EFFECT OF SINGLE AND MIXED STRAIN PROBIOTICS ON FEED INTAKE, DIGESTIBILITY, MILK YIELD, COMPOSITION AND METHANE EMISSION OF MILKING DAIRY COWS

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A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Master of Science Degree in Animal Nutrition of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

This research thesis is my original work and to the best of my knowledge has not been presented for any qualification in any other university.

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Recommendation

This research thesis has been prepared with our supervision and submitted to Graduate School with our approval as University supervisors

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DEDICATION

I dedicate this work to my parents Mr. and Mrs.Bakesigaki Xavier, my siblings Frank, Flavia, Phionah, Florence, Brian, Mary and Shivan.

I dedicate this work to my husband Mr. Bahunde Stephen and my son Bahunde Xavi Brandon who have been my inspiration.

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ABSTRACT

Probiotics are widely used in diets of ruminants to modulate rumen metabolism which ultimately enhance nutrient utilization and animal performance. Methane gas emission by ruminants results in 4-12% loss in gross energy of the feed energy ingested by the animal. Mixed strain/species probiotics may enhance the capability of colonizing the gastro-intestinal tract, combining the different mechanisms of each strain/species in a synergistic way. Experiments were conducted to determine the effect of single and mixed strain probiotics on feed intake, digestibility, milk yield and methane emission of dairy cows. Feed intake was measured by weighing feed offered and feed refusal. *In-vitro* gas production was measured at 0, 3, 6, 12, 24, 36, 48, 72 and 96 hr to determine the potential digestibility of the feed. Gas produced during *in-vitro* gas production was siphoned from each sample and taken for rumen methane analysis using a GC-flame ionization detection (FID) gas chromatography. To determine milk yield and composition; fifteen lactating Friesian dairy cows were fed with five experimental diets: T1 (Basal diet - control), T2 (40g *Lactobacillus plantarum* + basal diet), T3 (40g S*accharomyces cerevisiae* +basal diet, T4 (20g *Saccharomyces cerevisiae* + 20g *Lactobacillus plantarum* + basal diet) and T5 (40g *Saccharomyces cerevisiae* + 40g *Lactobacillus plantarum* + basal diet) each having three replicates in a multiple linear model. Initial weight, parity, stage of lactation, breed and age of each cow was fitted as covariates during data analysis. Data was subjected to analysis of variance using General Linear Model of SAS and mean separation done using Tukey's (HSD) test at $P<0.05$ significant level. Results of feed intake showed that probiotic supplementation had significant effect ($P<0.05$) on feed intake with the highest feed intake observed in T5 (11.011±0.66KgDM/day) and the lowest feed intake in T1 (9.533±0.66KgDM/day). Calculated OMD% was significant ($P<0.05$) and ranged from 59.560±2.499 to 38.773±2.499 MJ/KgDM. ME differed significantly ($P<0.05$) with the highest in T5 (8.050 ±0.425 MJ/KgDM) and lowest in T2 (4.517±0.425 MJ/KgDM). Concentration of short chain fatty acid (SCFA) differed significantly and ranged from 0.750±0.045 in T5 to 0.407± 0.045 in T2. Milk yield was significantly affected with the highest milk yield at 4.6 L and the least was from T5 at 3.8 L. No significant effect on milk composition was noted between the five dietary treatments. A combination of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* increased feed digestibility and decreased rumen methane emission when used in modulation in dairy cows. *Lactobacillus plantarum* had no significant effect on dry matter intake.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CO_2e	Carbondioxide Equivalent
CH_4	Methane
DFM	Directly Fed Microbes
FAO	Food and Agriculture Organisation of the United Nations
GDP	Gross Domestic Product
GHG	Greenhouse gas
GLM	General Linear Model
GIT	Gastro-Intestinal Tract
LAB	Lactic Acid Bacteria
OMD48	Organic Matter Digestibility at 48 hr
OMD	Organic Matter Digestibility
SAS	Statistical Analysis System
SCFA	Short Chain Fatty Acids
SNF	Solids Non Fat
UNFCC	United Nations Forum for Climate Change

CHAPTER ONE
INTRODUCTION

1.1 Background Information

The rapid growth of the human population globally has been associated with a growing demand for food of plant and animal origin (Boeckel *et al.,* 2015). It is estimated that by 2050 the number of people in the world will reach 9 billion (Béné *et al*., 2015; Searchinger *et al.,* 2014). There is an increasing demand for food of plant and animal origin due to increasing human population. The livestock sector accounts for 40% of the world's agriculture Gross Domestic Product (GDP) (Gaughan *et al*., 2009). It employs 1.3 billion people, and creates livelihoods for one billion of the world's population living in poverty (Gaughan *et al.,* 2009). Global livestock production has increased substantially over the years, a direct consequence of the growing world population and the increased demand for food (Thornton, 2010).

About 2 million rural households in Kenya produce milk (Wambugu *et al.,* 2011). With about 1,800 liters per cow and year, average annual milk production per cow on smallholder dairy farms is low (Kavoi *et al.,* 2010). Low cow productivity is also associated with high greenhouse gas (GHG) emission intensity (Weiler *et al.,* 2014). In 2010, Kenya's livestock emitted about 16.6 million tons of carbon dioxide equivalents (CO_2e), of which about 20% was from dairy cattle (Wilkes *et al*., 2019). Measures to increase cow productivity thus include increasing fodder production and improved feeding practices, improving animal health and welfare through better housing and preventive veterinary practices, and the use of higher yielding breeds (Quddus *et al.,* 2012). Adoption of practices that increase cow productivity can reduce the GHG intensity of dairy production (Capper *et al.,* 2009).

Livestock production is one of the major contributors of greenhouse gases such as methane (CH_4) and carbon dioxide (CO_2). These gases contribute greatly to global warming, environmental degradation and pollution (Carlsson-Kanyama *et al.,* 2009). Dairy farming contributes 20% of total global greenhouse gas (GHG) emissions from the livestock sector, with enteric methane (CH_4) being the largest source at 39% of dairy emissions (Gerber *et al.,* 2013). Methane has a greater global warming effect (about 23 times) more than carbondioxide (Allen *et al.,* 2003). Currently, livestock production faces a great challenge of increasing production to meet global demand for agricultural products and at the same time reducing environmental impact (Ugbogu *et al.,* 2019). Given the significance of CH_4 as a GHG, reducing enteric CH_4

emissions from dairy cows whilst maintaining levels of milk production could prove to be an important strategy for countries to meet reduction targets in global greenhouse gas emissions (Hristov *et al*., 2013).

For that reason, scientists are looking for solutions allowing intensification of food production, with simultaneous reduction of production costs, and in compliance with high standards of composition and safety, for animals, people and the environment (Markowiak & Śliżewska, 2018). These interconnected issues are creating immense pressure on the planet's resources (Godfray *et al*., 2010). There is need for high quality animal science research to help come up with strategies for sustainable production systems that can help to meet rising demand for livestock products in an environmentally and socially responsible way (McDermott *et al.,* 2010). Feed additives have been used by mankind for a longtime to improve the efficiency in feed utilization by farmers. Types of feed additives used affect animal health and increased production of high nutritive value meat, eggs, milk and fish (Markowiak & Śliżewska, 2018) with a pronounced example of probiotics as feed additive.

Probiotics are commonly defined as viable microorganisms that confer a beneficial effect on the health of the host animal when they are ingested, and their health benefits are strain-specific and not species-specific or genie-specific (Anadón *et al.,* 2016). The mechanism by which probiotics influence their beneficial effects includes; maintaining a beneficial microbial population in the gastrointestinal tract, improving digestion, feed intake, altering bacterial metabolism by increasing digestive enzyme, decreasing bacterial enzyme activity, neutralizing toxin and stimulation of the immune system (Musa *et al.,* 2009).

