ADDITION OF ACTIVATED CARBON IN A CATTLE DIET TO MITIGATE GHG EMISSIONS AND AMELIORATE INGESTED TOXINS

A thesis submitted by

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Abstract

Emissions from dairy and beef productions represent around 40% of methane emissions worldwide. In this study both *in-vitro* and *in-vivo* approaches are used to study the effect of Powdered Activated Carbon (PAC) on reducing Greenhouse gas (GHG) emissions from dairy cattle. Since ancient times various forms of carbon have been used as a remedy for ingestion of toxins and digestive ailments in animals and humans. We chose to study surface area effects as a focus, with commercially available quality assured products readily available i.e. PAC. Just prior to *in-vivo* PAC addition trials the dairy herd suffered from an incident with Lantana poisoning of 50 heifers on agistment, as a result additional work on the amelioration of toxins was included in this study.

The first *in-vitro* study (Chapter 3) added three concentrations of PAC (0%, 0.5% and 2%) to a dairy cattle pellet diet and two types of rumen liquid (grain and grass-fed) were used in this study. The reduction of methane (CH_4) emissions were from 61% to 69% for all of the PAC concentrations in both grain and grass-fed cattle. The reduction of GHG emissions from grain and grass-fed dairy cattle was between 25% and 20% for 2% and 0.5% of PAC respectively. Production precursors of volatile fatty acids (VFAs), of acetic, butyric and propionic acids were slightly positive, influenced by adding PAC concentrations to both feeds.

The second *in-vitro* study (Chapter 4) investigated the inclusion of powdered activated carbon (PAC) at (0.5% and 2%) dry matter (DM) on GHG emissions and key rumen health pre-cursors for a dairy cattle forage diet. Four types of forage diet were used. The 2% PAC concentration reported the highest GHG reduction (30 - 42%) and CH_4 emissions reductions were (54% - 69%), slightly outperforming the 0.5% PAC concentration. While emissions were substantially abated, production was not greatly affected, with concentrations of volatile fatty acids (acetate, propionate and butyrate) not differing significantly ($P<0.05$) in relation to the PAC concentrations. The pH did not show a significant ($P<0.05$) increase for oaten and barley hay, while it did differ significantly ($P<0.001$) when it was added to forage sorghum silage diets.

The third *in-vitro* study (**Chapter 5)** tested the effect of PAC on decreasing Lantana toxins in the diet of dairy cattle, reducing GHG emissions and improving rumen fermentation. Initially four concentrations of PAC (0, 0.1, 0.4 and 1g) were added to a Lantana extract i.e. berry, leaf or flower, to determine their base Lantadene adsorption rates for a nominally lethal Lantana dose (1% weight by weight (w/w) of diet). PAC addition achieved Lantadene reductions of (0.5 to 0.58 mM/g) with a combined Lantadene A and B concentration of 2.19mM/g. Three PAC concentrations (0%, 0.5% and 2 %) DM were then added to a dairy cattle diet, with and without Lantana extract (leaf) to evaluate the effects on greenhouse gas emissions and rumen fermentation, with VFAs measured as productivity indicators. GHG and CH_4 emission reductions were between (36% - 37%) and (40% - 47%) respectively for all PAC concentrations compared to control. In summary, a 0.5% PAC modified dairy cattle diet appears sufficient to ameliorate a typical lethal Lantana dose (1% W/W), with production levels unchanged ($P<0.05$). PAC's high surface area appears to be responsible for reducing Methanogenic flora species, resulting in GHG and CH_4 reductions of 36 - 37% and 40 - 47% respectively.

The fourth study was an *in-vivo* study (Chapter 6) to determine the effect of Powder Activated Carbon (PAC) at 0.5% by dry matter (DM) of diet on the enteric methane emissions and performance of dairy cattle when incorporated into a concentrated pellet. These results were obtained from 180 dairy cattle located in Brymaroo, Queensland (Qld), Australia. The addition of PAC improved daily milk production by 3.43% on average for the herd. PAC supplementation significantly increased the ($P<0.05$) milk protein by 2.63% and milk fat was significantly increased ($P<0.001$) by an average of 6.32%.

PAC concentration contributed to reducing CH_4 emissions observed ($P<0.001$) before, during and after milking. The PAC also slightly reduced the amount of CO_2 before, during and after milking. It can also be concluded from flux meter tests that the PAC did not significantly reduce CH_4 from the manure emissions of dairy cattle, although these emissions are small by contrast with those eructated from the rumen. The presence of 0.5% PAC in the diet significantly reduced ($P<0.001$) the CO_2 emissions from 1631 ppm to 1464 ppm from the manure of dairy cattle.

In addition, 16S rRNA Gene Sequencing was performed to determine the collective population of prokaryotic bacteria and Archaea as well as to characterize the specific Methanogenic communities between dairy cattle manure fed one of two pelleted diets; either a basic diet or supplemented with 0.5% PAC. There was a significant decrease (P<0.001) in the proportion of Proteobacteria (57% to 23%). There was a significant decrease in the proportion of members of the genera Methanobrevibacter from (83% to 51%) with a concurrent significant increase in the genera of the family Methanocorpuscuralceae (from 12% to 42%). The percentages of the other two minor genera also showed a minor increase, although their proportion remained low (Methanosphaera an observed increase from (3% to 4%) and members of the Methanomassiliicoccacea increasing (from 2% to 3%).

Certification of thesis

This thesis is the work of Mohammed Sabah Al-Azzawi except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

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List of acronyms & abbreviations

AQUAPLAN	Australia's National Strategic Plan for Aquatic Animal Health
ADF	Acid Detergent Fibre
ANOVA	Analysis of Variance
C	Carbon
CH_4	Methane
CO_2	Carbon Dioxide
CO	Carbon monoxide
GHG	Green House Gas
hr	Hour
LI-COR CO_2	Multi path laser CO_2 detector
CP	Crude Protein
DM	Dry Matter
DTT	Dithiothreitol
N	Nitrogen
O_2	Oxygen
PAC	Powdered Activated Carbon
BC	Biochar
AC	Activated Charcoal
FTIR	Fourier Transform-Infrared Spectroscopy
VOCs	Volatile Organic Compounds
P	Calculated Probability
pH	Hydrogen ion concentration (log scale)
CBC	Chicken Manure Biochar
RBC	Rape Straw Biochar
Cd	Cadmium
S	Sulphur
SD	Standard Deviation
SO_2	Sulfur Dioxide
N_2O	nitrous oxide
NH_3	Ammonia

NH^{+}_{4}	water-soluble ammonium
NO^{-}_{3}	nitrates
BW	Body Weight
kg	kilogram
g	Gram
mL	Millilitre
μm	Micrometre
CWVC	Wood Vinegar Compounds
ppm	Parts Per Million
WBC	White Blood Cell
BUN	Blood Urea Nitrogen
H_2S	Hydrogen Sulphide
BV	bamboo Vinegar
PC	Positive Control
ALT	alanine aminotransferase
AST	aspartate aminotransferase
TP	total protein
TG	triglycerides
TC	total cholesterol
HDL	high density lipoprotein
GLU	glucose

CHAPTER 1

1.1 Introduction

Biochar is the general term for a carbon product produced by heating biomass, such as wood, nutshell and plant residue in an oven with limited oxygen (O_2) (Nartey et al. 2014; Gui et al. 2020; Anto et al. 2021). The physical and chemical properties of biochar, such as porosity and stability, are strongly influenced by the conditions set during the biochar production process (Anto et al. 2021; Chen et al. 2021; Sekar et al. 2021). These factors include the chemical components of biomass, temperature, duration and amount of O_2 in the biomass (Ponnusamy et al. 2020; Tomczyk et al. 2020; Shrestha et al. 2021). Biochar production can be tailored to increase the specific surface area of the material by introducing a high level of porosity (Liu et al. 2020; Toková et al. 2020; Qu et al. 2021). If the biochar surface area is increased to greater than 1000m^2/gram it may be termed activated carbon rather than simply biochar. This porous structure provides a stable habitat for the infiltration of microorganisms (Aguayo-Villarreal et al. 2017; Cao et al. 2020; Das et al. 2020). Various articles, all using biochars and activated carbons of varying characteristics from different biomass sources, prepared in different ways, have revealed general benefits to animals from the addition of biochar in their diet.

Biochar is used effectively in many agricultural processes. Increases in crop production have also been associated with the application of biochar, mainly because it enhances the soil structure, increases porosity, reduces bulk density and aids water retention (Kroeger et al. 2021; Kwoczynski et al. 2021; Lopes et al. 2021). Furthermore, biochar optimizes the chemical conditions of soil, such as the total organic carbon content and improving the biological community in the soil (Cole et al. 2021; Xu et al. 2021)

Many researchers in Australia have reported that agriculture releases 16% of total anthropogenic GHG emissions (Charmley et al. 2008), while livestock produces approximately 70% of agricultural sector emissions (Peters et al. 2010). For example, beef cattle feedlots face considerable pressure to improve the management of manure to avoid environmental damage and the effects on human health (Archibeque et al. 2007; Cole et al. 2005; Miller and Berry 2005; Pandrangi et al. 2003).

In recent years, many researchers have shown that reducing CH_4 production has many advantages; for example, this reduction plays an important role in improving food quality and economic benefits as well as enhancing environmental conditions. However, meat and dairy production will lead to a significant increase in CH_4 emissions (Teoh et al. 2019).

In ruminants, typically, cellulosic feed materials are digested in the rumen by microbial fermentation. This process generates approximately 80% of methane and it releases up to 20% of the methane from the decomposition of manure (Vergé et al., 2007). Traditionally, these percentages have been changed in the dairy system from 60 to 100% enteric, while the decomposition of manure is between 0% and 40%. That change is normally dependent on dairy breeding, digestibility, feed types and animal housing (Rotz et al., 2010). According to the Food and Agriculture Organization (FAO), in 2010, in the United States, the CH_4 produce from milk production and grassland around 50% and 80% of GHG emissions, respectively. However, a proposed and new method to reduce GHG emissions from ruminants is adding biochar or activated carbon to the diet.

Biochar is also commonly used to reduce or control ammonia levels in animal production when used in flooring, to improve the farming system environment (Al-Kindi et al. 2017; Mirheidari, A. et al. 2019; Winders et al. 2019).

The majority of animal studies have focused on the benefits of biochar for ruminant animals such as beef cattle (Leng et al. 2012; Teoh et al. 2019; Winders et al. 2019), dairy cows (Hansen et al. 2012; Saleem et al. 2018) and other livestock including goats (Al-Kindi et al. 2017). This is primarily due to their interest in the ability of biochar supplementation to mitigate greenhouse gas production in the form of methane produced in the rumen (McFarlane et al. 2017; Saleem et al. 2018; Teoh et al. 2019). Reducing methane emissions improves feed energy use and system efficiency while lessening its significant contribution to climate change.

Hansen et al. (2012) evaluated the effect of biochar on CH_4 production from buffered rumen fluid that was collected from Jersey heifers. In that study, different types of biochar *in-vitro* and gasified, wood-based and straw-based biochar were employed. In their study, biochar at a concentration of 9% was added to the feed DM. They reported that using biochar decreased CH_4 emissions by 11% – 17% compared with those of the control. Another study was presented by (Leng et al. 2012), in which 0.6% of

biochar was added to the feed for cattle. Based on the study, using biochar reduced methane emissions by 22%. This demonstrated that the surface area of biochar can significantly increase methane oxidation and microbial growth efficiency. Preston and Leng (1987) reported that biochar has several benefits for animal production.

More recently, Mirheidari, A. et al. (2019) presented a study to show the effect of biochar on the diet of dairy ewes. In that study, two types of biochar: walnut shell and chicken manure, were used to evaluate the biochar in-vitro. Different concentrations of 0.5, 1.0 and 1.5% were used in their study. The result from in-vitro experiments indicated that using different levels of biochar in the diet decreased methane production and ammonia concentration and improved milk production. They suggested that using biochar with dairy ewes can be used to increase rumen metabolism and ultimately the productivity of animals. However, Teoh et al. (2019) suggested that further studies are required to evaluate the effect of biochar on different types of forage compositions.

Current studies for estimating GHG emissions from enteric fermentation and manure decomposition lack validation, particularly for dairy cattle. The main reason for that is these studies are conducted in artificial and highly controlled environments. Thus, the ability to reduce emissions and examine biochar/activated-carbon application efficiency on GHG emissions and rumen fermentation are limited by a lack of *in-vivo* studies which have not been adequately validated for dairy cattle.

1.2 Aims of Study

This research aims to investigate the efficacy of high surface area biochars categorised as powdered activated carbon (PAC) on improving rumen fermentation, milk production, health and performance of dairy cattle and to reduce GHG emissions by adding PAC to the diet of dairy cattle. Thus, the work presented in this focuses on how adding different concentrations of PAC can to a decrease in GHG and CH_4 as well as to improve rumen fermentation and dairy cattle productivity.

Prior work has identified that PAC is readily producible on-farm from a range of sources and details of this are outside the focus of this study. In addition in order to meet strict Animal ethics guidelines PAC with prior certification was required, hence the use of commercially available coconut shell derived PAC in this study at a cost of $2/kg AUD.

While there are potentially numerous benefits to soil from manure with PAC included, this study focusses on gaseous emissions eructated and from manure within the dairy itself.

1.3 Research Questions

This thesis will focus on the following question:

1. How does PAC impact on rumen efficiency and dairy cattle productivity?
2. Are there any negative impacts on dairy cattle's health?
3. What level of PAC is needed to effectively reduce GHG emissions?
4. Do PAC additions in feed reduce nutrition leaching from manure?

1.4 Objectives

The work presented in this thesis focuses on adding different concentrations of PAC to the diet (w/w) of dairy cattle to assess its effect on rumen fermentation. At the same time, it will give an increased understanding of greenhouse gas emissions from Australian dairy cattle. The following contributions will answer the research questions and achieve the following objectives:

1- To investigate the effects of PAC on milk production by monitoring precursor acetate ($C_2H_3O^{2-}$) concentration (as an indicator of milk fat) in both the *in-vitro* experiment and the *in-vivo* experiments.

2. To identify changes in the rumen process from methanogenesis to the methanotrophic process with the increased microflora activity of biochar addition by monitoring methane emissions under *in-vitro* experimental conditions.

3. To determine the effects of PAC on the health of dairy cattle by monitoring the general condition (such as animal productions) of animals, using milk production as an indicator of the efficiency of the digestion in the rumen and butyric acid production in *in-vitro and in-vivo* experiments.

4. To obtain current levels of GHG emissions, especially CH_4 and CO_2 for types of Australian dairy herds during milking as well as the emissions released from manure.

1.5 Hypotheses

The addition of PAC to dairy cattle feed will increase the efficiency of digestion through greatly improved microflora activities in the rumen and may help the maintenance of a healthy rumen, due to the greater surface area, allowing for increased populations of beneficial(non-Methanogenic) bacteria to grow. The enhanced rumen microbe populations will increase feed efficiency, resulting in greater milk productivity. As an odourless, tasteless additive, a small amount of PAC incorporated into the diet of animals will not affect the palatability nor consumption of the feed.

1.6 Research Outcomes and Significance

This research project aims to study the efficacy of PAC in improving rumen fermentation, milk production and animals' health and performance and reducing GHG emissions. Thus, this study will lead to a reduction in GHG by adding PAC to the diet of dairy cattle. Famers can use these studies for improving animal productivity. Moreover, these studies will address the effects of the PAC on milk production in relation to quality and quantity. Furthermore, they will help to enhance microflora activity in the rumen, resulting in an increase in microbial protein. In addition, these studies will help to reduce greenhouse gas (GHG) emissions. Thus, the significance of this research project is: firstly that PAC will be produced using agricultural residues that will reduce agricultural waste and emissions; secondly, that feeding PAC to dairy cattle will improve livestock health, stabilize rumen activities and potentially reduce stress on the digestive system of dairy cattle; finally, incorporating PAC into the diet of dairy cattle will decrease GHG emissions. Thus, this research will help the farmer to reduce the cost of improving animal productivity.

Based on the experimental results, the proposed studies in this thesis can achieve high performance through improved rumen fermentation. Figure 1.1 shows these studies which have been conducted successfully with high performance using the proposed studies. Moreover, they can reduce GHG emissions by adding PAC to the diet of dairy cattle. Thus, PAC has shown a significant impact on dairy cattle production.

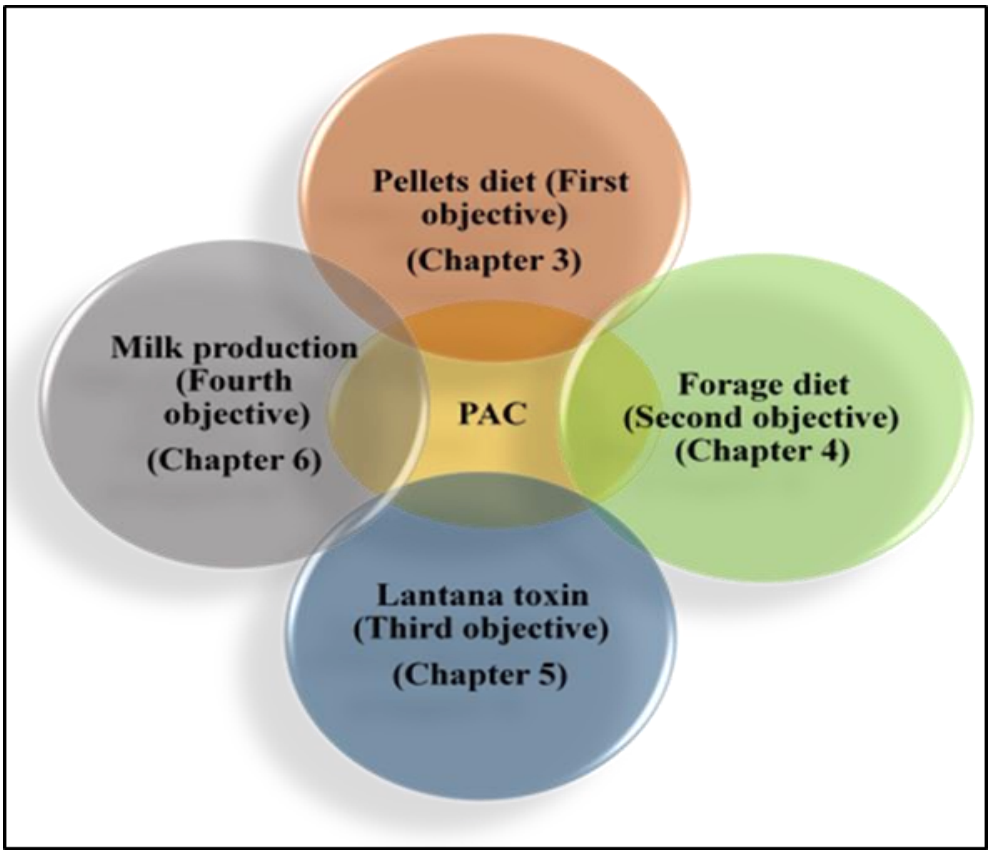


Figure 1.1: Study types conducted in each chapter for PAC addition to dairy cattle diet.

1.7 Thesis Structure

This thesis consists of six chapters and each chapter provides important information on that study. The rest of the thesis is structured as follows:

Chapter 2: Provides an overview of biochar. Firstly, this chapter introduces brief details about the background knowledge of the biochar. This chapter also introduces brief concepts of biochar, including its physical and chemical properties. This chapter also introduces the effect of biochar on animals such as live weight and productivity. This chapter then focuses on the effect of biochar on different types of animals.

Chapter 3: Integrates the effects of the addition of powdered activated carbon (PAC) to a pellet diet of dairy cattle on GHG emissions and rumen fermentation. This chapter introduces a suitable study by using PAC to reduce GHG emissions and enhance digestibility. This chapter also provides a study to evaluate the effect of PAC on two types of rumen liquid that fed grain and grass.

Chapter 4: Introduces a new study based on forage diets for studying the effect of PAC on GHG emission and rumen fermentation. The SPSS software program is used in this study to analyse all the obtained results. This study was used to investigate the effect of PAC on forage diets as a way to improve digestibility and reduce GHG production as well as to evaluate the effect of PAC on fibre digestion

Chapter 5: Provides an in-vitro rumen study of the effects of activated powdered carbon on Lantana toxics in the rumen. This study used different parts of Lantana such as berry, flower and leaf; Fourier transform-infrared spectroscopy (FTIR) is used to measure adsorption of Lantana toxins. This chapter also studies the effect of PAC on GHG and CH_4 and rumen fermentation.

Chapter 6: Investigates an in-vivo study of additions activated powdered carbon to the diet of dairy cattle to improve milk production, reduce GHG emissions and enhance microflora. This chapter also investigates suitable equipment to detect GHG emissions before, during and after milking of dairy cattle and from soil based faecal emissions.

Chapter 7: Presents an overall summary of findings of this study.

CHAPTER 2

2.1 Background

The main purpose of this research is to study the effect of powdered activated carbon (PAC) or biochar (BC) on the reduction of GHG emissions and the improvement in rumen fermentation efficiency in dairy cattle. The results of this study could be used to enhance dairy cattle productivity and reduce pollution in the environment. However, information on the effect of PAC on milk production in dairy cattle is limited. Therefore, the scope of this literature search will be extended to such diverse areas as soil and other livestock. In order to understand BC properties, this chapter begins in Section 2.1 by introducing the main properties of BC, which are correlated with the production process. Section 2.2 provides the effect of the addition of biochar on soil quality, manure and animal production. A key aspect of the literature was inconsistency in the BC results, to improve our chances of reliable results a key focus was to look at the surface area characteristics of the BC used. In order to have a broad understanding of the effect of the addition of biochar on soil quality, manure and animal production, this chapter also provides an overview (literature) of studies. A brief summary of the previous research is provided below.

2.2 Properties of Biochar (BC) vs Activated Carbon

This subsection discusses the physical and chemical properties of BC. Generally, all activated carbons can be classified as BC but with specific properties. Activated carbons have their surface area enhanced using acids or hydroxide treatments, 900°C steam or combinations of these. Subsequently, the specific surface area of the biochar is increased from 300 m^2/g (BC) to > 1000 m^2/g i.e. activated carbon (Li, X. et al. 2021; Shrestha et al. 2021). The surface area of BC is affected by its porosity. The size of micropores in BC ranges from < 0.9 nm to < 2 nm, whereas, that of macrospores ranges from < 2 nm to > 50 nm) (Chen et al. 2021; Durai et al. 2021)

The macrospores have been shown to act a surface for the microbes to grow. The smaller pores have been shown to be involved in the absorption and transport of molecules. A high level of porosity increases the surface area of BC. Moreover, the porous structure provides a stable habitat for the infiltration of organisms (Anto et al. 2021). Figure 2.1 shows the surface and pores of the jarrah wood BC via SEM.

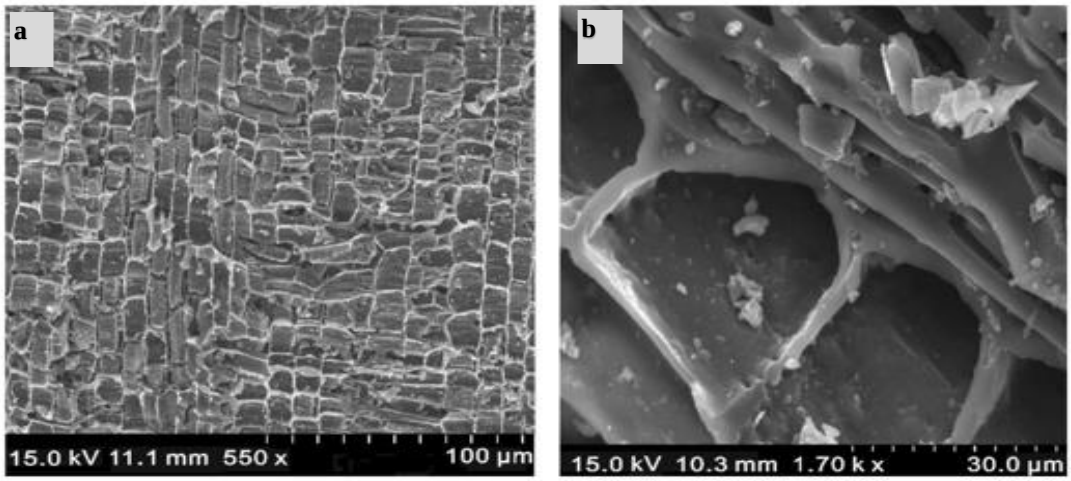


Figure 2.1: (a) Jarrah wood surface (b) Close-up of pores in the biochar using SEM (Joseph et al.2015).

The surface area of BC can generally be improved by increasing the pyrolysis temperature; for instance, an increase in temperature helped improve the porosity of wood activated carbon (Tomczyk et al. 2020; Chen et al. 2021). The temperature at which the biomass is treated has been shown to produce different results during the pyrolysis stage. The formation of microspores, during the dihydroxylation reaction, has been shown to be strongly related to pyrolysis temperature, with increases in porosity via cracking, resulting in a three-fold increase in surface area of the BC. Studies have used a wide range of temperature (200 to 700 °C) during the production of BC (Fan et al. 2020; Chen et al. 2021; Sekar et al. 2021).

At higher pyrolysis temperatures, often the holding time required is relatively short. However, this also depends on the size of the biomass particles being pyrolysed. Large biomass particles require longer time for the heat to soak through and to allow uniform carbon geophilic platelet formation (James R et al. 2020; Peterson et al. 2020). It has been suggested that flash carbonization (350 to 650 °C) is optimal to produce BC, with 50% of the biomass forming BC and the remaining 50% forming syngas (Daramy et al. 2020; Wang et al. 2020).

Ponnusamy et al. (2020) has reported that an increase in pyrolytic temperature from (600 to 900 °C) increased the surface area of BC from 2800 m^2/g to 3500 m^2/g. Leng et al. (2020) compared the effect of different temperatures (400 and 900 °C) on the surface area of BC and found that the surface area increased from 120 m^2/g to 460

m^2/g. While the specific biomasses used vary there is a general positive trend in BC surface area when treatment temperature increases, at least up until around 1000°C.

2.3 Chemical properties of biochar and stability

Biochar is a carbonaceous material that consists of different kinds of polycyclic aromatic hydrocarbons and other functional groups, depending on biomass type and treatment applied (Hao et al. 2020; Ukalska et al. 2020). The initial properties of the biomass feedstock can affect the stability over time, of the resulting BC. The resulting hydrophilic and hydrophobic properties allow the BC to react with soil and contribute to its chemical makeup (Li, M. et al. 2021; Murtaza et al. 2021; Xu et al. 2021)

It has also been reported that low temperatures allow the volatile organic compounds (VOCs) to reduce and block the pores and thus reduce the adsorption potential of BC (Guo et al. 2021; Kroeger et al. 2021). The stability of BC can also be affected by its porosity. An increase in surface area of BC also increases the nutrient retention capacity due to its ability to bind cations and anions (Kroeger et al. 2021; Tian et al. 2021).

Through the findings of recent studies, it is apparent that the chemical and physical properties of BC can have a significant effect when applied to different soil types. An increase in temperature helps improve the recalcitrant chemical characteristics of wood derived activated carbon (Feng et al. 2021).

Because of their molecular structure, BC can display a high degree of chemical and microbial stability, providing space for the growth of beneficial soil microorganisms (Das et al. 2020; Jien et al. 2020). Therefore, BC as a feed additive for animals will increase the population of beneficial microbes in the rumen and improve productivity. Figure 2.2 shows the internal surface of different types of BC at different pyrolysis temperatures. These pictures were taken at University of Southern of Queensland by using Fourier Transform Infrared (FTIR) Spectroscopy to show how the pyrolysis temperate changes the surface effective area of the resulting BC.

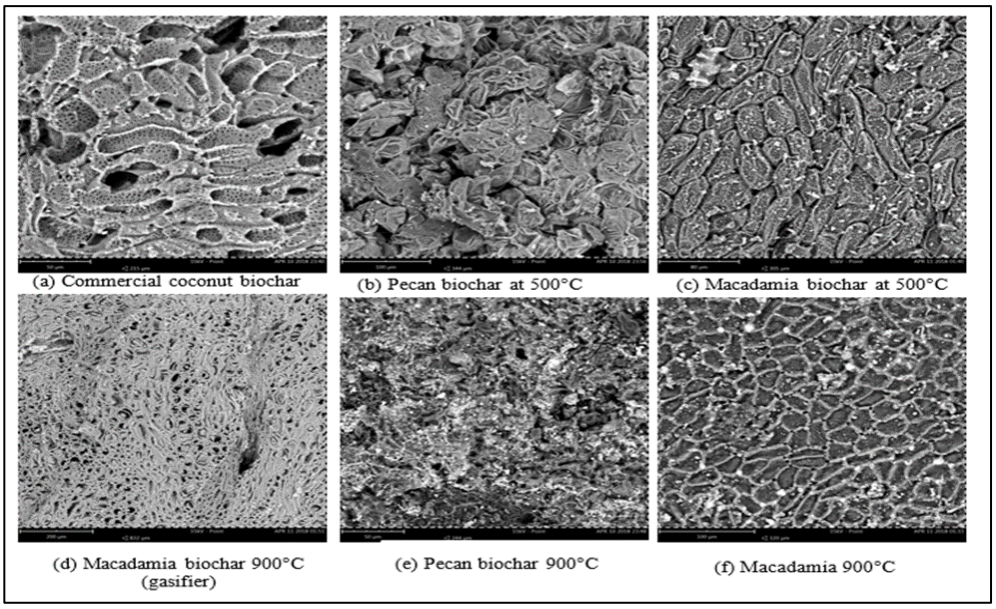


Figure 2.2: internal surface pores of different biochars (authors own images).

2.4 Biochar background literature

In this section, we focused on reviewing the effect of BC on non-ruminants (chicken, fish and pigs), ruminants (sheep, goats and cattle) and soil quality. A brief summary of the previous research is provided below.

2.4.1 Addition of biochar to animal diets

In animal husbandry, BC can be added to animal diets as a possible way to reduce GHG emissions and enhance rumen microflora (Al Kindi 2015a; McFarlane et al. 2017; Mirheidari, A. et al. 2019). In the Dairy industry, the increase in milk production depends on the end products of rumen fermentation, which is affected by rumen microbes as these microbes help break down feed, increasing the production of volatile fatty acids (VFAs) and reducing GHG emissions (Iqbal et al. 2009; Tapio et al. 2017). The protozoa in the rumen are important in the production of proteins via consumption of proteins, whereas bacteria have the ability to break down feed, produce VFAs, ammonia and increase organic matter digestibility (Williams and (Dahiya et al. 2015). Biochar can be produced from different materials, such as peat, wood and coconut shell (Man et al. 2020; Ponnusamy et al. 2020; Anto et al. 2021). The papers collected for the study in this chapter were classified based on the type of animals, such as sheep, goat, cattle, poultry, pig and fish; a summary of the results of previous studies on BC to enhance animal productivity are presented in Table 2.1.

Table 2.1: Summary table of effects of biochar on animal productivity

Animal	Biochar	Impacts							Reference
Poultry	Control	0	Total body weight (kg)	2.38	Average weight gain (g)	1.71	Feed intake (kg)	4.27	Kana et al. (2011)
	Maize cobs	0.20%		2.67		2		NS	
		0.40%		2.66		1.98		4.52	
		0.60%		2.52		NS		NS	
	Canarium Seed	0.20%		2.48		NS		NS	
		0.40%		2.44		NS		NS	
		0.60%		2.47		NS		NS	
Poultry	CWVC	0	Total body weight gain (g)	275	Average weight gain (g)	6.25	Feed intake (kg)	NS	Mongkol et al. (2001)
		1%		304.29		9.53		NS	
		3%		318.75		8.34		NS	
		5%		NS		NS		NS	
Cattle	Biochar	0	Total body weight (g)	94.3	Average weight gain (g/day)	103	CH4 (PPM)	84.5	Leng et al. (2012a)
		6%		97		196		64	
Goats	Bamboo biochar	0	Digest-ibility of DM	560	Digest-ibility DM (g/day)	566	Digestibility CP (g/day)	501	Van et al. (2006)
		0.5g/kg (BW)		624		626		616	
		1g/kg (BW)		638		634		652	
		1.5g/kg (BW)		NS		NS		NS	
Pigs	Bamboo biochar	0	Final body weight (kg)	109.67	Average daily weight gain	0.75	Feed intake (kg)	NS	Chu et al. (2013 a)
		0.30%		116.83		0.877		NS	
		0.60%		113.33		0.817		NS	
		0	Cortisol (mg/d L)	4.35		NS		NS	
		0.30%		2.44	Tri-glyceride (mg/d L)	61.4	Blood urea nitrogen (mg/d L)	NS	
		0.60%		2.4		52.17		NS	
Pigs	Control	0	Final body weights (kg)	109.67	Average daily gains	0.75	Feed efficacy (g/g)	0.29	Chu et al. (2013 b)
	Antibiotic	0.30%		117.18		0.913		0.36	
	Bamboo biochar	0.30%		116.83		0.877		0.34	
	Bamboo vinegar	0.30%		114.83		0.857		0.36	
Fish	Bamboo biochar	0	Final weight (g)	2.88	Average daily gains (g)	1.71	Survival rates (%)	77.3	Quaiyum et al. (2014)
		0.50%		3.02		1.85		84	
		1%		3.21		2.03		82	
		2%		3.67		2.5		84	
Fish	Activated charcoal	0	Final body weight (g)	12.9	Weight gain (%)	101.7			Pirarat et al. (2015)
		1%		15.87		190.04			
		2%		17.08		211.5			
		3%		14.26		158			
Fish	Bamboo charcoal	0	Weight gain (%)	105.9	Feed intake (g)	1.56	Feed efficacy (%)	0.85	Thu et al. (2010)
		0.0004%		NS		NS		NS	
		0. 01%		NS		NS		NS	
		0. 1%		NS		NS		NS	
		1%		NS		NS		NS	
		4%		128.9		1.77		0.95	

Table Legend: NS: not significantly different; BW: body weight.; CP: curd protein.; CWVC: charcoal powder including wood vinegar compounds

2.4.2 Addition of biochar to the diet of ruminants

George et al. examined the effect of activated charcoal (produced at 450°C) on the digestion of bitter-weed (*Hymenoxys odorata DC.*) in lambs (George et al. 2000). This study included three experiments. In the first experiment, the lambs were fed subacute levels (0.26% body weight (BW)) of bitterweed and activated charcoals at different levels (0, 0.5, 1 and 1.5 g/kg BW). The lambs refused to eat bitter weed after day 10 of the experiment. In the second experiment, the lambs were administered (by gavage) 0.264% BW of bitterweed and different levels of activated charcoal. They were then administered milo (*Sorghum* sp.) by gavage. The results revealed that the intake of milo significantly increased to 296g and 303g when activated charcoal was administered at levels 1 g/kg and 1.5 g/kg BW compared with 216g at 0% activated charcoal. This increase indicated that biochar enhanced the digestion of feed by enhancing microflora and improving the absorption of nutrients from the gastrointestinal tract.

