarbon
21. Tricyclene	000508-32-7	9.494	Hydrocarbon
22. Eicosane	000112-95-8	22.6864	Hydrocarbon
23. Nonane	000111-84-2	9.6736	Hydrocarbon
24. 2,3-Dimethyl-1-hexene	16746-86-4	14.7352	Hydrocarbon
25. 4-Octene	014850-23-8	8.8449	Hydrocarbon
26. 4-methyl-Cyclohexene	000591-47-9	11.1292	Hydrocarbon
27. 2-Octene	013389-42-9	6.8511	Hydrocarbon
28. 1-Methylcyclohexa-2,4-diene	004313-57-9	11.1292	Hydrocarbon
29. Undecane	001120-21-4	11.3754	Hydrocarbon
30. 3-methyl-Cyclopentene	001120-62-3	12.0249	Hydrocarbon
31. Skatole	003856-25-5	17.2882	Indole
32. Camphene	000079-92-5	10.7039	Monoterpene
33. α-Pinene	000080-56-8	10.3905	Monoterpene

34. α- cis-Bergamotene	018252-46-5	10.6816	Monoterpene
35. δ-Carene	013466-78-9	19.3905	Monoterpene
36. Limonene	000138-86-3	11.7113	Monoterpene
37. α-Thujene	002867-05-2	9.2029	Monoterpene
38. Terpinolene	000586-62-9	9.5165	Monoterpene
39. Geranyl linalool	68931-30-6	10.3227	Monoterpene
40. p-Cresol	000106-44-5	13.1452	Phenolic
41. β-Pinene	000127-91-3	10.7035	Monoterpene
42. 3-propyl,Phenol	000621-27-2	16.3256	Phenolic
43. α-Copaene	003856-25-5	17.1538	sesquiterpene
44. Camphor	000076-22-2	13.7945	Sesquiterpene
45. β-cis-Guaiene	000088-84-6	20.1553	Sesquiterpene
46. Caryophyllene	000087-44-5	17.7812	Sesquiterpene
47. cis-Calamenene	072937-55-4	19.5732	Sesquiterpene
48. cis-Cadina-1(6),4-diene	020085-11-4	19.2144	Sesquiterpene
49. γ- Cuprenene	029621-78-1	19.304	Sesquiterpene
50. γ-Muurolene	024268-39-1	19.5508	Sesquiterpene
51. Amorpha-4,7(11)-diene	486998-53-2	20.4463	Sesquiterpene
52. t-Cadina-1,4-diene	246522-85-0	21.8349	Sesquiterpene
53. β-Cubebene	013744-15-5	18.9236	Sesquiterpene
54. γ- Patchoulene	000508-55-4	19.3492	Sesquiterpene
55. Selina-3,7(11)-diene	006813-21-4	18.7217	Sesquiterpene
56. Italicene	94535-52-1	20.0657	Sesquiterpene
57. Disulfide, dimethyl	000624-92-0	4.6788	Thiol
58. 1-Propene-1-thiol	000870-23-5	21.6109	Thiol
59. Phenol	000108-95-2	11.6222	phenollic
60. 3-Methoxybenzyl alcohol	006971-51-3	11.4652	alcohol
61. 2- Nonanone	000821-55-6	12.921	ketone
62. 2-Decanone	000693-54-9	13.9961	ketone
63. 2-Heptylfuran	003777-71-7	17.445	Others
64. Phenol, 3,5-dimethyl-	000108-68-9	15.0043	phenollic

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Article

A Comparative Investigation of Volatile Organic Compounds of Cattle Rumen Metabolites using HS-SPME and PoraPak-Q Odor Trapping Methods

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Abstract: In the rapidly increasing field of metabolomic research, fast and accurate trapping of volatile odor compounds from biological samples is critical. Here a comparative evaluation of HS-SPME and PoraPak-Q adsorbent odor trapping methods followed by GC-MS analysis were used to determine volatile compounds in cattle rumen. Compared to the PoraPak-Q adsorbent, the HS-SPME method was more effective in trapping diverse metabolites including low molecular weight and highly volatile compounds such as carbon dioxide, acetic and butyric acid. Additionally, using the HS-SPME method, shorter trapping times were achieved (30 minutes) whilst the PoraPak-Q adsorbent required longer time extending beyond 1 hour for effective volatile trapping. In the context of metabolomics analysis from biological samples, the two different methods vary in determination of chemo-diversities, qualitatively and abundance of shared odor, and time required to trap odors, which are critical in such studies.