Probiotics are widely used in diets of ruminants to modulate rumen metabolism which ultimately may enhance nutrient utilisation and animal performance (Mutsvangwa *et al.,* 2010). Probiotics have been shown to promote growth, improve efficiency of feed utilisation, protect the host from intestinal infection and stimulate immune responses in farm animals (Ezema, 2013). With an increased feed utilization, methane reduction may be reduced as well.*Saccharomyces cerevisiae* additives may exert positive effect on the digestibility, especially fibre components probably by stimulating cellulolytic microbial populations in the rumen (Patra, 2012). Addition of probiotics to the diets of lactating cows has been known to improve performance of ruminants possibly because of their action on increasing digestion efficiency (Qadis *et al*., 2014).

In adult ruminants, most probiotics used have been selected to target the rumen compartment, which is the main site of feed digestion. The rumen microbial ecosystem consists of a wide diversity of strictly anaerobic bacteria, ciliate protozoa, anaerobic fungi, and archaea which are responsible for degradation and fermentation of 70-75% of the dietary compounds (Chaucheyras-Durand *et al.*, 2010). The rumen has a complex microbial ecology, where polysaccharides and protein ingested by the host are degraded by rumen micro-organisms resulting in production of short chain fatty acids and synthesis of microbial protein, which are used by the host as energy and protein sources (Bajagai *et al.*, 2016). In recent years, there has been an increasing interest to manipulate the rumen microbial ecosystem to increase the efficiency of the rumen fermentation processes to improve animal productivity (Wanapat *et al.*, 2013).

The microbial composition of probiotic products ranges from a single strain to mixed-strain or species composition. Many commercial products use mixed-strain probiotics, although the benefits of using more than one strain or species in a single product has not been clearly established (Bajagai *et al.*, 2016). It has been recognized that functionality of mixed-strain and mixed species probiotics could be more effective than that of single/mono-strain probiotics with a possibility of a synergistic effect. The advantages of administering mixed-strain/species probiotics may include the enhanced capability of colonizing the gastrointestinal tract and to combine the different mechanisms of action of each strain in a synergistic way (Agazzi *et al.*, 2014). The mixed-strain probiotics have a broad-spectrum effect from the different strains against infections and could increase their beneficial effects of probiotics due to their synergistic adhesion effect (Adjei-Fremah *et al*., 2018).

Probiotics used must be safe in order to optimize for their use. The mode of action of probiotics as microbial additives to feed is still under study. Probiotics used should be able to maintain the stability and protection of the rumen ecosystem. They should be able to influence the course of digestive and metabolic processes and the immunological response (Chichlowski *et al.*, 2007). Consequently, properties of these probiotics should lead to increased productivity.

Selection of new probiotic organisms involves strains and even genie of microorganisms demonstrating the most beneficial or the most specific effects. The assessment focuses mostly on safety and the benefit-to-risk ratio associated with the use of a given probiotic strain (Markowiak & Śliżewska, 2018). Microorganisms used for production of probiotic animal formulas are

isolated from individuals belonging to the species for which they are intended, because part of health beneficial effects is probably species specific. Due to that procedure, the obtained biological material is maximally adapted to the conditions present in the alimentary tract of the given species of animals (Zhang *et al*., 2018). Moreover, probiotic cultures added to feed are resistant to temperatures and pressures used in the process of pelleting, and to humidity and the effect of adverse substances during feed handling and storage, such as heavy metals or mycotoxins (Anekella, 2012). Probiotic products may contain one or more selected microbial strains. Microorganisms used as feed supplements in the European Union are mostly bacteria (Wassenaar *et al.,* 2008). Most often they are gram-positive bacteria belonging to the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*. Also, some fungi and yeast strains of *Saccharomyces cerevisiae* are probiotics. Bacteria belonging to the genie *Lactobacillus* and *Enterococcus* are components of the natural microbiota of the animal alimentary tract, and are usually present in amounts of 10^7–10^8 and 10^5–10^6 CFU/g, respectively (Guder, 2019).

The global methane emission from all sources has been estimated as 500-600 Million tones/year About 50% of the total global emission of methane is through anthropogenic activities of which significant or major contributor is found to be livestock sector (Sejian, 2011). Estimated values of methane emission from domesticated animals varied widely in different reports from 70-220 Million tones/year (Renuka *et al.,* 2013). The large variation in values attributed to the methodology adopted and assumption made in estimating the per animal emission rate (Casey & Holden, 2005). Livestock production systems play a significant role in Kenyan economy by contributing a significant amount of food (milk, meat); fibre (wool, fur & hair); skin and manure (Herrero *et al.,* 2013). Amount of feed consumed and its digestibility are two important factors, which determine the total methane production. This is attributed to the poor-quality roughages/feed available to the animals (Dung *et al*.,2019).

Methane one of the potent GHG in trapping the warmth, is mainly contributed by anthropogenic activities including rearing of livestock and manure management. Strategies and research effort are required to be directed towards mitigating the methane emission from enteric fermentation of feed in ruminants and manure management to reduce the rate of methane gas emission in the atmosphere (Abatenh *et al.*, 2018).

The technologies that can reduce the amount of methane production in rumen or total release of methane into atmosphere are useful for efficient use of feed and making the environment more favorable. In addition, this will go a long way in guaranteeing sustainable livestock production system globally. Several options have been considered for mitigating methane production and emitting in atmosphere by the livestock (Hook *et al.*, 2010). Methane has relatively short life (10-12 years) in the atmosphere as compared to other GHGs, for example CO_2 has 120 years and therefore strategies to reduce the methane in atmosphere offer effective and practical means to slow global warming. Decreased emission rate of only 10% will stabilize methane concentration in atmosphere at present level (Ulyatt *et al*., 2001)

Energy loss in ruminant livestock through enteric methane emissions has been identified as a major problem not only because of the impact on climate change but also owing to the considerable effect on animal productivity (McMichael *et al.*, 2007). Ruminant animals, particularly cattle produce significant quantities of methane via enteric fermentation of feed both in the foregut and hindgut. Compared to other ruminants, cattle contribute the most to methane gas emissions due to their greater body size, energy intake, and population size and account for 61% of the emissions attributed to all domestic animals (Broucek, 2014). Furthermore, enteric methane energy loss accounts for 2% to 12% of total gross energy (GE) intake in ruminants and which is significant (Eckert *et al.*, 2018; Subepang *et al*., 2019). In addition, methane gas energy loss reduces the efficiency of feed energy utilization and dairy cattle productivity. Therefore, the use of feeding strategies that reduce enteric methane emission is a priority in improving animal productivity and environmental sustainability

1.2 Statement of the Problem

Increased intensive farming practices have led to the use of highly fermentable carbohydrate (concentrates) as a feed constituent in dairy cattle leading to severe cases of rumen/lactic acidosis sometimes even leading to death of animals. In an attempt to address this, and other problems associated with low feed utilisation efficiency, the use of probiotics has become inevitable. Probiotics are known to have a positive impact on feed utilisation through increased intake, improved digestibility, increase milk yield and composition. However, these results have been found to be inconsistent and show wide variations that depend on the strain(s) of probiotic used. There is, therefore, a need to determine if single or mixed strain probiotics use

can improve efficiency of feed utilisation and reduce energy loss of ruminants in the form of methane gas emissions.

1.3 Objectives

1.3.1Overall Objective

The overall objective of this study was to contribute to food and nutrition security through improved production of lactating dairy cows offered probiotic-based diets.

1.3.2 The specific objectives of the study were to: -

i. To determine the effects of single strain and mixed strain probiotics on feed intake and digestibility of lactating dairy cows.
ii. To evaluate the effect of single and mixed-strain probiotics on rumen methane gas emission of lactating dairy cows.
iii. To determine the effects of single strain and mixed strain probiotics on milk yield and composition of lactating dairy cows.

1.4 Research hypotheses

The following Null hypotheses (H_0) were postulated for this study: -

i. Single and mixed strain probiotics have no significant effect on feed intake and digestibility of feed in lactating dairy cows.
ii. Single strain and mixed strain probiotics have no significant effect on the rumen methane gas emission in lactating dairy cows.
iii. Single and mixed strain probiotics have no significant effect on milk yield and composition in lactating dairy cows.