The third experiment used sixteen lambs divided into two treatment groups. The first treatment consisted of feeding the lambs 20% crude protein and 1 g/kg BW of activated biochar mixed with the diet. In the second treatment, the lambs were not fed activated biochar. The lambs that were fed 20% crude protein and 1g/kg BW of activated biochar consumed 302g/day of bitterweed, whereas the lambs that did not receive activated biochar consumed 297g/day of bitterweed. The addition of biochar to the diet improved digestion and enhanced microfloral action, thereby improving the rumen health and feed intake in the lambs (George et al. 2000). However, further investigation is required to evaluate the effect of biochar on digestive function and animal health. We believe that the problem associated with bitterweed as a feed can be solved by feeding lambs sufficient activated biochar that can alter the rumen environment, control pathogenic bacteria and break down bitterweed.

Biochar has also been reported to have beneficial effects on the health of cattle. Leng et al. (2012a) studied the effect of biochar on growth and feed conversion in local yellow cattle (Table 2.1). The basal diet was compared with the diet with 6% biochar, 6% potassium nitrate and 1.83% urea. The biochar produced from rice husk in a gasifier stove at pyrolysis temperature of 400°C helped increase pores and surface area. The average weight gain significantly increased by 14% with the addition of 6% biochar. However, feed intake was not affected by the addition of biochar. This study

demonstrated that biochar increased microbial habitat, enhanced microbial growth efficiency and improved essential amino acid contents. These results can be extended to other species of cattle (Leng et al. 2012a). As rice can be a toxin accumulator its use as a biomass source requires care before inclusion in any animal diet. In contrast, biochar as a feed additive for livestock requires more studies before its economic application. It has been suggested that extensive studies on the use of biochar should be carried out and that these study results should be available for other researchers and farmers.

Matthew et al. (2001) studied the effect of feeding activated charcoal on the productivity of goats. This study included three experiments. The first experiment consisted of 20 goats; 10 goats per treatment. The first treatment was without activated charcoal and the second group was fed 1 g/kg w/w of activated charcoal in an aqueous solution. Every morning, the goats in the second treatment group received 1 g/kg BW activated charcoal (by gavage) after being mixed with 500 mL of distilled water and 100g juniper. The results showed that activated charcoal increased the intake of red berry juniper during the initial period of exposure.

The second experiment commenced two weeks after the first experiment (Matthew et al. 2001). This trial contained two treatments: the first treatment was a control without activated charcoal (the goats were fed Ashe juniper). The goats in the second treatment group were fed 1 g/kg BW activated charcoal and 100 g of Ashe Juniper for 10 d. The results showed that the intake of Ashe juniper was not affected by the addition of biochar. The third experiment started two weeks after the second experiment and continued for ten days. The goats were fed 200g of red berry or Ashe Juniper mixed with biochar for 2 h daily. The difference in feed consumption indicated that the goats preferred Ashe Juniper with biochar (20g/kg BW) rather than red berry juniper (3 g/kg BW). Nevertheless, the increased intake of red berry juniper and Ashe Juniper with biochar required greater effort to reduce their limited intake. This could be remedied by using different types and concentrations of biochar as well as using biochar produced at different pyrolysis temperatures.

Van et al. (2006) suggested that bamboo charcoal has a significant effect on the growth of goats (Table 2.1). Bamboo charcoal was added at different levels 0, 0.5, 1.0 and 1.5g/kg BW to the diet of goats. The results revealed that the addition of bamboo charcoal at a concentration of 0.5 and 1.0g/kg DM had a significant effect on the

digestibility of dry matter, organic matter and curd protein. Furthermore, the digestibility of dry matter increased significantly by 11% and 14% by the addition of 0.5 and 1.0 g/kg DM biochar, respectively. The digestibility of organic matter increased significantly by 11% and 12% with the addition of 0.5 and 1.0 g/kg BM biochar, respectively. The content of curd protein increased by 23% and 30% with the addition of 0.5 g/kg and 1.0 g/kg BM biochar, respectively. These results indicate that bamboo charcoal improved digestibility by influencing rumen stability and intestinal sensitivity. However, Van et al. (2006) did not mention the temperature that was used to produce bamboo charcoal and perhaps even more importantly if the bamboo surface area was high compared to BC in other studies.

Adsorption treatment with biochar as a non-digestible carrier is an important technique to avoid ingested harmful substances being absorbed from the gastrointestinal tract or changed by the microbial community in the rumen. The chemistry and microbial community of the ruminant faeces change with the addition of biochar to the ruminant diet. This has been attributed to the changes in the microbial community in the rumen and intestine (Van et al. 2006; Thu et al. 2010; Chu et al. 2013). Thu et al. (2010) reported that biochar can affect anti-nutrients and fermentation products in the rumen and improve the absorption of nutrients through the cell membrane.

Methane emission is an inevitable consequence of cattle and goat production and biochar can help reduce this. Biochar addition significantly enhanced the ratio of methanotrophic microorganisms (that can consume methane) to methanogens in paddy soils (Feng et al. 2012), demonstrating that the addition of biochar to the diet of animals can increase microbial population and internal surface area in the rumen.

An *in vitro* study evaluated the effect of biochars (gasified, wood-based and straw-based biochars) on CH_4 production from buffered rumen fluid; the rumen fluid was collected from two fistulated Jersey heifers. Biochar at a concentration of 9% was added to the feed DM. Biochar decreased CH_4 emissions by 11%–17% compared with that of the control because the habitat and growth of microbes were affected by biochar porosity (Hansen et al. 2012). However, this study did not consider that biochar might adsorb nutrients and other minerals and vitamins. We believe that biochar produced at a high temperature, such as 900 °C, can increase the surface area and reduce the ability of biochar to absorb nutrients.

In vivo studies have suggested that feeding cattle 0.6% biochar reduced methane emission (P<0.066) by 22%. This proved that the surface area of biochar can significantly increase methane oxidation and microbial growth efficiency (Leng et al. 2012a). Increasing the population of methanotrophs in the digestive system can increase oxidation and microbial habitat in the rumen. In addition, Preston and Leng (1987) found that biochar has several benefits on animal production, including increased feed conversion efficiency, microbial habitat, microbial growth efficiency and essential amino acid content.

McFarlane et al. (2017) studied the effect of type and size of biochar on *in vitro* rumen fermentation of orchard grass hay (*Dactylis glomerata*) due to the improvements in microbial fermentation in the rumen. The study focused on the effect of biochar on the nutritive value, *in vitro* digestibility, VFA production and GHG emission. Biochar was produced from three tree types of chestnut oak (*Quercus prinus* L.), yellow poplar (*Liriodendron tulipifera*) and white pine (*Pinus strobus* L.) at a pyrolysis temperature of 1110 °C. Biochars of two particle sizes (< 178 μm, fine; 178 μm, coarse) were used. Biochars were added to the basal diet containing orchard grass hay at a rate of 81 g/kg DM. The content of VFAs was not affected by the type and particle size of biochar. The reduction in GHG emissions by coarse biochar was more than that by fine biochar. These results necessitate further studies to evaluate the effect of type and size of biochar before using it as a feed additive for ruminants. We believe that McFarlane et al. (2017) used a very high temperature (1110°C), which resulted in a reduced surface area and not an activated biochar, therefore the outcome was not significant. Studies to identify the best pyrolysis temperature, type and size of biomass help to obtain better activated biochar.

Prasad et al. (2000) conducted a study to investigate the effect of charcoal addition to the concentrate mixture on rumen digestibility in cattle. Charcoal was added to the diet at a rate of 1 and 2 g/kg BW. The study showed that the addition of charcoal to the concentrate mixture did not have negative effects on digestibility efficiency, nitrogen-free extract and the content of VFAs, serum protein, urea, albumin and minerals. Furthermore, charcoal, when added at a rate of 1 and 2 g/kg BW, did not show a significant effect on digestibility in the rumen. We believe that increasing the rate of biochar addition to up to 2% might increase the surface area, thus enhancing the

number of fibrinolytic bacteria in the rumen and improving the digestibility of fibre; this might have a significant effect on digestibility.

Lee et al. (2000) studied the effect of biochar on *in vitro* ruminal fermentation efficiency and nutrient absorption. Charcoal was added at different rates (0%, 0.25% and 0.50%) to different types of diet (roughage/concentrate ratio; 8:2, 6:4, 4:6 and 2:8), respectively. The ruminal pH did not change significantly with the addition of charcoal to the diet. The percent of propionate, ruminal degradation of DM, crude protein, neutral detergent fibre (NDF), acid detergent fibre (ADF) and hemicellulose tended to increase with the addition of charcoal to the diet. Greenhouse gas emission decreased with the addition of 0.50% charcoal to the concentrate diet, whereas the emissions tended to increase with high levels of roughage diets. However, biochar addition to the diet of ruminants requires further studies before its inclusion in the commercial diet of livestock. We believe that adding activated charcoal to different types of diet (roughage and concentrate) might be beneficial in enhancing the microflora and reducing GHG emission.

Overall, these studies demonstrate that biochar can increase microbial habitat, enhance microbial growth efficiency and improve essential amino acid contents and reduce GHG emissions. We believe that biochar can effectively mitigate GHG emissions through C and N recycling by the rumen microbes, which can be utilized for microbial growth. However, further studies are necessary to understand the mechanisms underlying GHG emissions and to verify whether biochar addition can increase the quality and quantity of milk and animal productivity before using biochar as a feed additive for ruminants.

2.4.3 Use of biochar in the diet of poultry

Biochar has been routinely used as a feed supplement to improve various aspects of poultry production. Reported research has focused primarily on the benefits to chickens, with very limited information available for other species of poultry such as ducks (Ruttanavut et al. 2009). Various biochars have been investigated, made from an array of materials including wood, maize cobs, bamboo, woody green waste, plant seeds, cow bone, or recycled broiler litter, often mixed with other compounds and

prepared under different conditions (Kutlu et al. 2001; Kana et al. 2011; Raphal et al. 2014).

The addition of biochar to poultry dietary feed varies widely as it is often based on the regional availability and economical benefit compared to conventional or commercial feed sources. Such biochars are often used to improve areas of animal health and environment, such as improvements in nutrition, growth and egg production, control of pathogens, diseases, toxin deactivation and control of ammonia emissions from faecal deposits.

Wood (oak) charcoal is often included in poultry nutrition due to the claim that it prevents or medicates fatty liver syndrome and improves food conversion efficiency in layer hens or broilers. Initial research by (Hasan et al. 2001) verified that 5% of dietary wood charcoal significantly improved food intake, body weight gain and food conversion efficiency in young broilers, during the initial four weeks of supplementation, but that this beneficial effect was temporary and disappeared by the end of the six week trial. There was also no detrimental effect on abdominal fat, weight, carcass dry matter, fat or protein content. Faecal analyses showed that dietary charcoal had increased dry matter, fat and fibre content in a dose related manner, indicating increased fat excretion when the broilers received charcoal supplementation. Similar results were reported by (Kutlu et al. 1999), with an improvement in broiler performance when fed 2.5% DM of charcoal, either for the initial three weeks or for all six weeks of the trial. Again the benefits were age dependent, being more pronounced in the starter diets. The addition of charcoal in the initial three weeks or last three weeks did not significantly improve food conversion efficiency or body weight gain. By the end of the study the groups receiving charcoal at 2.5% in the starter and/or finisher diets showed significantly higher body weight gains, carcase weight and numerically higher food intake and food conversion efficiency, than the control group receiving non-charcoal diets. It was questioned whether the presence of charcoal may have a negative effect on fat and fat related nutrient absorption, leading to nutrient deficiencies, thus limiting optimum growth, as charcoal supplementation tended to reduce the abdominal fat width and percentage.

Further investigation by Kutlu et al. (2001) into the effects of supplementing the diets of both broiler hens and laying hens with wood (oak) charcoal, at different levels (0, 0.25%, 0.5% and 1% charcoal/feed for broilers plus 0.1%, 0.2% or 0.4% charcoal/feed

for layers) and different times (phased feeding). Their results confirmed a temporary gain, as previously noted. The addition of charcoal to the diet was of value in improving broiler performance only during the first 28 days, after which there was little benefit. A significant improvement was noted if charcoal was added to the diet of broiler hens during the first 28 days of the fattening period. Birds fed charcoal showed higher feed intake and weight gain, along with increased overall carcass weight, compared to the control group that received no charcoal. Supplementation for a longer time period showed no beneficial gains for the older birds, mirroring earlier results.

In addition, when 34 week old younger laying hens were fed dietary supplementation with wood charcoal (but at lower levels of 10, 20 or 40g/kg), a significantly reduced number of cracked eggs was recorded, in line with the dose applied. However at this lower rate there was no improvement to feed intake or laying performance of the hens, i.e. the mass of: eggs, albumin and shell thickness all remained unaffected (Kutlu *et al.,* 2001). Other gains to egg production have been reported (Yamauchi et al. 2010). An increase in collagen in the egg yolk has also been reported when 67 weeks old laying hens were fed either of two diet supplemented (at 9.9%) with wood charcoal powder containing wood vinegar liquid (Yamauchi et al. 2010).

Increasing levels of bamboo charcoal powder with vinegar liquid in a basal diet was also shown to incrementally improve the growth performance of Aigamo ducks (Ruttanavut *et al.,* 2009). There were also beneficial changes in intestinal morphology, specifically increased villus height, villus area, epithelial cell area and increased cell mitosis. However, at the highest supplementation of 1% there was no significant improvement in feed intake, with gain or feed efficiency.

Biochar has been shown to improve the production performance of chickens. Samanya et al. (2001) studied the influence of dietary charcoal powder, including wood vinegar compounds (CWVC), on poultry productivity. Single comb white leghorn chickens (130-d-old) were fed diets with varying levels of CWVC (0%, 1%, 3% and 5%). Charcoal was produced from broad-leaf trees at 300-450 °C. The CWVC at levels 1% and 3% significantly increased the total body weight (BW) by 11% and 16%, respectively. Feed intake was not significantly affected with the addition of CWVC. Moreover, the functions of villi and ileum were activated when the chickens were fed a diet containing 1% CWVC, which was detected by morphological changes.

However, feed consumption was not significantly enhanced. Thus, feeding chickens biochar was shown to enhance digestion by improving nutrient absorption through the cell membrane and microbial action in the intestine of chickens (Anjaneyulu 1993). The beneficial effects of charcoal were confirmed by another study. Jiya *et al.* (2014) studied the effect of activated charcoal on carcass yield, organoleptic and serum biochemistry of broiler chickens. Activated coconut shell charcoal was added at different levels 0%, 0.5%, 1%, 1.5% and 2% of feed. The results showed that feeding activated charcoal improved feed efficiency, health and nutrient utilization.

Tanka *et al.* (2016) studied the effect of the addition of 4% biochar (woody green waste biochar produced at 550°C), bentonite and zeolite on the diet of layer chickens as an alternative to antibiotics to eliminate diseases. Biochar and bentonite significantly affected the intestinal bacterial community in chickens. Biochar and zeolite reduced the population of Proteobacteria more efficiently than bentonite. Both the treatments reduced the need for antibiotics.

Islam *et al.* (2014) also reported benefits to ducks fed a diet supplemented with up to 1% of a 50:50 mix of sea tangle (an edible brown seaweed) and charcoal. Antibiotics are frequently used in overcrowded poultry production, as it reduces the incidence of disease and improves growth rate, feed efficiency and meat quality. Supplementation with the unconventional feed resource had no adverse effect on the overall growth performance of the growing ducks, which closely mirrored the gains from antibiotic supplementation. Hence, the biochar/seaweed mix was a more acceptable alternative to the common practice of supplying antibiotics as a growth promoter (Islam *et al.*, 2014).

These results proved that biochar, bentonite and zeolite can be utilized to specifically decrease the population of some poultry zoonotic pathogens by maintaining the diversity of microbiota in chickens. Biochar can change the intestinal microbial community by increasing the surface area of the digestive system; this might enhance the growth of microbes, thus, improving digestive efficiency. Researchers believe that biochar can be used to improve the quality of feed by binding to or breaking down toxins.

Watarai (2005) proved the protective efficacy of activated charcoal (made from oak) containing wood vinegar liquid against intestinal infection with the pathogen *Salmonella enterica. S. enterica* is a common pathogen and routinely causes human

gastroenteritis via the ingestion of contaminated chicken eggs and meat. Routine vaccination does not fully inhibit bacterial growth in chickens, as indicated by faecal detection. Addition of the charcoal: vinegar mix to the diet of chickens effectively absorbed *S. enterica* but had a slower binding capacity of *Eneterococcus faecium.* The presence of the wood vinegar inhibited the growth of *S. enterica* but enhanced normal bacterial flora including *E. faecium* and *Bifidobacterium thermophilum.* The presence of harmful substances such as organochlorine pesticides, heavy metals, radionuclides and mycotoxins is sometimes found in poultry feed (Kan 1994). Some research has been completed, investigating the ability of biochars to remove the detrimental effect of these harmful substances. Waibel et al. (1972) investigated the efficacy of charcoal to mitigate the effects of the environmental contaminant dithiothreitol (DTT), known to affect birds and egg production. Neither DTT (when fed at 300ppm) or charcoal affected egg weight or shell thickness. The presence of 3% charcoal effectively reduced DDT deposition in both hen abdominal fat and egg yolk. However, there was also a reduction in yolk pigmentation due to the charcoal. Aspergillus species produce various aflatoxins, which contaminate food and animal feeds worldwide, causing serious health problems and livestock production losses.

Kana et al. (2010) studied the effectiveness of biochars to mitigate aflatoxin BI toxicosis, when young male broiler chicks were fed a peanut meal diet containing ~22 ppb of aflatoxin B1, a fungal toxin commonly produced by *Aspergillus flavus*. Two types of biochar (produced from maize cob or the seed of African elemi (*Canarium schweinfurthii*)) were added at different levels up to 0.6% to the toxic diet. Overall, maize cob biochar resulted in superior results compared to the seed biochar. The addition of 0.2%, 0.4%, or 0.6% maize biochar resulted in significantly higher final body weight than the control containing aflatoxin. However, improvement in final BW with the addition of seed biochar was gained only at the highest supplementation of 0.6%. Moreover, adding > 0.6% biochar actually had a negative effect and decreased the average final BW. The average weight gain significantly increased by 17% and 16% with the addition of biochar A at levels 0.2% and 0.4%, respectively. Feed intake increased by 6% with the addition of 0.4% biochar A. Thus, the improvement in live BW and BW gain was achieved with the beneficial effect of biochar on the intestinal bacterial flora competing with the host for available nutrients, thus improving

digestion (Kana et al. 2011). Therefore, further studies are necessary to investigate the interaction between biochar and digestive enzymes and epithelial lining in chicken.

Kana *et al.* (2014) further investigated the ability of biochar to influence growth performance in broiler chickens that were fed a diet laced with aflatoxin B1 toxin (AFB1 at 36 ppb or 60 ppb). Aflatoxins are poisonous carcinogenic and hepatotoxic compounds produced by *Aspergillus* fungal contaminants that can grow in animal food and feedstock. The presence of aflatoxins in poultry feed adversely effects production, causes a reduction in growth rate and feed efficiency, decreases egg production and quality, has a negative effect on survival and hatchability and increases susceptibility to disease (Andretta et al. 2011). Since the gastrointestinal tract is the first organ to be affected by any toxin, addition of biochar (made from *Canarium* seeds or cow horn, both common in Africa) was to the diet itself. As expected, there were indications of liver damage and increases in white blood cell (WBC) count and haematocrit with increasing level of AFB1. Horn charcoal supplementation at 0.4% also significantly reduced the serum parameters and could be used as protective agent in poultry to alleviate the harmful effects of aflatoxin B1. Compared to the aflatoxin diet alone, the live BW and weight gain improved by 20% and 18% with the addition of 0.4% bone charcoal and 0.2% *Canarium* charcoal, respectively. Both the biochars increased the size of villi in the jejunum, thus improving the function of villi through increased surface area for digestion in the small intestine. This enhanced the digestive system of broiler chickens. Overall, partial protective effects were noted in broilers of experimental groups in which 0.4% charcoal was added but not when added at 2%. The addition of biochar at higher levels may provide a higher protective level.

Biochar supplementation is common practice for poultry in Japan and China, while the Australian industry is beginning to investigate the benefits. In several states, Spotty Liver Disease, caused by *Campylobacter hepaticus*, is emerging as a disease of concern on layer farms (Willson et al. 2019). The addition of biochar, bentonite or zeolite at 4% feed, has been shown to effectively reduce many bacterial species, including some potential pathogens including Campylobacterales such as *C. hepaticus* and the human pathogen *C. jejuni* (Prasai et al. 2016). A reduction in other pathogenic bacteria including *Gallibacterium anatis, Bacteroides dorei* and *Clostridium aldenense* and *Heliobacter pullorum* (which is associated with poultry liver lesions and several human diseases: gastroenteritis, irritable bowel syndrome (IBD) and

Crohn's disease) was also noted. The addition of biochar did not cause any significant alterations to the overall richness and diversity of the intestinal bacterial community, which is beneficial to the poultry, indicating an effective method to control the major poultry pathogens as a viable alternative to antibiotic use. The use of antibiotics to suppress pathogenic bacteria is banned in many countries, including Australia due to the increase in antibiotic resistance and the consequences on human health (Samanya et al. 2001).

2.4.4 Use of biochar in a Porcine diet

Biochar has also been used as a feed supplement to improve various aspects of pig production. Pig production plays a large role in the provision of food to regional communities in many countries, primarily as intensive stall farming, although free range pig production is gaining popularity.

As previously mentioned, many toxic substances and fungal contaminations are present in animal feed ingredients, because they are heat-stable during the manufacturing heat process (MacLachlan et al. 2013; Guerre 2016). Toxic weed seeds (such as castor oil, Mexican poppy, potato weed, sesbania pea, thorn apple and jute) can also penetrate feed mixes. These toxins disturb animal growth performance and cause the animal to become more susceptible to disease and stress.

Mekbungwan *et al.,* (2004) first reported that piglets fed a diet supplemented with charcoal powder and wood vinegar showed improved feed efficiency and daily body weight gain compared to those on the control diets. Interestingly, those fed 5% biochar and vinegar actually mirrored the control, indicating that at higher levels, any potential benefit was lost. Moreover, close examination of the intestinal villi revealed significant adaptive histological changes. Villi were longer, with an increased surface area available for absorption of nutrients when biochar was added at 1-3%. The duodenal villus tip surface had clearer cell outlines, larger cells and cells enlarged further into the lumen, than those of the control group, suggesting that the charcoal elevated the absorptive function of intestinal villi and epithelial cells in all small intestinal parts. Such beneficial changes were not found in the high 5% diet, further supporting the belief that there is an effective supplement range that could improve animal growth.

Intestinal morphology can be markedly affected by the diets fed to animals (Langhout *et al.,* 1999). Such changes to the villi strongly suggest that the charcoal and wood

vinegar are elevating the absorptive function of the villi. Since the intestine is the predominant site for nutrient absorption, long villi are associated with increased surface area, resulting in greater absorption of nutrients while increased cell mitoses indicates rapid function. Lee *et al.* (2011) investigated the changes in Finishing Pigs when their basal diet was supplemented with differing concentrations of Stevia and/or Charcoal. The group supplemented with both stevia and charcoal (at 0.3%) showed the highest meat quality traits and storage characteristics compared to all other groups receiving either stevia or charcoal alone or the control group. This included a higher pH, water holding capacity and lower drip loss than those in the control group. Meat from this group also showed lower lightness and yellowness values and higher redness colour value than the control, which resulted in a redder surface meat colour. Marbling and colour scores also improved. In the panel test, this meat also had a higher tenderness and juiciness scores than the control group. Overall, addition of charcoal towards the end of production significantly improved both the quality and storage characteristics of the meat product.

Chu *et al.* (2013 a) also reported that adding bamboo biochar powder to the diet of fattening pigs improved their growth performance, feed efficiency and beneficial faecal microflora composition ($P < 0.05$). The biochar also concurrently decreased the production of noxious gas emissions, which is a common problem in intensive pig farming. Bamboo biochar has superior physical properties, with higher adsorption capacity than other biochars. This is primarily due to the special complex network of micro-pores present in bamboo stems resulting in fourfold more cavities, threefold more mineral content and fourfold better absorption rate than normal wood charcoal (Zhao et al. 2008). Overall, benefits were noted with the treatments compared to the control, although there was no statistical gain by the increase in bamboo charcoal. The addition of bamboo charcoal to the basal diet (at 0.3% and 0.6%), fed over 42 days, significantly improved both the average daily weight gain (by 17% and 11%) and final body weight gain (7% and 3%) respectively, compared to the basal diet containing mainly corn, wheat and soybean meal. Both treatments also exhibited a significant effect on feed efficiency when compared with the diet not supplemented with bamboo biochar. Although the average daily feed intake decreased marginally with the addition of bamboo charcoal this did not negate the overall gains. Addition of either level of bamboo biochar also improved the environment of the intestine, with a measurable

increase in beneficial bacteria (*Lactobacillus* spp.) with a concomitant decrease in the harmful faecal microflora (*E. coli* and *Salmonella* spp) and total anaerobic bacteria.

Overall, there was no measurable effect on most blood parameters or various blood cells, as all remained the same. There were also no increases in IgM and IgA levels (which would indicate infection). Small decreases in lactose dehydrogenase LDH (which plays an important role indicating cell damage or disease), triglyceride and blood urea nitrogen BUN (with elevated levels indicating disease) levels were detected in lower levels of charcoal and the cortisol concentration with either addition of bamboo charcoal. The elevated levels of IgG and lowered cortisol levels in the supplemented groups suggest that the addition of bamboo biochar in the diet may also protect pigs from infection and reduce stress.

Moreover, there was a significant decrease in the levels of faecal acids (including lactic acid, propionic acid, butyric and isovaleric acid) with an overall decrease in faecal pH in both Biochar (BC) treatments. This beneficial decrease in odour-inducing compounds suggests that bamboo charcoal can effectively neutralize anti-nutrients related to feed and can provide an economical way to suppress the production of noxious substances, gases and acid compounds. This may be due to the higher absorption capacity attributed to the micropores present in the bamboo stem and charcoal, which give a four-fold increase in cavities and absorption rate over wood charcoal (Asada et al. 2002). From the environmental perspective, supplementing the diet with bamboo charcoal resulted in a significant decrease in the release of the noxious gases; ammonia, methane, amine and hydrogen sulphide, the higher level of bamboo showing greater decreases. Such odor-inducing compounds are prevalent in livestock facilities and represent a major concern in intensive farming.

The gains measured in the supplemented groups were achieved by the bamboo charcoal and may be due to improved nutrient utilisation and researchers believe that bamboo charcoal can enhance the gastrointestinal system, enhancing the function of the intestine by improving the function of the intestinal villi and epithelial cells.

Similar results with bamboo have been reported by a number of other researchers. *Song et al.* (2014) reported similar benefits when pigs were grown on diets supplemented with bamboo powder (1%-3%). Significant gains in feed intake, feed efficiency and body weight occurred. They also noted positive reductions in odour inducing compounds (ammonia, methane, hydrogen sulphide and acetic acid) with

increasing amounts of bamboo powder, compared to the absence of such biochar. In contrast to other reports, there was no significant gain per day, nor in final body weight. There were also no changes in volatile fatty acids (VFA) between the control diets and those fed bamboo powder. Supplementation with bamboo powder also effectively increased numbers of beneficial bacteria, but, as harmful bacteria were never detected, the ability of the bamboo powder to decrease these numbers was not determined.

For biochar to be effective in pig production, it is important to supplement a diet with at least 1% biochar. Supplementation of pig diets with lower levels of charcoal fails to significantly improve animal growth and welfare and reduce noxious gas production overall. Kwak *et al.* (2017) reported no improvement when feeding organic charcoal at 0.1%. While the addition of organic charcoal (at 0.1%) did result in increased feed intake and lower ammonia emissions, it failed to improve dry matter digestibility, improve crude protein, immune response or reduce H_2S gas production at these low levels.

The effect of addition of bamboo charcoal or bamboo vinegar, added as an alternative to dietary antibiotics, showed similar gains on pig performance (Chu et al. (2013 b). The addition of antibiotics is commonplace in intensive farming, but although economic benefits are gained in the form of growth promotion and improved health and welfare, indiscriminate use of antibiotics has caused a number of critical challenges, such as the build-up of antibiotic-resistant pathogens and residues of antibiotics in livestock products. These challenges have fuelled public concern regarding cross transfer of resistance to humans plus residual antibiotics in pork increasing human bacterial resistance, resulting in the ban of general antibiotic use in animal feeds. Hence, finding an alternative method of disease control is imperative. Either treatment, 0.3% bamboo charcoal (BC), or 0.3% bamboo vinegar (BV) improved daily weight gain ($P < 0.05$), DWG and feed efficiency, compared to the control diet (C) and was as effective as 0.3% antibiotic supplementation (positive control, PC). The average daily weight gain increased by 17% and 14% with the BC and BV treatments, respectively, similar to the 22% gain in the antibiotic PC. The feed efficiency significantly increased by 18% and 25% with the BC and BV treatments, respectively, comparable to the 27% gain using antibiotics. The final body weight also significantly increased, by 7% and 5% with the BC and BV treatments, respectively, while the antibiotic control increased by 7%. It was evident that the addition of bamboo

biochar enhanced the overall growth of beneficial bacteria (such as lactic acid bacteria) and reduced the growth of harmful bacteria (such as coliform bacteria and *Salmonella* spp.), levels that were comparable to the control group receiving the antibiotic supplements, but were lower compared to the control group.

As before, there were no significant changes to total protein, cholesterol (total, HDL or LDL). However, levels of LDH, BUN and cortisol in the blood were all significantly lowered, compared to the control and were similar to levels in fed antibiotics. There were no changes in IgM levels, whereas the levels of IgA and IgG in the blood of pigs were elevated, thus reducing stress in the pigs.

Faecal pH and noxious gas emission from faeces also improved overall. Ammonia, methane, amine and hydrogen sulphide levels were significantly lower than the control and the faecal pH was higher. While levels of propionic, isobutyric and valeric acids remained unaltered, levels of lactic, acetic, butyric and isovaleric acids were lower in both the bamboo charcoal and the vinegar diet, compared to both the control and the antibiotic-supplemented diet.

Therefore, the use of bamboo biochar (or vinegar) is an effective and safe alternative to generalised antibiotic use for pig production, but provides protection in a different manner. Bamboo charcoal has a physical function in the gastrointestinal tract, while bamboo vinegar has a chemical function derived from various organic acids it contains (Chu et al. 2013 b).

The addition of rice husk biochar or a rice distillers yeast by-product, as a supplement to a forage based diet (consisting of ensilaged banana and taro) for pigs also showed improved growth rates and feed conversion benefits (Sivilai et al. 2018). Significant weight gain of greater than 20% occurred with either supplement, without affecting feed intake, which remained unaltered. Feed conversion also improved more than 10% over the control group. No extra benefit was gained by combining the two supplements together.

2.4.5 Use of biochar in Fish diet

Aquaculture is a more recent and specialised industry confronting some unique problems not faced in land-based livestock production. In such a water-based medium, with high stock numbers, environmental and health implications can be serious if not tightly monitored. Feed is usually in the form of tiny particles (Masser et al. 1992)

and the decomposition of these particles consumes oxygen and produces ammonia and other toxic substances, which results in poor water quality. The resulting reduced health due to fish stress can cause disease outbreaks with high fatality rates (Zhou et al. 2018). There is limited knowledge on the effect of charcoal on fish growth and performance. Various biochars have been used to mitigate such concerns and have overall improved the health of fish and eliminated these problems. However, the required biochar dose can be unique to the species of fish.

Quaiyum et al. (2014) studied the effect of feeding biochar made from bamboo, to striped catfish (*Pangasius hypophthalmus*) in an aquarium setting. Catfish are a common fish within local Bangladesh local markets, providing a cost-effective protein source for poorer people. However, waste products and feed costs are limiting factors for the growth and survival of this fish. The bamboo charcoal was added to the diet of fish at four different levels (0%, 0.5%, 1% and 2%). The bamboo charcoal demonstrated a positive effect on final weight, daily weight gain and survival rate. The weight gain increased ($P<0.05$) by 12%, 17% and 67% with the addition of 0.5%, 1% and 2% bamboo charcoal respectively, compared to the diet without biochar. The presence of biochar in the diet, regardless of the percentage added, also resulted in a significant increase (84%, 82% and 84%) in the survival rate of the fish. There was also an observed decrease in the ammonia concentration, with increasing charcoal levels. Histological observations confirmed an increase in villus height and area in intestinal segments with increasing charcoal supplementation, similar to that reported for chicken and pigs. These results indicated that bamboo biochar can be an effective feed additive to eliminate ammonia from the aquarium water and to enhance the intestinal function of fish by enhancing the villus function.

Similarly, the addition of four levels of bamboo biochar (0%, 0.5%, 1% and 2%) to the diet of striped catfish significantly improved the final weight and average daily weight gain (Rawnak et al. 2014). The final weight increased (by 30%, 47% and 31%) and the average daily weight gain also significantly improved (by 33%, 51% and 33%), with the addition of 0.5%, 1% and 2% bamboo biochar, respectively. The survival rate of *P. hypophthalmus* again increased by over 87%, even with the lowest addition of bamboo biochar, mirroring the results reported by Quaiyum *et al.* (2014). These results confirm that bamboo biochar can increase the final weight and average daily weight gain by increasing the absorption of nutrients through the cell membrane by increasing

surface area and activating intestinal microbes. Lan et al. (2012) also obtained similar results feeding rice biochar and/or charcoal (at 1% DM) to tank raised striped catfish. Growth rates increased 36% by adding biochar to the feed and by 44% with charcoal. Fish gained more flesh, with weight to length ratio increasing by 25%. Moreover, supplementary feeding with biochar also improved water quality, with lower levels of ammonia nitrogen, nitrites, phosphates and chemical oxygen demand.

Jahan *et al.* (2014) also reported that the addition of bamboo charcoal improved growth of pangasaiid catfish (*Pangasianodon hypophthalmus*) and reduced ammonia levels in the water medium. Aquaculture of this species is rapidly increasing in Bangladesh, due to the high market demand. However, the common high stocking density causes serious fish health issues primarily due to the resulting high accumulation of toxic nitrogenous wastes. Of the three treatments tested (0.5%, 1% and 2% added bamboo biochar), fish fed a diet supplemented with 1% charcoal had significant average daily weight gains and final weight gain, compared to the other diets including the control. The survival of the fish was also significantly better, as was the water quality, especially ammonia elimination.

The addition of activated charcoal in rice husk diets improved growth for juvenile mud catfish (*Clarias gariepinus*) (Lawal et al. 2016). Rice husks are a common agricultural by-product of rice milling in Nigeria. They may be an environmental problem and are rarely used in feed due to their fibre content, poor nutritive value and bulkiness and are hence a cheap source for charcoal production. When supplied at 2.5% or 5% of the biochar improved growth performance nutrient utilisation, resulting in a significant improvement in growth. The presence of biochar in the diet did not significantly alter the average feed intake. There was an improvement in the protein efficiency ratio. However, biochar supplied at higher levels (7.5% of diet) did not improve growth. Supplementation of biochar into the diet of freshwater catfish provides an effective method to remove toxins present in the feed ingredients and remove ammonia and nitrogen by absorption.

