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Influence of accessions, metabolite ion features, storage and processing conditions on efficacy of Moringa plant extracts to inhibit enteric methane emission

By

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Declaration

I, **Addisu Endalew Zeru**, state that this thesis, which I hereby submit for the degree of PhD in Animal Science at the University of Pretoria, is my work, except where references have been made, and it has not been submitted by me for any other degree at any university or institution.

Signature_____

Addisu Endalew Zeru

Preface

The thesis has been completed under the supervision of Prof Abubeker Hassen in the Department of Animal Sciences at the University of Pretoria in South Africa. Prof Zeno Apostolides from the Department of Biochemistry, Genetics and Microbiology at the University of Pretoria and Dr Julius Tjelele from the Range and Forage Sciences at Agricultural Research Council (ARC) co-supervised the PhD research. The study was initiated with the general objective to improve our understanding for developing consistent Moringa plant leaf extract products that can be used effectively as an additive to mitigate enteric methane (CH_4) emissions from ruminants. The background information and need for the study are justified in the general introduction of this thesis. Detailed literature reviews are discussed in Chapter 1 and cover important aspects such as fermentation of feeds in the rumen; methanogenesis in the rumen; the influence of molar proportions of volatile fatty acids on CH_4 production; secondary plant metabolites (SPMs) in Moringa and their role in modulating rumen fermentation. The influence of growing environments, adaptability, leaf yield, disease and pest tolerance of Moringa on secondary plant metabolite (SPM) production and composition, which affect their bioactivities are also reviewed in this chapter. A statement of the problem, objectives and working hypotheses of the thesis are provided at the end of the literature review. The experiments that were conducted as part of the PhD research have been structured into four separate manuscripts organised as Chapters 2, 3, 4 and 5, which have been published or are ready for submission to peer-reviewed journals for publication:

1. Zeru, A. E.; Hassen, A.; Apostolides, Z.; Tjelele, J., 2022. Establishing relationships between agronomic traits of Moringa accessions and *in vitro* gas production characteristics of a test feed incubated with or without Moringa plant leaf extracts (Chapter 2). Published in *Plants*, 11(21):2901; https://doi.org/10.3390/plants11212901.
2. Zeru, A. E.; Hassen, A.; Apostolides, Z.; Tjelele, J., 2022. Screening of candidate bioactive secondary plant metabolite ion features from *Moringa oleifera* accessions associated with high and low enteric methane inhibition from ruminants (Chapter 3). Published in *Metabolites*, 12(6):501; https://doi.org/10.3390/metabo12060501.
3. Zeru, A. E.; Hassen, A.; Apostolides, Z.; Tjelele, J., 2022. Investigating the associative effects of binary cocktails of *Moringa oleifera* accession extracts mixed at various

proportions on *in vitro* rumen fermentation parameters (Chapter 4). To be submitted for publication in *Animal Feed Science and Technology*.

4. Zeru, A. E.; Hassen, A.; Apostolides, Z.; Tjelele, J., 2022. Evaluating the effect of postharvest processing and storage conditions on the efficacy of *Moringa oleifera* leaf extract to inhibit *in vitro* methane production (Chapter 5). To be submitted for publication in *Animal Feed Science and Technology*.

Chapter 2 investigated the relationships between agronomic traits of Moringa accessions, total phenolics, and total flavonoids and the *in vitro* gas production characteristics of a test feed incubated with or without the plant extracts of the accessions. This study identified the most effective and adaptable accessions in terms of CH_4 inhibition potential by using their agronomic traits. The third chapter screened the candidate bioactive SPM ion features associated with high and low enteric CH_4 inhibition characteristics of Moringa plant extracts by selecting four Moringa accessions studied in Chapter 2. In Chapter 4, the two best CH_4-inhibiting Moringa accessions that were identified in Chapters 2 and 3 were mixed in various proportions to test the effectiveness of binary cocktail extracts on *in vitro* CH_4 inhibition. In Chapter 5, the effects of postharvest processing conditions (i.e. drying methods, drying temperatures and extraction solvents) and storage environments (i.e. storage temperatures and storage light conditions) are evaluated for their effects on the efficacy of *M. oleifera* plant leaf extract to inhibit enteric CH_4 production from ruminants. General conclusions, recommendations and critical reviews of the thesis are synthesized in Chapter 6.

The write-up of the thesis was organized according to the authors' instructions in Animal Feed Science and Technology and the requirements of the University of Pretoria.

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Mr Demissew Teshome, a friend and colleague at the Forest and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, was by my side throughout. I truly appreciate Mrs Firehiwot Eshetu's efforts as a sister to conserve my happiness and health after I arrived in Pretoria and Ms Makida Mahri's support during the rumen fluid collection. Moral encouragement given by Dr Ashenafi Shibeshi and Mr Melese Reta during the challenges was also appreciated.

My wife, Habtam Mengistie, took full responsibility for our children during my absence. Hence, I am grateful to Habtam and my children Mahlet, Bisrat and Yeabisra for their tolerance, sacrifices and peace of mind throughout. I want to transfer many thanks to my family, including Mr Aderaw Belay, Mr Solomon Zeru, Mr Behailu Mamo and Mrs Zomanesh Belay, who are too many to list their names for their support in hosting me and keeping the family bonded.

Last, I would like to thank my God for empowering me with wisdom, courage, strength, knowledge and perseverance that allowed me to overcome the day-to-day challenges.

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ADL	Acid detergent lignin
AHI	Average intensity of the *m/z* ion features in higher methane inhibition
$AlCl_3$	Aluminium chloride
ALI	Average intensity of the *m/z* ion features in lower methane inhibition
ANOVA	Analysis of variance
ARC	Agricultural research council
C_2/C_3	Acetate to propionate ratio
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CE	Catechin equivalent
CH_4	Methane
ChAE	Chlorogenic acid equivalent
CO_2	Carbon dioxide
$CoCl_2.6H_2O$	Cobalt chloride hexahydrate
DE	Diosgenin equivalent
DM	Dry matter
$FeCl_3.6H_2O$	Ferric chloride hexahydrate
GAE	Gallic acid equivalent
GC	Gas chromatography
H_3PO_4	Orthophosphoric acid
IPCC	Intergovernmental panel on climate change
IVOMD	*In vitro* organic matter digestibility
OMD	Organic matter digestibility
$KC_2H_3O_2$	Potassium acetate
KH_2PO_4	Potassium phosphate monobasic
m.a.s.l	Meters above sea level
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MIF	Metabolite ion feature
MIFs	Metabolite ion features

$MnCl_2.4H_2O$	Manganese (II) chloride tetrahydrate
Na_2HPO_4	Sodium hydrogen phosphate dibasic
$Na_2S.9H_2O$	Sodium sulphide nonahydrate
$NaHCO_3$	Sodium bicarbonate
NDF	Neutral detergent fibre
NH_4HCO_3	Ammonium bicarbonate
PC	Principal component
PCA	Principal component analysis
ppm	Parts per million
psi	Pounds-force per square inch unit
QE	Quercetin equivalent
RCBD	Randomized complete block design
RE	Rutin equivalent
SAS	Statistical analysis software
SEM	Standard error of the mean
SPM	Secondary plant metabolite
SPMs	Secondary plant metabolites
TAE	Tannic acid equivalent
TGP	Total gas production
TVFA	Total volatile fatty acids
UPLC-MS	Ultra-performance liquid chromatography-mass spectrometry

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ABSTRACT

Developing healthier and more practically applicable intervention strategies for methane (CH_4) inhibition emitted from ruminants has been a priority issue globally. Among the strategies, the use of natural medicinal plants containing bioactive secondary plant metabolites (SPMs) is increasing owing to the cost-effectiveness, environmental suitability and safety value of producing healthy organic animal products of the interventions. Since multiple complex factors affect their bioactive SPMs and bioactivities, the study of wider influencing factors of the extract activities may probably increase the efficacy and sustainability of the final products during application. Thus, this study was initiated i) to investigate the relationships of agronomic traits and SPMs of Moringa accessions with *in vitro* gas production characteristics from ruminants, ii) to identify potential bioactive SPMs ion features responsible for high and low CH_4 inhibition from ruminants, iii) to investigate the effectiveness of varying proportions of binary Moringa accession extract cocktails on *in vitro* CH_4 inhibition, and iv) to evaluate the effects of postharvest processing and storage conditions on the efficacy of Moringa extracts to inhibit enteric CH_4 production from ruminants. For the study, twelve Moringa accessions were raised at the University of Pretoria and transplanted in the field at the Roodeplaat experimental site of the ARC in Pretoria, South Africa. The leaf samples from individual plants from these accessions were harvested in the fifth month of transplanting to the field. Then, a series of studies were conducted to develop Moringa leaf extract products that were effective and consistent when used as additives for mitigating enteric CH_4 emission in ruminants.

In the study that investigated the relationships of agronomic traits of Moringa accessions with *in vitro* gas production characteristics, most of the agronomic traits, total flavonoids and total phenolics varied among these accessions of *M. oleifera*. All accessions reduced the total gas (TGP) and CH_4 volume compared with the control, incubated without Moringa plant extracts. Hence, among the twelve accessions, those designated A3 (7633), A8 (7717) and A11 (Pretoria) exhibited superior *in vitro* antimethanogenesis and organic matter digestibility (OMD) with equivalent or superior performances in adaptability in the field. The subsequent study was aimed at identifying potential bioactive secondary plant metabolite ion features responsible for high and low CH_4 inhibition from ruminants. In this study, the *m/z* ion features (MIFs) 4.44_609.1462 and 4.53_433.1112 were linked with higher CH_4 inhibition, whereas the MIF 9.06_443.2317 and MIF 15.00_487.2319 were linked with lower CH_4 inhibition. The secondary MIFs associated with higher CH_4 inhibition can be considered potential secondary MIFs markers for the standardization of plant extracts and commercialization of Moringa varieties that provide extracts effective in antimethanogenic activity from ruminants. In another study, the effectiveness of varying proportions of binary Moringa accession extract cocktails of the two highest CH_4-inhibiting accessions was investigated for their effect on *in vitro* fermentation parameters and CH_4 inhibition. The cocktails were prepared by mixing the selected accessions extracts after freeze-drying, milling, and extracting with methanol as 100:0, 80:20, 60:40, 50:50, 40:60, 20:80, and 0:100 proportions (A3:A11) and applied to anaerobically incubated *E. curvula* hay with 50 mg extract per kg substrate. Thus, the application of Moringa accession extracts in the form of cocktails provided better inhibition of CH_4 with comparable or greater *in vitro* organic matter digestibility (IVOMD) of the substrate feed compared with those values recorded for single Moringa accessions. Thus, a cocktail of $A3_{50}A11_{50}$ showed favourable associative effects on propionate, CH_4 inhibition, total volatile fatty acids (TVFA), CH_4/TVFA and acetate to propionate ratio (C_2/C_3), whereas $A3_{60}A11_{40}$ exhibited associative effects on TVFA, acetate, and CH_4/TVFA. Thus, the binary combination of the two selected *M. oleifera* accession extracts at 50% mix exhibited higher benefits of antimethanogenesis and propionate production with a decrease in C_2/C_3 ratio than when were used the two Moringa accessions in a single form or other cocktails were mixed in various proportions. In the last study, the effects of various postharvest processing and storage conditions were evaluated for their effect on the efficacy of *M. oleifera* extract to inhibit CH_4 production from ruminants. The studied postharvest factors

include drying methods (sun, freeze, oven, and shade), drying temperatures (25, 45, 60, and 80 °C), extraction solvents (absolute methanol, 70% methanol, 70% ethanol, and 70% acetone), storage temperatures (4, 15, 25, and 35 °C) and storage light conditions (light and dark). The TGP, CH_4 inhibition, total phenolics and total flavonoids differed significantly among the drying methods, drying temperatures and extraction solvents. The freeze- and sun-drying methods compared with shade- and oven-drying methods, drying temperatures of 25 °C and 45 °C compared with 60 °C and 80 °C, and 70% acetone among the extraction solvents recorded higher total phenolics and total flavonoids with better antimethanogenic activities. Storage temperatures of 4 °C and 15 °C also maintained higher total phenolics, total flavonoids and IVOMD with lower CH_4/TGP and CH_4/IVOMD than those recorded at 25 °C and 35 °C of storage. Dark storage showed lower TGP and CH_4 production with better total phenolics, total flavonoids and IVOMD than light storage. Hence, among the postharvest factors, freeze-drying methods, drying temperature of 45 °C, 70% acetone, the storage temperature of 4 °C and dark storage conditions were preferable processing and storage conditions to maintain higher antimethanogenesis, total phenolics and total flavonoids with equal or improved IVOMD from the Moringa plant extract products. The variation recorded among these Moringa accessions when grown in the same environment in terms of SPMs, agronomic performances, and different *in vitro* fermentation parameters generally indicate the presence of wider genetic sources among Moringa varieties. These could in turn show that superior accessions can be further improved to develop commercial varieties suitable as a source of plant extract products that can be used for mitigating enteric CH_4 production. The total phenolics, total flavonoids and the subsequent antimethanogenic activities are influenced crucially by the various postharvest treatments of Moringa leaf/powder. This infers the need for the establishment of appropriate postharvest processing methods, storage conditions and time for a specific purpose and application to utilize optimally Moringa as a dietary additive effectively throughout the year to inhibit CH_4 emission from ruminants. However, further investigations are needed on the relationship of agronomic traits with gas production characteristics using long-term adaptability performance at different agro-ecologies, stages of plant growth, plant density, season of harvesting and parts of the plant. The identified MIFs as potential candidates for higher and lower CH_4 inhibition of Moringa accessions needed to be studied for their pathways and mode of action. The different organic solvents with various extraction aqueous levels and each storage temperature for longer storage

periods at various time points to fix a cut-off storage time for each storage temperature need also be conducted in the future.

Keywords: agronomic traits; acetate to propionate ratio; drying methods; drying temperatures; extraction solvents; *in vitro* organic matter digestibility; storage light; total flavonoids; total gas; phenolics

GENERAL INTRODUCTION

Ruminant animals make a substantial contribution to food security in the world. However, their emissions of carbon dioxide (CO_2), CH_4 and nitrous oxide (N_2O) are a global concern (Gerber *et al.*, 2010; Liu *et al.*, 2021). The Food and Agriculture Organization of the United Nations revealed that ruminant animals (i.e. buffalo, cattle, sheep, goats, etc.) contributed to 11.1% to 19.6% of anthropogenic greenhouse gases (GHGs) (Blaustein-Rejto & Gambino, 2023). However, the estimates are widely varied among reports due to the variations in the source of emission included, the years used for the estimate, global warming potential values used for CH_4 and NO_2 estimation and method of analysis. Thus, ruminants appear to be a higher emitter of CH_4 to the atmosphere than any other single source of anthropogenic CH_4 (Black *et al.*, 2021). Methane has twenty-three times higher global warming potential than CO_2 and has approximately twelve years of net average atmospheric lifetime (Scholtz *et al.*, 2020). A portion of the gross energy intake of ruminant animals will also be lost as a result of CH_4 production during fermentation and this affects their productivity negatively (Hristov *et al.*, 2013). Hence, the Intergovernmental Panel on Climate Change (IPCC) has frequently cautioned about the urgency of CH_4 mitigation (Legg, 2021) and the European Commission (EC) launched the global CH_4 pledge to decrease its emission by 30% in the coming ten years period (EC, 2021).

Several studies reported intervention strategies to decrease CH_4 production in ruminant animals (Chapman *et al.*, 2017; Medjekal *et al.*, 2017; Neubauer *et al.*, 2018). However, most of them are not effective or might not be acceptable to producers and consumers owing to their negative effects on application, safety and product quality. As a result, most researchers have shown increasing interest in the use of additives based on natural medicinal plant products to provide healthy organic animal products for human consumption (Jayanegara *et al.*, 2020; Durmic *et al.*, 2021; Harahap *et al.*, 2022). This growing interest in medicinal plants is attributed to their richness in bioactive SPMs, which have effective antimicrobial action for efficient feed fermentation and inhibition of CH_4 emission in the rumen with minimal or no adverse effect on animal products (Calabrò *et al.*, 2012; Lin *et al.*, 2018). Previous studies showed that a substantial decrease in CH_4 production (up to 50%) was achieved from ruminants with SPMs such as tannins and saponins (Patra & Saxena, 2010; Goel & Makkar, 2012). Tannins commonly have binding properties for microbial enzymes and proteins, which may impede enteric

protozoa and related methanogen activities (Liu *et al.*, 2011), whereas saponins directly inhibit the bioactivities of rumen protozoa and associated symbiotic methanogens (Goel & Makkar, 2012). The addition of other SPMs such as myricetin, rutin, flavone, naringin, quercetin, and kaempferol flavonoids also inhibited CH_4 in the range of 5–9 ml/g substrate feed (Oskoueian *et al.*, 2013). Similarly, the inclusion of *p*-coumaric, caffeic, catechin hydrate and trans-cinnamic acids resulted in the inhibition of CH_4 without an adverse effect on volatile fatty acids (VFA) and TGP (Giuburunca *et al.*, 2014).

Moringa oleifera is one of the promising medicinal plants that have many biomedical applications because it contains substantial amounts of SPMs such as phenolics, flavonoids, tannins, saponins, alkaloids, glucosinolates, essential oils, phytates and oxalates (Sudha *et al.*, 2020; Bhalla *et al.*, 2021; Fidrianny *et al.*, 2021; Teclegeorgish *et al.*, 2021; Kashyap *et al.*, 2022). However, numerous factors affect the bioactive components of plants and their subsequent biological activities during application for various purposes. Genes are the most basic internal element, which strictly regulates the biosynthesis and accumulation of bioactive SPMs (Li *et al.*, 2020; Szepesi, 2021). Hence, the variation in total phenolics, total flavonoids and various phenolic compound concentrations observed between and within species of *M. stenopetala, M. oleifera, and M. peregrina* (Egypt) were attributed to plant genetics (Abo El-Fadl *et al.*, 2020). However, the regulatory mechanisms of the SPMs production pathways are susceptible to environmental factors such as infestation by disease and insect pests, defoliation by herbivorous animals, exposure of the plants to environmental stresses such as drought, extreme temperatures, flooding, salinity, and chemical toxicity (Li *et al.*, 2020; Szepesi, 2021).

Leaves of Moringa are perishable and they need to be dried or pulverized or extracted to maintain the composition and concentration of beneficial bioactive SPMs within a reasonable period for easier management and longer shelf life. Previous research showed that the efficacies of the processed products are highly reliant on the drying method, drying temperature, extraction solvent and storage environment (Potisate *et al.*, 2015; Ademiluyi *et al.*, 2018; Gatahi & Nyoro, 2021). Moringa leaves dried under 50% shade net and room temperature exhibited superior total polyphenols and terpenoids contents compared to those obtained in shade-dried and oven-dried Moringa, whereas the oven-dried leaves recorded the highest total glucosinolates concentration (Gatahi & Nyoro, 2021). In contrast, the shade-dried leaves were

contaminated by toxic microbes such as *Escherichia coli*, staphylococcus, moulds, and yeast (Gatahi & Nyoro, 2021). *Moringa oleifera* also showed the highest antioxidant activity when extracted in 50% methanol extraction solvent with better enzymatic inhibition in 100% ethyl acetate and phenolic profiling in 100% methanol solvents (Rocchetti *et al.*, 2020). Compared with acetone, chloroform and ethanol extraction solvents, the aqueous-extracted leaves of *M. oleifera* maintained the highest contents of flavonoids, whereas methanol extract recorded the highest phenolics (Gull *et al.*, 2016). Furthermore, the dried Moringa leaf powder and extract need to be stored to supply these products for a longer period. However, the composition, concentration and bioactivities of most of the bioactive compounds are highly sensitive to storage conditions (i.e. storage light, humidity and storage temperatures) (Vongsak *et al.*, 2013a; Zhang *et al.*, 2017). Moringa powder stored at 15 $^{\circ}$C retained higher antioxidant activity and total phenolics with lower changes in green colour after two months of storage than the powder stored at 35 $^{\circ}$C (Potisate *et al.*, 2015). Different bioactive compounds at various concentration levels are associated with the multiple benefits of *M. oleifera* (including enteric CH_4 inhibition) and thus there is a need to determine optimal growing, extraction and storage conditions of the final product to obtain consistent results.

Therefore, this PhD thesis research was conducted with the aim to improve our understanding by establishing the main factors influencing the efficacy of plant extracts to develop Moringa plant extract products that could be commercialized as a dietary additive to reduce enteric CH_4 emissions in ruminant animals. Hence, various subsequent specific studies were conducted to evaluate how the Moringa accessions and their MIFs, associative effects of mixtures of *Moringa* accession extracts, and various postharvest processing and storage conditions affect the efficacy of the final Moringa extract product to inhibit enteric CH_4 emission from ruminant animals.

CHAPTER 1

Review of literature

1.1 Introduction

A large amount of CH_4 emitted from ruminant animals affects the environment and productivity of animals adversely (Liu *et al.*, 2021; Khanal *et al.*, 2022). This higher emission can be associated with the anaerobic fermentation with diverse microbes found in the rumen (Black *et al.*, 2021). Thus, several CH_4 mitigation interventions were established in various studies. However, the use of natural plant products abundant in SPMs has been preferable to produce healthy organic animal products (Jayanegara *et al.*, 2020; Durmic *et al.*, 2021; Harahap *et al.*, 2022). However, the synthesis and storage of SPMs and subsequent bioactivities of natural plant-based mitigation strategies are highly dependent on plant genotypes, growing environments and the exposure of plants to other biotic factors (i.e. herbivores, pests, microorganisms, invaders, grazers, and parasites) (Zhu *et al.*, 2014; Srivastava *et al.*, 2018; Li *et al.*, 2020; Mishra *et al.*, 2020; Szepesi, 2021). Postharvest processing and storage management conditions are also likely to affect the concentrations and bioactivities of phytochemicals in Moringa plant extracts (Ademiluyi *et al.*, 2018; Gąsecka *et al.*, 2020). But little is known about how the variations in plant genetics, growing environments, and postharvest management conditions can influence the efficacy of Moringa plant extracts to inhibit CH_4 emissions. Hence, reviewing the various works in this chapter aimed at providing a detailed understanding of the ranges of influencing factors with their limitations that can affect the development of consistent Moringa leaf extract products.

1.2 Fermentation of feeds in the rumen

Ruminant animals have enormous advantages in the use of poor-quality roughage feeds, unfit for human consumption, through anaerobic enteric microbial fermentation (Black *et al.*, 2021). Rumen microbes primarily include bacteria, methanogens, protozoa, and fungi with estimated population counts of 10^{10}, 10^9, 10^6, and 10^3–10^7 cells/ml, respectively (Galmessa *et al.*, 2019; Newbold & Ramos-Morales, 2020). These microbes degrade non-structural carbohydrates (sugars such as glucose, fructose, and sucrose), structural carbohydrates (cellulose, pectin,

fructan and hemicellulose), proteins, and non-protein nitrogen components to VFA, ammonia and other fermentation by-products (CO_2 and CH_4). The VFAs are directly absorbed through rumen epithelium and satisfies the major energy demand of the animal while ammonia will eventually be assembled into microbial protein to give higher nutrient-dense ingredients (i.e. amino acids) to the host animal because of a symbiotic relationship of rumen microbes with the host animals (Belanche *et al.*, 2021; Soltan & Patra, 2021; Leahy *et al.*, 2022). The rumen provides environments conducive to the microbes and required substrates, whereas the microbes supply the nutrients (mainly VFAs and microbial protein) to generate energy and amino acids for the host animals (Russell & Rychlik, 2001). Short-chain VFAs are largely absorbed across the rumen wall, which covers more than 70% of the energy demand and major carbon requirements of ruminant animals (Janssen, 2010; Patra, 2013; Soltan & Patra, 2021). The microbial degradation of dietary proteins, non-protein ammonia-N, amino acids and oligopeptides provide the microbial protein for the host. Thus, the ingested plant proteins and these microbial proteins are used as a source of dietary amino acids for ruminant animals.

Along with the microbial protein and main VFAs produced during feed fermentation in the rumen, formate, ethanol, lactate, succinate, water and different gases (CO_2, NH_3, CH_4, and H_2) are released to various extents in the process (Moss *et al.*, 2000; Soltan & Patra, 2021). The formations of these products have their advantages and disadvantages in the fermentation process. Mainly, the productions of CO_2 and CH_4 are ineffective for the host animals and affect the environment negatively (Soltan & Patra, 2021). Thus, approximately from 5% to 7% of dietary GE intake and 16 to 26 g/kg of dietary DM intake of the animals in the form of CH_4 (Hristov *et al.*, 2013) and a substantial amount of protein as ammonia-N (Galmessa *et al.*, 2019; Fouts *et al.*, 2022) have been lost from ruminant animals. Other studies reported a loss of 8–14% of digestible energy intake or 2–12% of gross energy, which is equivalent to average emissions of 28 L or 20 g of CH_4 per kg dry matter (DM) feed intake (Okine *et al.*, 2004). However, the types and amounts of end products varied depending on the microbial population, types of feed (concentrates vs roughages), level of dietary fat, animal-related factors (type, body weight, level of production, growth rate, level of feed intake and rumen pH) and environmental conditions (Moss *et al.*, 2000; Patra, 2013; Getabalew *et al.*, 2019; Zhongming *et al.*, 2019).

1.3 Methanogenesis in the rumen

Enteric methane is the largest source of anthropogenic GHG emissions from ruminant production, which accounts more than 90% anthropogenic methane emission in the sector (Opio *et al.*, 2013). Thus, CH_4 formation in the rumen, methanogenesis, is an intrinsic process of feed fermentation with specialized obligate anaerobes called methanogens. These methanogens are a subgroup of the archaea, but not true bacteria. They are essential to microbial anaerobiosis and the normal functioning of the rumen ecosystem through interspecies H_2 transfer with bacteria, fungi and protozoa (Patra *et al.*, 2017). Using the principle of H_2 consumption and production in the rumen, the nature of rumen microbes was classified into three groups (Bodas *et al.*, 2012). Thus, the first group completely fermented the feed components to propionate, butyrate, ethanol, and lactate without being subject to the disposal of the H_2 and CH_4 production. The second group produces acetate and H_2 as the main products. However, their fermentation efficiency depends on the third group of bacteria, namely methanogenic microbes (i.e. H_2 users), which play a major role in enteric CH_4 production.

Methane is produced via various pathways of anaerobic fermentation. However, the H_2-utilizing pathway is the main precursor to methanogenesis (Figure 1.1). Volatile fatty acids and microbial proteins are products of rumen fermentation, as are significant amounts of reducing cofactors, such as flavin adenine dinucleotide (FADH), nicotinamide adenine dinucleotide phosphate hydrogen (NADP), nicotinamide adenine dinucleotide (NADH) (Knapp *et al.*, 2014). Thus, re-oxidation of these cofactors releases a substantial amount of H_2, although its accumulation in the rumen thermodynamically has a negative feedback on the process and will inhibit further re-oxidation of the cofactors, growth of microbes, synthesis of microbial protein, and overall extent of carbohydrate fermentation (Leahy *et al.*, 2022). The H_2-utilizing methanogens, free-living or endosymbionts inside protozoa use H_2 as a principal source of energy and prevent its accumulation in the rumen (Soltan & Patra, 2021; Kelly *et al.*, 2022; Leahy *et al.*, 2022). Thus, methanogenesis from H_2 and CO_2 is the simplest reaction to minimize the pressure owing to the accumulation of H_2 in the rumen and helps to keep the normal enteric feed fermentation process (Leahy *et al.*, 2022). However, its production affects the productivity of animals negatively and is increasingly a concern for the public owing to its environmental impacts. The degradation of dietary protein and assimilation into microbial protein, synthesis of lipids and bio-

hydrogenation of the fatty acids may also produce or utilize the reducing equivalents. However, the net volume might be too small (Knapp *et al.*, 2014).

Some methanogens also produce CH_4 in the rumen using other intermediate substrates, such as acetate, formate, methanol, methylamine, and other alcohols, obtained from feed fermentation (Morgavi *et al.*, 2010). Hydrogenotrophic methanogens use formate as a source of energy, reoxidize and utilize its electron to reduce CO_2 to CH_4 (Kelly *et al.*, 2022). However, formate is not easily detected in the rumen because of its nature, which has been rapidly metabolized and changed to CH_4. Hence, numerous rumen microbes have formate dehydrogenase genes to utilize H_2, which is more suitable for interspecies electron transfer at lower H_2 pressure. The formate fermentation pathway generally accounts for 18% of enteric CH_4 emitted from ruminants (Leahy *et al.*, 2022). During anaerobic enteric fermentation, acetic acid can also be used as an intermediate substrate for CH_4 production. The acetic acid-producing microbes use one of the products released in the fermentation pathway and thus are among the H_2-producing groups. Thus, CH_4 can also be produced from acetate to a lesser extent through the acetoclastic pathway or two-step syntrophic acetate oxidation and the hydrogenotrophic pathway (Fotidis *et al.*, 2013). But the low growth rate of acetate-utilizing methanogen, the limited importance of acetoclastic methanogenesis in the rumen, and the lower affinity of acetogens to H_2 assist in easy flushing of the available acetate before methanogenesis (Thauer *et al.*, 2008; Morgavi *et al.*, 2010). Furthermore, methylamine, methanol and other alcohols also contributed to a lesser amount to the total enteric CH_4 emitted from ruminant animals (Morgavi *et al.*, 2010). Figure 1.1 shows the major anaerobic H_2-producing and -utilizing fermentation pathways for a better understanding of the methanogenesis process in the rumen.

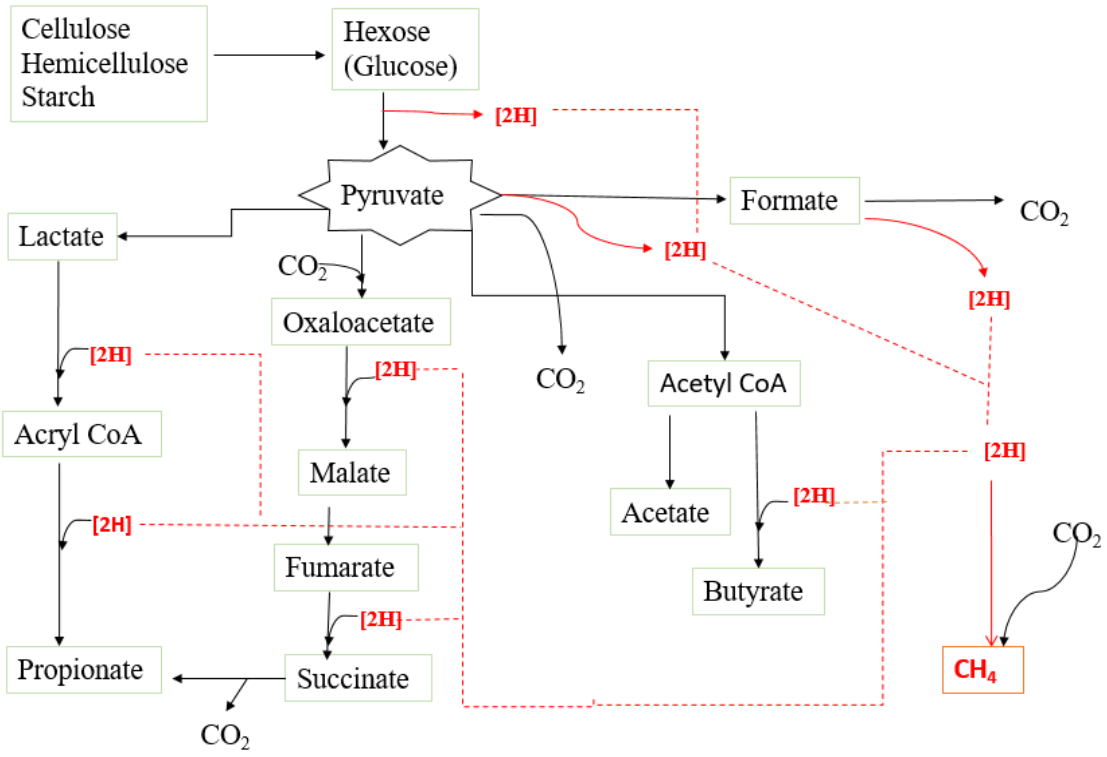


Figure 1.1 Carbohydrate fermentation in the rumen and methanogenesis via hydrogen transfer

Source: Mitsumori & Sun (2008)

1.4 Effect of volatile fatty acid molar proportions on methanogenesis

In a complex process of enteric fermentation, the types and amounts of VFAs made a substantial contribution to the amount and type of end products, including CH_4 volume. Along with the major VFAs (acetic, butyric and propionic acids); valeric, isovaleric, isobutyric, and 2-methyl butyric acids are also produced in the rumen fermentation to smaller extents (Ghimire, 2015; Wang *et al.*, 2020). Acetate production would supply more H_2 (twice) than butyrate, whereas the process of propionate production consumes the H_2. Hence, establishing relationships between CH_4 production with the proportions of VFAs plays a substantial role in the feeding interventions of ruminants to inhibit enteric CH_4 emission (Mirzaei-Aghsaghali & Maheri-Sis, 2011). Suppressing the H_2-producing reactions inhibits CH_4 production. However, microbial growth and subsequent carbohydrate fermentation might be affected negatively by this approach for CH_4 inhibition (Kelly *et al.*, 2022; Leahy *et al.*, 2022). Succinate and lactate (acrylate) fermentation pathways of propionate production from dicarboxylic acid, fumarate, malate or

Acryl-CoA are used as alternative sinks for hydrogen (Leahy *et al.*, 2022). However, large precursors of propionate are required and are expensive for animals. Thus, diverting the H_2 into propionate production via fumarate, malate or lactate might be an important feeding strategy to decrease the flow of H_2 to CH_4 production (Mitsumori & Sun, 2008). A mechanism that shifts the pattern of enteric fermentation from acetate to propionate decreases the available H_2 (Moss *et al.*, 2000; Kingston-Smith *et al.*, 2010). The amount of CH_4 volume produced per glucose fermented from possible fermentation pathways of VFAs can vary from 0 moles to 1 mole (Figure 1.2). Thus, there is no CH_4 production per glucose fermentation from the molar proportion of 1.33 propionate + 0.66 acetates, whereas 1 mole of CH_4 can be produced from the molar proportion of 2 acetates + 4 H_2 per mole of glucose fermented in the pathway. Among the VFAs produced, the propionate-favouring pathways are generally more competitive for hydrogen use and reduce CH_4 emission, whereas the acetate and butyrate-promoting pathways release the H_2 and increase enteric CH_4 production from enteric fermentation in ruminants.

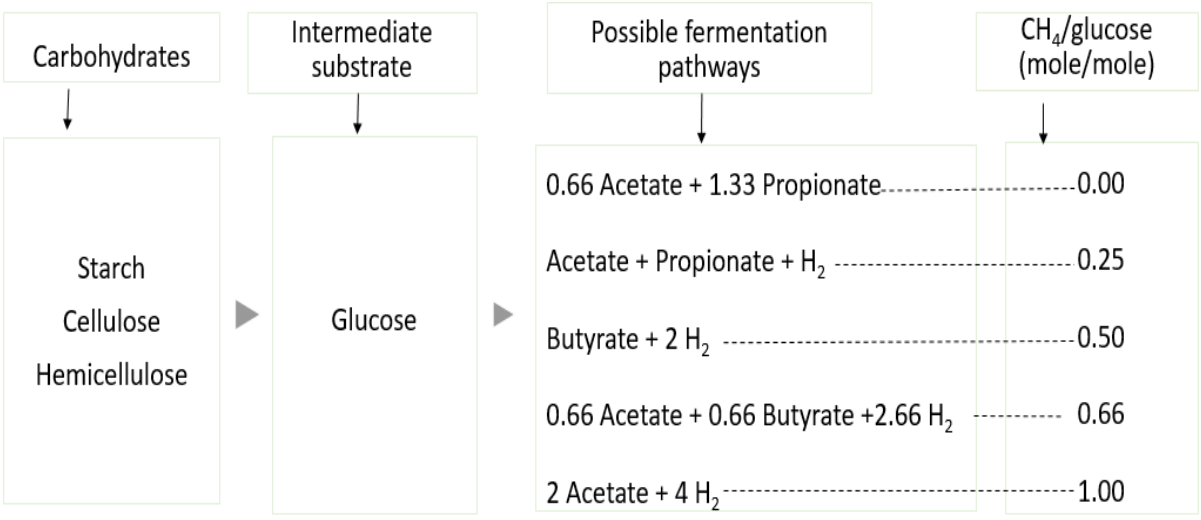


Figure 1.2 Carbohydrate fermentation and possible pathways of methane production via glucose, volatile fatty acids and hydrogen (CO_2, H_2O, and H^+ are not included in the pathways for simplicity)

Source: Janssen (2010)

1.5 Secondary plant metabolites in Moringa

Plants produce numerous low molecular weight organic compounds. Functionally, they can produce primary metabolites (involved in plant development, growth and reproduction), hormones (regulate the metabolic processes) and SPMs (mediate the interaction of the plant with their growing environments) (Erb & Kliebenstein, 2020). Hence, hundreds of thousands of SPMs are synthesized from the main glycolysis or shikimic acid pathway as potential regulators of plant defence in response to the stress conditions created by abiotic and biotic factors of the plant growing environments (Li *et al.*, 2020). These SPMs are the basis of many commercial herbal medicines and pharmaceutical drugs produced from medicinal plants. Hence, *M. oleifera* leaves are abundant sources of bioactive SPMs (i.e. tannins, saponins, phenolics, flavonoids, alkaloids, glucosinolates, phytates, and oxalate (Sudha *et al.*, 2020; Bhalla *et al.*, 2021; Fidrianny *et al.*, 2021).

1.5.1 Phenolic compounds

'Phenolic compounds are SPMs that are mostly present as derivatives of hydroxycinnamic acid (free-phenolics) and hydroxybenzoic acid (bound-phenolics), which have one or more hydroxyl groups connected to the aromatic ring, and can be found in plants as esters or glycosides' (Rahmanian *et al.*, 2014; Kashyap *et al.*, 2022). These compounds include simple phenols, lignins, tannins, coumarins, lignans, flavonoids and phenolic acids (Soto-Vaca *et al.*, 2012; Rahmanian *et al.*, 2014; Tsimogiannis & Oreopoulou, 2019). Phenolic compounds are abundant in Moringa while wide variations exist among studies in terms of composition and concentration within the same or different species. *Moringa oleifera* leaves grown in different countries showed different total phenolic contents of 29.3–53.5 mg CAE/g (Vongsak *et al.*, 2013b), 46.9–68.8 mg GAE/g (Ademiluyi *et al.*, 2018) and 1.1–2.1 mg GAE/g (Omede, 2016) (Table 1.1). Three Moringa species (*M. stenopetala*, *M. peregrina*, and *M. oleifera*) that grew in Egypt also exhibited total phenolic contents of 43.2–96.5, 53.4–116.6 and 48.7–108.7 mg GAE/g, respectively (Abo El-Fadl *et al.*, 2020). Hence, the variation reported among the studies for Moringa owing to the differences in the growing environment (Leone *et al.*, 2015a), stage of leaf maturity (Nouman *et al.*, 2016; Du Toit *et al.*, 2020), drying

temperatures (Park *et al.*, 2021), drying methods (Ademiluyi *et al.*, 2018), extraction methods (Rocchetti *et al.*, 2018) and genotypes (Abo El-Fadl *et al.*, 2020).

Table 1.1 Total phenolic contents reported in leaves of Moringa

Drying methods	Extraction methods	Value as dry weight	Country	Reference/s
Air-dried	Hexane	48.7 mg GAE/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Ethyl acetate	58.7 mg GAE/g		
	70% ethanol	108.7 mg GAE/g		
Air	Maceration (70% methanol) + air evaporation	97.0 mg GAE/g	Southern Tunisia	(Bennour *et al.*, 2019)
	Maceration (70% methanol) + rotary evaporation	80.9 mg GAE/g		
	Maceration (70% methanol) + oven evaporation	136.4 mg GAE/g		
Oven	Methanol using homogenizer	71.1–76.6 mg GAE/g	Mexico	(Castillo-Lopez *et al.*, 2017)
Air	Soxhlet with 80% methanol (crude extract)	1.6 mg GAE/g	Nigeria	(Omede, 2016)
	Crude extract + H_2O	1.1 mg GAE/g		
	Crude extract + ethyl acetate (3 x 100 ml)	2.1 mg GAE/g		
Air	80% methanol, stirred for 2 hours	28.1 mg GAE/g	Chad	(Leone *et al.*, 2015a)
		35.5 mg GAE/g	Algeria	
		25.5 mg GAE/g	Haiti	
Oven	Maceration, 70% ethanol	53.5 mg CAE/g	Thailand	(Vongsak *et al.*, 2013b)
	Maceration, 50% ethanol	29.3 mg CAE/g		
	Percolation, 70% ethanol	37.1 mg CAE/g		
	Percolation, 50% ethanol	32.8 mg CAE/g		
Freeze	Distilled water	68.8 mg GAE/g	Nigeria	(Ademiluyi *et al.*, 2018)
Air		59.4 mg GAE/g		
Sun		50.0 mg GAE/g		
Oven		46.9 mg GAE/g		

CAE: chlorogenic acid equivalent; GAE: gallic acid equivalent; NA: not available; TAE: tannic acid equivalent

1.5.2 Flavonoid compounds

'Flavonoid compounds are the major class of phenolics that comprise 15-carbon atoms with two aromatic rings connected by three-carbon bridges' (Bodas *et al.*, 2012). Flavonoids are amply found in vegetables, fruits and beverages, and are usually connected to a sugar moiety as glycosides or in free forms (Makita *et al.*, 2016). The responses to microbial infections of plants trigger the synthesis and accumulation of flavonoid compounds, which attract pollinator insects, regulate cell growth, and generally act against abiotic and biotic stresses (Dias *et al.*, 2021). In some studies, *M. oleifera* leaves exhibited plentiful total flavonoid content of 12.3–25.1 mg IQE/g (Vongsak *et al.*, 2013b), 25.0–62.5 mg/g (Ademiluyi *et al.*, 2018) and 31.2–62.8 mg QE/g (Abo El-Fadl *et al.*, 2020).

Major flavonoid compounds (Table 1.2) obtained in leaves of Moringa include quercetin, rutin, quercitrin, catechin, epicatechin, luteolin, apigenin, naringenin, Isorhamnetin, naringin, kaempferol, and myricetin in various concentrations (Rocchetti *et al.*, 2018; Bennour *et al.*, 2019; Abo El-Fadl *et al.*, 2020; Rocchetti *et al.*, 2020; Kashyap *et al.*, 2022). Moringa grown in Nigeria showed abundant amounts of rutin (91.1 mg/g), kaempferol (43.9 mg/g), epicatechin (43.4 mg/g), quercetin (17.8 mg/g) and catechin (6.1 mg/g) (Ademiluyi *et al.*, 2018). *Moringa oleifera* grown in Southern Tunisia also exhibited rutin (2.7–6.2 mg/g) and quercitrin (1.8–3.8 mg/g) as the principal flavonoid constituents as well as other flavonoids detected in smaller concentrations of less than 0.04 mg/g dried extract (Bennour *et al.*, 2019). In a comparison study of *M. stenopetala, M. oleifera* and *M. peregrina,* hesperidin (3.9 mg/g) was found to be the principal flavonoid in leaves of *M. oleifera*. In addition, 3-OH tyrosol (1.4 mg/g), rutin (0.34 mg/g), quercetin (0.07 mg/g), naringin (0.05 mg/g), rosmarinic (0.05 mg/g) and other flavonoids in smaller concentrations ranging from 3 to 11 µg/g dried extract were recorded in *M. oleifera* (Abo El-Fadl *et al.*, 2020).

In terms of their bioactivity, different sources of flavonoid compounds showed some promising antimethanogenic activity (Ebrahimi *et al.*, 2011; Zmora *et al.*, 2012; Oskoueian *et al.*, 2013). However, their antifungal, antiviral, antibacterial and antiprotozoal activities may generally depend on the structures of the flavonoids and have not been fully investigated for the benefits of using flavonoids for antimethanogenic activity.

Table 1.2 Total flavonoids, flavonoid metabolites and phenolic acids reported in leaves of Moringa

Bioactive compound	Drying method	Extraction method	Value as dry weight	Country	Reference
Total flavonoids	Air	Maceration (70% MeOH) + Air evaporation	31.7 mg QE/g	Southern Tunisia	(Bennour *et al.*, 2019)
		Maceration (70% MeOH) + Rotary evaporator	44.2 mg QE/g		
		Maceration (70% MeOH) + Oven evaporation	42.0 mg QE/g		
	Oven	MeOH using homogenizer	55.7-60.3 mg QE/g	Mexico	(Castillo-Lopez *et al.*, 2017)
	Air	Hexane	62.8 mg QE/g	Egypt	(Abo El-Fadl *et al.*, 2020)
		Ethyl acetate	51.0 mg QE/g		
		70% EtOH	31.2 mg QE/g		
	Air	Soxhlet, 80% MeOH (crude extract)	920.0 µg QE/g	Nigeria	(Omede, 2016)
		Crude extract + H_2O	660 µg QE/g		
		Crude extract + ethyl acetate (3 x 100 ml)	130.0 µg QE/g		
	Air	Maceration, 96% EtOH	10.5 mg QE/g	NA	(Fachriyah *et al.*, 2020)
	Oven	Maceration, 70% EtOH	25.1 mg IQE/g	Thailand	(Vongsak *et al.*, 2013b)
		Maceration, 50% EtOH	12.3 mg IQE/g		
		Percolation, 70% EtOH	18.0 mg IQE/g		
		Percolation, 50% EtOH	14.6 mg IQE/g		
	Different	Distilled water	25.0–62.5 mg QE/g	Nigeria	(Ademiluyi *et al.*, 2018)
Catechin	Air	MeOH	40.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Different	Distilled water	6.0–30.0 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
Epicatechin	Different	Distilled water	18.6–43.4 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
Luteolin	Air	Maceration, 70% MeOH	1.9–4.3 µg/g	Southern Tunisia	(Bennour *et al.*, 2019)

Table 1.2 *Cont.*

Bioactive compound	Drying method	Extraction method	Value as dry weight	Country	Reference/s
Apigenin	Air	MeOH	3.9 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
Naringenin	Air	MeOH	260 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Air	Maceration, 70% MeOH	0.7–1.6 µg/g	Southern Tunisia	(Bennour *et al.*, 2019)
Kaempferol	Different	Distilled water	9.6–43.9 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
	Air	MeOH	5.6 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Air	Maceration, 70% MeOH	0.3–2.3 µg/g	Southern Tunisia	(Bennour *et al.*, 2019)
Quercetin	Different	Distilled water	17.8–62.2 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
	Air	Maceration, 70% MeOH	1.8–3.8 mg/g	Southern Tunisia	(Bennour *et al.*, 2019)
	Air	MeOH	660.0 mg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Air	Maceration, 70% MeOH	8.6–10.8 µg/g	Southern Tunisia	(Bennour *et al.*, 2019)
Rutin	Air	MeOH	340.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Air	Maceration, 70% MeOH	2.7–6.2 mg/g	Southern Tunisia	(Bennour *et al.*, 2019)
	Different	Distilled water	70.2–91.1 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
Hesperidin	Air	MeOH	3.9 mg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
Rosmarinic			50.0 µg /g		
Hispertin			10.0 µg /g		
Phenolic acids					
Gallic acid	Air	Maceration, 70% MeOH	5.8–10.7 µg/g	Southern Tunisia	(Bennour *et al.*, 2019)
	Oven	MeOH using homogenizer	1.24 mg/g	Mexico	(Castillo-Lopez *et al.*, 2017)
	Air	MeOH	0.01 mg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Different	Distilled water	41.1–60.1 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
Chlorogenic acid	Oven	MeOH using homogenizer	590.0 µg/g	Mexico	(Castillo-Lopez *et al.*, 2017)
	Air	MeOH	320.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Different	Distilled water	62.4–79.5 mg/g	Nigeria	

Table 1.2 *Cont.*

Bioactive compound	Drying method	Extraction method	Value as dry weight	Country	Reference/s
Caffeic acid	Air	MeOH	5.9 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Oven	MeOH using homogenizer	503.0 µg/g	Mexico	Castillo-Lopez *et al.*, 2017)
	Different	Distilled water	58.7–78.9 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
Ellagic acid	Air	MeOH	32.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Different	Distilled water	5.9–31.0 mg/g	Nigeria	(Ademiluyi *et al.*, 2018)
Ferulic acid	Oven	MeOH using homogenizer	536.0 µg/g	Mexico	(Castillo-Lopez *et al.*, 2017)
	Air	MeOH	15.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
	Air	NaOH + HCl + ethyl acetate + Na_2SO_4	66.0 µg/g	Chad	(Leone *et al.*, 2015a)
			89.0 µg/g	Algeria	
			97.0 µg/g	Haiti	
trans-ferulic acid	Air	Maceration, 70% MeOH	3.7–11.9 µg/g	Southern Tunisia	(Bennour *et al.*, 2019)
Isoferulic acid	Air	MeOH	9.3 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
Salicylic acid	Air	MeOH	76.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
			3.0 µg/g	Haiti	
		NaOH + HCl + ethyl acetate + Na_2SO_4	1.0 µg/g	Chad	(Leone *et al.*, 2015a)
			2.0 µg/g	Algeria	
Coumaric acid	Oven	MeOH using homogenizer	1.1 mg/g	Mexico	(Castillo-Lopez *et al.*, 2017)
p-coumaric acid	Air	MeOH	12.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
Alpha-coumaric acid	Air	MeOH	7.5 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
O-caffeoylquinic acid	Air	Maceration, 70% MeOH	7.7–13.7 mg/g	Southern Tunisia	(Bennour *et al.*, 2019)
Quinic acid	Air	Maceration, 70% MeOH	1.8–3.3 mg/g	Southern Tunisia	(Bennour *et al.*, 2019)
P-OH benzoic	Air	MeOH	690.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)
Benzoic	Air	MeOH	184.0 µg/g	Egypt	(Abo El-Fadl *et al.*, 2020)

EtOH: ethanol; GAE: gallic acid equivalent; IQE: isoquercetin equivalent; MeOH: methanol; NA: not available; QE: quercetin equivalent; RE: rutin equivalent

1.5.3 Phenolic acids

These compounds are a subclass of plant polyphenolic metabolites that contain one carboxylic acid group and commonly exist in bound form as glycosides, esters, and amides. However, they may be found infrequently in free forms (Kumar & Goel, 2019). They are derived from hydroxycinnamic acid (i.e. *p*-coumaric, caffeic, chlorogenic, ferulic, and sinapic acids) or hydroxybenzoic acids (i.e. *p*-hydroxybenzoic, vanillic, syringic and protocatechuic acids) (Kumar & Goel, 2019). Most of the studies presented in Table 1.2 reported numerous phenolic acids with different concentrations in *M. oleifera* leaves and their glycoside derivatives. The Moringa grown in Nigeria contains 79.5 mg/g chlorogenic acid, 78.9 mg/g caffeic acid, 43.2 mg/g gallic acid and 5.9 mg/g ellagic acid (Ademiluyi *et al.*, 2018). The *M. oleifera* grown in Mexico recorded 1.2 mg/g gallic acid, 1.1 mg/g coumaric acid, 0.6 mg/g chlorogenic acid, 0.5 mg/g ferulic acid and 0.5 mg/g caffeic acid contents (Castillo-Lopez *et al.*, 2017). Leaves of *M. oleifera* grown in Egypt also showed 3.2 mg/g of chlorogenic acid, 0.3 mg/g of *E*-vanillic acid, 0.2 mg/g of benzoic acid, 0.1 mg/g of salicylic acid, and 0.1 mg/g of P-OH benzoic acid with poorly detectable amounts of other phenolic acids (Abo El-Fadl *et al.*, 2020). However, the three comparative species (*M. stenopetala*, *M. oleifera* and *M. peregrina*) did not maintain similar concentrations of these phenolic acids, which indicates variability among Moringa species. Superior 4-*O*-caffeoylquinic acid (7.7–13.7 mg/g) and quinic acid (1.8–3.3 mg/g) were recorded in Moringa grown in Southern Tunisia compared with poorly detected phenolic acids (less than 4 mg/100 g) (Bennour *et al.*, 2019).