Keywords: Gas chromatography-Mass spectrometry, Headspace Solid Phase Microextraction, PoraPak-Q adsorbent, Volatile odor compounds

Introduction

Cattle rumen houses some of the most diverse and complex microbial colonies [1] capable of breaking down fibrous plant materials. Hence both ruminants and humans are able to derive energy and food from the interactions of rumen microbiome and plants [2], popularly called fermentation. However, this process has been attributed to production of numerous metabolites including greenhouse gases such as CO_2 and CH_4 [3]. With the growing attention on such volatile organic compounds, little information exists on suitable techniques available for their extraction from biological matrices. Presently, many bioanalytical research like metabolomic studies prefer sample extraction procedures that demand relatively smaller sample volumes, rapid, high throughput, non-depletive, and easy to interphase with existing analytical instrumentation [4,5]. Thus, choosing which odor collection method to use is of particular importance.

In the recent years, different odor collection techniques like HS-SPME [6], HS-SPME Arrow [7], stir-bar sorptive (SBSE) extraction [8] dynamic

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headspace extraction [9], PoraPak-Q adsorbent extraction [10] followed by Gas Chromatography have been used to determine volatile odors from various matrices including food products and urine [11-13].

In the present study, we compared the performances of HS-SPME and PoraPak-Q adsorbent, both applied in the direct headspace extraction method using the same rumen sample. Here, we show that the two methods demonstrated both qualitative and quantitative differences in relation to chemodiversity, relative abundance and trapping duration required.

Materials and methods

Sample collection

Rumen fluid was collected from five randomly selected freshly slaughtered male boran cattle (*Bos indicus)* from choice meats abattoir located in Kahawa west, Nairobi County. A minimum of 1 litre of rumen fluid was collected in sterile airtight freeze-resistant glass jars. The samples were kept in a cooler ice box and transported to the chemistry research laboratories at the chemical ecology unit based at the International Center of Insect Physiology and Ecology (*icipe*), Duduville campus, Kasarani, Nairobi County where experimental studies were conducted. From the same cattle, rumen fluid was aliquoted into 200 ml each in two jars for odor trapping by the two methods.

Headspace-SPME (HS-SPME) trapping

Volatile rumen metabolites were trapped from 200 mL freshly obtained ruminal fluid samples in airtight 1L glass odor collection jars (Sigma Scientific, USA) by using the HS-SPME technique. A general purpose 65 µm PDMS/DVB (polydimethyl siloxane/divinylbezene, Supelco, Bellefonte, PA, USA, SPME fibers were used for this study (Fig. 1B). This type of SPME fibers are suitable for trapping of low molecular weight volatiles, amines including nitro-aromatic compounds. Additionally, more volatile polar analytes, like alcohols, are adsorbed more efficiently and released faster on this type of fiber. Prior to analysis, the HS-SPME fibers were conditioned by heating at 250°C (injector port temperature) for half an hour on a gas chromatography system GC-HP-7890B, Agilent Technologies, USA) and later introduced into the sample headspace for volatile metabolite adsorption (Fig. 1D). The volatiles were adsorbed on a 65 µm PDMS/DVB (polydimethyl siloxane/divinylbezene), stableflex 24Ga, manual holder SPME fiber (Fig. 1B-C, Supelco, Bellefonte, PA, USA). The extractions were carried out at 37°C for 5, 15, 30 minutes and 1 hour, after equilibration of the samples for 15 minutes at the same temperature, ensuring a homogeneous temperature for sample and headspace.