Pirarat *et al.* (2015) evaluated the influence of activated charcoal on the production of Nile tilapia (*Oreochromis niloticus*) in tanks. Tilapia are the world's most important fresh, warm water farmed fish due to their rapid growth and they are valued as a good source of protein. As such, tilapia aquaculture is an important industry in Thailand. This industry faces ongoing issues including infection, feed contamination with

harmful chemicals or toxins, improper waste product disposal and inappropriate management practices. When activated charcoal was added to the diet of a group of Tilapia at different levels (1%, 2% and 3%) compared to a control group, the addition at all levels significantly (P<0.05) affected the final body weight, weight gain and feed efficiency ratio. The final body weight increased (by 23%, 32% and 11%) and the weight gain increased (by 190%, 211% and 158%) with the addition of 1%, 2% and 3% biochar to the diet, respectively. There were also beneficial changes in intestinal morphology. Generally the higher the activated charcoal in the diet, the greater the improvement in the villus height, indicating improved gut function. The foregut and midgut villus height of the 3% and 2% activated charcoal-supplemented diet groups were similar, but were significantly higher than the control group. Overall, 2% biochar was found to be the optimum level to increase the production of Nile tilapia. The increase in growth with the addition of biochar to the diet may be due to the reduction in ammonia and nitrogen contents in the aquarium water, which is a well-documented function of activated charcoal, although this was not measured. Furthermore, the enhancement in intestinal function was probably due to the microbes in the digestive system; consequently, increasing the utilization and absorption of nutrients across the cell membrane (Mekbungwan et al. 2004; Mui et al. 2006; Thu et al. 2010a) .

Michael *et al.* (2017) examined the effect of the addition of different levels of commercial wood charcoal to the diet of a related species of fresh water red tilapias commonly grown in Egypt (*O. mossambicus* x *O. niloticus*). The results revealed that the commercial wood charcoal had several benefits. It significantly increased weight gain by 8% and 12% with the addition of 3% and 4% wood charcoal, respectively compared to the control. The protein efficiency also increased, (by 24% and 25%) and the feed conversion ratio was enhanced (by 18% and 20%) with the addition of 3% and 4% wood charcoal, respectively.

Salt water fish species also benefit from the addition of biochar. Thu *et al.* (2009) investigated the effect of bamboo charcoal (BC) in the basal diet of (at 0.0004%, 0.01%, 0.1%, 1% and 4%) tiger pufferfish (*Takifugu rubripes*) raised in tanks. Pufferfish is a Japanese delicacy (known as fugu). This important finfish species is grown in both floating coastal seas cages (with the consequential disadvantage of nitrogenous waste into the coastal environment) or a closed recirculating system, which removes the environmental issue but prevents the optimum growth rate due to

poor water quality caused by ammonia excretion. The results revealed that the highest dose of bamboo charcoal (4%) resulted in the best improvements, with significant increases in feed intake, weight gain, feed efficiency ratio and protein efficiency ratio by 13%, 22%, 12% and 13%, respectively. Fish fed a 4% BC diet showed a significantly lower ammonia excretion rate than those fed 0.004% BC or control diet. Moreover, nitrogen retention was also significantly higher than control and other BC diet groups. In contrast to other papers, the addition of BC had no effect on survival, which was 100% for all groups.

Thu et al. (2010a) also found that comparable improvements were gained for Japanese saltwater Flounder (*Paralichthys olivaceus*), with a similar high market value. This flounder is commonly grown in a water recirculating system but faces serious health issues. The addition of bamboo charcoal at all levels (0.25%, 0.5%, 1%, 2% and 4%) to the diet of juvenile Japanese flounder (*Paralichthys olivaceus*) significantly improved weight gain, feed efficiency ratio and protein efficiency, compared to the control. In contrast to striped catfish (where 4% BC was the most effective), a much lower dose of 0.5% BC was more suited to give maximum growth performance. BC at 0.5% increased weight gain, feed efficiency ratio and protein efficiency by 18%, 13% and 8%, respectively. Higher doses of BC lead to lower gains in all measured parameters. In addition, all diets containing any BC significantly decreased the levels of ammonia excreted into the water compared to the control diet. At the same time, nitrogen retention was significantly higher in all BC diets, compared to the control diet. Hence the addition of charcoal effectively addressed a number of serious fish health problems that are commonly associated with flounder aquaculture, due to the high accumulation of nitrogenous waste products.

Recently, Mabe *et al.* (2018) studied the effect of feeding juvenile common carp (*Cyprinus carpio* L.) dietary bamboo charcoal (BC) on growth performance and health. This species of carp is widespread in China where it accounts for over 10% of freshwater aquaculture production. Although it has a rapid growth rate and a high tolerance to environmental and stocking rates, the carp are prone to disease outbreaks, resulting in economic losses. The BC was added to the diet at increasing levels (0.5%, 1%, 2% and 4%). In contrast to other reports, the added BC exhibited no significant improvements in the growth performance of juvenile carp. However, the addition of 4% BC to the diet of juveniles significantly increased the activity of alanine

aminotransferase (ALT) and aspartate aminotransferase (AST) and the content of total protein (TP), triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL) and glucose (GLU) in the serum, all of which indicate healthy liver functions and impact the fish's overall health and immunity. Improvements were also observed in the intestinal villus length and goblet cell numbers at 4% supplementation. These results proved that the addition of 4% BC significantly increased the health of juvenile carp. They concluded that BC could be used as a feed additive because it has no adverse effects on growth or the gastrointestinal tract but enhanced other health parameters.

Comparing all literature reviewed, we noted that overall biochar significantly improves the production and wellbeing of fish raised in intensive aquaculture farms, similar to the benefits of feeding biochar to ruminants or other monogastric animals. However, the amount of required biochar supplementation seems to be species specific. The additional surface area provided by charcoal or rice biochar are likely to provide additional habitat for biofilm attachment and increase the rate of solubilisation of solid feed materials through more efficient microbiota in the digestive tract of the fish. Hence the role of the microbiota in the digestive tracts of fish appears to be similar to those described for humans and animals in general although research needs to be completed to prove this (Romero et al. 2014).

It is also noted that most reports involve small holder fish farming from small or developing countries with little published information about the possible benefits to large sea-based commercial fishing from Japan. Aquaculture within Australia alone is a billion-dollar industry. Over forty freshwater, brackish and marine species are commercially produced in Australia, including such high value species as pearl, salmonids, freshwater finfish, prawns, tuna and oysters. Maintaining a sustainable aquaculture practice is vital to ensuring its survival and growth. Aquaculture operations, particularly those that operate in, or discharge into, public waters, are required to comply with stringent environmental controls monitored on an ongoing basis by state agencies. While Australia's National Strategic Plan for Aquatic Animal Health (AQUAPLAN) aims to improve biosecurity, emergency disease preparedness, surveillance, treatment and education, there have been some serious disease outbreaks and much public debate about such mass scale farming (Wang et al. 2015). It would be beneficial to clarify how the addition of biochar to feed would improve the

performance and health of fish in this industry and help to meet the above aims and concerns.

2.5 Chapter summary

In brief, this chapter summarises the application of biochar in representative monogastric and ruminant farming systems. It supports the premise that feeding monogastric and aquatic animal's dietary and ruminant charcoal or biochar has numerous benefits. The studies showed positive effects of biochar on parameters such as feed efficiency, feed intake, digestion, weight gain and growth performance (Boonanuntanasarn et al. 2014; Quaiyum et al. 2014; Ma et al. 2015). In addition, biochar increased microbial habitat, enhanced microbial growth efficiency, improved blood composition, essential amino acid content, improved intestinal villi function, enhanced beneficial bacterial population (such as lactic acid bacteria) and reduced harmful bacterial populations (such as coliform bacteria and *Salmonella* spp.). This is achieved by enhanced intestinal cell membrane function and increased beneficial microbe community by reduced surface tension, thus improving the intestinal villus height and increasing nutrient absorption and utilization (Thu et al. 2010b).

However, the opportunity exists to further understand the influence of biochar on such livestock and the interaction between biochar and microbes in the digestive system. We believe that further studies could reveal significant untapped opportunities for commercial dairy cattle farming, thus helping to reduce feed cost in the future, improve the well-being of animals and address ongoing environmental concerns around emissions. Thus, in the next chapter, a new study is conducted to show the effect of biochar on a cattle pellet diet. This study is also compared with several existing studies in prior literature.

CHAPTER 3

In-vitro Laboratory experiments

Summary: In the following series of experimental work an invitro approach using a so-called gas production system, initially adapted from ((Menke 1988; Blu et al. 1993).

3.1 Dairy Cattle Pellet Diet

Investigations to reduce methane (CH_4) emissions from beef cattle have two primary goals. The first is to reduce the negative environmental impact on global warming; the second is to reduce ingested diet energy loss in the animals (Moumen et al. 2016; Preston et al. 2017; Tan et al. 2017). Researchers have previously tried different strategies to reduce CH_4 emissions from ruminants by using feed additives such as tannins (Grainger et al. 2011; Deighton et al. 2014; Hristov et al. 2015; Taylor et al. 2017) and biochar to improve microbial growth efficiency (Hansen et al. 2012; Leng et al. 2012a; McFarlane et al. 2017). Other researchers have shown a beneficial change in fertility and foetal growth (Hagemann et al. 2018; Diatta et al. 2020).

Biochar has long been reported as having beneficial effects on the digestive health of cattle and other livestock. Biochar is a term used for carbon compounds that are produced under different pyrolysis temperatures. It is made from a variety of feedstocks such as wood products, animal manures, agricultural residues and other organics (Jiang et al. 2020; Kroeger et al. 2021). The characteristics of biochar (degree of surface area and porosity) depend on the production procedures. The beneficial effects of biochar include: improvement of soil quality and water, enhancement of microflora in the rumen, reduction of GHG emissions and, as a result, mitigation of global warming (Diatta et al. 2020; Jien et al. 2020). Biochar addition to paddy soils significantly enhances the ratio of methanotrophic microorganisms (that consume methane) to methanogens (Kroeger et al. 2021; Sekar et al. 2021).

Various in-vitro animal studies have also shown benefits. When wood-based and straw-based biochars were added to Jersey heifer rumen fluid as feed dry matter (DM) at a concentration of 9% CH_4 emissions were decreased by 11%–17% compared with those of the control because the habitat and growth of microbes were affected by biochar porosity (Hansen et al. 2012). Leng, Preston, et al (2012) established that

adding 1 % of biochar into cattle rumen fluid caused a significant reduction in methane production 11% and 13%, however; there were no further benefits from increasing the biochar level to between 2% and 5%.

The efficacy of biochar has also been shown with live animals. Preston et al. (1987) found that biochar had many benefits on animal production, such as increasing efficiency of feed conversion, increasing microbial habitat, enhancing microbial growth efficiency and improving essential amino acids. Leng et al. (2012) studied the effect of 6% rice husk biochar on growth and feed conversion in local yellow cattle compared to the basal diet. The average weight gain significantly increased by 14% with the addition of 6% biochar while feed intake remained unaffected. This study also demonstrated that biochar increased microbial habitat, enhanced microbial growth efficiency and improved essential amino acid contents. These results can be extended to other species of cattle (Leng et al. 2012). However, because biochar can be produced from different food sources, the effect on a specific source as a feed additive for livestock requires more study before its economic application. However, there is still a lack of investigation into the efficacy of various PACs on the mitigation of GHG emissions and how it modifies rumen fermentation in dairy cattle. Therefore, the objective of this research was to determine the effects of a specific coconut powdered activated carbon (PAC); on reduction of GHG emissions and enhancing rumen fermentation in dairy cattle.

3.2 Materials and methods

3.2.1 Powdered activated carbon (PAC)

In this study, PAC or Biochar was purchased from Activated Carbon Technologies Pty Ltd, Victoria, Australia. This PAC had enhanced properties over standard biochar granules, such as a high adsorption capacity and fine particle size ensuring the most efficient contact time and adsorption profile. Table 3.1 shows the properties of PAC, Figure 3.1c shows an SEM image of the typical surface profile of PAC.

Table 3.1: Properties of PAC

Property	Value
Pyrolysis temperature	1000 °C
Surface area	1000 m^2/g
Total pore	0.35 mL/g
Density	0.35-0.45 g/mL
Moisture and Ash	5%
Iodine	1000 m^2/g
Particle size	100sh

3.2.2 Rumen liquid collection

An *in-vitro* technique was used to estimate the effects of PAC additions on GHG emissions and feed digestibility. All researchers received a Q-fever vaccination to avoid bacterial infection caused by *Coxiella burnetii*. The rumen liquid samples were collected from an abattoir rather than from fistulated cattle, to avoid ethical concerns. Samples were sourced from a local meat processor at Oakey Beef, Queensland, Australia. Two types of rumen liquid samples were collected i.e. either grain or grass-fed dairy cattle. Three of each cattle type had their rumens sampled, by placing on a table, the paunch membrane material sliced open and rumen liquid promptly collected. The rumen contents were gently squeezed through cheesecloth to eliminate feed particles and approximately 1000 ml of extracted rumen liquid was collected in sterile containers using two layers of cheesecloth sealed and placed into an insulated container at 39°C, suitable for the growth of microbes (Chaudhry 2008). Figure 3.1 shows the surface area of the rumen wall: (a) grain fed cattle; (b) grass-fed cattle.

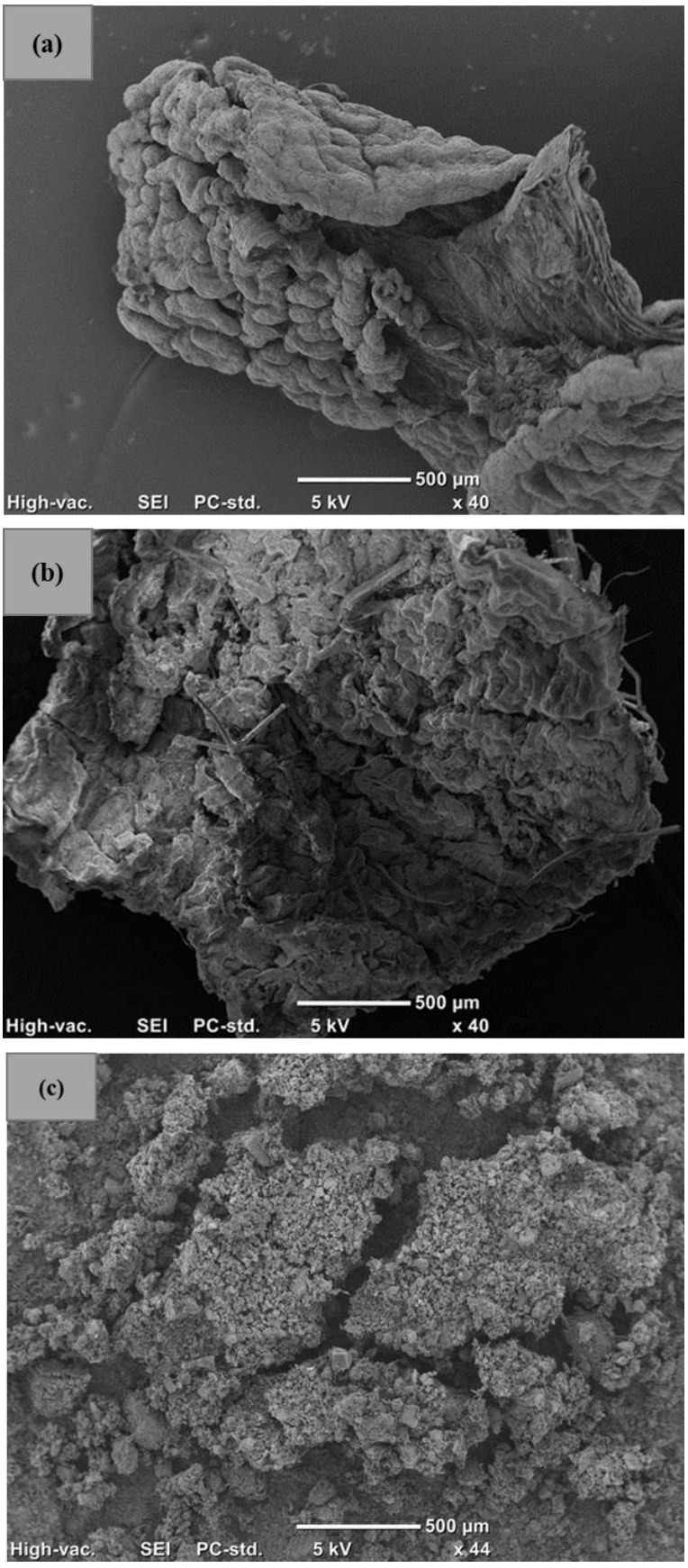


Figure 3.1: Rumen wall: (a) Grain fed, (b) grass-fed, (c) PAC surface

3.2.2 Buffer solution preparation

Maintaining the pH of the rumen plays an important role in enhancing the rumen fermentation efficiency because it affects the persistence and stability of the microflora in the rumen. The typical rumen pH range is between 5.5 to 7.5 but the pH is affected by feeding frequency and type of diet (Franzolin et al. 2010). The in-vitro experiments have used a buffer solution to maintain the ideal pH (6.8) for microflora and enzymes (Qadis et al. 2014). The materials for preparing the rumen buffer solution are Sodium bicarbonate ($NaHCO_3$) and Ammonium bicarbonate (NH_4HCO_3). In this experiment, the following procedures were followed: two litre bottles were used to make buffer solution in two steps. $NaHCO_3$ (70g) was added to 1 litre of water, bottled in two litre capacity bottles. NH_4HCO_3 (8g) was then added into 1 litre of water that in a nominal one litre capacity bottle. $NaHCO_3$ and NH_4HCO_3 solutions were then left to dissolve fully overnight. NH_4HCO_3 (500 ml) and $NaHCO_3$ (1000 ml) were mixed to make the volume up to two litres with water. The pH was around 8-8.2 at this stage, with the buffer mineral solution then flushed with CO_2 to reduce the pH to around 7.2.

3.2.3 In-vitro incubation and sampling procedure

In this experiment, three concentrations of PAC: 0%, 0.5% and 2% were added to the diet of dairy cattle. During the experiments, each concentration of PAC was added separately with three replicates into this diet at 0.6g dry matter (DM). In this paper, the effect of PAC on GHG emissions and rumen fermentation was tested. This study included diet pellets fed as shown in Table 3.2. The pellets were commercially made by Ridley Agriproducts pty ltd at their Toowoomba Queensland plant.

Table 3.2: Commercial Dairy cattle pellet analysis

Ingredient	As fed	Dry matter at 89%
Total Crude Protein (minimum)	16.0 %	18.0 %
Crude Protein (minimum)	13.1 %	14.7 %
Equivalent Crude Protein (maximum)	2.9 %	3.2 %
Urea (maximum)	1.0 %	1.1 %
Crude Fibre (maximum)	12.0 %	13.5 %
Crude Fat (minimum)	1.5 %	1.7 %
Calcium (minimum)	1.0 %	1.1 %
Phosphorus (minimum)	0.5 %	0.56 %
Magnesium (minimum)	0.4 %	0.45 %
Salt (maximum added)	0.5 %	0.56 %
Copper (added)	45 mg/kg	50 mg/kg
Zinc (added)	150 mg/kg	170 mg/kg

3.2.4 Measurement of GHG emissions

The buffered mineral solution and rumen liquid were mixed into beaker (500ml) as 25% of rumen liquid and 75% of buffer solution. Then, 35 ml of the rumen solution was added to 0.6 g of the pellet diet of dairy cattle for each treatment, with different percentages (0%, 0.5% and 2%)/ DM of PAC. Rumen liquids that were accessed from grain and grass-fed dairy cattle were used in this trial. The resulting solution was kept in the injectors of the gas production system to determine 6 hours of gas production during the rumen fermentation (DePeters et al. 2003). Data were recorded manually for each hour. The positional change of the pistons in these injectors was detected as the scale changing of volume. The detected volume changes were used to quantify the gas emissions from the rumen liquid with different PACs.

3.2.5 Methane (CH_4) emissions and Chemical analysis

In this section, the measurements of CH_4 were firstly explained and then the chemical analysis was discussed. All the measurements of CH_4 were tested according to the method described by Demeyer et al. (1988). In this study, the buffered mineral solution and rumen liquid were mixed in the backer lab as 25% of rumen liquid and 75% of buffer solution. Then, three additions of 35 ml of the rumen solution were added to 0.6g of the pellet diet for each treatment, with different percentages of PAC: 0%, 0.5% and 2% /DM and two types of rumen liquids: grain and grass liquids. The samples were kept in glass serum vials (100ml) that were closed with rubber stoppers and crimped. They were placed into the shaking water bath at 39˚C for 48h. A 500 Microlitre (uL) gas sample was taken from each glass serum vial and injected into the multi-gas analyser (Gas Chromatograph (GC), Kanagawa, Japan, Shimadzu Corporation (www.shimadzu.com/an/) and GC-2014).

Regarding chemical analyses, the volatile fatty acids (acetic, propionic and butyric acids) along with feed digestibility measurements were carried out. Firstly at the end of the incubation trial, all the volatile fatty acids (VFAs) were measured (by Chromatograph, Kanagawa, Japan, Shimadzu Corporation (www.shimadzu.com/an/) 2014), based on the method illustrated by Wuyi (2006). Finally, the contents of the treatments were centrifuged at 3000r/min and 4°C for 20 minutes. The centrifuged samples were dried in a forced-air oven at 55˚C for 72 hours. The dried samples were sent to a laboratory at the University of Queensland (UQ) to determine and measure the digestive feed components (organic matter digestibility (OM), neutral detergent fibre digestibility (NDF), acid detergent fibre digestibility (ADF), acid detergent lignin digestibility (ADL), cellulose digestibility and hemicellulose digestibility) as described by Redondo-Cuenca et al. (2008). Figure 3.2 (a-f) shows the equipment of laboratory.

Figure 3.2: *In-vitro* experiment: (a) 5-digit balance, (b) multi-gas analyser (Gas Chromatograph (GC), (b) Shaking water bath, (d) fibre digestibility analyser, (e) lignin digestibility analyser and (f) spectrophotometer.

3.2.6 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS-version 23) software was used to analyse the experimental data that was distributed normally (Swan 1995) and involved the analysis of variance (ANOVA). Means of cone index were compared for significance using the least significant differences approach (Duncan) at (P=0.05) level of probability, for PAC, GHG emissions and feed type (grain and grass-fed) data. Orthogonal polynomial contrasts were applied to examine the linear and quadratic responses to PAC and feed types (grain and grass-fed) and compare treatments to the control. Significance was shown at $P \leq 0.05$ and a trend at $0.05 < P < 0.10$ unless otherwise stated.

3.3 Results of PAC addition on Emissions (grain and grass-fed diet)

This research has presented the effect of three factors: time, PAC concentration and feed type on total GHG and CH_4 emissions from dairy cattle.

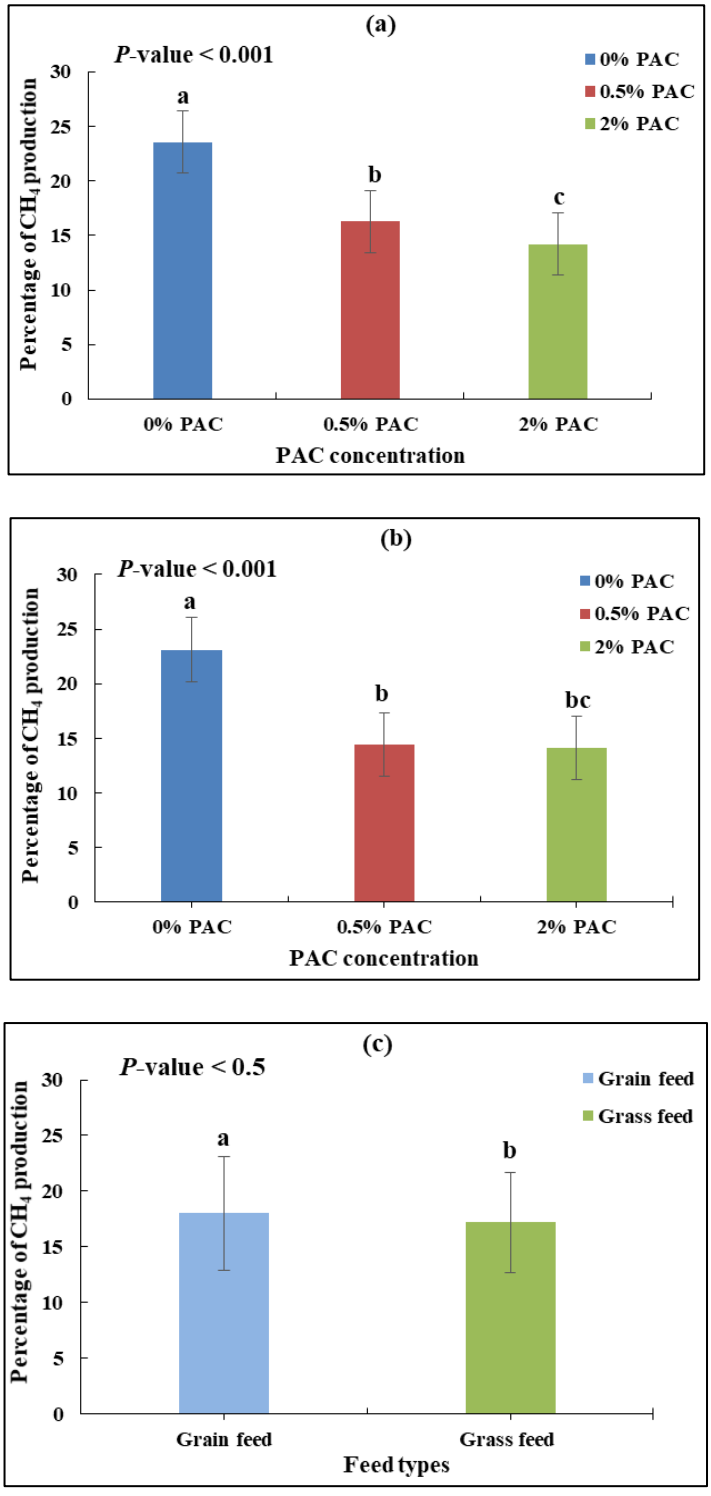


Figure 3.3: Effect of PAC on CH_4 emissions for: (a) Grain fed and (b) grass-fed (Mean ±SD).

Figure 3.3 shows the effect of PAC addition at various concentrations on methane (CH_4), emissions from grain and grass-fed dairy cattle. In Figure (3.3a), the percentages of CH_4 emissions were 23.56%, 16.28% and 14.19% when the PAC quantity were added at 0%, 0.5% and 2% respectively to grain fed cattle. Consequently, the reductions of CH_4 were 69% and 60% at 0.5% and 2% of PAC respectively. In the grass-fed case, Figure (3.3b) shows the percentage of CH_4 emissions as 23.10%, 14.43% and 14.13% when the PAC quantity added was 0%, 0.5% and 2% respectively. The respective reduction in CH_4 emitted was 62% and 61% at 0.5% and 2% of PAC addition. Thus, the behaviour of the CH_4 emissions was similar in both types of dairy diet i.e. grain and grass-fed as shown in Figure (3.3c).

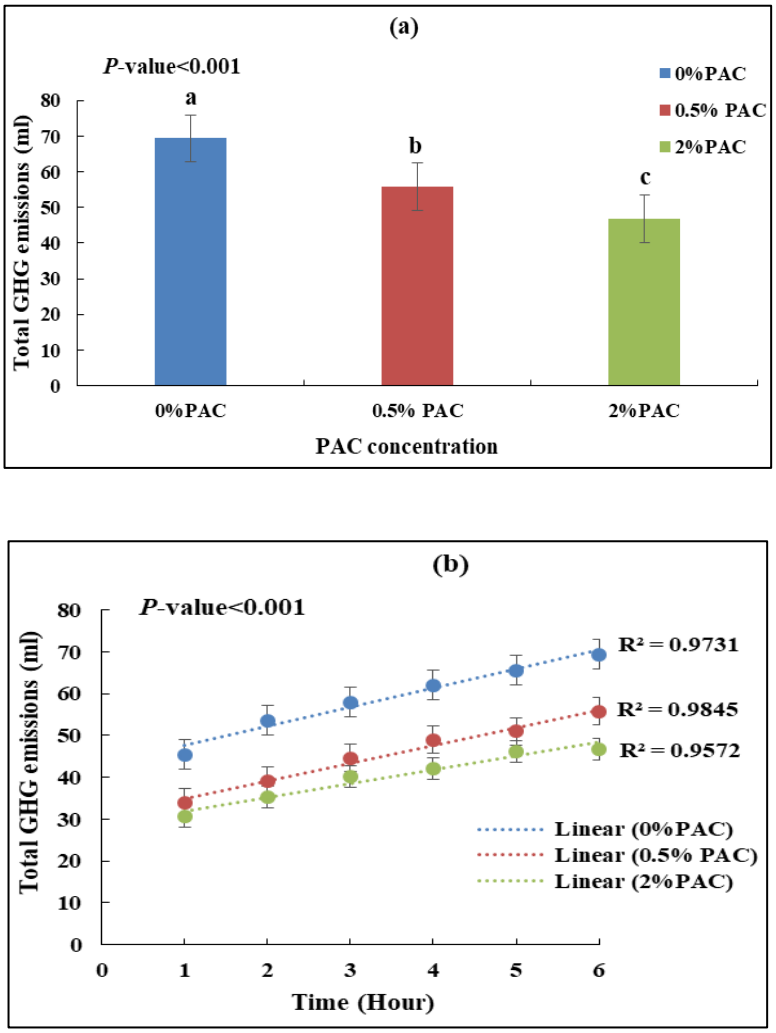


Figure 3.4: Effect of PAC addition on (a) Total GHG emissions and (b) Cumulative GHG emissions at hourly intervals over a six hr period for a grain fed dairy cattle diet.

Figure 3.4 shows the effect of PAC concentrations on cumulative GHG emissions and reduction from the dairy cattle (grain fed). In Figure (3.4a), the Total GHG emissions with no PAC was the highest amount as a comparison with the total GHG emissions at 0.5% and 2% of PAC concentrations (grain feed). The total GHG emissions were 69.4%, 55.8% and 46.8% at 0%, 0.5% and 2% of PAC respectively.

The amount of the total GHG emissions per hour was from (45.4 ml to 69.4 ml) for 0% of PAC and between (34 ml and 55 ml) and (30.6 ml to 46.8 ml) for 0.5% and 2% of PAC respectively, over hourly intervals up to six hours. Consequently, the reductions in the total GHG at 0.5% and 2% of PAC were 14% and 23% respectively, as shown in Figure (3.4b). For further evaluation of the proposed study, the correlation of determination (R^2) was used to show the relationship between GHG emissions and time for all PAC concentrations as shown in Figure 4(b). Based on the obtained results in Figure 3.4b, it can be seen that there is an agreement between GHG emissions and time. The outcomes were R^2 = 0.97, 0.98 and 0.95 for 0%, 0.5% and 2% of PAC respectively. These results confirm that the PAC had a higher impact on GHG emission in grain fed. The proposed study provided better results compared with other results in the literature.

Figure 3.5(a) clearly indicates that for grass-fed cattle, the total GHG emissions have significantly dropped due to the addition of PAC. These were 68%, 48% and 43.8% at 0%, 0.5% and 2% PAC respectively, as shown in Figure 3.5(a). The cumulative GHG emissions were from (44.8 ml to 68.4 ml), (32.6 ml to 48 ml) and (27.8 ml to 43.8 ml) for 0%, 0.5% and 2% of PAC respectively, over hourly intervals up to six hours. Furthermore, Figure (3.5b) shows that the reductions of the total GHG emissions in the grass-fed cattle were 20% and 25% at 0.5% and 2% of PAC respectively. For further evaluation, correlation coefficient (R^2) was used to show the relationship between GHG emissions and time for all PAC concentrations as shown in Figure (3.5b). Based on the obtained results displayed in Figure (3.5b), it can be seen that there is a linear agreement between GHG emissions and time. The outcomes were R^2 = 0.99, 0.98 and 0.99 for 0%, 0.5% and 2% of PAC respectively.

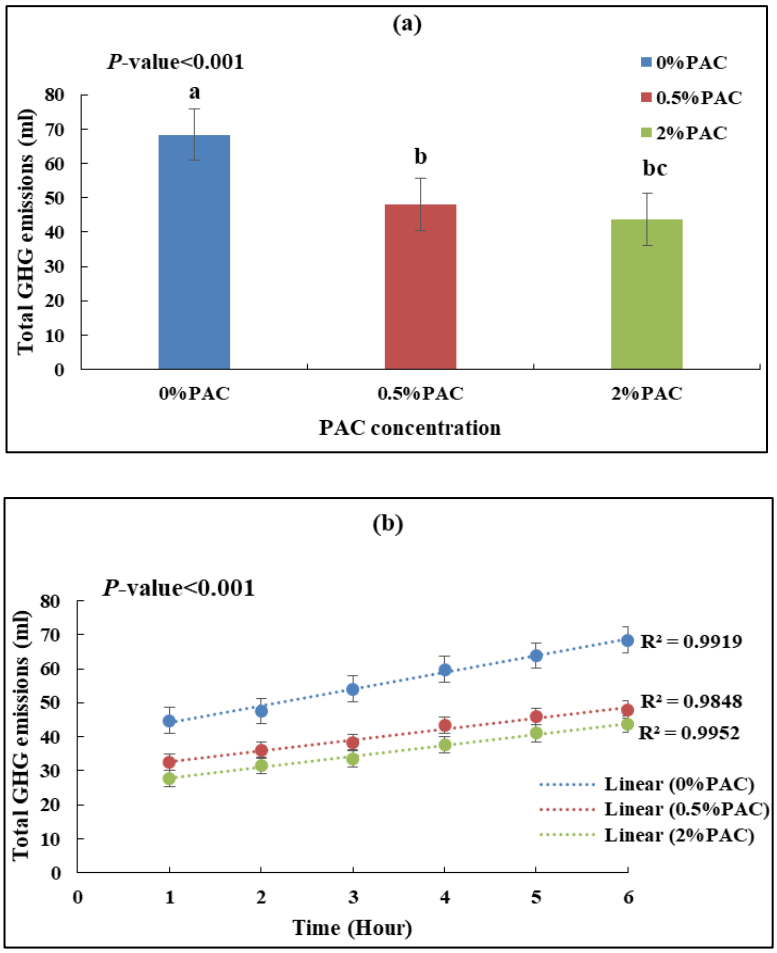


Figure 3.5: Effect of PAC addition on (a) Total GHG emissions and (b) Cumulative GHG emissions at hourly intervals over a six hr period for a grass-fed dairy cattle diet.