1.5 Justification

There was no doubt that profitability is the main objective in commercial dairy farming. Demand for livestock products due to population increase had tremendously increased over years without any corresponding increase in available land for farming. Milk is one such livestock product whose production has not kept pace with increasing demand and especially in developing countries such as Uganda. There is therefore a need for intensification to increase production while optimizing use of the available feed resources without endangering the

environment. Probiotics may optimize feed utilization through increased efficiency in digestion and intake, and possibly reducing greenhouse gas emission. The information on use of single strain and mixed-strain probiotics could give clear indication on what farmers need to do to optimize feed utilization efficiency of available feed resources. This could lead to finding better management decisions for intensively managed animals to enhance their productivity. It was envisaged that adopting appropriate strains of probiotic would be instrumental in ensuring that use of feed resources is optimized with maximum benefits while ensuring sustainability of a healthy environment through reduction in greenhouse gas emission.

CHAPTER TWO
LITERATURE REVIEW

2.1 Background Information

The world's human population is expected to reach more than 9 billion by 2050, posing enormous food security challenges, particularly for developing countries. Moreover, economic growth has increased the demand for livestock products putting pressure on the livestock sector to produce more with limited resources (Bajagai *et al.*, 2016). The world food economy is increasingly being driven by the shift in diet and food consumption patterns towards more livestock products, especially with increasing per capita disposable income. In the last few decades in the developing countries of Asia; where the bulk of the world population increase has taken place, consumption of meat has been growing at over 4 percent per annum, and that of milk and dairy products between 2 to 3 percent per annum (Steinfeld *et al.*, 2006). Over the last 30 years, consumption of meat, milk and eggs in low-and middle-income countries has more than tripled. Population growth, urbanization, increase in per capita income and globalization continue to fuel the "livestock revolution", offering numerous business opportunities for many livestock producers (FAO, 2018).

Livestock sub-Sector plays an important role in the national economy of Kenya with a direct contribution of around 42 percent to the agricultural Gross Domestic Product (GDP) and 12 percent to the national GDP (Salami *et al.*, 2010). It supplies the domestic requirements of meat, milk, dairy products, eggs, and other livestock products while accounting for about 30 per cent of the total marketed agricultural products. Animal nutrition and feeding plays an important role in the productivity of livestock and is also a key economic input in this sector. Efficient and sustainable use of available feed resources is a major factor in improving livestock productivity and use of probiotics could be one way that can be used to increase feed utilization efficiency to enhance productivity in the livestock sector, especially in ruminants (Azzaz *et al.,* 2015; Ondarza *et al.,* 2010).

The gastrointestinal tract of domestic ruminant animals mainly cattle, sheep and goat are inhabited by diverse and complex microbial communities including bacteria, protozoa, fungi, archaea and viruses (Herderson *et al.,* 2013). In the last three decades, there has been numerous research studies to characterize the gut and rumen microbiota population and understand their importance on ruminant nutrition and health (Malmuthuge *et al.,* 2015). In dairy cows, the

rumen, which is the main fermentation chamber contains different microbial communities; about 100 billion bacteria, protozoa, methanogens and other anaerobic fungi. The major microbial groups in the rumen are *Prevotella, Selenomonas, Streptococcus, Lactobacillus* and *Megasphaera* (Kamra *et al.,* 2015). The rumen is also predominantly inhabited by fiber-degrading bacteria such as *Fibrobacter, Ruminococcus, Butyrivibrio* and *Bacteriodes* (Koike *et al*., 2009). These native microbial groups have important function in the digestion and fermentation of dietary polysaccharides by the host. In ruminants, variation in the rumen microbiota between individual animals has been reported (Weimer, 2015). There is a growing research interest in the application of beneficial microbes/probiotics in ruminant production to help balance the gut microbiota, and as possible alternative to antibiotic use through improved gut health (Adjei-Fremah *et al.,* 2018).

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Gill *et al.,* 2009). In ruminants, probiotics are administered to target the rumen where they have an effect on rumen fermentation especially on feed digestibility and degradability and rumen microbiota (Kumar *et al.,* 2014). Probiotic positively affect celluloysis and synthesis of microbial protein during digestion, and stabilizes rumen pH and lactate levels. In addition, probiotics are able to enhance nutrient absorption. Direct-fed probiotic have been shown to reduce ruminal acidosis (Retta, 2016).

Lactic-acid bacteria strains such as *Lactobacillus, Bifidobacterium, Bacillus, Saccharomyces* and *Enterococcus* are commonly used as probiotics in functional foods and animal enteric infection (Hazallah & Belhadj, 2013). These beneficial microbes consist of different species of microorganisms such as bacteria and yeast and they may be used as single or multi-strain. The multi-strain probiotics have a broad-spectrum effect from different strains against infections and could increase their beneficial effects of probiotics due to their synergistic adhesion effect (Chapman *et al.,* 2012).

Recent studies suggest that utilization of probiotics as feed supplement for ruminants improves growth performance, production, and enhance health and overall wellbeing of the animals. Applications of probiotics have been shown to reduce the negative environmental impact such as methane emission associated with ruminant production (Gaggìa *et al.,* 2010).

Lactobacillus plantarum and *Saccharomyces cerevisiae* have a potential to be used as single and or mixed strain probiotics (Gerardi *et al.,* 2019). This is because they are good

inhabitants of the host organism and have the ability to adhere and colonize the epithelial cells of the gut (Ouwehand *et al.*, 1999). They are able to grow and survive in the host, do not affect the indigenous gut microbiota population of the host (Sánchez *et al.*, 2017). They ably adapt to the environment of the gut and locate a suitable niche in the rumen (such as epithelium, fluid or feed), while exerting positive effects on the host (Adjei-Fremah *et al.*, 2018).

2.2 Mode of Action of Single and Mixed-Strain(s) of Probiotics

Probiotic activity in various livestock is not well defined. Supplementing livestock feed with probiotics is based primarily on potential beneficial post-ruminal effects (Ghazanfar *et al.*, 2017)**.** Certain probiotics might also have beneficial effects in the rumen, in particular helping to prevent ruminal acidosis (Jouany, 2006).

Probiotics action in the host organism include; regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function, expression of bacteriocins, enzymatic activity inducing absorption and nutrition, immunomodulatory effects, inhibition of pro-carcinogenic enzymes and interference and interference with the ability of pathogens to colonize and infect the mucosa (Harzallah *et al.*, 2013; Lutgendorff *et al.*, 2008; Nava *et al.*, 2005; Zhang *et al.*, 2019). In ruminants, the mechanism of probiotics metabolism is dependent on the strain of the micro-organism used (Adjei-Fremah *et al.*, 2018).

It is important to highlight that probiotic are live microorganisms and that their beneficial effects may be affected by a myriad of conditions such as species, host microbial species, and diet (Kaur *et al.*, 2002). Influencing factors that determine the effects of probiotics have been described by and include dosage, timing, specific strain of probiotic, and animal conditions. If the purpose of the probiotic is to target the rumen, it must be active and remain viable in such environment (Chaucheyras-Durand *et al.*, 2008). Because of these requirements, the research is limited to a few genera such as *Enterococcus, Lactobacillus, Bifidobacterium, Streptococcus, Bacillus,* and *Propionibacterium.* These bacteria are most commonly used as DFM for ruminants and are classified as lactic acid producing (LAB), lactic acid-utilizing (LUB) (Seo *et al.*, 2010). Different probiotics affect the gastrointestinal tract through diverse pathways.

Lactic acid-producing bacteria have four common modes of action in ruminants: constant lactic acid supply, adaptation to the lactic acid accumulation, stimulation of lactate utilizing bacteria (LUB), and stabilization of pH (Mayra-Makinen *et al.*, 2004; Seo *et al.*, 2010). The LUB have five modes of action that include: conversion of lactate to volatile fatty acids (VFA),

production of propionic acid, decrease methane production, increase feed efficiency, and increase rumen pH (Bhatia & Yang, 2017). The rationale for feeding LAB such as *Enterococcus* and *Lactobacillus* is that the activity of these bacteria could create a low steady concentration of lactate in the rumen. This provides a constant stimulation of LUB to prevent accumulation of lactate and reduce the risk for acidosis (Thomas, 2017). In addition, these bacteria can also decrease the amount of hydrogen available for methane production.