Headspace-PoraPak-Q adsorbent trapping

Prior to extraction, PoraPak™-Q 50-80 mesh 30 mg adsorbents (Fig. 1A, Sigma Scientific, VCT USA) were cleaned and conditioned with 1 mL of GC-MS-grade hexane, followed by same amount of dichloromethane (DCM). Volatiles were collected from 200 mL freshly obtained ruminal fluid sample in an airtight 1L glass odor collection jar (Sigma Scientific, USA) using a dynamic headspace volatile extraction technique comprised of a portable volatile collection pump, PoraPak™-Q adsorbent (Sigma Scientific, USA) [13,14]. The PoraPak-Q adsorbent was attached to a Teflon tube connected to a portable odor collection pump and placed onto the sample headspace (Fig. 1E). Clean air was pumped at the rate of 2.5 L/min on the sample to facilitate volatile equilibration at the headspace and a pull set at 2 L/min to draw volatiles through to the adsorbent for adsorption. The experiment was set to run for 5 minutes, 15 minutes, 30 minutes and 1 hour respectively each time using newly acquired clean PoraPak-Q adsorbent. Adsorbed volatiles were desorbed from the PoraPak-Q adsorbent by passing through 300 µl GC-MS-grade hexane and the eluate was collected into a 1.5 mL vial.

GC-MS analysis

HS-SPME adsorbed volatiles and PoraPak-Q adsorbed volatiles were analyzed by Gas chromatography GC-HP-7890A, Agilent technologies, USA) coupled with quadruple mass analyzer Mass spectrometry system (MS-

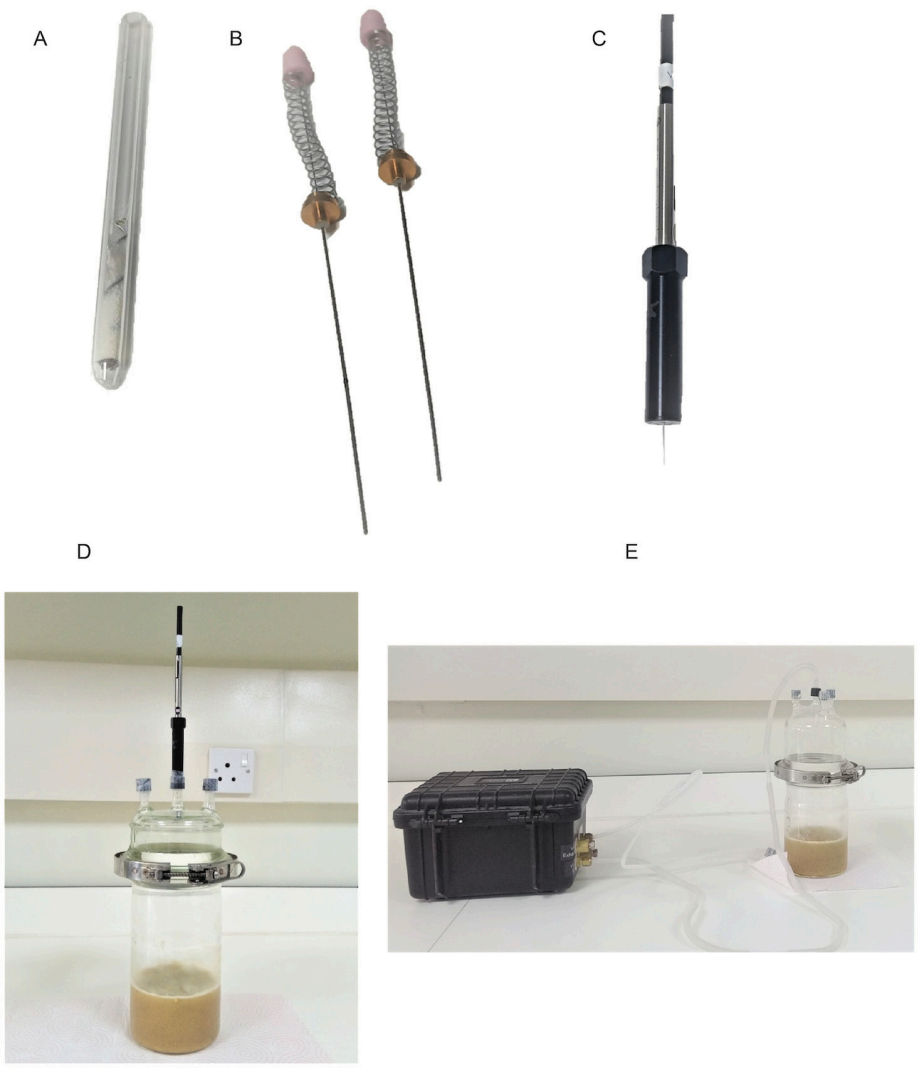

Figure 1. Photo showing odor extraction materials and methods used in this study: (A) PoraPak-Q adsorbent (B) SPME fibers (C) SPME holder (D) HS-SPME rumen odor collection setup and (E) Dynamic Head space with PoraPak-Q adsorbent set up