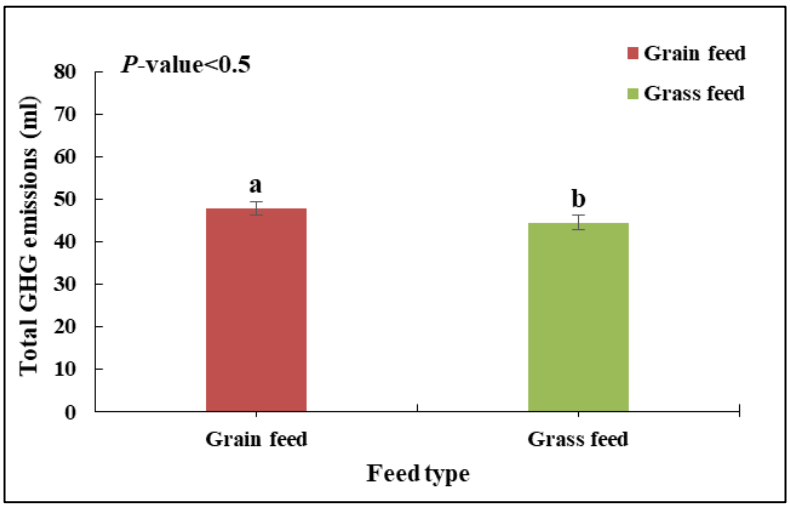


Figure 3.6: Effect of feed types (grain and grass-fed) on total GHG emissions (Mean ±SD).

These results also confirm that as expected due to the lower inherent feed value, PAC had a higher impact on GHG emissions in grass-fed cattle. This study provided greater results when compared with other results in literature. Indicating the variability of the total GHG emissions to have a similar behaviour in both types of dairy cattle (grain and grass-fed) Figure 3.6. As a result, as shown in Figures 3.4(a-c) and 3.5(a and b), the PAC may have a positive effect on dairy products such as milk (fat and protein in milk).

3.4 Results of grain and grass-fed production pre-cursors

Figures 3.7(a-f) show the relationship between the added PAC concentrations and productivity precursors for both grain and grass-fed dairy cattle. Figure 3.7 (a), (b) and (c) represent the effect of PAC on acetate, propionate and butyrate respectively, as shown for grain fed cattle. Figure 3.7 (d), (e) and (f) represents the effect of PAC on acetate, propionate and butyrate respectively for a grass-fed cattle diet. Overall, according to Figure 3.7 (a-f), PAC has a small positive influence on the acetate, propionate and butyrate acids.

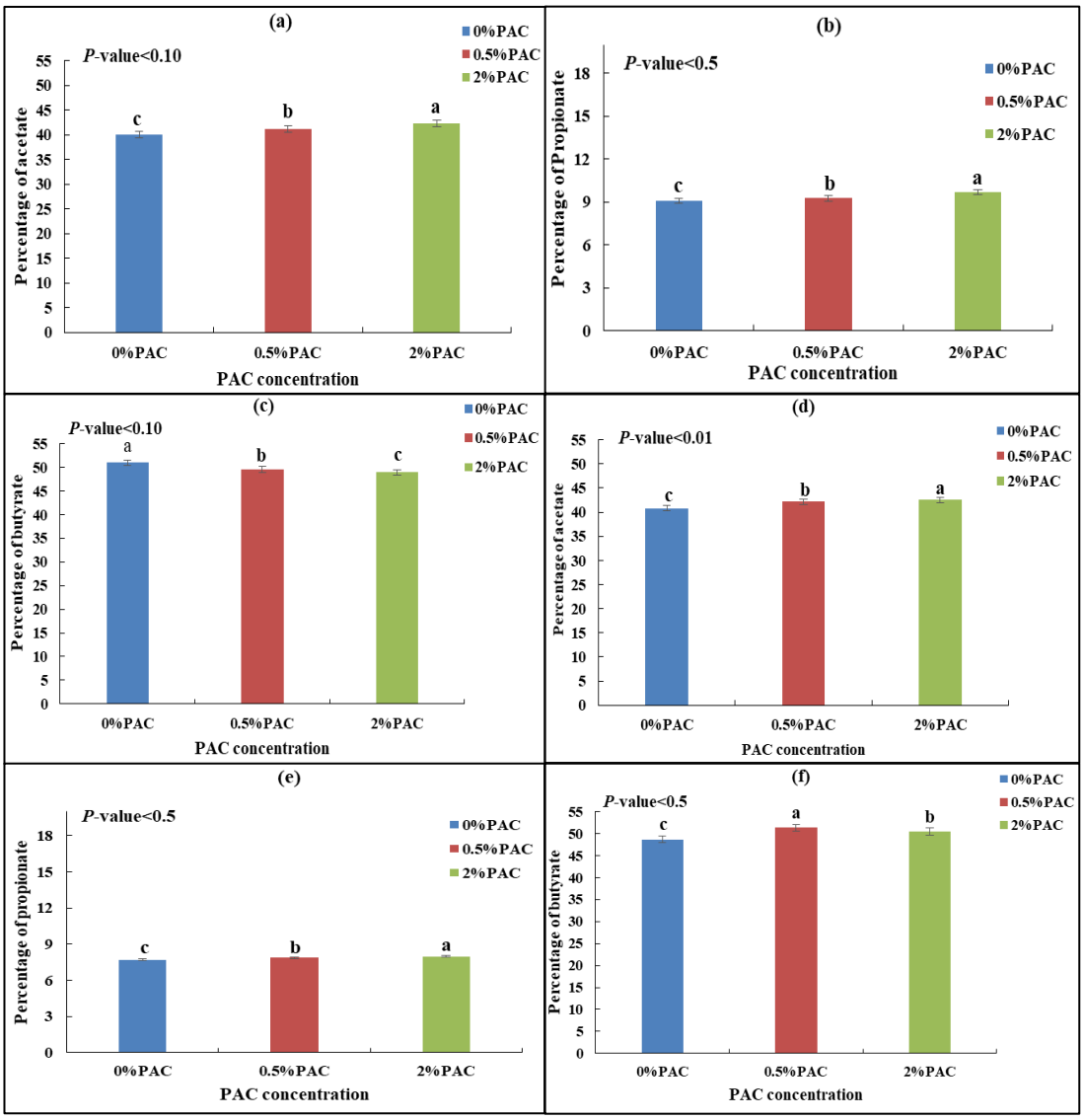


Figure 3.7: Effect of PAC on acetate, propionate and butyrate in the dairy cattle: (a), (b) and (c): grain fed, (d), (e) and (f) grass-fed, (Mean ±SD).

Table 3.3 shows the effect of PAC concentrations on the digestibility parameters regardless of the feed types. The Dry Matter (DM) % did not differ between control and 2% PAC. However, there is a significant (P<0.001) increase in the DM around 3% at 0.5% PAC compared to control and 2% PAC. Organic matter (OM) data are reported in Table 3.3. A quadratic increase (P= 0.01) was observed for OM with the 2% PAC treatment having the greatest OM (91.98%). Similarly, DM tended (P=0.03) to increase quadratically. A linear decrease (P=0.06) was observed for NDF, with no PAC having the lowest digestibility (27.42%). However, neutral detergent fibre (NDF) increased significantly (P<0.001), with 0.5% and 2% PAC inclusion of 38.03% and 39.33% respectively. A tendency was observed for a quadratic increase in NDF (P=0.05). A linear and quadratic (P=0.01) increase in acid detergent fibre (ADF) and acid detergent lignin (ADL) digestibility was observed with the inclusion of 0.5% and 2% PAC additions of diet DM. Cellulose digestibility (%) was significantly increased (P<0.1; Table 3.3) by adding 2% PAC. A quadratic (P= 0.43) increase in cellulose digestibility was observed with the inclusion of 2% PAC. Hemicellulose digestibility was increased significantly (P<0.001) by adding 0.5% and 2% of PAC. Hemicellulose digestibility had a quadratic (P=0.06) increase with 0.55 and 2% in PAC treatments as shown in Table 3.3.

Table 3.3: Effects of Feed type in a dairy diet on digestibility (*in-vitro* experiment).

	PAC inclusion % /DM			*P*-values[1]		
Item (%)	**0**	**0.5**	**2**	**SEM**	**Lin**	**Quad**
DM	26.3	23.38	26.48	0.56	0.34	0.03
OM	91.43	90.42	91.98	0.21	0.05	0.01
NDF	27.42	38.03	39.33	2.42	0.06	0.05
ADF	16.7	16.63	19.92	0.53	0.01	0.01
ADL	5.55	6.28	8.65	0.34	0.001	0.001
Cellulose	11.15	10.34	11.24	0.35	0.63	0.43
Hemicellulose	10.72	21.42	19.45	2.34	0.21	0.06

[1]Linear and quadratic orthogonal polynomial contrasts, Dry matter (DM), Organic matter (OM), Neutral detergent fibre (NDF), acid detergent fibre (ADF) and Acid detergent lignin (ADL).

Table 3.4: Effect of PAC additions on diet components of dairy cattle.

Item (%)	**Type of feed**	***P*-valuses[1]**			**Type of feed**	***P*-values[1]**		
	Grain feed	**SEM**	**Lin**	**Quad**	**Grass feed**	**SEM**	**Lin**	**Quad**
DM	24.61	0.75	0.34	0.35	26.18	1.10	0.66	0.001
OM	91.23	0.23	1.00	0.29	91.32	0.44	0.25	0.001
NDF	41.03	4.13	0.001	0.001	28.82	0.86	0.18	0.25
ADF	18.18	0.09	0.02	0.001	17.33	0.28	0.35	0.06
ADL	6.6	0.06	0.02	0.001	7.06	0.36	0.01	0.01
Cellulose	11.55	0.37	0.03	0.002	10.27	0.25	0.008	0.04
Hemicellulose	22.89	2.6	0.02	0.001	11.5	0.66	0.18	0.37

[1]Linear and quadratic orthogonal polynomial contrasts, Dry matter (DM), Organic matter (OM), Neutral detergent fibre (NDF), acid detergent fibre (ADF) and Acid detergent lignin (ADL).

3.5 Discussion of PAC effects on grain and grass-fed diets

Two types of dairy cattle rumen liquids (grain and grass-fed) and three concentrations of PAC (0% - 2%) were used in this study. Our results showed that PAC can strongly reduce CH_4 and total GHG emissions in both types of feed (grain and grass-fed). It is worth mentioning that PAC reduced overall GHG and CH_4 emissions, which is consistent with previous studies where other PAC varieties reduced GHG emissions arising from ruminal microbial fermentation *in-vitro* (Hansen et al. 2012; Leng et al. 2012; Vongkhamchanh et al. 2015; Preston et al. 2017; Saleem et al. 2018). Saleem et al. (2018) suggested that PAC could have the potential to alter ruminal microflora and decrease GHG emissions. Saleem et al. (2018) examined three different (Jack pine) PAC concentrations (at 0.5%, 1.0% and 2.0% of diet/ DM) and observed that CH_4 was significantly decreased by 24% at 0.5% of PAC compared to a high-forage diet. The addition of PAC at 1% to a cassava root meal and urea substrate also decreased methane emissions by 11%-13% (Hansen et al. 2012). A net reduction in methane production could have been anticipated because of the relatively high specific surface area of PAC that was produced at 550°C (Leng et al. 2012a). Lehmann et al. (2009) said that the supportive capacity of PAC can be affected by very high temperatures (900°C) and the type of biomass used to make PAC. In addition, the differences in responses of PAC among studies is due to variation in chemical composition, biomass feedstocks, or size and porosity of the pyrolysis of each PAC product, which may change the fermentation process in the rumen in varying degrees (Hansen et al. 2012; McFarlane et al. 2017).

Our *in-vitro* results also showed that PAC had slightly improved the percentage of acetate, propionate and butyrate acid in the rumen of grain and grass-fed cattle. These results differed when compared with the previous study (McFarlane et al. 2017) which showed that acetate, propionate and butyrate were not influenced by PAC (wood chips) concentrations in rumination. However, there is a lack of *in-vitro* research about the effect of different varieties of PAC on VFAs when fed to dairy cattle.

Reports on the effects of PAC on forage digestion are contradictory. In our *in-vitro* study, PAC slightly enhanced diet components (DM, OM, NDF, ADF, ADF, ADL, Cellulose and Hemicellulose). The PAC used in this study and by Leng et al. (2012a) could involve circumstances that enhanced the digestibility efficiencies of rumen microflora while other tested PAC varieties did not enhance the digestibility of feed

in the rumen used by (Hansen et al. 2012; McFarlane et al. 2017). Al Kindi (2015b) found that adding activated charcoal to the diet of goats significantly increased (P<0.001) the faecal concentrations of NDF and ADF. The authors attributed the digestibility improvements to the ability of PAC to increase the surface area in the rumen in a way that could make a change in the community of microflora in the rumen, resulting in more effective digestion. It can be concluded that there are still limited data available on the effects of PAC additions on the digestibility parameters of feed in dairy cattle.

3.6 Chapter summary

In this chapter 3, our findings indicate that adding PAC to either (grain or grass fed) rumen fluid from dairy cattle significantly reduced the GHG and CH_4, and slightly improved the digestibility of feed. However, the CPAC did not affect production or ratio of VFAs. The PAC could be playing a role in altering the microflora community in the rumen by increasing its surface area. Our results mirror other research, with PAC showing a capacity for reducing GHG emissions and significantly improving rumen fermentation of feed. Some limitations, such as the mechanism of PAC in the rumen also requires further research to provide a deeper understanding. This ability should now be tested to determine if incorporating PAC as a feed additive in different forage diets results in comparable benefits in an *in-vivo* on farm situation; the aim of our future work.

CHAPTER 4

4.1 Forage Diet Background

The Food and Agriculture Organization of the United Nations reported that methane (CH_4) mitigation strategies have significant environmental, food security and economic importance (Gerber et al. 2013). The digestion of low quality grass feed in the rumen released CH_4 emissions by ruminal methanogens (Deppenmeier 2002; Broucek 2014). The livestock produced 40% of greenhouse gas (GHG) emissions globally, the major GHG emissions produced by cattle which is produced 77% (Gerber et al. 2013). The process of generating methane from rumen represents an estimated 2%–12% which is considered gross energy intake wastage (Van Haarlem et al. 2008; Ramin et al. 2013; Broucek 2014).

In the last few years, various attempts have been made by ruminant nutritionists using chemical feed additives: antibiotics, ethane inhibitors and defaunating agents, to reduce methane (CH_4) production, promote animal productivity and improve feed utilization (Mao et al. 2010; Patra et al. 2012; Joseph et al. 2015; Tapio et al. 2017; Ungerfeld 2018; Xiao et al. 2018; Van Wesemael et al. 2019). Although these studies obtained relatively good results, they had a side effect. Therefore, it is essential to develop new strategies to increase the efficiency of rumen metabolism, to decrease CH_4 production and improve animal performance.

In the last decade, biochar was proposed by researchers as a new feed addition to ruminant feed to improve animal performance (Leng et al. 2012). More specifically, biochar is defined as a natural substance that could be added to animal diets as a possible way to enhance rumen fermentation (Leng et al. 2012). It is produced from agricultural residues, wood waste and weeds that are slowly burned with restricted oxygen (Lehmann et al. 2011).

Hansen et al. (2012) evaluated the effect of biochar on CH_4 production from buffered rumen fluid. In that study, different types of biochar in vitro, gasified, wood-based and straw-based biochar were employed. In their study, biochar at a concentration of 9% was added to the feed DM. They reported that using biochar decreased CH_4 emissions by 11%–17% compared with the control. Another study was presented by

Leng et al. (2012), in which 0.6% of biochar was added to the feed for cattle. Based on the study, using biochar reduced methane emissions by 22% proving that the surface area of biochar can significantly increase methane oxidation and microbial growth efficiency. Preston and Leng (1987) reported that biochar has several benefits for animal production.

More recently, Mirheidari, A: et al. (2019) presented a study to show the effect of biochar on the diet of dairy ewes. In that study, two types of biochar, including walnut shell and chicken manure, were used to evaluate the biochar in *in-vitro* experiments, using different concentrations of 0.5%, 1.0% and 1.5%. The results from in vitro experiments indicated that using different levels of biochar in the diet decreased methane production and ammonia concentration and improved milk production. They suggested that using biochar with dairy ewes may increase rumen metabolism and ultimately the productivity of animals. However, Teoh et al. (2019) suggested that further studies are required to evaluate the effect of biochar on different types of forage compositions. Therefore, the objectives of this paper were to investigate the effect of powdered activated carbon (PAC) additions to different sources of forage diet, (oaten hay, forage sorghum silage, barley hay and mixed diet (forage sorghum silage and pellets)), on the reduction of GHG emissions, thus improving rumen fermentation.

4.2 Materials and methods

A robust *in-vitro* experiment was conducted to study the effects of powdered coconut biochar on GHG emissions and rumen fermentation. All the experiments were carried out in a laboratory at the University of Southern Queensland. First, the experimental design is discussed, then the measurements associated with GHG emission and rumen fermentation are explained.

4.2.1 Powdered activated carbon (PAC)

As per chapter 3, **Section 3.2.1**

4.2.2 Rumen liquid collection

As per chapter 3**, Section 3.2.2**

4.2.3 Buffer solution preparation

As per chapter 3, **Section 3.2.3**

4.2.4 *In-vitro* incubation and sampling procedure

In this experiment, three concentrations of PAC: 0%, 0.5% and 2% / DM of dairy cattle's diet were used. During the experiments, each concentration of biochar was added separately with three replicates into different dairy cow diets: oaten hay, forage sorghum silage, barley hay and mixed diet (forage sorghum silage and pellets) at 0.6g (DM). The effect of powdered activated carbon (PAC) on GHG emissions and rumen fermentation was tested. Table 4.1 shows the ingredients of forage.

Table 4.1: Proximate analysis of dairy cattle diet

Ingredients	**Feed types**		
	Oaten hay	Barley hay	Forage sorghum
Dry Matter %	95.3	96.1	96.0
Organic Matter %	90.0	88.6	90.0
Ash %	10.0	11.4	10.0
Crude Protein %	1.82	18.1	10.8
NDF %	53.7	40.7	64.8
ADF %	27.6	22.0	38.8
Lignin %	2.7	1.9	3.4

4.2.5 Measurement of GHG and CH_4 emissions

As per chapter 3, **Section 3.2.5 and 3.2.6**

4.2.6 Statistical analysis

To evaluate the performance of experimental data, *in-vitro* data were statistically analysed in this study as a completely randomized design, using SPSS-version 24 software, (ANOVA) (Swan et al. 1995), with 12 treatments and three replicates (4 types of feed samples x ferments per treatments). Means were compared using the least significant differences (Duncan) at significance level of $P \leq 0.05$. Cohen's *d* test was used to measure the effect size correlations of PAC additions to the various diets Setiawan (2019).

The equation below is used to calculate Cohen's *d* measurement:

$$(M_2 \; or \; M_3 - M_1)/SD_{pooled} \ldots\ldots\ldots\ldots\ldots\ldots 1$$

M_1: Control (no PAC)

M_2: treatment2 (0.5% PAC)

M_3: treatment3 (2% PAC)

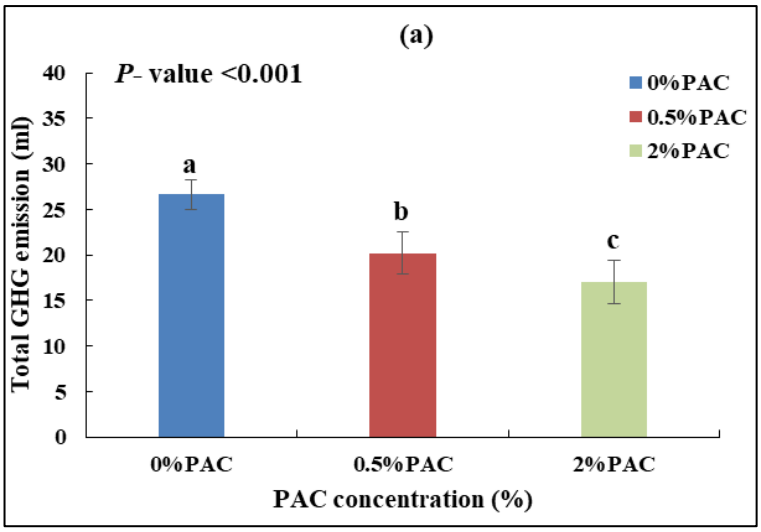
SD_s: average of standard deviations for both M_1 and M_2 or M_3

4.3 Results

4.3.1 PAC addition on a forage diet emissions

A number of tests were conducted in this study to evaluate the effect of biochar on the total gas production from oaten hay, barley hay, forage sorghum silage and mixed forage sorghum silage and pellets as well as on volatile fatty acids (VFAs) and feed fermentation. The tests used different concentrations of PAC (0%, 0.5% and 2%).

The total GHG emissions had a strong negative correlation (P<0.001) with PAC additions. Figure 4.1(a) showed the corresponding results of testing the effect of different concentrations of PAC on total GHG emissions from oaten hay. The results indicated that the lowest value of GHG of 17.05 ml registered at 2% of PAC, followed by 20.22 ml at 0.5% of PAC concentration, followed by 26.67 ml at 0% of PAC. Statistically, GHG emissions were 6.44 ml and 9.61 ml at 0.5% and 2% respectively, which had a strong negative correlation (P<0.001) with PAC additions. Moreover, Figure 4.1(b) showed the GHG emissions reduction values measured over hourly intervals up to six hours. It is worth noting that GHG emissions over a six hour interval had a strong negative correlation (P<0.001) with PAC additions Figure 4.1(b). Overall, PAC additions to the oaten hay showed a strong negative correlation with (Cohen's *d in the range of* = -1.04 to -1.54).



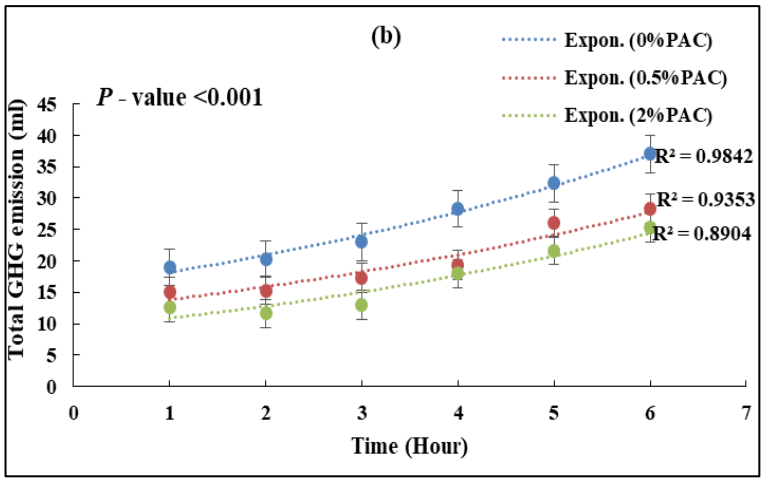


Figure 4.1: Effect of PAC on (a) Total GHG emissions and (b) Cumulative effect measured over hourly intervals for six hours for an oaten hay diet.

The effect of PAC on the barley hay was also employed in this study, as shown in Figure 4.2(a). Based on the obtained results in Figure 4.2(a), total GHG emissions had a strong negative correlation (P<0.001) with PAC additions. The total GHG emissions were 21.28 ml, 15.00 ml and 13.39 ml at 0%, 0.5% and 2% of PAC, respectively. Consequently, the reductions in the total GHG emissions at 2% of PAC were the greatest as a comparison with 0% and 0.5% of PAC. For further evaluation, the effect of PAC concentrations on total GHG emissions was also measured in this work over hourly intervals up to six hours, as shown in Figure 4.2(b). The amount of the total GHG emissions per hour was from (15 ml to ≈ 30 ml) for 0% of PAC and between (11ml to 20 ml) and (10 ml to 18 ml) for 0.5% and 2% respectively. Consequently, total GHG emissions had a strong negative correlation (P<0.001) with PAC additions over hourly intervals up to six hours. Overall, PAC additions to the barley hay showed a strong negative correlation (Cohen's d= -1.44 to -1.79).

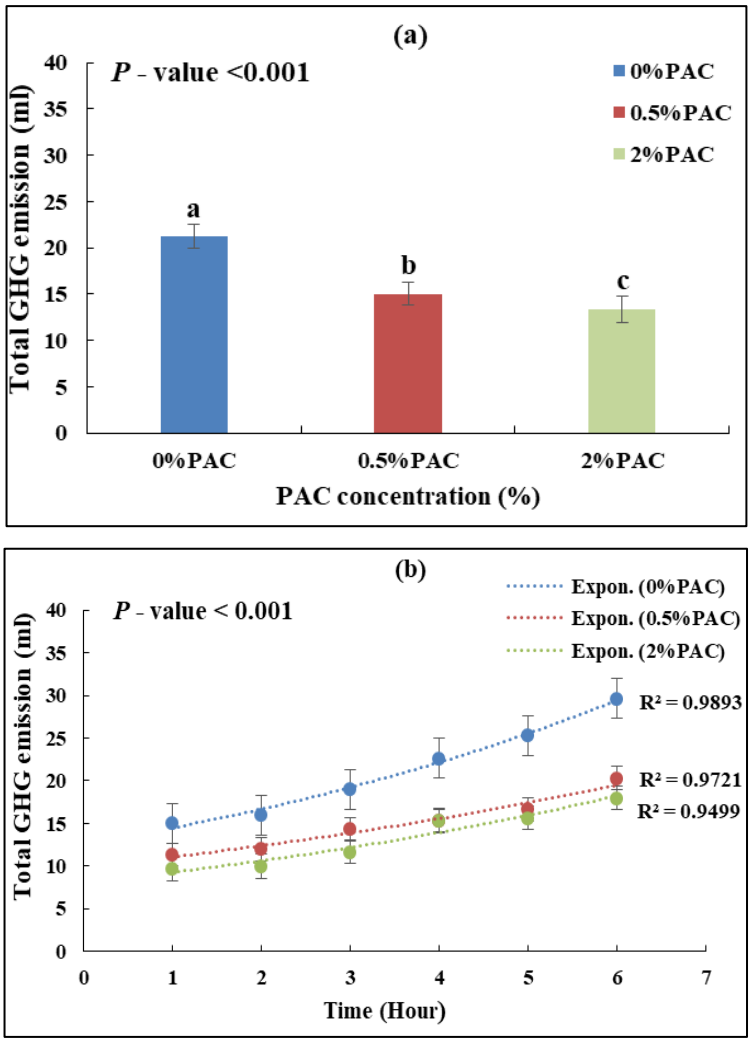


Figure 4.2: PAC addition effects on Barley hay diet (a) total GHG emissions and (b) Cumulative effect measured hourly for six hours.

The effect of different PAC concentrations on cumulative GHG emissions from forage sorghum silage was also used in this study, as shown in Figure. 4.3(a). Results showed that GHG emissions had a strong negative correlation ($P< 0.001$) with PAC additions. The GHG emissions were 24ml, 19ml and 14ml for 0%, 0.5% and 2% of PAC respectively. Consequently, the highest reduction in GHG emissions was 10 ml by 2% of added PAC. However, to shed more light on the experimental results, six hours of incubation have been used to evaluate the effects of PAC on GHG emissions from sorghum forage Figure 4.3(b). The greater reduction of 10ml - 19ml of GHG emissions was achieved when 2% of PAC was added over six hours interval to the diet. Overall, PAC additions to the forage sorghum silage showed a strong negative correlation (Cohen's d = -1.31 to -2.95).

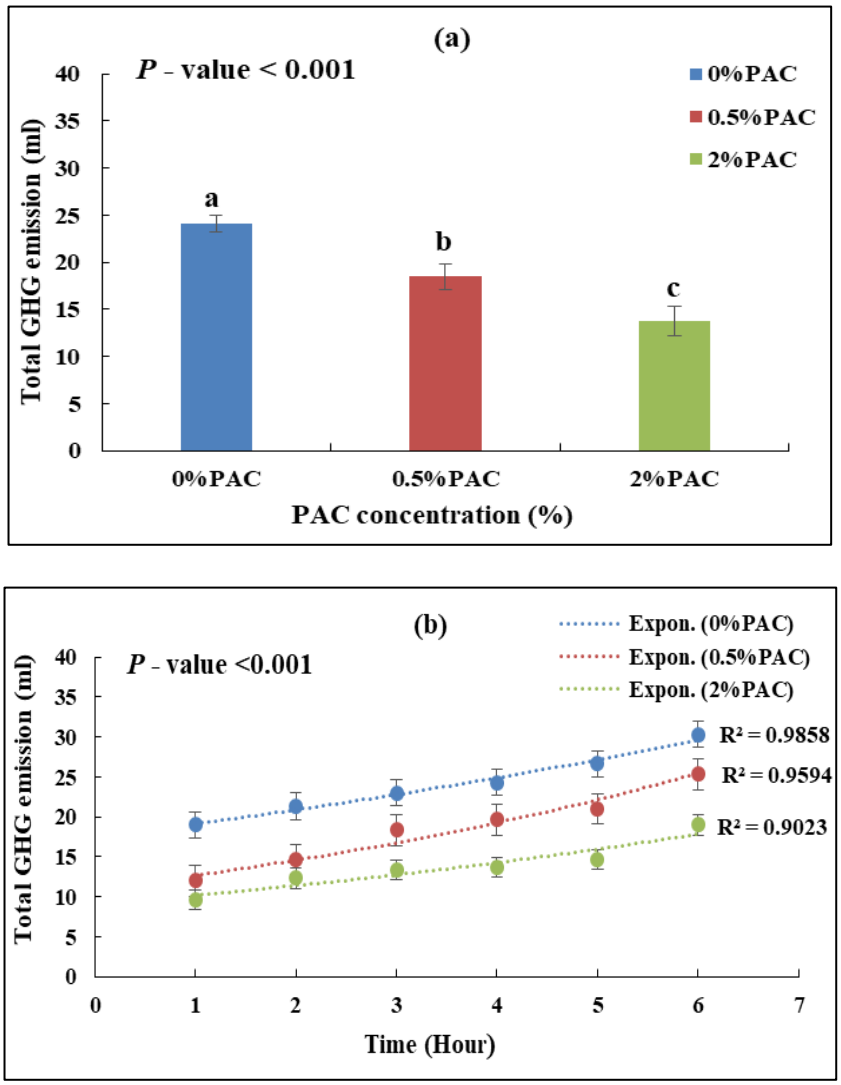


Figure 4.3: (a) Effect of PAC on (a) Total GHG emissions from forage sorghum silage and (b) Cumulative effect measured over hourly intervals for six hours.

The effect of PAC percentages on cumulative GHG emissions from mixed (4:1) forage sorghum silage and pellets was also tested, Figure. 4.4(a). GHG emissions had a strong negative correlation (P<0.001) with PAC additions. Our findings show that adding 2% of PAC had the highest reduction in GHG emissions which was 15ml, while the second GHG emissions reduction was 12ml when the percentage of PAC was 0.5%. In addition, the effect of the interaction between PAC concentrations and incubation time on the GHG emissions was also tested in this work.

The results showed that GHG emissions had a strong negative correlation with PAC additions (0.5% and 2%) to a mixed diet over six hours, as shown in Figure 4.4(b). The greater reduction of 13ml - 16ml was achieved when 2% of PAC added over six hours of an interval to the diet. Overall, PAC additions to the forage sorghum silage and pellets diet showed a strong negative correlation (Cohen's *d*= -2.72 to -3.09).

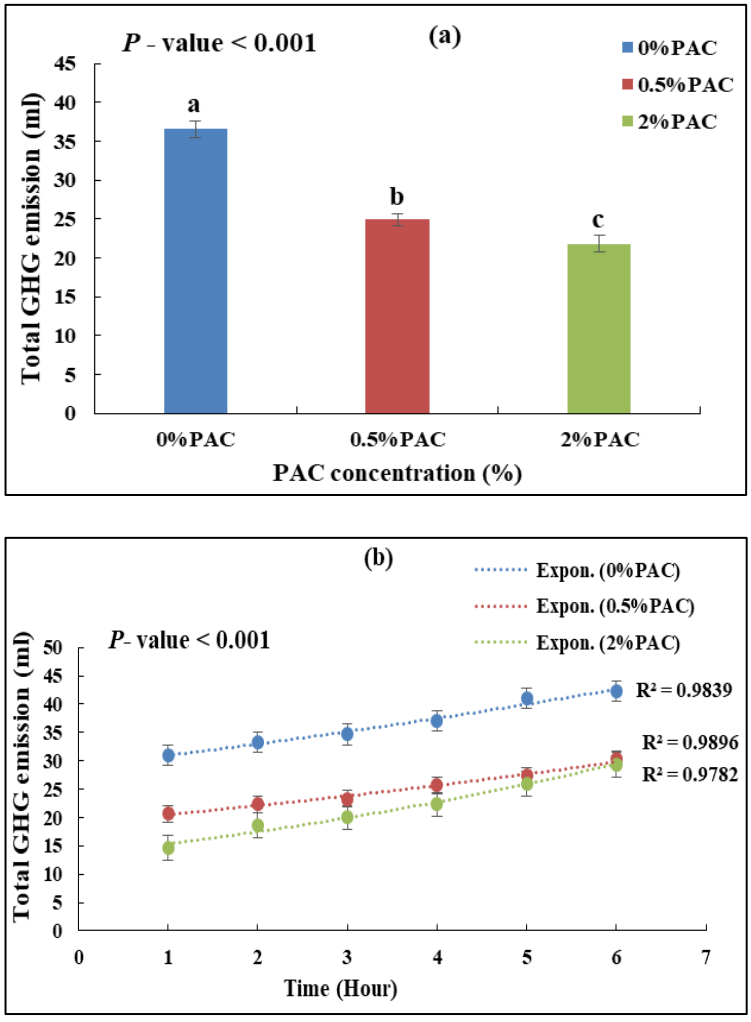


Figure 4.4: Effect of PAC on: (a) total GHG emissions from forage sorghum silage and pellets and (b) Cumulative effect of PAC measured at hourly intervals over six hours.

4.3.2 PAC amelioration of Mixed Diet Emissions

As with CH_4 emissions from the variety of feed resources (oaten hay, barley hay, forage sorghum silage and (forage sorghum silage and pellets)), had a strong negative correlation with PAC (P<0.001; Figures 4.5(a, b, c and d). Based on the obtained results in Figure 4.5 (a), the minimum CH_4 emissions were 6% and 17% with 2% and 0.5% concentrations of PAC were, respectively, while the maximum CH_4 emissions of 22% with 0% concentration of PAC was recorded from oaten hay. All these results were achieved using the number of experiments *in-vitro.*

The effect of PAC supplementation on CH_4 emissions from barley hay was also investigated and used in this paper. The results of the experiment were presented in Figure 4.5(b). They showed that CH_4 had a strong negative correlation (P<0.001) with PAC addition. The value of CH_4 emissions of 25%, 19% and 12% were reported with 0%, 0.5% and 2% concentrations of PAC, respectively; the highest reduction of CH_4 was 52% with 2% concentration of PAC. The values of CH_4 emissions and % reduction were 9% and 14% when 2% concentration of PAC was used with forage sorghum silage, as shown in Figure 4.5(c). Based on these values, it is clear that CH_4 emissions had a strong negative correlation (P<0.001) with PAC additions to forage sorghum silage.

Finally, Figure 4.5(d) showed the effect of PAC on CH_4 emissions from a mixed diet (forage sorghum silage and pellets). In this experiment, 0.5% and 2% of PAC were added for treatment. The results demonstrated that the PAC influenced CH_4 emissions, but that CH_4 emissions had a strong negative correlation (P<0.001) with PAC additions. Moreover, the values of CH_4 emissions were 17%, 14% and 4% with three concentrations of PAC 0%, 0.5% and 2%, respectively. Overall, PAC addition to the various diets showed a strong negative correlation (Cohen's d= -1 to -15).