Saccharomyces cerevisiae have six different modes of action: reduction of ruminal oxygen, inhibition of excess lactic acid, supplying organic acids and vitamin B growth factors, increase microbial activity and numbers in rumen, and increase ruminal end products (Elghandour *et al.*, 2015; Seo *et al.*, 2010). *Saccharomyces cerevisiae* frequently increases bacterial numbers in the rumen (Seo *et al.*, 2010). In addition, it can compete with starch utilizing bacteria for fermentation preventing lactate build up. Because of their pH regulation and oxygen scavenging actions, *Saccharomyces cerevisiae* create better conditions for cellulolytic activity by leading to increased forage utilization. *Saccharomyces cerevisiae* in ruminants stabilizes the pH of rumen and therefore favor the growth of cellulolytic bacteria sensitive to low pH.

Oxygen scavenger property of *Saccharomyces cerevisiae* in rumen helps to protect obligate anaerobes from the air ingested in rumen along with feed intake and water (Sheikh *et al.*, 2017). *Saccharomyces cerevisiae* is widely used in commercial ruminant production because they tend to improve milk yield of dairy cows and live weight gain of growing cattle with positive results being more pronounced in animals on highly fibrous basal diets. Available products vary widely in both the strain of *Saccharomyces cerevisiae* used and the number and viability of yeast cells present (Beauchmin *et al.*, 2008).

The use of *Saccharomyces cerevisiae* has been introduced to ruminants feeding on fibrous roughages because yeast culture action can utilize part of free sugar in the rumen and also create a fermentation shift due to rapid and extensive degradation of fibrous material. Furthermore, the yeasts can secrete some metabolites that are useful to other rumen microorganisms. Yeast culture contains B-Complex vitamins, amino acids, and organic acids, particularly malate, which stimulates growth of other rumen bacteria that digest the cellulose (Kashongwe *et al.*, 2017).

Lactic acid bacteria (LAB) are gram-positive, acid-tolerant, either rod-shaped (bacilli) or spherical (cocci) bacteria. These bacteria are naturally found in the gastrointestinal tract (GIT) of the ruminants, produce lactic acid as the major metabolic end product of carbohydrate fermentation (Sun *et al.,* 2014). The positive effects of LAB on dairy animal's performance have been reported, but the actions of LAB are species and strain specific and depend on their availability and viability in the host animal Gastro-Intestinal Tract (GIT) (Arena *et al*., 2017). There are many mechanisms that have been proposed to explain how the LAB bring about their positive effects including: antagonist the harmful bacteria in GIT through production of lactic acid and bacteriocins; compete with pathogens for adhesion and nutrients sites; stimulate animal's immune response through activate phagocytosis and natural killer cells; detoxification of GIT toxins and stimulating digestive enzymes production and secretion (Bajaj *et al.,* 2021; Brashears *et al*., 2005; Naidu *et al*., 1999). LAB might help for prevention of ruminal acidosis, by allowing the ruminal microflora to adapt to the presence of lactate in the rumen. As the energy issue is so critical for dairy animals in early lactation, LAB inclusion could promote nutrients uptake through decrease the thickness of the inflamed intestinal wall, as well as improve feed efficiency by reducing the amount of energy used for GIT tissue turnover (Xu *et al*., 2017).

Lactobacillus plantarum requires Vitamins; $B_{6,}$ riboflavin, thiamine, nicotinic acid and pantothenic acid for growth (Pallotta, 2019) and which may be supplied by the *Saccharomyces cerevisiae* when fed together as a mixed culture. The use of *Lactobacillus spp*. culture may overcome the nutrient imbalances due to dietary changes in the rumen in early and mid-lactation for high yielding dairy cows by its constant level of lactic acid production to rumen microbiota which allows lactate utilizing bacteria to sustain a metabolically active population (Oyebade, 2021). However, there have been limited studies using *Lactobacillus* culture in lactating dairy cow (Habeeb, 2017). Supplementation of lactobacilli may be useful in the close-up dry period of lactation when intake is depressed and animals are stressed (Stella *et al*., 2007). Cows supplemented with lactobacilli in the transition period produced higher milk yield and had lower blood non-etherified fatty acids, but higher blood glucose than the untreated cows (Gado *et al.,* 2007).

Research concerning the effects of probiotics on performance of lactating dairy cows are limited. Even in the reported studies the probiotics are fed together with other additives making it difficult to elucidate the effects of the probiotics themselves (Yirga, 2015).

There are indications that probiotics might have beneficial effects through manipulating ruminal fermentation, which would aid in the prevention of ruminal acidosis (Krehbiel *et al*., 2003). Owens *et al.* (1998) suggested that acute or chronic acidosis due to the ingestion of excessive amounts of readily fermentable carbohydrates (like starch) was a prominent production problem for ruminants fed high-concentrate diets. Ruminal acidosis was characterized by a decrease in ruminal pH to 5.6 or below for subacute, 5.2 or below for acute and high ruminal concentrations of total volatile fatty acids (VFAs; subacute acidosis) or lactic acid (acute acidosis). Lactate-producing bacteria (like *Lactobacillus* and *Enterococcus* species) might help prevent ruminal acidosis, potentially by allowing the ruminal microorganisms to adapt to the presence of lactate in the rumen (Elghandour *et al.*, 2015; Ghorbani *et al.*, 2000; Yoon & Stern, 1995).

Probiotic bacteria are not typical ingredients but rather living cells that can rapidly respond and adapt to changing conditions in their environment. Numerous factors from culture preparation and preservation, conditions in consumer product matrices, and genetic, dietary, cultural, and health differences between consumers can affect probiotic cell activity and probably influence the specific host–microbe interactions required for probiotic effects in the digestive tract (Terpou *et al*., 2019). These exogenous factors have typically not been considered in the design of human and animal studies on probiotics. Probiotic efficacy depends on various factors such as microbial species composition (like single or mixed strain) and viability, administration level, application method, frequency of application, overall diet, age, overall farm hygiene, and environmental stress factors (Mountzouris *et al.*, 2010).

Although responses to probiotics have been positive in many experiments, enhancing our understanding of the mode of action would improve our ability to select and apply probiotics to ruminant diets appropriately for improved livestock production (Krehbiel *et al.*, 2003).

The mode of action of mixed-strain(s) of probiotics can be more complex and varied than single-strain probiotics. The combination of different strains can enhance the benefits of each individual strain and provide a broader spectrum of health benefits. However, the effectiveness

of mixed-strain probiotics depends on the selection of compatible strains, optimal doses, and synergy between strains.

2.3 Effect of Single and Mixed-Strain Probiotics on Feed Intake

The effects of applying a bacterial inoculant to silage immediately before feeding on silage intake, digestibility, degradability and rumen volatile fatty acids concentrations in growing beef cattle showed that the application of lactic acid bacteria to a well preserved grass silage before feeding did not significantly affect silage dry matter (DM) intake (Keadyt & Steenf, 2006).

Direct-fed microbials fed to lactating dairy cows have been reported to increase DMI (Nocek *et al*., 2002). However, this response is inconsistent as other studies do not report improvements on DMI in ruminants (Raeth-knight *et al*., 2007), but reported a positive effect on feed efficiency. Not observing an increase in DMI, but an increase in output means that animals utilize more nutrients from the amount of DM that they consumed, therefore increasing productive efficiency. This is supported by the reports of Alzahal *et al.* (2014); Nocek *et al*. (2002), and Qiao *et al.* (2010), who indicate that feeding DFM increases nutrient digestibility.

In ruminant animals, the application of yeasts *(Saccharomyces cerevisiae*) in the form of live culture, or dead cells with culture extracts, has proved successful in beneficially modifying rumen fermentation. Yeast cultures may stimulate forage intake by increasing the rate of digestion of fibre in the rumen in the first 24 hours after its consumption. The improvement in early digestion and intake could be brought about by alterations in the numbers and species of microorganisms in the rumen (Chaucheyras-Durand *et al*., 2012). The increase in forage intake may result in improved live weight gain, milk yield and milk fat content, although the effects are often low in dairy cows (Yirga, 2015).