Regarding their biological functions, phenolic acids, such as caffeic, *p*-coumaric, catechin hydrate, and trans-cinnamic from different sources exhibited CH_4 inhibition without any substantial effect on the VFAs, pH and total gas produced in the rumen (Giuburunca *et al.*, 2014). Similarly, several metabolites having functional groups of phenolic acids exhibited strong antimicrobial activities (Leone *et al.*, 2015b; Haque, 2018). However, the antimethanogenic activity of many essential specific phenolic acids is poorly understood yet.

1.5.4 Alkaloids and glucosinolates

Alkaloids and glucosinolates are among the bioactive compounds abundantly available in *M. oleifera* (Leone *et al.*, 2015b; Fachriyah *et al.*, 2020; Hossain *et al.*, 2020; Kashyap *et*

al., 2022). Alkaloids are naturally occurring organic compounds that contain hydrogen, carbon and nitrogen as main elements (Babbar, 2015). However, they may also have sulfur, oxygen and, more infrequently, phosphorus, bromine and chlorine. Alkaloids can be produced from polyketide and terpenoid pathways, purine and histidine pathways and shikimate pathways (Gutiérrez-Grijalva *et al.*, 2020). Plant alkaloids have essential bioactivities in traditional medicines as antitussives, sedatives and purgatives and in pharmacology for the preparation of modern drugs to treat cancer (Alasvand *et al.*, 2019), diabetes (Ma *et al.*, 2019) and Neurodegenerative illnesses (Hussain *et al.*, 2018). A study conducted in Nigeria revealed alkaloid contents of 12.8, 13.4, 5.0 and 10.6 mg/g in the freeze-dried, air-dried, sun-dried and oven-dried leaves of *M. oleifera*, respectively (Ademiluyi *et al.*, 2018).

Glucosinolates are synthesized from amino acids and are largely abundant in *M. oleifera*. Some include benzyl, 4-hydroxy benzyl (2.4 mg/g), 4-(α-L-rhamnopyranosyloxy) benzyl (33.9–59.4 mg/g), 4-O-(α Lacetylrhamnopyranosyloxy) benzyl isomer 1 (2.8–5.2 mg/g); isomer 2 (1.2–1.5 mg/g) and isomer 3 (17.4–50.2 mg/g) (Amaglo *et al.*, 2010). However, alkaloids and glucosinolates are commonly distrusted in their application for animal nutrition and antimethanogenic activities or are not clearly understood yet.

1.5.5 Other secondary plant metabolites

Tannins are water-soluble phenolic SPMs that precipitate alkaloids and proteins, which usually comprise hydrolysable (mixtures of esterified simple phenol) and condensed tannin (condensed flavonoids) (Altemimi *et al.*, 2017). They have generally lower palatability (voluntary uptake) and poor digestibility and are considered anti-nutritional factors in animal nutrition. However, their antimethanogenic activities have increased the attention of researchers (Bodas *et al.*, 2012; Akhtar *et al.*, 2022). Tannins are found abundantly in the leaves of *Moringa oleifera*. Thus, studies revealed total tannin content of 6.8–10.2 mg GAE/100 g (Hossain *et al.*, 2020), 12.0–20.6 mg/g (Teixeira *et al.*, 2014; Akhtar *et al.*, 2022), 5-6 mg GAE/100 g (Ademiluyi *et al.*, 2018), and 1.3–1.7 mg/g (Omede, 2016). Studies also comprehensively showed the presence of strong antimethanogenic activities of tannins in ruminants and up to 50% enteric CH_4 inhibition (Patra & Saxena, 2010; Goel & Makkar, 2012).

With a wide range of heterogeneity, saponins are found largely in plants, including Moringa (Yang *et al.*, 2021). They are a subclass of terpenoids and, hence, are also known as triterpene glycosides. Several studies showed total saponin content of 3.5 mg/g (Arwani *et al.*, 2019), 7.9 mg/g (Indriasari *et al.*, 2019) and 12.0–20.6 mg/g (Akhtar *et al.*, 2022) in leaves of Moringa. Ademiluyi *et al.* (2018) revealed saponin content of 16.4 mg/g in freeze-dried, 16.4 mg/g in the air-dried, 10.9 mg/g in sun-dried, and 7.3 mg/g in oven-dried leaves of *M. oleifera*. Saponins have the potential to interact with the phospholipids and cholesterol components of the cell membrane owing to their hydrophilic and lipophilic nature (Lorent *et al.*, 2014). Biologically, they have cytotoxic, hypolipidemic, and anti-diabetic effects, which have played important roles in the development of folk medicines and modern drugs (Elekofehinti *et al.*, 2021). Thus, they have increased the attention of animal nutritionists and exhibited up to 50% reduction of CH_4 production from ruminants, which might be attributed to their antimicrobial activities on protozoa and associated symbiotic methanogens (Bodas *et al.*, 2012). However, they are known as an anti-nutritional factor in animal feeds (Akhtar *et al.*, 2022).

Furthermore, oxalate contents of 4.5–10.5 mg/g (Pallavi & Dipika, 2010; Teixeira *et al.*, 2014) and 6.7–10.0 mg/g (Ademiluyi *et al.*, 2018), and phytates contents of 58.5–89.8 mg/g (Ademiluyi *et al.*, 2018) were found in leaves of *M. oleifera.* These large amounts of phytates and oxalates are considered anti-nutritional factors (Akhtar *et al.*, 2022). However, the antimethanogenic and rumen modulation potentials of these metabolites have not been reported.

1.6 The role of Moringa in rumen modulation and methane inhibition

Moringa has been used, mainly the leaves, as a fodder tree or feed supplement in animal nutrition (Safwat *et al.*, 2014; Aharwal *et al.*, 2018; Mahfuz & Piao, 2019; Shankhpal *et al.*, 2019). Leaves of Moringa have more highly digestible protein with balanced amino acid composition. However, the higher ruminal degradability of its protein decreases the undegradable protein amount for ruminant animals (Babiker *et al.*, 2017). Thus, leaves of Moringa contained 18–30% crude protein (CP) with 47% bypass protein and adequate amino acid profile, 44% carbohydrate, 45% nitrogen-free extract, 15–29% crude fibre, 3–6% ether extract, and 9% ash contents (Teixeira *et al.*, 2014; Aharwal *et al.*, 2018; Shankhpal *et al.*, 2019; Akhtar *et al.*, 2022). Animals supplemented with leaves of Moringa exhibited up to

30% increases in body weight and milk production (Valdivié *et al.*, 2017). Similarly, increases in milk yield by 9.2% and fat content by 7.4% were observed because of the addition of Moringa leaf to the diet of dairy cattle (Shankhpal *et al.*, 2019). The presence of readily fermentable nitrogen and energy in *M. oleifera* also promotes the synthesis of microbial protein in the rumen (Shankhpal *et al.*, 2019). *Moringa oleifera* leaf silage completely replaced soybean meal with desirable effects on ruminal fermentation (Morsy *et al.*, 2022).

The rumen microbial activities, rumen fermentation, CH_4 emission and the overall performances of animals are influenced by the bioactive SPMs of plants (Saucedo-Pompa *et al.*, 2018). Thus, many SPMs are abundant in Moringa, which makes it one of the promising plants for ruminal modulation and inhibition of CH_4 production from ruminants (Ku-Vera *et al.*, 2020; Kashyap *et al.*, 2022). *Moringa oleifera* leaf extracts studied at 10, 100 and 1000 µg/500 mg DM diets were an effective natural replacement for monensin to enhance the efficacy of dietary nutrients with inhibited CH_4 production from ruminants (Soltan *et al.*, 2014). Similarly, *M. oleifera* inhibited protozoal population, total CH_4, CH_4 production per body weight gain, and urine nitrogen (N) excretion compared with monensin (Soltan *et al.*, 2018). Moringa leaf extract reduced CH_4 without significant effects on protozoal counts, ruminal degradability, ammonia concentrations and gas production equivalent compared with monensin (Soltan *et al.*, 2019). Various dosages of reconstituted Moringa leaf extracts also exhibited 4.5%, 5.2%, 28.7% and 29.3% CH_4 inhibition at 100, 75, 50 and 25 mg/L, respectively (Akanmu & Hassen, 2017). These studies generally highlighted the benefit of using Moringa extracts to modulate rumen fermentation and inhibit enteric CH_4 production from ruminants. However, evidence is not sufficient about the effects of various specific SPMs on rumen fermentation parameters and antimethanogenic potentials.

1.7 Adaptability and performance of Moringa

1.7.1 Growing environment of Moringa and its effect on secondary plant metabolites

Moringa oleifera is indigenous to India, Nepal and Pakistan, and is among the most cultivated and studied species in the Moringaceae family (Paliwal *et al.*, 2011; Leone *et al.*, 2015b). Its multiple potentials as nutritional, medicinal and biofuel functions attract farmers and researchers globally, though mainly in African and Middle East countries (Salaheldeen

et al., 2015; Shah *et al.*, 2016; Babiker *et al.*, 2017). *Moringa stenopetala* also belongs to the Moringaceae family and is indigenous to north-east African countries (i.e. Ethiopia, Kenya and Somalia) (Leone *et al.*, 2015b). *Moringa stenopetala* also exists in the wild in northern India (Yisehak *et al.*, 2011). However, its distribution and cultivation are not as widely known as *M. oleifera.*

Moringa favours growing in annual rainfall from 760 to 2500 mm and temperature ranges from 25 to 35 °C (Nouman *et al.*, 2014; Fredalette, 2018). However, it can grow in wider environmental ranges with annual precipitation from 250 to 3000 mm (Chukwuebuka, 2015) and temperature fluctuations from –1 °C to 48 °C for shorter periods (Anwar *et al.*, 2007). It likes sandy loam or loamy soils of slightly acidic to neutral pH, and cannot tolerate waterlogged soils for longer periods (Horn *et al.*, 2022). In terms of altitude, it needs to grow below 600 meters above sea level (m.a.s.l), although it can grow up to 1200 m.a.s.l (Nouman *et al.*, 2014; Fredalette, 2018). Therefore, Moringa generally favours more heat and sunlight but does not tolerate heavy clay soils with waterlogging and frost for an extended period (Horn *et al.*, 2022).

Several studies revealed significant relationships between the growing environmental factors of various plants and the biosynthesis and accumulation of their SPMs (Verma & Shukla, 2015; Li *et al.*, 2020; Szepesi, 2021). However, the response of Moringa SPMs to growing environmental factors was not investigated in this way. Hence, it is apparent that all plants, including Moringa, interact with sunlight, rainfall, drought, humidity, temperature, soil, salinity, air, and magnetic fields in their surroundings. Thus, any change in these factors from the plants' optimum requirement level may alter the normal physiological and biochemical functions (Ma *et al.*, 2019). The exposure of plants to extreme environmental conditions affects their normal growth and development adversely. This situation usually creates secondary stresses on the plants, which stimulate the production of SPMs, which affects their growth, development and survival (Verma & Shukla, 2015; Kurepin *et al.*, 2017; Sharma, 2018). The regulatory mechanisms of SPM production pathways are vulnerable to changes in environmental factors, which certainly affect the manifestation of genes involved in their production pathways and storage (Li *et al.*, 2020; Szepesi, 2021).

1.7.2 Growth and leaf yield of Moringa and their relationship with secondary plant metabolites

Moringa is a fast-growing, deciduous, evergreen softwood tree that can grow up to 2 m in 116 days (Patricio & Palada, 2017). However, plant survival, growth, branching habit, leaflet shape and size, time to flowering, pod length and width and leaf yield varied among the Moringa species (Radovich, 2011; Yang *et al.*, 2015). *Moringa oleifera* accessions showed seedling survival rates of 75–100% (central Philippines) (Patricio & Palada, 2017) and 100% (Ethiopia) (Samuel *et al.*, 2016). *Moringa stenopetala* accessions grown in Ethiopia maintained a 97% seedling survival rate (Samuel *et al.*, 2016). However, losses of 20–30% of Moringa seedlings might happen in the first year, although the remaining seedlings may regenerate three to five new shoots after each cutting (Ponnuswami, 2012).

The leaf yield of Moringa showed a wide range of variations among the studies. However, most of them are difficult to compare owing to their differences in growing environments and management factors. The smallholder farmers of Limpopo in northern South Africa obtained leaf yields of Moringa ranging from 527 kg/ha (lower planting density) to 2867 kg/ha (higher planting density) (Mabapa *et al.*, 2017). Various accessions of Moringa grown under tropical regions of the Central Philippines with 2–3 harvests at 3333 plants/ha provided a 3–6 tons/ha leaf yield (Patricio & Palada, 2017). Fresh leaf biomass of 9.4, 15.6, 24.8, and 30.0 tons/ha using planting density of 10000, 20000, 30000, and 40000 plants/ha, respectively and yields of 22.4, 20.4, 30.0 tons/ha at 4, 6, and 8 weeks harvesting intervals were obtained in Central Philippines, respectively (Patricio *et al.*, 2015). In this study, a greater plant density of 40000 plants/ha and a harvesting frequency of eight-week intervals were preferable to obtain a higher leaf biomass yield. Thus, the yield and growth performances of Moringa are reliant on plant spacing and cutting interval (Gadzirayi *et al.*, 2019; Eshete *et al.*, 2022), watering (Azam *et al.*, 2020), fertilization (Mendieta-Araica *et al.*, 2013), harvesting season, stage of plant growth, and environmental factors (Mabapa *et al.*, 2017; Korsor *et al.*, 2019).

The growth and leaf yield performances of Moringa are expected to have a direct or indirect relationship with the synthesis and accumulation of SPMs. However, no study reported such relationships specific to Moringa. Secondary plant metabolites can be produced through special controlling pathways and exceptional transport routes in certain cells, tissues and

organs of the plant (Belkheir *et al.*, 2016). However, the concentrations of those components over the growth and developmental stages depend on the plant species, plant parts and type of SPMs (Li *et al.*, 2020), because most of the factors that affect the growth of plants influence the initiation of specific cell structures that participate in the production and storage of SPMs (Broun *et al.*, 2006). Higher SPMs were reported in the reproductive growth stages of plants (Geng *et al.*, 2011), although another study showed greater amounts of SPMs in the vegetative growth stages (Zhu *et al.*, 2014). In the same way, various bioactive SPMs indicated higher relative abundance at different stages in the same plant. For instance, *Magnolia officinalis* bark showed the highest abundance of quercetin, hyperin and chlorogenic acid in a 13-year-old plant, magnolol in a 10-year-old plant, and rutin in a 7-year-old plant (Yang *et al.*, 2012). The production and storage of SPMs had generally positive relationships with the growth and yield parameters of woody and perennial medicinal plants. Thus, when the plants have higher growth periods, their stem diameter, plant height, root diameter and biomass yield increase, which makes the plants more vigorous and increases their metabolism and storage of SPMs (Li *et al.*, 2020). This result implies the presence of higher possibilities of establishing positive relationships between growth and leaf yield parameters of Moringa with its composition and concentration of the SPMs. However, this relationship has not been supported with enough evidence in Moringa and thus there is a need for a comprehensive study to maximize the benefits of SPMs without compromising the leaf yield.

1.7.3 Diseases and pests of Moringa and their relationships with secondary plant metabolites

Moringa is fairly described as being resistant to most common diseases (Gatan, 2020). However, some studies reported the prevalence of the disease. Diseases include twig canker *(Fusarium pallidoroseum),* fruit rot (*Cochliobolus hawaiiensis),* root rot (*Diplodia sp.),* leaf spot *(Cercospora moringicola),* anthracnose (*Colletotrichum chlorophyti*), fusarium wilt (*Fusarium oxysporium*), dieback (*Fusarium semitectum*), and damping off *(Rhizoctonia solani)* (Carbungco *et al.*, 2017; Patricio & Palada, 2017; Gatan, 2020). In addition, about 24 fungal morphospecies were detected in Moringa (Carbungco *et al.*, 2017), in which *Aspergillus flavus* Link, *Aspergillus niger* van Tieghem, *Fusarium oxysporum* Schlecht emend Snyder Hansen, *Alternaria alternata* (Fr.) Keissl, and *Rhizopus stolonifera* (Ehrenb.) Vuill, *Macrophomina phaseolina* (Tassi) Goid were identified (Mridha & Barakah, 2017).

The incidences of pests, such as leaf caterpillar (*Noorda blitealis*), pod fly (*Gitona distigma*), budworm (*Noorda moringae*), bark borer or bark caterpillar (*Indarbela tetraonis),* longhorn beetle (*Batocera rubus*), red mite (*Tetranyctrus urticia*), leaf-footed bug (*Leptoglossus phyllospus*) and whitefly (*Bermisia* sp.) were reported in most Moringa-growing countries (Faleono *et al.*, 2017; Mridha & Barakah, 2017; Patricio & Palada, 2017). In addition, aphids (*Aphis gossypii*), leaf-eating weevils (*Myllocerus* spp.) and bud midges (*Stictodiplosis moringae)* were identified as minor pests (Mridha & Barakah, 2017).

Like all other living things, Moringa can be exposed to biotic challenges, such as pathogenic bacteria, viruses, fungi and pests in its lifetime (Carbungco *et al.*, 2017; Patricio & Palada, 2017; Gatan, 2020). Hence, plants make physiological and biochemical changes in response to the biological attacks that promote the production and accumulation of SPMs as adaptive mechanisms of the plants to compete, survive and reproduce (Matilla, 2018; Erb & Kliebenstein, 2020; Divekar *et al.*, 2022). However, the syntheses of SPMs are controlled strictly by the interaction of signalling molecules involving the phytohormones (Mishra *et al.*, 2020). Some SPMs induce hormonal defence signalling pathways or assist in the quick detection of attacks and rapidly answer to changing situations because of pathogenic diseases and pests (Divekar *et al.*, 2022). These SPMs help to control the defence-signalling pathways against the stress developed because of the occurrence of disease and pests (Isah, 2019; Mishra *et al.*, 2020). Mostly defence response genes have been upregulated to the increasing level of SPMs accumulation. Subsequently, the number of receptors is enhanced, and the cells become more sensitive to the hormone and permit higher cellular activities. The production of SPMs generally increases the survival and competitiveness of the plants under challenging conditions (Matilla, 2018; Mishra *et al.*, 2020). As a result, plants commonly synthesize and accumulate various types of SPMs in response to stress because of disease and pests as defence mechanisms (Srivastava *et al.*, 2018; Mishra *et al.*, 2020). However, the relationships between disease and pests and the production and accumulation of SPMs in Moringa have not been studied directly.

1.8 Factors affecting production and bioactivities of secondary plant metabolites

1.8.1 Genetics of the plants

Genes strictly control the biosynthesis processes of SPMs in plants (Li *et al.*, 2020; Szepesi, 2021). However, their compositions and concentrations vary among plant species, cultivars,

varieties, ecotypes, plant parts and individual plants (López *et al.*, 2007; Isah, 2019). *Moringa oleifera* accessions grown in Algeria, Haiti and Chad exhibited total phenolics of 3552, 2545, and 2813 mg/100 g, ferulic acid of 8.9, 9.7 and 6.6, mg/100 g and salicylic acid of 0.2, 0.3 and 0.1 mg/100 g, respectively (Leone *et al.*, 2015a). These variations are associated with genotype and environment interactions. The *Moringa stenopetala, M. oleifera* and *M. peregrina* grown in Egypt also differed in terms of their total flavonoids, total phonics, and the concentrations of 36 specific phenolic compounds (Abo El-Fadl *et al.*, 2020). Different varieties and cultivars of berries also exhibited different types, contents and bioactivities of the SPMs (Skrovankova *et al.*, 2015). Genotype was also reported to be the basic determining factor for the difference in the biosynthesis and accumulation of SPMs as well as antioxidant properties observed in green coffee and wheat (Rodrigues *et al.*, 2015; Shamloo *et al.*, 2017). Several findings generally indicated that the constituents, yields and subsequent bioactivities of the SPMs are highly dependent on genotype.

1.8.2 Effects of growing environments

Plants always interact with abiotic factors in their surroundings and changes in the abiotic factors alter the normal physiological and biochemical functions (dos Santos *et al.*, 2022). The exposure of plants to extreme abiotic factors usually creates secondary stresses on the plants and alters gene expression, which stimulates the biosynthesis and accumulation of SPMs (Verma & Shukla, 2015; Li *et al.*, 2020; Szepesi, 2021). If there is any change in the concentration and composition of the plant components, it is apparent that the subsequent bioactivities of the plant products will also be affected.

a) Temperature effects

Naturally, every plant has its temperature requirement zone. Thus, temperatures out of the requirement level affect their growth, development and productivity negatively (Shamloo *et al.*, 2017). High temperatures usually decrease the photochemical efficacy of their photosystem II (Anderson *et al.*, 2021). This situation triggers mostly the synthesis of SPMs (Naghiloo *et al.*, 2012; Li *et al.*, 2020). The responding genes may be upregulated or downregulated to elevated temperatures (Li *et al.*, 2016). The plants substantially change their molecular processes (water uptake, metabolic reactions and cellular dehydration) and physiochemical processes when they induce very low temperatures, which slowly leads to adaptation or acclimation (Hasanuzzaman *et al.*, 2017). Not only does the stress caused by

low temperatures affect the normal growth and productivity of plants negatively, but it also influences the production and storage of SPMs in various plants (Verma & Shukla, 2015). This seems opposite to the effect observed in high-temperature stress. However, the plants may develop a survival mechanism known as cold acclimation to low-temperature stress (Ashrafi *et al.*, 2018).

b) Drought effects

Water is essential for plant productivity, in which plant metabolism and bioactive SPMs are influenced by the changes in water availability and the occurrence of drought (Sanchez *et al.*, 2012; Kapoor *et al.*, 2020). Water deficiency reduces the water absorption in plants and affects the biological functions of growth, yield and development adversely (Ashrafi *et al.*, 2018). However, stress because of drought generally promotes biosynthesis and accumulation of SPMs. The genes and proteins involved in these processes during drought stress are not well understood (Ashrafi *et al.*, 2018; Li *et al.*, 2020). Despite severe stress owing to drought generally increasing flavonoid and phenolic contents, the growth of plants is affected negatively as the level of stress increases (Azhar *et al.*, 2011; Li *et al.*, 2020). In grapevines (*Vitis vinifera* L.), various genes involved in the biosynthesis of phenolic compounds were enhanced in response to low to moderate drought stress conditions (Król *et al.*, 2014). However, these high energy-demanding processes are restricted when the plants are exposed to moderate to severe drought stress conditions. Correspondingly, the basic enzymes that contributed to the biosynthesis of baicalin in *Scutellaria baicalensis* Georgi were promoted by a certain level of drought stress, which increased its production and accumulation (Cheng *et al.*, 2018). Plants also increased the accumulation of quercetin, rutin, betulinic acid, artemisinin and glycyrrhizin in response to the exposed drought stress conditions (Verma & Shukla, 2015; Hosseini *et al.*, 2018). However, the production and storage of SPMs are increased mostly under drought stress, and the responses to the level of water scarcity are reliant on plant species and types of compounds.

c) Effect of light

Plant photosynthesis and growth require an appropriate level of light intensity (Li *et al.*, 2018). Both deficiency in and excessive irradiance of sunlight can affect the net photosynthetic rate, growth of plants, synthesis and storage of SPMs (Kong *et al.*, 2016; Szymańska *et al.*, 2017; Li *et al.*, 2018). However, higher irradiance sometimes might be

useful for the growth of plants and the biosynthesis of SPMs (Zhang *et al.*, 2015). The *Erigeron breviscapus* leaves grown under shade exhibited lower amounts of flavone glycosides compared with those grown in the sun (Zhou *et al.*, 2016). Similarly, higher light intensities increased essential oil contents (Kong *et al.*, 2016; Li *et al.*, 2018). However, fully irradiated *Flourensia cernua* produced lower amounts of sabinene, b-pinene, bornyl acetate, borneol, Z-jasmone, and camphene than those values recorded under partially shaded growth conditions of plants (Estell *et al.*, 2016). Accordingly, the production and storage of SPMs in response to the photoperiod and light intensity requirements might be varied among the plant species (Zhou *et al.*, 2016; Li *et al.*, 2018).

d) Soil and salinity effects

Soils that contain higher salt contents affect nutrient absorption, photosynthesis and plant growth negatively, which induces hyperosmotic stress and nutritional imbalance (Banerjee & Roychoudhury, 2017). However, an increasing SPMs concentration or a decreasing one might be obtained in response to specific ion toxicity or salinity-related osmotic stress (Akula & Ravishankar, 2011). *Plantago ovata* grown in salinity stress conditions exhibited higher alkaloid compounds and tannins, phenolics, proline, saponins, and flavonoid contents (Haghighi *et al.*, 2012; Verma & Shukla, 2015). However, the tolerance of salt stress may be induced by multilevel changes in the plant metabolome, transcriptome and proteome, accompanied by the response in molecular changes (Banerjee & Roychoudhury, 2017). Thus, evaluating the association between active constituents of medicinal plants with the soil microbial community helps to provide systematic direction for growing and managing medicinal plants with the highest bioactive SPMs contents. However, speculation from a single study exposed to one stress and interpretation of SPMs differences as abiotic factors induced by the complex internal plant system is difficult. Hence, the interest of many recent studies in medicinal plants is in developing a wide range of resistance to multiple stresses.

1.8.3 Postharvest processing and storage conditions

Plants are highly perishable and need to be processed (dried or pulverized or extracted) for easier management and longer shelf life. Along with the various plant factors, the composition and efficacy of Moringa SPMs are affected considerably by postharvest drying methods (Ademiluyi *et al.*, 2018), drying temperatures (Gąsecka *et al.*, 2020), extraction

solvents (Urías-Orona *et al.*, 2017), storage temperature and storage light conditions (Li *et al.*, 2012).

a) Effect of drying methods

Most plants are highly perishable and need to be dried and pulverized for easier management and storage for year-round supply. However, the drying methods and temperatures affect significantly the active components and bioactivities (Ademiluyi *et al.*, 2018; Gatahi & Nyoro, 2021). Ademiluyi *et al.* (2018) recorded the highest content of total phenolics in freeze-dried leaves of *M. oleifera* (68.8 mg/g), followed by air-dried (59.4 mg/g), sun-dried (50.0 mg/g), and oven-dried leaves (46.9 mg/g) (Ademiluyi *et al.*, 2018). The leaves of Moringa dried at room temperature and under 50% shade net exhibited superior polyphenols and terpenoids compared to those dried in an oven and a greenhouse. However, the oven-dried leaves showed the highest glucosinolates, followed by greenhouse drying. In contrast, the shade-dried leaves developed toxic microbes (i.e. *Escherichia coli*, staphylococcus, moulds, and yeast), which may affect the bioactive ingredients and their subsequent bioactivities negatively (Gatahi & Nyoro, 2021). Similar to this result, Sauveur & Broin (2010) warned that air-drying could not be guaranteed to maintain the highest recommended moisture (10%), making it mould-free and retaining the safety of the products (Sauveur & Broin, 2010). Compared to the sun-dried and oven-dried ethanolic extract, air-dried Moringa extract showed superior inhibition of *Staphylococcus typhi*, *Staphylococcus aureus*, and *Escherichia coli* (Hussein *et al.*, 2015). Five accessions of African eggplants (*Solanum aethiopicum*) showed the highest bioactive compounds in the freeze-drying method, compared with oven, vacuum and solar drying methods (Mbondo *et al.*, 2018). Oven-dried peels of *Citrus sinensis, Citrus reticulate and Citrus paradisi* exhibited higher essential oil contents compared with those dried at ambient temperature and with fresh peel (Kamal *et al.*, 2011). Similarly, the air-dried *Cosmos caudatus* showed the highest antioxidant activity, which was followed by the freeze- and oven-dried *Cosmos caudatus* (Mediani *et al.*, 2014; Hussein *et al.*, 2015). However, freeze-dried tomatoes and ginger showed the best antioxidant properties compared with the sun-, oven- and vacuum-dried tomatoes and ginger (Gümüşay *et al.*, 2015). The freeze- and air-dried *Annona reticulata* leave exhibited superior antioxidant, anti-inflammatory and antibacterial activities to the oven-dried leaves.

b) Effect of drying temperatures

Drying temperatures play a substantial role in the stability, efficacy and bioactivity of dried plant materials. *Hericium erinaceus* and *Leccinum scabrum* samples dried at 70 °C maintained the lowest total phenolics, organic acid, ergosterol contents and free radical inhibition, followed by 40 ˚C, 20 ˚C and their fresh samples (Gąsecka *et al.*, 2020). Less frequently, leaves dried quickly at a higher temperature (110 ˚C) maintained superior bioactive SPMs and antioxidant activity compared to those dried at 4 ˚C, –20 ˚C and room temperature (Vats, 2016). Basil leaves dried at 45 ˚C retained higher SPMs constituents compared with those dried at 55 ˚C and 65 ˚C. However, the study did not indicate the subsequent bioactivities of the products (Parma *et al.*, 2018). Individual plants may have their safe drying temperature requirements, and understanding these temperature zones would improve significantly the stability, efficacy and bioactivity of medicinal plant products. Moringa dried at 60 ˚C maintained better protein, vitamin C and ß-carotene, total phenolics and antioxidant activity compared with the leaf dried at 70 ˚C and 80 ˚C (Razzak *et al.*, 2021). As the drying temperatures were raised from 30 ˚C to 70 ˚C, the crude protein, beta-carotene and vitamin C contents were decreased from 284.4 to 198.6 mg/g, 52.2 to 49.5 mg/g and 27 to 25 mg/100 g, respectively, with increased carbohydrate, fibre and ash contents (Kucha *et al.*, 2015). Drying Moringa leaves above 55 ˚C changed the leaf to brown, although the study did not indicate the effect of this colour change on subsequent biological activities (Sauveur & Broin, 2010). However, *Azadirachta indica* leaves dried quickly at a higher temperature (110 ˚C) showed superior bioactive SPMs and antioxidant activity less frequently compared to those dried at 4 ˚C, –20 ˚C and room temperature (Vats, 2016). These results imply that the drying temperatures may vary based on the purpose of the products and plant species.

c) Effect of extraction solvents

Several studies revealed that the choice of extraction solvent affected significantly the yield, composition and biological efficacy of the plant compounds (Urías-Orona *et al.*, 2017; Abo El-Fadl *et al.*, 2020). Thus, hexane, ethyl acetate, and ethanol 70% extracted leaves of *M. oleifera* extract grown in Egypt exhibited different total phenolic contents of 48.7, 58.7, and 108.7 mg GAE/g, respectively (Abo El-Fadl *et al.*, 2020). The three levels of methanol and ethanol (100%, 80% and 50% of organic solvents) maintained significantly different total

phenolics (55.9–226.2 mg ChAE/g), total flavonoids (17.6–23.5 mg CE/g) and antioxidant capacity of 101.0–245.3 α, α-diphenyl-β-picrylhydrazyl (DPPH)) (Urías-Orona *et al.*, 2017). These authors preferred 50% ethanol because it maintained the highest phenolics and antioxidant activity, whereas 100% methanol was a more effective solvent for phenolic profiling than 50% methanol and 100% ethyl acetate to extract leaves of *M. oleifera* (Rocchetti *et al.*, 2020). However, higher antioxidant and effective enzymatic inhibitory activities were obtained with 50% methanol and 100% ethyl acetate, respectively (Rocchetti *et al.*, 2020). Acetone (50%) extracted *Salacia chinensi* roots maintained higher yields of extracts, flavonoids and phenolic compounds, and better antioxidant activity than pure ethanol, acetone, methanol, water and their 50% aqueous solvents (Ngo *et al.*, 2017).

In the chromatographic separation of methanol and aqueous-extracted leaves of *M. stenopetala*, methanol had higher constituents of 3-Hydroxybenzoic acid, quercetin-3-*O*-rutinoside, hydroxycinnamic acid with better free-radical scavenging activity. However, the aqueous extracts maintained superior syringic, succinic, fumaric and chlorogenic acid contents compared to those recorded in the absolute methanol solvent (Dessalegn & Rupasinghe, 2021). Moringa fruits extracted with water, 70% ethanol and hexane as a solvent suppressed the growth of *Candida albicans* by 89.9%, 57.9% and 9.0%, respectively (Nuryanti & Puspitasari, 2017). Therefore, the plant-solvent matrix played a remarkable role in the bioactive SPMs concentrations, constituents and subsequent bioactivities of the extracts. However, this would then need intensive research to obtain the most effective solvent for a plant species for a specific purpose.

d) Effect of storage environments

Medicinal plant products are infrequently used immediately after harvest and are usually kept for a period under various storage conditions before utilization. However, most bioactive compounds are sensitive to storage factors, which may decrease or destroy their biological efficacy (Vongsak *et al.*, 2013a; Zhang *et al.*, 2017). Moringa powder stored at 15 °C maintained higher total phenolics and antioxidant activity with lower changes in green colour after two months compared with that stored at 35 °C (Potisate *et al.*, 2015). Inter-Aide (2012) recommended storage of Moringa leaf powder for up to six months if stored below 24 °C in airtight containers protected from light and humidity. However, this study did not indicate the variation in effectiveness depending on the purpose of utilization of the stored

product. Moringa stored at 25 °C and 75% relative humidity for six months showed a lower decrease in three main antioxidant components (astragalin, isoquercetin and crypto-chlorogenic acid) (13–27%) and antioxidant activity (30%) compared with the decrease in antioxidant components (38–53%) and antioxidant capacity (50%) maintained at 40 °C and 75% relative humidity, respectively (kVongsak *et al.*, 2012). This indicates that storage at higher temperatures and humidity may result in undesirable losses of plant components and bioactivities. This conforms with the notion that the chemical changes during processing and storage are highly stimulated by light, higher temperature, oxygen and catalysts (Li *et al.*, 2012). Similarly, the walnut male inflorescence stored at 25 °C showed higher loss rates of flavonoid and phenolic contents compared with the values observed at 4 °C storage. However, the antioxidant activities and the rate of reduction of these compounds were different in extended storage periods (Zhang *et al.*, 2017). In a systematic evaluation of the storage conditions of blueberry extract (–20 °C to +35 °C for 60 days), different recoveries of phenolics and biological activities were found across the storage time and temperature (Srivastava *et al.*, 2007). The leaf extracts of *Anemopsis californica* stored at –20 °C and 4 °C for six months also exhibited higher total phenols and total flavonoids with better antioxidant activity than those maintained at 25 °C and 50 °C (Del-Toro-Sánchez *et al.*, 2015). However, previous studies did not indicate the efficacy of the extracts in terms of antimethanogenic activity when those ideal storage environments were changed under different practical storage conditions.

1.9 Conclusions and statement of the problem

1.9.1 Conclusions from the literature review

The high CH_4 emission from ruminants is attributed to anaerobic fermentation with diverse microbes found in the rumen and has been an increasing global concern because of its negative effect on the environment and the productivity of animals. Thus, developing an effective and friendly mitigation intervention with the environment to inhibit enteric CH_4 emissions from ruminants has attracted many researchers. Hence, the use of medicinal plants has benefited the production of healthy organic animal products without additional burdens on the environment. However, the effectiveness of this method is influenced by multiple factors. Thus, the genetic makeup of the plants, growing environments, plant exposure to biotic factors (herbivores, pests, microorganisms, invaders, grazers, and parasites) and the

postharvest processing and storage conditions are important aspects that can affect the production and accumulation of bioactive SPMs, which in turn affect their subsequent bioactivities during the application. However, little is known about how variations in plant genetics, growing environment, cocktails of the accessions extracts and postharvest management conditions can affect the efficacy of Moringa plant extracts to inhibit CH_4 emissions from ruminants.

1.9.2 Statement of the problem

The composition and concentration of SPMs in Moringa and their subsequent biological activities are affected by several multifaceted factors. The genetic makeup of the plants, including the presence of specific genes and groups, is the most basic internal element that regulates the biosynthesis and accumulation of SPMs (Li *et al.*, 2020; Szepesi, 2021), which affects the bioactivities of the plant products including enteric CH_4 inhibition. It was evident that the variations in total phonics, total flavonoids and various phenolic SPMs recorded in *M. stenopetala, M. peregrina* and *M. oleifera* grew in Egypt were attributed to plant genetics (Abo El-Fadl *et al.*, 2020). However, the regulatory systems of the pathways, biosynthesis, concentration and storage of SPMs are sensitive to the changes in the growing environment, stage of maturity, postharvest handling of the leaf, extraction solvents and storage condition of the plant products (Li *et al.*, 2020; Szepesi, 2021). Although past research revealed the differences among varieties of Moringa in their bioactive SPMs composition and concentrations, there is a dearth of comprehensive and conclusive studies to comprehend the variations when different accessions of Moringa are grown in the same environment. The antimethanogenic effects of individuals or groups of SPMs associated with a single or combination of plant extracts from accessions are also hardly understood in previous studies. The effect of various postharvest treatments, such as drying methods (Ademiluyi *et al.*, 2018), drying temperatures (Gąsecka *et al.*, 2020), extraction solvents (Urías-Orona *et al.*, 2017), storage temperatures and storage light conditions (Li *et al.*, 2012) on the efficacy of enteric CH_4 inhibition from ruminants and sustainability of the final products were not established sufficiently when used as dietary additives. Therefore, it is essential to conduct additional research i) to establish the relationships of agronomic traits and SPMs of Moringa accessions with *in vitro* gas production parameters, ii) to identify key bioactive SPMs ion features responsible for high and low CH_4 inhibition characteristics of the varieties, iii) to determine whether there are associative effects from the use of mixtures

of *Moringa* accession extracts, and iv) to quantify the effects of postharvest processing and storage conditions on the efficacy of Moringa extracts to inhibit enteric CH_4 emission from ruminants. Thus, the production of such data will help us to improve our understanding of the main factors that influence the composition and concentration of the bioactive SPMs associated with enteric CH_4 inhibition. This would lead to the production of standardized, effective and consistent Moringa plant extract products that can be used as dietary additives to reduce enteric CH_4 emissions from ruminants.

1.10 Working hypotheses and objectives

1.10.1 Hypotheses

To improve our understanding and ultimately produce standardized and consistent Moringa plant extract additives, the working hypotheses were formulated:

HO1: Agronomic traits of Moringa accessions did not have significant relationships with *in vitro* gas production characteristics of a test feed incubated with the respective accessions leaf extracts.

HO2: *Moringa oleifera* accessions did not differ significantly in the bioactive SPM ion features contributed to high and low enteric CH_4 inhibition from ruminants.

HO3: The effectiveness of binary cocktails prepared from two promising CH_4-inhibiting Moringa accession leaf extracts did not differ in their *in vitro* enteric fermentation characteristics when mixed in various proportions.

HO4: Moringa leaf or powder treated with different postharvest processing and storage conditions did not affect significantly the efficacy of the leaf extracts to inhibit enteric CH_4 production.

1.10.2 Objectives

The general objective of this study is to improve our understanding by establishing the main factors influencing the efficacy of plant extracts to develop a standardized and consistent Moringa leaf extract product suitable for commercialization for inhibition of enteric CH_4 emission from ruminants.

Therefore, the specific objectives are:

1. To establish the relationships between agronomic traits of Moringa accessions and *in vitro* gas production characteristics of *E. curvula* hay test feed incubated with their leaf extracts
2. To identify the potential bioactive SPM ion features responsible for high and low CH_4 inhibition characteristics of *M. oleifera* plant extracts when used as an additive to inhibit enteric methane emission from ruminants
3. To investigate the effectiveness of binary cocktails prepared from promising Moringa accession leaf extracts mixed in various proportions to exploit additive or synergic effects in inhibiting CH_4 emission
4. To quantify the effects of postharvest processing and storage condition treatments of *M. oleifera* leaf or powder on the efficacy of the plant extract additives to inhibit enteric CH_4 production from ruminants

1.10.3 List of studies

To achieve these specific objectives and test the working hypotheses, a series of field and laboratory *in vitro* studies were conducted, and their findings were prepared as a research manuscript reported in Chapters 2-5 of this thesis.

1. Establishing relationships between agronomic traits of Moringa accessions and *in vitro* gas production characteristics of a test feed incubated with or without Moringa plant leaf extracts
2. Screening of candidate bioactive secondary plant metabolite ion features from *Moringa oleifera* accessions associated with high and low enteric methane inhibition from ruminants
3. Investigating the associative effects of binary cocktails of *Moringa oleifera* accession extracts mixed in various proportions on *in vitro* rumen fermentation parameters
4. Evaluating the effect of postharvest processing and storage conditions on the efficacy of *Moringa oleifera* leaf extracts to inhibit *in vitro* methane production

CHAPTER 2

Establishing relationships between agronomic traits of Moringa accessions and *in vitro* gas production characteristics of a test feed incubated with or without Moringa plant leaf extracts

Abstract

The use of medicinal plants and their extracts has attracted the attention of many researchers as a CH_4 mitigation strategy. This study evaluated the relationships of agronomic traits and SPMs of Moringa accessions with *in vitro* gas production measurements and feed digestibility from ruminants. Twelve Moringa accessions were treatments of this study and grown at the Roodeplaat experimental site of the ARC in Pretoria, South Africa. Agronomic traits, such as seedling survival rate, leaf yield, canopy and stem diameter, plant height, number of primary branches, plant vigour, greenness, chlorosis, and disease and pest incidences were recorded. The leaves were harvested in the fifth month after transplanting to the field. Freeze-dried leaves were extracted with methanol, and their total phenolic and total flavonoid contents were determined. The extract was applied at a dose of 50 mg/kg of DM feed for *in vitro* gas production studies. Most of the growth and agronomic traits, that is, seedling survival rate, leaf yield, canopy diameter, plant height, number of primary branches, the score of plant vigour, and greenness, total phenolics and total flavonoids differed significantly among the accessions except for stem diameter and chlorosis score. All accessions reduced significantly the *in vitro* total gas and CH_4 production from *E. curvula hay* treated with their leaf extracts compared with those produced from the control with equal or higher OMD. Higher CH_4 inhibition was obtained in *M. oleifera* accession 7633 (A3) (28.4%) and accession Pretoria (A11) (29.1%), whereas a lower inhibition was recorded in accession bulk (A1) (17.9%) and accession 7229 (A2) (18.2%). The total phenolics (0.62), total flavonoids (0.71), and most agronomic traits of the accessions correlated positively with the CH_4 inhibition potential of the accessions. *Moringa oleifera* accessions A3, accession 7717 (A8) and A11 resulted in higher *in vitro* CH_4 inhibition potential and improved OMD of the feed with equal or higher adaptability performances in the field. Thus, there is a possibility of selecting Moringa accessions for higher antimethanogenic activity without compromising the feed digestibility by selecting for higher total phenolics, total flavonoids and agronomic performance traits. There is a need

for further study to determine the long-term adaptability in different agro-ecologies, harvesting seasons, ages and parts of the plant with concurrent antimethanogenesis efficacy when used in the diet of ruminant animals.

Keywords: methane mitigation; *in vitro* organic matter digestibility; ruminants; total flavonoids; total gas; total phenolics

2.1 Introduction

Ruminant animal production has been among the most important sectors of livestock and has a pivotal role in sustainable food supply and economic benefits (Adams *et al.*, 2021). However, CH_4 produced in ruminants as a by-product of anaerobic microbial fermentation in the rumen has increasingly been a shadow on production (Black *et al.*, 2021; Khanal *et al.*, 2022). Methane emitted from ruminants not only has a global warming effect but also results in energy loss and reduces the productivity of animals (Hristov *et al.*, 2013). Thus, the development and implementation of applicable strategies to reduce enteric CH_4 production and to improve the dietary energy use efficacy of animals are urgently required (Legg, 2021). In this regard, the use of medicinal plants and their extracts are suitable and relatively economical alternative feed additives in the ruminant feeding system to reduce enteric CH_4. The multipurpose functions of *M. oleifera* as an antioxidant, anticancerous, antidiabetic, and anti-inflammatory agent with antimicrobial properties and nutritional benefits have increased its demand and it has been intensively cultivated (Salaheldeen *et al.*, 2015; Shah *et al.*, 2016).

Plant survival, plant growth, adaptability and bioactivities interact with multiple factors (Rodrigues *et al.*, 2015; Skrovankova *et al.*, 2015). Thus, this genetic variability was implicated as a reason for the variation in plant growth, leaf yield and agro-morphological characteristics (leaflet size and shape, branching habit, time to flowering, pod width and pod length of the plants), and played a remarkable role for its application in ruminant production (Radovich, 2011; Palada *et al.*, 2017). Similarly, the distribution patterns of bioactive SPMs are different among genetics and plant parts, such as flowers, seeds, stems, leaves and roots, which are tissue- or organ-specifi**c** (Belkheir *et al.*, 2016; Li *et al.*, 2020). The leaves of three Moringa species (*M. oleifera, M. stenopetala. M. peregrina*) grown in Egypt showed different total phonics (48.7–108.7 mg GAE/g**),** total flavonoids (31.2–62.8 mg QE/g) and phenolic compound concentrations (Abo El-Fadl *et al.*, 2020). *Moringa oleifera* accessions

grown in Algeria, Chad and Haiti differed in their total polyphenols (25.5–35.5 mg/g), salicylic acid (1.4–3.3 µg/g) and ferulic acid (66.1–96.9 µg/g) contents (Leone *et al.*, 2015a). The variation of these SPMs was linked to genotype and environment interactions. A study conducted on three varieties of *Labisa pumila* Benth leaf extracts showed different antioxidant activities (Akula & Ravishankar, 2011). Hence, the biosynthesis processes of SPMs are strictly controlled by genes (Li *et al.*, 2020; Szepesi, 2021), which influence the antimethanogenic potentials of the plants. Accessions of Moringa species may have different adaptability and growth performances, which affect the gas production characteristics of feeds when their extracts are used as an additive in ruminants.