5975C, Agilent technologies, USA) with a slight variation in sample injection. Whereas HS-SPME adsorbed volatiles were introduced into the GC-MS instrument by manual injection of the SPME fiber into GC-MS inlet port, 1 µl of PoraPak-Q adsorbed volatile eluate were automatically injected to the GC-MS using an auto sampler (7683B series, Agilent Technologies, USA). Desorption of the HS-SPME volatiles were conducted at the injection port fitted with straight inlet liners without glass wool, 2 mm id (Agilent technologies, USA) at 250°C for 2 minutes in splitless mode [15]. Chromatographic separation of the volatiles was achieved by HP-5MSI, 30m X 0.25 mm i.d, 0.25 µm thick capillary column (J & W scientific, USA) immobilized with 5% (phenyl methyl silicone) as the stationary phase. Helium gas (99.99% purity, Air Products & Chemicals, USA, through local supplier Chemigas Ltd Kenya) was used as the carrier gas at a flow rate of 1.2 mL per minute. Initial oven temperature was programmed at 30°C where it was held for 5 minutes followed by a progressive increase at the rate of 10°C/min to 280°C where it was held at an isothermal state for 10.5 minutes. The mass spectrometry (MS) detector was operated in the

scan mode within a mass range of 16 to 550 *mz* at 1 scan s^{-1}, with electron energy of 70 eV. The MS ion source was set at 230°C while the mass quad set at 150°C. The total analysis time was 35 minutes.

Data processing

After chromatographic analysis, all the data were analyzed with the Agilent MSD productivity chemstation software for GC and GC/MS systems (Agilent technologies, USA). The integration was done with probability based matching algorithm, initial peak width set at 0.034 and initial threshold of 15.7. Individual compounds were identified by computer-aided comparison based on their retention times and respective mass spectral data against the MSD library (NIST, 2005, NIST 05a, and Adams MS HP, USA). The compounds were considered as correctly identified when their spectra showed a minimum probability match factor >80% [16,17]. Compounds that appeared in at least 3 out of 5 cattle rumen samples were considered as positively present in cattle rumen.

Statistical analyses

To establish the variations in the identified volatile organic compounds extracted by the two extraction techniques, chromatogram profiles were assessed based on the relative abundance of individual compounds and later analyzed using PAST statistical software Version 4.02. Additionally, the number of identified volatiles extracted by both HS-SPME and PoraPak-Q adsorbent including their mean values and standard error at different extraction durations were calculated from the five cattle rumen sample replicates using independent t-test of GraphPad Prism version 9.

Results and discussion

In the present study, two methods were used to provide an overview of the chemical diversity exhibited by cattle rumen. Observed GC-MS chromatograms (Fig. 2) shows significant variation in the type and relative abundance of volatile compounds extracted by both HS-SPME and PoraPak-Q adsorbent. The study reveals presence of diverse chemical entities harbored by cattle rumen. Greenhouse gases (carbon dioxide), volatile fatty acids (acetic & butyric), phenolic compounds (p-cresol), monoterpenes (limonene, camphene), and hydrocarbons (eicosane) which are metabolic products of ruminal fermentation induced by microorganism [18].

Generally, popular volatile odors like p-Cresol, limonene, camphene were the most abundant in both methods. Such volatiles which are mainly analogous to cattle, have been demonstrated to affect the host-vector interaction [13] by acting as chemical cues for host-seeking vectors [13,19]. Using the HS-SPME-GC/MS, we show the presence of CO_2, a major greenhouse gas [20] and a chemostimuli for stable flies, mosquitoes, and other vectors [21,22], but was not detected in PoraPak-Q adsorbent (Fig. 2 and supplementary Fig. S1 & Table S1). Furthermore, short chained volatile fatty acids like acetic and butyric acid which are essential in energy metabolism and protein synthesis in livestock [3] were determined exclusively by HS-SPME-GC/MS technique.