Overall, the effect of single and mixed-strain probiotics on feed intake is likely to vary depending on the specific strains used, the dose and duration of supplementation, and the species and age of the animal. Therefore, it is important to carefully select and evaluate the efficacy of probiotics for each specific application

2.4 Effect of Single and Mixed-Strain Probiotics on Feed Digestibility

Saccharomyces cerevisiae was metabolically active in the rumen and the small intestine after ingestion. Several modes of action have been proposed. Yeast can be considered to be facultative anaerobes and therefore have the ability to scavenge oxygen from the rumen making ecosystem more favorable for growth and activity of the obligate rumen anaerobic microbes, especially cellulolytic bacteria. It also has the ability to increase cellulolytic activity in the rumen and increases nutrient digestibility, especially in the case of basal diets high in fibre. Yeasts regulates the rumen pH and limit acidosis risks through regulating both of lactate producing and lactate utilizing bacteria. *Saccharomyces cerevisiae* are also a rich source of nutrients like peptides, vitamins, organic acids and cofactors which may be required by the rumen bacteria (Kassa, 2016).

The more feed an animal consumes each day, the greater will be the opportunity for increasing its daily production. Probiotic supplementation has been found to increase feed intake and is known to influence the performance of ruminants. The reason for increased feed intake and performance is due to improved cellulolytic bacteria in the rumen-fed probiotics fortified diets and their positive effect on ruminal pH, leading to improved fiber degradation and dry matter intake (Retta, 2016). The increase in productivity is often associated with an increase in feed intake.

The positive effect and mode of actions of *Saccharomyces cerevisiae* products are generally considered to involve changes in rumen fermentation rates and patterns. Certain strains of active dry *Saccharomyces cerevisiae* are particularly effective at raising and stabilizing ruminal pH by stimulating certain populations of ciliate protozoa, which rapidly engulf starch and, thus, effectively compete with amylolytic lactate-producing bacteria (Chaucheyras-Durand *et al*., 2008; Fonty & Chaucheyras-Durand, 2006). A less acidic ruminal environment has been shown to benefit the growth and fiber-degrading activities of cellulolytic microorganisms *Saccharomyces cerevisiae* also has the potential to alter the fermentation process in the rumen in a manner that reduces the formation of methane (CH4) gas (Retta, 2016; Uyeno *et al*., 2015). Therefore, it is concluded that probiotic supplementation in the diet may result in improved nutrient digestibility.

Overall, while the specific mechanisms by which probiotics improve feed digestibility may vary depending on the species and the probiotic strain used, there is evidence to suggest that incorporating probiotics into animal feed can have a positive impact on nutrient utilization and overall animal health

2.5 Effect of probiotics on Volatile Fatty Acid Production

Volatile Fatty Acid (VFA) concentrations and ammonia have been observed to vary in several studies feeding DFM to dairy cows (Raeth-Knight *et al.,* 2007). Propionate is produced via two main pathways in the rumen: succinate pathway or the acrylate pathway. The succinate pathway is used when fermenting carbohydrates, lactate or succinate to produce propionate. The acrylate pathway uses lactate and acrylate analogues to produce propionate. One other pathway is the propanediol pathway used for deoxyribose sugars, however it is not common. Increase in propionate concentrations when feeding DFM with either *Propionic bacterium* or *Bacillus subtilis* have been reported (Baldwin *et al.,* 1963; Counotte *et al*., 1981; Louis *et al., 2017*; Reichardt *et al.,* 2014). However, Raeth-Knight *et al*. (2007) did not report any differences in ammonia or total VFA concentration. Since propionate is the precursor for gluconeogenesis, an increase in the concentration of this VFA can mean more glucose could be available for milk production (Weiss *et al.*, 2008).

When feeding DFM with LAB to dairy cows, the production of lactate could be maintained at a low, steady rate as opposed to rapid spikes with increased risk for acidosis (Nocek *et al.,* 2002) when feeding readily fermentable diets. The VFA concentrations in ruminants are variable based on the mode of action of each type of probiotic used (Qadis *et al.*, 2014). Probiotics enhance growth and/or cellulolytic activity by rumen bacteria and prevent ruminal acidosis by balancing the VFAs ratios in the rumen (El-Trwab *et al.,* 2016)

The rationale for feeding LAB such as *Enterococcus* and *Lactobacillus* is that the activity of these bacteria could create a low steady concentration of lactate in the rumen, thus providing a constant stimulation of LUB to prevent accumulation of lactate and reduce the risk for acidosis (Cord-Ruwisch, 2017). These bacteria can also decrease the amount of hydrogen available for methane production (Chen *et al*., 2020).

Saccharomyces cerevisiae frequently increase bacterial numbers in the rumen (Seo *et al.,* 2010) in addition, yeast can compete with starch utilizing bacteria for fermentation preventing lactate build up (Fonty & Chaucheyras-Durand, 2006). Because of their pH regulation and

oxygen scavenging actions, *Saccharomyces cerevisiae* create better conditions for cellulolytic activity by bacteria (Chaucheyras-Durand *et al*., 2012) leading to increased forage utilization

The specific mechanisms by which probiotics increase VFA production may vary depending on the species and the probiotic strain used. However, it is believed that probiotics can enhance the activity of beneficial bacteria in the gut, which in turn leads to increased fermentation of dietary fiber and increased production of VFAs.

Overall, incorporating probiotics into animal feed can have a positive impact on VFA production, which can lead to improved nutrient utilization and overall animal health

2.6. Effect of Single and Mixed-Strain Probiotics on Rumen Methane Emission in Lactating Dairy Cows

At the United Nations Climate Change Conference in 2015, a key component of the global agreement was to protect food production whilst also reducing greenhouse gas (GHG) emissions (UNFCC, 2015). Dairy farming contributes 20% of total global GHG emissions from the livestock sector, with enteric CH_4 being the largest source at 39% of dairy emissions (Gerber *et al.,* 2013). Given the significance of CH_4 as a potent GHG, reducing enteric CH_4 emissions from dairy cows whilst maintaining levels of milk production could prove an important strategy for countries to meet reduction targets in global emissions. Enteric $CH_4$is normally produced in the digestive tract by archaea microorganisms as a by-product of anaerobic fermentation (methanogenesis).

In ruminants, the use of feed additives such as probiotics are used to manipulate rumen microbial population and thus ruminal fermentation to maximize the efficiency of feed utilization to further increase ruminant productivity for products like milk, meat, and wool production (Tirado-González *et al*., 2018). With an increased efficiency in feed utilization, methane production may be reduced as well. Dietary modification was directly linked to changes in the rumen fermentation pattern and types of end products. Studies show that changing fermentation pattern is one of the most effective ways of methane abatement (Bodas *et al.,* 2012). Desirable dietary changes provide two-fold benefits like improved production and reduction in GHG emissions. Dietary manipulation by selecting and utilizing high composition of forages, strategic supplementation of forages, changing concentrate proportion with special emphasis on changing

carbohydrate composition should be considered as an immediate and sustainable methane mitigation approach of enteric CH_4 emitted from ruminant livestock (Haque, 2018).

Increasing efficiency in the digestibility of forage in the rumen needs to incorporate measures that can also reduce methane gas production (Benchaar *et al*., 2001). Such a strategy has the dual benefit of not only releasing more feed energy to the host animal for metabolism but also helps to reduce the greenhouse gas emissions that have been blamed for the predicted changes in climate particularly, the global warming. This is crucial given that enteric fermentation of high fibre forages results in approximately 4-12% of feed gross energy being lost as CH_4 (Eckert *et al.,* 2018; Migwi *et al*., 2013).

The adaptive or coping strategies can assume various forms focussing on dietary manipulation approaches that enhance livestock productivity under a changing climate (Eckard *et al*., 2010). Such measures are necessary to avoid loss in body condition, low live weight and ultimately low animal production performance. To reduce the gas emission by dairy cattle, feed additives such as probiotics can be used (Ferket *et al.,* 2002).