The gas production and antimethanogenic potential of the Moringa accession extracts may also be associated with their agronomic and growth performances. One study stated that there was an increased accumulation of SPMs with higher biomass, stem diameter, root diameter and plant height in woody and perennial medicinal plants (Li *et al.*, 2020). This may result in better antimethanogenic potential during their application in rumen modulation. Hence, determining the optimal harvesting stage may help to obtain the highest metabolite composition, yield of SPMs and bioactivity, including the antimethanogenic activity specific to Moringa plants. Phenolics and flavonoids are abundant in Moringa and showed various bioactivities (Kashyap *et al.*, 2022). Several studies reported phenolic and flavonoid compounds as the main active components against bacteria, protozoa and fungi (Dos Santos *et al.*, 2018; Bouarab-Chibane *et al.*, 2019; Shamsudin *et al.*, 2022). Similarly, strong antimicrobial activities were also obtained from SPMs with functional groups of phenolic acids, phenolics and terpenoids (Zhang *et al.*, 2011; Leone *et al.*, 2015b; Haque, 2018), which thereby affect the antimethanogenic potential of the extracts in different ways. The inclusion of some commercial flavonoids (quercetin, flavone, myricetin, rutin, kaempferol, and naringin) also exhibited 5–9 ml/g DM CH_4 inhibition, although most of them affected feed digestibility negatively, except for quercetin and naringin (Oskoueian *et al.*, 2013). Some phenolic acids (i.e. caffeic acid, *p*-coumaric acid, trans-cinnamic acid, and catechin hydrate acids) also inhibited CH_4 production with no significant effect on total gas, VFAs profile and pH (Giuburunca *et al.*, 2014). These imply that the antimethanogenic potentials of Moringa can be correlated with total phenolics and total flavonoid contents. However, the relationship between gas production characteristics and antimethanogenic potential with its agronomic traits and the contents of total phenolics and total flavonoids

has been poorly understood. Thus, generating such information for Moringa is crucial to identify more productive varieties of Moringa accessions for specific areas and to increase its antimethanogenic efficacy with a co-benefit of improving feed digestibility.

Thus, the current study was conducted mainly to investigate whether the agronomic traits, total phenolic and total flavonoid contents of Moringa accessions could be different when they grew in the same environment because of genetic variation, which affects the *in vitro* fermentation characteristics of ruminant feed. This would increase our understanding of influencing factors to develop a consistent Moringa leaf extract product to mitigate CH_4 from ruminants. However, to the best of the author's knowledge, variations in plant growth and agronomic traits, total phenolics and total flavonoids have not been associated with the gas production and antimethanogenic potential of Moringa accessions in previous studies. Therefore, this study evaluated the relationships of agronomic traits and SPMs contents of Moringa accessions grown in the subtropical climate of Pretoria, South Africa with *in vitro* gas production characteristics of *Eragrostis curvula* hay incubated with Moringa accession leaf extracts.

2.2 Materials and methods

2.2.1 Plant growth and agronomic data measurement

Ten *M. oleifera* accessions and one *M. stenopetala* accession were imported from the gene bank of the International Centre for Research in Agroforestry (ICRAF) in Kenya (Table 2.1). In addition, the seeds of one *M. oleifera* accession were collected from a private farmer in Pretoria, South Africa. Seedlings of the Moringa accessions were raised in a glasshouse at the University of Pretoria Experimental Farm. Seeds were sown on seedling trays in the glasshouse on 27 August 2018 and most of them germinated from 14 to 28 days after sowing. Moringa seedlings can be transplanted within 3–6 weeks of germination (Leone *et al.*, 2015b). Thus, vigorous seedlings were transplanted to the field at the Roodeplaat experimental site of the ARC in the subtropical climate of Pretoria, South Africa, located at 25°44′30″ S, 28°15′30″ E on 18 October 2018, that is, 4–6 weeks after germination. The experimental site was prepared before the transplanting date, that is, cleaned, fenced, prepared to a fine tilth, and blocked into three blocks that consisted of 12 plots per block with a plot size of 8 m^2 (2 m × 4 m). Thus, each of the study accessions was placed randomly

on a plot in the block and grown in triplicate following a randomized complete block design (RCBD). Each plot comprised 15 seedlings in three rows, which are five seedlings per row, with a 1 m plant distance between rows and within the row. There was a 1.5 m distance between the plots in a block. The plants were irrigated three times a week using a sprinkler and cleaned continuously for weeds. The seedling survival rate, leaf yield, plant height, canopy diameter, stem diameter, number of primary branches, the score of plant vigour, greenness and chlorosis, and pest and disease incidence traits were recorded for agronomic performance evaluation of these accessions. The data collection techniques of most study parameters were adopted from methods of the International Livestock Research Institute (ILRI) for the 'Evaluation of forage legumes, grasses and fodder trees for use as livestock feed' (Tarawali *et al.*, 1995). The data on agronomic traits were recorded approximately in the fifth month after transplanting to the field, which coincided with the end of the summer season for 2019.

Table 2.1 The germplasm of Moringa accessions imported from the International Centre for Research in Agroforestry in Kenya and collected in Pretoria, South Africa

Species name	Accession code/number	Collection area	Country of origin
Moringa oleifera	A1 (Bulk)	Meru	Kenya
Moringa oleifera	A2 (7229)	Machakos	Kenya
Moringa oleifera	A3 (7633)	Segou	Mali
Moringa oleifera	A4 (7632)	Bamako	Mali
Moringa oleifera	A5 (7627)	NA	Kenya
Moringa oleifera	A6 (3295)	Mbbololo	Kenya
Moringa oleifera	A7 (5536)	Busia	Kenya
Moringa oleifera	A8 (7717)	Ramogi	Kenya
Moringa oleifera	A9 (7316)	Kibwezl	Kenya
Moringa oleifera	A10 (7216)	Ramisi	Kenya
Moringa oleifera	A11 (NA)	Pretoria	South Africa
Moringa stenopetala	MS (NA)	NA	Kenya

NA: not available

The percentage of seedling survival rate (% SSR) was calculated from the total number of seedlings transplanted to the field on the transplanting date. Thus, $\%\,\mathrm{SSR} = \left(\frac{\mathrm{TNSD1}-\mathrm{NSCT}}{\mathrm{TNSD1}}\right) \times 100$, where % SSR is the percentage of plant survival rate; TNSD1 is the total number of seedlings transplanted to the field on day 1; and NSCT is the stand count of the plants at five months from the transplanting date. The data of plant height (metre stick), stem diameter (calliper), canopy diameter (metre stick) and primary branches were taken

from three to five randomly selected individual plants per plot approximately in the fifth month of transplanting to the field. The score of leaf greenness was given to each plot by comparing the plants with 100% green leaves using a scale of 10 (0 to 9) from light green to deep green. The score value of 0 was given for plots containing the light green plants, whereas 9 was given for plots containing the highest deep green plants (Tarawali *et al.*, 1995). Similarly, plant vigour was assessed by considering how healthy they looked in the field, and each of the plots was scored on a scale of 10 (0 to 9), where a scored value of 0 was given for plots containing the least vigorous plants, and 9 was given for plots containing the highest vigorous plants. The accessions were monitored closely at weekly intervals for signs of pests and disease. However, no incidences of disease or pests were observed in the study period.

To determine the leaf yield, all the leaves were harvested approximately in the fifth month of transplanting, except for three to four leaves left to promote the growth of leaves for the next harvest. Two samples per plot or six samples per accession, 72 total samples, were collected for the study. The fresh leaves were weighed immediately after harvest and converted to kg of fresh leaf yield per ha. A subsample of the leaf was also taken to determine its moisture content to determine kg of leaf DM yield per ha. The subsample was oven-dried at 105 $^{\circ}$C for 24 hours until a constant weight was attained, reweighed (DWss) and estimated the dry weight of the leaf. Thus, $\mathrm{DWL} = \frac{\mathrm{DWss}}{\mathrm{FWss}} \times \mathrm{FWt}$, where DWL is the dry weight of the leaf per plot in g; DWss is the dry weight of the leaf subsample in g; FWss is the fresh weight of the leaf subsample in g and FWt is the total fresh weight of the leaf per plot in g.

2.2.2 Plant extraction and determination of total phenolic and total flavonoid contents

The harvested leaves were dried with a freeze drier for five days and milled with a 1-mm sieve in a milling machine. Approximately 50 g of the dried leaf powder was suspended in 500 ml methanol at a ratio of 1:10 *w/v* (Eloff, 1998) for 96 hours in a shaker. The mixture was filtered through a 150-µm aperture sieve (Vickers, Durban, South Africa) and placed in fume chambers for 48 hours until semidried. The semidried crude extracts were further dried using a freeze-drier until a constant weight was attained and kept in the refrigerator at 4 °C until further use.

One gram of this crude extract was taken from each sample and macerated with 15 ml of 50% methanol to determine the total phenolic content, which was determined according to the Folin–Ciocalteu method (Madaan *et al.*, 2011; Shah *et al.*, 2016). The solution was extracted three times with the same amount of 50% methanol, filtered with Whatman paper, and the volume of the volumetric flask was brought up to 100 ml with 50% methanol. To prepare a gallic acid standard, 25 mg of gallic acid was dissolved in 100 ml of 50% methanol (250 µg/ml). The solution containing the standard was further diluted to 12.5, 25, 50, 100, 150 and 200 µg/ml A 1 ml aliquot of the sample and each dilution of the standard were taken in separate test tubes and diluted with 10 ml of distilled water. Then, 1.5 ml Folin–Ciocalteu reagent was added and incubated at room temperature for 5 min. Four millilitres of 20% (w/w) sodium carbonate (Na_2CO_3) were added and adjusted with distilled water up to the mark of 25 ml, agitated and left for 30 min at room temperature. Each of the samples and the standard were conducted in triplicate, and their absorbance was measured at 765 nm with a UV/VIS spectrophotometer (ANALYTIK JENA AG, Model: SPEKOL 1300, Germany) against blank distilled water. Eventually, the absorbance readings of the samples in the spectrophotometer were calculated with a standard curve and expressed as mg GAE/g of the extract. Thus, $\text{total phenolics (mg GAE/g)} = \frac{\text{GAE} \times \text{D} \times \text{V}}{\text{W}}$, where GAE: gallic acid equivalent (mg/ml); D: dilution factor; V: volume of extract (ml); and W: weight of the plant extract (g) used for the preparation of stock solution (Shah *et al.*, 2016).

The total flavonoid content was determined with an $AlCl_3$ colourimetric assay (Madaan *et al.*, 2011; Bag *et al.*, 2015). Approximately 20 mg of quercetin was dissolved in 100 ml methanol and diluted to 20, 40, 80, 120, 150 and 200 µg/ml with methanol. Aluminium chloride (10%) and 1 M potassium acetate were prepared with distilled water. Then, 0.5 ml of each extract solution and each dilution of the standard were added to separate graduated test tubes in triplicate. To each of the test tubes, 1.5 ml methanol, 0.1 ml aluminium chloride solution, 0.1 ml potassium acetate and 2.8 ml distilled water were added and mixed well. Blanks were prepared similarly by replacing the aluminium chloride solution with distilled water. After 30 min of incubation at room temperature, the absorbance of the mixture was measured at 415 nm against the blank with the UV/VIS spectrophotometer. Then, absorbance readings of the samples in the spectrophotometer were calculated and expressed as mg QE/100 g of the extract. Thus, $\text{total flavonoids (mg QE/g)} = \frac{\text{QE} \times \text{D} \times \text{V}}{\text{W}}$, where QE: quercetin equivalent obtained from the standard curve (mg/ml); D: dilution factor; V:

volume of the extract stock solution (ml); and W: weight of the plant extract (g) (Bag *et al.*, 2015).

2.2.3 Medium preparation and in vitro incubation

As described by Mould *et al.* (2005), buffer, macromineral and micromineral solutions were prepared before the incubation day and kept in the refrigerator at 4 °C (Goering & Van Soest, 1970). Incubation for gas measurement was performed with 120 ml serum bottles fitted with modified needle syringe taps that could be opened and closed. Owing to the limited capacity of the Incoshake/incubator, 24 samples from each block (two samples per plot) in the field were incubated in triplicate bottles. Thus, 78 bottles, including three control and three blanks, were incubated in each run. Hence, the variation in the rumen fluids used in each incubation and the samples because of the blocks in the field coincided and was accommodated by the RCBD. A previous *in vitro* study had been conducted at four doses (25, 50, 75 and 100 mg extract per kg DM) to determine the dose of certain medicinal plant extracts, including *M. oleifera* and recommended 50 mg extract per kg DM feed for effective antimethanogenic activities (Akanmu & Hassen, 2017). They used subsequently this single dose in other studies and obtained good CH_4 inhibition potential (Akanmu, 2018; Akanmu *et al.*, 2020), which was confirmed by our preliminary studies. Thus, the extract was applied at a dose of 50 mg extract per kg DM feed in the study (Akanmu & Hassen, 2017). To prepare the recommended dose, the well-grounded powder of the dried extract was reconstituted with distilled water (5 mg/1000 ml). Then, 4 ml of the reconstituted extract was added to each bottle containing 400 mg *E. curvula* except for the blanks and the control during incubation. Distilled water, buffer, macromineral and micromineral solutions, tryptone and resazurin solution (0.1%; w/v) were mixed early in the morning of the incubation day. The mixture was bathed at 39 °C and bubbled continuously with CO_2 until the incubation process was completed. Rumen fluid was collected from three ruminally cannulated Pinzyl (Pinzgauer cross Nguni) steers adapted to *E. curvula* grass hay for approximately 14 days before the start of the research. The collection was conducted according to the approved protocol by the Animal Ethics Committee of the University of Pretoria, South Africa (No. EC039-18). Rumen fluid was collected before the morning feeding of each incubation day. Then, it was transported into the laboratory with a preheated thermos flask under anaerobic conditions within 10–15 min and placed in a water bath set at 39 °C. L-cysteine and $Na_2S.9H_2O$ were added to the medium 10 to 15 min before the

rumen fluid was added. When the solution was sufficiently reduced and changed to colourless, the rumen fluid was sieved with four layers of cheesecloth and mixed with the medium containing the buffer at a 15:25 (v/v) rumen fluid to medium ratio. Ultimately, 40 ml of this mixture was added to all incubation bottles, including the controls and blanks. The bottles were closed with rubber stoppers and transferred to an incubator set at 39 °C and 120 revolutions per minute (rpm). After the inoculum had been added to all incubation bottles, the taps were opened for a few seconds to release any gas built up during the addition of the inoculum and to set a similar starting point. Six runs, two runs per block, were conducted to determine the total gas and CH_4 production.

In vitro organic matter digestibility was incubated with two modified digestion phase techniques (Tilley & Terry, 1963; Engels & Van der Merwe, 1967). In the first phase of incubation, 200 mg *E. curvula* grass hay containing extracts of the accessions (50 mg extract/kg DM feed), artificial saliva solution, urea and rumen fluid was incubated under anaerobic conditions for 48 hours at 39 °C with test tubes fitted with modified stoppers. In the second phase, an acid and pepsin solution was prepared from 20 ml of 32% HCl and 8 g of pepsin by dissolving them in 2000 ml of distilled water. Then, 20 ml of the prepared acid and pepsin solution was added after gently decanting the liquid on the top of the incubation tubes and incubated for another 48 hours at 39 °C. After 96 hours of the total anaerobic incubation period, the residual plant materials were oven-dried at 100 °C for 18 hours and ashed following a standard procedure (Engels & Van der Merwe, 1967). In the same way, the incubation was repeated independently three times to determine the IVOMD of *E. curvula* hay treated with extracts of the accessions.

2.2.4 Determination of in vitro total gas, methane and organic matter digestibility

The total gas was measured with a pressure transducer attached to a digital data logger at 3, 6, 12, 24 and 48 hours of incubation (Theodorou *et al.*, 1994). The gas produced during incubation in the bottles was released to the transducer, and the value on the digital data tracker was recorded in pound force per square inch (psi) unit. To obtain the cumulative gas pressure readings of the whole incubation period, each time point reading recorded in psi was added and converted to ml. Thus, $V_x = V_j P_{psi} \times 0.068004084$, where V_x is the gas volume in ml at 39 °C in ml; V_j is the headspace of the incubation glass bottle in ml; and P_{psi} is the cumulative pressure recorded by the gas monitor system software (ANKOM, 2014).

Syringes fitted with stopcocks, which corresponded to the incubation bottles, were prepared before the commencement of the research for the collection of CH_4 samples. Similar to total the gas pressure reading, the CH_4 samples were collected at 3, 6, 12, 24 and 48 hours. Methane concentration in each sample was analysed by gas chromatography (GC) equipped with a flame ionization detector. Methane samples were injected into the GC by a pull and push method, and the area recorded from the GC was converted into parts per million (ppm) using the CH_4 standard curve. The CH_4 concentration captured in the headspace was converted to ml by multiplying the total gas produced (ml) by the per cent CH_4 in the sample: CH_4 (ml) = total gas produced (ml) × % CH_4 concentration. Blanks were used to correcting the total gas and CH_4 produced by the inoculums. Finally, the total gas and the CH_4 produced within 48 hours of incubation from the substrate feed treated with leaf extracts of the accessions were expressed as ml/g DM incubated feed (Ghamkhar *et al.*, 2018).

The IVOMD was determined following the two-digestion-phase *in vitro* incubation techniques as described above in section 2.2.3. Thus, the residual plant materials left after 96 hours of total anaerobic incubation were oven-dried at 100 °C for 18 hours, measured, and ashed. The percentage of IVOMD was calculated as $\%\ \text{IVOMD} = \frac{\text{OMFS}-(\text{UDR}-\text{Blank})}{\text{OMFS}} \times 100$, where IVOMD: *in vitro* organic matter digestibility (%); OMFS: the organic matter of the feed sample; UDR: undigested residue.

2.2.5 Statistical analyses

A randomized complete block design was applied for the statistical analyses of the agronomic traits and *in vitro* fermentation characteristics to accommodate the variations because of the difference in rumen fluids used in each incubation and the sample variation because of the block effect in the field. Before one-way analysis of variance (ANOVA), the data were checked for assumptions of the ANOVA. Significant differences were determined using treatment as a fixed effect: $Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$, where Y_{ij} is the response of treatment i observed in block j; μ is the grand mean; α_i is the effect of i^{th} treatment; β_j is the effect of the j^{th} block; and ε_{ij} is the random error. Means were compared using the Tukey test when the F test of the parameters showed significant differences among accessions (P <0.05). Correlation, multivariate principal component analysis (PCA) and hierarchical cluster analysis (HCA) were also carried out to determine the relationships of agronomic traits and SPMs with the *in vitro* fermentation characteristics of accessions. SAS version 9.4 (SAS,

2013) and PAST free software (Hammer *et al.*, 2001) were used to conduct the statistical analyses in the study.

2.3 Results

2.3.1 Agronomic performances of Moringa accessions during the establishment year

The agronomic performances of the Moringa accessions at five months of the establishment year are illustrated in Table 2.2. The accessions were significantly (P <0.01) different in their seedling survival rate in the field and ranged from 45% in *M. oleifera* A10 to 78% in A2. The accession collected from a private farmer in Pretoria (A11), South Africa, was expected to show the highest survival rate. However, some accessions collected in various areas of Kenya (A2, A5, and A8) and Mali (A3 and A4) exhibited equal or higher seedling survival rates than A11. *Moringa stenopetala* showed a relatively poor survival rate (59%) compared with most of the *M. oleifera* accessions. Most of the plant agronomic traits of the accessions, such as canopy diameter, plant height, number of primary branches and fresh and dry leaf yield, varied significantly *(P* <0.05) except for the stem diameter and chlorosis score. In this stage of growth, *M. oleifera* A3 (188 cm), A2 (164 cm), A4 (160 cm) and A5 (166 cm) were higher than other accessions, whereas A10 (105 cm) from *M. oleifera* and *M. stenopetala* (47 cm) were the shortest among the accessions in plant height (Table 2.2). *Moringa oleifera* A1, A2, A3, A5 and A8 revealed significantly higher leaf yields than the other accessions. In terms of the amount of fresh leaf yield, the highest yield was obtained from A2 (2981 kg/ha, 828 kg/ha), followed by A5 (2149 kg/ha), A3 (2144 kg/ha), A8 (2107 kg/ha) and A1 (2063 kg/ha). Similarly, *M. oleifera* A2 (828 kg/ha) showed the highest amount of dry leaf yield, followed by A3 (608 kg/ha), A8 (604 kg/ha), A5 (590 kg/ha) and A1 (553 kg/ha). However, all these accessions had equivalent amounts of leaf yield statistically.

Table 2.2 The mean values of agronomic and growth performances of Moringa accessions at five months of the establishment year

Accession number/name/code	PH (cm)	SD (cm)	CD (cm)	FLY (kg/ha)	DLY (kg/ha)
Bulk (A1)	153.9 ^{A}	2.3	72.9 ^{AB}	2063.8 ^{AB}	553.2 ^{AB}
07229 (A2)	163.6 ^{A}	2.8	78.8 ^{AB}	2981.3 ^{A}	827.6 ^{A}
07633 (A3)	188.1 ^{A}	3	105.3 ^{A}	2144.6 ^{AB}	608.4 ^{AB}
07632 (A4)	160.3 ^{A}	2.6	95.7 ^{AB}	1573.3 ^{AB}	470.5 ^{AB}
07627 (A5)	166.2 ^{A}	2.5	82.6 ^{AB}	2149.2 ^{AB}	589.8 ^{AB}
03295 (A6)	127.6 ^{AB}	1.9	68.2 ^{AB}	590.4 ^{B}	168.2 ^{B}
05536 (A7)	125.9 ^{AB}	2	69.1 ^{AB}	1458.3 ^{AB}	423.0 ^{AB}
07717 (A8)	150.8 ^{A}	2.5	74.5 ^{AB}	2107.5 ^{AB}	604.1 ^{AB}
07316 (A9)	138.3 ^{AB}	2.1	74.3 ^{AB}	1697.9 ^{AB}	468.2 ^{AB}
07216 (A10)	105.1 ^{AB}	1.6	58.9 ^{B}	805.0 ^{B}	220.2 ^{B}
Pretoria (A11)	146.7 ^{A}	2.1	69.1 ^{AB}	1975.4 ^{AB}	552.4 ^{AB}
M. stenopetala (MS)	46.7 ^{B}	1.7	60.0 ^{B}	1452.9 ^{AB}	421.2 ^{AB}
p value	0.002	0.073	0.009	0.006	0.009
Accession number/name/code	**NPB**	**PVS**	**PGS**	**PCS**	**SS (%)**
Bulk (A1)	0.5 ^{B}	5.3 ^{AB}	5.67 ^{AB}	2.7	52.5 ^{AB}
07229 (A2)	1.4 ^{AB}	7.0 ^{A}	6.00 ^{AB}	2.7	78.1 ^{A}
07633 (A3)	2.7 ^{A}	7.7 ^{A}	6.33 ^{A}	3.7	74.0 ^{AB}
07632 (A4)	0.8 ^{B}	7.0 ^{A}	6.33 ^{A}	4	75.2 ^{AB}
07627 (A5)	0.2 ^{B}	6.3 ^{A}	5.00 ^{AB}	3.3	74.1 ^{AB}
03295 (A6)	0.7 ^{B}	5.7 ^{AB}	5.33 ^{AB}	2.7	61.9 ^{AB}
05536 (A7)	0.5 ^{B}	4.7 ^{AB}	5.33 ^{AB}	3.3	51.9 ^{AB}
07717 (A8)	0.7 ^{B}	7.0 ^{A}	6.33 ^{A}	3.3	75.4 ^{AB}
07316 (A9)	1.1 ^{B}	5.7 ^{AB}	6.33 ^{A}	4	58.2 ^{BC}
07216 (A10)	0.3 ^{B}	4.3 ^{AB}	5.67 ^{AB}	3	44.9 ^{B}
Pretoria (A11)	0.9 ^{B}	6.0 ^{AB}	5.67 ^{AB}	4	73.7 ^{AB}
M. stenopetala (MS)	1.3 ^{AB}	2.0 ^{B}	4.33 ^{B}	1.7	59.2 ^{AB}
p value	0.001	0.006	0.027	0.328	0.0053

The mean values with different superscript letters within a column are significantly different at $P < 0.05$. PH: plant height; SD: stem diameter; CD: canopy diameter; NPB: number of primary branches; FLY: fresh leaf yield; DLY: dry leaf yield; PVS: plant vigour score; PGS: plant greenness score; PCS: plant chlorosis score; SS: seedling survival rate (%)

The scored values of plant vigour and greenness were also significantly ($P < 0.05$) different among the accessions (Table 2.2). *Moringa oleifera* A2, A3, A4, A5, A8 and A11 exhibited significantly higher scored values of plant vigour, which indicates their healthiness and appearance of their overall performances in the field. The *M. oleifera* A10 and *M. stenopetala* had the lowest plant vigour performances in the field. Most of the accessions that showed superior plant vigour performance also had higher greenness values. However, some accessions, such as A9 and A10, which scored lower on plant vigour, exhibited better

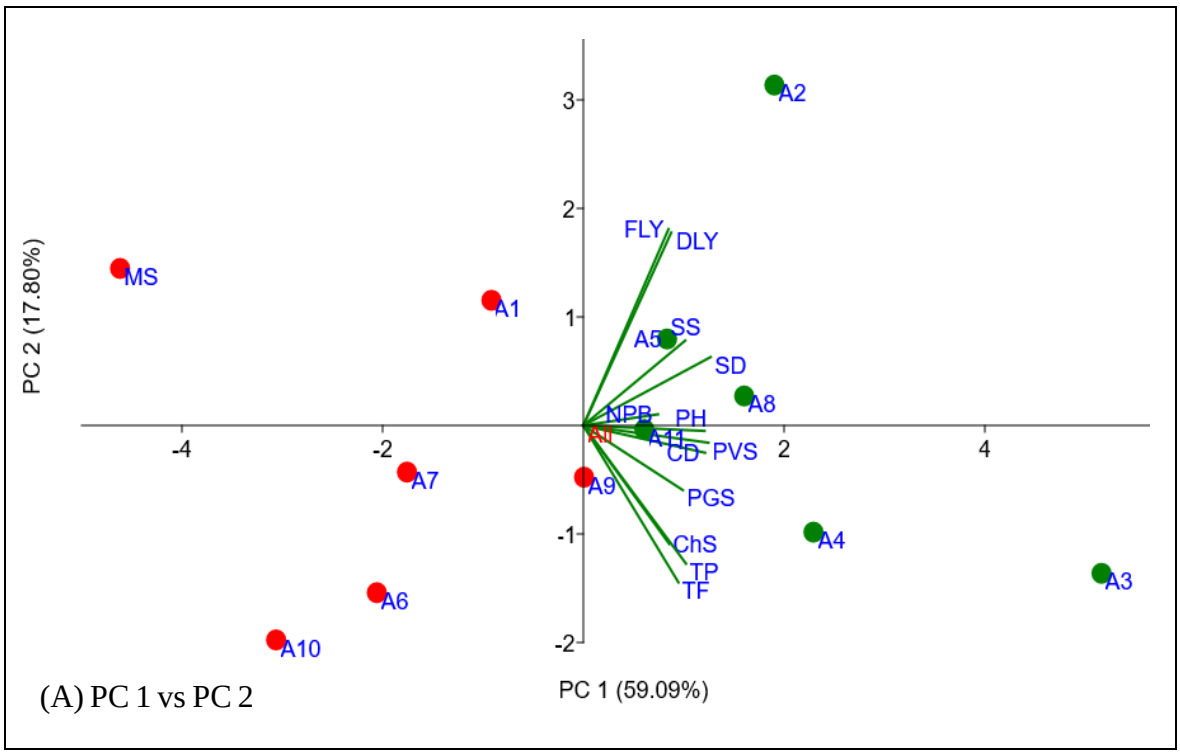
greenness values. The problem of chlorosis was also noted in most of the accessions, starting from the fourth month of transplanting and gradually increasing from the bottom (older leaves) to upwards (young leaves), although severity did not vary significantly (P >0.05). However, identifying the cause of chlorosis was not within the scope of the current study and thus not known, though ageing of the leaves might be the cause. Hence, optimizing the leaf harvesting age of Moringa plant growth might be essential to obtain quality leaves with increased leaf production. In this study, diseases and pests were also assessed regularly. However, no incidence was observed within five months of the experimental period. Generally, *M. oleifera* A2 and A3 seemed to be superior in most of the agronomic traits, whereas *M. oleifera* A6 and A10 and *M. stenopetala* showed inferior performances in most of the parameters (Table 2.2).

The principal component loadings of the agronomic traits and scores of the accessions obtained from the PCA are presented in Table 2.3 and Figure 2.1. The first three major PCs explained 87% of the total variation among the accessions. Principal component analysis clarified the variation of accessions in terms of their agronomic performances in the field. Thus, PC 1 alone accounted for 59% of the total variation and was correlated positively with all agronomic traits of the study. However, the plant height, stem diameter, canopy diameter and score of plant vigour were the major parameters responsible for PC 1. Principal components 2 and 3 were responsible for 18% and 10% of the total variation in accessions, respectively. Most of the parameters were correlated negatively with PC 2, although dry leaf yield, fresh leaf yield, number of primary branches, stem diameter, and seedling survival correlated positively. The fresh leaf yield (51%), dry leaf yield (50%) and total flavonoid content (−40%) were the main parameters responsible for PC 2. Figure 2.1 also illustrates the scatter dot plots of the accessions and biplot distribution of the agronomic parameters recorded at five months of the establishment year. Thus, the score plot distribution of the accessions seemed to divide them into two main groups, as referenced to PC 1 with 59% variation. The vectors of all agronomic traits and total phenolic and total flavonoid contents lay in the same direction with the accessions represented with green dots, and opposite to the accessions denoted by red dots. Hence, accessions marked with green dots (i.e. A2, A3, A4, A5, A8 and A11) showed higher performances in most of these agronomic traits, total phenolic and total flavonoid contents than those accessions marked with red dots.

Table 2.3 The principal component loadings of the agronomic traits of the Moringa accessions recorded at five months of the establishment year

Agronomic Traits	PC 1 × 100	PC 2 × 100	PC 3 × 100
Canopy diameter	33.9	−7.0	16.8
Chlorosis score	23.9	−30.5	−41.2
Dry leaf yield	24.5	49.7	−1.7
Fresh leaf weight	23.7	50.6	−3.9
Number of primary branches	20.8	2.9	66.6
Plant greenness score	27.7	−16.7	−29.4
Plant height	33.7	−1.4	−28.0
Plant vigour score	34.8	−4.4	−23.9
Stem diameter	35.4	17.6	4.4
Seedling survival rate	28.4	21.9	7.2
Total flavonoids	26.5	−40.4	25.0
Total phenolics	28.6	−35.6	25.7
Eigenvalue	7.1	2.1	1.2
% variance	59.1	17.8	10.1

PC: principal component



(A) PC 1 vs PC 2

Figure 2.1 *Cont.*

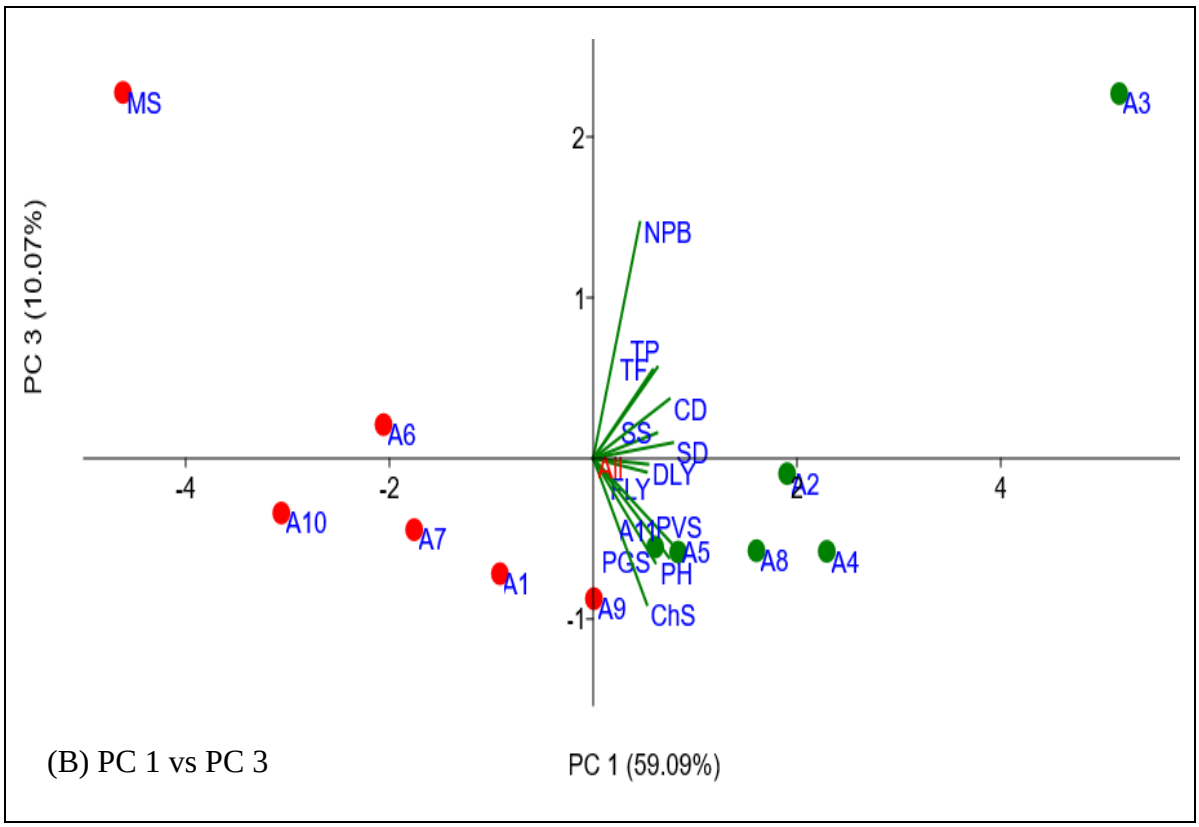


Figure 2.1 Principal component analysis scatter plots of the Moringa accessions and biplot of agronomic traits at five months of the establishment year

CD: canopy diameter; PH: plant height; NPB: number of primary branches; SD: stem diameter; FLY: fresh leaf yield; DLY: dry leaf yield; PVS: plant vigour score; PGS: plant greenness score; ChS: plant chlorosis score; TP: total phenolics; TF: total flavonoids. The green and red dots can be used to differentiate the accessions to higher and lower agronomic traits in their field performances, respectively. (A) Refers to the principal component analysis results with the principal component 1 and principal component 2, whereas (B) shows the results of principal component analysis using the principal component 1 and principal component 3.

2.3.2 In vitro total gas, methane production and organic matter digestibility

On a DM basis, *E. curvula* grass hay incubated in this study contained 93.1% DM, 3.9% ash, 8.6% crude protein content (CP), 80.0% neutral detergent fibre (NDF), 41.0% acid detergent fibre (ADF), and 8.3% acid detergent lignin (ACDL). Hence, the total gas production (TGP) from anaerobically incubated *E. curvula* hay treated with leaf extracts of all Moringa accessions differed significantly (P <0.01) from 160.0 ml/g DM to 172.6 ml/g DM (Table 2.4). However, this result was lower than the TGP from untreated *E. curvula hay* with any extract, hereafter referred to as 'control' (203.8 ml/g DM). The feed fermentation kinetics were not affected negatively by the extracts, which was proved with their equal or higher IVOMD compared with the control. Consistently, the total CH_4 produced from the substrate feed treated with these accession extracts also varied from 4.5 ml/g DM in *M. oleifera* A11 to 5.2 ml/g DM in A1. This result was significantly lower compared with the CH_4 released from the control (6.3 ml/g DM). The CH_4/IVOMD of the substrate feed and their percentage of antimethanogenic potential were also substantially different among the accessions. Hence, the percentage of CH_4 inhibition recorded among the accessions ranged between 18% (A1) and 29% (A11). However, six *M. oleifera* accessions (i.e. A1, A2, A6, A7, A9 and A10) showed statistically similar and lower methane inhibition potentials compared with other five *M. oleifera* accessions (i.e. A3, A4, A5, A8, and A11).

Table 2.4 The *in vitro* fermentation characteristics of *Eragrostis curvula* hay incubated with leaf extracts from Moringa accessions

Accession name/number /code	Mean value ± SEM				
	TGP (ml/g DM)	CH_4 (ml/g DM)	CH_4/IVOMD (%)	IVOMD (%)	CH_4 inhibition (%)
Bulk (A1)	170.5 ± 1.2 ^{BCD}	5.2 ± 0.1 ^{B}	8.8 ± 0.4 ^{BCD}	59.3 ± 0.4 ^{A}	17.9 ± 2.0 ^{B}
07229 (A2)	172.7 ± 1.6 ^{B}	5.2 ± 0.2 ^{B}	8.7 ± 0.4 ^{DE}	60.0 ± 0.9 ^{A}	18.2 ± 2.0 ^{B}
07633 (A3)	160.8 ± 2.2 ^{E}	4.6 ± 0.2 ^{C}	7.6 ± 0.4 ^{F}	60.03 ± 1.0 ^{A}	28.4 ± 2.1 ^{A}
07632 (A4)	164.4 ± 0.5 ^{CDE}	4.7 ± 0.1 ^{C}	8.7 ± 0.2 ^{DE}	54.2 ± 0.7 ^{BC}	25.9 ± 2.0 ^{A}
07627 (A5)	163.7 ± 2.8 ^{DE}	4.7 ± 0.2 ^{C}	8.8 ± 0.2 ^{CD}	53.7 ± 0.9 ^{C}	26.0 ± 2.3 ^{A}
03295 (A6)	169.9 ± 3.4 ^{BCD}	5.1 ± 0.2 ^{B}	9.4 ± 0.4 ^{BCD}	53.8 ± 0.5 ^{C}	20.6 ± 3.4 ^{B}
05536 (A7)	170.4 ± 2.7 ^{BCD}	5.0 ± 0.2 ^{B}	9.4 ± 0.4 ^{BC}	53.7 ± 0.9 ^{C}	20.9 ± 2.7 ^{B}
07717 (A8)	164.2 ± 1.6 ^{CDE}	4.7 ± 0.2 ^{C}	8.1 ± 0.2 ^{EF}	58.4 ± 0.5 ^{A}	26.6 ± 2.3 ^{A}
07316 (A9)	170.6 ± 1.9 ^{BC}	5.0 ± 0.1 ^{B}	9.2 ± 0.2 ^{BCD}	54.3 ± 1.1 ^{BC}	21.4 ± 2.0 ^{B}
07216 (A10)	170.2 ± 1.6 ^{BCD}	5.0 ± 0.2 ^{B}	9.2 ± 0.4 ^{BCD}	54.7 ± 1.3 ^{BC}	20.9 ± 2.8 ^{B}
Pretoria (A11)	164.1 ± 0.4 ^{CDE}	4.5 ± 0.2 ^{C}	7.9 ± 0.3 ^{F}	57.6 ± 1.0 ^{A}	29.1 ± 2.6 ^{A}
M. stenopetala	175.0 ± 1.1 ^{B}	5.2 ± 0.2 ^{B}	9.5 ± 0.2 ^{B}	54.7 ± 0.9 ^{BC}	18.8 ± 2.1 ^{B}
Control	203.8 ± 5.3 ^{A}	6.3 ± 0.2 ^{A}	11.7 ± 0.4 ^{A}	54.7 ± 0.4 ^{BC}	–

The mean values with different superscript letters within a column are significantly different (P <0.01)**.** CH_4: methane; IVOMD: *in vitro* organic matter digestibility; SEM: standard error of the mean; TGP: total gas produced

The IVOMD of the *E. curvula* hay fermented with extracts of these Moringa accessions was considerably different (P <0.01) and ranged from 54% (A5) to 60% (A3) (Table 2.4). The IVOMD obtained from the *E. curvula* hay fermented with extracts of these accessions was higher than or equal to that of the control (55%). Based on their effect on IVOMD, these accessions seemed to be divided into two distinct groups, as indicated in Figure 2.2. Thus, the five *M. oleifera* accession extracts of A1 (59%), A2 (60%), A3 (60%), A8 (59%) and A11 (58%) improved significantly (P <0.01) the IVOMD. The other accessions, such as A4 (54.2%), A5 (53.7%), A6 (53.8%), A7 (53.7%), A9 (54.3%), A10 (54.7%) and *M. stenopetala* (54.7%), showed equal IVOMD with the control, *E. curvula* hay (54.7%) incubated without any plant extract additive. *Moringa oleifera* A1 and A2 established a superior IVOMD to the control. However, their antimethanogenic potentials were lower than those of most of the accessions. On the other hand, A4 and A5 exhibited higher CH_4 inhibition but did not improve the IVOMD of the feed. None of the accessions showed a decrease in IVOMD compared with the control, which infers that none of the accession extracts affected the feed fermentation kinetics negatively. However, five of these

accessions improved the IVOMD of *E. curvula* hay compared with the control, and only A3, A8 and A11 provided the two benefits of higher CH_4 inhibition potential and improved feed OMD (Figure 2.2). *Eragrostis curvula* hay treated with *M. stenopetala* extracts exhibited higher total gas and CH_4 production with lower CH_4 inhibition potential and the IVOMD recorded for *M. stenopetala* plant extract compared with most *M. oleifera* accession extracts. This shows the presence of wide genetic variability within the Moringa accessions and between the species, which eventually influences the effect of extracts on their anti-methanogenic potential and feed digestibility.

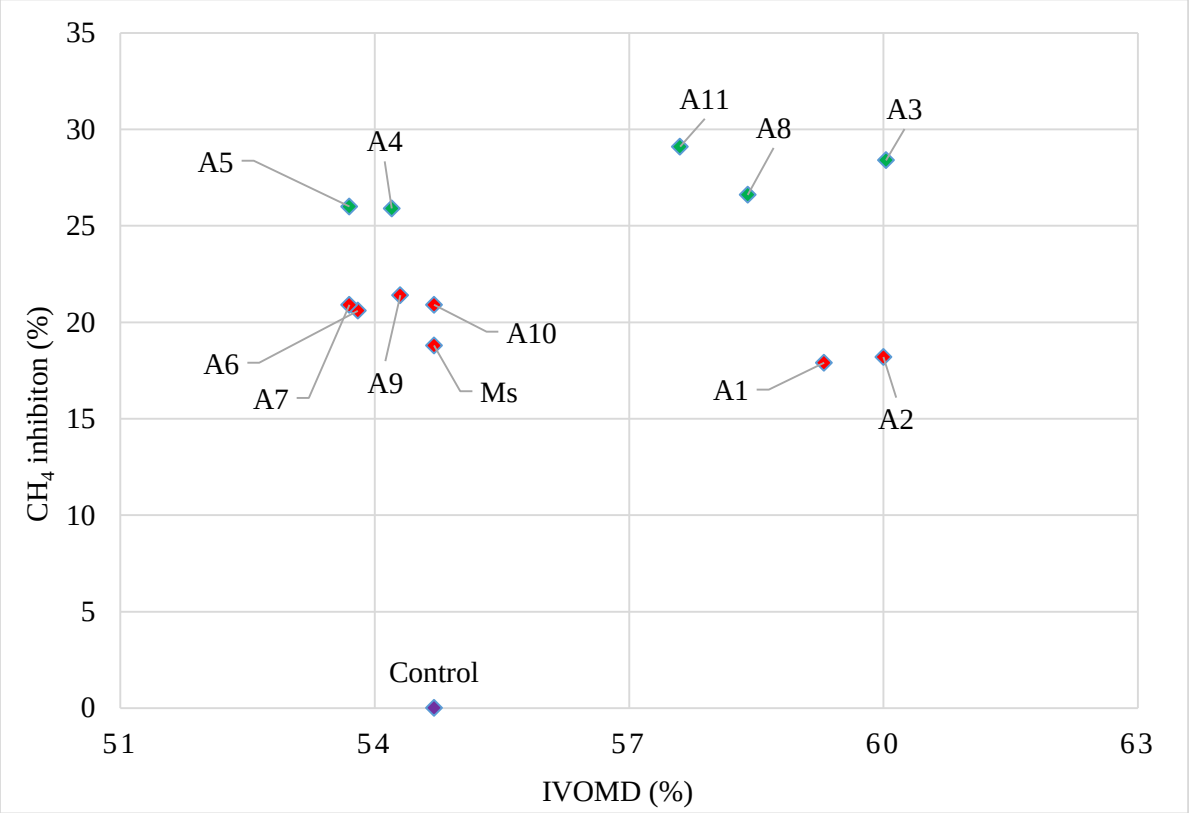


Figure 2.2 Scatter diagram of methane inhibition vs *in vitro* organic matter digestibility of *Eragrostis curvula* hay fermented with the leaf extracts of Moringa accessions

Where A1, A2, A3, etc., indicate the various *Moringa oleifera* accessions and MS is the *Moringa stenopetala*; green diamonds show higher methane inhibitor accessions, whereas red diamonds indicate lower methane inhibitor accessions; and IVOMD is the *in vitro* organic matter digestibility of the substrate feed in the study.

2.3.3 Total phenolic and total flavonoid contents

The accessions differed significantly (P <0.01) in total phenolic and total flavonoid contents in the study (Table 2.5). The highest total phenolic content was obtained in *M. oleifera* A3 (4698 GAE/100 g extract), followed by A8 (3998 GAE/100 g extract), A4 (3927 GAE/100 g extract) and A11 (3800 GAE/100 g extract), whereas *M. oleifera* A2 (3489 mg GAE/100 g) maintained the lowest total phenolics. The total flavonoid content also varied significantly from 1834 mg QE/100 g extract (A1) to 2915 mg QE/100 g extract (A3). *Moringa oleifera* A3 also showed the highest total flavonoid content, followed by A4 (2391 mg QE/100 g extract), A5 (2311 mg QE/100 g extract), A11 (2275 mg QE/100 g extract) and A8 (2250 mg QE/100 g extract). *Moringa oleifera* A3, A4, A8 and A11 exhibited higher contents of the SPMs referred in this study, whereas A1 and A2 of *M. oleifera and M. stenopetala* were inferior in these metabolites. Along with the *M. oleifera* A3, A8 and A11, A4 and A5 maintained relatively superior total flavonoid and total phenolic contents and antimethanogenic potential compared with other accessions. However, A4 and A5 did not improve the OMD of the substrate feed. In contrast, A1 and A2 recorded lower total phenolic and total flavonoid contents, whereas they improved IVOMD compared with the control. This result infers that IVOMD is not only dependent on the total phenolic and total flavonoid contents, but might be affected by other SPMs in a specific way. However, these SPM contents correlated significantly with total gas, CH_4, CH_4/IVOMD production and CH_4 inhibition potentials of the accessions. Hence, the CH_4 inhibition potential of accessions showed significant positive Pearson correlation coefficients of 0.71 and 0.62 with total flavonoid and total phenolic contents, respectively (Table 2.5).