The number of extracted volatile compounds increased progressively with increased extraction time, thus more volatile compounds were extracted at 1 hour extraction period than 5 minutes for both HS-SPME and PoraPak-Q adsorbent (Fig. 3B). Moreover, there was notable variation in the number of extracted volatile compounds between the two techniques at different extraction durations (Fig. 3A&B). Whereas trapping time affect efficiency, absorptive affinity of odor compounds on the adsorbent, and subsequently the analysis performance [23], this study reveals that it might also affect the odor diversity. More volatile odors were determined as the extraction period was extended for both methods (Fig. 4).

The optimal trapping period for each method differed based on the compounds' competitive absorption on the adsorbents. For instance, at 30 minutes of extraction time, the SPME fiber captured most of the metabolites and extending this time further yields no qualitative significance. However, PoraPak-Q adsorbent demands extended trapping period running beyond 1 hour (Fig. 3 & 4). Considering the higher extraction

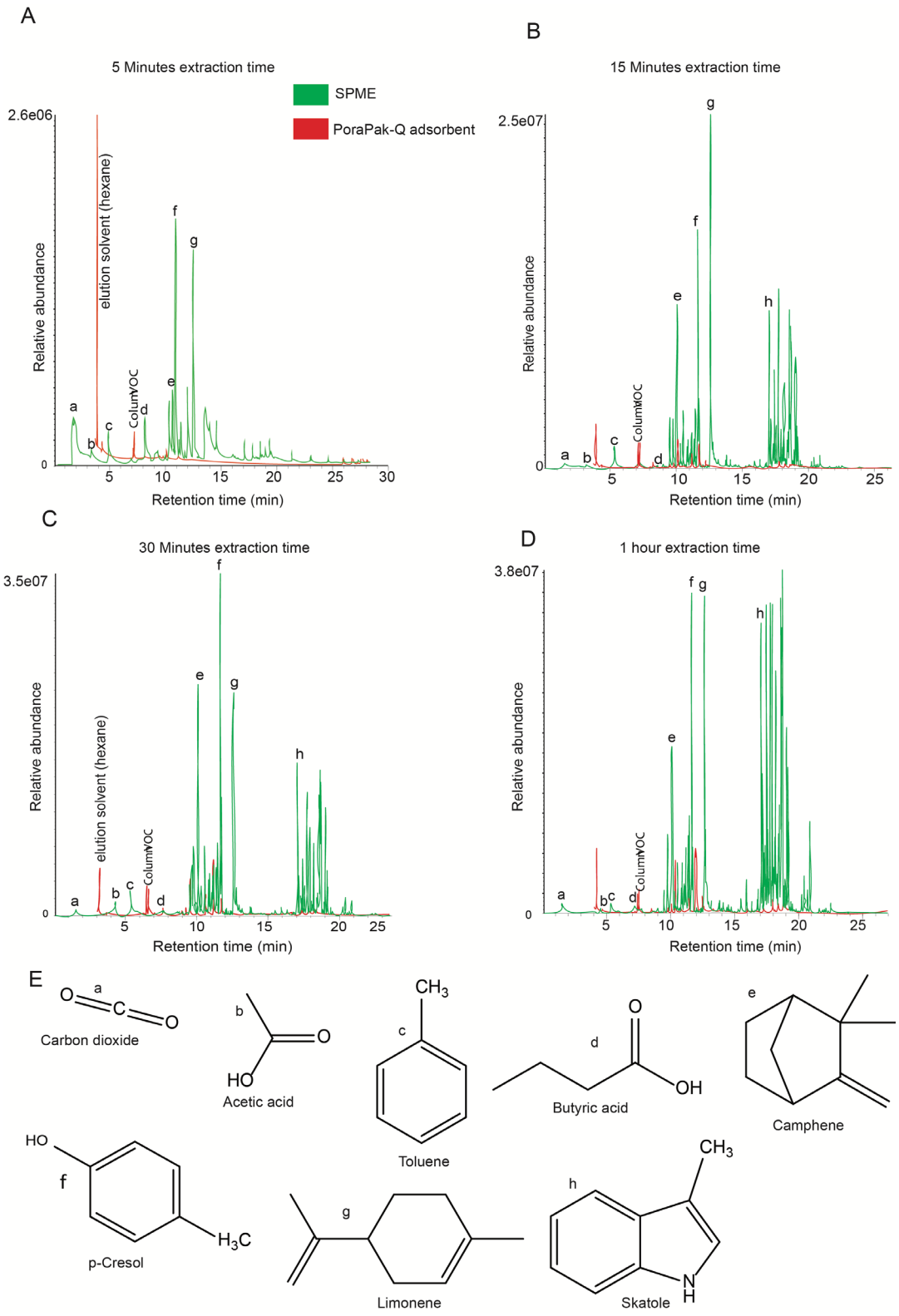

Figure 2. Representative GC-MS chromatogram of cattle rumen volatile odor profile trapped using HS-SPME and PoraPak-Q extracted volatiles from rumen fluid for 5 minutes (A), 15 minutes (B), 30 minutes (C) and 1hr (D) extraction times, (E) structure of some selected compounds