One of the alternatives for reduction of methane gas production by ruminants that has drawn considerable attention in recent years is the use of yeasts, as one type of direct fed microbes or probiotics. Yeast products have been used as feed additives for ruminants to improve production performance (increase of growth rate, meat, and milk) and to alleviate acidosis thus improving animal health and welfare (Darabighane *et al.,* 2019). As a natural feed additive, yeasts contribute to balance and stabilize rumen microbiota, to maintain a favorable pH and enhance the formation of fermentation end-products in the rumen, and to improve ammonia utilization by ruminal bacteria (Elghandour *et al*., 2020).

This effect might be dependent on dosage of probiotic, strain of probiotic and diet composition. The previously conducted studies point to insignificant effects of probiotic products on reduction of CH_4 production of dairy and beef cattle. This study evaluated the impact of single and mixed strains of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* of methane emission in lactating dairy cows (Adesogan, 2009)

Therefore, there is a need to develop efficient feeding strategies to reduce methane gas production by using an appropriate strain(s) of probiotics, which affect the environment and also improve feed digestion and nutrient metabolism in the animal.

Studies suggest that both single and mixed-strain probiotics have the potential to reduce rumen methane emission in lactating dairy cows. However, further research is needed to determine the optimal probiotic strain(s) and dosages to achieve maximum reduction in methane emission, as well as to investigate the long-term effects of probiotic supplementation on animal health and productivity

2.7 Effect of Single and Mixed Strain(s) Probiotics on Milk Yield and Composition

The supplementation of animal feed with probiotics has a beneficial effect on subsequent milk yields, fat and protein content. *Saccharomyces cerevisiae* supplementation was reported to increase milk yield in dairy cows mainly due to increase in number of cellulolytic bacteria, fiber degradation and changes in volatile fatty acid in the rumen (Rai *et al.,* 2013). In the traditional milk products, microbes are selected for their enhanced ability to grow and produce organic acids in milk. In case of probiotics, microbes are mainly selected on the basis of their potential health associated properties (Rai *et al.,* 2013).

The effects of feeding yeast and propionic bacteria to dairy cows on milk yields, composition and reproduction in combination with yeast increased actual milk yield, solids corrected milk and 4% fat corrected milk production by 8.5–16.6% (above controls) in mixed parous cows but not primiparous cows during mid but not early lactation (Lehloenya *et al*., 2008).

Dairy cows fed probiotic based diets had their milk yield increase by about 0.75–2.0 Kg/day (Yirga, 2015). In general, an increase in milk yield has been a consistent response, whereas changes in milk composition have been variable. In support of these observations, Gomez-Basauri *et al.* (2001) reported that cows fed Lactic acid bacteria produced more milk compared to control. The authors reported that milk yield increased over time for Lactic acid bacteria-fed cows, whereas control cows maintained constant milk yield. Furthermore, there are reports that suggest probiotics fed alone or in combination with fungal cultures might be efficacious for increasing milk production in lactating dairy cows (Gaggìa *et al*., 2010). Dietary supplementation of *Lactobacillus* bacteria was reported to increase milk production and reduced somatic cells count in milk (Chen *et al.,* 2017). However, more research is needed before recommendations can be made on the use of a combinations of fungal cultures and lactic acid bacteria

The use of Lactobacillus sp. culture may overcome the imbalances due to dietary changes in the rumen in early lactation for high yielding dairy cows by its constant level of lactic acid production to rumen microbiota which allows lactate utilizing bacteria to sustain a metabolically active population (Santra *et al*., 2003). Probiotics supplemented animals have a beneficial effect on subsequent milk yields, fat and protein content. The increase in milk production, milk Solids-Not-Fat and milk protein percentages in dairy cows were associated with the numbers of cellulolytic bacteria, fiber degradation and changes in volatile fatty acid in the rumen observed an increase in milk production when feeding cows, a mixture of Lactobacillus acidophilus (McCarthy *et al.*, 1989)

Studies suggest that the effect of probiotics on milk yield and composition may vary depending on the specific strain(s) used and the dosages administered. Further research is needed to determine the optimal probiotic supplementation strategies to achieve maximum benefits in milk production and composition. Additionally, individual variation among cows and differences in management practices can also influence the response to probiotic supplementation.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Site

The experiment was conducted at Tatton Agriculture Park (TAP), Egerton University, Njoro. TAPis located approximately within longitude 36º36'E and latitude 0º22'S and at an elevation of 2,238 metres above sea level. The site receives annual rainfall ranging from 1000 – 1200 mm with a bimodial distribution pattern with long rains in April-July and short rains in October-November. The temperature of the locality varies between 19 to 22^{o}C (Egerton University Weather Station, 2018, unpublished data). Proximate analysis of the feed samples and *in-vitro* gas production procedures were done at the Animal Nutrition Laboratory, Department of Animal Science of Egerton University.

3.2 Experiment 1: To determine effects of single and mixed strain probiotics on feed intake and digestibility in lactating dairy cows

3.2.1 Selection of Animals

To determine feed intake, fifteen (15) lactating dairy cows (8 Friesian and 7 Guernsey) in their early and mid-lactation stage with average milk yield of 4.5 L of milk per day and an average live weight of 440±50 Kg were used in the study. The cows were subjected to five (5) dietary treatments with three dairy cows being allocated to each dietary treatment. All cows were sprayed with Duo Dip 55% EC at the start of the experiment to control ticks and other external parasites. To ensure all animals shared the same housing environment, all animals were kept in a zero-grazing unit with each animal being allocated its own individual cubicle (**Plate 3.1**). Cows in all groups were individually fed on their allocated experimental diets; while water and mineral salt were offered *ad libitum.*

Plate 3.1 Experimental animals housed in the zero-grazing unit

3.2.2 Preparation of probiotic cultures

a) Culturing *Saccharomyces cerevisiae*

The starter yeast culture of *Saccharomyces cerevisiae* was purchased from a local retail outlet (Menengai Agrovet) in 400g package (Diamond V xpc 400 g). Four hundred grammes (400g) of the dairy meal were measured and placed in an anaerobic jar and 400 ml of water added to the dairy meal in a ratio of 1:1 and mixed to make a slurry. This was autoclaved at 121^{o}C at 1 atmosphere (atm) in a TUR OFFNEN type 23 autoclave for 15 minutes so as to sterilize it. The slurry was then left to cool and its pH adjusted to 4.0 using citric acid. Thereafter 5g of *Saccharomyces cerevisiae* were then mixed with the slurry and incubated in the oven for seven days at 32^{o}C. This was done to test for the viability of cell culture. After seven days; a sample of the slurry with grown yeast cells was diluted with peptone water. 3.75 g of peptone were mixed in 250 ml of distilled water. 18 plates were prepared each with 20 ml; plates were plated by pouring the mixture in the plates and kept in the oven at 32^{o}C for seven days. They were then removed for colony counting.

b) Culturing *Lactobacillus plantarum*

Resuscitating cells (*Lactobacillus plantarum* isolates)

Lactobacillus plantarum isolates provided by the Microbiology Laboratory in the Department of Dairy and Food Science & Technology, Egerton University were taken through a resuscitation process to make them viable. MRS broth was used as the nutrient media. 2.08 g of

broth was dissolved in 40 ml of distilled water. The solution was autoclaved for 15 minutes at 121^{o}C (1 atm) in a TUR OFFNEN type 23 autoclave so as to sterilize it. The solution was then allowed to cool to room temperature. *Lactobacillus plantarum* cells were then placed into the solution, and incubated in an oven at 37^{o}C for 16 hr after which the solution was checked for turbidity which is an indicator of live cells. This was positive and after 24 hr, the cells were ready for culturing.

Media preparation

Potato Dextrose Agar (PDA) was used as the growth media. 19.5g of PDA was dispersed in 200 ml of water. The solution was autoclaved for 15 minutes and allowed to cool. 20 ml of solution was poured in each petri dish (8) and allowed to settle and solidify in readiness for inoculation (**Plate 3.2 and Plate 3.3**). The cells were inoculated by streaking and then placed in an anaerobic jar; transferred to the oven at 37^{o}C. After 16 hr the cells were harvested and introduced into the probiotic diet.