Table 2. 5 Total phenolic and total flavonoid contents of Moringa accession extracts (mean ± SEM) and their Pearson correlation coefficients with major *in vitro* fermentation characteristics

Accessions number/name	Total phenolics (mg GAE/g extract)	Total flavonoids (mg QE/g extract)
Bulk (A1)	35.1 ± 0.8 ^{CD}	18.3 ± 0.7 ^{D}
07229 (A2)	34.9 ± 2.6 ^{CD}	18.9 ± 1.7 ^{CD}
07633 (A3)	47.0 ± 0.6 ^{A}	29.2 ± 0.7 ^{A}
07632 (A4)	39.3 ± 0.7 ^{BC}	23.9 ± 0.4 ^{B}
07627 (A5)	36.7 ± 1.2 ^{BCD}	23.1 ± 1.2 ^{B}
03295 (A6)	36.6 ± 0.9 ^{BCD}	21.6 ± 0.8 ^{BCD}
05536 (A7)	35.9 ± 1.6 ^{BCD}	21.0 ± 0.4 ^{BCD}
07717 (A8)	40.0 ± 0.9 ^{B}	22.5 ± 1.3 ^{B}
07316 (A9)	35.8 ± 0.1 ^{BCD}	20.8 ± 0.8 ^{BCD}
07216 (A10)	37.7 ± 1.0 ^{BC}	21.9 ± 1.5 ^{BC}
Pretoria (A11)	38.0 ± 0.9 ^{BC}	22.8 ± 1.1 ^{B}
M. stenopetala (MS)	32.7 ± 1.8 ^{D}	18.3 ± 1.4 ^{D}
Pearson correlation coefficients (*2-tailed*)		
Total gas	−0.71 *	−0.73 *
CH_4	−0.62 *	−0.71 *
% CH_4 inhibition	0.62 *	0.71 *
IVOMD	0.32	0.11
CH_4/IVOMD	−0.67 *	−0.62 *

The means of accessions with different superscript letters within a column are significantly different at P <0.01. The Pearson correlation coefficients with superscript * indicate the presence of significant correlations between fermentation parameters and the metabolites at P <0.01. IVOMD: *in vitro* organic matter digestibility; QE: quercetin equivalent; GAE: gallic acid equivalent

2.3.4 Associating agronomic traits of Moringa accessions with in vitro fermentation characteristics

The Pearson correlation analysis results for agronomic traits with the *in vitro* fermentation characteristics of *E. curvula* hay treated with the Moringa accession plant extracts are presented in Table 2.6. Total gas was correlated with most agronomic traits, such as canopy diameter, plant seedling survival, plant height, stem diameter, vigour and plant greenness negatively and significantly (P <0.05), but not with leaf yield and the number of primary branches of the accessions (Table 2.6). However, the total CH_4 produced (negatively) and % CH_4 inhibition (positively) showed a significant correlation only with the seedling survival rate and leaf greenness score of the plants, and most agronomic traits were negatively and significantly associated with the CH_4/IVOMD, except for the chlorosis score

(P <0.05). The IVOMD of the tested *E. curvula* hay was also positively and significantly correlated with the fresh and dry leaf yield, the number of primary branches, plant height, and stem diameter of the accessions at P <0.05. The total flavonoid and phenolic contents of the accessions were also correlated positively with several agronomic traits, such as canopy diameter, the number of primary branches, plant height, stem diameter, as well as the score of plant vigour and greenness (P <0.05). Hence, this correlation analysis generally showed a positive association of the CH_4 inhibition potentials of the accessions with higher agronomic performances of the accessions.

Table 2.6 Correlation of agronomic traits of Moringa accessions with total phenolic and total flavonoid contents and *in vitro* fermentation characteristics of *Eragrostis curvula* hay treated with their extracts (Pearson correlation, 2-tailed)

Agronomic Traits	SPMs		*In vitro* fermentation characteristics				
	TP	TF	TGP	CH_4	IVOMD	CH_4/IVOMD	% CH_4 Inhibition
CD	0.45 **	0.44 **	−0.48 **	−0.31	0.20	−0.37 *	0.30
DLY	0.04	−0.07	−0.21	−0.14	0.42 **	−0.37 *	0.14
FLY	0.03	−0.08	−0.18	−0.11	0.45 **	−0.36 *	0.11
NPB	0.42 *	0.38 *	−0.24	−0.25	0.40 *	−0.42 **	0.25
PCS	0.20	0.25	−0.37 *	−0.24	−0.01	−0.19	0.25
PGS	0.36 *	0.26	−0.43 **	−0.43 **	0.22	−0.47 **	0.43 **
PH	0.43 **	0.36 *	−0.48 **	−0.30	0.36 *	−0.46 **	0.30
PVS	0.45 **	0.43 **	−0.46 **	−0.31	0.32	−0.44 **	0.31
SD	0.33 *	0.26	−0.37 *	−0.24	0.36 *	−0.41 *	0.24
SS	0.29	0.29	−0.37 *	−0.36 *	0.27	−0.46 **	0.36 *

The Pearson correlation coefficients with superscript ** indicate significant correlations at P <0.01, whereas * shows significant correlations at P <0.05. CD: canopy diameter; DLY: dry leaf yield; FLY: fresh leaf yield; IVOMD: *in vitro* organic matter digestibility; NPB: number of primary branches; PCS: plant chlorosis score; PGS: plant greenness score; PH: plant height; PVS: plant vigour score; SD: stem diameter; SPMs: secondary plant metabolites; SS: seedling survival rate; TF: total flavonoids; TGP: total gas production; TP: Total phenolics

The principal component (PC) loadings and scatter plot of Moringa accessions using their different agronomic traits and *in vitro* fermentation parameters are presented in Table 2.7 and Figure 2.3. Thus, the first three major PCs explained approximately 83% of the total variations of the accessions on these parameters. Principal component 1 alone contributed to 57% of the total variation and was positively correlated with most of these parameters.

However, it was correlated negatively with TGP, CH_4 volume, CH_4/TGP and CH_4/IVOMD. Plant height (26%), stem diameter (26%), plant vigour (27%), canopy diameter (26%), TGP (−28%), CH_4 (−26%), CH_4/IVOMD (−28%), % CH_4 inhibition (26%), total phenolics (26%) and total flavonoids (27%) contributed better to PC 1. Principal components 2 and 3 were responsible for about 18% and 8% of the total variation obtained in the PCA, respectively. Principal component 2 correlated negatively with CH_4 inhibition, CH_4/IVOMD, chlorosis score, total phenolic and total flavonoid contents and correlated positively with other parameters. Most of the variation in PC 2 was attributed to dry leaf yield (40%), fresh leaf yield (41%), IVOMD (36%) and CH_4/TGP (30%), whereas PC 3 was highly reliant on CH_4/TGP (39%), plant greenness (45%), plant height (30%) and seedling survival (29%). The PCA elucidated the overall relationship of agronomic traits, total phenolics and total flavonoids of the accessions with the *in vitro* fermentation characteristics of *E. curvula* hay treated with their extracts (Figure 2.3). Thus, the biplots of CH_4 volume, TGP, CH_4/TGP and CH_4/IVOMD were laid in the same direction as the score plot of the accessions, which was characterized by lower CH_4 inhibition potential (A1, A6, A7, A9, A10, MS). However, these biplots were placed opposite to the score plots of accessions (A3, A4, A5, A8 and A11) that showed higher CH_4 inhibition. The vectors of most agronomic traits, % CH_4 inhibition, total phenolics and total flavonoids were laid in the same direction with accessions grouped as higher CH_4 inhibitors (A3, A4, A5, A8, and A11) as referenced to PC1, which infers the positive relationship of the mentioned parameters. Hence, in this PCA, it is apparent that the higher CH_4 inhibition potential obtained from A3, A4, A5, A8 and A11 is positively associated with most agronomic traits (plant height, seedling survival, leaf greenness, plant vigour, canopy diameter and stem diameter) and the total phenolics and total flavonoids contents found in their extracts. These results are accompanied by the results of positive and significant Pearson correlation coefficients of most agronomic traits with the CH_4 produced per unit of IVOMD illustrated in Table 2.6. However, only leaf greenness and seedling survival from agronomic traits (Table 2.6) and total phenolic and total flavonoid contents (Table 2.5) were significantly correlated with the total CH_4 volume produced.

Table 2.7 Principal component loadings of the agronomic traits of the Moringa accessions and *in vitro* fermentation characteristics of *Eragrostis curvula* hay treated with their extracts

Parameters	**PC 1 × 100**	**PC 2 × 100**	**PC 3 × 100**
Canopy diameter	26.2	6.9	20.9
CH_4	−25.6	27.4	22.3
% CH_4 inhibition	25.6	−27.6	−22.0
CH_4/IVOMD	−28.1	−3.0	21.8
CH_4/TGP	−20.7	29.9	38.5
Chlorosis score	21.7	−21.2	24.7
Dry leaf yield	17.5	39.9	−23.2
Fresh leaf yield	16.8	41.0	−22.0
IVOMD	15.8	35.8	−9.4
Number of primary branches	15.9	15.8	−8.2
Plant greenness score	21.2	5.9	45.2
Plant height	26.2	11.5	29.8
Plant vigour score	27.5	8.5	26.1
Stem diameter	26.4	25.0	10.0
Seedling survival rate	23.8	12.2	−29.2
Total flavonoids	25.0	−24.9	8.6
Total gas	−27.8	20.8	3.0
Total phenolics	26.0	−15.1	13.9
Eigenvalue	10.3	3.3	1.4
% variance	57.0	18.2	7.9

PC: principal component; IVOMD: in vitro organic matter digestibility; TGP: total gas produced

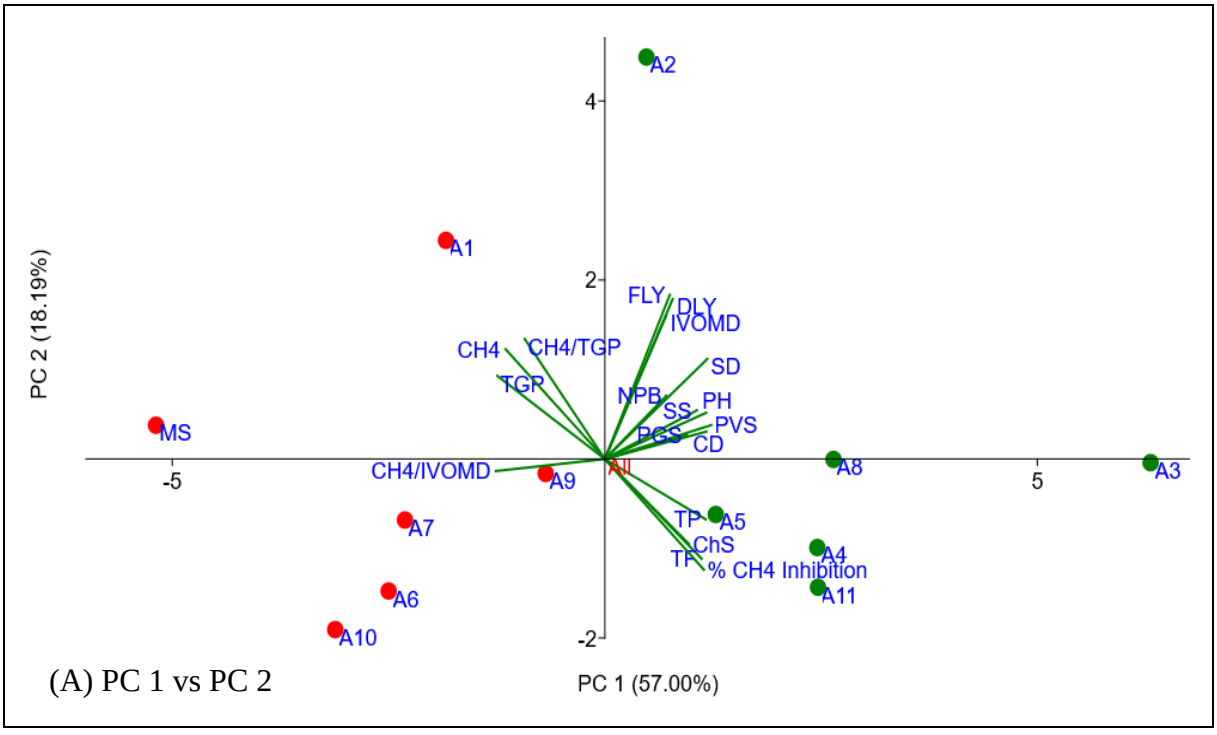


Figure 2.3 *Cont.*

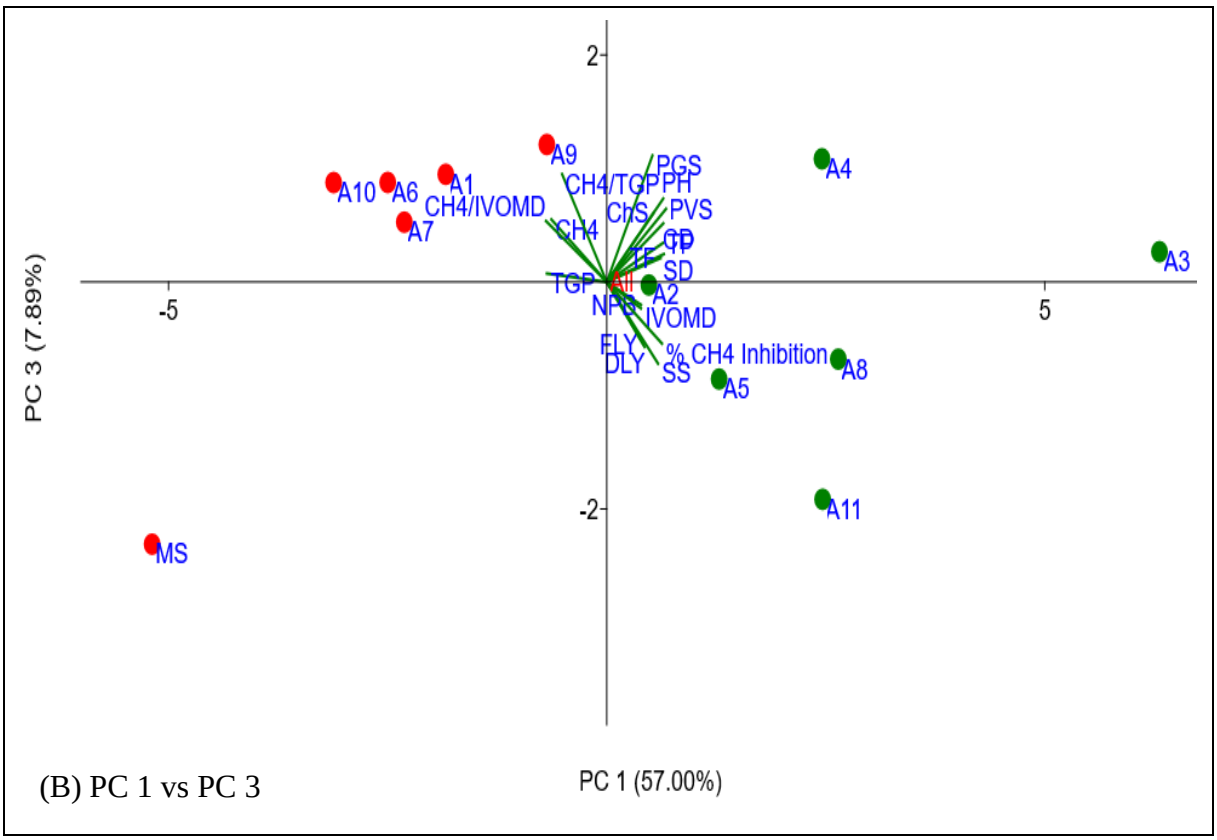


Figure 2.3 Principal component analysis scatter plots of the Moringa accessions, which show the relationship of the different agronomic traits with the *in vitro* fermentation characteristics

Where CD: canopy diameter; CH_4: methane; DLY: dry leaf yield; FLY: fresh leaf yield; IVOMD: *in vitro* organic matter digestibility; NPB: number of primary branches; PCS: plant chlorosis score; PGS: plant greenness score; PH: plant height; PC: principal component; PVS: plant vigour score; SD: stem diameter; SPMs: secondary plant metabolites; SS: seedling survival rate; TF: total flavonoids; TGP: total gas production; TP: Total phenolics. Green and red dots represent the accessions and are used to differentiate them, as the green dots indicate higher methane inhibition and agronomic performances, whereas those denoted by red dots exhibit lower methane inhibition and agronomic performances in the field. **(**A**)** refers to the principal component analysis results using the principal component 1 and principal component 2, whereas **(**B**)** shows the principal component analysis results using the principal component 1 and principal component 3.

The HCA, illustrated in Figure 2.4, showed two main cluster trees and supported the results obtained from the scatter plot distribution of PCA output. *Moringa oleifera* A1, A2, A6, A7, A9, and A10 and MS clustered in one of the main cluster trees, whereas the other accessions of *M. oleifera* (A3, A4, A5, A8 and A11) were grouped on the other main cluster tree of the HCA. This relationship study showed the presence of a positive correlation between most agronomic traits and higher CH_4 inhibition characteristics of the accessions, in which more adaptable accessions in the field exhibited better inhibition of CH_4 production. However, *M. oleifera* A2, which showed superior agronomic performance in the field and inferior CH_4 inhibition potential compared to most studied accessions, seemed to be an exception, and this study could not identify and explain the reasons for its variation.

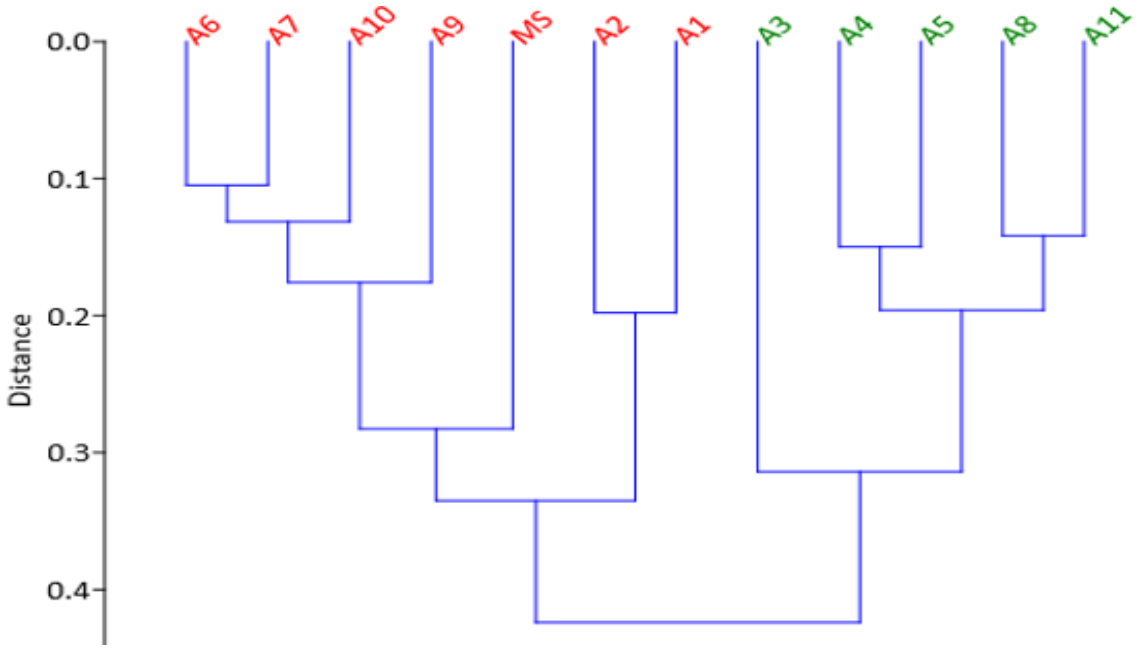


Figure 2.4 Hierarchical clustering of Moringa accessions using all the *in vitro* fermentation characteristics and agronomic traits

Similar to the PCA, accessions represented by green show higher CH_4 inhibition and agronomic performance, whereas those denoted by red exhibit lower CH_4 inhibition and agronomic performances in the field.

2.4 Discussion

Moringa oleifera accessions and *M. stenopetala* grew in the subtropical climate of Pretoria, South Africa, and exhibited different agronomic performances in the field. The seedling survival rates of *M. oleifera* varied from 45% (A10) to 78% (A2), whereas *M. stenopetala* showed a 59% survival rate. This rate was lower than the 100% survival reported in *M. oleifera* and 97% in *M. stenopetala* at the Bako Research Centre in Ethiopia (Samuel *et al.*, 2016). However, we could not guarantee the comparison because of uncertainty about the similarity of environmental conditions and soils or seedling growth mediums of the mentioned study and the current study. A higher survival rate of 75–100% was also revealed in *M. oleifera* accessions grown in the tropical climate of the central Philippines (Patricio & Palada, 2017). The maximum plant height growth (1.88 m) obtained in *M. oleifera* A3 in the fifth month of growth in the field was lower than the 3 m height attained in the third month in Zimbabwe (Leone *et al.*, 2015b). Moringa accessions were also different in their leaf greenness score in the current study. The variation might be attributed to the difference not only in nitrogen (N) content but also in potassium (K) content (Rorie *et al.*, 2011; Hou *et al.*, 2020) and chlorophyll molecules (Virtanen *et al.*, 2020). Thus, darker green leaves indicated higher N contents, whereas lighter green leaves showed greater K rates (Hou *et al.*, 2020). Another study conducted on rice supported this idea and correlated closely the colour of leaves with N concentration (Wang *et al.*, 2014). However, the study of the variation in nitrogen content and its cause was not within the scope of the current study and may be affected by many factors, including the soils of the growing area. Hence, it is apparent that the difference in nitrogen content affects the growth and other plant components of the accessions and subsequently their biological activities. After the fourth month of transplanting the Moringa accessions to the field, the leaves started to change to yellow and gradually increased from the bottom (older leaves) upwards (young leaves). However, although the current study did not identify the cause of the observed problem, previous studies associated it with water stress, root damage, pest damage, herbicide use, ageing of leaves, high soil pH and nutrient deficiencies (i.e. iron, zinc, nitrogen, manganese, or phosphorus) (NA, 2020). Hence, professional interventions to optimize the growing condition, harvesting stage and nutrient management might be essential to increase production with quality leaves. However, these need to be investigated and confirmed in future studies. In addition, genetics might be responsible for the difference in adaptability among accessions in the current study. However, the genotype effect can also be influenced

by environmental factors such as temperature, light, humidity, water, nutrients and soil characteristics (Gang *et al.*, 2015; Kumar & Goel, 2019).

All Moringa accession plant extracts of the current study reduced CH_4 production, which corresponds with several studies that illustrated that Moringa was a potent medicinal plant to reduce enteric CH_4 from ruminants (Leone *et al.*, 2015b; Akanmu & Hassen, 2017). However, it is not possible to be certain of the similarity of accessions between these studies and the current one. The antimethanogenic potential obtained in the current study (18–29%) agreed with the CH_4 inhibition results of 4.5% (100 mg/L), 5.2% (75 mg/L), 28.7% (50 mg/L) and 29.3% (25 mg/L distilled water) reported using the various dosage levels of *M. oleifera* leaf extracts (Akanmu & Hassen, 2017). However, the lower CH_4 inhibition potentials of 4.5% (100 mg/L) and 5.2% (75 ml/L) might be attributed to the difference in the dosage levels of the extract (Akanmu & Hassen, 2017). Plant extract bioactivities are highly reliant on the application doses of the SPMs and their thresholds of minimum and maximum activities (Macheboeuf *et al.*, 2008; Burrell-Saward *et al.*, 2017; Gokulan *et al.*, 2019). Different antimicrobial activities were also reported among the biological differences in Moringa (Leone *et al.*, 2015a; Ghamkhar *et al.*, 2018). However, none of the previous studies indicated the direct effects and variations of varieties, ecotypes, cultivars, individual plants and plant parts of Moringa on antimethanogenic potential and digestibility. In the current study, none of the Moringa accession leaves extracts affected the feed digestion characteristics and kinetics of fermentation negatively. However, the TGP from anaerobically incubated *E. curvula* hay treated with various Moringa accession extracts was decreased compared with those produced from the control. This was evidenced by their equal or higher OMD when compared with the control, which corresponded with the earlier findings (Dey *et al.*, 2014; Akanmu & Hassen, 2017). The lack of significant improvement in OMD and an adverse effect in any of the Moringa accessions implies that Moringa leaf extracts have stimulatory or no effects on the microbes involved in feed digestibility.

The *M. oleifera* accessions and *M. stenopetala* species of the current study maintained significantly different SPM contents of total phenolics and total flavonoids. The studied accessions showed total phenolics ranging from 34.9 to 47.0 mg GAE/g extract. This total phenolic content is higher compared with total phenolics grown in Chad (28.1 mg GAE/g), Algeria (35.5 mg GAE/g) and Haiti (25.5 mg GAE/g) (Leone *et al.*, 2015a). However, it was

lower than 108.7 mg GAE/g (Abo El-Fadl *et al.*, 2020) and 80.9–136.4 mg GAE/g dried extract (Bennour *et al.*, 2019) reported in Egypt and Southern Tunisia, respectively. Leone *et al.* (2015a) also associated the polyphenolic variations recorded among accessions with their genotypes and environment interaction in the area. However, the genotypic variability of Moringa accessions may be the sole factor responsible for the wide range of phenolic values obtained in the current study. The total flavonoids also varied significantly among accessions in the current study, which ranged from 18.3 to 29.2 mg QE/g extract. This yield might be comparable with 18.0–25.1 mg IQE/g total flavonoid contents reported in Thailand (Vongsak *et al.*, 2013b) and higher than 6.6–13.1 mg QE/g total flavonoids found in Nigeria (Omede, 2016). However, it was lower than the total flavonoids of 31.7–44.2 mg QE/g reported in Southern Tunisia (Bennour *et al.*, 2019), 31.2–62.8 mg QE/g in Egypt (Abo El-Fadl *et al.*, 2020) and 55.7–60.3 mg QE/g in Mexico (Castillo-Lopez *et al.*, 2017). The *M. stenopetala* showed 32.7 GAE/g total phenolic and 18.3 QE/g total flavonoid contents, which are lower compared with those contents obtained in most accessions of *M. oleifera* in the current study. However, these SPMs recorded in *M. stenopetala* were statistically equivalent to the values maintained in *M. oleifera* A1 and A2. Thus, the difference in total phenolic and flavonoid contents obtained in Moringa accessions of the current study indicates the presence of wide ranges of genetic variability among accessions, which play a major role in the variation of SPMs compositions and contents in Moringa (Leone *et al.*, 2015b). This could affect the biological activities of the extracts, including gas production characteristics and antimethanogenic potentials, in different ways. However, the process and accumulation of the SPMs are highly affected by stress and defence response signalling in plant-growing environments, such as extreme temperature, drought, flooding, light, soil fertility and salinity (Verma & Shukla, 2015; Yang *et al.*, 2018; Isah, 2019), and their biosynthesis processes are strictly controlled by genes (Li *et al.*, 2020; Szepesi, 2021). This result is consistent with the findings of previous studies (Yang *et al.*, 2018; Isah, 2019), and the variations are generally associated with the highest diversification in many characteristics within the *M. oleifera* accessions (Leone *et al.*, 2015b).

This study also tried to investigate the relationship between various agronomic performances of Moringa accessions in the field with *in vitro* gas production characteristics. Thus, higher CH_4 inhibitor accessions correlated positively with most agronomic performances (plant height, leaf greenness, plant vigour, canopy diameter and stem diameter) of the accessions.

The positive relationships of most agronomic traits of the Moringa accessions with higher CH_4 inhibition characteristics of their extracts are supported by another study that found a higher accumulation of SPMs with increased plant height, stem and root diameter and biomass yield in woody and perennial medicinal plants (Li *et al.*, 2020). These could ultimately result in better antimethanogenesis during their application in rumen modulation. This may imply that accessions that showed higher adaptability to the growing environment were able to produce higher SPMs in response to that environment, which increased the bioactivity of the extracts, including gas production characteristics, antimethanogenesis and OMD. However, measurements and determinations in the current study were carried out on five-month-old leaves, so the results cannot be considered exhaustive for a tree's life cycle. Thus, additional information on Moringa leaves is necessary to explain the possible sources of variation linked to the season effect (within and among years) and Moringa plant ages of the same accession. Further study on the relationship of agronomic practices that increase the growth and yield parameters of the plant, such as fertilizer application, watering frequency and soil characteristics, with antimethanogenesis vs. harvesting stage, also needs to be established

The antimethanogenic activity of accessions in the current study might partly be attributed to the total phenolic and total flavonoid contents. This can be proved by the recorded positive correlation coefficients of antimethanogenesis with the total phenolic (0.62) and total flavonoid (0.71) contents. However, more information about SPMs and major molecules in the extracts is needed to understand the active ingredients and how the molecules interact. Concurrently, *M. oleifera* A3, A8 and A11 contained relatively superior total phenolic and total flavonoid contents and exhibited a higher decrease in CH_4 production with improved digestibility. However, this relationship was not true for all accessions and the *in vitro* OM digestibility of incubated feed was not significantly correlated with these SPMs in the current study. In agreement with the recorded significant positive correlation coefficients of the CH_4 inhibition potential with phenolic and flavonoid contents of this study, several studies explained that these compounds are the main active components against bacteria, protozoa and fungi (Dos Santos *et al.*, 2018; Bouarab-Chibane *et al.*, 2019; Shamsudin *et al.*, 2022). Similarly, many studies stated strong antimicrobial activities from SPMs with functional groups of phenolic acids, phenolics and terpenoids (Zhang *et al.*, 2011; Leone *et al.*, 2015b; Haque, 2018), which affects the antimethanogenic potential of the extracts in different ways. The inclusion of some flavonoids (flavone, myricetin, naringin, rutin,

quercetin, and kaempferol) at 4.5% w/w DM of the substrate decreased *in vitro* CH_4 production by 5 to 9 ml/g DM while improved digestibility was obtained only in quercetin and naringin (Oskoueian *et al.*, 2013). These authors, Oskoueian *et al.* (2013), associated the antimethanogenic activities of the flavonoids with decreased microbial protein synthesis efficacy and specific enzymes (i.e. filter paperase, xylanase, carboxymethyl cellulase, β-glucosidase activities, and purine content). Thus, the effect may vary depending on the structures of the flavonoids. This might be part of the reason that not all accessions in the current study that recorded higher total phenolics and total flavonoids showed similar CH_4 inhibition and improvement in IVOMD of the feed. Therefore, IVOMD is not only dependent on the total phenolic and total flavonoid contents, but also might be affected by other SPMs, which agrees with the non-significant correlation coefficients of IVOMD with these SPMs as revealed by the Pearson correlation coefficients. In addition, the mechanism of increased *in vitro* digestibility recorded in some of these accessions (A1, A2, A3, A8, and A11) may be partly attributed to the antimicrobial and laxative properties reported in Moringa, which makes the rumen environment more conducive to beneficial organisms and enzymes involved in substrate digestion (Harry-Asobara & Samson, 2014). Based on the findings of this study, four accessions (two higher and two lower CH_4 inhibitors) were promoted to the next stage of the metabolomics study to identify the candidate metabolite ion features responsible for their variation in enteric CH_4 inhibition from ruminants (Zeru *et al.*, 2022b). Thus, the published paper (Zeru *et al.*, 2022b) is a continuation of this study, which focused on the identification of the responsible metabolite ion features to higher and lower CH_4 inhibition characteristics recorded in Moringa accessions. However, the identified metabolite ion features need to be investigated for their detailed pathways and mode of biological action using pure compounds in the future.

2.5 Conclusions

The Moringa accessions grown in Pretoria, South Africa, showed different agronomic performances, total phenolics, total flavonoids, CH_4 inhibition potential and organic matter digestibility. Plant extract from *M. oleifera* accession 07633 (A3), 07717 (A8) and Pretoria (A11) resulted in higher *in vitro* CH_4 inhibition with a co-benefit of improved feed organic matter digestibility with equal or higher agronomic performances in the field compared with the other accessions. The positive relationship of total phenolics, total flavonoids and most agronomic traits of the Moringa accessions with CH_4 inhibition potentials obtained in the

study improve knowledge and understanding during the production, standardization, commercialization and utilization of the Moringa accession plant extracts as dietary CH_4 mitigation additives. These positive relationships suggest that there is a possibility of selecting Moringa accessions for higher CH_4 inhibition potential by selecting for higher plant height, canopy diameter, stem diameter, number of primary branches, plant greenness, plant vigour, leaves yield and seedling survival. However, the reported results are not exhaustive for a tree's life cycle and further study is required on a long-term adaptability performance in different agro-ecologies, seasons of harvest, age and parts of the plant with concurrent *in vitro* and *in vivo* antimethanogenic efficacy. This in turn will help to identify the possible sources of variation linked to the season effect (within and among years) and different Moringa plant ages of the same accession and sustain the efficacy of the plant extract.

CHAPTER 3

Screening of candidate bioactive secondary plant metabolite ion features from *Moringa oleifera* accessions associated with high and low enteric methane inhibition from ruminants

Abstract

This study evaluated the relationships of bioactive SPM ion features of *M. oleifera* accessions with antimethanogenesis to identify potential MIFs that were responsible for high and low CH_4 inhibition from ruminants. Plant extracts from 12 Moringa accessions were evaluated at 50 mg/kg DM feed for gas production and CH_4 inhibition. Subsequently, the accessions were classified as low and high enteric CH_4 inhibition groups. Four of twelve accessions (the two lowest and the two highest CH_4 inhibitors) were used to characterize them in terms of MIFs. A total of 24 samples (12 from lower and 12 from higher CH_4 inhibitors) were selected according to their CH_4 inhibition potential, which ranged from 18% to 29%. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and untargeted metabolomics with univariate and multivariate statistical analyses with MetaboAnalyst were used in the study. Although 86 MIFs showed (P <0.05) variation between the higher and lower CH_4 inhibition groups and lay within the detection ranges of the UPLC-MS column, only 14 were significant in the volcano plot. However, Bonferroni correction reduced the candidate MIFs to ten, and their R^2 value with CH_4 production ranged from 0.39 to 0.64. Eventually, MIF 4.44_609.1462 and MIF 4.53_433.1112 were identified as bioactive MIFs associated with higher CH_4 inhibition, whereas MIF 9.06_443.2317 and MIF 15.00_487.2319 were associated with lower CH_4 inhibition with no significant effect on *in vitro* organic matter digestibility of the feed. Plant breeders could use these MIFs as potential markers to develop new *M. oleifera* varieties with high CH_4 inhibition characteristics. However, further investigation on identifying their names, structures, and detailed biological activities of these bioactive SPMs needs to be carried out for future standardization, commercialization, and application as dietary CH_4 mitigation additives.

Keywords: gas production; *in vitro* organic matter digestibility; methane inhibition; methane emission; metabolite ion features; relationships; volcano plot

3.1 Introduction

Methane is the second most important greenhouse gas next to CO_2, and livestock, mainly ruminant animals, that rely on low-quality roughage feed contribute 21% (16% from enteric fermentation and 5% from animal waste) of its anthropogenic emission (Augenbraun *et al.*, 2010), as cited by Sholtz *et al*. (2020). Hence, the animal production sector has been highly threatened due to its global warming potential and negative effect on animal productivity. Therefore, the development of a suitable CH_4 mitigation strategy has been compelled to minimize the shadowing effect on the sector (Bodas *et al.*, 2012; Hill *et al.*, 2016; Medjekal & Bousseboua, 2016; Chapman *et al.*, 2017; McCann *et al.*, 2017; Neubauer *et al.*, 2018). However, the long-term CH_4 reduction effects have not been established as expected. Recently, most enteric CH_4 abatement strategies have focused on feeding and feed additives. The use of antibiotics and synthetic chemical feed additives is becoming less popular globally and in EU countries in particular because of concerns about long-term residual effects on human health (DemİrtaŞ *et al.*, 2018). Hence, the global scenario has shifted from the use of synthetic antibiotics to natural plants and their extracts to produce organic animal products free from dietary antibiotic residues (Rira *et al.*, 2015; Jayanegara *et al.*, 2020; Ku-Vera *et al.*, 2020).

Medicinal plants such as *M. oleifera*, which are rich in bioactive SPMs, are relatively inexpensive and could replace synthetic chemical feed additives safely (Kim *et al.*, 2012; Pal *et al.*, 2015; Jafari *et al.*, 2016; Akanmu & Hassen, 2017). The potential of medicinal plants to reduce CH_4 has been associated with the presence of nonnutritive SPMs, which have developed over years as a survival and defence mechanism against herbivores, pests, microorganisms, invaders, grazers, and parasites (Erb & Kliebenstein, 2020). Studies have generally associated the microcidal or microstatic action of SPMs with the capacity to form irreversible complexes with cholesterol in the microbial cell membrane (Francis *et al.*, 2002) and interfere with the bacterial cell membrane to disintegrate membrane structures that cause ion leakage and cell lysis, which ultimately reduced CH_4 emission (Bodas *et al.*, 2012). Thus, exploring various aspects of the biologically active SPMs in medicinal plants such as *M. oleifera* is crucial and highly demanded (Faehnrich *et al.*, 2021).

Several studies have tried to profile the SPMs constituents of *M. oleifera* because of its multipurpose functions (Amaglo *et al.*, 2010; Leone *et al.*, 2015a; Leone *et al.*, 2015b; Sudha

et al., 2020; Bhalla *et al.*, 2021; Fidrianny *et al.*, 2021). However, the composition, concentration, and bioactivities of SPMs may differ among plant species, varieties, ecotypes, cultivars, and individual plants of the same species and even among plant parts (López *et al.*, 2007). Studies have shown that leaf extracts of three varieties of *Labisa pumila* Benth illustrated different total phenolic and flavonoid contents and antioxidant activities (Akula & Ravishankar, 2011). *Moringa oleifera* accessions grown in Chad, Algeria, and Haiti showed substantial variation in total polyphenols and salicylic and ferulic acids (Leone *et al.*, 2015a). Similarly, Moringa accessions grown in China and India were different in composition and concentration of plant components (Lin *et al.*, 2019). *Moringa oleifera* leaves collected from Gauteng, Limpopo, and Mpumalanga in South Africa also exhibited different nutrient contents, metabolite profiles and antioxidant activities (Teclegeorgish *et al.*, 2021). Hence, these variations might be attributed to the genotype and growing environment or the effects of their interactions. However, many of the studies did not show variations in terms of SPMs between accessions when they were grown in the same environment and were used as a source of plant extracts for rumen modulation and antimethanogenesis.

The inclusion of flavonoids such as flavone, myricetin, naringin, rutin, quercetin, and kaempferol at 4.5% *w*/*w* DM of the substrate decreased *in vitro* CH_4 production by 5 to 9 ml/g DM (Oskoueian *et al.*, 2013). Luteolin-7-glucoside showed more promising results on CH_4 and NH_3 inhibition without compromising fermentation efficiency relative to quercetin, epicatechin, isoquercetin, catechin, epigallocatechin, epigallocatechin gallate, and gallocatechin (Sinz *et al.*, 2018). However, tannic acid, gallocatechin, and epigallocatechin gallate decreased gas production and IVOMD compared with the negative control (Sinz *et al.*, 2018). Hence, depending on the structure and functional group of the SPMs, their effects on antibacterial, antifungal, antiviral, antiprotozoal, and CH_4 production might take the form of inhibition or stimulation. Strongly inhibited growth and metabolism of rumen microbes were obtained from oxygenated monoterpene SPM, whereas slight inhibition and occasional stimulation of rumen microbial activities were reported in hydrocarbon monoterpene SPMs (Benchaar *et al.*, 2008). Plant SPMs with phenolic acid, phenol, and terpenoid functional groups also showed strong antimicrobial activities (Zhang *et al.*, 2011; Leone *et al.*, 2015b; Haque, 2018), which might influence the antimethanogenic potential of the extracts in diverse ways. Studies on the relationship of plant SPMs with antimethanogenic potential using Moringa as a model medicinal plant helped to establish knowledge about the active

SPMs responsible for antimethanogenesis. These SPMs can also be used as markers for the selection of commercial varieties that will be used as a source of plant extracts to mitigate enteric CH_4 from ruminants. However, several studies did not describe this aspect of medicinal plants and their components at the metabolite level except for some that showed the use of crude plant extracts (Melesse, 2011; Delgado *et al.*, 2012; Akanmu & Hassen, 2017; Ghamkhar *et al.*, 2018). Therefore, this study intended to establish the relationship between bioactive SPM ion features and the *in vitro* CH_4 inhibition characteristics of Moringa accessions. The intention was to identify potential MIFs that could be used as markers to select breeding varieties with high CH_4 inhibition characteristics. These could then be used as dietary additives to the ruminant feeding systems.

3.2 Materials and methods

3.2.1 Plant materials and preparation of crude extracts

Eleven accessions of *M. oleifera* and one accession of *M. stenopetala* were grown from October 2018 to February 2019 in the subtropical climate of Pretoria at the Roodeplaat experimental site of the ARC, Pretoria, South Africa, which is located at 25°44′30″ S, 28°15′30″ E. Before this study, plant extracts from these accessions were evaluated for *in vitro* gas production and CH_4-reducing potential when used as dietary additives. The accessions were classified into low and high CH_4 inhibition groups based on their CH_4 inhibition potential. Subsequently, four accessions were selected for this metabolomics study (Zeru *et al.*, 2022a). Two samples per plot or six samples per accession were collected in the fifth month after transplanting to the field. This coincided with the end of the summer season in 2019.

The leaves were freeze-dried for approximately five days until the leaves were attained a constant weight and milled to a 1 mm sieve size in a milling machine. Approximately 50 g of the dried leaf powder was suspended in 500 ml of methanol at a ratio of 1:10 (mass/volume) (Eloff, 1998) for 96 hours in a shaker. The mixture was filtrated and extracted through a 150 µm aperture sieve (Vickers, Durban, South Africa). The solution containing the extract was placed in fume chambers until it was semidried for approximately 48 hours. The semidried extracts were then dried until a constant weight was attained and kept at 4 $^{\circ}$C for further use.

3.2.2 Determination of in vitro total gas, methane, and organic matter digestibility

The total gas, antimethanogenic potential, *in vitro* organic matter digestibility (IVOMD) of *E. curvula* hay, and CH_4/IVOMD treated with these Moringa accession leaf extracts were determined in chapter 2 and imported into this chapter of the study (Zeru *et al.*, 2022a). Buffer, macromineral, and micromineral solutions were prepared according to simplified *in vitro* medium preparation procedures before the incubation day and kept in the refrigerator at 4 ˚C (Goering & Van Soest, 1970; Mould *et al.*, 2005). Hence, the buffer solution consisted of ammonium bicarbonate (NH_4HCO_3) and sodium bicarbonate ($NaHCO_3$), whereas the macromineral solution was prepared from sodium hydrogen phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), and magnesium chloride hexahydrate ($MgCl_2.6H_2O$). The micromineral solution was composed of calcium chloride dihydrate ($CaCl_2.2H_2O$), manganese chloride tetrahydrate ($MnCl_2.4H_2O$), cobalt chloride hexahydrate ($CoCl_2.6H_2O$), and ferric chloride hexahydrate ($FeCl_3.6H_2O$) (Goering & Van Soest, 1970). Early in the morning of the incubation day, the prepared micromineral, macromineral, buffer, and resazurin (0.1% *w*/*v*) solutions were mixed with tryptone and distilled water. Then, the mixed solution was bathed at 39 ˚C and bubbled continuously with CO_2 until the incubation process was completed to maintain anaerobic conditions. Before morning feeding on the incubation day, rumen fluid was collected from three ruminally cannulated Pinzyl (Pinzgauer cross Nguni) steers and adapted for 14 days, following procedures approved by the Animal Ethics Committee of the University of Pretoria (No. EC039-18), South Africa. L-cysteine and $Na_2S.9H_2O$ were added to the medium 10 min before the addition of rumen fluid (Mould *et al.*, 2005). When the solution was sufficiently reduced, the rumen fluid was filtered through four layers of cheesecloth and mixed with a buffer medium at a 15:25 ml rumen fluid-to-medium ratio. The dried extract was redissolved with distilled water and applied with a 50 mg/kg DM feed (Akanmu & Hassen, 2017) except for the blanks and the control. Then, 40 ml of the inoculum was added to all the bottles and incubated with an incubator or Incoshake set at 39 ˚C and 120 revolutions per minute (rpm).

The total gas produced during incubation was measured with a pressure transducer attached to a digital data logger at 3, 6, 12, 24, and 48 hours of incubation and recorded in pound-force per square inch (psi) (Theodorou *et al.*, 1994). The gas pressure was converted to ml as $V_x = V_j P_{psi} \times 0.068004084$, where V_x is the gas produced at 39 ˚C in ml; V_j is the

headspace of the incubation bottle in ml; and P_{psi} is the pressure recorded by the gas monitor system software (ANKOM, 2014).

Similarly, CH_4 samples were taken at 3, 6, 12, 24, and 48 hours of incubation and analysed by gas chromatography (GC) (8610C, SRI Instruments GmbH, Bad Honnef, Germany) equipped with a flame ionization detector. The CH_4 area obtained from the GC was converted into parts per million (ppm) using a standard curve and then changed into a percentage. Next, the CH_4 concentration was converted to ml by multiplying the total gas produced (ml) by the percentage of CH_4 in the sample as CH_4 (ml) = total gas produced (ml) × % CH_4 concentration. Eventually, the antimethanogenic potential of the plant extract was expressed as ml/g DM incubated feed (Ghamkhar *et al.*, 2018). In addition, the CH_4 inhibition potential was converted to a percentage compared with the control, and the accessions were classified into lower and higher CH_4 inhibition groups. Of the 12 accessions, 4 accessions (A3 and A11 to higher CH_4 inhibition, and A1 and A2 to lower CH_4 inhibition) containing 24 samples were selected (Table 3.1) to characterize the *m*/*z* ion features of Moringa accessions with CH_4 inhibition and identify the MIFs responsible for their antimethanogenic potential.

In addition, the IVOMD was determined with the modified two digestion phase techniques (Tilley & Terry, 1963; Engels & Van der Merwe, 1967) and imported to this part of the study to understand the relationship of these MIFs with feed digestibility. In the first phase of the incubation, 200 mg substrate feed, *E. curvula* grass hay, extracts of the accessions (50 mg extract/kg DM feed), artificial saliva solution, and urea and rumen fluid were incubated under anaerobic conditions for 48 hours at 39 ˚C using test tubes, which was fitted with modified stoppers. Artificial saliva was prepared from potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), and calcium chloride ($CaCl_2$) before the incubation day and kept in the refrigerator until use. In the second phase, an acid and pepsin solution was prepared from 20 ml of 32% HCl and 8 g of pepsin by dissolving them in 2000 ml of distilled water. Then, 20 ml of this solution was added after gently decanting the liquid on the top of the tubes and incubating for another 48 hours at 39 °C.

After 96 hours of total anaerobic incubation, the residual plant materials were oven-dried at 100 °C for 18–24 h, ashed, and weighed, and the percentage IVOMD was calculated:

$$\% \text{ IVOMD} = \frac{\text{OM of feed sample} - (\text{undigested residue} - \text{blank})}{\text{OM of feed sample}}$$

3.2.3 Ultra–performance liquid chromatography–mass spectrometry data analysis

To study metabolomics, approximately 100 mg of the crude extract of each sample (i.e. two samples per plot in the field; six samples per accession) used to determine *in vitro* CH_4 was redissolved in 10 ml of 70% methanol (10 mg/ml concentration) in the Department of Chemistry, University of Pretoria, South Africa. The mixture was then vortexed and centrifuged for 10 min. Then, 10 µL of the sample supernatant was taken and diluted again with 990 µL of 70% methanol. Approximately 1 ml of solution (0.1 mg/ml) was filtered with 0.2-micron syringe filters and transferred to the injection vial of UPLC–MS for analysis. UPLC–MS analyses were carried out with a Waters Synapt G2 quadrupole time-of-flight (QTOF) MS connected to a Waters Acquity UPLC (Waters, Milford, MA, USA).

The prepared six samples per the selected accession with the injection vials were subjected to both the positive and negative ion mode ionization of the UPLC–MS. The untargeted metabolomics data were transferred to Microsoft Excel and a MetaboAnalyst 5.0 system for univariate and multivariate statistical analyses. After clustering the accessions with their antimethanogenic potential as high or low CH_4 inhibition, two accessions (A3 and A11), which contained 12 samples and showed the best *in vitro* CH_4 inhibition (hereafter 'high'), and another two accessions (A1 and A2), which comprised 12 samples and recorded the lowest CH_4 inhibition, were selected as 'low' CH_4 inhibitors. Then, before the metabolomics analysis, the low and high CH_4 inhibition labelling of the samples was added to the UPLC–MS data. The MIFs in the column regeneration step (after 17 min) and the void volume (0–2 min) of the chromatogram were poorly resolved and ignored in all statistical analyses. Thus, the focus of the study was MIFs detected in the chromatogram between 2 and 17 min. A data preprocessing strategy for metabolomics was carried out to reduce the masking effect in data analysis. The values that were missing because of the limits of quantification in the detector were managed with a modified 80% rule. Thus, variables were excluded when the proportion of nonmissing elements accounted for less than 80% in each biological group (Yang *et al.*, 2015). In addition, as recommended in MetaboAnalyst 5.0, the quality of

LC/MS data was controlled by 20% relative standard deviation (RSD), and the ion features that showed low repeatability or greater than 20% RSD were removed before analysis. Next, the UPLC-MS data were subjected to continuous univariate and multivariate statistical analyses, such as a *t* test, fold change, volcano plot, and PCA with MetaboAnalyst 5.0. Initially, PCA was performed to explore the maximum variation to obtain the overview and classification. The volcano plot visualized the predictive component loading and identified important MIFs by fold change and *t* tests. After analysing the significant variation of each MIF intensity with the *t* test (P <0.05), the Bonferroni correction was applied to minimize the multiple testing problem or false discovery rate (Vinaixa *et al.*, 2012). The relationship (R^2 value) of MIF intensity with CH_4 production was determined with scatter plot analysis.