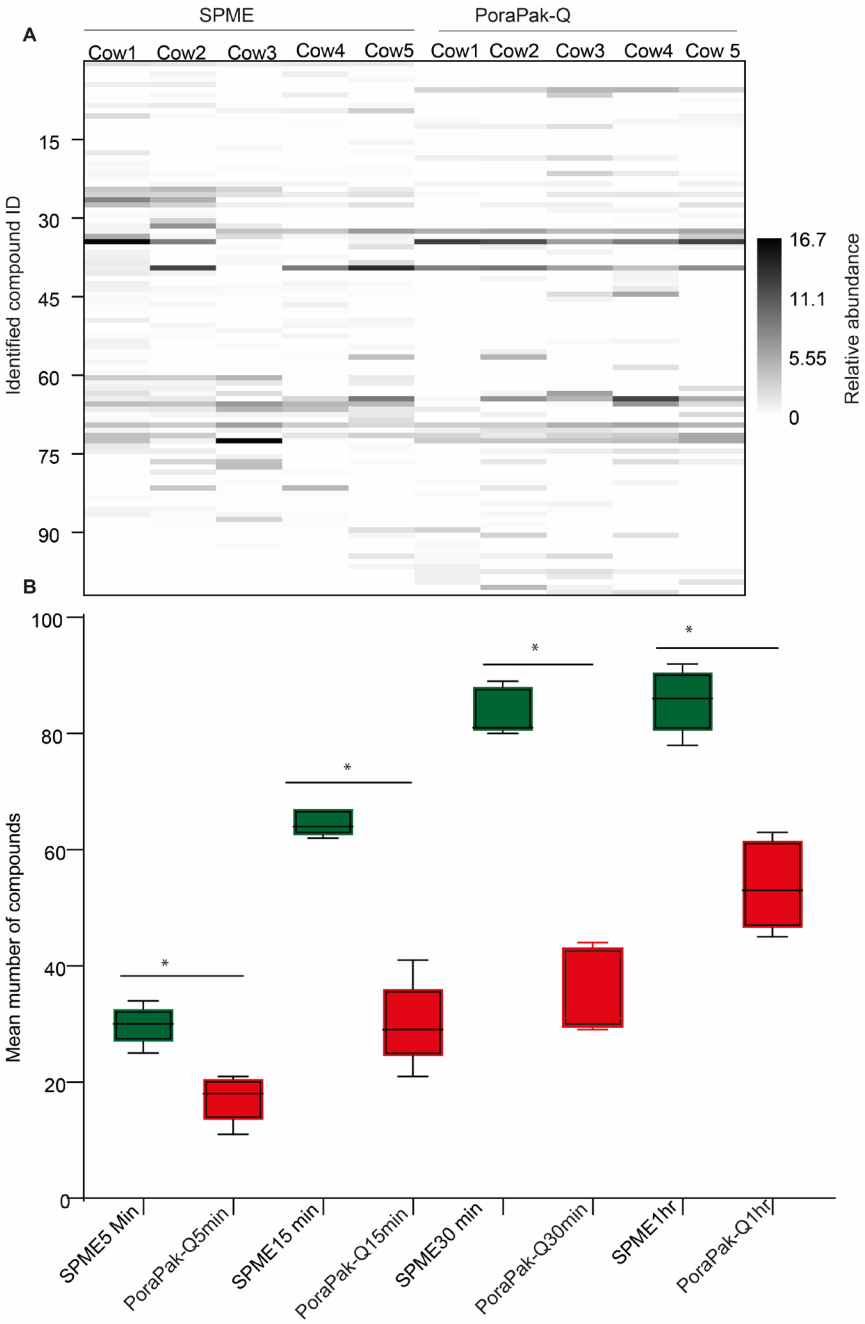

Figure 3. (A) Heat Map coded matrix showing relative percent contribution of individual volatiles to the total composition of analyzed compounds extracted by each method. (B) Box plot representation of mean number of identified compounds extracted by both HS-SPME and PoraPak-Q adsorbent at different time durations. *, $P < 0.001$ independent t-test, for 15 minute, Mann Whitney test, $P=0.008$, n=5

durations for most compounds, 30 minutes was the optimal trapping time duration for the direct insertion HS-SPME-GC/MS method and 15 minutes as the minimum time required for trapping odors from cattle rumen fluid. On the other hand, PoraPak-Q required at least 1 hour for significant odor trapping.

The simplicity and efficiency in quick sampling of HS-SPME method as demonstrated in the study qualifies the technique for investigation

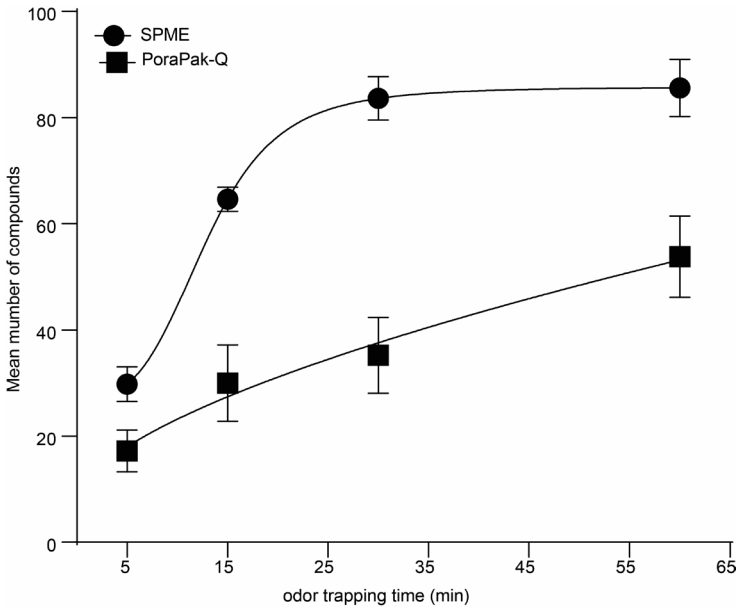

Figure 4. A nonlinear regression curve of the number of metabolites trapped as a function of odor collection time for the two methods

of odor, including highly volatile odors such as CO_2 profiles in biological matrices. Thus, it is well-suited for the study of biodegradation pathways of metabolites [5]. However, it demands extreme care especially when handling to avoid fiber breakage. Additionally, complete quantification of compounds is still a bottleneck when using HS-SPME technique, however it is possible to use this approach to correlate the relative abundances of compounds among samples when the same analytical procedure is used. The inability to elute and use the extract for other experiments, which can easily be done when PoraPak-Q used is the other drawback of using HS-SPME. Finally, the HS-SPME-GC/MS technique described here is a promising approach for bioanalytical and metabolomic studies which demand rapid, non-destructive, and efficient analysis methods.

Conclusions

Odors from host are emitted in a spatiotemporal dynamic in a small amount. HS-SPME success in trapping odors including greenhouse gases in a short period of time clearly shows their potential for spatiotemporal odor dynamics study and expand the range of bio-chemical research questions that is possible to address. However, a longer period of time is required for effective odor trapping when using PoraPak-Q adsorbent method.

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Supplementary data

Fig. S1 and Table S1 are given as supplementary information.

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