Plate 3.2 Culturing of *Saccharomyces cerevisiae* in Microbiology laboratory of Egerton University

Plate 3.3 Culturing of *Lactobacillus plantarum* in Microbiology laboratory of Egerton University

c) Colony count procedure

The test sample was diluted following the serial dilution technique. Each dilution bottle was filled with 9 ml of peptone water and 1ml of diluted sample was added to the solution making it up to 10 ml. Holding the bottle in the right hand, the cap was removed and the neck of the bottle flamed for sterilizing. One (1) ml of the sample was injected into the bottle and the cap replaced and the bottle was gently shaken for a uniform mixture. Ten dilution bottles were prepared. The 10^{th}, 9^{th} and 8^{th} dilution bottles were used to draw samples for inoculating in the petri dishes for colony count. The lid of the petri dish was slightly opened and the sample poured onto the petri dish and the lid replaced. The neck of the bottle was flamed and replaced with a cap. The Petri dishes were gently rotated to mix the culture and the medium thoroughly and to ensure that the medium covers the plate evenly. The Potato Dextrose Agar was allowed to completely gel. The plates were incubated at 32°C for *Saccharomyces cerevisiae* and for 7 days and 37°C/48 hr for *Lactobacillus plantarum*. After 7 days or 48 hr all colonies were counted using a magnifying colony counter. Colony-forming units (cfu) were counted and ranged from 1 x 10^7cfu to 1 x 10^{10}cfu. These were used to form probiotic diet where the autoclaved dairy meal was the carrier.

3.2.3 Formulation of the probiotic diet

400 g of Dairy meal mixed with 400 ml of water in a ratio of 1:1 was sterilized at 121^{o}C at 1.5 atm for 30 minutes using a TUR NUR WECHSELSTROM MELAG type 23 autoclave.

The autoclaved dairy meal was then left to cool. For *Saccharomyces cerevisiae*, the pH of the dairy meal was adjusted to 4.0 using citric acid measured using AD1020 pH/mV/ISE to attain optimum growing conditions (**Plate 3.4**). While for *Lactobacillus plantarum* the pH was maintained at 7. After inoculation, 20 g and 40 g of the probiotic diet were measured and placed into separate tubes to be added in the dairy meal as treatment procedures before feeding.

Plate 3.4 Formulation of the probiotic diet in Animal Science Laboratory of Egerton University

Dietary formulations

Cows in all groups were fed a diet composed of a basal diet consisting of 70% Rhodes grass hay and 30% dairy meal concentrate on dry matter basis. Treatments were supplemented with probiotics which were mixed with the dairy meal. Treatment 1 (T1) which was the control consisted of the basal diet (Rhodes grass hay and Dairy meal), Treatment 2 (T2) was basal diet + 40 g of *Lactobacillus plantarum*, Treatment 3 (T3) was basal diet + 40g of *Saccharomyces cerevisiae*, Treatment 4 (T4) was basal diet + 20 g of *Saccharomyces cerevisiae* + 20 g of *Lactobacillus plantarum* and Treatment 5 (T5) was basal diet + 40 g of *Saccharomyces cerevisiae* + 40 g of *Lactobacillus plantarum.*

Dietary treatment feeding management

Probiotics supplemented diets were given to the treatment groups continuously for 35 days. Animals were allowed 14 days of adaption period and 21 days of data collection. The control group received the basal diet with no probiotics supplement. Cows under the experiment were given 40g/day probiotics (containing averagely 1×10^{9} CFU/g of probiotics

supplementation), while (treatment 4 had 20 g of *Lactobacillus plantarum* with 1×10^5 cfu and 20 g of *Saccharomyces cerevisiae* with 1×10^5) mixed with the dairy meal that was offered twice daily in the morning (06:00 hr) and afternoon (16:00 hr) in equal amounts after milking. The live probiotics used in this study were *Lactobacillus plantarum* and *Saccharomyces cerevisiae.* The proportion of the mixed strain was 1:1. The dairy meal was offered twice daily during milking; 3 Kg per day per cow.

Experimental design

Three lactating dairy cows were randomly allocated to each of the five dietary treatments (**Figure 3.1**). The response variables that were measured included; feed intake, milk yield and milk composition.

ALLOCATION OF TREATMENT DIETS									
TRT 1		**TRT 2**		**TRT 3**		**TRT 4**		**TRT 5**
COW 1		COW 4		COW 7		COW 10		COW 13
COW 2		COW 5		COW 8		COW 11		COW 14
COW 3		COW 6		COW 9		COW 12		COW 15

Figure 3.1 Schematic representation of random allocation of animals to the dietary treatments

Recording Observations

3.2.4 Feed Intake

The feeds were offered twice daily to ensure availability of the basal ration at all times so as not to restrict intake due to unavailability of the basal diet. Feed were weighed before feeding and recorded. The days' refusals were weighed the next day in the morning before offering the animals fresh feed.

Feed intake was calculated as follows: -

Feed intake = Feed offered – Refusal

3.2.5 *In-vitro* Dry Matter Digestibility of Feed

One steer was used as a donor for rumen fluid. It was fed with the experimental diets T1, T2, T3, T4 and T5 with each dietary treatment being administered for 7 days before rumen liquor was collected for analysis on the 7^{th} day consecutively. Rumen fluid was collected at 08:00 hr before morning feeding (**Plate 3.5**). One litre of rumen fluid from the donor animal was stored under anaerobic conditions in a thermos flask. This was filtered through two layers of cheese-

cloth to obtain strained rumen fluid which was then flushed with carbon dioxide (CO_2) to maintain anaerobic conditions. Part of the rumen fluid was analyzed for the volatile fatty acid profile. Rumen fluid was used in combination with buffers to simulate the action of saliva. The module glass was lubricated with petroleum jelly to ease the sliding of the piston and prevent gas escape then the silicon rubber closed with a plastic clip. The fermentative activity of the mixed microbial population of treatments was determined using the gas production technique as described by Menke *et al.* 1979. The rumen fluid and buffer medium were mixed in the ratio of 1:2 (v/v). Thirty (30) ml of the buffer-rumen fluid mixture were added into syringes holding the treatment diet samples, shaken gently and any air bubbles released.

The substrate was weighed to 0.200±0.02 g DM to contain approximately 0.14 g of DM of Rhodes grass hay and 0.06 g of DM of dairy meal concentrate (a pinch of probiotic for each treatment added) and dispensed into 100ml calibrated glass syringes fitted with pistons. Subsequently, 30 ml of buffered rumen fluid were dispensed into syringes containing substrate with different strains of probiotics and blank syringes without substrate. The syringes with the substrate and the those with blanks were incubated in a water bath maintained at 39^{o}C for 96 hr taking readings at 0, 3, 6, 9, 12, 24,48,36, 72 and 96 hr of incubation. The samples were run in duplicates. The gas produced was determined as the total increase in volume minus the mean blank value from the recorded gas production of all samples to give the net gas production. The net gas volumes data was then fitted in the equation of Ørskov & Mcdonald (1979) to determine the potential degradability of the feed. The model was fitted using NEWAY excel software version 6,

$$Y = a + b\,(1 - e^{-c\,t})$$

where:

Y= the volume of gas produced (ml) at time t,

a= the gas production from the immediately soluble fraction (ml),

b = the gas production from the immediately degradable fraction (ml) at time t,

a+b = the potential gas production (ml),

c = the rate constant of gas production (fraction/h)

Determination of organic matter digestibility (OMD) at 48 hr;

In-vitro organic matter digestibility at 48 hr was calculated from the equation:

OMD48 (%) = 18.53 + 0. 9239 (gas production at 48hr) + 0.0540 CP (Menke *et al.*, 1979)

Short chain fatty acids were determined from the equation;

SCFA (m Mol/200mgDM) =0.0222 GP-0.00425,

where GP is 24 hr net gas production (ml/200 mg DM).