3.2.4 Statistical analyses

One-way analysis of variance and the Tukey test for RCBD were conducted to determine the variations in total gas, CH_4 production, and IVOMD among these accessions using SAS version 9.4 (P <0.05). To investigate the variations and relationships of MIFs with antimethanogenic potential, continuous semiquantitative univariate and multivariate statistical analyses, such as *t* tests, fold change, volcano plots, and PCA, were carried out with MetaboAnalyst 5.0. The relationship (R^2 value) between *m/z* ion features intensity and CH_4 production, total gas production, and IVOMD was determined with scatter plot analysis. The Bonferroni correction was also calculated and applied at the corrected $P \leq 6.25 \times 10^{-3}$ in the negative ion mode and $P \leq 6.25 \times 10^{-4}$ in the positive ion mode (Vinaixa *et al.*, 2012).

3.3 Results

3.3.1 In vitro methane inhibition

The substrate feed, *E. curvula* hay, which was used for *in vitro* fermentation and CH_4 determination contained approximately 93.1% DM, 8.6% CP, 80.0% NDF, 41.0% ADF, 8.3% ADL, and 3.9% ash content on a DM basis. The total gas and total CH_4 and CH_4 yield per unit of OMD of *E. curvula* hay treated with the leaf extracts varied significantly (P <0.01). Their total CH_4 production and CH_4 yield per unit of OMD ranged from 4.5 ml/g DM (A11) to 5.2 ml/g DM (A1) and 7.6 (A3) to 8.8 ml (A1), respectively. Thus, the total CH_4 production and CH_4 yield per unit of OMD were significantly (P <0.01) decreased in

all accession extracts compared with the CH_4 produced in the control (6.4 ml/g DM; 11.7 m/g organic matter). Compared with the control, the recorded antimethanogenic potential (percentage CH_4 inhibition) of the accessions ranged from 18% (A1) to 29% (A11). Thus, the hierarchical cluster analysis (HCA) grouped the study samples into two broad cluster trees with high and low CH_4 inhibition with 80% similarity (Figure 3.1) using their antimethanogenic variation. The higher and lower CH_4 inhibition categories of the samples in their antimethanogenic potential (Figure 3.1; Table 3.1) involved drawing an arbitrary line through a continuous distribution. Thus, the 12 samples of *M. oleifera* A3 (07633) and A11 (Pretoria) lay in a higher CH_4 inhibition cluster tree, whereas the other 12 samples of A1 (bulk) and A2 (07229) belonged to the lower CH_4 inhibition cluster tree (Figure 3.1). In addition, the *E. curvula* hay treated with extracts of these accessions exhibited an IVOMD of approximately 58% to 60%. None of the four accessions recorded a negative effect on feed OMD and showed a small improvement compared with the OMD of the control (55%). Thus, the variations in these parameters might be attributed to the differences in the active compounds in the extracts, which were expressed as SPM ion features.

Table 3.1 *In vitro* fermentation characteristics of *Eragrostis curvula* hay treated with the leaf extracts of selected *Moringa oleifera* accessions

Accession	CO	Sample Code	TGP	CH_4	% IVOMD	% CH_4 Inh	CH_4/ IVOMD	CH_4 IG
Bulk (A1)	Kenya	A01p10_1	173.7	5.4	59.9	15.7	9.0	low
		A01p10_2	170	5.1	59.6	19.4	8.6	low
		A01p16_1	171.9	5.4	58.8	15.9	9.1	low
		A01p16_2	170.3	5.4	60.1	14.6	9.1	low
		A01p32_1	168.8	5.0	59.1	21.2	8.5	low
		A01p32_2	168.3	5.1	58.4	20.6	8.7	low
Mean ± SEM			170.5 ± 1.2 ^{B}	5.2 ± 0.1 ^{B}	59.3 ± 0.4 ^{A}	17.9 ± 2.0 ^{B}	8.8 ± 0.2 ^{B}	
07229 (A2)	Kenya	A02p09_1	175.1	5.4	60.4	15.4	8.9	low
		A02p09_2	174.6	5.4	60.2	14.9	9.0	low
		A02p23_1	171.9	5.3	58.1	16.7	9.1	low
		A02p23_2	173.3	5.3	59.2	17.2	8.9	low
		A02p34_1	170.3	4.9	59.3	22.5	8.3	low
		A02p34_2	170.7	5	62.8	22	7.9	low
Mean ± SEM			172.6 ± 1.6 ^{B}	5.2 ± 0.2 ^{B}	59.9 ± 0.9 ^{A}	18.1 ± 2.4 ^{B}	8.7 ± 0.4 ^{B}	
07633 (A3)	Mali	A03P11_1	163.6	4.6	59.0	27.2	7.9	high
		A03P11_2	162.8	4.7	60.2	26.6	7.8	high
		A03P19_1	161.4	4.7	58.6	26.4	8.0	high
		A03P19_2	162.3	4.7	59.2	25.8	8.0	high
		A03P31_1	157.1	4.2	62.1	33.7	6.8	high
		A03P31_2	157.5	4.4	61.2	30.6	7.2	high
Mean ± SEM			160.8 ± 2.2 ^{C}	4.6 ± 0.1 ^{C}	60.0 ± 1.0 ^{A}	28.4 ± 2.2 ^{A}	7.6 ± 0.4 ^{C}	
Pretoria (A11)	South Africa	A11P01_1	164.3	4.5	56.3	29.1	8.0	high
		A11P01_2	165.2	4.5	55.73	29.6	8.1	high
		A11P15_1	163.6	4.8	58.4	25.2	8.2	high
		A11P15_2	164.2	4.8	59.34	24.8	8.2	high
		A11P33_1	163.7	4.3	58.25	32.3	7.4	high
		A11P33_2	163.7	4.3	57.51	33.2	7.4	high
Mean ± SEM			164.1 ± 0.4 ^{BC}	4.5 ± 0.2 ^{C}	57.59 ± 1.0 ^{B}	29.0 ± 2.5 ^{A}	7.9 ± 0.3 ^{C}	
Control			203.8 ± 5.3 ^{A}	6.4 ± 0.2 ^{A}	54.7 ± 0.4 ^{C}		11.3 ± 0.4 ^{A}	

The mean values of the accessions with different superscript letters along the column are significantly (P <0.01) different. CO: country of origin; TGP: total gas production (ml/g DM); IVOMD: *in vitro* organic matter digestibility; A1P10_1 in the sample code referring to accession 1, plot 10, sample 1, etc.; SEM: standard error of the mean; CH_4 IG: CH_4 inhibition group: % CH_4 Inh: % CH_4 inhibition

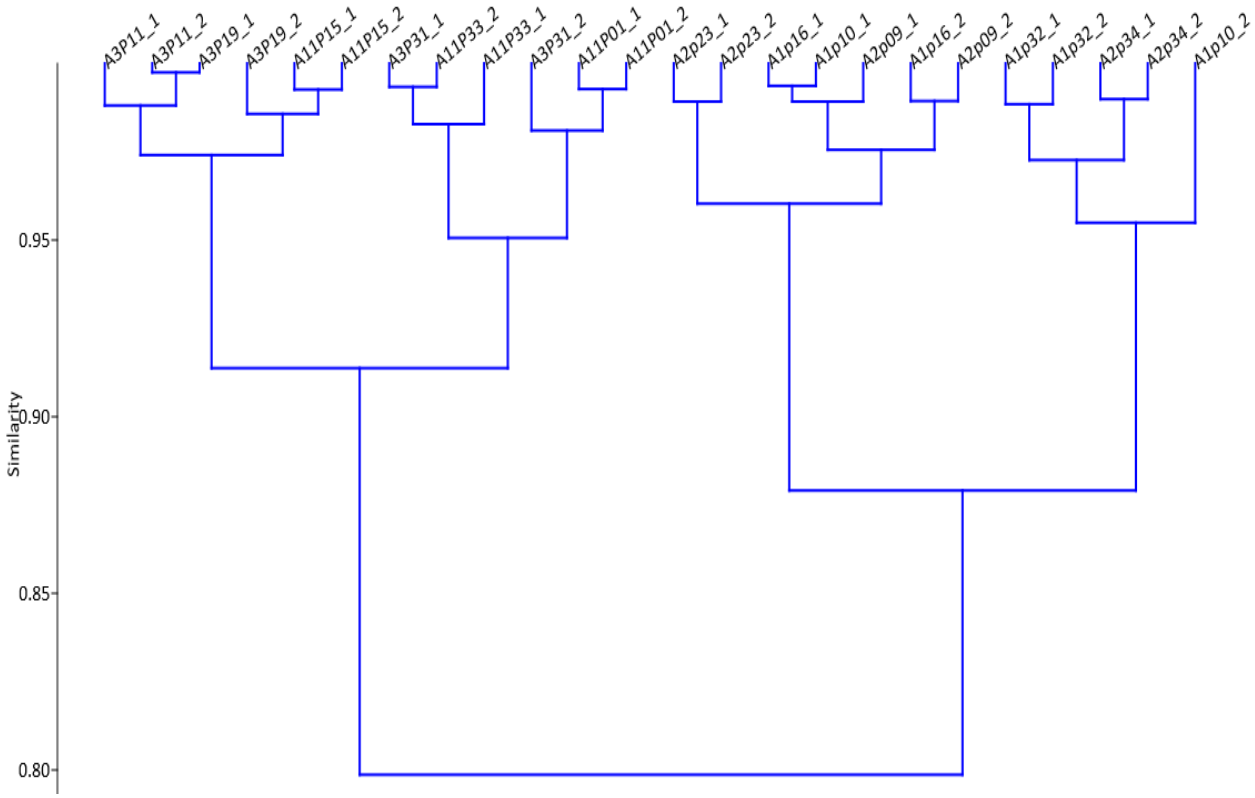


Figure 3.1 Hierarchical cluster analysis of Moringa accession samples using total methane produced from *Eragrostis curvula* hay treated with their extracts and the percentage methane inhibition potential

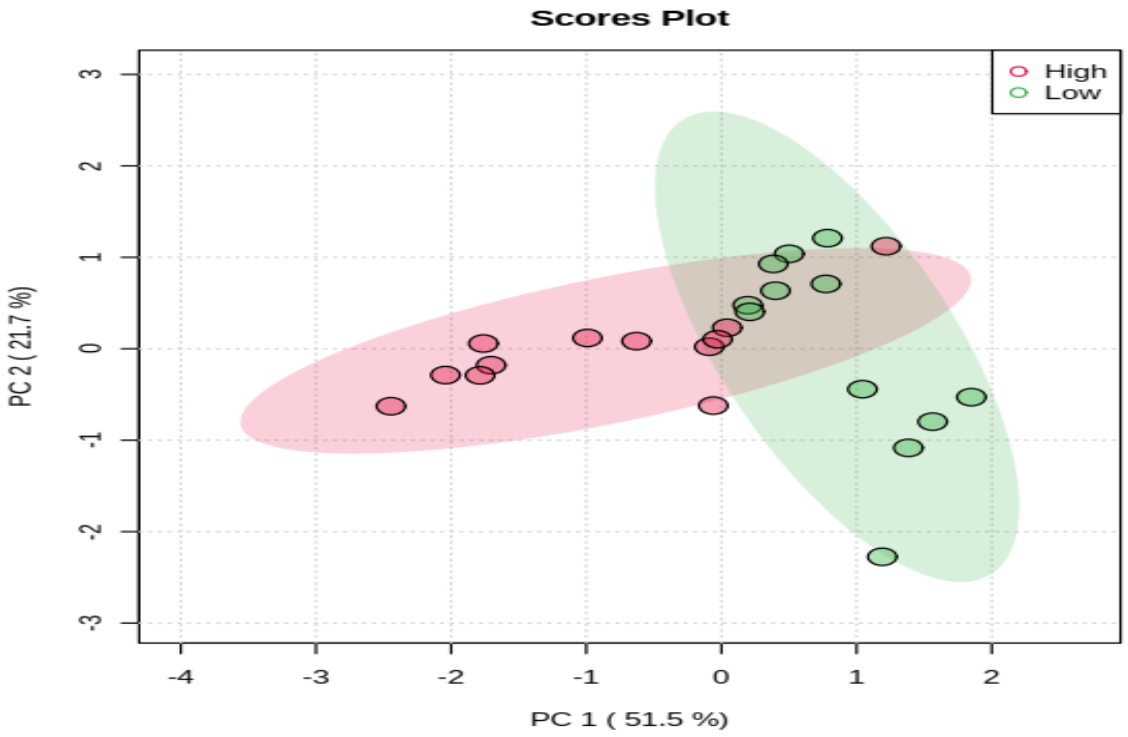
In the figure, A1P10_1 refers to accession 1, plot 10, and sample 1; A1P10_2 refers to accession 1, plot 10, sample 2, etc., where A is the accession of the sample and P is the plot number of the sample collected in the field.

3.3.2 Characterizing the m/z ion features of Moringa accessions with methane inhibition

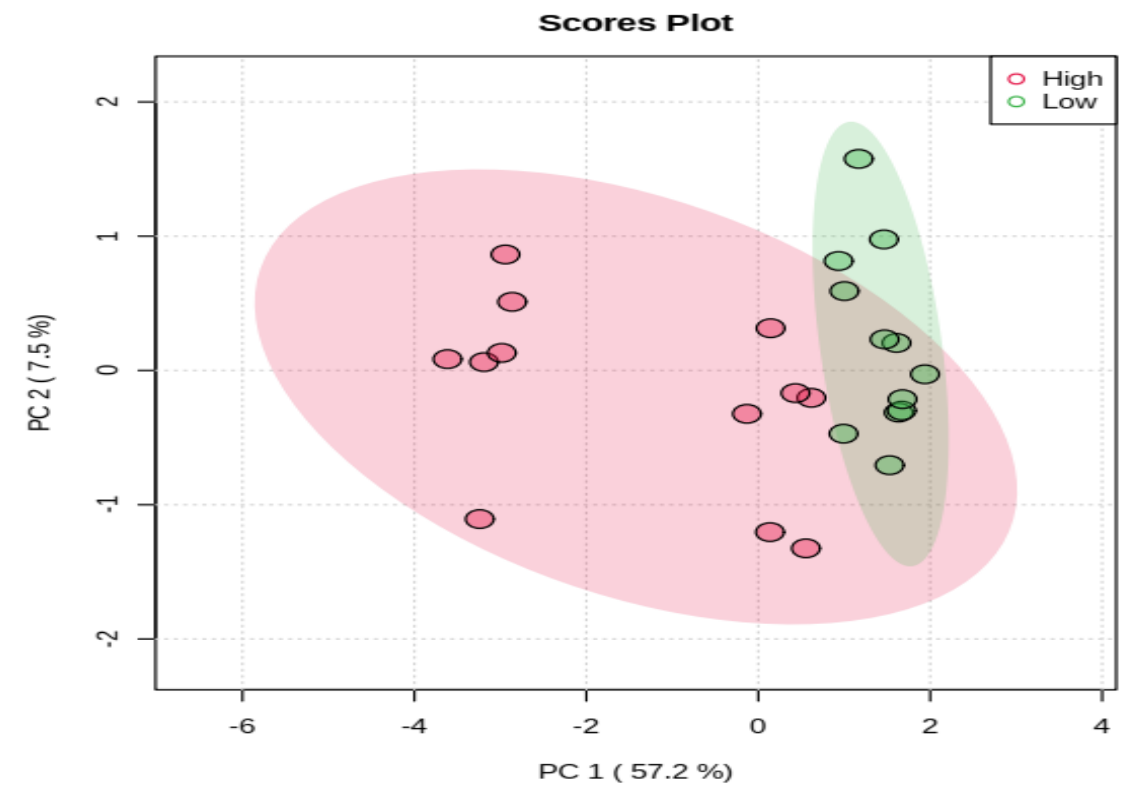
A significant decrease in CH_4 production was obtained from all accessions of Morinaga leaf extracts compared with the control. Hence, their antimethanogenic potential was significantly (P <0.01) different. This antimethanogenic variation was expected because of the difference in composition and concentration of the plant components, which were represented as MIFs in this study. Large numbers of MIFs were detected at the initial step of the screening from the UPLC–MS data of the accessions (data not shown). However, only 161 (33 in the negative and 128 in the positive ion mode) were selected for MetaboAnalyst using the detection range of 2 to 17 min and a significant level of intensity between the two CH_4 inhibition groups before data processing (P <0.05). The MetaboAnalyst outputs of the

MIFs containing data obtained from UPLC–MS in the positive and negative ion modes are illustrated in Figures 3.2–3.6. During the metabolite data analysis, PCA (Figure 3.2) clustered the samples into high and low CH_4 inhibition groups and explained approximately 73.2% in the negative ion mode and 64.7% in the positive ion mode and of the total variations of the samples. The volcano plot (Figure 3.3) showed the importance of the MIFs in a model, which makes the difference between the two sample groups, whereas the boxplot (Figure 3.4) illustrated the abundance of *m*/*z* ion features in the samples, as well as a 95% confidence interval around the median of each group and their mean abundance in each group.

Among the 161 MIF markers uploaded into MetaboAnalyst, eight MIFs in the negative ion mode and 78 MIFs in the positive ion mode were significantly different between the high and low CH_4 inhibition sample groups using the *t* test at $P < 0.05$ (all data not shown). The volcano plot, that is, drawn from fold change threshold (x) 2 and the *t* tests threshold (y) 0.05 selected 14 MIFs (six in the negative ion mode and eight in the positive ion mode) that varied significantly between high and low inhibition samples (Figure 3.3). Using the Bonferroni correction, five MIFs in the negative mode (corrected $P \leq 6.25 \times 10^{-3}$) and seven MIFs in the positive ion mode (corrected $P \leq 6.4 \times 10^{-4}$) were significantly different between the two CH_4 inhibition groups. As illustrated in Figure 3.3, the Bonferroni corrected value of the negative ion mode is 2.2 (log0.00625 = –2.204), whereas the positive ion mode is 3.2 (log 0.00064 = –3.19). Hence, using these two horizontal lines, there is no MIF above 2.2 in the negative ion mode associated with higher CH_4 inhibitors, while five MIF markers are above 2.2 associated with lower CH_4 inhibitors. In the positive ion mode, the MIF 4.53_433.1112 associated with higher CH_4 inhibitors and five MIFs associated with lower CH_4 inhibitors lay above the 3.2 horizontal line (Figure 3.3). Thus, the MIFs that showed significant variations in all the tests were promoted to the next stage of associating them with higher and lower CH_4 inhibition potential. However, some MIFs that did not vary significantly with Bonferroni corrected values but showed significant variations in the other tests were considered in the selection because of their higher relative abundance for easy practical application and higher relationship value (R^2 value) of the intensity with CH_4 inhibition potential.

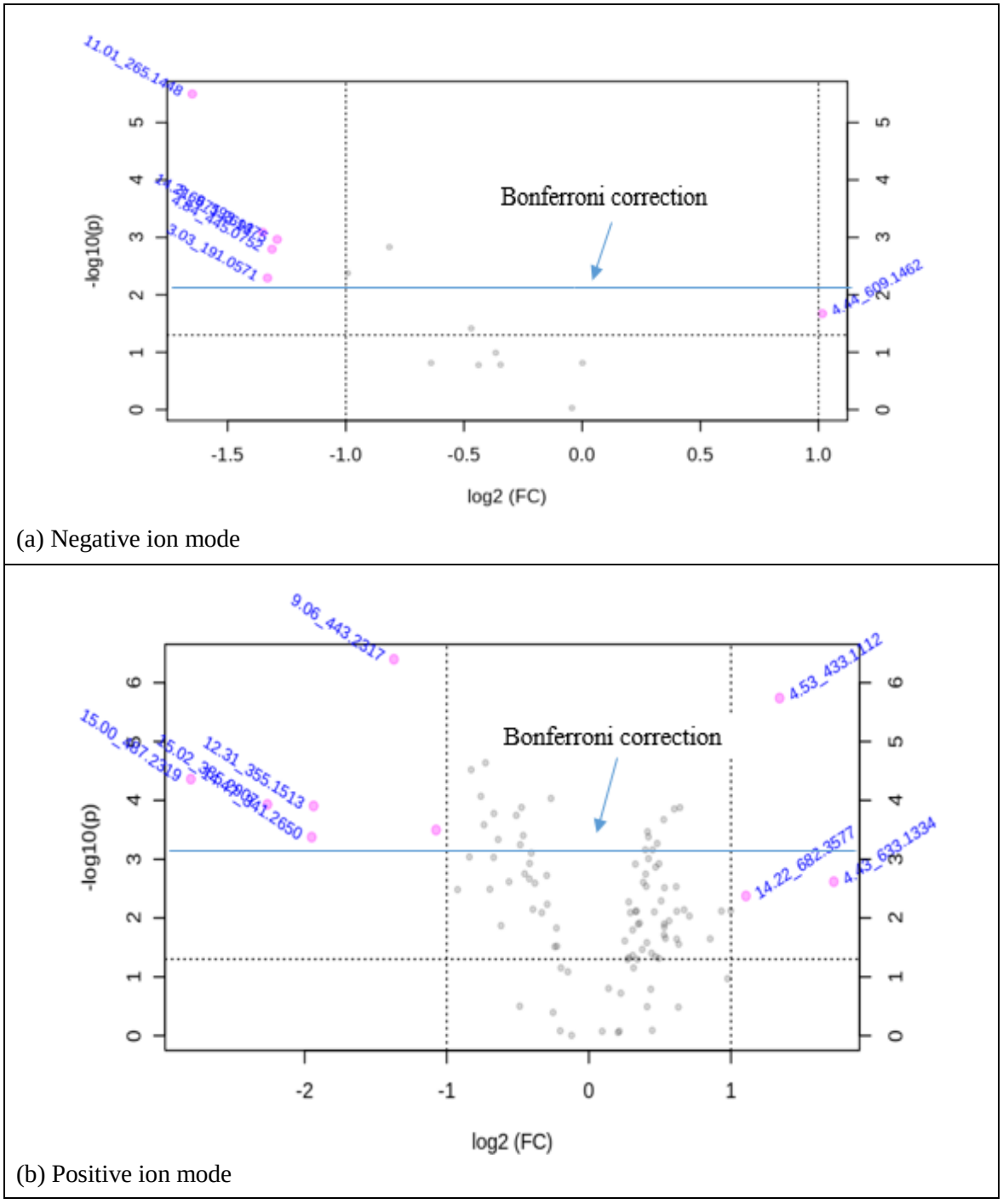


(a) Negative ion mode



(b) Positive ion mode

Figure 3.2 Principal component analysis score plot between the selected principal components that grouped the samples into high and low methane inhibition

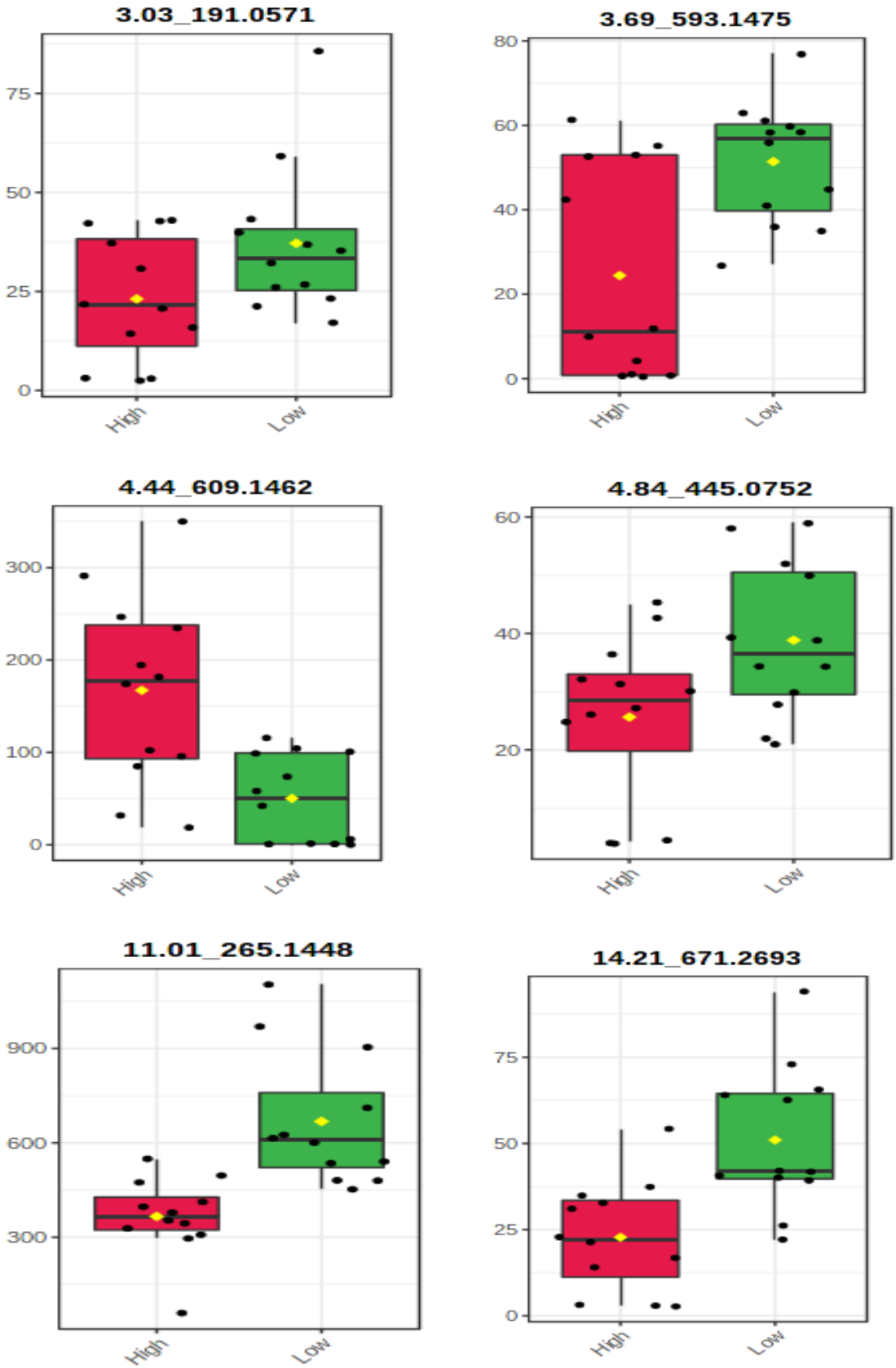


(a) Negative ion mode

(b) Positive ion mode

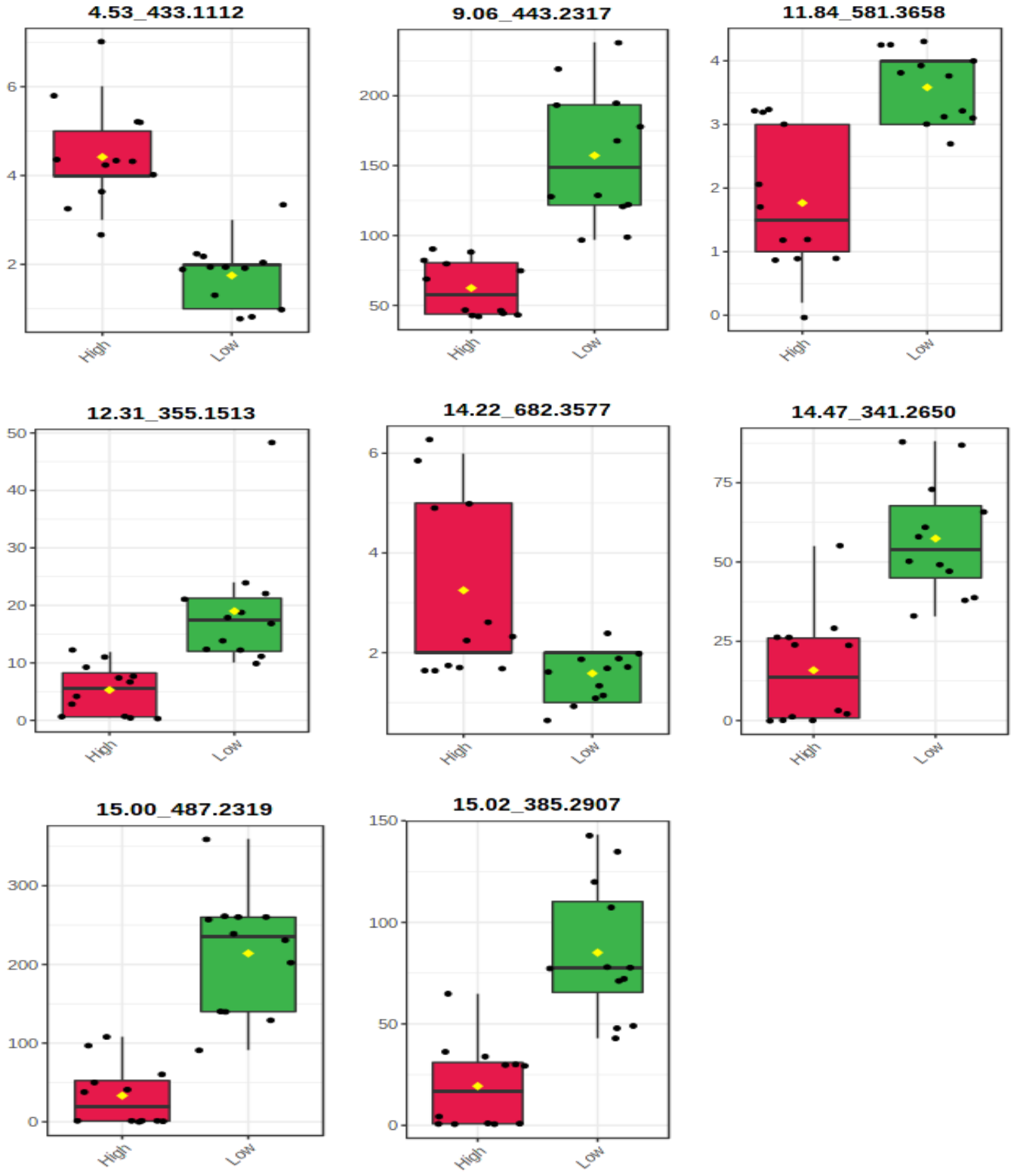
Figure 3.3 Important *m/z* ion features selected by volcano plot with fold change threshold (x) 2 and *t* tests threshold (y) 0.05

The red circles/dots in this volcano plot represent ion features above the threshold. Both fold changes and p values are log-transformed. The further its position is away from the (0,0), the more significant the feature is. The direction of comparison is 'high CH_4 inhibition'/'low CH_4 inhibition'. The overlapping *m/z* ion features in a and b are identical to the MIFs shown in the box plots of Figures 3.4 a and b below.



(**a**) Identified in negative ion mode

Figure 3.4 *Cont.*



(b**)** Selected in the positive ion mode

Figure 3.4 Boxplot of the selected *m/z* ion features in the negative ion mode (a) and positive ion mode (b)

Black dots represent the abundance of the selected ion feature from all samples. The red and green boxes indicate the 95% confidence interval around the median of each group, defined as +/−1.58*/QR/sqrt (n), which can be used to evaluate differences between groups. If the boxes do not overlap, the medians are probably different. The mean concentration of each group is indicated with the yellow diamond. The direction of comparison is 'high CH_4 inhibition'/'low CH_4 inhibition'.

3.3.3 Associating the m/z ion feature contribution to high methane inhibition

The potential candidate *m/z* ion features responsible for high CH_4 inhibition and their summary data are shown in Table 3.2. The relationship (R^2 value) of MIF intensity associated with higher CH_4 inhibition with CH_4 volume, total gas, IVOMD, and CH_4 yield per IVOMD was calculated with a scatter plot (Figure 3.5). However, several MIFs seemed to be associated with high CH_4 inhibition characteristics of the samples, and most of them showed a lower relationship value and were not statistically significant in the subsequent analysis. Thus, after long screening steps, one MIF in the negative ion mode (P <0.05) and two MIFs in the positive ion mode (corrected $P \leq 6.4 \times 10^{-4}$) were selected as candidates for higher CH_4 inhibition potentials of the accessions. The relationship values determined with scatter plot analysis between the MIF intensities with CH_4 produced per DM feed incubated and CH_4 yield per organic matter digested were 0.42 and 0.41 in MIF 4.44_609.1462 and 0.54 and 0.43 in MIF 4.53_433.1112, respectively (Figure 3.5). In addition, the Pearson correlation analysis correlated negatively and significantly (P <0.01) for the MIF 4.44_609.1462 and MIF 4.53_433.1112 intensities with total CH_4 production and CH_4 yield per OMD (Table 3.2). In terms of the magnitude of ion feature intensity, MIF 4.44_609.1462 from the negative ion mode showed relatively higher abundance in higher CH_4 inhibition sample groups compared with MIF 4.53_433.1112 and MIF 14.22_682.3577 in the positive ion mode. However, MIF 4.53_433.1112 exhibited the highest R^2 value (0.54) and correlation coefficient (−0.66). Hence, the higher signal strength obtained in MIF 4.44_609.1462 might provide an advantage for analysis and practical application, although concentration is not the sole determining factor of their antimethanogenic potential. It was proven from MIF 4.53_433.1112, however, that its level of intensity was low, and it exhibited a greater relationship (R^2 value) with CH_4 production than was observed in MIFs with greater intensity. This result indicated not only that metabolite concentration played a crucial role in their antimethanogenic potential but also that the specific action of the SPMs

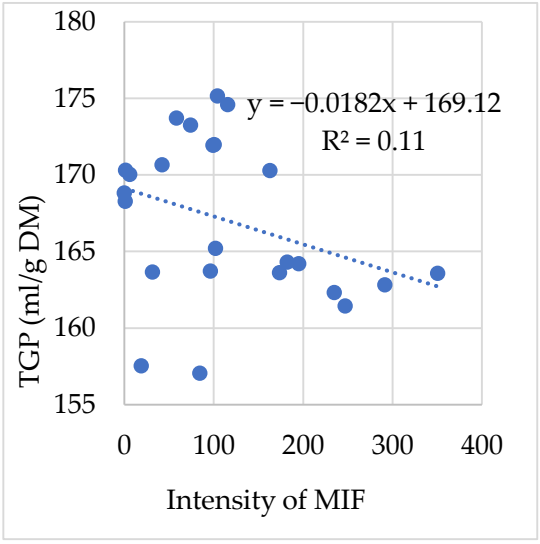
and their thresholds of maximum and minimum dose-dependent influence might determine their biological activities, including antimethanogenesis. The potential *m/z* ion features identified for their contribution to higher CH_4 inhibition were found in high and low CH_4 inhibition sample groups of the study. The variations in their effect on the two groups might be attributed to differences in their concentration. However, several MIFs seemed to contribute positively to the higher CH_4 inhibition potential of the samples with different levels of contribution. The three MIFs illustrated in Table 3.2, individually or combined, provided the most responsibility for the higher CH_4 inhibition potentials of Moringa accessions. However, MIF 4.53_433.1112 showed significant variations after the Bonferroni correction at corrected $P \leq 6.4 \times 10^{-4}$ and seemed to have the greatest responsibility for the higher inhibition of CH_4 recorded in the study. However, MIF 4.44_609.1462 could be a promising candidate in the future because of its larger intensity, which might be easier for analysis and practical applicability. It also had a relatively good R^2 value and correlation coefficient.

This study also associated these MIFs with other major fermentation characteristics of ruminant animals, such as total gas production (TGP) and IVOMD, to determine their overall effect on fermentation kinetics and animal performance. Thus, the MIFs selected for their contribution to higher CH_4 inhibition correlated negatively and significantly with CH_4 production, TGP and CH_4/IVOMD. However, MIF 14.22_682.3577 was not significantly correlated with CH_4/IVOMD at $P < 0.01$ (Table 3.2). However, MIF 4.44_609.1462 and MIF 4.53_433.1112 were not significantly ($P > 0.05$) correlated with IVOMD, except MIF 14.22_682.3577, which correlated negatively and significantly ($P < 0.05$) with IVOMD. Thus, MIF 14.22_682.3577 is not of interest in this study because of its negative effect on digestibility and nonsignificant reduction in CH_4 yield per unit of organic matter digested, which might decrease the animal's performance during its application. The relationships scatter plot analysis between MIF 4.44_609.1462 and MIF 4.53_433.1112 intensities versus TGP (ml/g DM) showed R^2 values of 0.11 and 0.42 (Figure 3.5). Thus, the contribution to higher CH_4 inhibition potential obtained in these *m/z* ion features with no effect on the IVOMD of the tested feed proved significantly that it did not influence fermentation kinetics or the productivity of the ruminant animals negatively. However, its detailed bioactivity needs to be confirmed by future studies.

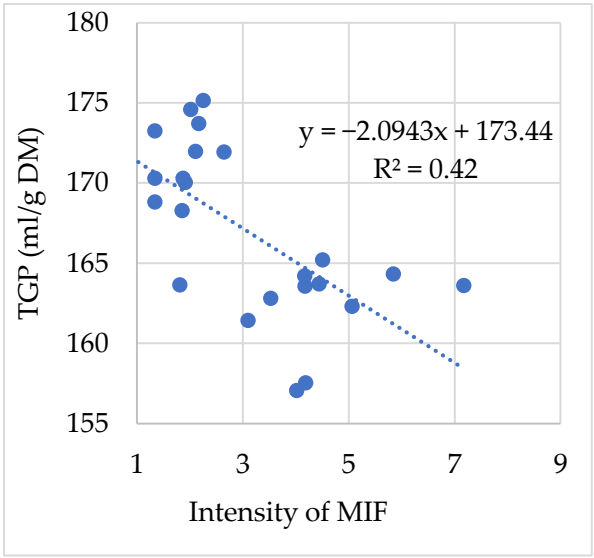
Table 3.2 Summary data of potential *m/z* ion features contributed to higher methane inhibition in Moringa accessions and their Pearson correlation with major *in vitro* fermentation characteristics

	***M/z* ion features (MIFs)**		
	Negative ion mode	**Positive ion mode**	
	4.44_609.1462	**4.53_433.1112**	**14.22_682.3577**
Detected mass (mol)	609.1462	433.1112	682.3577
Actual mass (mol)	609.1462	432.1112	681.3577
Retention time (sec)	4.44	4.53	14.22
ALI	63.89	1.81	1.54
AHI	167.66	4.10	2.83
Fold change	2.02	2.54	2.13
R^2-value with CH_4	0.42	0.54	0.25
p value	0.021	4.7×10^{-7}	0.0047
Pearson correlation coefficients of the selected MIFs with CH_4, total gas and IVOMD (2-tailed)			
CH_4	−0.48 *	−0.74 **	−0.50 *
Total gas	−0.63 **	−0.79 **	−0.37 *
IVOMD	0.09	−0.29	−0.46 *
CH_4/IVOMD	−0.59 **	−0.66 **	−0.37

Correlation coefficients with ** and * indicate significant correlations at P <0.01 and P <0.05, respectively. ALI: average intensity of the *m/z* ion features in the lower CH_4 inhibition sample group; AHI: average intensity of the *m/z* ion features in the higher CH_4 inhibition sample group; actual mass: detected mass minus one hydrogen atom (in the positive ion mode); IVOMD: *in vitro* organic matter digestibility (%); sec: second

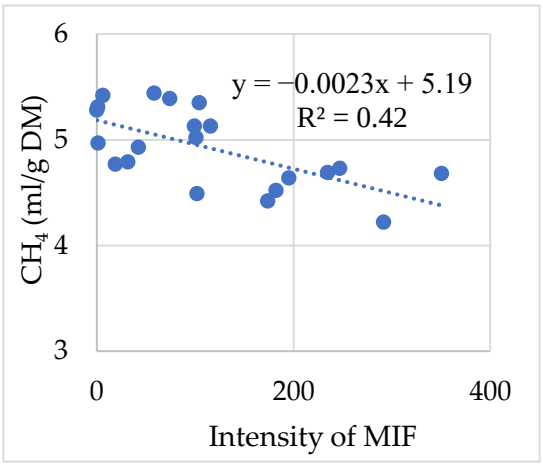


(a) TGP vs MIF 4.44_609.1462

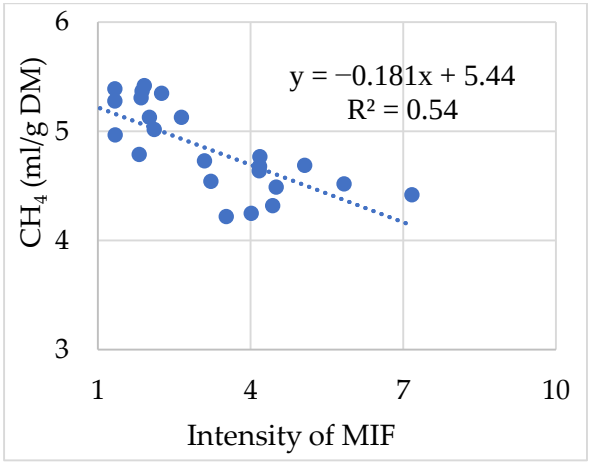


(b) TGP vs MIF 4.53_433.1112

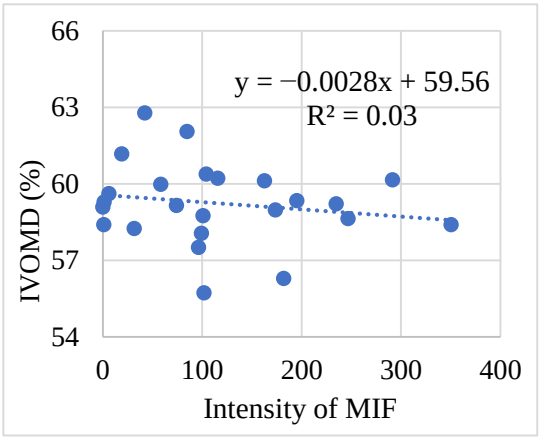
Figure 3.5 *Cont.*



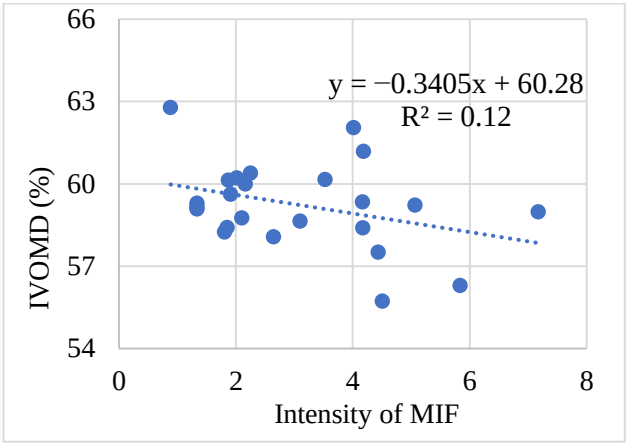
(c) CH_4 vs MIF 4.44_609.1462



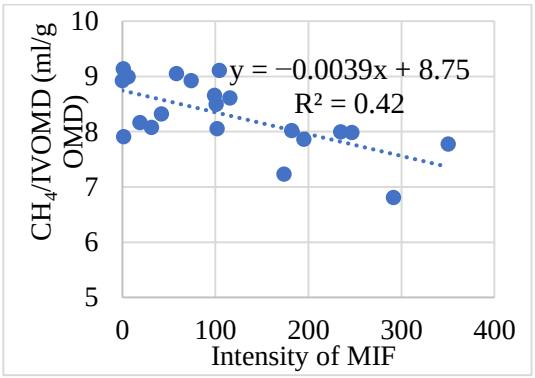
(d) CH_4 vs MIF 4.53_433.1112



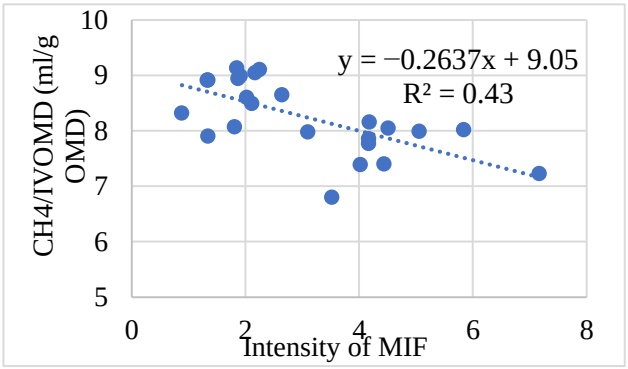
(e) IVOMD vs MIF 4.44_609.1462



(f) IVOMD vs MIF 4.53_433.1112



(g) CH_4/IVOMD vs MIF 4.44_609.1462



(h) CH_4/IVOMD vs MIF 4.53_433.1112

Figure 3.5 Scatter plot of selected *m/z* ion feature 4.44_609.1462 and 4.53_433.1112 contributed to higher methane inhibition, which showed the relationships of *m/z* ion feature intensities with *in vitro* total gas, methane production, organic matter digestibility, and methane yield per organic matter digestibility

Where CH_4 is the total methane produced; IVOMD is the *in vitro* organic matter digestibility of the substrate incubated; MIF is metabolite ion feature; and TGP is the total gas produced.

3.3.4 Associating the m/z ion features with their contribution to low methane inhibition

Among the MIFs that showed significant variation between the two groups of samples, most were associated with the lower CH_4 inhibition group (all data not shown). After analyses of the *t* test, fold change, PCA, and volcano plot, these candidate *m/z* ion features, for their contribution to lowering CH_4 inhibition, are presented in Table 3.3. Among the *m/z* ion features detected in the UPLC column that were significantly (P <0.05) different between higher and lower CH_4 inhibition in the *t* test, most MIFs intensities and total CH_4 production were positively associated with lower CH_4 inhibition characteristics of the samples, with R^2 values ranging from 0.03 to 0.64. However, the volcano plot and Bonferroni correction were used to explore the MIFs most responsible for lower CH_4 inhibition. Only seven MIFs (one in the negative ion mode and six in the positive ion mode) varied between the two sample groups at the corrected $P \leq 6.25 \times 10^{-3}$ in the negative ion mode and $P \leq 6.4 \times 10^{-4}$ in the positive ion mode. Thus, the Bonferroni correction reduced the candidates to seven and selected the MIFs that showed an R^2 value above 0.39 (Table 3.3). However, all the *m/z* ion features that were positively associated with lower CH_4 inhibition in the scatter plot analysis (Figure 3.6) and varied significantly by the *t* test and volcano plot between the two sample groups of the study were expected to affect methanogenesis with different levels of contribution. Among these *m/z* ion features, the highest Pearson correlation coefficients and relationship values (R^2 value) of CH_4 production with *m/z* ion features intensity were obtained in MIF 15.00_487.2319 (0.80; 0.64) and MIF 9.06_443.2317 (0.77; 0.59), respectively (Table 3.3; Figure 3.6). The *m/z* ion features selected for their contribution to the lower CH_4 inhibition characteristics of the accessions were included in both sample groups of the study with different levels of intensity. Hence, their variations in the antimethanogenesis effect might be associated with differences in concentration among accessions and within samples of the accessions.

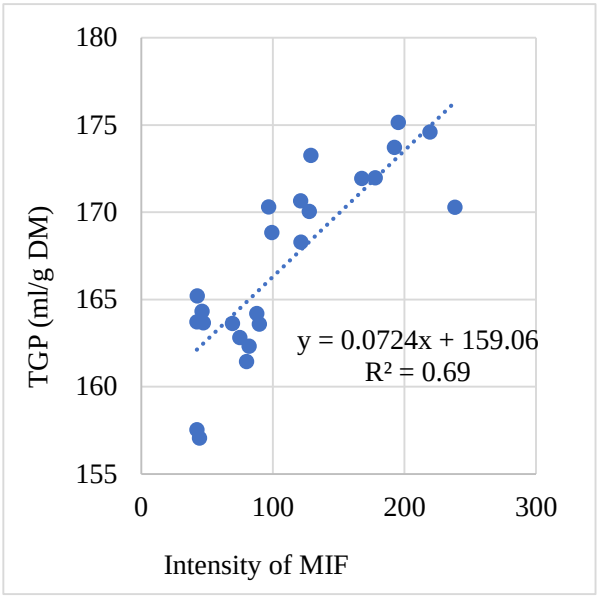
The seven *m/z* ion features selected for their contribution to lower CH_4 inhibition were also analysed for their correlation and relationship with *in vitro* TGP, IVOMD, and CH_4 per unit of OMD (Table 3.3). Thus, all seven *m/z* ion features were positively and significantly correlated with CH_4, TGP, and CH_4/IVOMD (P <0.05). However, they did not (P >0.05) correlate with the IVOMD of the substrate feed. In the scatter plot analysis, intensities of MIF 9.06_443.2317 and MIF 15.00_487.2319 showed relationship values (R^2 values) of 0.69 and 0.08 with TGP across samples of the study. These *m/z* ion features were also

positively and significantly (P <0.01) correlated with CH_4 yield per organic matter digested and showed a relationship (R^2 value) of 0.48 and 0.56, respectively. However, the nonsignificant correlation of these *m/z* ions features intensities with IVOMD proved that they did not affect the fermentation kinetics and productivity of the animals negatively. Therefore, the *m/z* ion features selected for their contribution to lower inhibitors of the accessions may have an antagonistic effect on the *m/z* ion features that are involved in higher CH_4 inhibition or may stimulate methanogens during ruminal fermentation with no effect on OMD. However, this study did not reveal enough evidence of the bioactivity and mechanism of action of the MIFs. The seven *m*/*z* ion features would have been selected as potential candidates, individually or combined, and might be the responsible MIFs, although MIF 9.06_443.2317 and 15.00_487.2319 could be the most promising *m*/*z* ion features for the lower CH_4 inhibitor Moringa accessions. However, identification of their names, structures and detailed biological activities will be needed in the future.