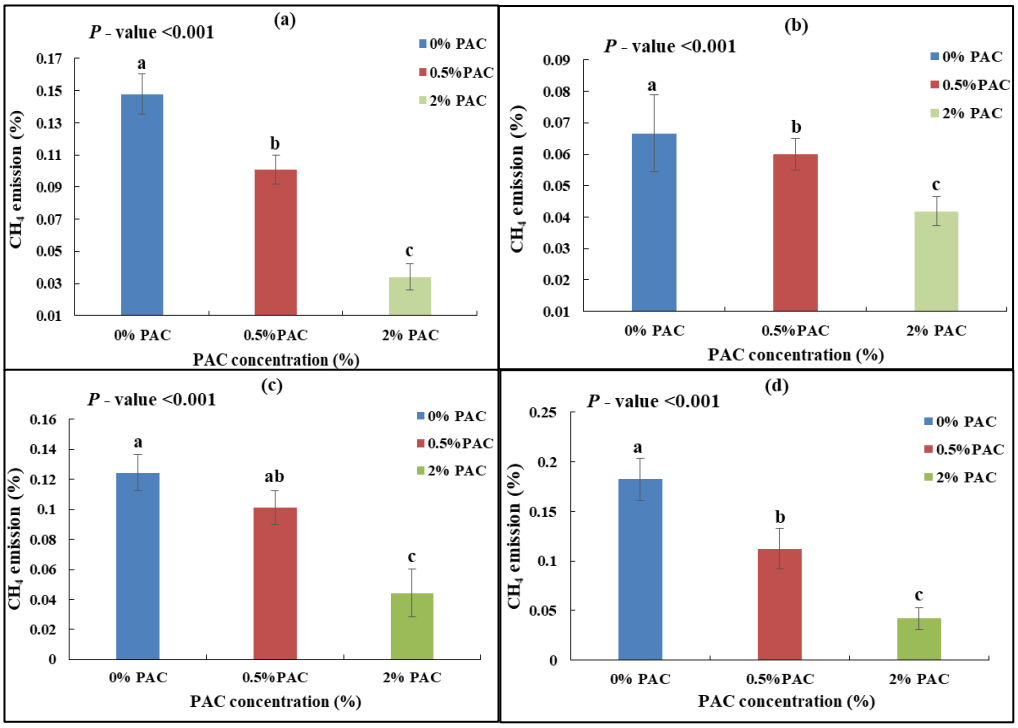


Figure 4.5: Effect of PAC on CH_4 emissions from (a) oaten hay, (b) barley hay (c) sorghum silage, (d) sorghum silage and pellets (Mean ± SD).

4.3.3 Volatile fatty acids (VFAs) effects with feed type.

To get a clear picture of the effect of adding PAC on volatile fatty acids (VFAs), different feed types with different concentrations (0, 0.5 and 2%) of PAC were used in this study. Our findings show that there was a non-significant change observed in adding PAC on VFAs (P<0.05) for all Duncan ANOVAs), as shown in Table 4.1.

To avoid the inconsistency in this research, the results of each type of feed (oaten hay, barley hay, forage sorghum silage and forage sorghum silage and pellets) were reported in terms of mean ± standard deviation over three concentrations. Table 4.1, shows that average VFA concentrations for the four types of feed were 33.11%, 33.33% and 33.33% for 0%, 0.5% and 2% PAC concentrations, respectively. The experimental results demonstrated that the addition of PAC on VFAs had no influence on their performance in relation to oaten hay and barley hay, forage sorghum and forage sorghum silage and pellets in relation to CH_4 and GHG emissions. Overall, the effect size of PAC additions on VFAs showed a strong negative correlation (Cohen's d= -0.98 to -3.94).

Moreover, it is clear that there was a strong negative correlation with increasing the level of PAC in the oaten hay, which led to increases in the degradability of crude protein (*P* =0.003), NDF (*P*=0.001), Lignin (*P*=0.001) and Hemicelluloses (*P*=0.001) as shown in Table 4.2. On the other hand, Ash, organic matter and cellulose showed a non-significant change, observed when the PAC was added (*P*=0.10, *P*=0.5 and *P*=0.10, respectively) as shown in Table 4.2. For example, the PAC virtual control of crude protein (5.50% vs 50.67%), NDF (76.03% vs 79.07%), Lignin (7.27%vs 7.30%) and Hemicelluloses (36.30% vs 39.23%) had greater degradability. Overall, the effect size of PAC addition on feed digestibility showed strong negative correlation (Cohen's *d*= -22.72 to - 16.52).

Table 4.1: *P*-value and (mean ± SD) results with PAC supplementation on VFAs in oaten and barley hays, forage sorghum and forage sorghum and pellets.

Feed type	**Items (acid)**	**PAC concentration % (Mean ± SD)**			***P*- Value <**
		0	**0.5**	**2**	
Oaten hay	Acetic	44.95 ± 4.34	44.85 ± 2.16	44.29 ± 0.55	.10
	propionic	6.08 ± 0.80	5.83 ± 0.30	5.96 ± 0.58	.10
	Butanoic	48.97 ± 3.82	49.32 ± 2.48	49.75 ± 0.85	.10
Barley hay	Acetic	44.79 ± 0.77	47.21 ± 1.93	50.06 ± 1.91	.10
	Propionic	5.99 ± 0.40	5.73 ± 0.08	6.01 ± 0.27	.5
	Butanoic	49.21 ± 0.96	47.05 ± 1.92	43.93 ± 1.86	.10
Forage sorghum	Acetic	48.49 ± 1.48	48.49 ± 2.07	50.90 ± 1.26	.5
	Propionic	5.60 ± 0.72	5.60 ± 0.43	5.57± 0.35	.10
	Butanoic	43.20 ± 0.80	45.92 ± 1.70	43.53 ± 1.56	.5
Forage sorghum & pellets	Acetic	53.28 ± 3.31	57.41 ± 3.00	58.40 ± 0.91	.001
	propionic	5.03 ± 0.58	4.87 ± 0.41	4.65 ± 0.10	.10
	Butanoic	41.69 ± 3.23	37.72 ± 2.69	36.96 ± 0.85	.5
Overall VFAs		**33.11± 1.75**	**33.33 ± 1.60**	**33.33± 0.92**	**.27**

P-value arising from Duncan ANOVAs are presented

Table 4.2: Effect of PAC addition on in-vitro forage degradability in dairy cattle

Feed type	Items	PAC concentration % (Mean ± SD)			*P*-value<
		0	0.5	2	
Oaten hay	Organic matter (%)	94.23±0.17	94.53±0.40	94.61±0.56	.10
	Ash (%)	5.67±0.27	5.57±0.18	6.03±0.24	.5
	Crude protein (DM) (%)	5.50±0.02	5.60±0.01	5.67±0.04	.003
	NDF (DM) (%)	76.03±0.23	78.43±0.17	79.07±0.33	.001
	ADF (DM) (%)	43.57±0.03	45.67±0.42	46.53±1.38	.05
	Lignin (DM) (%)	7.27±0.03	7.26±0.21	7.30±0.08	.001
	Cellulose (%)	32.46±0.05	32.76±1.25	32.54±0.50	. 10
	Hemicelluloses (%)	36.30±0.20	38.41±1.71	39.23±0.66	.001
Forage sorghum & pellets	Organic matter (%)	87.80±1.13	88.80±0.64	88.33±2.17	.10
	Ash (%)	12.27±1.21	11.20±0.64	12.03±1.92	.10
	Crude protein (DM) (%)	12.86±0.43	12.83±2.47	20.50±0.36	.01
	NDF (DM) (%)	67.93±0.78	70.53±0.34	71.03±0.89	.003
	ADF (DM) (%)	42.77±0.34	43.10±0.44	44.53±0.19	.05
	Lignin (DM) (%)	6.50±0.08	6.43±0.14	5.53±2.02	.10
	Cellulose (%)	25.16±0.46	27.43±0.32	26.50±2.56	.5
	Hemicelluloses (%)	36.27±0.78	36.67±1.03	39.16±1.71	.001

P-value arising from Duncan ANOVAs are presented

4.3.4 The effect of PAC on pH in different feed types.

This measurement was used to show the persistence and stability of the microflora in the rumen. From the results in Figure 4.6, we can see that all the feed types have reported reasonable results in terms of pH.

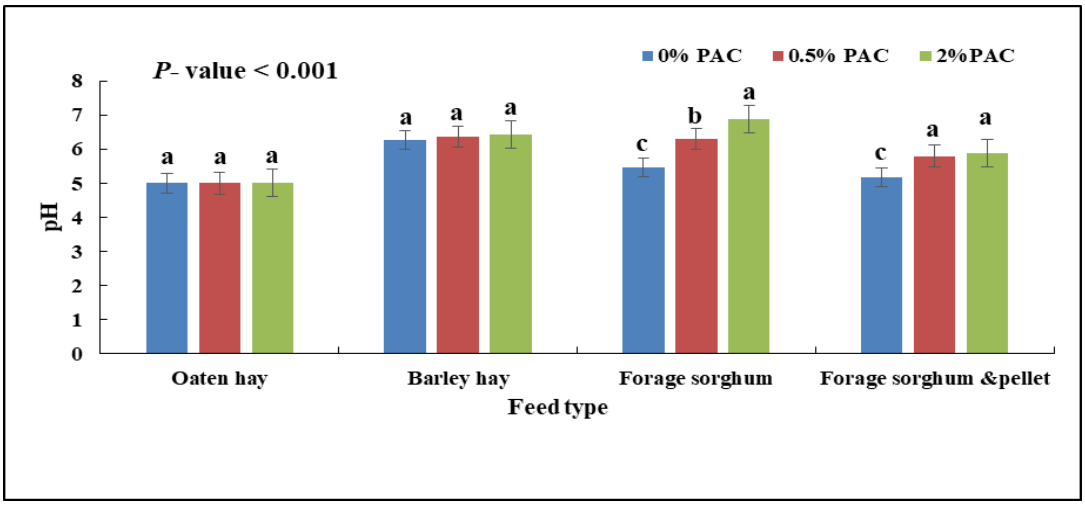


Figure 4.6: Effect of PAC addition on pH from oaten hay, barley hay, forage sorghum silage, and forage sorghum silage and pellets. (Mean ± SD).

4.4 Discussion: PAC addition on Emissions for various Forage diets

In this study, the effect of PAC on total GHG emissions, CH_4, feed fermentations and VFAs, from oaten hay, barley hay, forage sorghum silage and mixed diet (forage sorghum silage and pellets), was employed to study and analyse PAC characteristics *in-vitro*, which gives better results compared with other studies. Our findings show that total GHG and CH_4 emissions had a strong positive correlation with PAC additions to various diets but had no influence on VFAs.

Different types of concentration (0%, 0.5% and 2%) of PAC were added to each type of forage diets: oaten hay, barley hay, forage sorghum silage and mixed diet (forage sorghum silage and pellets). The total gas production (GHG) and CH_4 decreased with the addition of PAC to the forage diets. Thus, reducing the gas production with PAC in this experiment indicates that firstly the PAC has provided a large surface area for microbial communities. Secondly, the PAC has been become impregnated with microorganisms when there is organic matter in the rumen and it has led to an increase in the efficiency of redox reactions through transferring the electron directly between ruminal microbial species.

To the best of our knowledge, our results are agreement with those of (Leng et al. 2012; McFarlane et al. 2017; Cabeza et al. 2018), who found a significant reduction in total gas production by the addition of biochar to the oaten hay, barley hay, forage sorghum and mixed diet. They reported that a decrease in methane production in those studies could firstly be attributed to the gas sorption capacity of PAC because it has a large internal surface area. Secondly, methane production has the ability to stimulate microbial growth and methanotrophic microbes. In contrast, many researchers reported that the total gas production *in-vitro* increased with addition of biochar, for example, when it was added to the basal diet of orchard grass hay (Menke et al. 1979; Menke 1988).

For further investigation, the effect of PAC on VFAs production *in-vitro* was also investigated and evaluated in this study. Based on the obtained results, it was found that the VFAs were not significantly altered by adding different concentrations of PAC to the oaten hay, barley hay, forage sorghum and mixed diet. Therefore, our results agreed with those of other researchers: (Pereira et al. 2014; McFarlane et al. 2017; Cabeza et al. 2018; Mirheidari, A: et al. 2019; Teoh et al. 2019) who reported that the

PAC additions did not have a negative influence on the VFAs production. In contrast, other studies in the literature (Saleem et al. 2018) found that adding PAC to the artificial rumen liquid increased the production of VFAs during *in-vitro* experiments. These typically used high quality PAC, such as pyrolysis conditions, surface area and porous volume of PAC.

Our results from *in-vitro* experiments indicated that adding 0%, 0.5% and 2% concentrations of PAC to the oaten hay, barley hay, forage sorghum silage and mixed diet (forage sorghum silage and pellets) decreased total gas productions without any influence on VFAs concentration. These results could firstly provide an explanation as to why the PAC used in this study has an effect on CH_4 production. Moreover, it can be seen that there are many improvements in the digestibility of nutrients. These improvements have been achieved in this paper due to the positive role of PAC in manipulating the rumen microbial ecosystem to increase the efficiency of rumen metabolism and this ultimately leads to the productivity of animals.

In summary, examination and evaluation of the effect of PACs on the total GHG and CH_4 emissions from oaten hay, hay barley forage sorghum silage and mixed diet (forage sorghum silage and pellets) will permit a number of research studies. It will provide an important contribution to animal feeding by providing new research measurements on the effects of PAC for dairy cows. These research outcomes will be vital to enhance animal feeding as well as milk production.

4.5 Chapter summary

In chapter 3, a new study focused on *in-vitro* experiments was presented to show the effect of PAC on different feed types: oaten hay, barley hay, forage sorghum, and mixed diet (forage sorghum silage and pellets). In this research, three concentrations: 0, 0.5 and 2% of PAC were added to each feed types. Ultimately, our findings definitely showed that PAC can reduce total gas emissions (GHG and CH_4 emissions) by increasing the surface area in the rumen, enhancing microflora, and improving feed digestibility. In addition, the highest reduction of total gas emissions was achieved with 2% of PAC, indicating that there was a strong negative correlation with PAC addition. Thus, the concentrations of PAC should be carefully and accurately assessed for obtaining acceptable results as well as for assessing the viability of utilizing PAC as a feed additive in the diet of dairy cattle. Furthermore, the experimental results *in-*

vitro were also compared with other existing studies. The obtained results showed that the addition of PAC to the diet of dairy cattle *in-vitro* outperformed other supplements. Finally, this study found that the particle concentrations of PAC were most efficacious when incorporated in the diet of dairy cattle, leading to a reduction in GHG emissions and an increase in feed digestibility compared to small particle concentration.

CHAPTER 5

LABORATORY EXPERIMENTS

5.1 Introduction

One of the most common perennial shrubs in Queensland, Australia is Lantana (Lantana camara). Lantana was first discovered in 1841 and by 1897 it had spread widely, covering more than 5 million ha of Eastern Australia. While the cultivated form planted in gardens produces few seeds and is compact, the weedy form of Lantana found in the wild is a prolific seeder, which forms dense, impenetrable thickets via vegetative reproduction, overtaking native bushland and pastures (Anderson et al., 1983; Dayson, 1989; Fensham et al., 1994; Fensham, 1996; Batianoff and Franks, 1997). Consequently, Lantana is listed as a 'Weed of National Significance' and is regarded as one of the worst weeds in Australia because of its invasiveness, the potential for spread and its consequential debilitating economic and environmental impacts (Reed et al., 2011; Olsen, 2020; Ralph, 2020).

Lantana has serious effects on both natural flora and fauna. When eaten, it is toxic to most native fauna, such as kangaroos (Johnson and Jensen, 1998; Barik et al., 2020; Ralph, 2020). More than 1400 native fauna species are also negatively affected by Lantana invasion, including many endangered and threatened species, due to its allelopathic ability, releasing chemicals into the soil that prevent germination and growth of other plant species. This ability aids its spread and destroys other vegetation in pastures, causing fodder scarcity, which in turn compels native and domestic animals to eat it. It seriously affects biodiversity, causing habitat destruction and weakens the ecosystem generally, including within several World Heritage-listed areas in Queensland (Barik et al., 2020; Chowdhury et al., 2020; Demetriou et al., 2020; Devi and Khwairakpam, 2020; Ncube et al., 2020). Commercially, it competes for resources with and reduces the productivity of agriculture, pastures and forestry plantations. It currently covers about 60% of pasture in Queensland (Osunkoya et al., 2020; Wilson et al., 2020). Some studies have reported that Lantana is now spreading across the remainder of Australia, being pollinated easily by honey bees (Ncube et al.,

2020; Wilson et al., 2020). In fact, based on the latest world statistics, Lantana is one of the world's most common noxious weeds (Lüi, 2011).

Importantly, Lantana is highly poisonous and is responsible for heavy mortality rates of livestock. In addition, it has a high impact on most wild animals, who are often present in the same pastures (Catterall, 2020). Both ruminant and non-ruminant animal species are susceptible to Lantana toxicity, including cattle, buffalo, sheep, goats, pigs, horses, ostriches, camels and kangaroos (Parimoo et al., 2015; Kumar et al., 2016; Worku and ZelekeTessema, 2019; McKenzie, 2020). Most cases of Lantana poisoning occur when new stock are introduced into Lantana-infested areas. Stock bred on Lantana-infested country have learned to avoid Lantana unless forced to eat it due to lack of other fodder. Many studies have been reported that cattle are highly susceptible to Lantana poisoning. The effect of Lantana toxins on livestock is varied and depends on the species, age, size and body condition of an animal. The type and condition of the plant also influences the level of toxicity, with green fruit toxic whereas ripe berries are largely safe to consume (McKenzie, 2020) (Sharma et al., 2007).

This toxicity is due to an array of compounds, including mono and sesquiterpenes (curcumenes and safrole), iridoid glycosides (theveside, geniposide), flavonoids, napthoquinones, coumarins (umbelliferone, methylcoumarin), salicylic acid, with triterpenes as Lantadenes (Lantadene A to D) being the most toxic (Achhireddy et al., 1985; Jain et al., 1989; Singh et al., 1989; Rathinasabapathi et al., 2005; Sharma et al., 2007). Lantadenes are pentacyclic triterpenes, which are produced in the leaves, stems, flowers and fruits. There are over 30 different colour forms of Lantana and not all the Lantana varieties produce these poisons; the red flower variety (L. camara var. aculeate) is the most toxic (Sharma et al., 2007) while Helidon white, Townsville prickly orange, pink variety of Lantana camara, usually grazed by animals in New Zealand, are non-toxic (Hart et al., 1976a; Hart et al., 1976b; Black and Carter, 1985). The location and levels of Lantadenes differ within the part of the plant and change with age. The levels of Lantadenes in leaves and stems (the primary fodder for stock) increases with age, with the highest level of Lantadenes present in mature leaves (Sharma et al., 1981; Sharma et al., 2007). In mature Lantana leaves, Lantadene A represents the highest amount at 806mg/100 g dry wt (followed in decreasing order as Lantadene B, 522; Lantadene C, 425; Lantadene D, 177; reduced Lantadene A, 29 and reduced Lantadene B, 19). Lantadenes are absent from roots (which contain different

triterpenoids including oleanolic, oleanonic and lantanolic acids, while the green immature fruits are poisonous due to different toxins, but once ripened they are safe for birds, animals and humans to eat.

Lantana has negative effects on livestock, initially causing sickness and decreasing productivity (Sharma et al., 2007; Diaz, 2011). Upon ingestion, these poisons can cause liver damage, with subsequent consequences including photosensitization, jaundice, rumen stasis and depression, usually resulting in mortality within 1-4 weeks after symptoms occur (Sharma et al., 1981; Quinn et al., 2014). Lantana toxicity results in the release and absorption of toxins in the gastrointestinal tract; the hepatic phase resulting in cholestasis, hyperbilirubinaemia and hyperphylloerythrinaemia. The Lantana toxins are absorbed through all regions of the gastrointestinal tract (GIT) including the stomach, small intestine and large intestine but the animals rapidly stop feeding in about 2 h. Rumen stasis occurs quickly within 4-6 of ingesting Lantana and the contents stop moving into the duodenum, causing the rumen contents to increase in toxicity as the toxins are released from the ingested leaves. This is primarily due to neural inhibition from the damaged liver, which becomes evident within the same period of 4-6 hours (Sharma et al., 2007).

Constipation begins and within the next 24–48 hours the animals become sedated and photosensitive, resulting in the eyelids, muzzle and other hairless parts becoming swollen due to sunburn (Yadava and Verma, 1978; Sharma et al., 1981b). Cell injury results from the accumulation of bilirubin and phylloerythrin (Sharma et al. 2002) and in severe cases the animal dies. If the rumen contents are emptied the animals show rapid recovery (McSweeney and Pass, 1982).

Research has revealed that the toxins Lantadene A and B have a strong antimicrobial activity to a number of human pathogens, including Bacillus subtilis, Escherichia coli, Micrococcus pyrogenes, Pseudomonas aeruginosa and Staphylococcus aureus (Sousa and Costa, 2012). However, there is conflicting information about their effects on rumen microflora. (McSweeney and Pass, 1983) reported that protozoal and bacterial populations of the rumen decreased with Lantana poisoning and starvation, but suggested that this change was due to anorexia and rumen stasis and not by a direct effect on rumen microorganisms themselves. In contrast, Lantana and Lantadene were reported to have no antibacterial activity on most of the rumen microflora (Sharma et al., 2007). Sharma et al. (2000) have reported that no biotransformation of Lantadene

to 3-hydroxy derivatives or any other metabolite occurs using in-vitro rumen fluid, suggesting the absence of a suitable enzymatic system for biotransformation of such compounds. The effect of Lantana poisoning specifically on methanogens responsible for rumen GHG production has not been investigated.

One known way to alleviate Lantana poisoning in livestock is to use activated charcoal or clay. Pass and Stewart (1984) reported that feeding powdered activated charcoal mixed with an electrolyte solution by stomach tube to cattle and sheep could successfully bind Lantana poisons within the rumen and prevent mortality. Similar results were reported by McKenzie (1991) for Lantana poisoned calves, with calves fed activated charcoal recovering three days faster than those fed bentonite clay.

This study firstly investigates the in-vitro ability of coconut derived PAC on the absorption of Lantana toxin from the rumen fluids and secondly, whether the presence of Lantana toxins affects growth and function of rumen microflora, rumen fermentation and Methanogenic GHG production.

5.2 Materials and methods

The materials other than those described previously in **section 3.2.1** and the proposed additional methods are briefly described in this section.

5.2.1 Lantana collection and extraction of Lantadene

Mature Lantana was collected from Toowoomba Qld, Australia in February 2019. Lantana sampling was random throughout the infected field. Lantana samples (leaves, flowers or berries) were oven dried at 55 °C for 48 h and ground into a fine powder of approximately 1 mm average particle size, with the help of a grinder, as shown in Figure 5.1(a – c). Lantadenes were extracted by the protocol described by Sharma et al. (2000) with certain modifications as shown in Figure 5.1(d). The Lantana samples (100g) were mixed with 500mL methanol (CH_3OH) and placed into an intermittently shaking water bath at 30ºC for 24 hours, the liquid was filtered through two layers of muslin cloth. The residue was extracted once again with 200mL methanol for 24 hours then filtered through two layers and combined with the first extract. This resulted in 100g Lantana extract in 700 mL of methanol.

To determine the levels of lantadene that the PAC could potentially absorb, the combined lantadene extract was treated with PAC to remove the excess amount of chlorophyll and Lantadene from the extract using different concentrations of PAC (0, 0.1, 0.4 and 1g were added to 30 mL of extract.). The lantadene samples were treated with PAC for 6 hours in a rotary shaker at 100 rpm. The samples were collected after 6 hours and filtered through Whatman paper number (1-22).

Samples were analysed for Lantadene on a Shimadzu Nexera UPLC System using a Phenomanex C18 Kinetix column (50 mm x 2.1 mm x 2.6 µm) with gradient elution (A= MilliQ Water with 0.1% Formic Acid; B = Acetonitrile with 0.1% Formic Acid). The flow rate was 0.8 mL/min, column temperature was 40 °C and the gradient profile was as follows: 50% B to 100% B over 5mins; hold 100% B, 2.5 mins; hold 50% B 2.5 mins. Samples were injected as either 1µL or 5 µL aliquots. Total Lantadene was quantified using a PDA detector (215 nm) using a series of Lantadene A external standards 0.01mM-1mM. The absorbance of Lantadene B was assumed to be the same as Lantadene A. Peaks eluted at approximately the same retention time and were integrated as one peak. The presence of Lantadene B was confirmed by LC-MS. Standards were injected as 5µL injections. Samples were injected as either 1µL

injections (S1-S72) or 5uL injections (S73-S108). Limit of Quantification = 0.01 mM. It was noted that all peaks usually followed the same trend as Lantadene. Standards were prepared using 5mg of lantadene A (Rehmannic acid; [22fl (Z)]-2-methylisocrotonoyloxy-3-oxoolean-12-en-28-oic acid,) (98%) from (Kemix Pty Ltd, Melbourne, Australia) as shown in Table 5.1 for detecting Lantadene A and B concentration by HPLC-DAD. Fourier transform-infrared spectroscopy (FTIR), which measures the molecular geometry and vibrational frequencies of a chemical, was carried out following the method of (Diwivedi et al. 2009). Figure 5.1(e - f) clearly show the effect of Lantana toxicity on dairy cattle health. The sample used for FTIR measurements of PAC was made up of 1g of PAC suspended in 30mL of methanol after 6 hours of shaking.

Table 5.1: standard production and serial dilutions used.

Molarity (moles/Litre=M)	**Lantadene A (g/L)**	**milligrams/mL (mg/mL)**	**Parts Per Millions (ppm)**	**Number of serial dilutions**
1 M	552.796	552.796	552.796	/
0.1M	55.2796	55.2796	55.279	/
0.01M	5.52796	5.52796	5.527	/
10^{-3} M	5.52796×10^{-1}	5.52796×10^{-1}	552	1
10^{-4} M	5.52796×10^{-2}	5.52796×10^{-2}	55.2	2
10^{-5} M	5.52796×10^{-3}	5.52796×10^{-3}	5.2	3
10^{-6} M	5.52796×10^{-4}	5.52796×10^{-4}	5.2×10^{-1}	4
10^{-7} M	5.52796×10^{-5}	5.52796×10^{-5}	5.2×10^{-2}	5

Figure 5.1: (a) fresh Lantana, (b) drying lantana in the oven, (c1): powdered lantana flowers (c2); lantana leaves powdered and (c3): powdered lantana berry (d) Lantadene leaf extract (e) and (f) physical effect of lantana poisoned dairy heifers.

5.2.2 Rumen liquid collection

Method as per Chapter 3 **Section 3.2.2**

5.2.3 Buffer solution

In this study, the buffer solution preparation was used to stable the rumen pH. This step is important to microflora activity. Maintaining pH of the rumen plays an important role in enhancing the rumen fermentation efficiency because it affects the persistence and stability of the microflora in the rumen. More details are provided in Chapter 3, **Section 3.2.3**.

5.2.4 In-Vitro incubation and sampling procedure

Four treatments were performed: the first treatment (control) had rumen fluid 35 mL, 0.6g ground pellets and no PAC or Lantana (La), (No PAC & La). The second treatment had rumen fluid 35 mL, 0.6g ground pellets (feed) with 1% powdered Lantana leaves/dry matter (DM) of pellets and without PAC (No PAC with 1% La). The third treatment had rumen fluid 35 mL, 0.6g ground pellets with 1% powdered Lantana leaves/DM with 0.5% PAC. The fourth treatment had rumen fluid 35 mL, 0.6g ground pellets with 1% powdered Lantana leaves/ DM and 2% PAC. The buffer mineral solution was added to each treatment at a ratio of 1:4 rumen liquid: buffer solution. Each treatment had three replicates of this diet at 0.6g/DM in the form of commercially made animal diet pellets, from Ridley Agriproducts Pty ltd. The effect of PAC on *in-vitro* GHG emissions and rumen fermentation was tested in this paper. The chemical components of Lantana plant constituents are shown below in Table 5.2.

Table 5.2: chemical components of Lantana parts (proximate analysis).

Items (%)	Type of Lantana parts		
	Leaf	**Flower**	**Berry**
Dry matter	71	76	69
Organic matter	88.6	91.4	94.8
Ash	11.4	8.6	5.2
Crude protein (DM)	21.7	13.0	7.3
NDF (DM)	35.1	27.7	61.2
ADF (DM)	21.1	21.8	50.9
Lignin (DM)	9.7	10.0	22.0
Cellulose	14.0	6.0	10.2
Hemicelluloses	11.5	11.7	28.9

5.2.5 Measurement of GHG

All GHG emission measurements were tested according to the method described by Makar (2004) as described in **Section 3.2.5**.

5.2.6 Measurement of methane (CH_4) emission and chemical analysis

CH_4 measurements and chemical analysis were tested according to the methods described by Makar (2004) as mentioned in **Section 3.2.6**.

5.2.7 Statistical analysis

The statistical package for the Social Sciences (SPSS-version 23) software was used to analyse the *in-vitro* experimental data (Swan 1995) and involved the analysis of variance (Seshadri et al.). Means of cone index were compared for significance using the least significant differences (Duncan) at 5% level of probability for the PAC concentration, feed type (grain and grass-fed), rumen fermentation and absorption Lantadene A and B data and GHG emissions.

5.3 Experimental results

In this study, initial experiments were conducted to evaluate the effectiveness of coconut PAC on adsorption of Lantana toxins, specifically lantadene, from different Lantana plant parts; berry, flower and leaf. The chemical control reference readings were firstly carried out by Fourier transform-infrared spectroscopy (FTIR). The sample used for FTIR measurements of PAC was made up of 1g of PAC suspended in 30mL of methanol after 6 hours of shaking. The FTIR spectrum of PAC alone without Lantana (with methanol as a solvent) is shown in Figure 5.2a. FTIR results for powdered of Lantana leaf, flower and berry alone (without PAC) are presented in Figures 5.2 (b-d) and were very similar, regardless of plant part.

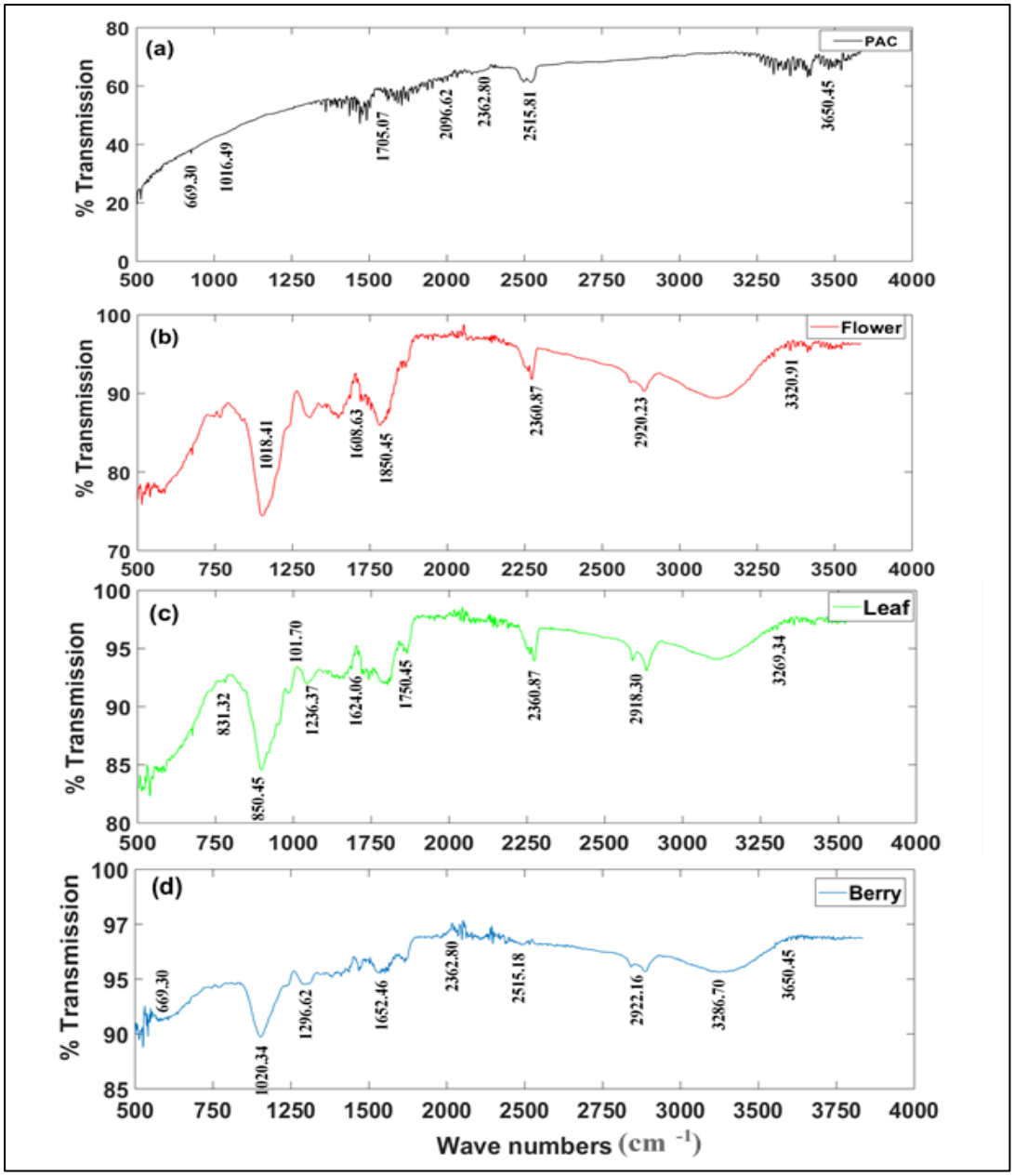


Figure 5.2: FTIR spectra for: (a)1g PAC and 30ml of methanol after 6 hours shaking, (b)powdered lantana flower, (c)powdered lantana leaf, and (d)powdered lantana berry.

The FTIR spectra of the soaked PAC with methanol showed that most of the prominent peaks fall below a wavenumber of 3000 cm^{-1} with a few above this mark, indicating that the mixture structure is mostly of an aliphatic nature with the existence of few unsaturated constituents. The broad peak at 3209.55 cm^{-1} is likely to be associated with the hydroxy group (OH stretch) resulting from the interaction of methanol with PAC. The peak at 1705 cm^{-1} suggests the presence of a small amount of carboxylic structures. The prominent peak in the range of 1900-2300 cm^{-1} is linked with the presence of cyanide structures. The neighbouring peak at 2515 cm^{-1} is an indication of hydride

vibration such as S-H, Si-H and P-H. B-H etc. The peaks at lower frequencies, the wavenumber of 669 cm^{-1} and 1016 cm^{-1} indicate alkyne and carbon-halogen structure particularly C-F.