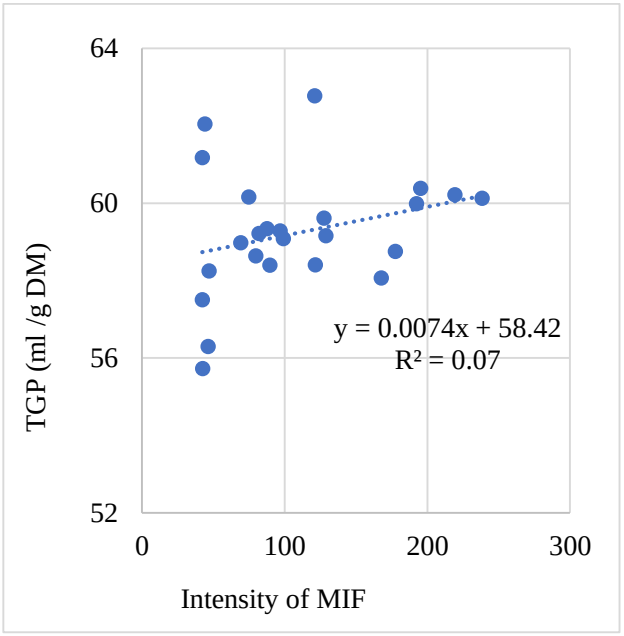
Table 3.3 Summary data of potential candidate *m/z* ion features contributed to lower methane inhibition in the accessions and their Pearson correlation coefficients with *in vitro* fermentation characteristics

	***M/z* ion features**						
	Negative ion mode			**Positive ion mode**			
	11.01_265.1448	**9.06_443.2317**	**11.84_581.3658**	**12.31_355.1513**	**14.47_341.2650**	**15.00_487.2319**	**15.02_385.2907**
Detected mass (mol)	265.1448	443.2317	581.3558	355.1513	341.2650	487.2319	385.2907
Actual mass (mol)	265.1448	442.2317	580.3658	354.1513	340.2650	486.2319	384.2907
Retention time (sec)	11.01	9.06	11.84	12.31	14.47	15.00	15.02
ALI	668.2	157.2	3.5	18.9	57.4	214.0	84.9
AHI	394.1	58.5	1.8	5.2	15.9	33.1	19.1
Fold change	0.3	0.4	0.5	0.3	0.3	0.1	0.2
R^2-value with CH_4	0.45	0.59	0.42	0.48	0.39	0.64	0.54
p value	3.2×10^{-6}	4.75×10^{-7}	2.9×10^{-4}	1.1×10^{-4}	4.2×10^{-4}	4.27×10^{-6}	1.2×10^{-4}
Pearson correlation coefficients of the selected MIFs with CH_4, TGP and IVOMD (2-tailed)							
CH_4	0.62 **	0.77 **	0.65 **	0.69 **	0.63 **	0.80 **	0.74 **
TGP	0.66 **	0.65 **	0.51 *	0.58 **	0.52 **	0.68 **	0.64 **
IVOMD	0.15	0.26	0.44	0.29	0.33	0.18	0.32
CH_4/IVOMD	0.58 **	0.69 **	0.52 **	0.61 **	0.53 **	0.75 **	0.66 **

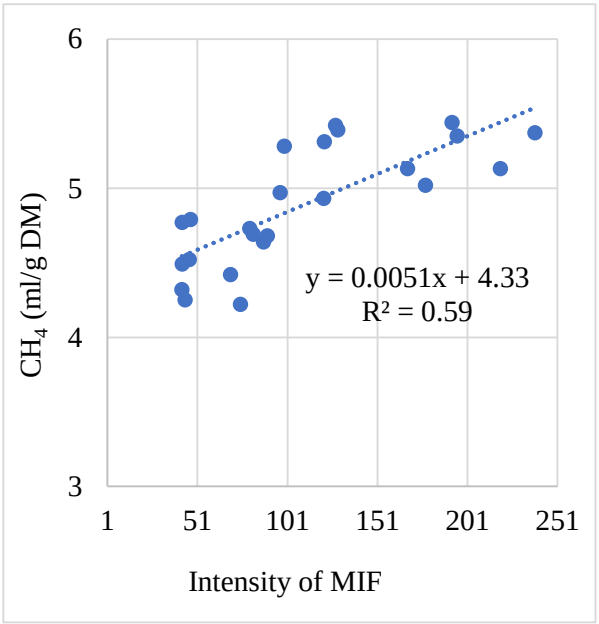
The MIFs intensities between higher and lower CH_4 inhibition sample groups are significantly different at the corrected $P \leq 6.25 \times 10^{-3}$ in the negative ion mode and $P \leq 6.4 \times 10^{-4}$ in the positive ion mode. Pearson correlation coefficients with ** and * are significant at $P < 0.01$ and $P < 0.05$ (2-tailed), respectively. ALI is the average intensity of the *m/z* ion features in the lower CH_4 inhibition sample group, whereas AIH is the average intensity of the *m/z* ion features in the higher CH_4 inhibition sample group. Actual mass indicates the detected mass minus one hydrogen atom (in the positive ion mode); TGP is total gas produced (ml/g DM); and IVOMD is *in vitro* organic matter digestibility (%)



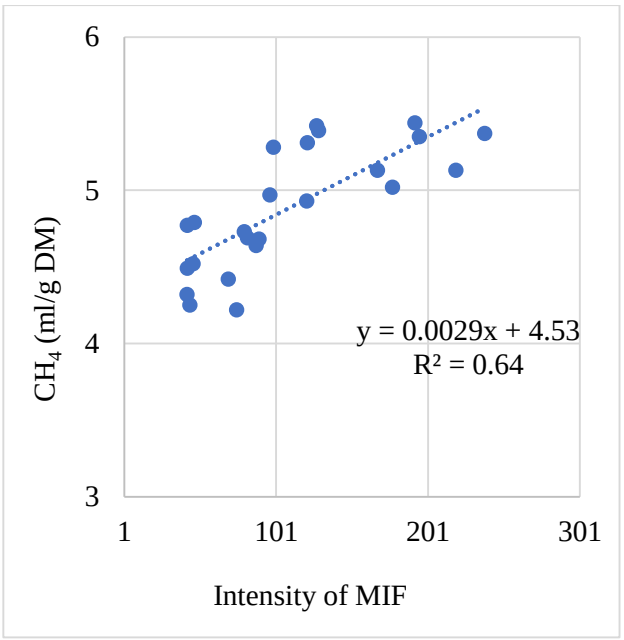
(a) TGP vs MIF 9.06_443.2317



(b) TGP vs MIF 15.00_487.2319

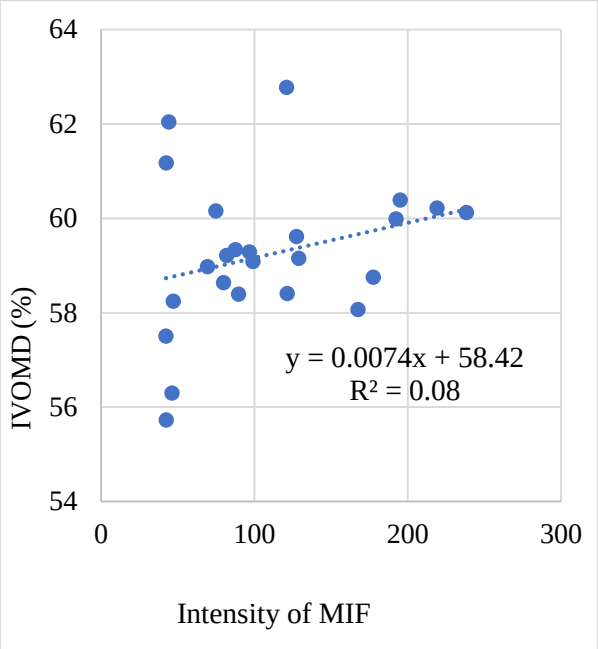


(c) CH_4 vs MIF 9.06_443.2317

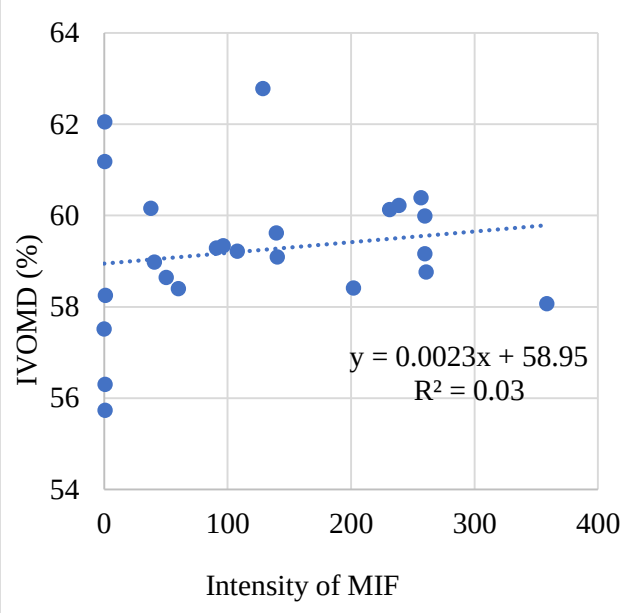


(d) CH_4 vs MIF 15.00_487.2319

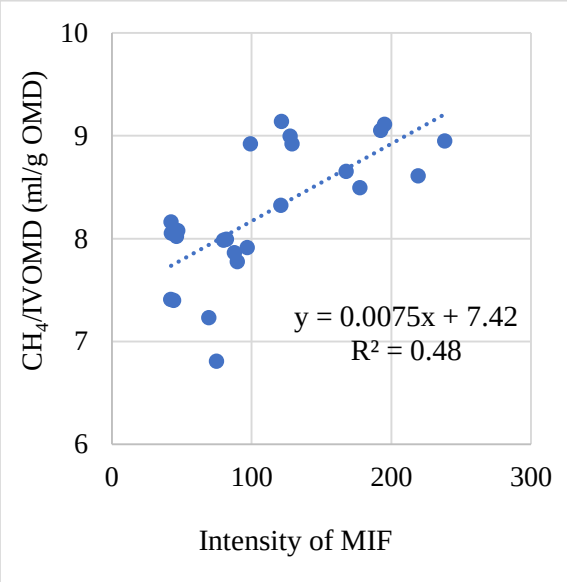
Figure 3.6 *Cont.*



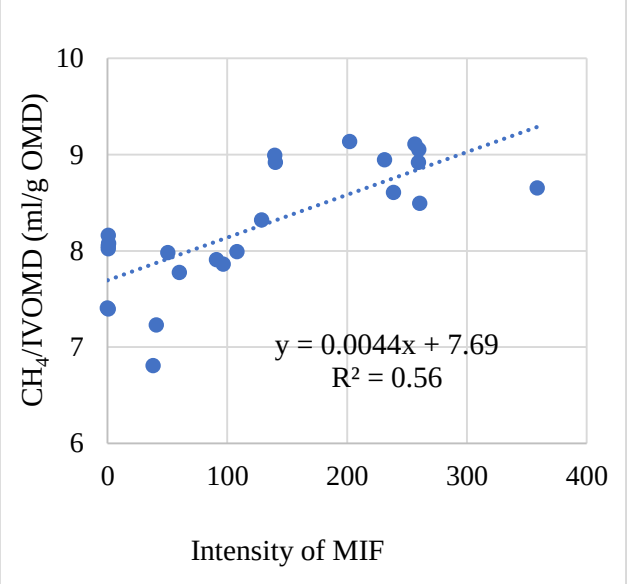
(e) IVOMD vs MIF 9.06_443.2317



(f) IVOMD vs MIF 15.00_487.2319



(g) CH_4/IVOMD vs MIF 9.06_443.2317



(h) CH_4/IVOMD vs MIF 15.00_487.2319

Figure 3.6 Scatter plot of the selected *m/z* ion features 9.06_443.2317 and 15.00_487.2319 contributed to lower methane inhibition, which showed the relationship of their intensities with *in vitro* total gas, methane production, organic matter digestibility, and methane per organic matter digestibility across samples

Where CH_4 is the total methane produced; IVOMD is the *in vitro* organic matter digestibility of the substrate incubated; MIF is metabolite ion feature; and TGP is the total gas produced.

3.4 Discussion

Secondary plant metabolites are produced by stress and defence response signalling in plant growth (Faehnrich *et al.*, 2021), although their composition and concentration are determined by the species, physiology, developmental stage, and environmental factors such as light, temperature, water, soil fertility, and salinity (Yang *et al.*, 2018; Isah, 2019; Li *et al.*, 2020). However, some metabolite ion features were not found in all samples, and others varied in intensity, which confirmed the variations in the accessions in terms of composition and concentration, even when they were grown in the same environment. Among the 10 potential candidate *m*/*z* ion features selected after screening all were included in both sample groups with different concentration levels. However, the intensity of some MIFs within the group was below the limits of detection, and a zero value was recorded in the detector. Similarly, among the 122 metabolite constituents identified in a previous study of Moringa accessions grown in China and India, 118 were shared components. However, four compounds were detected in only one of the accessions (Lin *et al.*, 2019).

The bioactive SPMs denoted by *m*/*z* ion features in this study affected methanogenesis in various ranges by suppressing the bioactivity of active compounds involved in CH_4 inhibition or by changing the pathways that encourage CH_4 production. Thus, the MIFs in higher CH_4 inhibitors reduced CH_4 per unit of organic matter digested because of their increasing effect or no effect on OMD. On the other hand, increased total CH_4 production and CH_4 yield were found in the lower CH_4 inhibitor accessions compared with the higher inhibitor group. However, all samples reduced the total CH_4 production and CH_4 yield per unit of OMD with improved IVOMD of the substrate feed compared with the control, and the magnitude of the antimethanogenic effect varied within accessions. This might be because of the cumulative effect of all the compounds in the sample. Thus, the biological activity of the *m*/*z* ion features that contributed to higher inhibition might have dominated those that were responsible for lower CH_4 inhibition. The benefits of decreasing total CH_4 production and CH_4 yield per organic matter digested were recorded in all accessions with a significant improvement in IVOMD. Thus, it is apparent that this antimethanogenic variation and improvement in IVOMD are attributed to the difference in the compounds that existed in the plants and their extracts. This is supported by several studies in which SPMs were reported as antioxidant and antimicrobial agents that act against bacteria, protozoa, and

fungi (Francis *et al.*, 2002; Bodas *et al.*, 2012; Cieslak *et al.*, 2013; Al_husnan & Alkahtani, 2016; Lelario *et al.*, 2018; Othman *et al.*, 2019; Teclegeorgish *et al.*, 2021).

The effects of *m/z* ion features in the samples of the study might be attributed to the variation in the dose-dependent influence of the SPMs and their thresholds of maximum and minimum activities (Macheboeuf *et al.*, 2008; Burrell-Saward *et al.*, 2017; Gokulan *et al.*, 2019). There were variations within the SPMs in their minimum inhibitory concentration to rumen microbes and among microbial species in their sensitivity to metabolite action. This might be part of the reason that the MIF 4.53_433.1112 level of intensity was very low, whereas it exhibited comparable and higher antimethanogenesis with the *m/z* ion features having larger intensity. Of the 10 potential *m/z* ion features selected all were found in both high- and low-inhibition sample groups. Thus, their variation on the effect of antimethanogenesis might be associated with the level of concentration in the samples. Consequently, along with the type, structure, and functional group of the SPMs affecting their antimethanogenic potential, the concentration of the specific *m/z* ion features substantially influenced their effect on CH_4 production.

The variations in CH_4 inhibition may also be associated with differences in selective direct inhibition and toxic effects (for example, condensed tannin on methanogens or saponins on protozoa) of the compounds or depression of the microbial metabolic processes involved in methanogenesis (Goel & Makkar, 2012). Some SPMs such as essential oils and tannins reduce CH_4 production through the depression of rumen fermentation (Zhou *et al.*, 2020; Cardoso-Gutierrez *et al.*, 2021; Rossi *et al.*, 2022). However, this type of nonspecific action is less important because it results in lower efficiency of feed utilization and ultimately reduces the productivity of animals. However, all the MIFs selected for higher CH_4 inhibition potential and lower CH_4 inhibitors did not reduce significantly the IVOMD of the feed. Hence, they would not affect the productivity of ruminant animals negatively during their ultimate application. Furthermore, volatile fatty acids can be used as a substrate during methanogenesis by methanogens (Lee *et al.*, 2021). Hence, the mechanisms of metabolites action varied in the manipulation of diverting hydrogen (H_2) into propionate production via lactate, fumarate, and malate pathways, which decrease the flow of H_2 into CH_4 production, whereas some SPMs are involved in favouring H_2 into the acetyl-CoA and formate pathway, which produces more acetate and may increase CH_4 (Mitsumori & Sun, 2008). However, the biological activities of most SPMs are generally associated with the potential of the

specific metabolite intruding into the bacterial cell membrane and disintegrating the membrane structure that causes ion leakage. This in turn formed irreversible complexes with cholesterol in the protozoal cell membrane (Francis *et al.*, 2002; Bodas *et al.*, 2012), which ultimately reduced CH_4 production. Therefore, the observed higher CH_4 inhibition without a negative effect on OMD indicates that the inhibition potential obtained in these accessions might be partly associated with changing the H_2 pathway towards propionate production or may play a role in the direct inhibition of the methanogens involved in CH_4 production. This feature increases the interest of the MIFs selected in this study. However, further detailed investigations are warranted to annotate these *m*/*z* ion features and elucidate their pathway and mechanism of action on CH_4 production.

3.5 Conclusions

The study confirmed that there are variations of *M. oleifera* accession plant extracts in *m*/*z* ion features intensities, composition, and antimethanogenesis (18–29%) even when grown in the same environment. However, several SPM ion features were detected in the initial stage of screening: three *m*/*z* ion features for high CH_4 inhibition and seven for low CH_4 inhibition, individually or combined, potentially contributing to the antimethanogenic variation in these accessions. Among them, the bioactive *m*/*z* ion features 4.53_433.1112 and 4.44_609.1462 had higher CH_4 inhibition, whereas the *m*/*z* ion features 9.06_443.2317 and 15.00_487.2319 had lower CH_4 inhibition and were selected as potential markers for Moringa. The promising *m*/*z* ion features selected for both higher and lower CH_4 inhibitors did not affect the fermentation and productivity of the animals adversely. Hence, the use of these *m*/*z* ion features as potential markers to standardize and select through breeding varieties for high CH_4 inhibition characteristics would increase the benefit without compromising the feed quality when used as dietary additives in ruminant feeding. However, further detailed investigations on matching their name, structure, and biological activities need to be carried out using a multidisciplinary approach before application in CH_4 mitigation strategies.

CHAPTER 4

Investigating the associative effects of binary cocktails of *Moringa oleifera* accessions extracts mixed in various proportions on *in vitro* rumen fermentation parameters

Abstract

Moringa is an important medicinal plant with multiple uses, and the leaf extracts of some accessions have shown high enteric CH_4 inhibition properties. Improving our understanding of the bioactivities of the key SPMs (e.g. Hesperidin and Isovitexin) involved in antimethanogenesis of the promising accessions and factors that influence the level of activities of sole accessions or cocktail extracts would probably increase the efficacy and sustainability of the final products. Therefore, this study was conducted to investigate the effectiveness of using binary cocktails prepared from two promising Moringa accession leaf extracts, mixed in various proportions, to exploit additive or synergic effects in inhibiting CH_4 emission. Two promising CH_4-inhibiting Moringa accessions (accession A3 and A11) were selected from earlier studies conducted at the University of Pretoria. The leaves were freeze-dried, milled, and extracted with methanol. Then, the cocktail extracts were prepared by mixing the selected accessions as 100:0, 80:20, 60:40, 50:50, 40:60, 20:80, and 0:100 proportions (A3:A11) and applied to anaerobically incubated *E. curvula* hay with 50 mg extract per kg substrate. Cocktails exhibited significantly (P <0.05) different *in vitro* TGP, total CH_4, OMD, acetate, propionate, CH_4 inhibition, and C_2/C_3 ratio. All binary cocktails or sole accession extracts reduced the TGP and CH_4 volume compared with the control with a co-benefit of enhanced IVOMD. The cocktails resulted in equal or higher IVOMD (52–58%) and antimethanogenesis (13–18%) compared with the IVOMD (53–54%), and antimethanogenesis (12–13%) recorded in the sole accession extracts, whereas the control produced the highest contents of acetate and TVFA. Most of the binary cocktails maintained higher antimethanogenic potential than the single plant extracts without affecting the kinetics of fermentation negatively, which proved from equal or higher OMD recorded in the control and sole accession extracts. Associative effects were recorded in the cocktail $A3_{50}A11_{50}$ (propionate, CH_4 inhibition, TVFA, CH_4/TVFA and C_2/C_3) and cocktail $A3_{60}A11_{40}$ (TVFA, acetate, and CH_4/TVFA). When applied as an additive in ruminant feeding, the 50% binary combination of the selected *M. oleifera* accession extracts appears to have multiple benefits on higher antimethanogenesis and propionate production with a decrease in C_2/C_3 ratio. The tendency for higher antimethanogenic activity observed in the

cocktails, though all not supported by the chi-square test, might be generally associated with the difference in the key bioactive SPM ion features, total phenolics and total flavonoids found in the cocktails. However, the repeatability, the complex biochemistry undertaken in the mixture and detailed mechanisms of action need to be confirmed using a comprehensive untargeted metabolomics approach with *in vivo* animal evaluation that integrates observation on rumen microbial population dynamics and metabolomics analysis of the diet, rumen fluid, faecal and urinary excretion as well as animal products.

Keywords: acetate; acetate to propionate ratio; methane inhibition; *in vitro* organic matter digestibility; propionate; volatile fatty acids

4.1 Introduction

Methane has recently been a concern worldwide because of its great negative impact on the environment (Liu *et al.*, 2021). However, methane has shorter atmospheric lifetime (12 years) compared with 100-200 years atmospheric lifetime of CO_2, it has twenty-three times more global warming effect than CO_2 and has been the second important GHGs next to CO_2 (Scholtz *et al.*, 2020). Ruminants (i.e. buffalo, cattle, sheep and goats) appear to be a higher emitter of CH_4 to the atmosphere than any other sole anthropogenic source of CH_4, which is associated with the anaerobic fermentation with diverse microbes found in the rumen (Black *et al.*, 2021). The CH_4 produced by ruminants has not only a negative impact on the environment but also affects the available energy and productivity of the animals adversely (Hristov *et al.*, 2013). Hence, establishing sustainable mitigation interventions for enteric CH_4 emission, including the development of dietary additives, has been inevitable and a priority issue globally. Among the strategies, the use of natural medicinal plants containing bioactive SPMs is increasing because of the cost-effectiveness and environmentally friendly nature of the interventions (Haque, 2018; Poornachandra *et al.*, 2019; Canul-Solis *et al.*, 2020; Ku-Vera *et al.*, 2020; Durmic *et al.*, 2021). However, several factors, such as plant genetics, growing environment and postharvest conditions, substantially affected the key bioactive SPMs composition, concentration and antimethanogenic efficacies (Verma & Shukla, 2015; Nuryanti & Puspitasari, 2017; Li *et al.*, 2020; Szepesi, 2021).

The use of plant extract cocktails of different plant species or accessions might be more or less effective than applying in a single species/accession form. A blend mixed with a ratio of 6.25:6.25:0.125 ml/L (FB:SM:EG) resulted in an associative effect on *in vitro* CH_4

inhibition tested among the four cocktails prepared from *Ficus bengalensis* (FB) leaf extract (as a source of tannins), *Sapindus mukorossi* (SM) fruit extracts (as a source of saponins) and *Eucalyptus globulus* (EG) oils (as a source of essential oils) (Singh *et al.*, 2018). Similarly, among the five cocktails (1:0, 3:1, 1:1, 1:3, and 0:1; tannins:saponins) prepared from *Swietenia mahogani* extract (tannin) and *Sapindus rarak* fruits (saponins) and added in high fibre and high concentrate substrates, a cocktail mixed at 1:3 ratio (tannins:saponins) recorded favourable associative antimethanogenic activity in high concentrate diet (Jayanegara *et al.*, 2020). In a different study, cocktails of essential oils, tannins and bioflavonoids mixed at a ratio of 1:2.5:0.1 also reduced CH_4 production (Rossi *et al.*, 2022). The binary cocktails of *Camellia sinensis* leaf, *Vitis vinifera* seed and *Uncaria gambir* leaf extracts with *Acacia mearnsii* bark extract also showed 3-5 times more CH_4 reduction than a decrease caused by acacia extract alone (Sinz *et al.*, 2019). However, the modes of action of the key specific SPMs involved in various combinations of these studies lack sufficient explanations.

Moringa oleifera leaf extracts showed 5–29% antimethanogenic variations because of the difference in dosage levels (Akanmu & Hassen, 2017). Similarly, several studies also linked the different bioactivities of plant extracts to the variation in the dose with the thresholds of the active ingredients (maximum and minimum concentration) to their bioactivities (Burrell-Saward *et al.*, 2017; Gokulan *et al.*, 2019). The dose-response of various fermentation parameters on pure and plant-extracted essential oils (2.5, 5.0, 10.0, and 25.0 mmol/L) exhibited significantly different amounts of total gas, hydrogen, methane, ammonia, acetate, propionate, butyrate, and valerate (Macheboeuf *et al.*, 2008). However, these SPMs might also work with synergistic modes of action in terms of the recorded non-linear higher antimethanogenic activity. Furthermore, there are also variations within the microbes to SPMs action and among the SPMs in their lowest inhibitory quantity or dose to the microbes (Li *et al.*, 2013; Yin *et al.*, 2021).

Thus, studying the various cocktails of Moringa accessions that vary in the key bioactive SPMs concentrations (i.e. total phenolics, total flavonoids, and SPM ion features) and mode of action (anti-protozoa, anti-methanogen, etc.) is suggested to exploit potential synergic effects as compared with applying them in a sole form. However, not all mixtures have positive synergistic effects, and sometimes the cocktails may have antagonistic or only additive effects on the traits of interest. An adequate understanding of the factors that

influence the level of activities of plant extract from individual accessions or a combination of plant extracts from different accessions may increase the efficacy and sustainability of the final products. To this end, studies are lacking to quantify the effect of varying proportions of Moringa accession extract mixtures, with known key SPMs, on rumen fermentation and antimethanogenic effect. However, Akanmu (2018) reported favourable associative effects on various *in vitro* fermentation parameters and CH_4 inhibition using the binary cocktails prepared from *M. oleifera* plant extract with some other medicinal plant extracts. Therefore, the current study investigated the effectiveness of binary cocktails prepared from various proportions of two best CH_4-inhibiting *M. oleifera* accession extracts on rumen fermentation parameters.

4.2 Materials and methods

4.2.1 Preparation of the cocktails and incubation medium

Among the twelve Moringa accessions studied previously at the University of Pretoria (Zeru *et al.*, 2022a), two promising CH_4-inhibiting accessions (i.e. accession 07633 (A3) and accession Pretoria (A11)) were selected for this study. Another subsequent study conducted by Zeru *et al*. (2022b) also associated the MIFs with low and high CH_4 inhibition and identified the key secondary MIFs involved in the high and low CH_4 inhibition potential of the Moringa accessions. In the subsequent study by Tshiyoyo *et al.* (2022, unpublished), the two key MIFs selected as responsible for higher CH_4 inhibition characteristics for these accessions were identified as Hesperidin and Isovitexin (Tshiyoyo *et al.*, 2022). The extracts used for *in vitro* incubation in this study was similar with the extracts used in Chapter 3 for *in vitro* incubation and MIFs identification, which was prepared as follows. Thus, leaf samples were freeze-dried for five days, milled (1-mm sieve) and extracted with methanol using a 10:1 (volume/mass) solvent-to-plant ratio for 96 hours in the Incoshake (Eloff, 1998). The solution containing the extract was strained with a 150-µm aperture sieve (Vickers, Durban, South Africa), semidried in a fume hood for two days and further freeze-dried up to the extract kept to a constant weight. During incubation, 5 mg of the dried crude extracts of the selected accessions (A3 and A11) were reconstituted with 1 L of deionized water. The various binary cocktails were prepared by mixing varying proportions of the two accessions with the following proportions as 100:0 (sole A3), 80:20, 60:40, 50:50, 40:60, 20:80, 0:100 (sole A11) proportions of A3:A11, as presented in Table 4.1.

Table 4.1 Binary cocktails were prepared for the study by mixing varying proportions of the selected accessions

Blends	Proportion in the mixture
Accession 07633 ($A3_{100}$)	100% A3 + 0% A11
$A3_{80}A11_{20}$	80% A3 + 20% A11
$A3_{60}A11_{40}$	60% A3 + 40% A11
$A3_{50}A11_{50}$	50% A3 + 50% A11
$A3_{40}A11_{60}$	40% A3 + 60% A11
$A3_{20}A11_{80}$	20% A3 + 80% A11
Accession Pretoria ($A11_{100}$)	0% A3 + 100% A11

For the total gas and CH_4 determination, solutions of the buffer and minerals were prepared before the incubation day following the procedures stated by Menke & Steingass (1988). Thus, the micromineral solution contained calcium chloride dihydrate ($CaCl_2.2H_2O$), cobalt chloride hexahydrate ($CoCl_2.6H_2O$), ferric chloride hexahydrate ($FeCl_3.6H_2O$) and manganese chloride tetrahydrate ($MnCl_2.4H_2O$) (Goering & Van Soest, 1970). The macromineral solution consisted of potassium phosphate monobasic (KH_2PO_4), magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and sodium hydrogen phosphate dibasic, whereas the buffer solution was prepared from sodium bicarbonate ($NaHCO_3$) and ammonium bicarbonate (NH_4HCO_3 (Na_2HPO_4) (Mould *et al.*, 2005). In addition, a resazurin solution (0.1%) was prepared from 0.1 g by dissolving it in 0.1 L distilled water before the incubation day.

Similarly, artificial saliva and urea (8.68 g/L H_2O) solutions were prepared for IVOMD determination before the incubation day (Akanmu, 2018). The artificial saliva was composed of sodium bicarbonate ($NaHCO_3$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), calcium chloride ($CaCl_2$), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), and sodium chloride (NaCl). Finally, all the solutions prepared to determine *in vitro* total gas, CH_4 and organic matter digestibility were kept at 4 ˚C.

4.2.2 In vitro incubation for determination of total gas and methane

For the collection of rumen fluids, three cannulated steers of the Pinzgauer cross Nguni (pinzyl) were adapted to *E. curvula* grass hay (substrate feed) for fourteen days. The substrate feed was incubated using serum bottles of 120 ml volume fixed with needle syringe

taps that can be opened and closed during total gas recording and CH_4 sample collection. The prepared solutions of buffer, macromineral, micromineral and resazurin were mixed with deionized water and tryptone during incubation. During the process, the medium was flushed continuously with carbon dioxide to avoid microbial changes owing to contamination with oxygen (O_2). The rumen fluid was then harvested from the steers and transported to the nutrition laboratory using a warmed thermos flask (Akanmu & Hassen, 2017). Sodium sulphide nonahydrate and L-cysteine were mixed with the medium before the rumen fluid was added to the mixture. When the medium changed to colourless, the rumen fluid was transferred to the medium at a 25:15 (volume/volume) medium-to-rumen fluid ratio. Then, each of the incubation bottles was filled with 40 ml of the mixture, closed with a stopper, and moved to the Incoshake set at an oscillatory motion of 120 rpm and 39 $^{\circ}$C. Immediately after finishing the inoculum addition to all the incubation bottles, a similar starting point was set by opening the taps for a few seconds and releasing the gas produced during inoculum addition. In each incubation/run, treatments were incubated in the Incoshake using a complete randomized design (CRD) with four replications and repeated three times (runs) in the study.

In vitro total gas was recorded using a pressure transducer PX4200-015GI (Omega Engineering Inc., Laval, QC, Canada) fitted with a digital data tracker (Tracker 220 series indicators; Omega Engineering Inc., Swedesboro, NJ, USA) (Akanmu *et al.*, 2020). Data of the pressure readings were taken at 3, 6, 12, 24 and 48 hours of incubation (Theodorou *et al.*, 1994). The gas pressure recorded at each time in psi was added to calculate the whole incubation period. Then the gas pressure recorded in psi was changed to gas volume in ml with the conversion formula stated in the ANKOM technology user guide: $V_x = V_j P_{psi} \times 0.068004084$, where V_x: gas volume at 39 $^{\circ}$C (ml); V_j: headspace of incubation bottles (ml); and P_{psi}: total pressure over the incubation period (ANKOM, 2014).

Concurrently, CH_4 samples from each incubation bottle were collected with the syringes fitted with stopcocks after each time point gas reading. The samples were injected into the gas chromatography (GC) (8610C, SRI Instruments GmbH, Bad Honnef, Germany) (Akanmu *et al.*, 2020). The recorded CH_4 area in the GC was changed to ppm with the standard curve and then to a percentage. The produced CH_4 volume in terms of ml was calculated using its age and total gas recorded from the substrate feed: CH_4 (ml) = total gas produced (ml) × % CH_4 concentration (Akanmu & Hassen, 2017). Finally, the CH_4 produced

through a 48 hours incubation period was converted to ml/g DM incubated feed for statistical analyses (Ghamkhar *et al.*, 2018).

4.2.3 Measurement of the volatile fatty acids

Volatile fatty acids (*i.e.* butyrate, isobutyrate, propionate, acetate, valerate, and isovalerate) were determined from similar incubation bottles used for the analyses of TGP and CH_4. After recording the total gas and collection of CH_4 samples for 48 hours of incubation, all the bottles were transferred into a freezer to stop the fermentation process. Twenty-five per cent of Orthophosphoric acid (25% H_3PO_4; weight/volume) was prepared from 85% H_3PO_4 to preserve the samples until analysis. A subsample (15 ml) of the incubated material was taken from each bottle and mixed with H_3PO_4 (3 ml). This subsample was kept at a freezer temperature (-20 °C) until analysed by the GC (SCION Instruments, SCION GC-456 Series, EH547FA, Scotland, UK) (Ottenstein & Bartley, 1971). Analysis of the VFA in the GC was conducted following the procedures stated in the quality assurance for animal feed analysis laboratories (Balthrop *et al.*, 2011) and by Webb (Webb, 1994). The GC has a flame ionization detector, an auto-sampler, and a CP-WAX 58 (FFAP) CB column with a 0.53 mm internal diameter, 25 m length and 2.0 µm thickness. The sample (1 µl) was injected into a capillary column of the GC. Initially, the oven temperature of the GC was kept at 80 °C for 2 min and then gradually increased to 195 °C at a rate of 30 °C/min. Helium as a carrier gas, hydrogen and purified air as combustion gases were used in the GC at a flow rate of 25, 30, and 300 ml/min, respectively. The detector and injector were set at 250 °C with a 1:100 split ratio. Each VFA in the samples was detected by comparing its retention time with those of a standard VFA reprocessed on Compass software and quantified with an external standard method (Piet & John, 2019). The determined VFAs were expressed as millimolar (mM) using the linear regression of the peak area of the standard as the y-intercept and the concentration of the standard as the x-intercept, as stated in Equations 1 and 2 below.

$$y = c + mx \quad \text{..... Equation 1}$$

$$x = \frac{(y-c)/m}{Mr} \times 100 \times DF \quad \text{..... Equation 2}$$

Where x is the amount of each VFA in the sample as millimolar (mM); c is the y-intercept; Mr is the molar mass of each VFA; DF is the dilution factor; m is the slope; and 1000 is the conversion factor of ml to L.

4.2.4 Determination of in vitro organic matter digestibility

The modified two digestion-phase techniques were used to determine the IVOMD of the substrate feed in the study (Tilley & Terry, 1963; Engels & Van der Merwe, 1967). Initially, 200 mg substrate was added in triplicate tubes with extracts of the sole accessions and cocktails (50 mg per kg DM substrate). The prepared urea solution (2 ml) was sprayed in all incubation tubes, including the blanks and controls. The artificial saliva and rumen fluid were mixed at a ratio of 3:1 (volume/volume). Then, 20 ml of the medium was transferred to each incubation tube fitted with blue stoppers and incubated for 48 hours in the Incoshake set at 39 $^{\circ}$C. In the second phase, a fresh solution was prepared by dissolving 8 g pepsin and 20 ml (32% HCl) in 2 L distilled water. After centrifugation and gentle removal of the liquid found in the incubation tubes, 20 ml of this solution was transferred into each tube and incubated for another 48 hours. The substrate residues left after 96 hours total incubation period were oven-dried at 100 $^{\circ}$C for 18 hours, measured for their dry weight, ashed and calculated for % IVOMD (Engels & Van der Merwe, 1967). Each treatment was incubated with triplicate bottles and the whole incubation was repeated three times for the study.

4.2.5 Statistical analyses

The data were subjected to ANOVA for significant variations of a parameter among the treatments. Means were compared with the Tukey test at P <0.05 using the SAS version 9.4 (SAS, 2013). Associative effects (either positive or negative) in the binary cocktails on various response parameters were determined using the chi-square test χ^2. Thus, the presence of a significant variation between the observed and expected values of a parameter exhibited an associative effect (either positive or negative) between accession extracts used in the cocktail. However, the absence of a significant variation between the observed and expected value of a parameter only showed a linear or additive effect of the cocktail.

The expected value (EV) and associative effect (AE) of a parameter in each cocktail were calculated:

$$\mathrm{EV} = \sum(\mathrm{OV\ of\ A3} \times \%\ \mathrm{A3}\ +\ \mathrm{OV\ of\ A11} \times \%\ \mathrm{A11})$$

$$\mathrm{AE} = \frac{(\mathrm{OV} - \mathrm{EV}) \times 100}{\mathrm{EV}}$$

Where OV is the observed value of the mixture; EV is the expected value of the mixture; and A3 and A11 are the selected *M. oleifera* accession 07633 and accession Pretoria, respectively.

In addition, the significance level of a test was determined by relating the critical values with the calculated χ^2 *v*alues:

$$\chi^2 = \sum \frac{(\mathrm{OV}-\mathrm{EV})^2}{\mathrm{EV}}$$

Furthermore, the general relationships of the different parameters in the cocktails were also explored using the Multivariate PCA (Hammer *et al.*, 2001).

4.3 Results

4.3.1 In vitro rumen fermentation characteristics

The substrate feed, *E. curvula* grass hay, incubated in this study was similar to that used in the previous chapters and contain 93.1% DM, 3.9% ash, 8.6% CP, 80.0% NDF, 41.0% ADF, and 8.3% ADL on a DM basis. The *in vitro* rumen fermentation results of *E. curvula* grass hay incubated with the two selected *M. oleifera* accession extracts as a sole and their binary cocktails at various proportions are presented in Table 4.2. Most of the fermentation parameters monitored in this study differed significantly (P <0.05) among treatments. The *E. curvula* grass hay treated with two selected accession leaf extracts as a sole and their binary cocktail at varying proportions reduced the TGP and CH_4 volume compared with the *E. curvula* incubated with no plant extract additive (control). In this study, the binary cocktail prepared at 50:50 ($A3_{50}A11_{50}$) proportion produced the lowest amounts of TGP and CH_4 compared with the control, sole accessions extracts and binary cocktails of the two Moringa accessions at 80:20 ($A3_{80}A11_{20}$) and 20:80 ($A3_{20}A11_{80}$) proportions. However, the

lowest volumes of TGP and CH_4 recorded for the $A3_{50}A11_{50}$ binary cocktail were not different from those recorded in $A3_{40}A11_{60}$ and $A3_{60}A11_{40}$ cocktails. Compared with the control, the IVOMD was significantly different among the cocktails, and all Moringa plant extract-added treatments recorded equal or improved OMD. This means that none of the two Moringa accessions plants extracts as a sole or their various proportion binary cocktails affected the fermentation kinetics of the feeds adversely. The Moringa cocktails at $A3_{80}A11_{20}$ (58%) and $A3_{60}A11_{40}$ (55%) proportion recorded higher IVOMD, whereas the cocktails at $A3_{20}A11_{80}$, $A3_{40}A11_{60}$ and $A3_{50}A11_{50}$ proportions showed equivalent IVOMD compared with the value recorded in the control. The IVOMD recorded in a binary cocktail at $A3_{20}A11_{80}$ (52%) proportion was lower than those obtained in $A3_{80}A11_{20}$ (58%) and $A3_{60}A11_{40}$ (55%) although it was not statistically lower than the IVOMD (48%) recorded in the control.

Table 4.2 *In vitro* fermentation results and their different indices obtained from *Eragrostis curvula* hay fermented with sole and binary cocktails at the various proportions of the two *Moringa oleifera* accession leaf extracts

Parameters	**Control**	**$A3_{100}$**	**$A3_{80}$ $A11_{20}$**	**$A3_{60}$ $A11_{40}$**	**$A3_{50}$ $A11_{50}$**	**$A3_{40}$ $A11_{60}$**	**$A3_{20}$ $A11_{80}$**	**$A11_{100}$**
TGP (ml/g DM)	190.0 ^{A}	172.7 ^{B}	171.2 ^{B}	168.7 ^{BC}	163.9 ^{C}	168.7 ^{BC}	170.2 ^{BC}	172.6 ^{B}
CH_4 (ml/g DM)	13.2 ^{A}	11.6 ^{B}	11.4 ^{B}	11.3 ^{BC}	10.8 ^{C}	11.3 ^{BC}	11.5 ^{B}	11.5 ^{B}
IVOMD (%)	47.9 ^{B}	53.0 ^{AB}	58.4 ^{A}	55.5 ^{A}	54.4^{AB}	54.8 ^{AB}	51.7 ^{B}	54.2 ^{AB}
TVFA (mM)	146.8 ^{A}	112.3 ^{BC}	117.3 ^{B}	123.4 ^{B}	117.2 ^{B}	105.7 ^{BC}	104.7 ^{BC}	91.1 ^{C}
Acetate (mM)	105.4 ^{A}	79.4 ^{BC}	80.7 ^{BC}	86.0 ^{B}	75.3 ^{BCD}	69.3 ^{CD}	69.1 ^{CD}	59.1 ^{D}
Propionate (mM)	28.7 ^{A}	22.9 ^{BC}	25.8 ^{AB}	25.7 ^{AB}	29.5 ^{A}	25.3 ^{AB}	23.3 ^{BC}	20.8 ^{C}
Butyrate (mM)	9.7	8.4	8.8	9.3	9.4	8.5	9.9	9.3
Other VFA (mM)	3.1 ^{A}	1.6 ^{B}	2.9 ^{AB}	2.6 ^{AB}	2.4 ^{AB}	2.4 ^{AB}	2.1 ^{AB}	1.9 ^{AB}
Different indices								
% CH_4 inhibition	–	12.2 ^{B}	13.4 ^{B}	14.1 ^{AB}	18.2 ^{A}	14.4 ^{AB}	13.1 ^{B}	12.9 ^{B}
CH_4/TGP (%)	7	6.7	6.8	6.7	6.6	6.7	6.8	6.7
CH_4/IVOMD (%)	27.6 ^{A}	21.9 ^{B}	19.9 ^{B}	20.5 ^{B}	19.9 ^{B}	20.7 ^{B}	22.3 ^{B}	21.2 ^{B}
CH_4/TVFA (%)	9.0 ^{C}	10.3 ^{BC}	9.8 ^{BC}	9.3 ^{BC}	9.2 ^{BC}	10.8 ^{B}	11.0 ^{B}	12.7 ^{A}
C_2/C_3	3.7 ^{A}	3.5 ^{AB}	3.2 ^{BC}	3.4 ^{AB}	2.6 ^{D}	2.8 ^{CD}	3.0 ^{BCD}	2.9 ^{CD}

Different superscript letters of the means within a row indicate significant variation among the treatments (P <0.05). C_2/C_3: acetate to propionate ratio; CH_4: Methane (ml/g DM); IVOMD: *in vitro* organic matter digestibility; Other VFA: other volatile fatty acids (isobutyrate + isovalerate + valerate); TGP: total gas produced (ml/g DM); TVFA: total volatile fatty acids (acetate + butyrate + propionate + valerate + isobutyrate + isovalerate)

Most of the *in vitro* VFA measurements, i.e. TVFA, propionate, acetate and the sum of valerate, isovalerate and isobutyrate, hereafter referred to as other VFA production, recorded

for the Moringa leaf extracts (sole or binary cocktails) were significantly different among the treatments except for butyric acid. The control maintained the highest TVFA volume (146.8 mM). The sole $A11_{100}$ extract produced significantly (P <0.01) lower TVFA compared with those values recorded for the binary cocktails at $A3_{50}A11_{50}$, $A3_{60}A11_{40}$ and $A3_{80}A11_{20}$ proportions, whereas all the cocktail extracts maintained statistically equivalent amounts of TVFA with sole $A3_{100}$ Moringa plant extract. The cocktails differed significantly (P <0.01) in acetate and propionate concentrations in the study. Hence, the control produced not the highest TVFA acetic acid in the study. The acetate contents obtained from Moringa plant extract treated *E. curvula* hay (59–86 mM) were significantly lower than the value (105 mM) recorded for the control. Although the binary Moringa plant cocktail at $A3_{60}A11_{40}$ (86 mM) proportion seemed to have a higher acetate; it was statistically not different to the values recorded for sole $A3_{100}$ (79.4 mM) and the binary cocktail of the two Moringa accessions at $A3_{80}A11_{20}$ (81 mM) and $A3_{50}A11_{50}$ (75 mM) proportions. In this study, propionic acid also varied significantly (P <0.01) from 23 mM in the $A3_{20}A11_{80}$ cocktail to 30 mM in the $A3_{50}A11_{50}$ cocktails. Although the binary cocktail at $A3_{50}A11_{50}$ proportion resulted in higher propionic acid (30 mM), it was not statistically different to the propionic acid produced from the cocktails at $A3_{40}A11_{60}$ (25 mM), $A3_{60}A11_{40}$ (26 mM), and $A3_{80}A11_{20}$ (26 mM) proportions and the control (29 mM). Relatively, the propionic acid volume recorded for the sole Moringa leaf extract of the accession $A11_{100}$ was lower than the propionic acid obtained in most binary cocktails. But the concentration of the other VFAs recorded for the cocktails was statistically similar to the other VFAs found in the control. The exception was for the sole extract of $A3_{100}$ (2 mM), which was fairly lower compared with those produced in the control (3.1 mM).

Plant extracts from the sole accessions provided significantly (P <0.05) different CH_4 inhibition, CH_4/TVFA and C_2/C_3 fermentation indices compared with those values obtained in some of the cocktails (Table 4.2). Among the cocktail, however, only % CH_4 inhibition and C_2/C_3 showed significant differences. The antimethanogenic potentials of the cocktails ranged from 13% ($A3_{20}A11_{80}$) to 18% ($A3_{50}A11_{50}$), while the CH_4 inhibition for the sole Moringa plant extracts was recorded as 12% ($A3_{100}$) and 13% ($A11_{100}$). The binary cocktails of Moringa plant extracts at $A3_{50}A11_{50}$ (18%), $A3_{60}A11_{40}$ (14%) and $A3_{40}A11_{50}$ (14%) proportion resulted in higher CH_4 inhibition compared with those maintained in cocktails of $A3_{20}A11_{80}$ (13%) and $A3_{80}A11_{20}$ (13%) proportion as well as the sole extract $A3_{100}$ (12%) and $A11_{100}$ (13%). All Moringa plant extracts (sole or cocktails) reduced CH_4 yield per unit

of organic matter digested compared with the control. The cocktails also recorded significantly (P <0.01) different C_2/C_3 ratios. In the study, the cocktail $A3_{50}A11_{50}$ and control showed the lowest and the highest C_2/C_3 ratio, respectively.

4.3.2 Determining the associative effects of the cocktails using a Chi-square analysis

Results of the chi-square test for determining the associative effects of binary Moringa plant extract mixed at various proportions are presented in Table 4.3. However, significant differences were recorded for most parameters when the two sole Moringa plant extracts were compared with their various binary cocktails, only the binary cocktails mixed at $A3_{50}A11_{50}$ and $A3_{60}A11_{40}$ proportions recorded significant associative effects on five and three parameters of the study, respectively. Among the cocktails, the 50% binary cocktail of the two *M. oleifera* accessions ($A3_{50}A11_{50}$) resulted in significant positive associative effects in terms of % CH_4 inhibition, propionic acid and TVFA production with significant negative associative effects on C_2/C_3 ratio and CH_4/TVFA. The binary cocktail of the two Moringa plant extract mixed at $A3_{60}A11_{40}$ proportion also showed significant (P <0.05) positive associative effects on acetic acid and TVFA with a negative associative effect on CH_4/TVFA. However, no associative effects were observed in this specific mixture on CH_4 inhibition, propionate production and C_2/C_3 ratio. Other than these parameters and two cocktails, none of these binary cocktails showed significant associative effects in terms of TGP, total CH_4 volume and IVOMD of the incubated substrate feed.

Table 4.3 Associative effect of cocktails of varying proportions of the selected two *Moringa oleifera* accession extracts on different fermentation parameters of the substrate feed incubated with their extracts

Parameters	**$A3_{20}A11_{80}$**	**$A3_{40}A11_{60}$**	**$A3_{50}A11_{50}$**	**$A3_{60}A11_{40}$**	**$A3_{80}A11_{20}$**
Acetic acid	9.6	2.8	8.9	21.1 *	7.1
Butyric acid	8.7	–2.9	7.8	6.5	3.2
C_2/C_3	0.3	–11.7	–18.7 *	5.0	–5.9
CH_4	–0.5	–2.0	–6.5	–2.0	–1.2
CH_4 inhibition	3.3	17.6	47.8 **	9.1	10.6
CH_4/IVOMD	4.2	–3.8	–7.9	–5.5	–9.6
CH_4/TGP	0.9	0.3	–1.5	0.3	–0.4
CH_4/TVFA	–9.8	–7.9	–18.8 *	–17.9 *	–9.5
IVOMD	–4.2	2.0	1.5	3.7	9.6
Other VFA	56.4	76.9	114.2	69.8	50.0
Propionic acid	9.7	16.8	34.5 *	15.8	14.2
TGP	–1.4	–2.3	–5.1	–2.3	–0.9
TVFA	10.0	6.3	15.3 *	18.9 **	8.5

The chi-square test values with superscripts * and ** show significant associative effects in the cocktails at P <0.05 and P <0.01, respectively. C_2/C_3: acetate to propionate ratio; IVOMD: *in vitro* organic matter digestibility; Other VFA: other volatile fatty acids (isobutyrate + isovalerate + valerate); TGP: total gas produced; TVFA: total volatile fatty acids (acetate + butyrate + propionate + isobutyrate + isovalerate + valerate)

4.3.3 Analysis of the relationships using a multivariate approach

The scores of these Moringa plant extracts (sole and cocktails) and principal component (PC) loadings of fermentation parameters of a substrate (*E. curvula* hay) incubated with the sole or binary extracts are illustrated in Table 4.4. Three main PCs explained 92% of the total variation that existed in the study. The principal component 1 (PC 1) alone accounted for 55% of the total variation and was positively correlated with TGP, CH_4, TVFA, acetate, propionate, butyrate, other VFA, CH_4/TGP, CH_4/IVOMD, and C_2/C_3. This PC was also correlated negatively with IVOMD, CH_4 inhibition, CH_4/TVFA, and the key SPM ion features (MIFs) used in this study. A higher (positive) PC 1 value was associated with the control treatment, which was characterised by higher total gas production (30%), CH_4 (31%), CH_4/IVOMD (31%), CH_4/TGP (31%), and MIF 4.53_433.1112 (32%). In contrast, the lower (negative) PC 1 values were associated with the sole $A11_{100}$ and $A3_{100}$ extracts and binary cocktails mixed at $A3_{80}A11_{20}$, $A3_{50}A11_{50}$, and $A3_{40}A11_{60}$ proportions. Thus, the negative scored values in the PC 1 were characterised by higher IVOMD (–26%), MIF

4.53_433.1112 (–32%), MIF 4.44_609.1462 (–24%) and MIF 9.06_443.2317 (–29%) values. In the study, PC 2 explained approximately 22% of the total variation and its higher values were mainly reliant on the values of $A3_{50}A11_{50}$ (1.9), $A3_{80}A11_{20}$ (1.9), and $A3_{60}A11_{40}$ (1.5) treatments, which characterised by relatively higher values of TVFA, acetate, propionate, CH_4 inhibition, other VFA, MIF 4.44_609.1462 and MIF 15.00_487.2319. The lower PC 2 values were largely attributed to $A11_{100}$ (–3.6) and $A3_{20}A11_{80}$ (–1.9) treatments and characterised by the parameters higher scored values of CH_4/TVFA (–45%), butyrate (–14%) and CH_4/IVOMD (–12%). Principal component 3 explained 15% of the total treatment variations. Its higher positive values were highly linked with the sole $A3_{100}$ and to a smaller extent with the binary cocktails mixed at $A3_{80}A11_{20}$ and $A3_{60}A11_{40}$ proportions. These higher positive values are mainly characterised by higher scored values of the parameters of C_2/C_3 (42%), MIF 4.44_609.1462 (26%) and MIF 15.00_487.2319 (33%). On the other hand, the lower or negative PC 3 values were mainly attributed to the cocktails of $A3_{50}A11_{50}$ (–2.9) and $A3_{20}A11_{80}$ (–0.5), which were mainly characterised by the parameters of % CH_4 inhibition (–52%), butyrate (–36%) and propionate (–30%) values.

Table 4.4 Scores for the selected sole Moringa accessions and their varying proportion cocktails and the principal component loadings of the response parameters

	Scores		
Treatments	**PC 1 × 100**	**PC 2 × 100**	**PC 3 × 100**
A3	–109.1	34.2	279.5
A3(80)	–107.8	187.3	96.6
A3(60)	–28.3	153.7	32.4
A3(50)	–156.4	193.8	–287.4
A3(40)	–113.5	–17.1	–22.3
A3(20)	–18.1	–189	–54.1
A11	–123.6	–364	–34.2
Control	656.8	1.2	–10.5
Parameters	**Principal component loadings**		
	PC 1 × 100	**PC 2 × 100**	**PC 3 × 100**
TGP	30.1	–9.9	16.8
CH_4	30.7	–9.0	16.5
IVOMD	–25.8	17.7	4.6
TVFA	26.5	29.7	1.2
Acetate	26.8	27.2	11.1
Propionate	14.7	37.5	–30.9
Butyrate	14.7	–13.7	–36.9
Other VFA	19.1	35.0	–4.4
% CH_4 inhibition	–1.7	25.3	–52.5
CH_4/TGP	30.6	–6.6	16.0
CH_4/IVOMD	30.8	–12.4	6.5
CH_4/TVFA	–14.2	–45.1	5.5
C2/C3	22.1	8.0	42.0
M1	–32.1	0.4	2.2
M2	–24.3	25.6	25.8
M3	–28.5	18.0	18.8
M4	–16.1	33.9	33.3
Eigenvalue	9.4	3.8	2.5
% variance	55.3	22.3	14.8

M1: secondary plant metabolite ion-feature 4.53_433.1112; M2: secondary plant metabolite ion-feature 4.44_609.1462; M3: secondary plant metabolite ion-feature 9.06_443.2317; M4: secondary plant metabolite ion-feature 15.00_487.2319; C_2/C_3: acetate to propionate ratio; IVOMD: *in vitro* organic matter digestibility; Other VFA: other volatile fatty acids (isobutyrate + isovalerate + valerate); PC: principal component; TGP: total gas produced; TVFA: total volatile fatty acids (acetate + butyrate + propionate + isobutyrate + isovalerate + valerate)

The distribution of the scores of the cocktails and vectors of these parameters across the two main PCs (PC 1 vs PC 2) is illustrated in Figure 4.1. In the figure, TGP, CH_4 volume, TVFA, acetate, CH_4/TGP, CH_4/IVOMD, and C_2/C_3 lay closer to each other and in the same direction

as the control, as referenced to PC 1. These results indicate the positive relationship between the mentioned parameters and their higher production in the control compared with the sole and cocktail extracts of the study. The cocktails denoted by red dots ($A3_{50}A11_{50}$, $A3_{60}A11_{40}$ and $A3_{80}A11_{20}$) appear to maintain higher IVOMD, CH_4 inhibition and propionic acid compared with the cocktails represented by green dots. This infers the presence of closer positive relationships among these parameters, which seemed to correspond with the ANOVA and mean comparison analyses result. The higher antimethanogenic activity and propionate production observed at $A3_{50}A11_{50}$, $A3_{60}A11_{40}$ and $A3_{80}A11_{20}$ appear to be more influenced by the key MIFs 4.44_609.1462, 15.00_487.2319 and 9.06_443.2317 than the MIF 4.53_433.1112. However, their mechanism of action needs detailed investigations.

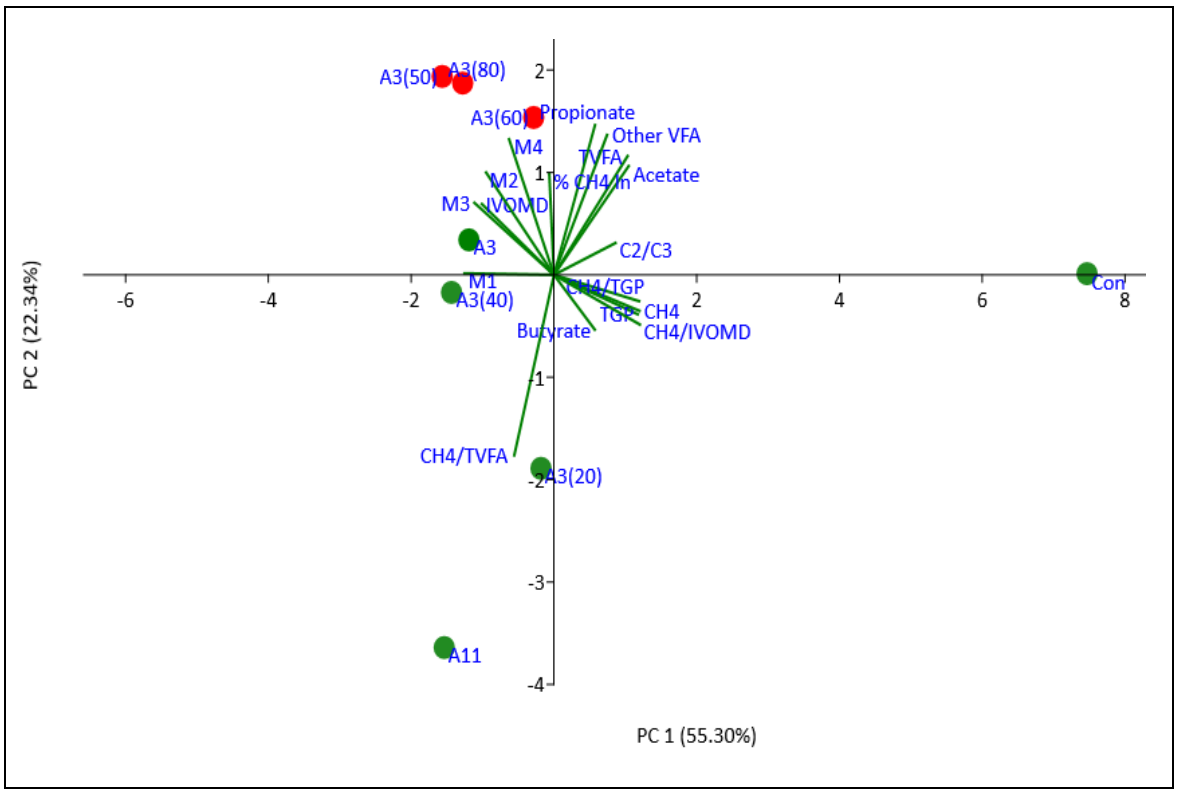


Figure 4.1 Scatter plots of the treatments and biplots of the parameters in the principal component analysis

In this figure, A3 (20) refers to the cocktail prepared at a proportion of 20% A3 + 80% A11; A3 (40) mixed at 40% A3 + 60% A11; etc; PC is the principal component. M1, M2, M3 and M4 are the selected secondary plant metabolite ion features 4.53_433.1112, 4.44_609.1462, 9.06_443.2317 and 15.00_487.2319, respectively. IVOMD is the *in vitro* organic matter digestibility; Other VFAs refer to other volatile fatty acids (i.e. isobutyrate + isovalerate + valerate); C_2/C_3 is the acetate to propionate ratio; TGP is the total gas produced; TVFA is

the total volatile fatty acids (acetate + butyrate + propionate + isobutyrate + isovalerate + valerate) produced from the incubated substrate.

4.4 Discussion

Cocktail extracts in this study maintained equivalent or higher CH_4 inhibition potentials (i.e. 13.1% obtained in $A3_{20}A11_{80}$ to 18.2% in $A3_{50}A11_{50}$) compared with the inhibition recorded for sole accession extracts (i.e. 12.2% gained in $A3_{100}$ and 12.9% in $A11_{100}$). The current study exhibited higher antimethanogenic activity (12.2–18.2%) of *M. oleifera* leaf extract compared with 4.5–5.2% CH_4 reductions recorded at doses of 75–100 mg/L, whereas it was lower than 28.7–29.3% inhibition recorded when used it at doses of 25–50 mg/L (Akanmu & Hassen, 2017). The antimethanogenic variations observed between the present and previous study reported by Akanmu and Hassen (2017) might be partly associated with the variations in the application dose, genotypes, MIFs types and concentrations, rumen fluid, buffer nitrogen, and NDF content of substrate feed samples (Maccarana *et al.*, 2016; Yáñez-Ruiz *et al.*, 2016; Zeru *et al.*, 2022b). In an *in vivo* study, *M. oleifera* leaf extract inhibited equivalent amounts of CH_4 produced per body weight gain and total CH_4 production as recorded for monensin (Soltan *et al.*, 2018). The higher antimethanogenesis obtained in the current study did not affect the total gas production adversely, which was proven by improved OMD of the Moringa plant extract-treated substrate compared with the control. This implies that none of the Moringa plant extracts (either sole or binary cocktails) tested in the current study affect negatively the fermentation kinetics, which minimizes the feed energy loss by the animals (Ungerfeld, 2018). Compared with the control, the improved OMD of the substrate feed treated with all extracts of *M. oleifera* accessions in the current study confirms enhanced IVOMD reported in previous studies for Moringa plant extracts (Akanmu & Hassen, 2017; Zeru *et al.*, 2022a).

In the current study, the significant CH_4 inhibition recorded in the χ^2 test between the expected vs observed values for a binary cocktail of Moringa plant extracts at a 50:50 ($A3_{50}A11_{50}$) proportion revealed a favourable associative effect on antimethanogenic activity. However, the other binary cocktails tested in the present study maintained linear additive effects in terms of antimethanogenesis. In agreement with the present study, *Moringa oleifera* leaf extract mixed with different medicinal plant leaf extracts also showed associative effects in terms of various *in vitro* fermentation parameters, i.e. *M. oleifera* + *Aloe vera* in TGP/IVOMD; *M. oleifera* + *Tithonia diversifolia* in CH_4/TVFA; and *M.*

oleifera + *Jatropha curcas* in CH_4 volume, CH_4/IVOMD and CH_4/TGP productions (Akanmu, 2018). However, previous studies did not show sufficient evidence about the effectiveness of different Moringa varieties or accessions cocktails compared with those used in a sole form. In contrast, a number of studies conducted other plant combinations revealed supportive associative effects for methane inhibition. Thus, Jayanegara *et al.* (2020) tested five cocktails prepared from *Swietenia mahogani* extract as tannin and *Sapindus rarak* fruits extract as saponins, and reported favourable associative antimethanogenesis at a ratio of 1:3 (tannins:saponins) combination, which agreed with the current study. Similarly, the binary cocktails of *Camellia sinensis* leaf (grape seed), *Vitis vinifera* seed (green tea) and *Uncaria gambir* leaf (gambier) extract with *Acacia mearnsii* bark (acacia) extract also exhibited three to five times more CH_4 reduction than the reduction caused by acacia alone (Sinz *et al.*, 2019). These authors generally associated the CH_4 inhibition potential obtained in these cocktails with the synergistic activities of the bioactive SPMs found in the mixture.

The desirable associative effect of the cocktails might be connected with the variations in terms of total phenolics and total flavonoids (Zeru *et al.*, 2022a) as well as different key bioactive MIFs concentrations (Zeru *et al.*, 2022b). These two reported consecutive studies showed that the two selected Moringa accessions differed significantly in their total phenolics (38.0–46.9 mg GAE/g), total flavonoids (22.8–29.2 mg QE/g) and intensity of key MIFs identified as potential candidates for the higher (MIF 4.44_609.1462) and lower CH_4 inhibition characteristics (MIF 9.06_443.2317 and MIF 15.00_487.2319). Hence, the desirable associative antimethanogenic activity was recorded at 50:50 proportions when the total phenolics, total flavonoids and the mentioned MIFs (i.e. 4.53_433.1112, 4.44_609.1462, 9.06_443.2317 and 15.00_487.2319) reached to their average concentrations or intensities in the mixture. These imply that applying the selected two accessions at 50:50 proportions may attain the bioactive MIFs involved in higher CH_4 inhibition (MIF 4.44_609.1462 and MIF 4.53_433.1112) at their thresholds of maximum antimethanogenic activities. In contrast, those MIFs selected as responsible for lower CH_4 inhibition potentials of Moringa accessions (MIF 9.06_443.2317 and MIF 15.00_487.2319) can be achieved to their thresholds of minimum antimethanogenic effects in this cocktail. These results can be accompanied by the principle that some SPMs may have unfavourable antimethanogenic effects via direct stimulation of the methanogenesis process or by suppressing the active SPMs involved in antimethanogenesis. Thus, applying medicinal

plants in the form of mixtures helped to play a role in better antimethanogenesis in both ways, that is, by maintaining the active SPMs to their thresholds of maximum and minimum bioactivities for better efficacy of the ultimate products to the traits of interest, antimethanogenic activity (Li *et al.*, 2013; Yin *et al.*, 2021). In line with this idea, other prior studies also attributed the various bioactivities of plant extracts to the variation in the dose and thresholds of the bioactive ingredients found in the plant extracts (Burrell-Saward *et al.*, 2017; Gokulan *et al.*, 2019). Similarly, the response of different parameters for the cocktails of several past studies associated with the biochemical properties, types and concentrations of the active SPMs found in the single plant species/accession or the combination of plant extracts (Goel *et al.*, 2008; Li *et al.*, 2013; Singh *et al.*, 2018). In agreement with the findings of the current study, the dose response for different pure and plant-extracted essential oils (2.5, 5.0, 10.0, and 25.0 mmol/L) also differed significantly in terms of total gas, hydrogen, methane, ammonia, acetate, propionate, butyrate, and valerate (Macheboeuf *et al.*, 2008). However, the biochemistry undertaken in the mixture and detailed mode of action with an untargeted metabolomics approach using all SPMs needs separate and comprehensive study in the future.

The favourable associative antimethanogenic activity recorded in the cocktail at 50:50 proportion of the selected two accessions might be further associated with the observed variation in molar proportions of VFAs. This cocktail resulted in non-linear positive effects on propionic acid and total VFA production as well as a negative associative effect on the C_2/C_3 ratio. This might be owing to the shift of fermentation that enhances propionate production and the associated decrease in the available H_2 to the methanogenesis pathway (Moss *et al.*, 2000; Kingston-Smith *et al.*, 2010). The propionate production pathway consumes H_2, whereas the acetate production pathway releases more H_2 than the butyrate production pathway during enteric fermentation in ruminants (Mirzaei-Aghsaghali & Maheri-Sis, 2011). Lower CH_4 production may imply the presence of faster degradation of the fibre components and increased passage rates of the substrate. Similarly, other studies also partly associated the increased propionate obtained in the saponins and *Humulus lupulus* extract with the change in H_2 pathways from CH_4 to propionate production and a reduced C_2/C_3 ratio (Flythe & Aiken, 2010; Patra & Saxena, 2010). However, the propionate favouring fermentation pathway sometimes needs large precursors and is expensive for animals (Leahy *et al.*, 2022). The decrease in acetate production at higher concentrations of plant extracts is also generally linked to the reduced ruminal protozoa and associated

methanogen activities owing to their binding properties and may sometimes affect animals' productivity negatively (Liu *et al.*, 2011).

4.5 Conclusions

All sole *M. oleifera* accession extracts, that is, 7633 ($A3_{100}$) and Pretoria ($A11_{100}$) and their varying binary cocktails (A3:A11; 20:80, 40:60, 50:50, 60:40, and 80:20) reduced the *in vitro* total gas and CH_4 production with an equivalent or higher OMD compared with the control. This study revealed many additive effects with some associative effects in binary combinations of the selected two Moringa leaf extracts. Thus, the binary cocktail of the two Moringa accessions at $A3_{50}A11_{50}$ resulted in many beneficial non-additive relationships or associative effects in terms of higher antimethanogenic activity, propionic acid, total volatile fatty acids and lower acetate/propionate ratio. The cocktail $A3_{60}A11_{40}$ also maintained non-linear effects on acetic acid, total VFA, and CH_4/TVFA. The recorded positive associative bioactivity on antimethanogenesis in the 50% cocktail generally corresponds well with non-linear increases in propionate and TVFA production and decreases in CH_4/TVFA and acetate/propionate ratios. Therefore, a 50% binary cocktail of the two selected *M. oleifera* accession plant extracts can be recommended as an additive, as it provides a higher benefit of antimethanogenesis without affecting the feed fermentation adversely when compared with the sole accession leaf extracts or other binary cocktails. However, the repeatability, the complex biochemistry undertaken in the mixture and detailed mechanisms of action need to be confirmed using a comprehensive untargeted metabolomics approach with *in vivo* animal evaluation that integrates observation on rumen microbial population dynamics and metabolomics analysis of the diet, rumen fluid, faecal and urinary excretion as well as animal products.

CHAPTER 5

The effect of postharvest processing and storage conditions on the efficacy of *Moringa oleifera* leaf extracts to inhibit *in vitro* methane production

Abstract

Leaves of *M. oleifera* are highly perishable and need to be processed for longer shelf life and storage to ensure a year-round supply of plant extract while maintaining their efficacies for various bioactivities. A series of studies were conducted to evaluate the effects of postharvest drying methods, drying temperatures, extraction solvents, storage temperatures, and storage light conditions on the efficacy of Moringa extract to inhibit CH_4 production from ruminants. In the first study, samples of Moringa leaves were dried in freeze-, shade-, sun-, and oven-drying methods, whereas the leaves were dried using an oven set at 25, 45, 60 and 80 $^{\circ}$C drying temperatures for the second study. In the third study, the plant extracts were prepared using 100% methanol, 70% acetone, 70% methanol and 70% ethanol to evaluate the effect of extraction solvents on the efficacy of plant extracts to inhibit enteric CH_4 emission. In the fourth study, the powder of Moringa plant extract was stored at 4, 15, 25 and 35 $^{\circ}$C, and under dark and fluorescent light storage conditions for six months. The drying methods, drying temperatures and extraction solvents differed significantly in terms of TGP and CH_4 inhibition, total phenolic and total flavonoid contents. Freeze- and sun-drying methods compared with shade- and oven-drying methods, as well as drying temperatures of 25 $^{\circ}$C and 45 $^{\circ}$C compared with 60 $^{\circ}$C and 80 $^{\circ}$C, showed higher total phenolics, total flavonoids and antimethanogenic activities. Similarly, 70% acetone among the studied extraction solvents resulted in better results in these parameters. Storage temperatures of 4 $^{\circ}$C and 15 $^{\circ}$C maintained higher total phenolics, total flavonoids and IVOMD with lower CH_4/TGP and CH_4/IVOMD than those recorded at storage temperatures of 25 $^{\circ}$C and 35 $^{\circ}$C. Dark storage also showed lower TGP and CH_4 production with superior total phenolics, total flavonoids and IVOMD to light storage or the control. Similar to the dark storage treatment, storage of the powder under the light for six months still reduced TGP, CH_4, CH_4/TGP and CH_4/IVOMD compared with the control. The use of 70% acetone, freeze-drying method, drying of the leaf with an oven at 45 $^{\circ}$C, and storage at 4 $^{\circ}$C under the dark conditions resulted in better antimethanogenesis effect, total phenolics and total flavonoids with equal or improved OMD. Hence, these postharvest processing methods and storage conditions are recommended to utilize Moringa plant

extracts effectively throughout the year with a minimum negative effect or none on the efficacy of the plant extracts to inhibit CH_4 emission in ruminants. However, there is a need to evaluate different organic solvents with various extraction aqueous levels and to evaluate storage temperatures for longer periods at different time points to fix a cut-off time for each storage temperature.

Keywords: antimethanogenesis; drying method; drying temperature; extraction solvent; *in vitro* organic matter digestibility; storage condition; total gas; total flavonoids; total phenolics

5.1 Introduction

Enteric CH_4 emission from ruminant animals reduces the utilization of the available feed energy for production, and its effects on global warming require effective management intervention (EC, 2021; Legg, 2021). Among the strategies, the use of medicinal plants as dietary additives has recently increased the attention of researchers (Haque, 2018; Poornachandra *et al.*, 2019; Canul-Solis *et al.*, 2020), because of their suitability, economic and safety values in producing healthy organic animal products (Jayanegara *et al.*, 2020; Durmic *et al.*, 2021; Harahap *et al.*, 2022). The bioactivities, including antimethanogenic activities of medicinal plants, are highly associated with the SPMs contents and compositions (e.g. phenolics, flavonoids, tannins, and saponins) (Bodas *et al.*, 2012; Rocchetti *et al.*, 2020; Kashyap *et al.*, 2022). Thus, studies reported the relationships between total phenolic and total flavonoid contents with the various bioactivities in medicinal plants (Chandra *et al.*, 2014; Aryal *et al.*, 2019; Phuyal *et al.*, 2020). The antimethanogenic activities of Moringa accessions correlated significantly with their total phenolic (0.62) and total flavonoid (0.70) concentrations (Zeru *et al.*, 2022a). The addition of some flavonoids inhibited CH_4 production by 5–9 ml/g (Oskoueian *et al.*, 2013). Similarly, the combination of anthraquinone with fumaric acid and the inclusion of phenolic compounds as a feed additive showed methane reduction with no negative effect on other gas fermentation characteristics (Ebrahimi *et al.*, 2011; Giuburunca *et al.*, 2014).

However, several factors that are not fully explored affect the utilization and effectiveness of medicinal plant products in rumen modulation and antimethanogenesis. Hence, postharvest processing (i.e. drying methods, drying temperatures and extraction solvents) and storage (i.e. storage temperatures and storage light) conditions have substantial roles in

the efficacy of medicinal plants (Urías-Orona *et al.*, 2017; Ademiluyi *et al.*, 2018; Gąsecka *et al.*, 2020). Leaves of plants in general and Moringa in particular are highly perishable and need to be dried, pulverized or extracted for longer shelf life. However, the efficacies of these products are reliant on the drying method, drying temperature and extraction solvent (Ademiluyi *et al.*, 2018; Gatahi & Nyoro, 2021). A study of drying methods of Moringa showed better polyphenols and terpenoids in 50% shade net and room temperature and the highest glucosinolates in the oven-drying method, whereas the shade-dried leaves developed toxic microbes (Gatahi & Nyoro, 2021). Similarly, the air-dried leaves of Moringa exhibited the highest inhibition of *Escherichia coli, Staphylococcus aureus and Staphylococcus typhi* growth compared with that recorded in the sun- and oven-dried leaves (Hussein *et al.*, 2015). Drying Moringa leaves above 55 °C also changed the leaf colour to brown (Sauveur & Broin, 2010).

The extraction solvents are essential postharvest processing factors that affect SPMs and bioactivities of Moringa products (Urías-Orona *et al.*, 2017; Abo El-Fadl *et al.*, 2020). Moringa extracted with 50% ethanol maintained the highest phenolics and antioxidant activity among three aqueous levels of methanol and ethanol (100%, 70% and 50%) organic solvents (Urías-Orona *et al.*, 2017). In a different study, the highest phenolics and flavonoid contents were recorded in methanol and aqueous-extracted leaves of *M. oleifera*, respectively (Gull *et al.*, 2016). This indicated that the effectiveness of the solvent might vary depending on the types of the target compounds and the intended use of the products. Furthermore, Moringa powder or extract needs to be stored for a year-round supply. However, most bioactive SPMs are sensitive to storage conditions such as light, humidity and temperatures (Vongsak *et al.*, 2013a; Zhang *et al.*, 2017), which in turn affect the reliability and efficacy of the stored Moringa plant products. Moringa powder stored at 15 °C provided higher total phenolics and free radical scavenging activity with lower changes in green colour after two months of storage compared with the powder stored at 35 °C (Potisate *et al.*, 2015). Studies generally indicated that the efficiency of postharvest processing and storage conditions might be varied depending on the types of bioactive SPMs needed and the intended use of the products. However, the response to modulating rumen fermentation and antimethanogenesis from ruminants to Moringa products treated under various processing and storage conditions has not been fully understood. Thus, this study evaluated the effect of postharvest processing and storage conditions of Moringa leaves or powder on the efficacies of the extracts in modulating rumen fermentation and

antimethanogenic activity in ruminants. Such information helps to optimize processing and storage techniques for Moringa plant extracts with consistent bioactive components and better efficacy when used as dietary additives to reduce enteric CH_4 emissions from ruminants.

5.2 Materials and methods

5.2.1 Plant material collection and extract preparation

Two- to three-month-old leaves of *M. oleifera* grown after the winter season were collected early in the morning at Lefakong Farm, Bosplaas, North West, South Africa, which is privately owned. Immediately after collection, the leaves were transported to the laboratory using an icebox and divided into various sample groups for the study of different postharvest processing and storage treatments. Four leaf samples were dried using freeze-drier (4 days), shade (12 days), sun (1.5 days) and oven (2 days) drying methods. The other portions of the leaf samples were dried at 25, 45, 60 and 80 $^{\circ}$C in an oven to study the effect of drying temperatures. The methods followed to evaluate drying temperatures, which commonly range from 30 to 80 °C, on the nutritional and medicinal values of Moringa were used in this part of the study (Sauveur & Broin, 2010; Kucha *et al.*, 2015; Razzak *et al.*, 2021). In terms of their suggestions, a range of drying temperatures (lower, medium and higher) were selected to evaluate their effect on the antimethanogenic activity of Moringa. However, the drying periods for each method and temperature were widely variable in previous studies, it was determined by measuring repeatedly the weight of the plant material in the drying process until a constant weight was maintained (Kumar *et al.*, 2020). The dried leaf samples were milled to pass through a 1-mm sieve. Then, the freeze-dried Moringa powder samples were stored at 4, 15, 25 and 35 $^{\circ}$C for six months using incubators to evaluate the effect of postharvest storage temperatures. Similarly, other samples of the freeze-dried powders were stored in the dark and fluorescent light at room temperature for six months using an airtight container. The storage temperatures, light and length of storage used in this study were determined partly based on Inter-Aide (2012), which suggested that Moringa leaf powder can be stored for up to six months below 24 $^{\circ}$C in airtight containers protected from light and humidity, and partly on the availability of incubators for six months' storage.

The dried powders were extracted following Akanmu & Hassen (2017). Thus, approximately 30 g of the powder that had been subject to each of the processing and storage

conditions was suspended in 300 ml solvent using a plant/solvent ratio of 1:10 (mass/volume) for 96 hours in the Incoshake (Eloff, 1998). All the postharvest samples were extracted using absolute methanol, whereas 70% ethanol, 70% acetone, 70% methanol and absolute methanol were used to evaluate the effect of extraction solvents on the efficacy of the plant extracts. After suspending the mixtures in a shaker for 96 hours, the solution was extracted under a 150-µm sieve and placed in a fume chamber for approximately 2–3 days until semidried. Ultimately, the semidried extract was further dried in a freeze-drier until a constant weight of the extract was maintained and preserved at 4 °C.

5.2.2 Ruminal fluid collection and in vitro incubation

Macromineral, micromineral and buffer solutions were prepared separately before incubation day and were stored at 4 °C until used (Goering & Van Soest, 1970; Menke & Steingass, 1988). Deionized water, micromineral, macromineral, buffer, tryptone and resazurin (0.1%; weight/volume) were mixed during incubation and bathed in warm water at 39 °C. From mixing the solutions to the end of the inoculum addition to the bottles, the whole incubation process was bubbled with CO_2. Three ruminally cannulated steers were adapted to the substrate feed for approximately fourteen days. The crude extract was redissolved (5 mg/1000 ml) with deionized water to prepare the recommended dose level of the extracts (50 mg/kg DM substrate). Then, 4 ml of the solution was transferred to each incubation bottle. The rumen fluid was then harvested from the steers and transported to the nutrition laboratory using a warmed thermos flask (Akanmu *et al.*, 2020). L-cysteine and $Na_2S.9H_2O$ were mixed with the medium before the rumen fluid was added to the mixture. When the medium changed to colourless, the rumen fluid was transferred to the medium at a 25:15 (volume/volume) medium-to-rumen fluid ratio. Then, each of the incubation bottles was filled with 40 ml of the mixture, closed with a stopper, and moved to the Incoshake set at an oscillatory motion of 120 rpm and 39 °C (Figure 5.1A). Immediately after finishing the inoculum addition to the bottles, a similar starting point was set by opening the taps for a few seconds and releasing the produced gas during inoculum addition. In each incubation or run, treatments were incubated in the Incoshake using a complete randomized design with four replications and repeated three times.

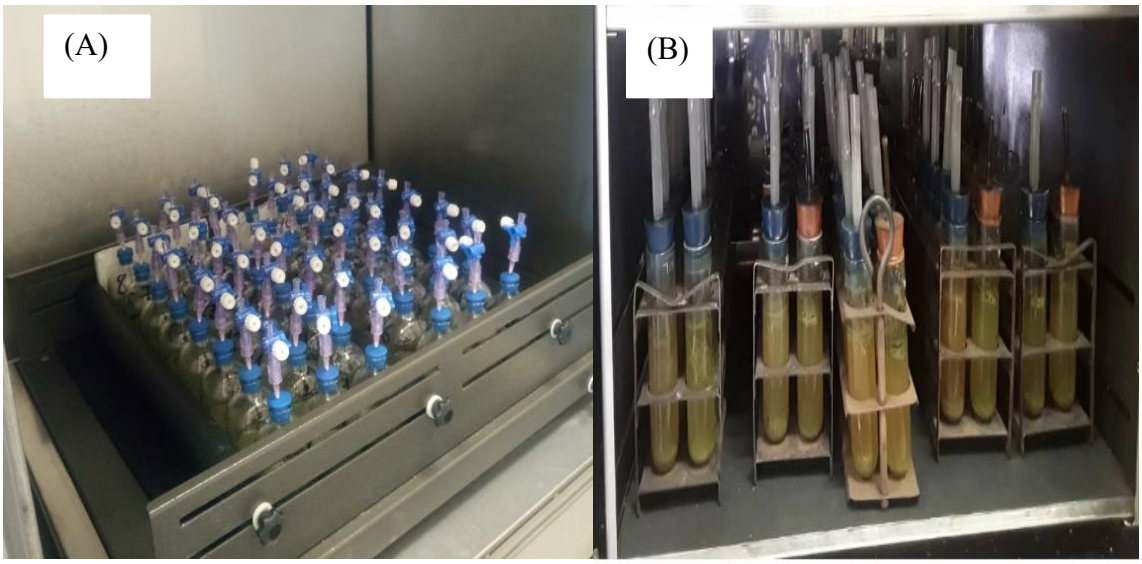


Figure 5.1 *In vitro* incubation, where (A) indicates the incubation used for determining the gas, and (B) refers to the incubation used to determine organic matter digestibility

The modified two-digestion-phase techniques were used to determine the IVOMD of the substrate feed (Tilley & Terry, 1963; Engels & Van der Merwe, 1967). Before commencing the incubation, artificial saliva and urea solution were prepared and kept at 4 °C. Initially, 200 mg of *E. curvula* grass hay was incubated under anaerobic conditions for 48 hours with extract cocktails (50 mg extract per kg DM substrate), urea, artificial saliva, and rumen fluid. Approximately 2 ml of the urea solution was sprayed in each incubation tube containing the substrate. The artificial saliva and rumen fluid were mixed at a ratio of 3:1 (volume/volume). Eventually, 20 ml of the medium was transferred to each incubation tube fitted with blue stoppers and incubated for 48 hours in the Incoshake set at 39 °C (Figure 5.1B). In the second phase, a fresh solution was prepared by dissolving 8 g pepsin and 20 ml (32% HCl) in 2 L distilled water. After centrifugation (2500 rpm for 15 min) and gentle removal of the liquid in the incubation tubes, 20 ml of the solution was transferred to each tube and incubated for another 48 hours. The substrate residues left after 96 hours of total incubation were oven-dried at 100 °C for 18 hours, measured for their dry weight, ashed and calculated for % IVOMD (Engels & Van der Merwe, 1967). Each treatment was incubated with triplicate bottles and the whole incubation was repeated three times for the study.

5.2.3 Measurement of in vitro total gas, methane and organic matter digestibility

The total gas was recorded using a semi-automated pressure transducer (PX4200-015GI, Omega Engineering Inc., Laval, QC, Canada) fitted with a digital data tracker (Tracker 220 series indicators; Omega Engineering Inc., Swedesboro, NJ, USA) (Akanmu *et al.*, 2020). Data of the pressure readings were taken at 3, 6, 12, 24 and 48 hours of incubation (Theodorou *et al.*, 1994). The gas pressure recorded at each time in psi was added to calculate the whole incubation period. Then, the gas pressure recorded in psi was changed to gas volume in mlwith the conversion formula stated in the ANKOM technology user guide: $V_x = V_j P_{psi} \times 0.068004084$, where V_x: gas volume at 39 ˚C (ml); V_j: headspace of incubation bottles (ml); and P_{psi}: total pressure over the incubation period (ANKOM, 2014).

Immediately after recording each time point's total gas reading, CH_4 samples from each incubation bottle were collected using the syringes fitted with stopcocks. The CH_4 samples collected with syringes were injected into the gas chromatography (GC) (8610C, SRI Instruments GmbH, Bad Honnef, Germany) with a pull-and-push technique. Initially, the CH_4 concentration was recorded in the GC in the form of area concentration, changed to parts per million (ppm) using the standard curve equation and then to percentage. The CH_4 volume in terms of ml was calculated using its percentage and the total gas recorded from the substrate feed: CH_4 (ml) = total gas produced (ml) × % CH_4 concentration (Akanmu *et al.*, 2020). Finally, the CH_4 produced through 48 hours of incubation was converted to ml/g DM incubated feed for statistical analyses (Ghamkhar *et al.*, 2018). In addition, the percentage of CH_4 inhibition potential of the extracts was determined: $\% \text{ MI} = \frac{(\text{TMC}-\text{TME})}{\text{TMC}} \times 100$, where % MI: % CH_4 inhibition; TMC: total CH_4 produced in the control over the incubation period (ml/g DM); TMT: total CH_4 produced by the extract over the incubation period (ml/g DM).

To determine the IVOMD, the substrate residues left after 96 hours of total incubation were oven-dried at 100 ˚C for 18 hours, measured for their dry weight, ashed and calculated for percentage IVOMD following the procedures of Engels & Van der Merwe (1967). Each treatment was incubated with triplicate bottles and the whole incubation was repeated three times for the study.

5.2.4 Total flavonoids and total phenolics determination

The total flavonoid content was measured with aluminium chloride ($AlCl_3$) colourimetric assay (Madaan *et al.*, 2011; Bag *et al.*, 2015). Approximately 20 mg quercetin standard was dissolved in 100 ml methanol. Then, the dissolved standard was further diluted to 20, 40, 80, 120, 150 and 200 µg/mlusing the same solvent. In addition, 10% $AlCl_3$ and 1 mole of potassium acetate ($KC_2H_3O_2$) were prepared using deionized water before starting the experiment. Approximately 0.5 ml of each dilution of the standard and the samples were added to separate test tubes in triplicate and mixed with 0.1 ml of $AlCl_3$ solution, 0.1 ml of $KC_2H_3O_2$, 1.5 ml of methanol and 2.8 ml of deionized water. Concurrently, blanks were prepared using deionized water instead of the $AlCl_3$ solution. Absorbance was taken at 415 nm against the blank using a UV/VIS spectrophotometer (ANALYTIK JENA AG, Model: SPEKOL 1300, Germany) after incubating the solution for 30 minutes at room temperature. Then, the total flavonoid content was determined from the readings:

$\text{Total flavonoids (mg QE/g)} = \frac{\text{QE} \times \text{D} \times \text{V}}{\text{W}}$, where QE: quercetin equivalent (µg/ml); D: dilution factor; V: volume of the extract solution (ml); W: weight of the plant extract (g) (Bag *et al.*, 2015).

The total phenolic content was determined using the Folin-Ciocalteu reagent (Madaan *et al.*, 2011; Shah *et al.*, 2016). Approximately 1 g of the crude extract was extracted three times using 15 ml methanol (50%), filtered with Whatman paper, and brought to 100 ml with 50% methanol. Then, 25 mg of the gallic acid standard was dissolved in 100 ml methanol (50%) and further diluted to 12.5, 25, 50, 100, 150 and 200 µg/ml with 50% methanol. Each dilution of the standard and 1 ml of the sample was placed into a separate test tube and further diluted with 10 ml of deionized water. After adding 1.5 ml Folin Ciocalteu reagent, the tubes were held for 5 minutes at room temperature. Four millilitres of sodium carbonate (Na_2CO_3) (20%; weight/weight) was added to each test tube, which was filled with deionized water to 25 ml and incubated for 30 minutes at room temperature. The absorbance of each sample was taken at 765 nm against the blank with a UV/VIS spectrophotometer (ANALYTIK JENA AG, Model: SPEKOL 1300, Germany). Finally, the readings obtained in the spectrophotometer were converted into mg gallic acid equivalent (GAE) total phenolics per g extract using a standard curve equation:

Total phenolics (mg GAE/g) $= \frac{\text{GAE} \times \text{D} \times \text{V}}{\text{W}}$, where GAE: gallic acid equivalent (µg/ml); D: dilution factor; V: volume of the extract (ml); W: weight of the plant extract (g) (Madaan *et al.*, 2011; Shah *et al.*, 2016).

5.2.5 Statistical analyses

The effects of the treatments were analysed using the RCBD. To accommodate the difference in the rumen fluids used in each incubation, the various runs were regarded as blocking factors in RCBD using the model treatment as a fixed effect: $Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$, where Y_{ij} is the response of treatment i observed in block j; μ is the grand mean; α_i is the effect of i^{th} treatment; β_j is the effect of the j^{th} block; and ε_{ij} is the random error. Statistically significant variations were determined with ANOVA and means were compared with the Tukey test (P <0.05) using SAS version 9.4 (SAS, 2013). After standardizing the data, analyses of the Pearson correlation and multivariate PCA were carried out using the mean data to comprehend the general relationships of factors over parameters of interest using PAST software (Hammer *et al.*, 2001).

5.3 Results

5.3.1 The effect of drying methods

Total flavonoid, total phenolic contents and *in vitro* fermentation characteristics of the *M. oleifera* leaf dried with various methods are presented in Table 5.1. The total flavonoid and total phenolic contents differed significantly (P <0.05) among the drying methods. The freeze-drying method (24.3 mg QE/g) maintained the highest total flavonoid content, followed by the sun (22.4 mg QE/g), shade (21.3 mg QE/g) and oven (19.3 mg QE/g) methods. Similarly, the freeze-drying method kept significantly (P <0.05) the highest total phenolics (37.8 mg GAE/g), whereas the oven-drying method retained the lowest total phenolics (33.5 mg GAE/g) in the study. Thus, the freeze-drying method generally showed the highest contents of both total phenolics and total flavonoids, whereas the oven-drying method exhibited the lowest amount of both of these compounds.

Table 5.1 Total phenolic and total flavonoid contents of *Moringa oleifera* leaves dried under various methods and *in vitro* enteric fermentation results of *Eragrostis curvula* hay treated with their extracts

	Mean ± SEM				
Parameters	**Freeze drying**	**Sun drying**	**Shade drying**	**Oven drying**	**Control**
Total flavonoids (mg QE/g)	24.3 ± 0.5 ^{A}	22.4 ± 0.3 ^{B}	21.3 ± 0.3 ^{C}	19.4 ± 0.3 ^{D}	–
Total phenolics (mg GAE/g)	37.8 ± 0.5 ^{A}	36.4 ± 0.2 ^{B}	35.0 ± 0.7 ^{B}	33.5 ± 0.1 ^{C}	–
TGP (ml/g DM)	164.3 ± 5.2 ^{C}	164.4 ± 4.4 ^{C}	169.5 ± 4.6 ^{B}	170.9 ± 5.1 ^{B}	195.3 ± 6.6 ^{A}
CH_4 (ml/g DM)	4.3 ± 0.3 ^{C}	4.3 ± 0.3 ^{C}	4.5 ± 0.4 ^{B}	4.5 ± 0.4 ^{B}	5.0 ± 0.3 ^{A}
IVOMD (%)	59.5 ± 1.0	60.3 ± 1.2	60.4 ± 0.8	59.8 ± 1.3	60.5 ± 1.5
CH_4/TGP	2.6 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	2.7 ± 0.2	2.6 ± 0.1
CH_4/IVOMD	7.2 ± 0.6 ^{B}	6.9 ± 0.6 ^{B}	7.4 ± 0.8 ^{AB}	7.5 ± 0.9 ^{AB}	8.2 ± 0.6 ^{A}
CH_4 inhibition (%)	14.3 ± 2.0 ^{A}	15.4 ± 2.1^{A}	10.3 ± 5.0 ^{B}	10.3 ± 4.9 ^{B}	–

The means of a parameter with different superscript letters within a row are significantly (P <0.05) different. GAE: gallic acid equivalent; IVOMD: *in vitro* organic matter digestibility; QE: quercetin equivalent; CH_4: methane; SEM: standard error of the mean; TGP: total gas production

Moringa leaves dried under various methods also differed in their effect on total gas and CH_4 production. Although the Moringa plant extracts from all methods reduced total gas and CH_4 production compared with those produced from untreated *E. curvula* hay (control), the sun-drying and freeze-drying methods produced significantly lower TGP and CH_4 compared with shade-drying and oven-drying methods. Other *in vitro* fermentation parameters exhibited significant (P <0.01) variations among the drying methods except for IVOMD and CH_4/TGP. Similarly, the CH_4 inhibition potential of Moringa leaves varied among drying methods and was higher in the sun- and freeze-dried Moringa. Thus, the lowest CH_4 volume or the highest CH_4 inhibition potential recorded for the methods appeared to correspond with the highest concentration of total phenolic and total flavonoid corresponds. However, the CH_4 yield per unit of organic matter feed digested did not differ (P >0.05) among the methods, and the freeze- and sun-drying methods exhibited lower CH_4 yield per unit of organic matter feed digested as compared with the CH_4 yield recorded in the control.

5.3.2 Drying temperatures

The total flavonoids, total phenolics and *in vitro* fermentation parameters of *M. oleifera* leaves dried with oven temperatures are summarized in Table 5.2. They differed significantly (P <0.05) in total flavonoid (18.2–24.7 mg QE/g) and total phenolic (29.3–39.3 mg/ GAE/g) contents. The drying temperatures of 25 °C and 80 °C maintained significantly (P <0.05) the highest and lowest total flavonoid contents. Moringa leaves dried at 25 °C and 45 °C also maintained statistically higher total phenolics compared with those dried at 60 °C and 80 °C. Thus, the leaves dried at 25 °C and 80 °C exhibited the highest and lowest contents of total phenolics and total flavonoids. However, the total phenolic content obtained at 25 °C was similar to the value recorded at 45 °C. As the drying temperature increased from 25 °C to 80 °C, both the total flavonoid and total phenolic contents seemed to decrease linearly except for the increase in total flavonoids content observed at 60 °C compared with 45 °C.