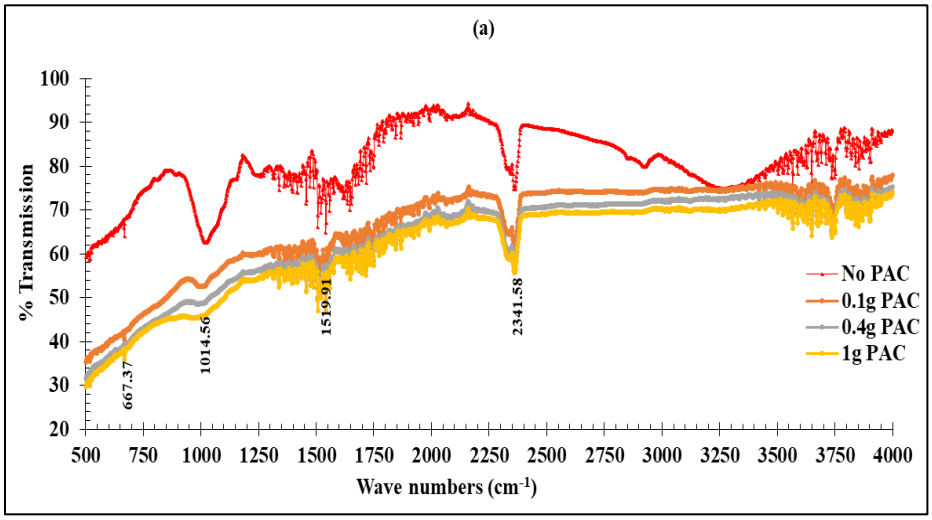
All three Lantana plant extracts (flower, leaf and berry) exhibited similar FTIR spectra with some minor shifting in the peaks. As the previse causes the prominent peaks to fall under the 3000 cm^{-1} region, it can be said that the structure is predominantly of an aliphatic nature. The broad peak at 3200 cm^{-1} or slightly above, together with the intense peak in the range of 1000-1200 cm^{-1} is a strong indication of hydroxy (alcohol) presence and this most likely to be attributed to the use of methanol as solvent. The peak at 1600 cm^{-1} suggests the presence of carbonyl structure C=O. Absorbance at 2360 cm^{-1} is normally related to the absorption of atmospheric CO_2, however, this is unlikely due to the capacity of FTIR spectroscopy to eliminate the background noise. Some studies ascribed the absorption at this wavenumber to the presence of asymmetric stretching of OH. The peak at 2900 cm^{-1} range corresponds to the saturated aliphatic structure particularly the stretch of C-H in asymmetric methylene. The peak right next to it represents the symmetric stretching of the same structure.

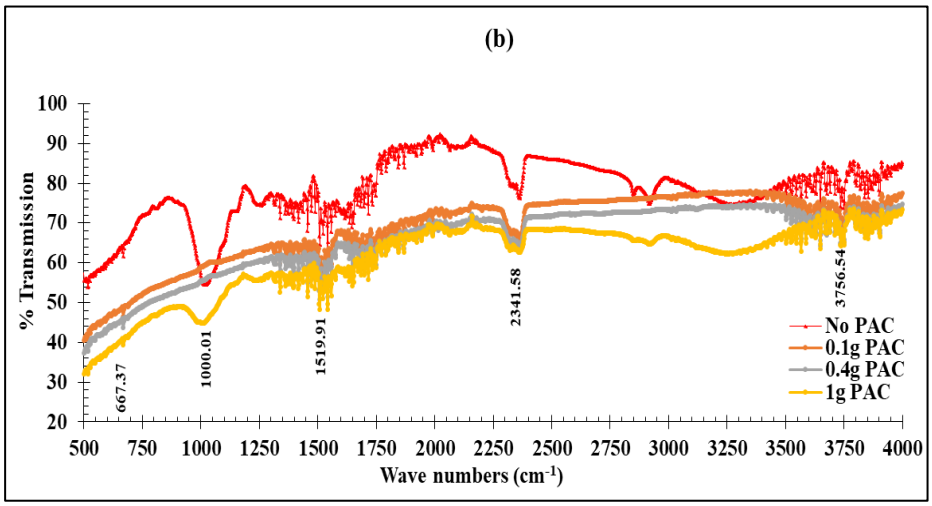
5.3.1 Effect of concentration and treatment time on Lantana interaction with PAC.

The concentration of lantadene (the quantitative results) in different samples was measured and then analysed using HPLC. The levels in the bushes on the farm were comparable to those reported by Sharma et al. (2000). In mature Lantana leaves, lantadene A represents the highest amount at 806mg/100 g dry weight (followed in decreasing order as lantadene B, 522; lantadene C, 425; lantadene D, 177; reduced lantadene A, 29 and reduced lantadene B, 19).

The effectiveness of PAC to remove toxins from the Lantana extracts was also determined as follows. Different concentrations of PAC were added to each 30 mL sample of Lantana extract, (0, 0.1, 0.4 and 1 gram of PAC). The FTIR spectra of these mixtures are shown in Figure 5.3 a-c. Overall, the higher the amount of PAC, the lower the resulting transmission, indicating a significant removal of Lantana-extract chemicals with increasing PAC quantity.

It can be seen from this figure that the hydroxyl (alcohol) peak at 1000-1200 cm^{-1} range maintained its presence in all the treatment scenarios and this is expected as the reaction medium was methanol. It seems that there is a slight effect of Lantana on the cyanide structure of PAC reflected by the slight shift in the peak to 2341cm^{-1}. The disappearance of the peak at 2515cm^{-1} suggests that exchange activities might have occurred with the hydride structure facilitating the adsorption of lantadenes. The presence of crowded peaks at the 3700cm^{-1} regions indicates the presence of oxygen-containing functional groups in a particular hydroxy stretch of primary alcohol (Hinterstoisser et al. 2000). This could be related to the interaction of methanol with Lantana and PAC forming a new structure linked by functional groups. There is an obvious dip at 1519 cm^{-1} and 2342 cm^{-1}.





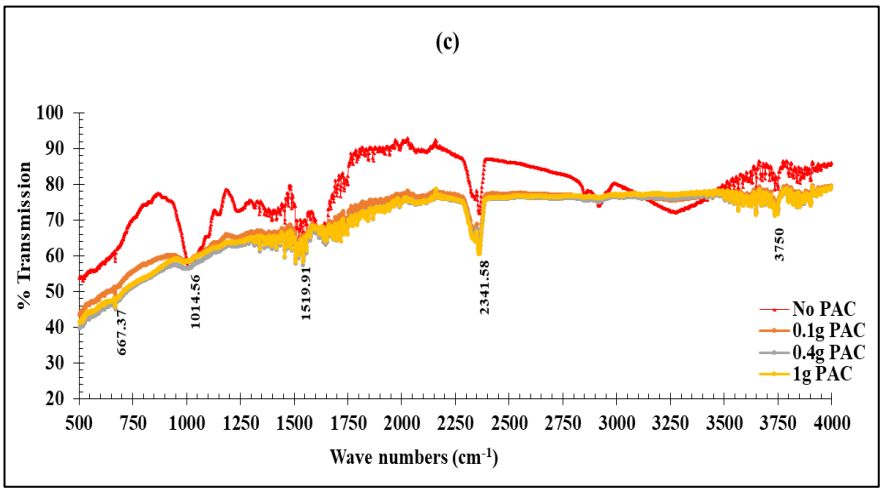


Figure 5.3: FTIR spectra of different concentrations of PAC with Lantana after 6 hr reaction time: (a) berries, (b) flowers and (c) leaves.

For further evaluation, the effect of time on Lantana interaction with powdered activated carbon was also used in this study. Similar spectra were obtained with varying reaction times and fixing the amount of the PAC used to1 gram, as illustrated in Figure 5.4 a-c. It can be noticed that 0.1g and 0.4g of PAC had almost the same spectra which are different than that of the 1g suggesting that the interaction of Lantana with the former two concentrations was almost the same, suggesting. However, when increasing PAC to 1g, the interaction appears to be more palpable. The same trend applies to the treatment time. This means that among the treatment conditions applied in this study, 1g of PAC for 6 hours has the best absorption outcome. Higher levels of PAC would eventually remove all chemical constituents. In summary, based on the obtained results in Figure 5.4 a-c, the PAC shows the greatest potential for reducing CH_4 production without loss of degradability.

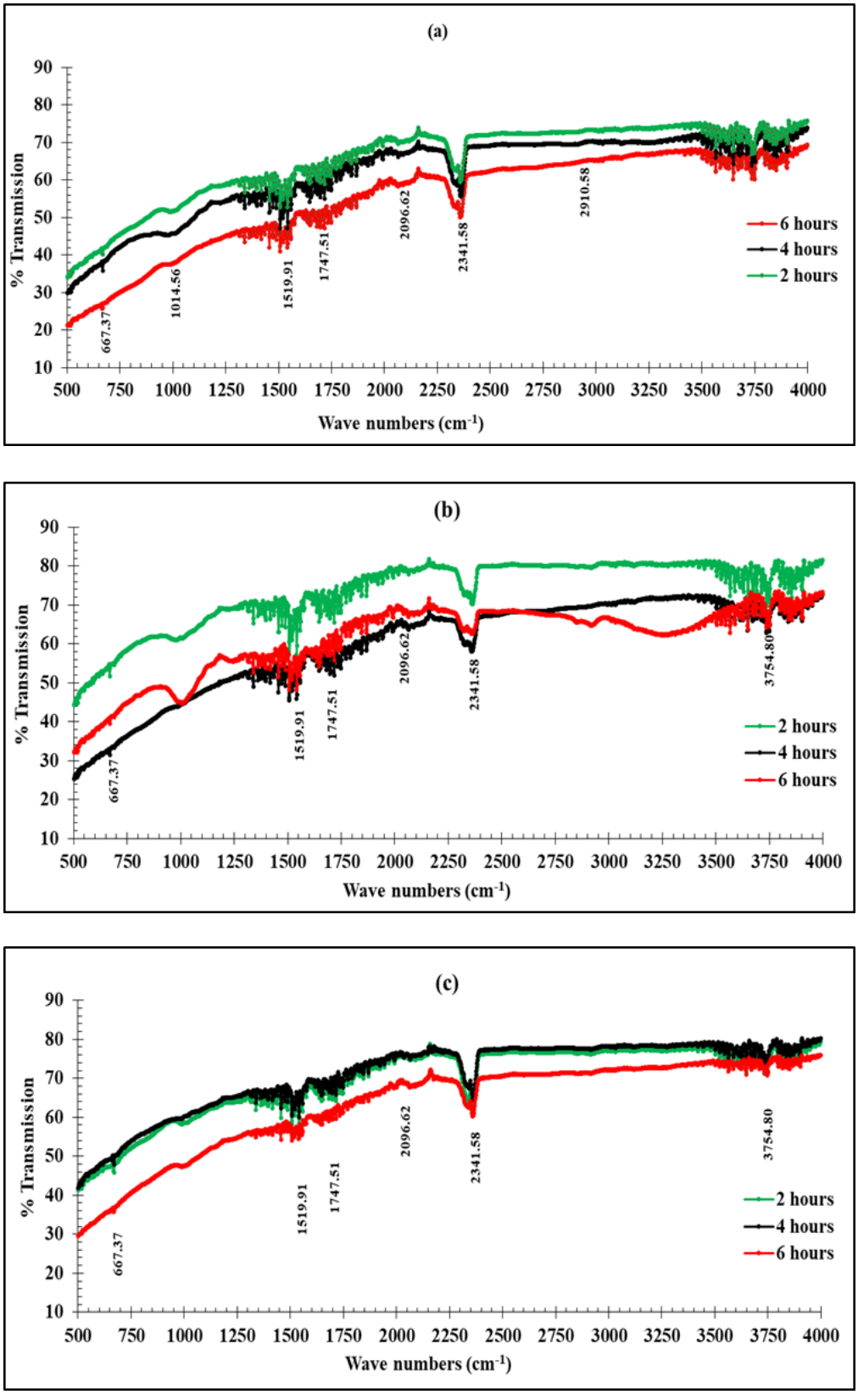


Figure 5.4: FTIR spectra of different treatment time for 1g of PAC with lantana: (a) berries, (b) flowers and (c) leaves.

5.3.2 Effect of PAC on the absorption of Lantadene A+B from Lantana leaves and flowers.

Figure 5.5(a) shows the performance of different PAC concentrations on the reduction of lantandene A and B in the Lantana leaves. It was obvious that the 1g of PAC added to 30 mL Lantana leaf extract had a substantial (P<0.001) reduction in the concentrations of lantadenes A and B. The concentrations of lantadenes A and B were 2.19mM, 2.16mM, 214 mM and 1.61mM by adding 0g, 0.1g, 0.4g and 1g of PAC, respectively. Figure 5.5b shows the effect of different PAC concentrations on the reduction of lantandene A and B of Lantana flower extract. Overall, with different concentrations (0g, 0.1g, 0.4g and 1g) of PAC, the reductions of lantandene A and B were significantly (P < 0.001) lower than the control group. The concentrations of lantandene A and B were 0.30 mM, 0.29 mM, 0.27 mM and 0.24 mM by adding 0g, 0.1g, 0.4g and 1g of PAC respectively. In summary, applying 1g of PAC can result in a significant reduction of lantadenes A and B in Lantana leaf and flower, while the concentration of lantadenes A and B in Lantana berry cannot be detected reliably by HPLC. It is postulated that PAC removal of lantadanes A and B from leaves was inhibited by high levels of colour presented, specifically with the presence of chlorophyll. However, the flower samples we saw had a much lower natural production of lantadenes A and B due to the lower levels of colour present.

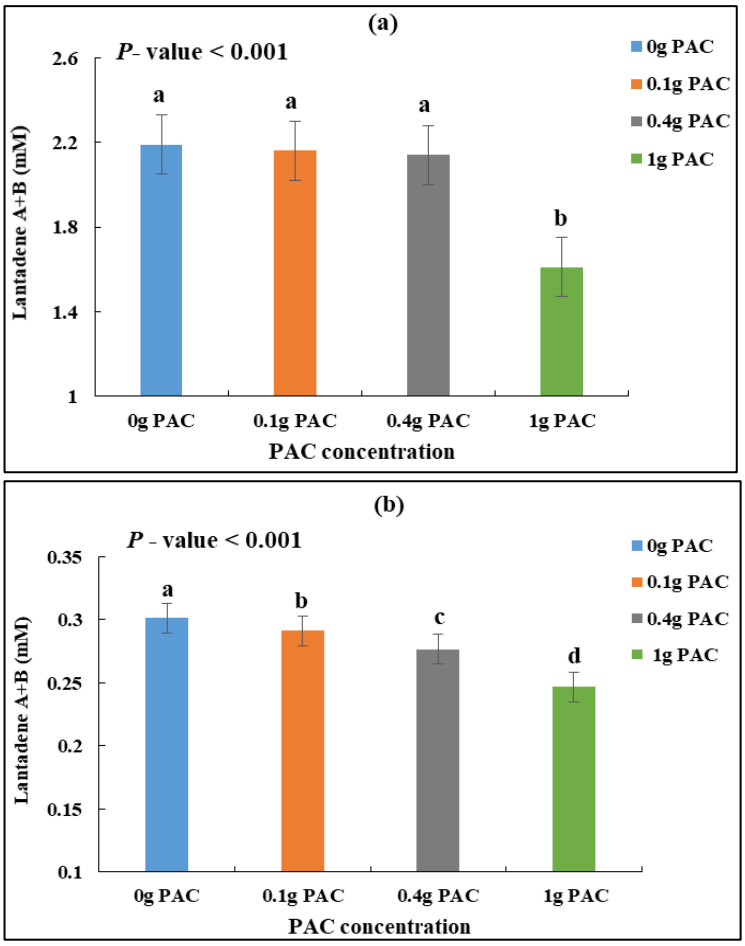


Figure 5.5: Effect of PAC level on Lantadene A+B absorption in (a) leaves and (b) flowers.

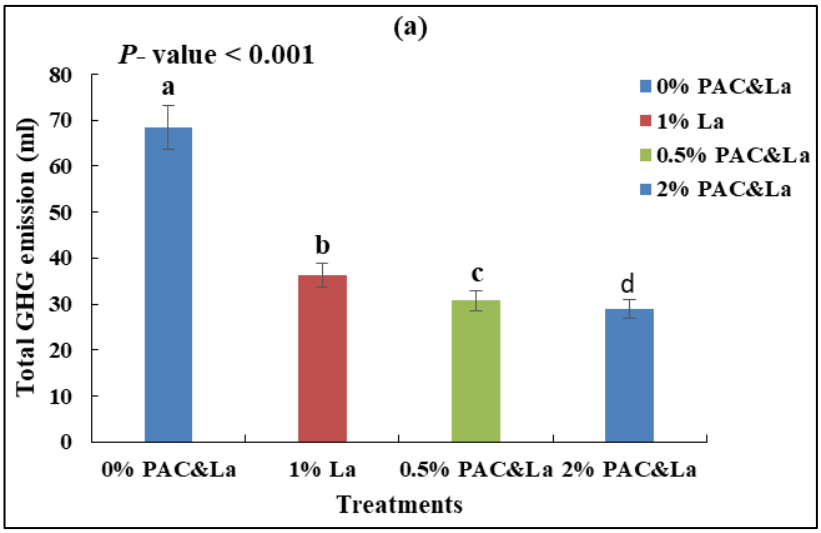
5.3.3 Effect of PAC on GHG and CH_4 emissions

Finally, the effect of PAC on GHG and CH_4 emissions was studied. In the next section, the obtained results will illustrate and discuss these effects in detail.

Figures 5.6 a-c present the effect of PAC on GHG and CH_4 emissions measured *in-vitro* with dairy cattle rumen fluid containing 1% (DM) of Lantana leaves. The findings showed that the best results were achieved with 2% PAC concentration compared to a control of 0% PAC (no Lantana and no PAC) and 0.5% PAC. However, the addition of PAC at any concentration had a significant reduction (P <0.001) in GHG and CH_4 production compared to the control treatments (no PAC or La). The addition of 1% Lantana caused a consistent reduction in GHG emissions by around 32%. The reduction in GHG emissions was also significant (P<0.001) over a six-hour interval between each two PAC concentrations, as shown in Figure 5.6a. The results indicated that the lowest amount of GHG of 29 mL registered at 2% of PAC, followed by 30.76

mL at 0.5% PAC, then the highest value of emissions was 66.4mL and 36.33mL at 0% PAC and 1% Lantana, respectively. The reductions of GHG were 37% and 36% by adding 0.5% and 2% of PAC respectively compared to the control (0% PAC and 0% Lantana). The reductions in GHG emissions were 6% and 7% at 0.5% and 2% compared to Lantana treatment.

For further evaluation, the effect of PAC on Lantana plant parts at hourly intervals (0-6) hours was tested in this study. Figure 5.6b shows the GHG emissions reduction over a six hour period. As mentioned earlier, the same concentrations, 0% (PAC and La), 1% La, 1% La, 0.5% PAC and 1% La and 2% PAC, were used. The results indicated that the lowest yield was with a 2% concentration of PAC. Lastly, Figure 5.6c shows the effect of PAC addition on CH_4 emissions from a dairy cattle diet. The results demonstrated that PAC affected ($P< 0.001$) CH_4 emissions. Moreover, three concentrations of PAC (0% PAC and La), (1% La), (1% La, 0.5% PAC) and (1% La and 2% PAC) respectively yielded values of CH_4 emissions of 0.23%, 0.15%, 0.07% and 0.06%. The CH_4 reduction shown was 47% and 40% by adding 0.5% and 2% of PAC respectively. To our knowledge, this is the first report describing the effects of PAC incorporated into the dairy cattle diet along with 1% (DM) Lantana leaves at various PAC treatment levels to treat Lantana toxicoses *in-vitro*. Our results indicate that PAC significantly reduced Lantadene A and B in the Lantana leaves and flowers. These results suggest that PAC may be an effective amelioration of GHG, along with toxic effects in a ruminant diet.



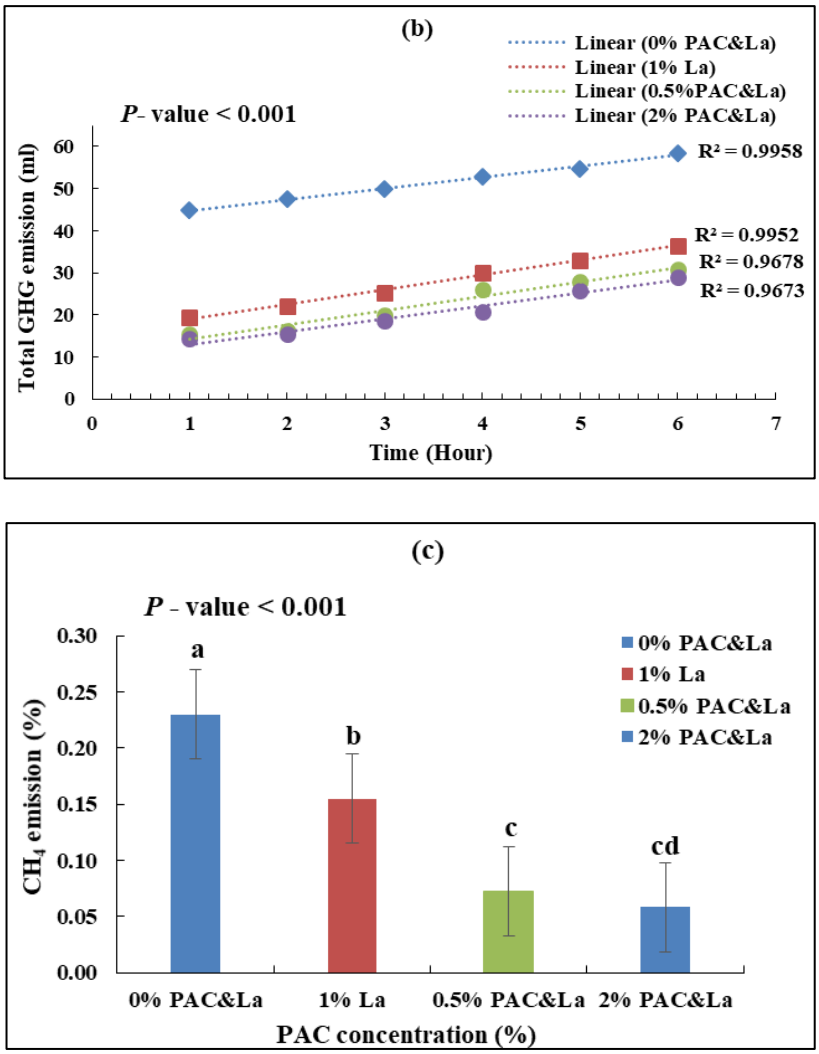


Figure 5.6: Effect of PAC addition on: (a) total GHG emissions; (b) GHG emissions over hourly intervals up to six hours :and (c) CH_4 emissions in dairy cattle: (T1: 0% PAC& Lantana, T2: 1%Lantana and 0%PAC, T3: 0.5% PAC & 1%Lantana, and T4: 2% PAC &1%Lantana).

5.3.4 The effect of PAC on VFAs, pH and feed fermentation in the in-vitro rumen from the diet (pellets and 1% Lantana leaf).

This study observed the effect of PAC on VFAs, pH and feed fermentation for the addition of Lantana into a dairy pellet diet as shown in Tables 5.3 and 5.4. Four concentrations of PAC were used and applied to study the effect of PAC on Lantana (leaves). These experiments (Treatments) were 0% (no PAC and La), 1% L, 0.5% PAC with 1%L and 2% PAC with 1%La. Furthermore, all experiments were conducted *in-vitro* and all the obtained results were statistically analysed using the SPSS software program. The obtained results were recorded in terms of mean and standard deviation (STD). Our findings show that there was no significant effect (P<0.05) for PAC addition to a dairy cattle diet with a toxic Lantana dose, in regard to VFAs, pH and

feed fermentation (for all Duncan ANOVAs, as shown in Tables 5.3 and 5.4). We can also observe that in Tables 5.3 and 5.4 there were some minor decreases in the results overall when the concentration of PAC was increased to 2%. For this reason, adding 0.5% PAC to the dairy diet was considered the best to maintain productivity.

Table 5.3: Effect of PAC on VFAs and pH in a dairy cattle diet

Items (%)	**Treatments (Mean ± STD)**				***P*-value**
	T1	**T2**	**T3**	**T4**	
Acetic acid	54.20±1.48	55.16 ±0.92	55.65±1.81	56.34±0.51	< 0.10
Propionic acid	5.60±0.72	5.11± 0.64	5.04± 0.92	4.60 ± 0.14	> 0.10
Butyric acid	41.20±0.80	39.72±1.53	39.31±0.92	37.91±0.29	< 0.10
pH	5.11±0.2	5.10±0.1	5.23±0.06	5.33±0.06	< 0.10

T1: 0% PAC& Lantana, T2: 1%Lantana and 0%PAC, T3: 0.5% PAC & 1%Lantana and T4: 2% PAC &1%Lantana

Table 5.4: Effect of PAC on feed digestibility in dairy cattle diet.

Items	**Treatments, (Mean ± STD)**				***P*- value**
	T1	**T2**	**T3**	**T4**	
Organic matter (%)	91.43±0.29	89.39±0.30	89.59±0.09	89.56±0.06	< 0.5
Ash (%)	10.57±0.26	10.33±0.14	10.47±0.14	10.54±0.06	< 0.5
Crude protein (DM) (%)	20.77±1.42	20.34±0.49	20.41±0.21	20.76±0.36	< 0.5
NDF (DM) (%)	24.42±1.25	23.68±0.49	24.26±0.21	23.44±0.36	<0.1
ADF (DM) (%)	13.63±0.35	13.19±0.19	13.46±0.49	14.34±0.14	< 0.001
Lignin (DM) (%)	4.55±039	3.32±0.10	3.80±0.37	4.36±0.40	< 0.1
Cellulose (%)	9.08±0.26	9.88±0.39	9.67±0.46	9.84±0.36	< 0.10
Hemicelluloses (%)	10.79±1.23	10.49±0.26	10.79±0.60	9.10±0.18	< 0.01

T1: 0% PAC& Lantana, T2: 1%Lantana and 0%PAC, T3: 0.5% PAC & 1%Lantana and T4: 2% PAC &1%Lantana

5.4 Discussion

PAC has been shown to be an important factor in improving rumen fermentation and reducing total gas emissions. This process may also lead to improved feed digestibility as well as increased dairy industry production. One of the most important benefits of PAC dietary supplementation is reducing the associated toxins of Lantana. Thus, this study focused on the toxins found within Lantana.

During our research, the private farm being used for our project experienced an episode of Lantana poisoning within young heifers, resulting in a high mortality rate. This halted our proposed on-farm research. Activated charcoal is often given to livestock that is showing signs of Lantana poisoning, with supportive therapy, as an effective method of treatment (Pass et al. 1984). Thus, the first objective in this study was to determine the amount of Lantana toxins, namely Lantadene (A and B) that could be absorbed by our coconut PAC. In this research, different parts of the Lantana plant such as berry, flower and leaf were used to evaluate the effect of PAC adsorption. Consequently, this study firstly focuses on measuring the chemical control reference readings of Lantana by using Fourier transform-infrared spectroscopy (FTIR). Our results in **Section 5.3** showed that Lantana plant components exhibit more or less the same FTIR spectra with some minor shifting in the peaks. Secondly, studying the effect of PAC on GHG, CH_4 and VFAs when it was added to Lantana leaves. Our results indicate that firstly the PAC tested was effective in reducing lantandene A and B from the Lantana leaf and flower extracts. Secondly, more Lantadenes were removed when the level of PAC was increased.

These results also have suggested that addition PAC might be highly variable in its ability to alleviate the Lantana toxic effects in dairy cattle, if not supplied at the required dose to effectively remove the Lantadene toxins from the rumen fluid *in-vivo*. Some studies reported that the current recommended dose for Lantana poisoning is drenching with a slurry containing 2.5 kg activated charcoal in 20 litres of electrolyte replacement solution for cattle (500 g in 4 litres for sheep and goats (Pass et al. 1984). That means that the activated charcoal is considered an effective but expensive poisoning antidote, particularly if the antidote is purchased in small quantities, typically $50 per 200g aimed at pets rather than livestock. If purchased in bulk quantities circa 500kg, the cost of PAC is around $8/kg, meaning that a treatment of 2.5kg is about $20, which is not high when compared to the general cost of a milking

cow in Queensland. Given that an average Qld cow produces $760 of milk per year (2016 Figs) so $2274 over three years from milk alone, PAC appears to be a cost-effective therapy for Lantana poisoning if landholders purchase in bulk.

Sharma (2017) reported using charcoal to decolourise the Lantana extract, prior to measuring Lantadene levels. This use almost certainly removed a significant portion of the Lantadenes from their samples, giving far lower results than the true level of Lantadenes in the plant. However, based on the above explanations, this is the first *in-vitro* study for dairy cattle dietary PAC effect on Lantana toxicity and further investigation will be required to clarify the effect of PAC on Lantana toxicity in *in-vitro* and *in-vivo.* In particular, our findings show that the addition of PAC (2%) to Lantana leaves significantly reduced GHG and CH_4 production. Moreover, it did not have any negative effect on the digestibility of feed, VFAs and pH, which remained similar to the controls. The lowest total gas production was achieved with 2% PAC. In order to strengthen the *in-vitro* studies, the investigation of the VFAs was also carried out in this research. It was evident from the obtained results that the VFAs production was not affected by adding PAC. Thus, PAC shows the greatest potential for reducing CH_4 production without loss of degradability.

To highlight more results, a variety of studies have been reported that PAC has the ability to reduce total gas production. Bhatta et al. (2013) reported that the tannins in *Lantana Camara* did not affect methane production (*in-vitro*), while other plants, such as *Sapindus laurifolia*, have a potential to suppress *in-vitro* methanogenesis. However, their comparison is limited since the paper does not identify whether the Lantana cultivar was of the toxic or benign variety. Biotransformation of toxic feed by rumen microflora is the first line of defence. While biotransformation of Lantadenes by rumen microflora has been reported not to occur (Sharma et al. 2003) other toxins can be detoxified by rumen microflora. Microbial detoxification by ruminal anaerobes has been reported for mimosine (Rodrigues 2014) and oxalates (Phaikaew et al. 2012).

Some research has shown that saponin- containing plant extracts suppress methane emission by reducing protozoal counts and changing the rumen fermentation parameters. Saponins, a form of pentacyclic triterpenes can have contradictory effects on rumen microflora. The addition of tea saponins (TS) in animal diets has been suggested to be an effective way to inhibit methanogenesis and hence has implications not only for global environmental protection but also for efficient animal production.

They have been reported to have an antiprotozoal effect, but little effect on the methanogen population in the rumen of sheep. Although the population remained unaffected, the presence of the tea saponins decreased Methanogenic activity indirectly due to the depressed ciliate protozoal population (Hristov et al. 2013). Around 10 to 20% of methanogens live in association with protozoa. Methanogenic archaea have been observed on the exterior surface of rumen ciliate protozoa (Vogels et al. 1980) and as endosymbionts within the ciliates where they simply provide a vehicle for retention of slower-growing methanogens in the rumen (Leng 2014).

While three different saponins also caused similar reductions in protozoal numbers, they also significantly affected methanogen numbers, in contrast to tea saponins. The saponins from *Sesbania* have been shown to significantly inhibit methanogens (78% reduction), which saponins from Fenugreek and *Knautia* had less effect, inhibiting only ~20% (Goel et al. 2012). However, it was shown that the decrease in methanogen population had no effect on methane production, suggesting that the remaining free, living Methanogenic microbes that do not associate with protozoa increased metabolism after the addition of saponins.

5.5 Chapter summary

This chapter used a methodology to study *in-vitro* the effect of PAC on absorption of Lantana toxins, GHG emissions and rumen fermentation. This study focused on the addition of PAC into the dairy cattle diet. In this research, different parts of Lantana such as fruit, flowers and leaves were used to evaluate the effect of PAC adsorption. The obtained results were analysed in the laboratory using Fourier transform-infrared spectroscopy (FTIR). The sample used for FTIR measurements of PAC was made up of 1g of PAC suspended in 30mL of methanol after 6 hours of shaking.

Several experiments were conducted in this study to evaluate the effect of PAC adsorption of Lantana toxic. The first experiment was conducted to study PAC concentration effects (0% to 2% with 1% Lantana) and treatment time effects on Lantana interactions with PAC.

The second experiment explored the effect of PAC on GHGs, VFAs, pH and feed fermentation using powdered Lantana-leaf to spike a dairy cattle diet. The performance of this study was evaluated in terms of *P-value* and the maximum peak

to peak FTIR observations. Our findings show that the addition of 0.5% and 2% PAC Lantana significantly reduced GHG and CH_4 production and it did not have any negative effects on the digestibility of the feed, VFAs or pH. The lowest total gas production was also achieved with 2% PAC, closely followed by the 0.5% PAC. In summary, PAC shows a great potential for reducing Lantana toxicity effects, GHG and CH_4 production, all without loss of productivity according to the precursor VFAs measured.

CHAPTER 6

FIELD EXPERIMENTS

6.1 Introduction

Methane produced by ruminants is a known contributor to greenhouse gas effects and global warming (IPCC, 2006). Ruminant methane emissions account for more than 50% of greenhouse gas (GHG) emissions released by milk production and this percentage can increase to around 80% in grassland fed scenarios (FAO, 2010). In ruminants, cellulosic feed materials are digested in the rumen by microbial fermentation that generates approximately 80% of the methane while the remainder is derived from the decomposition of manure (Vergé et al., 2007). These percentages are heavily influenced by dairy breed, food digestibility, feed types and animal housing, ranging from 60 to100% for enteric methane and between 0 to 40% for decomposition of manure (Rotz et al., 2010).

Farm livestock produces an enormous amount of manure that directly impacts agricultural land, soil, water and air quality by manure contamination, GHG emissions, nutrient leaching and odour (Larney et al. 2007; Holman et al. 2016). This manure is beneficial to soils, as an organic fertilizer, which reduces the amount of chemical fertilizer applied to the field and mitigates fertilizer runoff into waterways (Pandey et al. 2015; Pandey et al. 2018).

Elevated pathogens and pathogen indicator levels in manure have received considerable public attention because of associated public and animal health risks and the production of contamination (Zhang et al. 2015; Pandey et al. 2018). The supply of antimicrobials to livestock can also influence the levels of resistant bacteria in faeces and the feed ingredients in livestock diets can also vary the level of resistance in faeces microbiota (Zaheer et al. 2013; Vahjen et al. 2015) For these practical reasons, which collectively have a serious impact on agriculture, manure management in dairy farms and agricultural land is becoming more important, we hypothesized that the microbial complexity of dairy cattle manure should change with on farm manure treatment processes.

Supplementing cow diets with various biochars has been investigated as an alternative source method of improving soil fertility, available phosphorus and improvement of soil acidity (Joseph et al. 2015; Winders et al. 2019) . McHenry (2010) reported that feeding biochar to goats as a process of decreasing the input of chemical fertilisers to soil and improving soil properties through the deposition of the goat manure. Other uses of biochar include feeding sheep biochar for mitigation of toxicoses by absorption (Banner et al. 2000; George et al. 2000; Knutson et al. 2006; Rogosic et al. 2006), or feeding goats to improve nutrient digestibility (Mathew et al. 2001; Al-Kindi et al. 2017) and cattle (Leng et al. 2012a). However, there is a lack of information on the assessment and efficacy of powdered activated carbon (PAC) on the migration of GHG emissions during milking and performance of dairy cattle. Therefore, the objective of this study is to determine the effect Powdered Activated Carbon (PAC) at 0.5%/ dry matter (DM) of diet on the enteric methane (CH_4) emissions and performance of dairy cattle when incorporated into a concentrate pellet.