Table 5.2 Total phenolic and total flavonoid contents of *Moringa oleifera* leaves dried under various temperatures and *in vitro* enteric fermentation results of *Eragrostis curvula* hay treated with their extracts

Parameters	Mean ± SEM				
	25 °C	**45 °C**	**60 °C**	**80 °C**	**Control**
Total flavonoids (mg QE/g)	24.7 ± 0.3 ^{A}	22.5 ± 0.3 ^{B}	23.6 ± 0.3 ^{B}	18.2 ± 0.2 ^{C}	–
Total phenolics (mg GAE/g)	39.3 ± 1.4 ^{A}	37.3 ± 0.3 ^{A}	33.8 ± 0.2 ^{B}	29.3 ± 0.2 ^{C}	–
TGP (ml/g DM)	168.9 ± 4.6 ^{BC}	164.8 ± 4.0 ^{C}	173.7 ± 5.2 ^{B}	173.7 ± 5.7 ^{B}	195.3 ± 6.6 ^{A}
CH_4 (ml/g DM)	4.3 ± 0.2 ^{BC}	4.2 ± 0.2 ^{C}	4.5 ± 0.2 ^{B}	4.4 ± 0.3 ^{B}	5.0 ± 0.3 ^{A}
IVOMD (%)	59.9 ± 0.7	59.3 ± 0.4	59.4 ± 1.1	58.9 ± 0.1	60.5 ± 1.5
CH_4/TGP (%)	2.6 ± 0.2	2.6 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	2.6 ± 0.1
CH_4/IVOMD (%)	7.1 ± 0.5 ^{B}	7.0 ± 0.5 ^{B}	7.4 ± 0.4 ^{AB}	7.3 ± 0.6 ^{B}	8.2 ± 0.6 ^{A}
CH_4 inhibition (%)	14.3 ±1.6 ^{A}	16.5 ± 4.3 ^{A}	10.8 ±3.1 ^{B}	12.3 ± 4.1 ^{B}	–

The means with different superscript letters within a row show significant variation among the drying temperatures (P <0.05). GAE: gallic acid equivalent; IVOMD: *in vitro* organic matter digestibility; QE: quercetin equivalent; SEM: standard error of the mean; TGP: total gas production

The total gas, CH_4 and CH_4/IVOMD produced from *E. curvula* hay treated with extracts of Moringa after drying the leaves at 25, 45, 60, and 80 °C were significantly (P <0.05) lower compared with the control. The exception was CH_4/IVOMD at 60 °C. Compared with the control, the decrease in total gas and CH_4 volume differed significantly (P <0.05) among the oven-drying temperatures. Hence, Moringa leaves dried at 25 °C (14.3%) and 45 °C (16.5%)

generally showed higher CH_4 inhibition compared with 60 ˚C (10.8%) and 80 ˚C (12.3%). However, the total gas and CH_4 productions obtained at 25 ˚C were not lower than those recorded at 60 ˚C (10.8%) and 80 ˚C. The IVOMD, CH_4/TGP and CH_4/IVOMD were not significantly different among the drying temperatures. However, they were lower compared with those values recorded in the control. Thus, drying temperatures of 45 ˚C and 60 ˚C showed significant variations in most parameters (i.e. TGP, CH_4, and CH_4 inhibition) among the drying temperatures, but not in CH_4/IVOMD and total flavonoid content. On the other hand, though, the drying temperatures of 25 ˚C and 45 ˚C resulted in comparable effects on TGP, CH_4, CH_4/IVOMD and CH_4 inhibition, and these two drying temperatures did not differ in their effect on total flavonoid content.

5.3.3 Extraction solvents

Table 5.3 presents the total phenolic and total flavonoid contents of Moringa plant extracts and the *in vitro* fermentation characteristics of *E. curvula* hay treated with extracts of *M. oleifera* leaves obtained from various extraction solvents. The extraction solvents tested in this study affected significantly (P <0.05) the total flavonoid and total phenolic contents. The aqueous organic solvents, mainly 70% acetone and 70% ethanol, gave higher total flavonoid and total phenolic concentrations compared with the absolute methanol and 70% methanol, whereas the absolute methanol resulted in the lowest total phenolic content among these solvents.

Table 5.3 Total phenolic and total flavonoid contents of Moringa leaf extracts and the *in vitro* fermentation characteristics of *Eragrostis curvula* hay treated with the extracts obtained through the use of different extraction solvents

Parameters	Mean ± SEM				
	100% methanol	70% methanol	70% acetone	70% ethanol	Control
Total phenolics (mg GAE/g)	32.7 ± 1.3 ^{B}	37.8 ± 0.7 ^{B}	56.5 ± 0.5 ^{A}	54.9 ± 0.9 ^{A}	–
Total flavonoids (mg QE/g)	25.0 ± 0.2 ^{C}	27.2 ± 1.1 ^{B}	50.6 ± 2.2 ^{A}	49.3 ± 1.4 ^{A}	–
TGP (ml/g DM)	175.8 ± 7.4 ^{B}	171.9 ± 7.4 ^{BC}	168.1 ± 6.6 ^{C}	171.5 ± 6.9 ^{BC}	195.3 ± 6.6 ^{A}
CH_4 (ml/g DM)	4.8 ± 0.3 ^{B}	4.6 ± 0.4 ^{C}	4.4 ± 0.2 ^{C}	4.5 ± 0.3 ^{C}	5.0 ± 0.3 ^{A}
IVOMD (%)	58.5 ± 1.7	60.1 ± 1.9	61.7 ± 1.1	61.1 ± 1.9	60.5 ± 1.5
CH_4/TGP	2.7 ± 0.2	2.7 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.6 ± 0.1
CH_4/IVOMD	8.0 ± 0.8 ^{A}	7.4 ± 0.7 ^{B}	7.0 ± 0.6 ^{B}	7.3 ± 0.6 ^{B}	8.2 ± 0.6 ^{A}
CH_4 inhibition (%)	5.7 ± 4.1 ^{C}	9.6 ± 3.5 ^{B}	12.3 ± 3.2 ^{A}	10.2 ± 3.2 ^{B}	–

The means with different superscript letters within a row indicate significant variation among the extraction solvents (P <0.05). GAE: gallic acid equivalent; IVOMD: *in vitro* organic matter digestibility; QE: quercetin equivalent; SEM: standard error of the mean; TGP: total gas production

All the Moringa leaf extracts extracted with solvents (P <0.01) decreased the TGP and CH_4 volume compared with the untreated *E. curvula* hay control (Table 5.3). All the studied aqueous 70% organic solvents, that is, 70% methanol, 70% ethanol and 70% acetone recorded comparable CH_4 production. However, all these aqueous 70% organic solvents resulted in lower CH_4 volume compared with those produced in methanol-extracted Moringa leaves. Similarly, *E. curvula* hay treated with Moringa extracts extracted with aqueous 70% organic solvents showed a comparable effect on CH_4 yield per IVOMD digested. However, they all reduced CH_4 yield per IVOMD digested compared with the control or CH_4 yield per IVOMD digested recorded for methanol extraction solvent. The extraction solvents also differed significantly in antimethanogenic activity, ranging from 5.7% in absolute methanol to 12.3% in 70% acetone. However, CH_4/TGP and IVOMD were not significantly (P >0.01) different among the extraction solvents or when compared with the control. Therefore, all the aqueous 70% organic solvents exhibited superior reductions of CH_4 and CH_4/IVOMD with higher CH_4 inhibition compared to those values recorded in the absolute methanol. Among the aqueous 70% organic solvents, however, 70% acetone exhibited the highest CH_4 inhibition compared with 70% methanol and 70% ethanol and absolute methanol.

5.3.4 Storage temperatures

The total phenolics, total flavonoids and *in vitro* rumen fermentation results of the extracts recorded after storing the Moringa leaf powder at 4, 15, 25 and 35 ˚C for six months are summarized in Table 5.4. The total flavonoid and total phenolic contents varied significantly (P <0.01) among the storage temperatures, in which the Moringa powder stored at 4 ˚C maintained the highest contents of these SPMs. Although the powder stored at 35 ˚C showed the lowest concentration of total phenolics, the total flavonoids recorded at 35 ˚C were comparable with the powder stored at 25 ˚C. In addition, the powder stored at 35 ˚C exhibited a colour change starting from the fourth month of storage (Figure 5.2). However, this change was not associated with the observed *in vitro* fermentation characteristics. Unfortunately, the SPMs associated with the change were not determined as it was beyond the scope of this study.

Table 5.4 Total phenolic and total flavonoid contents of *Moringa oleifera* leaf powder recorded after treating the powder at different storage temperatures for six months and *in vitro* enteric fermentation results of *Eragrostis curvula* hay treated with their extracts

Parameters	Mean ± SEM				
	4 ˚C	15 ˚C	25 ˚C	35 ˚C	Control
Total flavonoids (mg QE/g)	28.8 ± 0.2 ^{A}	27.8 ± 0.4 ^{B}	26.8 ± 0.1 ^{C}	27.2 ± 0.6 ^{C}	–
Total phenolics (mg GAE/g)	42.8 ± 0.9 ^{A}	40.9 ± 0.2 ^{B}	40.6 ± 0.7 ^{B}	37.5 ± 0.9 ^{C}	–
TGP (ml/g DM)	172.6 ± 2.6 ^{B}	173.9 ± 1.1 ^{B}	172.2 ± 1.0 ^{B}	173.7 ± 1.5 ^{B}	199.1 ± 3.8 ^{A}
CH_4 (ml/g DM)	4.9 ± 0.3 ^{B}	5.0 ± 0.2 ^{B}	5.0 ± 0.3 ^{B}	5.1 ± 0.3 ^{B}	6.1 ± 0.4 ^{A}
IVOMD (%)	58.6 ± 0.8 ^{A}	57.7 ± 0.7 ^{A}	56.0 ± 0.5 ^{B}	55.3 ± 0.5 ^{B}	55.5 ± 0.5 ^{B}
CH_4/TGP (%)	2.9 ± 0.2 ^{B}	2.9 ± 0.1 ^{B}	2.9 ± 0.2 ^{B}	2.9 ± 0.2 ^{B}	3.0 ± 0.1^{A}
CH_4/IVOMD (%)	8.5 ± 0.7 ^{D}	8.7 ± 0.5 ^{C}	8.8 ± 0.6 ^{C}	9.2 ± 0.6 ^{B}	10.9 ± 0.6 ^{A}
CH_4 inhibition (%)	18.3 ± 1.1	17.2 ± 1.3	18.0 ± 0.4	16.4 ± 0.9	–

The means with different superscript letters within a row indicate significant variation among storage temperatures (P <0.05). GAE: gallic acid equivalent; IVOMD: *in vitro* organic matter digestibility; QE: quercetin equivalent; SEM: standard error of the mean; TGP: total gas production

The TGP, CH_4, CH_4/TGP and CH_4/IVOMD produced from *E. curvula* hay treated with Moringa extracts, from all Moringa leaf powder stored at these temperatures for six months, were lower compared with values recorded for these parameters from untreated *E. curvula* hay (control). However, most fermentation characteristics did not show significant differences in the study except for IVOMD and CH_4/IVOMD. The CH_4 yield per unit of

organic matter digested indicated a linearly decreasing trend with decreasing storage temperatures. Thus, Moringa leaf powder stored at 4 ˚C showed the lowest CH_4/IVOMD (8.5%), whereas that stored at 35 ˚C maintained the highest yield of CH_4/IVOMD (9.2%). Similarly, the IVOMDs of the *E. curvula* hay treated with Moringa stored at 4 ˚C (58.6%) and 15 ˚C (57.7%) were significantly higher compared with the IVOMD values recorded at 25 ˚C (56.0%) and 35 ˚C (55.3%) stored Moringa or the control (55.5%).

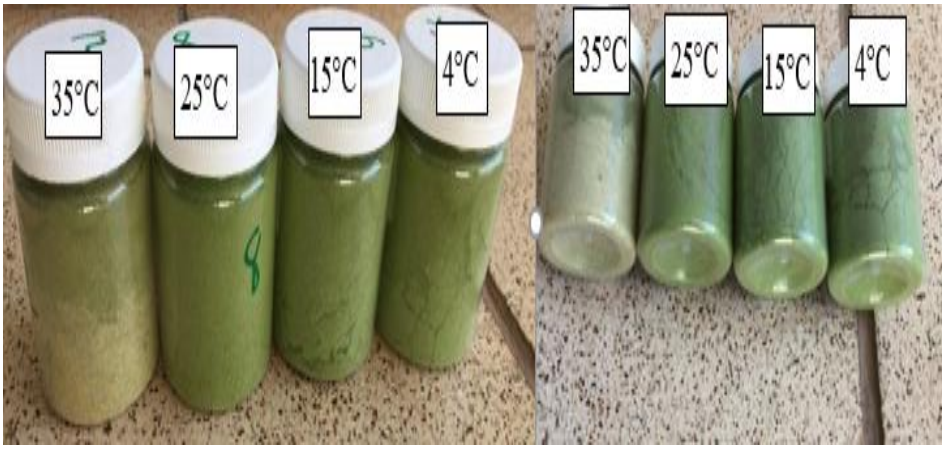


Figure 5.2 Storage of Moringa leaf powder at different temperatures and the observed colour change in the powder stored at 35 ˚C from the fourth month of the storage period

5.3.5 Storage light conditions

The total flavonoid and total phenolic contents and *in vitro* fermentation characteristics obtained after storing the Moringa powder under light or dark storage conditions for six months are presented in Table 5.5. Moringa powder stored in the dark for this period maintained significantly higher total flavonoid and total phenolic contents than those recorded in light-stored Moringa powder. Similarly, the *E. curvula* hay treated with Moringa stored in light or dark conditions reduced significantly (P <0.01) the total gas, CH_4, CH_4/TGP and CH_4/IVOMD production compared with the control, which was *E. curvula* hay treated without extract. Except for the total gas production, all of the *in vitro* fermentation results showed significant variations between the two storage conditions. Significantly higher reductions of CH_4, CH_4/TGP and CH_4/IVOMD values were found in dark storage compared with those values obtained in Moringa stored under light-exposed conditions. In addition, the dark-stored Moringa showed significantly (P <0.01) higher CH_4 inhibition (26.2%) and IVOMD (57.1%) compared with 19.7% CH_4 inhibition and 54.7% IVOMD obtained from light storage. Thus, the desirable fermentation results (i.e. reduced

CH_4 volume and CH_4/IVOMD with superior antimethanogenic activity and improved IVOMD) obtained in the dark storage are generally associated with higher total phenolic and total flavonoid contents.

Table 5.5 Total phenolic and total flavonoid contents of *Moringa oleifera* leaf powder recorded after treating the powder under light and dark storage conditions for six months and the *in vitro* enteric fermentation results of *Eragrostis curvula* hay incubated with the extracts

Parameters	Mean ± SEM		
	Dark storage	Light storage	Control
Total flavonoids (mg QE/g)	25.4 ± 0.4 ^{A}	22.7 ± 0.5 ^{B}	–
Total phenolics (mg GAE/g)	33.5 ± 0.3 ^{A}	29.8 ± 0.4 ^{B}	–
TGP (ml/g DM)	169.0 ± 0.9 ^{B}	173.9 ± 1.4 ^{B}	199.1 ± 3.8 ^{A}
CH_4 (ml/g DM)	4.5 ± 0.3 ^{C}	4.9 ± 0.3 ^{B}	6.1 ± 0.4 ^{A}
IVOMD (%)	57.1 ± 0.7 ^{A}	54.7 ± 0.4 ^{B}	55.5 ± 0.5 ^{B}
CH_4/TGP	2.6 ± 0.2 ^{C}	2.8 ± 0.2 ^{B}	3.0 ± 0.1 ^{A}
CH_4/IVOMD	7.8 ± 0.5 ^{C}	8.9 ± 0.6 ^{B}	10.9 ± 0.6 ^{A}
CH_4 inhibition (%)	26.2 ± 1.1 ^{A}	19.7 ± 1.1 ^{B}	–

The means with Different superscript letters within a row show significant variation between storage light conditions (P <0.05). GAE: gallic acid equivalent; IVOMD: *in vitro* organic matter digestibility; QE: quercetin equivalent; SEM: standard error of the mean; TGP: total gas production

5.3.6 Analysing the overall relationship between factors and response parameters using a multivariate approach

The overall relationships between different postharvest processing and storage conditions of Moringa leaf or the powder with the total phenolics, total flavonoids and *in vitro* fermentation characteristics were analysed using PCA analysis. The results of the PCA and Pearson correlation are summarized in Table 5.6 and Figure 5.3. Three principal components explained approximately 96% of the total variation in the study. Principal component 1 (PC 1) alone revealed 60% of the variations in the PCA. Total gas (41%), CH_4 (43%) and CH_4/IVOMD (42%) were the main parameters that correlated positively with PC 1 but correlated negatively mainly with total phenolics, total flavonoids, IVOMD, and CH_4 inhibition. Approximately 25% of the total variation was explained by PC 2, which was mainly correlated positively with CH_4 inhibition (53%), CH_4/TGP (42%) and total phenolic content (36%) and negatively with IVOMD (–47%). In addition, PC 3 alone explained approximately 11% of the total variation and was mainly correlated positively with total flavonoids (65%) and IVOMD (36%), and negatively with CH_4 inhibition (–44%).

Table 5.6 Principal component loadings using all parameters and correlation of total phenolics and total flavonoids with major *in vitro* fermentation results of the substrate feed treated with Moringa leaf extracts after the leaf or the powder treated with different postharvest processing or storage conditions

Parameters	**PC 1 × 100**	**PC 2 × 100**	**PC 3 × 100**
Total gas	40.8	–24.2	15.1
CH_4	43.5	10.4	26
IVOMD	–28.7	–46.5	36.2
CH_4/TGP	33.5	41.5	28.8
CH_4/IVOMD	42.4	25.4	7.5
% CH_4 inhibition	–22	52.6	–43.8
Total phenolics	–36.4	36.5	27.3
Total flavonoids	–30	26.1	65.1
Eigenvalue	4.8	2	0.9
% variance	60.3	24.9	10.8

Pearson correlation coefficients (*2-tailed*)

Parameters	**Drying method**		**Drying temperature**		**Extraction solvent**		**Storage temperature**	
	TP	**TF**	**TP**	**TF**	**TP**	**TF**	**TP**	**TF**
CH_4	–0.72	–0.79 *	–0.87 *	–0.69	–0.85 *	–0.76	–0.81 *	–0.61
IVOMD	–0.25	–0.17	0.26	–0.23	0.86 *	0.736	0.81 *	0.48
CH_4 inhibition	0.73	0.78 *	0.90 **	0.76	0.85 *	0.84 *	0.79 *	0.70

Correlation coefficients with * and ** show significant correlations at P <0.05 and P <0.01, respectively. IVOMD: *in vitro* organic matter digestibility; PC: principal component; TGP: total gas produced; TF: total flavonoids; TP: total phenolics

The principal component loadings of the PCA are supported by the significant Pearson correlations results obtained between the total phenolics or total flavonoids with fermentation parameters (CH_4 volume, CH_4 inhibition and IVOMD) in most postharvest treatments when the data of each postharvest treatment were analysed independently (Table 5.6). However, significant Pearson correlations were not observed when using the whole dataset of all postharvest factors together. The total phenolic content correlated significantly (P <0.05) with total CH_4 volume (negative) and CH_4 inhibition (positive) for Moringa treated with different drying temperatures, extraction solvents and storage temperatures. It was also correlated significantly and positively with IVOMD obtained from different extraction solvents and storage temperatures of the current study. The total phenolic contents obtained in most Moringa treated under different postharvest processing and storage conditions correlated significantly with the antimethanogenic activity of the extract. The total flavonoids correlated significantly with the CH_4 produced (negative) from Moringa

treated under different drying methods and with CH_4 inhibition (positive) obtained from Moringa extract treated under different drying methods and extraction solvents. Thus, the total phenolics showed several significant correlations with CH_4 inhibition and IVOMD for *M. oleifera* treated under different postharvest treatments compared with those recorded in the total flavonoid contents in the study.

The dot plots of the controls (control 1 and control 2) lay in a similar direction with the vectors of TGP, CH_4, CH_4/IVOMD and CH_4/TGP, as referenced to PC 1 (Figure 5.3). This infers the presence of positive relationships between TGP, CH_4, CH_4/IVOMD and CH_4/TGP to each other and higher values were recorded for these response parameters in the control treatment compared with the test feed treated with Moringa extract subjected to different postharvest and storage treatments. The direction and placement of TGP, CH_4, CH_4/TGP and CH_4/IVOMD also showed the presence of a higher reduction of these parameters in aqueous 70% organic solvents (red dots), drying methods (blue-violet dots), drying temperatures (chocolate dots) and dark storage (green dots) compared with those recorded in the controls (aquamarine dots), light storage (green dot), absolute methanol (red dot) and all storage temperatures (dark dots). On the other hand, the biplots of total phenolics, total flavonoids, CH_4 inhibition and IVOMD were placed in the same direction with the dot plots of all aqueous 70% organic solvents, drying methods, drying temperatures and dark storage, which indicate that their higher values in these treatments and the presence of positive relationships to each other. Conversely, the CH_4 inhibition and IVOMD were placed opposite to the dot plots of the absolute methanol, light storage and the controls as referenced to PC 1, which shows their lower values in the absolute methanol and light storage. But the presence of vector total flavonoids and total phenolics in a similar direction to CH_4 inhibition and IVOMD revealed the positive correlations of these SPMs with higher OMD and antimethanogenic potentials (Figure 5.3).

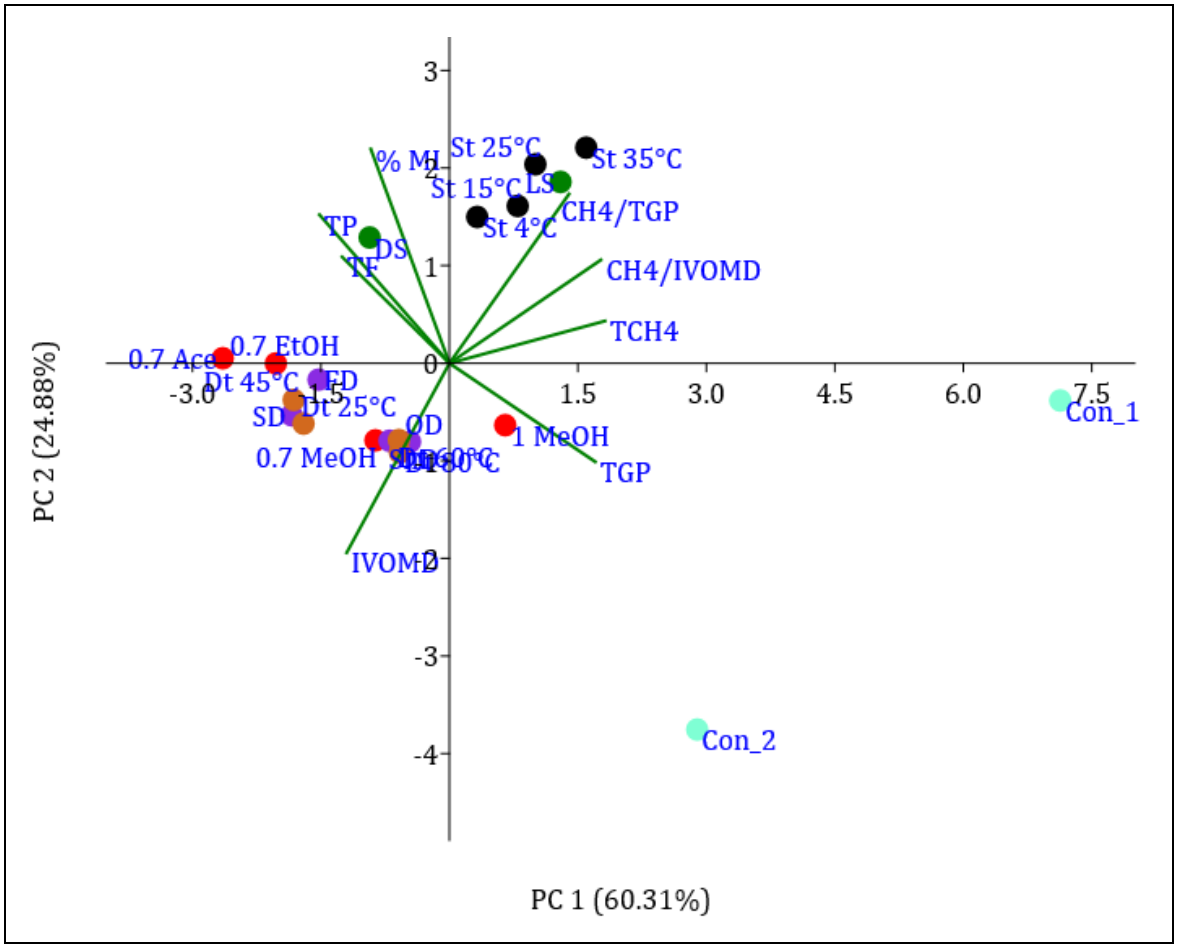


Figure 5.3 Principal component analysis using the total flavonoids, total phenolics and *in vitro* fermentation parameters of *Eragrostis curvula* hay treated with Moringa extracts after the leaf or powder treated with different postharvest processing or storage conditions

Where Con_1 is the control used during the study of postharvest processing conditions and Con_2 infers the control used during the study of postharvest storage conditions. The blue-violet dots are the drying methods; red dots are the extraction solvents; chocolate dots are the drying temperatures; green dots are the storage lights; black dots are the storage temperature; and aquamarine dots are the controls used in the study. 1MeOH: absolute methanol; 0.7MeOH: 70% methanol; 0.7Ace: 70% acetone, 0.7EtOH: 70% ethanol; % MI: percentage of methane inhibition; St: storage temperature, Dt: drying temperature: ShD: shade drying; SD: sun drying; FD: freeze drying; OV: oven drying; LS: light storage; DS: dark storage.

5.4 Discussion

5.4.1 The effect of postharvest drying methods

The drying method is among the main postharvest processing factors considered in this study owing to the variations in its effectiveness in maintaining the bioactive SPMs and subsequent bioactivities (Ademiluyi *et al.*, 2018; Gatahi & Nyoro, 2021). Thus, the freeze- and sun-dried Moringa in the present study showed higher total phenolic and total flavonoid contents with better antimethanogenesis compared with the shade- and oven-dried Moringa. Similarly, the highest total phenolic content was revealed in the freeze-dried, followed by air-, sun-, and oven-dried leaves of *M. oleifera* (Ademiluyi *et al.*, 2018). In contrast to the findings of this study, the highest antimicrobial activity of *Escherichia coli, Staphylococcus aureus and Staphylococcus typhi* were recorded in air-dried Moringa compared with the sun- and oven-dried extract (Hussein *et al.*, 2015). Moringa leaves dried under 50% shade net and room temperature maintained higher polyphenols and terpenoids contents compared with the air- and oven-dried leaves of Moringa, whereas the highest glucosinolates were found in oven-dried leaves (Gatahi & Nyoro, 2021). This indicates that the preference for the drying method might be dependent on the target bioactive compounds and the purpose of applications of a plant extract product. The same study reported that shade-dried leaves developed toxic microbes (i.e. *Escherichia coli*, staphylococcus, moulds, and yeast). In most previous studies of Moringa, the freeze-drying and air-drying methods seemed preferable for maintaining higher bioactive SPMs and for biological applications. However, the sun-dried Moringa in the current study showed comparable antimethanogenesis with the freeze-drying method.

The observed difference between the findings of the current study and those of earlier studies is associated mainly with the difference in the length of the drying period for sun drying versus air-drying. In line with this, shade-drying or air-drying may prolong plant enzymatic reactions and thus shade-drying may not guarantee the attainment of recommended moisture (10%) content that makes the product mould-free and safe (Sauveur & Broin, 2010). This seemed to be true in the current study because shade-drying took longer (12 days) compared with six (Sani Yabo, 2015), nine (Anuja & Ramkumar, 2017) and two days (Deepa *et al.*, 2021) reported earlier. Thus, a longer shade drying process may affect the plant extract products negatively owing to the extended enzymatic reactions or developed toxic microbes (i.e. *Escherichia coli*, staphylococcus, moulds, and yeast) (Gatahi & Nyoro, 2021). Similar

to the findings of the current study, quick drying maintained better bioactive compounds and better antioxidant activity than longer drying methods (Vats, 2016). The duration of the sun-drying period (1.5 days) in the current study was shorter compared with the duration of drying time reported by other studies, that is, four (Pallavi & Dipika, 2010), six (Anuja & Ramkumar, 2017) and two to three days (Hussein *et al.*, 2015). Less frequently, the current study can be supported by higher minerals (Mg, Fe, Zn, and K) and superior antioxidant capacity obtained in the sun-drying method compared to shade- and cabinet-drying methods (Setiaboma *et al.*, 2019). On the other hand, the differences may be linked to the influence of the drying method on various compounds, which agrees with the observed superior polyphenols and terpenoids under 50% shade net and room temperature, whereas the highest glucosinolates were recorded in oven-dried leaves (Gatahi & Nyoro, 2021). This implies that the sun-drying method showed a lesser effect on the antimethanogenic activity of SPMs and owing to this it might be preferable, in the absence of a freeze-drying facility, and the environmental conditions of the longer shade-drying process, that is, poor ventilation, higher humidity and an environment favourable to the development of moulds.

5.4.2 The effect of drying temperatures

The responses of bioactive components to the postharvest drying temperatures are significant. Thus, the effect of antimethanogenic activities of Moringa treated with the various drying temperatures was associated partly with the total phenolic and total flavonoid contents of Moringa extracts, which was proved by the positive correlation coefficient (0.78) with total phenolic contents recorded at various drying temperatures. Hence, the total flavonoid and total phenolic contents appear to decrease linearly as the drying temperature increases from 25 ˚C to 80 ˚C. The exception is the total flavonoid content observed at 45 ˚C and 60 ˚C. The colour change observed in the Moringa leaf dried at 80 ˚C in the current study appears to agree with the observed change in leaf colour to brown when the Moringa leaf is dried above 55 ˚C drying temperatures (Sauveur & Broin, 2010). However, this colour change was not associated with the plant components and their bioactivities. Similarly, the Moringa leaf dried at a lower temperature, 60 ˚C, maintained better nutritional contents (i.e. higher protein, vitamin C and ß-carotene), better free radical scavenging activity, and higher total phenolic contents compared with the leaf dried at 70 ˚C and 80 ˚C (Razzak *et al.*, 2021). The effects of drying temperature are partially associated with the response of membrane permeability of the SPMs (Thevissen *et al.*, 1996). These explained that the higher

temperature usually affects the binding property of cationic proteins to the negatively charged membrane surface and results in pore formation and protein degradation, which may decrease or lose the biological activities of the SPMs. Decreasing trends of crude protein, beta-carotene and vitamin C contents were recorded, whereas carbohydrate, fibre and ash contents were increased with the increase in drying temperature from 30 ˚C to 70 ˚C (Kucha *et al.*, 2015). However, the response of plants to the temperature ranges might be reliant on the species and purpose or target of the biological application of the plant extract products.

5.4.3 The effect of extraction solvents

The extraction solvent is another factor that substantially affects the SPMs and the bioactivities of Moringa products (Urías-Orona *et al.*, 2017; Abo El-Fadl *et al.*, 2020). In the current study, the extraction solvents showed different effects. Generally, the aqueous 70% organic solvents maintained higher total flavonoid and total phenolic contents with better antimethanogenic activity compared with the absolute methanol. Among the extraction solvents in the current study, 70% acetone resulted in the highest antimethanogenic potentials, whereas the absolute methanol-extracted Moringa exhibited the lowest antimethanogenesis. Several studies agree with the findings of the current study, which reported higher effectiveness in aqueous organic solvents compared with pure organic solvents (Ngo *et al.*, 2017; Urías-Orona *et al.*, 2017; Abo El-Fadl *et al.*, 2020). Furthermore, the aqueous 70% ethanol (108.7 mg GAE/g**)** retained the highest total phenolic content compared with absolute hexane (48.7 mg GAE/g) and ethyl acetate extracted leaves of *M. oleifera* (Abo El-Fadl *et al.*, 2020). Ethanol (50%) maintained the highest phenolic and antioxidant activity compared with three levels of methanol and ethanol (100%, 80% and 50% of organic solvents) (Urías-Orona *et al.*, 2017). Methanol (50%), 100% ethyl acetate and 100% methanol-extracted leaves of *M. oleifera* showed higher antioxidant activity, enzymatic inhibitory activity and phenolic profiling, respectively (Rocchetti *et al.*, 2020). In agreement with the highest antimethanogenesis obtained in 70% acetone in the current study, Ngo *et al.* (2017) found that 50% acetone was preferable to extract higher saponins, flavonoids, phenolics, and extractable solids with better antioxidant capacity than absolute acetone, methanol, ethanol, water and their 50% aqueous solvents. Consistently, acetone showed the highest scored value, calculated from the rate of extraction, ease of removal of the solvent, the number of inhibitors extracted, quantity and diversity of compounds

extracted, biological hazard and toxicity level in the bioassay compared with water, methanol, methylene dichloride, and ethanol extraction solvents (Eloff, 1998). Hence, the variation in total phenolics, total flavonoids and antimethanogenesis obtained in the studied solvents might be associated with the efficiency of the solvents to extract important lipophilic and hydrophilic SPMs, solvent volatility and their toxic properties to the rumen microorganisms (Eloff, 1998; Ngo *et al.*, 2017). Thus, acetone has the highest volatility, whereas methanol is more toxic than ethanol and acetone, which helps to minimize the residual effect of the solvent on the rumen microbes involved in rumen modulation and antimethanogenesis (Joshi & Adhikari, 2019). However, exhaustive evaluation of the aqueous organic solvents at various levels with the inclusion of more solvents needs to be conducted to obtain the most effective extraction solvent for antimethanogenic applications of Moringa plant extract as a dietary additive for ruminants.

5.4.4 The effect of postharvest storage conditions

The degrees of change in the chemistry of plant products and their subsequent biological activities are highly affected by the storage conditions, that is, temperature, light and humidity (Zhang *et al.*, 2017; Kashyap *et al.*, 2022). However, the antimethanogenic effect of Moringa did not show significant variations among the storage temperatures in six months' storage. But lower storage temperature seemed to be advantageous, as evidenced by the highest total flavonoid and total phenolic contents with the lowest CH_4 yield per unit of OM digested recorded in Moringa stored at 4 $^{\circ}$C, whereas the Moringa powder stored at 35 $^{\circ}$C showed the lowest total phenolics and the highest yield of CH_4 per unit of OM digested. Moringa stored at 4 $^{\circ}$C and 15 $^{\circ}$C exhibited higher IVOMD compared with storage at 25 $^{\circ}$C and 35 $^{\circ}$C. Most previous studies showed better biological activities at lower storage temperatures (Arendse *et al.*, 2014; Zhang *et al.*, 2017). Similarly, the Moringa powder stored at 15 $^{\circ}$C for two months showed lower decreases in total phenolics and changes in DPPH inhibition after compared with the powder stored at 35 $^{\circ}$C (Potisate *et al.*, 2015). Consistently, *Anemopsis californica* leaf extract stored for 180 days exhibited higher contents of total phenolics, total flavonoids and antioxidant activity at -20 $^{\circ}$C and 4 $^{\circ}$C than those recorded at 25 $^{\circ}$C and 50 $^{\circ}$C storage (Del-Toro-Sánchez *et al.*, 2015). Vongsak *et al.* (2013) also illustrated the higher susceptibility of bioactive compounds under storage conditions, which was confirmed by the variation in total phenolic and total flavonoid contents, IVOMD, and CH_4/IVOMD obtained in the current study. Storage of Moringa leaf

powder below 24 °C in airtight containers protected from light and Inter-Aide (2012) recommended humidity for up to six months. However, this study did not indicate the differences in the efficacy of the stored product because of the differences in the purpose of application.

Moringa powder stored in dark storage conditions for six months showed higher *in vitro* CH_4 inhibition and OMD than light-stored Moringa. These favourable *in vitro* fermentation results are associated partly with the higher total flavonoid and total phenolic contents obtained in dark storage than in light-stored conditions. These results confirmed that exposing Moringa powder to light during storage affected the total phenolic and total flavonoid contents and the subsequent bioactivities negatively, including the OMD and antimethanogenic efficacy of the extract. Moringa powder should be protected from humidity and light for more effective bioactivity after storage, because most bioactive compounds are sensitive to storage conditions, including light (Inter-Aide, 2012; Vongsak *et al.*, 2013a; Zhang *et al.*, 2017). The variations in SPMs contents antimethanogenesis and IVOMD obtained between Moringa powder stored in light and in darkness might be associated with the presence of catalysts, light, oxygen and temperature that are the main causes of chemical changes during storage and processing of animal feed additives (Li *et al.*, 2012).

5.4.5 The relationships between the studied factors and response parameters

The effectiveness of medicinal plant products is the result of the interaction effect of multiple factors that the plants face from growth until the time of application. Thus, relationship analysis might help us to identify systematically a factor that could explain a significant portion of the total variation and to prioritize these factors as part of management practice to obtain consistent effects. Thus, it is apparent that postharvest processing and storage management can affect the SPMs and the antimethanogenic activities differently. In the PCA score plot of the current study, the total flavonoids and total phenolics lay in a similar direction to CH_4 inhibition, which indicates the positive relationships of these SPMs with higher antimethanogenic potentials. In agreement with this finding, various studies associated the bioactivities of medicinal plants with their total phenolic and total flavonoid contents (Chandra *et al.*, 2014; Aryal *et al.*, 2019; Phuyal *et al.*, 2020). The concentration of total phenolics obtained from drying temperatures, extraction solvents, and storage temperatures of Moringa, and total flavonoid contents obtained from Moringa treated with

various drying methods and extraction solvents showed positive correlation coefficients with their antimethanogenic activities. The total phenolics from the drying methods and total phenolics of Moringa treated with oven and storage temperatures did not show a positive correlation with their antimethanogenic activities, which proves the variation of response variables to these postharvest factors. The total phenolic contents obtained in most postharvest-treated Moringa extracts in the current study correlated positively and significantly with the antimethanogenic activities of their extracts. This result also agrees with the significant positive correlation coefficients recorded between antimethanogenic activities and total phenolic (0.62) and total flavonoid (0.70) contents of Moringa accessions (Zeru *et al.*, 2022b). The application of some flavonoid compounds and the combination of anthraquinone and fumaric acid reduced the *in vitro* CH_4 production by 5–9 ml/g substrate and 25%, respectively (Ebrahimi *et al.*, 2011; Oskoueian *et al.*, 2013). The addition of phenolic compounds also inhibited CH_4 without affecting other fermentation characteristics adversely (Giuburunca *et al.*, 2014). However, the total phenolics and total flavonoids in the current study seemed to be highly affected by 70% acetone and 70% ethanol extraction solvents compared with other postharvest processing and storage conditions. Generally, the effect of plant products on a specific response variable is the result of the interaction of multiple factors and needs detailed investigation in a systematic approach to develop an effective management practice to get a consistent effect on the target bioactivity.

5.5 Conclusions

The lack of consistent results hindered the wider utilization of plant extracts as dietary additives to modulate rumen fermentation. Many postharvest processing methods and storage conditions affected the inherent properties of the plant extracts in terms of secondary plant metabolite concentrations and efficacy of the subsequent bioactivities of Moringa plant extracts. Thus, determining the optimum postharvest processing method and storage conditions of Moringa leaf is essential to ensure consistent efficacy of Moringa plant extracts to inhibit methane emissions. Among these postharvest factors, freeze-drying, followed by extracting Moringa leaves with 70% organic solvent, mainly 70% acetone, was found to be the optimal postharvest drying method and extraction solvent to ensure higher total phenolic and total flavonoid concentrations, which subsequently lead to higher CH_4 inhibition activities. In the absence of a freeze-drying facility, sun-drying or oven-drying at 45 $^{\circ}$C could be an alternative method to ensure higher antimethanogenic activities. Where Moringa leaf

powders must be stored for an extended period, they should be stored at a temperature of 4 °C and in a dark container to ensure higher methane inhibition activities. However, comprehensive evaluation of aqueous solvents with the inclusion of as many solvents as possible (*in vitro* and *in vivo*) and extended storage periods at different temperatures need to be conducted to standardize plant extracts to obtain consistent effects and establish their expiry dates.

CHAPTER 6

General conclusions, recommendations and critical evaluation

6.1 General conclusions and recommendations

The study evaluated a range of factors influencing the efficacy of Moringa plant extracts to develop reliable products of Moringa leaf extract that could be used as additives in ruminant feeding systems for inhibition of CH_4 without compromising ruminal fermentation and digestibility of feed. Hence, a series of experiments were conducted with different specific objectives. The conclusions and recommendations are provided below:

Moringa accessions that grew in the same environment differed in agronomic performances, total flavonoids, total phenolics, secondary plant metabolite ion feature intensities, and their effect on CH_4 inhibition and OMD of the *E. curvula* hay used as a test feed. Among these accessions, *M. oleifera* accession 07633 (A3), 07717 (A8) and Pretoria (A11) showed superior performances in most parameters. The antimethanogenic variations observed in these Moringa accessions are associated with four main bioactive secondary plant metabolite ion features, which included the MIF 4.44_609.1462 and MIF 4.53_433.1112 for higher CH_4 inhibition and the MIF 9.06_443.2317 and MIF 15.00_487.2319 for lower CH_4 inhibition. Thus, the selected MIFs responsible for the variation in methane inhibition of the accessions can be used to standardize Moringa plant extracts and commercialize promising varieties as a source of plant extract products as an additive for CH_4 inhibition in ruminants. The positive relationships of most agronomic traits, total flavonoids and total phenolics with antimethanogenic activity show the possibility of manipulating the growing environment and conditions to obtain Moringa plant extracts with higher CH_4 inhibition from the Moringa accessions. In addition, this study indicates the presence of wider genetic variability among Moringa accession genetic sources. Hence, the superior accessions can be improved to develop commercial varieties suitable as a source of plant extract products to mitigate enteric CH_4 production. However, detailed investigations of the bioactivity and mode of action of the main secondary plant metabolite ion features involved in methane inhibition and evaluating the repeatability of the performance of the selected accessions using animals need to be conducted to ensure the production of consistent effective plant extract products.

None of the cocktails of Moringa accession extracts affected the antimethanogenic activity and enteric fermentation kinetics of the test feed negatively. A binary cocktail mixed at 50% of the two selected Moringa accessions (*M. oleifera* A3 and A11) provided favourable associative effects in terms of higher CH_4 inhibition and propionate production with a decrease in the acetate/propionate ratio. This implies that applying the two selected Moringa accessions in the form of cocktails may increase the opportunity to optimize the plant extract products to get better CH_4 inhibition than when using these accessions in the sole source of plant extracts. The favourable antimethanogenesis recorded in a 50% cocktail of the selected accessions appears to be associated with the key MIFs 4.44_609.1462, 15.00_487.2319 and 9.06_443.2317. However, a comprehensive study using the untargeted metabolomics approach needs to be conducted to identify the most responsible bioactive SPMs involved in a non-linear antimethanogenic activity and understand the detailed chemistry or mechanisms of the metabolite action in the cocktails.

Leaves of *M. oleifera* are highly perishable and need to be processed first in powder form for longer shelf life and storage to ensure a year-round supply of plant extract while maintaining their efficacies for bioactivities. Hence, the leaf or powder of Moringa that was treated with different drying methods, drying temperatures, extraction solvents, storage temperatures and lights conditions were investigated, and it was found that these factors influenced significantly the total phenolic and total flavonoid contents, which affected enteric fermentation and CH_4 inhibition. Among the postharvest processing and storage conditions for Moringa, the freeze-drying method, 45 $^{\circ}$C drying temperature, 70% acetone extraction solvent, and storage of the plant extracts at t 4 $^{\circ}$C in the dark were preferable for superior antimethanogenic activity and other fermentation co-benefits. The variation in the effect of the drying methods might be attributed to the variation in the length of the drying period, which extends the plants' enzymatic activities and action of undesirable toxic microbes. Extraction solvents can also influence the efficiency of plant extracts owing to the variation they create in terms of the composition and concentration of lipophilic and hydrophilic bioactive compounds in the plant extract, which affects volatility and toxicity to the rumen microbes. The variations in the storage conditions of the plant extracts may be associated with the presence of catalysts, light, oxygen and higher temperatures that may induce chemical changes in key bioactive compounds in the plant powder during storage. This means that it is vital to optimize postharvest Moringa leaf processing methods, storage conditions and storage time of the plant products to utilize optimally Moringa plant extracts

as dietary additives throughout the year without compromising their efficacy of plant extracts to inhibit CH_4 emission from ruminants. Thus, comprehensive evaluation of aqueous solvents with the inclusion of as many solvents as possible (*in vitro* and *in vivo*) and extended storage periods under different temperatures is recommended to gain additional information that helps to produce standardized plant extracts that give consistent effects and to establish the expiry dates of the standardized plant extracts.

6.2 Critical evaluation

This study has some limitations in terms of the methodology that was adopted as well as the scope of data that were collected as part of this PhD research. Some of these limitations and recommendations are included in the critical evaluation section to benefit future research on Moringa for the extraction of beneficial bioactive compounds.

Recording of Moringa accession agronomic traits and leaf harvest for *in vitro* gas production characteristics was done in the fifth month after transplanting of Moringa seedlings to the field. Hence, the favourable relationships of higher CH_4 inhibition characteristics with most agronomic traits were not validated by using long-term adaptability data to verify the repeatability of the observed effect in order to sustain the efficacy of the plant extracts. This may be taken as a weakness of the current study. Most previous studies reported higher accumulations of SPMs and better bioactivities with increases in plant growth stage, root diameter, plant height, stem diameter, and biomass yield in woody and perennial medicinal plants. However, such activity results might be different owing to the interaction of plant genetics with the growing environment of the area. Thus, validation of the findings of the current study using long-term adaptability data recorded at different stages of plant growth, plant density, season of harvesting and parts of the plant used with a concurrent evaluation of antimethanogenic efficacy would provide strong evidence about the relationship of the agronomic traits with gas production characteristics. This result in turn helps to select the best Moringa accession with its recommended management conditions to get superior antimethanogenic potential. Besides, accessions of Moringa used in the present study were grown in one location or agro-ecology, and there is a need to grow them in multi-location sites to explain the possible sources of variation linked to the location or agro-ecology and verify the repeatability of the results.

The process of extraction used to produce the crude extracts has also a central role in subsequent bioactivities of the extracts. Extraction was conducted by dissolving the powder in the solvent, suspending the solution in a shaker for 96 hours and filtrating it with a 150-µm sieve. Thus, the solution was passed several times through a 150-µm sieve until it had no solid residue. But the researchers noticed that the sieve that was used for extraction passed fine plant materials and it was difficult to obtain a clear solution with this size of the sieve. This may decrease the efficacy of the active ingredients owing to the dilution effect of fine plant materials or loss of active ingredients during the process. Hence, future studies should consider two-stage extraction, that is, repeated filtration using a 150-µm sieve in the first phase (I), and subsequently straining the solution with another smaller sieve in phase II. This may resolve the dilution problem and increase extraction efficiency.

Extraction solvents have wide variations in their extraction efficiency. Thus, the inclusion of only four extraction solvents in a plant extract that has many known and unknown bioactive SPMs may be regarded theoretically as a weakness of the current study. However, it would have been difficult to manage more extraction solvents because of the inclusion of many postharvest factors at a time. Hence, the evaluation of many solvents with wide ranges of aqueous organic solvents (10–90%) needs to be investigated. Although 70% Ace showed the highest antimethanogenic activity among the four solvents tested in this study, it was not adopted in the generation of data for the other experiments. Hence, the repeatability of results for 70% acetone extraction needs to be validated by monitoring the antimethanogenic activity of Moringa leaf extract in the future.

The four bioactive secondary MIFs selected for higher and lower CH_4 inhibition characteristics of Moringa accessions were not characterized to the end level, and need a detailed description of their structures, names, pathways and mode of action. However, Tshiyoyo *et al.* (2022) identified Hesperidin and Isovitexin as the two key MIFs responsible for higher CH_4 inhibitors in a multidisciplinary approach. The two major MIFs responsible for lower CH_4 inhibition characteristics of the accessions were not yet known. The effectiveness of those MIFs in inhibiting CH_4 needs to be validated using *in vitro* gas production studies by applying a different dose of the two compounds and subsequently monitoring whether one can get a dose-dependent response.

Although the use of a binary cocktail, obtained by mixing the two promising Moringa accessions with higher antimethanogenic activity, resulted in a synergic effect, the key MIFs

responsible for higher CH_4 inhibition and the associative effect of the cocktails were not studied comprehensively. Thus, the failure to conduct an inclusive study following an untargeted metabolomics approach that may help to identify the best bioactive SPMs responsible for higher antimethanogenesis obtained in the cocktail is considered a drawback for the current study.

Under practical conditions, farmers and other role players in the ruminant livestock industries are obliged to store plant products under wider ranges of temperatures for longer periods. The current study, however, evaluated the effect of plant extract storage temperatures with only four storage temperature levels (4, 15, 25 and 35 $^{\circ}$C) for six months. These temperatures and length of time might not be adequate to obtain a conclusive result as biochemical changes may occur at extreme storage temperatures and longer storage. Hence, the biochemical changes at 15, 25, and 35 $^{\circ}$C and above are quite different. Continuous evaluation of each storage temperature at extended storage durations might be crucial to setting a cut-off storage time for each temperature. Thus, this approach helps to identify a safer length of storage time for the different environmental temperatures and may improve the effectiveness of Moringa plant extract product utilization without loss of efficacy within the recommended range of storage times.

All experiments conducted in this thesis were *in vitro* using the rumen fluid collected from three ruminally cannulated steers, which fed *E. curvula* hay as a basal diet. But most previous studies revealed the variability of the *in vitro* results with the variations attributed to the rumen liquor, collection date, type of animals, diet of the animal, etc. Hence, being unable to confirm the repeatability of promising laboratory results obtained in the current study using live animals and different animal diets might be the limitation and needs to be considered in future studies.

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