6.2 Materials and methods

6.2.1 Location and animals

In this research, the dairy cattle productivity and gas emissions measurements were taken from a commercial dairy farm that using a traditional milking system, located in Brymaroo, Queensland, Australia. The farm facilities use natural ventilation generally with some forced ventilation used in the milking shed area. Nearly 180 dairy cattle are free to forage during the day and have open air shelters available as required at night with free access to drinking water.

6.2.2 Powdered activated carbon (PAC)

In this chapter, a high activity microporous and adsorption capacity of powdered activated carbon (PAC) was used. PAC was purchased from Activated Carbon Technologies Pty Ltd, Victoria, Australia. More details are mentioned in chapter 3 **Section 3.2.1.**

6.2.3 Experimental design

All dairy cattle were fed a diet of pellets (Table 6.1). The daily cattle consumption was 6 kg of pellets diet feed and 40 kg of barley hay. Pellet diet feed (with and without PAC) was provided by Ridley Agriproducts Pty Ltd. The added PAC was added at 0.5% / dry matter (DM) of feed. Animal productivity and gas emissions (CO_2 and CH_4) of the dairy cattle were initially monitored without any PAC addition for four weeks to get baseline data. After that, PAC was added to their feed for six weeks and animal productivity and gas emissions were monitored for the last 4 weeks of each 6 week interval, i.e. after a two week adaptation period to allow animals to adjust to their assigned experimental diets. Similarly, the first 2 weeks of the subsequent 6 week intervals were used to adapt animals to their dietary changes with and without PAC addition. The purpose of this repeated series of feeding was to obtain accurate measurements and statistical validation.

Table 6.1: ingredients of dairy cattle diet.

Ingredient	**As fed**	**Dry matter at 89%**
Total Crude Protein	16.0 %	18.0 %
Crude Protein (minimum)	13.1 %	14.7 %
Equivalent Crude Protein	2.9 %	3.2 %
Urea (maximum)	1.0 %	1.1 %
Crude Fibre (maximum)	12.0 %	13.5 %
Crude Fat (minimum)	1.5 %	1.7 %
Calcium (minimum)	1.0 %	1.1 %
Phosphorus (minimum)	0.5 %	0.56 %
Magnesium (minimum)	0.4 %	0.45 %
Salt (maximum added)	0.5 %	0.56 %
Copper (added)	45 mg/kg	50 mg/kg
Zinc (added)	150 mg/kg	170 mg /kg

6.2.4 GHG emissions before, during and after milking dairy cattle.

The GHG measurements (CO_2 and CH_4) were conducted on a weekly basis at the dairy cattle farm using a portable diffuse flux meter with an LI-COR CO_2 detector (see Figure 6.1). The instrument contains a collecting chamber, personal digital assistant (PDA), a backpack and a robust container for shipping and transportation. The collecting chamber of the instrument was installed in the ventilation hatch of the milking area to cover all the GHG emissions areas. The approach used to measure emissions is an adaption of the polyethylene Tunnel method employed for sheep (Lockyer et al. 1995). The milking shed is covered on 3 sides to minimize effects of prevailing winds and uses forced ventilation to ensure consistent airflow and environment within the shed. Milking is done in the early morning and little variation was observed in wind speed was observed during the collection time of 5-6am over the interval.

Before field measurements were conducted, the CO_2 flux meter was calibrated using 0% and 4% CO_2 standard gases. The analytical error associated with a single measurement was about ±5% and the reproducibility was about ±10% for the range of 100–10,000 g m^{-2} day^{-1}. In addition to the standard calibration done at 1% or 10,000 ppm for CH_4, the fluxmeter was further calibrated using a more representative GHG levels with a 5L tedlar bag of inert N_2 and a similar Vessel with a 100ppm concentration of CH4, with average results within +/-1% over a 5 min sample period (i.e. average of 300 single point measurements over this period).

The GHG measurements were divided into three stages. The first stage measured the GHG 15 minutes before milking. In the second stage, GHG measurements were throughout the milking of around 180 dairy cattle. The third stage was the GHG measurements when the milking of the dairy cattle was finished and after the dairy cattle left the milking area. This farm experiment continued for 22 weeks. The usual time of starting experiments was 5:30 am. The dairy cattle productivity data (milk quality and quantity) were taken from the farm record. The GHG measurements were done with and without PAC supplements. Our Emissions monitoring has been made available in conjunction with an on-going Landcare community project with Animal ethics approval. (*"Use of Non-invasive Techniques to Estimate GHG Emissions on Working Farms*" CA2020/01/1338, 20/01/2020 – 24/05/2022).

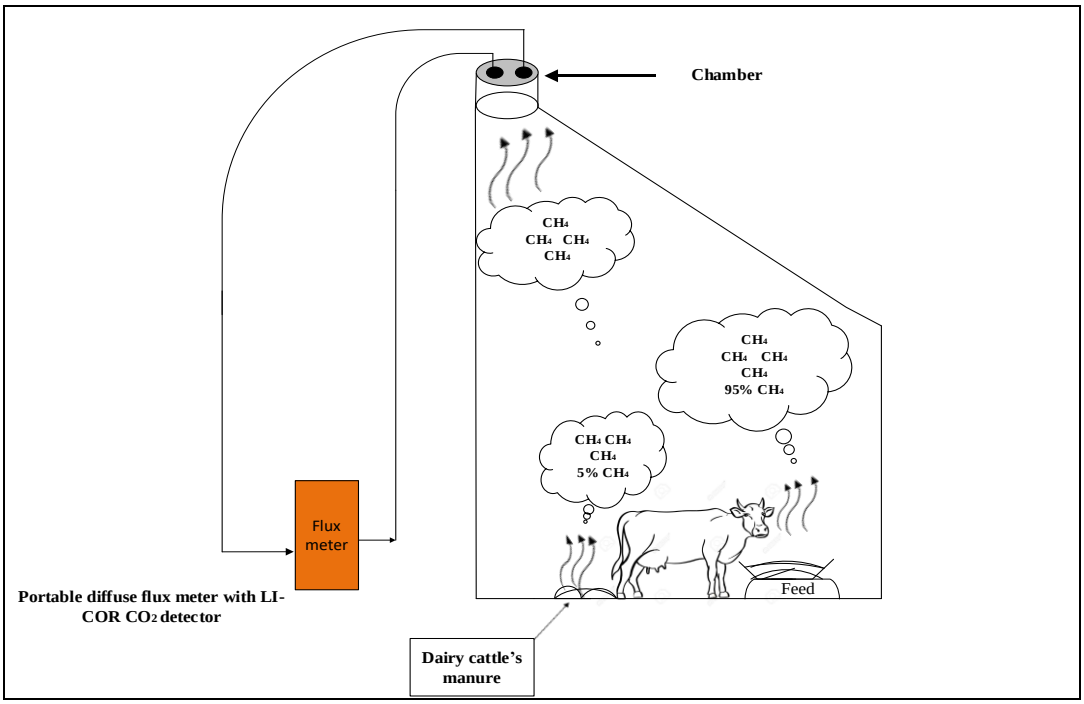


Figure 6.1: Measuring GHG emissions from milking area by flux meter with the LI-COR CO_2 detector.

6.2.5 Measuring GHG and CH_4 emissions from manure

GHG emissions (CO_2 and CH_4) from manure on the ground of the barn were measured by using a portable diffuse flux meter with the LI-COR CO_2 detector. Gas emissions from the manure of the dairy cattle had been monitored for four weeks before PAC supplementation. After that, GHG emissions were measured with PAC supplementation for four weeks. GHG emissions were then measured for another four weeks without PAC supplementation. The last four weeks been examined PAC supplementation. After each stage of measurements, the ground of the barn was cleaned completely before two weeks to obtain accurate data. The portable diffuse flux meter with the LI-COR CO_2 detector was located in the same area in the ground of the barn and the period of measurement was 15 minutes every week. In the last stage, the collected data were transferred using a computerized instrument part.

6.2.6 Manure collection

In this research, fresh manure samples were collected from the milking area for 4 cattle, using their unique dairy cattle ID tags. Five subsamples of each faeces pad were

col-lected to represent a single faecal pad for each cow. Faecal materials were placed in a small zip-lock plasticfood grade polyethylene bags after removing the air before sealing. All the manure samples were kept in a chilled thermally insulated container. After that, the manure samples with and without PAC supplements were sent to the laboratory in the UQ in Gatton for manure chemical components testing.

6.2.7 Archaeal and bacterial 16S rRNA gene sequencing

Five grams (g) of each faecal subsample collected as per section 6.2.6 from cattle diets with and without PAC (n=4 in both cases) were analysed. All the manure samples were kept in a chilled thermally insulated container and sent to the Federation University in Ballarat, Victoria (VIC), Australia for DNA extraction. DNA was extracted using the Power Soil kit following the manufacturing conditions and quality of the DNA assessed using nano-drop. Extracted DNA was processed at the Australian Genome Research Facility Ltd (Brisbane, Queensland, Australia) for microbial communities identification, using the Illumina MiSeq platform. Target 341F, 300 bp, Forward Primer CCTAYGGGRBGCASCAG

Reverse Primer GGACTACNNGGGTATCTAAT were used for PCR-amplification. Illumina Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5). Primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8.4), USEARCH (version 8.0.1623) and UPARSE software. Using USEARCH tools, sequences were quality filtered full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered and chimeric sequences were filtered using the “rdp_gold” database as a reference. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using QIIME. Diversity profiling amplicon generation and sequencing workflow are shown in Figure (6.2).

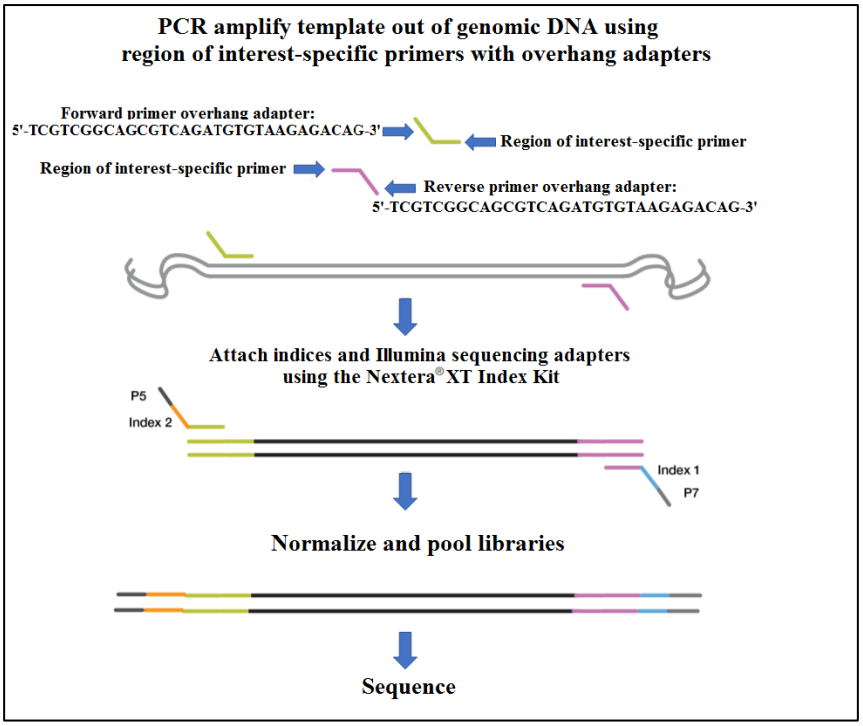


Figure 6.2: Diversity profiling amplicon generation and sequencing

6.2.8 Microbiome beta and alpha diversity analysis

For α-diversity indexes both rarefaction and non-filtering of the abundance table were performed using R studio. Measurements of alpha diversity were computed using the "Phyloseq" R package and the computed indices were compared for significance differences post PAC treatment using independent 2-group Mann-Whitney U Test = wilcox.test test using the ggrub Package. For the rarefaction method the set.seed (123) was used to initialize repeatable random subsampling.

For beta diversity, the Aitchison distance method was used with a centred log-ratio transformation approach as described by Gloor et al., (2017) for compositional datasets. Prior to transformation the dataset was filtered to only contain OUT that were detected at least 1 in 2 of the 8 samples. ps4 <- core (ps, detection = 1, prevalence = 2/n samples (ps)) Using this method to calculate ordination scores (eigenvalues) a redundancy analysis was selected and plotted. To generate a distance matrix, the Euclidean method was selected and the Adonis function from the Vegan R package was used to compute a PERMANOVA for identification of significant clustering based on treatment using $P<0.05$. The betadisper function from the Vegan R package was used to test for homogeneity.

6.2.9 Statistical analysis

The Statistical Package for the Social Sciences (SPSS-version 23) software was used to analyse the experimental data (Swan and Sandilands 1995) and involved the analysis of variance (ANOVA). Means of milk production, GHG emissions, Archaeal and bacterial diversity were compared using Duncan at 5% level of probability. Statistical analyses were graphically assessed by means of residual plots; and normalisation of data was not required. Linear regression analyses were used to describe the relationships between GHG emissions and times of measurement (before, during and after milking). The Co-factors were intervals of milking and PAC rates. Analytical values are reported as the mean ± standard deviation (SD).

6.3 Experimental results

A number of tests were conducted in this study to evaluate the effect of PAC on the total gas production and performance of dairy cattle. The tests used different concentrations of PAC (0 and 0.5%). The same methodology in Section 2 was used with all the experiments to extract and analyse the results. In this section, we will firstly show the obtained results and then we will briefly discuss the effect of the PAC on milk quality and quantity, GHG emissions and Microbial community.

6.3.1 The effect of PAC on dairy milk production

Figure 6.3 shows the effect of PAC at 0.5% by DM of diet on milk production quantity and quality for a working dairy farm. These results combine the milk production from 180 dairy cattle located in Brymaroo, Australia. Figure 6.3 (a) presents the daily milk production before and after adding 0.5% of PAC to their pellet diet. Although significant, this addition improved the daily milk production by 3.43% on average for the herd. Figure 6.3 (b) compares the percentage of milk protein with and without PAC. PAC supplementation significantly increased the (P<0.05) milk protein by 2.63% and milk fat was significantly increased (P<0.001) by an average of 6.32% as shown in Figure 6.3 (b).

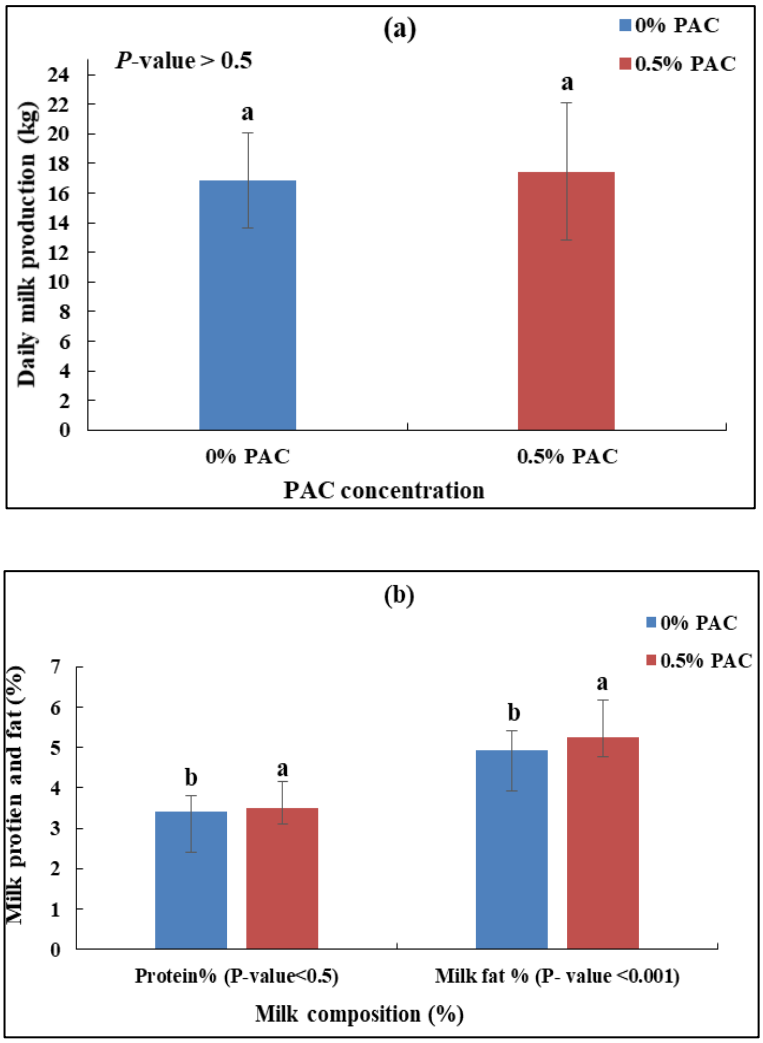


Figure 6.3: (a) Effect of PAC on daily milk production (kg), (b) Milk protein and fat (%).

6.3.2 Total GHG and CH_4 emissions before, during and after milking

CH_4 emissions were measured over four weeks (first test is called term1). CH_4 emissions were affected by all two factors: PAC concentrations and terms, as shown in Figure 6.4 (a-b). CH_4 measurements were taken under the same circumstances and conditions. Figure 6.4 (a) presents the effect of two percentages of PAC concentrations on the CH_4 emissions before, during and after milking processes in the dairy cattle farm. PAC concentration contributed to reducing the CH_4 emissions during milking from 25.03ppm to 16.55ppm (8.5 ppm reduction or 33.8%) when it was added into the feed with 0.5%.

Figure 6.4 (b) shows the effect of the interaction between two factors: PAC concentrations and terms. Before milking, in term one, the CH_4 emissions were reduced slightly from 3.84 to 3.14 ppm at 0.5% respectively, while they were reduced

significantly (P<0.001) in term two, with 4.59 ppm and 2.84 ppm at 0% PAC and 0.5% PAC respectively. CH_4 emissions during milking in term one also declined significantly (P<0.001) by adding 0.5% of PAC from 29.25 ppm to 15.97 ppm respectively. In term two, CH_4 emissions were also reduced significantly (P<0.001) from 20.81ppm to 17.13ppm by adding 0.5% PAC. The difference between terms one and two was 9.6ppm. Besides that, CH_4 emissions declined significantly (P<0.001) after milking dairy cattle in both terms (one and two). In term one, CH_4 production reduced from 6.10 ppm to 4.95 ppm by adding PAC. In term two, CH_4 dropped from 7.05 ppm to 4.24 ppm by adding PAC (Figure 6.4b).

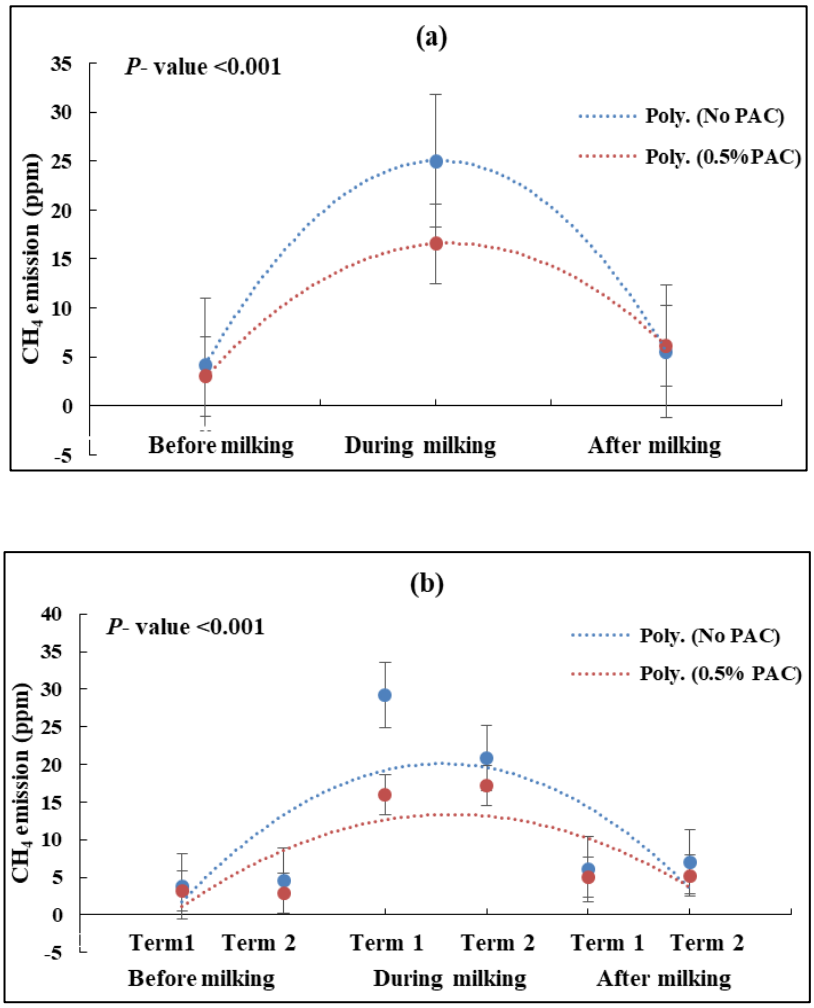
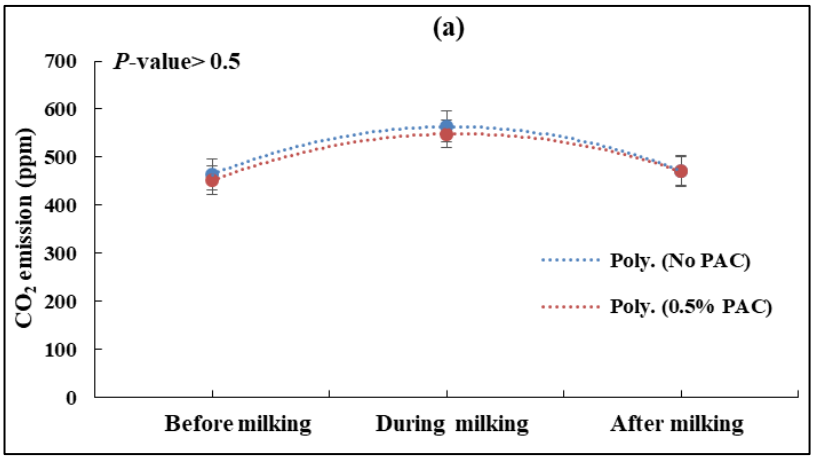


Figure 6.4: (a) Effect of PAC concentrations on the CH_4 emissions and (b) Effect of interaction between PAC, period and terms on the CH_4 emissions. Polynomial (Poly), Term1: First test (four weeks), Term 2: Second test (four weeks).

In the in-vivo experiment, following the two terms of on-farm measurement of CO_2 emissions before, during and after milking dairy cattle were measured. The PAC slightly reduced the amount of CO_2 before, during and after milking of dairy cattle as shown in Figures 6.5 (a and b). The amount of CO_2 before milking was 463 and 452 ppm at 0% and 0.5% PAC respectively, as shown in Figure 6.5a. The amount of CO_2 during milking was 563 and 5.48 ppm at 0% and 0.5% of PAC respectively as shown in Figure 6.5a. The amount of CO_2 after milking was 472ppm and 470ppm at 0% and 0.5% PAC respectively, as shown in Figure 6.5a.

Figure 6.5(b) shows the effect of interaction between PAC and terms (1 and 2) (before, during and after milking) on the CO_2 emissions from dairy cattle on the farm. Before milking, in term one, the CO_2 emissions increased slightly from 444 to 451 ppm at 0% and 0.5% of PAC respectively, while they were reduced significantly (P<0.5) in term two, in which it was 484 ppm and 452 ppm at 0% PAC and 0.5% PAC respectively. CO_2 emissions during milking in term one were increased by adding 0.5% of PAC from 440ppm to 451ppm, while in term two, CO_2 emissions were reduced slightly from 683 ppm to 646 ppm by adding 0% and 0.5% of PAC respectively. At the same time, CO2 emissions increased after milking dairy cattle in term one. In term one CO_2 production raised from 444ppm to 451ppm by adding 0% and 0.5% of PAC respectively, whereas, in term two, CO_2 did not affect production when 0.5% was added to the diet of dairy cattle, as shown in Figure 6.5(b).



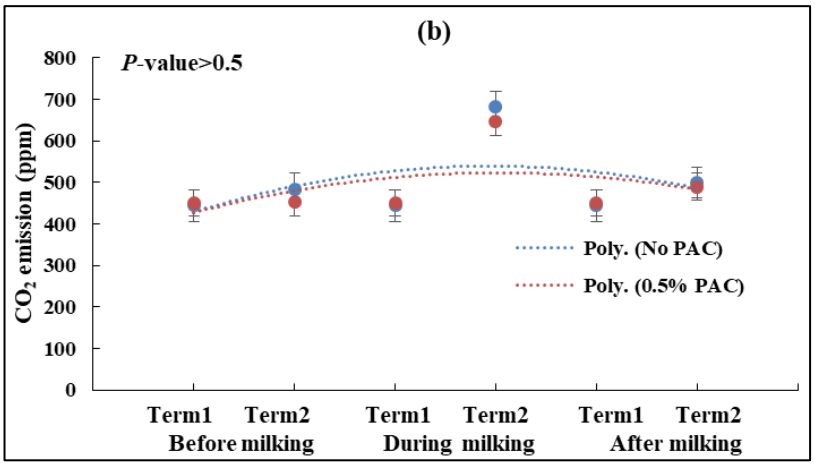


Figure 6.5: (a) Effect of PAC concentrations on the CO_2 emissions and (b) Effect of interaction between PAC, period and terms on the CO_2 emissions. Polynomial (Poly), Term1: First test (four weeks), Term 2: Second test (four weeks).

6.3.3 Total GHG and CH_4 emissions in dairy cattle manure.

We also tested the dairy cattle manure to investigate how the addition of PAC in the diet affected the GHG emissions and other parameters. GHG emissions were measured before and after the addition of PAC in the dietary pellets. Figure 6.6a shows the overall results of CH_4 emissions from manure with and without PAC. Although there was a slight increase in the manure CH_4 emissions, from 7.42 ppm to 7.49 ppm with the addition of 0.5% PAC, this was barely significant (P<0.10). In this study 3 manure test sites in the common area was studied to give an indication of any changes in manure emissions. In order to evaluate if this slight change is real or otherwise, a comparison of samples from specific cattle with and without treatment would be required.

Figure 6.6(b) presents the interaction between PAC and term on CH_4 emissions from dairy cattle manure before and after the cattle were fed PAC. CH_4 emissions in term one with and without PAC did not change. They were 9.14 ppm and 9.2 ppm at 0% and 0.5% of PAC respectively for the term one, while there was no effect in term two. In a word, it can be concluded that the PAC had not reduced CH_4 from the manure of dairy cattle.

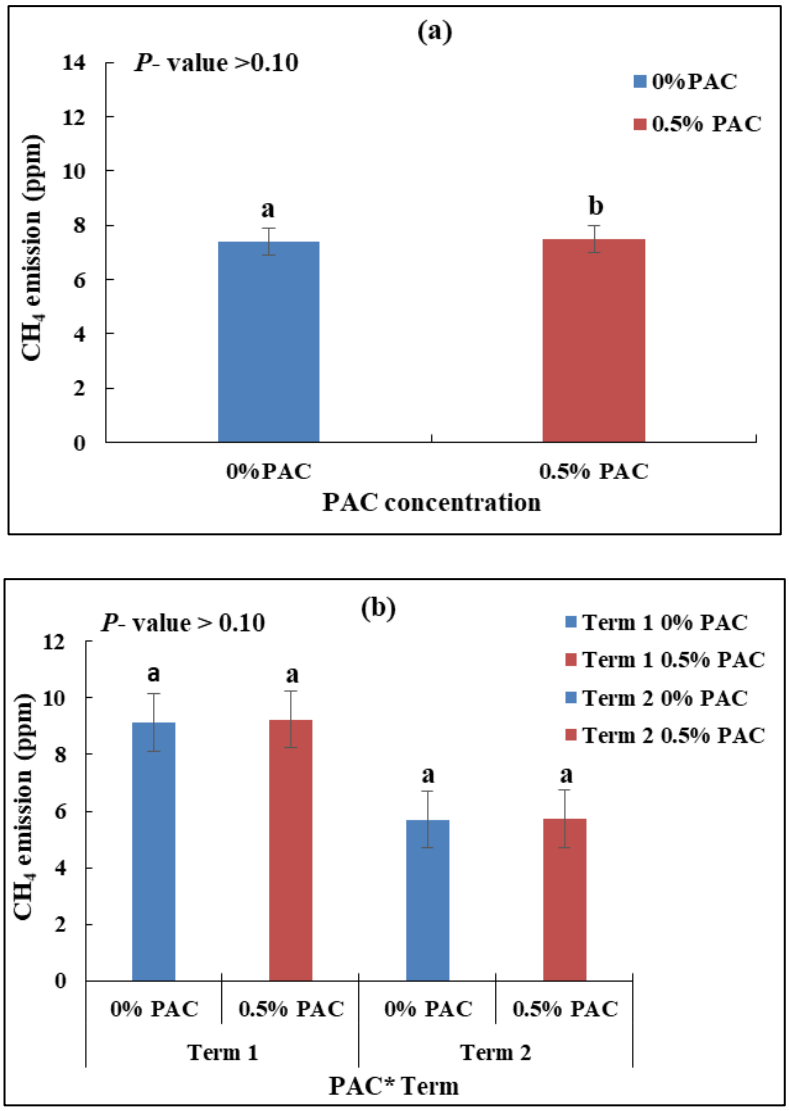


Figure 6.6: (a) Effect of PAC concentrations on the CH_4 emissions from manure and (b) Effect of interaction between PAC and terms on the CH_4 emissions from manure. Term1: First test (four weeks), Term 2: Second test (four weeks).

CO_2 emissions were affected by PAC concentrations over time and terms, as shown in Figure 6.7(a-b). Figure 6.7(a) presents the effect of two percentages of PAC concentrations on the CO_2 emissions from manure before and after being fed PAC. The presence of 0.5% PAC in the diet has significantly reduced (P<0.001) the CO_2 emissions from 1631ppm to 1464 ppm. Figure 6.7(b) shows the interaction effect of the PAC and terms to estimate the PAC behaviour. In term one, the CO_2 emissions dropped from (approximately 9.8% reduction) 1461ppm to 1318ppm at 0% and 0.5% PAC respectively. Similar results showed in term two and the effect of PAC was clear, as shown in Figure 6.7(b). CO_2 emissions were reduced from 1789ppm to 1609ppm.

The effect of PAC has had a similar trend in terms one and two as shown in Figure 6.7(b).

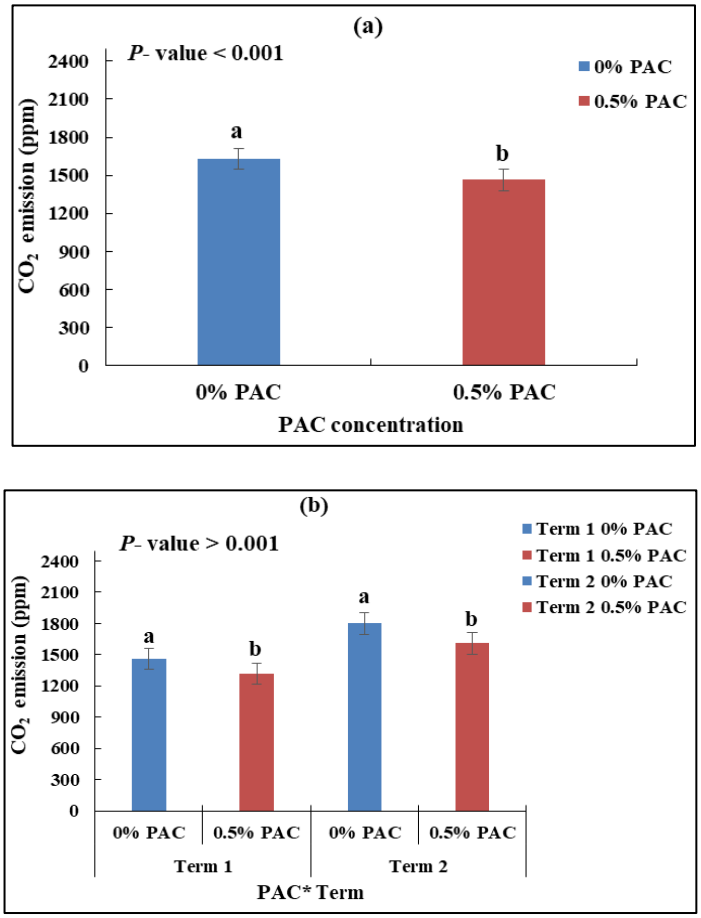


Figure 6.7: (a) Effect of PAC concentrations on the CO_2 emissions and (b) Effect of interaction between PAC and terms on the CO_2 emissions, from the manure dairy cattle manure. Term1: First test (four weeks), Term 2: Second test (four weeks).

6.3.4 The effect of PAC on the bacterial and archaeal microbial in dairy cattle's manure.

6.3.4.1 Microbial community analysis.

16S rRNA Gene Sequencing was performed by Next Generation Sequencing to determine the collective population of prokaryotic bacteria and *Archaea* to characterize the specific Methanogenic communities in dairy cattle manure from cattle fed one of two pelleted diets: either a basic diet or an equivalent diet supplemented with 0.5% PAC. By the use of an array of specific primer pairs, the Next Generation Sequencing captured a diverse range of bacteria in both manure groups. The sequencing and classification of microbiota following quality control methods resulted in an abundance table of 1379 taxa. The average library size was 27419.625 sequences with the lowest library size being 7814 sequences, the largest library size being 53750 sequences as shown in Figure 6.8.

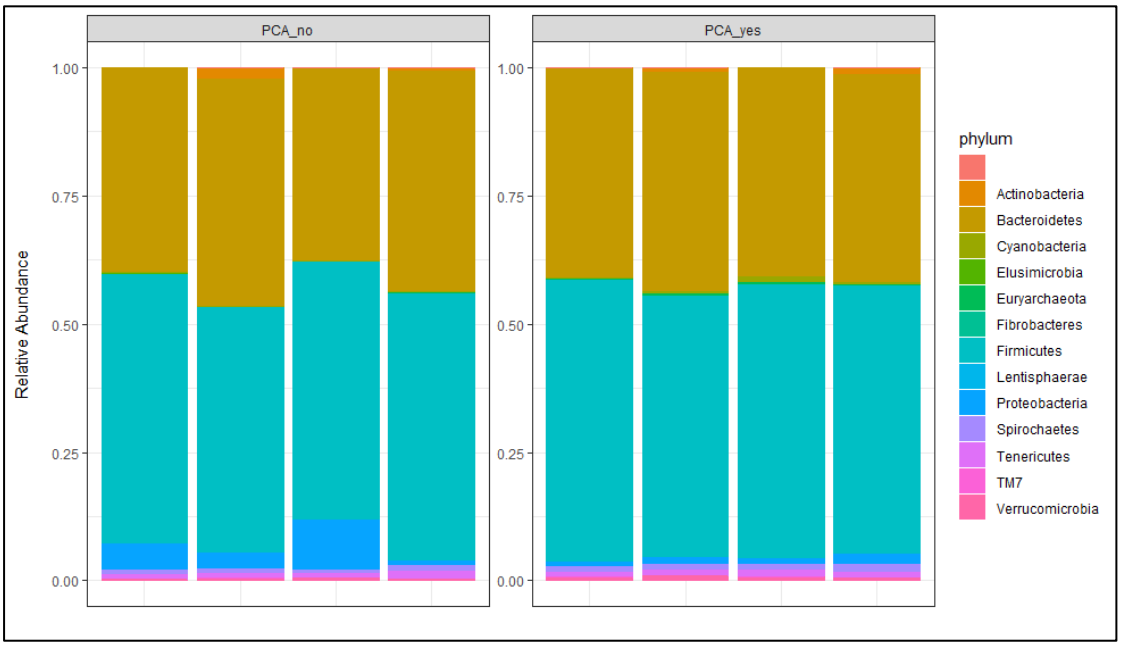


Figure 6.8: the sequencing and classification of microbiota with and without PAC additions.

The vast majority of bacteria in the manure samples (over 90%) belonged to two phyla: Bacteroidetes and Firmicutes (Figure 6.8). However, whether or not the diet was supplemented with PAC, there were no significant changes in the percentages representing Bacteroidetes and Firmicutes populations (53% to 51%; 42% to 41%respectively; Figure 6.9 (a and b).

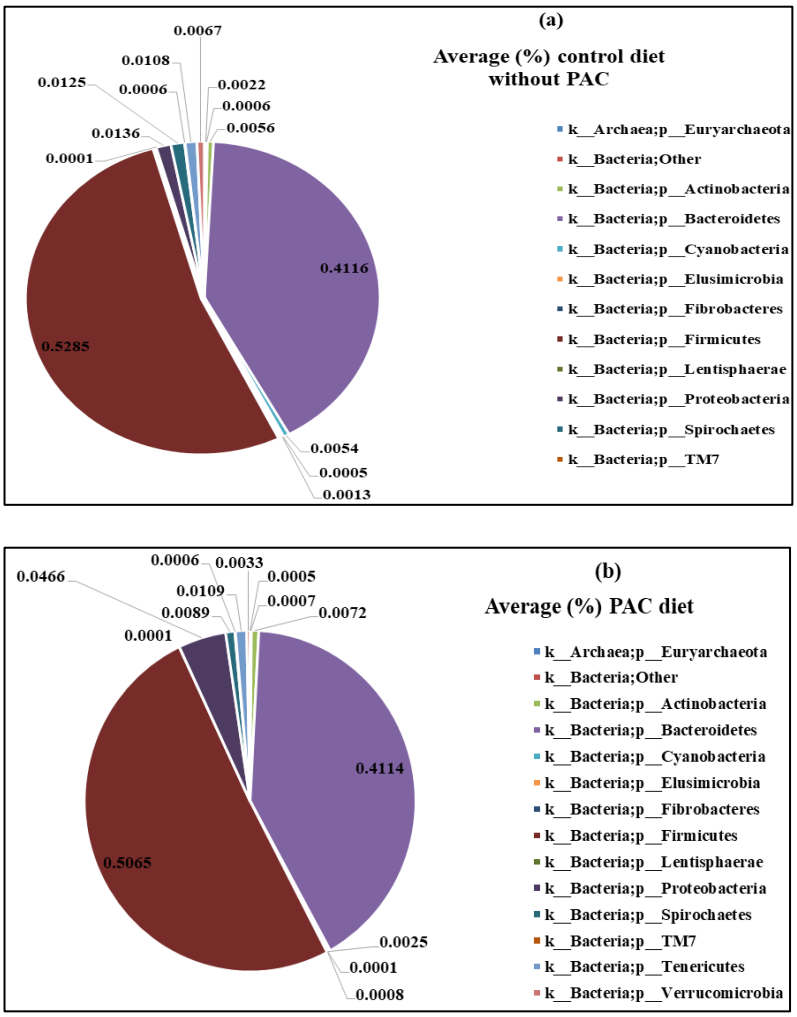


Figure 6.9: (a) Average pre of microbial community and (b) Average post of microbial community.

In contrast, there were also very small percentages of members from other phylums including Protoeobacteria, Spirochaetes, Tenericutes, Verrucomicrobia, Actinobacteria and Archeobacteria (ranging overall from 0% to 5%). Since there were no significant changes with the two major phyla (Bacteroidetes and Firmicutes), we removed them from the graph to allow better-quality comparison of changes within the minor phyla due to PAC supplementation (Figure 6.10(a) and (b)).

There were no significant changes in the levels of some of the minor phyla; specifically TM7, Actinobacteria and "other" groups (remaining at 1%, 9% and 1% respectively). Among the other minor phyla, there were some significant changes after PAC supplementation (Figure 6.10a and b). There was a significant decrease in the proportion of Proteobacteria (from 57% to 23%). All other phyla groups showed significant

increases: Spirochaetes (from 11% to 21%); Cyanobacteria (from 3% to 9%); Verrucomicrobia (from 4% to 11%); Tenericutes (from 13% to 18%); Elusimicrobia (from 0% to 1%); and Fibrobacteres (from1% to 2%) (Figure 6.10(a) and (b)).

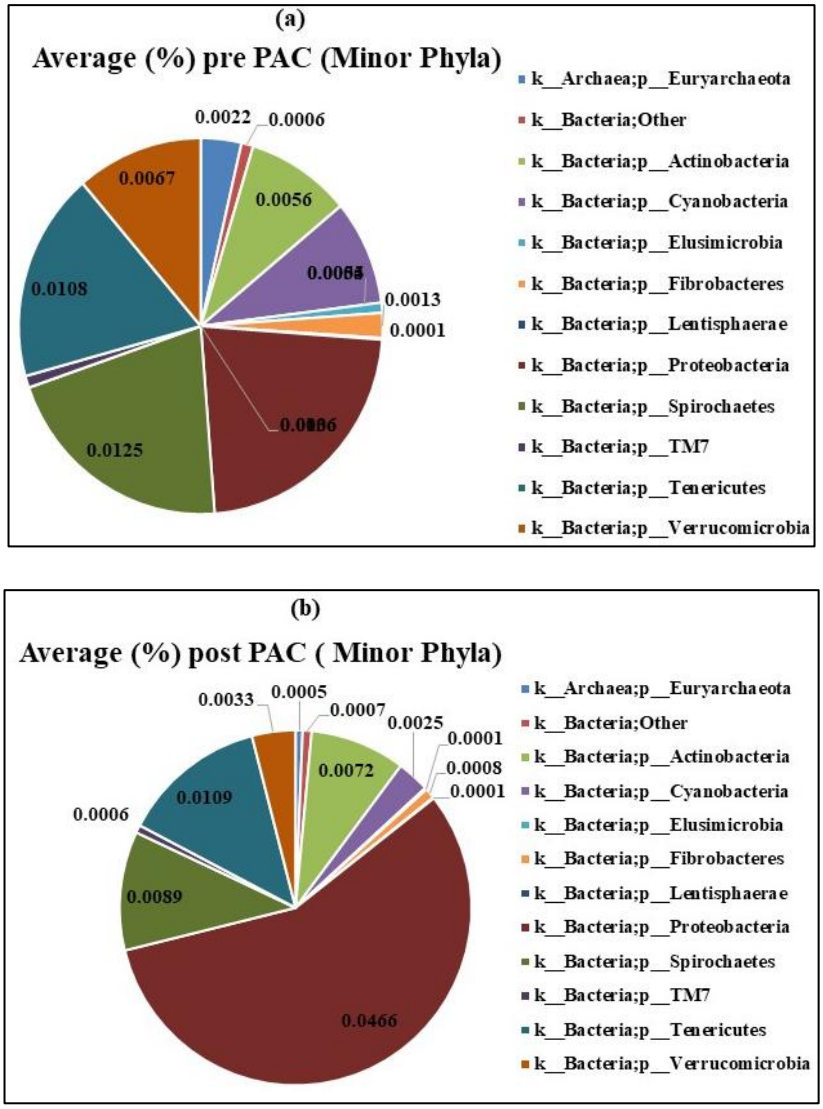


Figure 6.10: (a) Average pre PAC of microbial community (minor phyla) and (b) Average post PAC of microbial community (minor phyla).

Specifically for the members of the phylum Euroarchaeota within the Kingdom Archeae, which harbors the methanogen bacteria, there was a significant increase, when the diet was supplemented with PAC (from 1% to 4%). Figure 6.11a shows the methanogen species in dairy cattle manure with no PAC supplementation. The majority of archaea were members of the genera *Methanobrevibacter* (83%) and genera of the family Methanocorpusculaceae (42%) with very low levels of *Methanosphaera* (3%) and vadin CA11-related genera of the family Methanomassiliicoccaceae (2%). In contrast, there was a significant change within the

Methanogenic community in the manure from dairy cattle supplemented with 0.5% PAC (Figure 6.11(b)). There was a significant decrease in the proportion of members of the genera *Methanobrevibacter* (from 83% to 51%) with a concurrent significant increase in genera of the family Methanocorpuscuralceae (from 12% to 42%). The percentages of the other two minor genera also showed a minor increase, although their proportion remained low (*Methanosphaera* (an increase from 3% to 4%) and members of Methanomassiliicoccacea (from 2% to 3%).

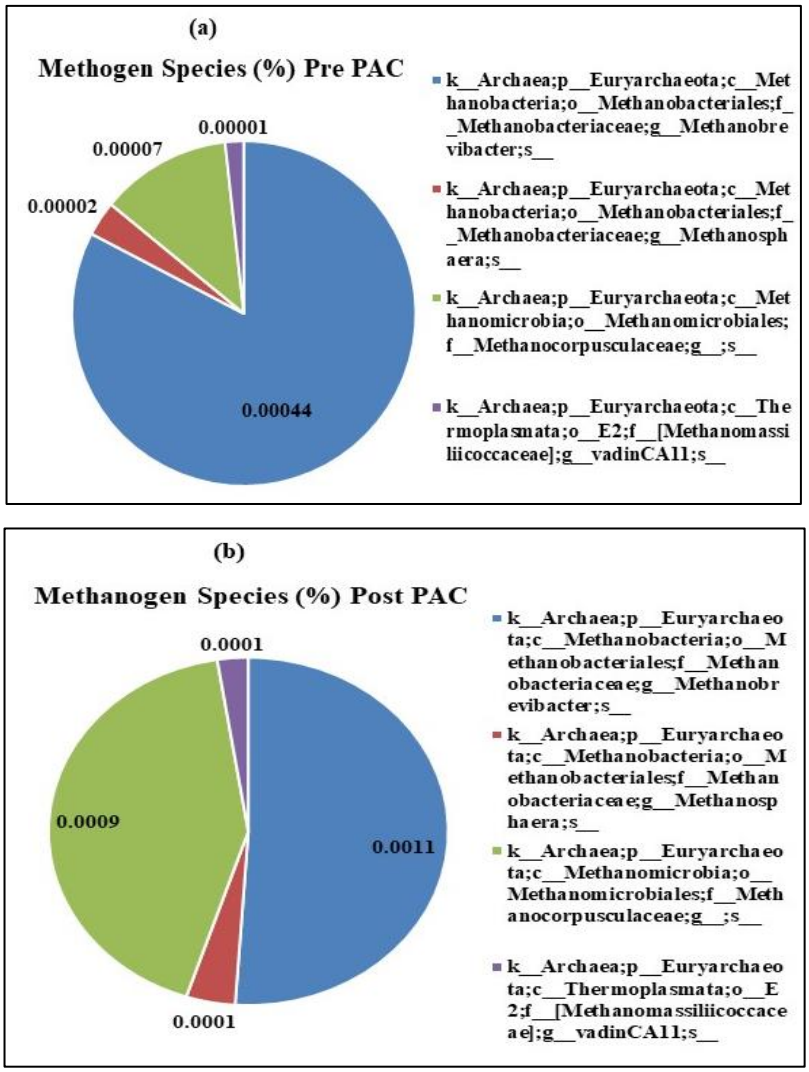


Figure 6.11: (a) Methanogen Species in the control diet (no PAC) and (b) Methanogen Species in a diet supplemented with 0.5% PAC.

Faecal samples (n=4) from randomly selected animals pre and post PCA supplementation were tested for changes in alpha and beta diversity. For alpha diversity, using both the rarefaction (Figure 6.12) and non-rarefaction approaches (supplementary file) there was an increase in the different methods used to measure

species richness. Rarefaction resulted in a reduced sample size of 7776 sequences in each sample for comparison. Significance increases in alpha diversity using the methods of Observed and Shannon index were identified (Figure 6.12).

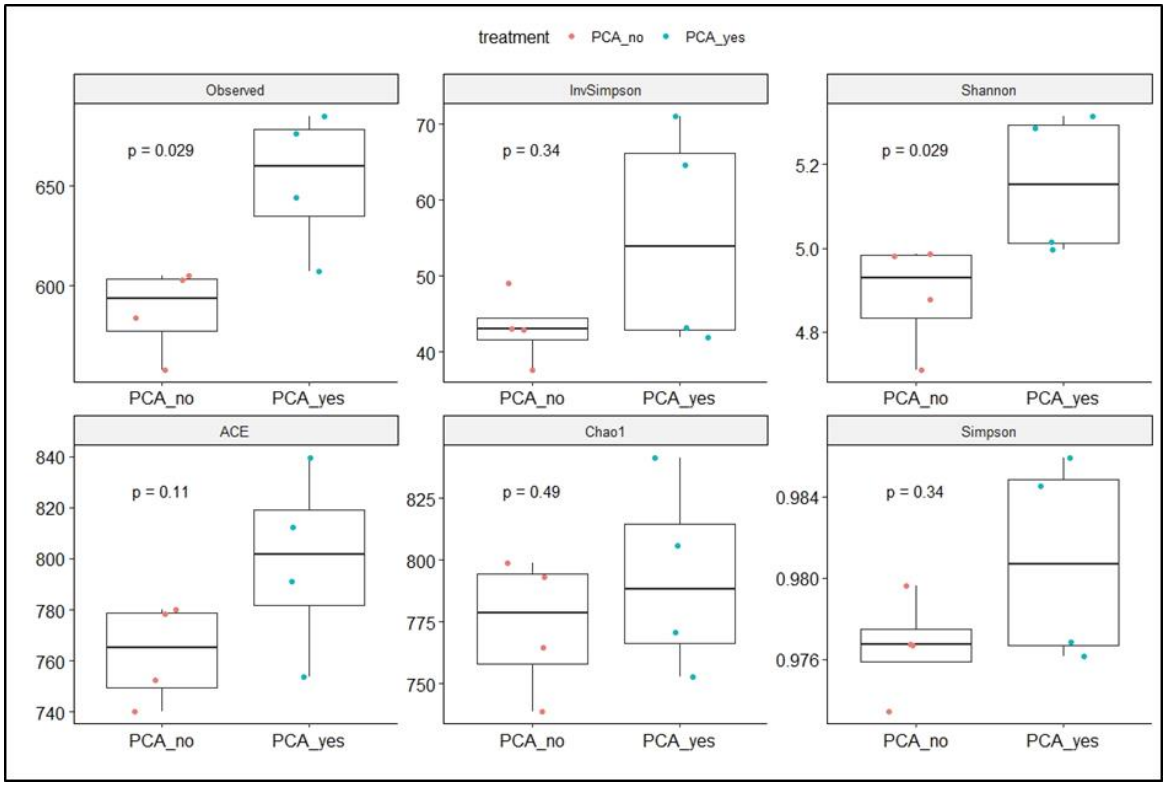


Figure 6.12: Alpha diversity calculations on a rarefaction dataset (p_3) following prevalence filtering (of 7814 sequences per sample.

PAC supplementation was also shown to result in a difference in overall microbiota composition between the two groups. Filtering of the dataset resulted in a reduced number of taxa (n=773). Redundancy analysis identified that 26% of the variation between the two datasets could be explained by the first component of (Figure 6.13(a)). Ordination analysis resulted in the unsupervised clustering into treatment groups as shown (Figure 13(b)). with a PERMANOVA indicating that treatment had a significant effect on microbiota composition ($r^2 = 0.22$, $p = 0.035$).

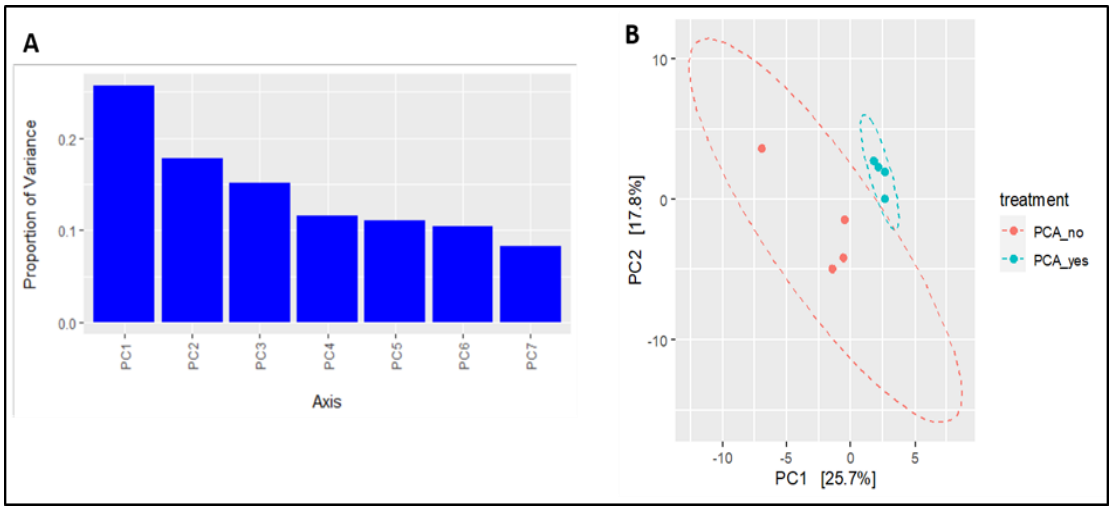


Figure 6.13: Overall microbiota composition difference between treatments (with and without PAC addition).

6.3.5 Chemical compounds of manure

Manure samples were collected to evaluate the effect of PAC on chemical compounds of manure as shown in Table 6.2. Dry matter (DM) is significantly increased (P<0.001) by adding 0.5% PAC. The percentages of DM were 88.56% and 89.11% at 0% and 0.5% PAC respectively in the dairy cattle manure. Organic matter also was increased (P<0.02) by 0.92% at 0.5% PAC in the dairy cattle manure, while Ash was reduced by 0.93% at 0.5% PAC. The percentage of curd protein (CP) significantly dropped (P<0.001) in the dairy cattle manure. The percentages of CP were 13.11% and 10.37% at 0% and 0.5% PAC respectively. Neutral Detergent Fibre (NDF) and lignin did not affect by adding PAC to the dairy cattle diet while acid detergent fibre (ADF) was significantly increased (P<0.001). ADF levels were 26.56% and 28.34% at 0% and 0.5% PAC respectively, as shown in Table 6.2.

Table 6.2: Effect of PAC on the chemical compound of dairy cattle's manure

Items	0% PAC	0.5% PAC	*P*<
Dry matter	88.56	89.11	0.001
Organic matter	77.7	78.62	0.02
Ash	22.3	21.37	0.02
Curd protein	13.11	10.37	0.001
NDF%	43.15	43.06	0.10
ADF%	26.56	28.34	0.001
Lignin%	9.63	8.99	0.1

6.4 Discussion

6.4.1 Milking production and GHG emissions.

To meet the growing demand for dairy products such as milk, methods of increasing milk production for herds are investigated. This study is focused on an important topic: whether the addition of 0.5% PAC could reduce the production or influence GHG and CH_4. Here we recorded the effect of adding 0.5% of a commercial PAC on milk quality and quantity in a private herd consisting of 180 cows. The biochar or powdered activated carbon (PAC) from a commercially prepared source was added to commercially made dietary pellets at 0.5% DW. This PAC, derived from coconut, has a high activity microporous powdered activated carbon and adsorption capacity. However, as far as we know, this is the first study showing the beneficial effects of PAC on dairy products and the reduction of GHG emissions in the dairy farm. The reduction of GHG emissions by adding Biochar (BC) to the diet corroborates our earlier findings in an *in-vitro* experimental incubation with rumen fluid and different varieties of PAC substrates, including the form used in this work.

Other reports using *in-vitro* PAC have also investigated the beneficial effects of biochar on GHG emissions (Hansen et al. 2012; Leng et al. 2012; Leng et al. 2012a; Cabeza et al. 2018; Saleem et al. 2018). Our findings showed that adding the 0.5% PAC did not affect dairy milk yield, although it significantly improved both the protein and fat concentrations in the milk. These improvements in milk quality could be reflected in the effect of PAC in altering rumen fermentation and biohydrogenation. Baumann et al. (2016) found that adding lipids in the form of soybean oil to the traditional diet had limited effects on milk fat and protein. Our reports show that the

addition of the PAC to the diet of cattle also had a significant effect on milk fat and protein.

There was a significant reduction in both carbon dioxide (CO_2) and methane (CH_4) emissions eructated from the dairy farm cattle when their pellet diet was supplemented with 0.5% PAC, compared to the control treatment (containing no PAC). Methane (CH_4) and carbon dioxide (CO_2) are two major greenhouse gases released by ruminants such as dairy cows. These types of gases are major sources of GHG emissions from animals. CH_4 is considered an important indicator of farming productivity because it is related to the conversion of feed to the product in livestock (Hill et al. 2016); the higher the levels of CH4 the greater the loss of feed. Enteric CH_4 emissions are produced by microbial fermentation of feed components. CH_4 is produced predominantly in the rumen (87%) and to a small extent (13%) in the large intestines (Kempton et al. 1976; Torrent et al. 1994).

Our results were consistent with those of Leng et al. (2012a) who found that adding 0.6% of biochar (Rice husk) to cattle diet reduced CH_4 emissions by 22%. Further research and development of novel technologies to better understand the efficacy of PAC on GHG emissions in dairy cattle farms are still required, such as increasing the levels of PAC in the pellets.

Several studies have also revealed that the addition of biochar to soil can effectively reduce GHG and CH_4 emissions from the soil, presumed to be due to macropores in biochar. (Wang et al. 2012; Lai et al. 2013; Xie et al. 2013; Martin et al. 2015). However, conflicting results found that PAC that was co-applied with manure to a basic soil did not reduce GHG emissions (Angst et al. 2014). This conundrum may be related to the differences in the structure of the biochar substrates.

6.4.2 Microbial community

The study also focused on comparisons of methanogen diversity and bacterial populations in pre-and post-treatment (post-diet supplemented with 0.5% PAC) in dairy cattle manure, allowing at least 14 days for the rumen microbial community to adapt to the new diet. Results showed that despite a small subset of samples analysed that PAC supplementation cause a significant increase in species richness and abundance (Alpha diversity) and resulted in a different microbiota composition (Beta diversity).

The microbiota of an animal's intestinal tract plays important roles in the animal's overall health, productivity and well-being. Using rDNA bacterial tag-encoded FLX amplicon pyrosequencing analysis, Dowd et al, (2008) revealed that dairy cows displayed a high diversity of bacterial species and genera in their faeces, including Clostridium, Bacteroides, Porpyhyromonas, Ruminococcus, Alistipes, Lachnospiraceae, Prevotella, Lachnospira, Enterococcus, Oscillospira, Cytophage, Anaerotruncus and Acidaminococcus spp. The general proportion of phyla determined in the manure with and without supplementation with PAC is similar to that reported by (Dowd et al. 2008; Durso et al. 2011). The microbiotia was dominated by members of the phyla Firmicutes, with 53% of the OTUs belonging to this taxonomic group. Bacteroidetes was the second dominant phylum, representing 41% of the OTUs and Proteobacteria (5% of the OTUs). Callaway et al. (2010) also reported Firmicutes and Bacteroidetes as the predominant phyla, with 25 most common genera accounted for over 85% of the faecal bacterial populations. Our results differ slightly to those reported by Ozutsumi et al (2014) who measured higher levels of Firmicutes (81.3%), lower levels of Bacteroidetes (14.4%), Actinobacteria (2.5%) and Proteobacteria (1.4%). Bhatt and Maheshwari (2019) recently reported supporting evidence to the above reports and also extended the knowledge of faecal flora by reporting the additional presence of a few fungal sp., (*Aspergillus* and *Trichoderma*), about 100 species of protozoa and 2 yeast species, members of the manure population that we did not investigate

None of these reports specifically investigated members of the phylum Euryarchaeota, containing the important Methanogenic bacteria. Our results specifically investigated the presence of members of the Phylum Euryarchaeota, explicitly four groupings of Methanogenic bacteria responsible for the production of the GHG methane.

Rumen methanogens are predominantly associated with protozoa, with more than 99% of all methanogens located either endosymbiotically and ectosymbiotically in the protozoal fraction with the small remainder found free living in the rumen environment (Vogels et al, 1980; finely et al., 1994). Rumen methanogens are divided into three functional groups; the hydrogenotrophs (*Methanobrevibacter, Methanomicrobium* and *Methanobacterium spp*) which are commonly 95% of the dung methanogens and convert hydrogen and/or formate to CH_4; the methylotrophs (*Methanosphaera spp* and members of the order Methanomassiliicoccales) which produce CH_4 from methyl

compounds such as methanol and methylamines; and the acetoclastic methanogens (such as *Methanosarcina*), which can utilise acetate to produce CH_4 in addition to the hydrogenotrophic and methylotrophic pathway and usually are 6.5% of dung methanogens.

Members of the archaea phylum representing all three of these groups were detected in the faeces from the control diet as well as the diet supplemented with 0.5% PAC. However, they represented only a minor proportion of the bacterial flora in the fresh cow faeces. There were significant changes to the Methanogenic population, with the archaeal population increasing from 1% to 4% when the cows were fed the supplemented diet containing 0.5% PAC. There were proportional changes within other Methanogenic groups as well, with a significant decrease in the proportion of members of the genera *Methanobrevibacter* (from 83% to 51%) with a concurrent three-fold increase in genus *Methanocorpusculum* in the family Methanocorpuscuralceae (from 12% to 42%). The percentages of the other two minor genera also showed minor increases, although their proportion remained low; *Methanosphaera* (increased from 3% to 4%) and members of the vadin CA11 genera in Methanomassiliicoccacea (from 2% to 3%).

The identified genera are identical and proportional to that reported by Rastogi et al. (2008) who reported Methanomicrobiales (81.7%), Methanobacteriales (11.8%) and Methanosarcinales at (6.5%). in fresh dung samples using *mcrA* sequencing (which detects the methyl coenzyme M reductase gene unique to methanogens).

We also detected members of the vadin CA11 genera (order Thermoplasmatales) in pre and post diet faeces. In our analyses, members of the vadin CA11 genus cluster, increased 50% from 2 to 3% in the PAC-supplemented diet. Members of the vadin CA11are a novel group of rumen archaeal sequences with no closely related cultivable isolates, which have been implicated in the reduction of methane emissions (Poulsen et al, 2013). CA11 members were also detected by Tajima et al. (2001) using archaeal 16S rRNA in rumen fluid and also identified by Kumar et al. (2015) in the dairy cattle rumen, but contributed to <1% of abundance.

Godon et al. (1997) reported that higher levels of hydrogenotrophic methanogens (93.5%) and acetoclastic methanogens were 6.5%. Within Methanomicrobiales, 81.7% were of the genus Methanocorpusculum and 6.5% of total clones were related to acetoclastic lineages belonging to Methanosarcinales.

The methanogen community of fresh dung should reflect the same trend as observed in a typical cattle rumen such as the greater abundance of hydrogenotrophic methanogens than the acetoclastic methanogens. The results of the present study corroborate earlier observations reported by Singh et al. (2011) wherein the fresh dung *mcrA* library 93.5% clones belonged to the hydrogenotrophic methanogens, while the acetoclastic methanogens representing merely 6.5% of the total cloneacetoclastic methanogens normally make up only a small percentage of total methanogen community in cattle rumen (Singh et al. 2011) .

The reduction in both methanogen species, Thermoplasmata and Methanomicrobia and an increase in the Methanosphaera in post-treatment PAC supplementation, compared to the control, suggests that the presence of PAC has affected the growth and function of the archaea members. Further research on the effect of PAC on the microbial community both within the rumen and in faecal samples is necessary.

The positive effect of Biochar (BC) on methanogen communities has been well documented in other environments (Huang, Yibin et al. 2019; Zhang et al. 2019). BC supplementation in rice paddies had little effect on bacterial diversity but significantly changed the archaeal community structure (Zhang et al. 2019). The abundances of Methanogenic archaea in the paddy were decreased by BC application, while the methanotroph abundances were increased after BC was applied, although the differences were not significant due to the large variations in a given treatment. This showed that BC addition decreased CH_4 emissions, which may be due to decreased Methanogenic archaea abundance; hence, CH_4 could be utilized by methanotrophs (Huang, Y. et al. 2019). Feng et al. (2012) reported that Methanogenic archaea were not inhibited by BC amendments but there was a decreased ratio of Methanogenic to methanotrophic microorganisms in paddy soils.

We highly recommend further investigations for using PAC at higher concentrations in the diet of dairy cattle because the results of our pilot trial show significant changes to the Methanogenic population. Higher concentrations of PAC within the diet have the potential to further mitigate GHG and CH_4 emissions. They may also further increase milk fat and protein production, which although increased in our trial, did not meet a statistically significant change.

6.5 Chapter summary

Feed supplementation with powdered activated carbon (PAC) is a promising method for reducing GHG and CH_4 emissions and increasing milk quality and quantity of dairy cattle. Two supplementations (0% and 0.5%) were tested, PAC was incorporated into a pelleted compound feed. When pelleted feed was supplemented with PAC, milk production increased by 0.07% on average for the herd. The PAC supplementation had a significant increase ($P<0.05$) in milk protein by 2.63%. Milk fat was significantly increased ($P<0.001$) to an average of an average of 6.32%.

The PAC supplementation had a significant increase ($P<0.05$) in milk protein by 0.03%/cow. Milk fat was significantly increased ($P<0.001$) to an average of 0.06%. PAC supplementation reduced CH_4 emissions significantly ($P<0.001$) before during and after milking of dairy cattle. The PAC slightly reduced the amount of CO_2 before, during and after milking of dairy cattle. The PAC did not reduce CH_4 emissions from the dairy cattle manure, while it had significantly reduced ($P<0.001$) the CO_2 emissions. There was a significant decrease ($P<0.001$) in the proportion of Proteobacteria and the genera Methanobrevibacter. However, the genera of the family Methanocorpuscuralceae was significantly increased by PAC addition. PAC did not affect Methanosphaera and Methanomassiliicoccacea. The successful incorporation of PAC into pelleted feed used in this study appears to be effective way to introduce a low emissions feed product into the Australian dairy industry.

CHAPTER 7

7.1 Conclusions

In this thesis, four studies were conducted to show the efficiency of adding PAC to the diet of dairy cattle. Our findings in this project have shown that PAC has had a significant impact on the improvement of rumen fermentation, milk production, animals' health and performance and the reduction of GHG emissions. Different concentrations of PAC were used successfully in this project. To consider the performance of those proposed studies, they were compared with recently reported studies. The following contributions have been made to answer the research questions and achieve the objective.

From *in-vitro* study, it can be seen that adding PAC concentration to pellet dairy diet has an influence on reducing GHG and CH_4 emissions and improving rumen fermentation. This study gives evidence on the effectiveness of using PAC as it is a quality assured product made at 900-1000°C, containing 99% carbon with an average surface area of 1000m^2/g, which can be incorporated into a concentrate pellet of dairy cattle.

An investigation was conducted to study the efficacy of adding PAC to the forage diet (oaten and barley hay, mega sweet forage sorghum and combined silage and pellet diets were considered) of dairy cattle. In this research, the experimental results demonstrated that the addition of PAC to dairy cattle diets decreased the resulting GHG and methane emissions, whilst improving feed digestibility compared with the control. While emissions were substantially abated, production was not affected, with concentrations of volatile fatty acids (acetate, propionate and butyrate) not differing significantly (P<0.05) in relation to the PAC concentrations. pH did not show significant (P<0.05) increase for oaten and barley hay, while it did differ significantly (P<0.001) when it added to forage sorghum diets. Clearly, the increased surface area effects of PAC addition allowed GHG and CH_4 reductions while maintaining production levels. From the experiments in Chapter 4, it can be observed that the proposed study worked very well compared with other existing studies. It produced good results with the highest GHG and CH_4 emissions reduction.

To decrease the Lantana toxin in the diet of cattle, to reduce GHG emissions and to improve rumen fermentation, the addition of PAC to Lantana toxin was investigated

and used in this study to show its impact on Lantana toxics in the rumen, as can be seen in Chapter 5. Moreover, in this study, many advances were achieved from that study to improve rumen fermentation performance of dairy cattle's diet.

PAC effects on reducing Lantana toxicity in ruminants is one of the more important subjects that have aroused wide interest from an increasing number of investigators. Our findings in Chapter 5 showed that the addition of PAC decreased GHG and CH_4 emissions compared with the control. It showed that the 1g treatment of PAC achieved a greater reduction in Lantadene A and B in leaves and flowers of Lantana. Our study showed the effect of PAC on absorbing or reducing Lantana toxin, more accurately than in previous studies in literature. This allows further work to look at inclusion of PAC as a safeguard against ingestion of Lantana toxins by juvenile or cattle new to the area on agistment. At the same time as being a safeguard the addition of PAC decreases GHG and CH_4 emissions while not affecting rumen fermentation. In summary, nominally identical production levels were achieved by increasing the rumen surface area with the PAC modified dairy cattle diet, allowing significant GHG and CH_4 reductions without harming productivity. Details of this study are given in Chapter 5.

An *in-vivo* study was conducted to determine the effect PAC at 0.5%/ dry matter (DM) of diet on the enteric methane emissions and performance of dairy cattle when incorporated into a concentrated pellet. The addition of PAC improved daily milk production and increased significantly ($P < 0.05$) and ($P < 0.001$) of milk protein and milk fat respectively.

PAC concentration has contributed to reducing CH_4 emissions significantly ($P<0.001$). In addition, 16S rRNA Gene Sequencing was performed to determine the collective population of prokaryotic bacteria and Archaea as well as to characterize the specific Methanogenic communities between dairy cattle manure fed one of two pelleted diets; either a basic diet or supplemented with 0.5% PAC. There was a significant decrease ($P<0.001$) in the proportion of Proteobacteria (from 57% to 23%). There is a significant decrease in the proportion of members of the genera Methanobrevibacter (from 83% to 51%) with a concurrent significant increase in genera of the family Methanocorpuscuralceae (from 12% to 42%). We recommend further investigations for using PAC in the diet of dairy cattle because it has the potential to mitigate GHG

and CH_4 emissions and to increase milk fat and protein production. Details of this study are given in Chapter 6.

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