Metabolizable energy was determined from the equation;

ME (MJ/Kg DM) = 1.06 + 0.1570 × Gas produced (ml/200 mg DM) + 0.0084 × CP (g/Kg DM) + 0.022 × EE (g/Kg DM) - 0.0081 × Ash (g/Kg DM), Menke *et al.* (1979).

Plate 3.5 Collecting rumen liquor for laboratory analysis

3.2.6 Chemical Analysis

Samples were milled to pass through a 1 mm screen in preparation for various chemical analyses and *in vitro* gas production procedure. The dry matter (DM) was determined by drying the milled sample at 105^{o}C overnight and ash was determined by igniting the dry samples in a Muffle furnace at 550^{o}C for 2 hr. The crude fibre content was analyzed using ether extraction method. Nitrogen content was measured by Kjeldahl method (AOAC, 2003). The Crude Protein (CP) was calculated as Nx6.25. The organic matter (OM) was calculated as the difference between DM and ash content. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the method prescribed by Van Soest *et al*. (1991).

3.2.7 Data Analysis

Data on feed intake and digestibility were analyzed using completely randomized design.

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

where:

Y_{ij} = Measurement of gas produced/ feed intake associated with effect i^{th} diet treatment

μ = overall mean

α_i = effect of i^{th} diet treatment, where *i*= {1, 2, 3, 3, 4, 5}

ε_{ij} = random error associated with Y_{ij}

Statistical Analysis

The data were subjected to Analysis of variance (ANOVA) using General linear model (GLM) of Statistical Analysis Systems (SAS 2009) Computer package. Mean separation were done using Tukey's test at significant level of 0.05

3.2.8 Volatile fatty acid analysis

After 96 hr of fermentation, ruminal fluid was collected for clarification. Ruminal fluid was clarified by centrifugation at 13,000 x g at 4^{o}C for 10 minutes. 2-3 drops of Hydrochloric acid were added to lower the pH to 4-5 and stop the fermentation process. Concentration of the VFAs (Acetate, butyrate and propionate) using Gas chromatography varian STAR 3400 cx. 1µl of the sample was injected into the GC system with CX series. That was equipped with a Flame ionization detector with Nitrogen as a carrier gas with the column temperature kept at 80-150^{o}C, injector kept at 170^{o}C and detection temperature at 180^{o}C. The analysis was isothermal for 13 minutes. VFA were quantified from the chromatograph peak areas using calibration (**Plate 3.6).**

Plate 3.6 SHIMADZU, GC-9A) control panel

Statistical analysis

Effect of the treatments (probiotics) on volatile fatty acid (VFA) production were tested by analysis of variance using the general Linear Model procedure of the SAS (2003) as;

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

where:

Y_{ijk} = measurement of volatile fatty acid production due to effect i^{th} diet treatment

μ = overall mean

α_i = effect of i^{th} diet treatment, where i = {1, 2, 3, 3, 4, 5}

ε_{ijk} = random error associated with Y_{ijk}

3.3 Experiment 2: To evaluate effects of single and mixed-strain probiotics on rumen methane emission in lactating dairy cows

3.3.1 *In-vitro* gas production studies

In vitro studies were conducted to identify the best probiotic strain that reduces methane gas emission in lactating dairy cows. The techniques to be used in the experiment were *in-vitro* dry matter degradability (IVDMD) and *in vitro* gas production. From experiment Two; gas produced from the *in vitro* digestibility test were taken for further analysis using a chromatography test to identify the constitution of methane in the gas.

Suitable aliquot of gas collected from Gas-tight culture bottles (250 ml capacity) consisting rumen contents and feed samples, were withdrawn from the tip of the incubation bottles using glass modules and composition of gas in the headspace of bottles determined using gas chromatography.

The methane gas (CH_4) analysis was performed by GC-flame ionization detection (FID) using a gas chromatograph (SHIMADZU, GC-9A) equipped with a Hayesep Q packing column (2.44 M_1/8 in._2.0mm ID) (Bhatta *et al*., 2007; Johnson *et al.,* 1995; Situala *et al.*, 1992).

3.2.2 Procedure

The gas samples collected from the dietary treatments were run in Gas Chromatography (SHIMADZU, GC-9A) by injecting 1µL of the sample into injection port using Gas chromatography varian STAR 3400 cx. The samples were run for 10 minutes. The peak areas and retentions of the methane were calculated and reported by the digital processor. The percentage of the methane gas composition were calculated by expressing each peak area as a percentage of the total peak area.

3.2.3 Sample preparation

After 96 hr, gas produced from the *invitro* gas production technique process was collected into a gas vial and taken to a GC ionisation flame for further analysis. 1µl of gas was sampled from the gas and injected into the GC flame with injection temperatureat 120^{o}C, injector kept at 150^{o}C and detection temperature at 180^{o}C. The methane composition of the headspace was measured by gas chromatography (state model). An external standard with known concentration composition of methane was run; its retention time, area peaks and injection volume determined.

3.2.4 Statistical model

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

where:

Y_{ijk} = measurement of methane emission associated with effect i^{th} diet treatment

μ = overall mean

α_i = effect of i^{th} diet treatment where $i = \{1, 2, 3, 3, 4, 5\}$

ε_{ijk} = random error associated with Y_{ijk}

3.2.5 Data Analysis

The data were subjected to Analysis of variance (ANOVA) using General linear model (GLM) of Statistical Analysis Systems (SAS 2009) computer package. Mean separation were done using Tukey's test at significant level of 0.05

3.3 Experiment 3: To determine effect of single strain and mixed-strain probiotics on milk yield and composition of lactating dairy cows

3.3.1 Milk yield

Cows were milked twice daily from their stalls in the morning at 06:00 hr and in the afternoon at 16:00 hr; and the milk yield was recorded. Milk yield was recorded daily for 35 days. Daily milk yield was used to calculate weekly average milk yield per cow throughout the experimental period.

3.3.2 Milk composition

Milk samples (approximately 50 ml) from individual cows were collected on the last day of feed adjustment, end of week one, end of week 2 and end of week 3. The two samples collected on the same day were combined at a ratio of 1:1 (volume: volume) to ensure a fair representation of the milk composition of the specific sample day. The samples were stored at 4°C until further analysis.

The lactoscan SP technique which comply with WASO 9622 / IDF 141:2013 and AOAC, (2000) official method 972.16 was used to run milk composition tests. The milk composition parameters that were measured included; Butter fat content, protein, total solids, solids-not fat (SNF) and lactose.

3.3.3 Data Analysis

The data on feed intake, digestibility, milk yield and milk composition were analysed using the linear model. Parity, stage of lactation, breed and age of the animal were registered as covariates.

$$Y_{ijklmn} = \mu + \alpha_i + B_j + S_k + Age_l + P_m + e_{ijklmn}$$

where;

Y_{ijklmn}= the observation on the i^{th} *treatment on the* cow belonging to the j^{th} breed, k^{th} stage of lactation, of the l^{th} age and was in m^{th} parity

μ = the underlying population mean

α_i = Effect of the treatment

B_j = Effect of the breed

S_k = Effect of stage of lactation

Age_l = Effect of the age

P_m = Effect of parity

$e_{ijklmnn}$ = the random residual effect

Statistical Analysis

The data were subjected to Analysis of variance (ANOVA) using General linear model (GLM) of Statistical Analysis Systems (SAS 2009) computer package. Mean separation were done using Tukey's test at 5% significance level.

CHAPTER FOUR

RESULTS

4.1 To determine the effect of single and mixed strain probiotics on feed intake and digestibility in lactating dairy cows

The results of chemical analysis of the basal diet are shown in **Table 4.1**.

Table 4.1 Results from chemical analysis of the basal diet and concentrates used in the study

	Composition				
Ingredient	DM (%)	CP	Non-Digestible fibre	EE	Ash
Rhodes grass hay	91.5	9.65	75.5	1.9	6.3
Dairy meal	91.4	17.4	54.6	10.5	7.5

The results of feed intake Kg DM/d of cows fed on probiotic supplemented diet are shown in **Table 4.2**

Table 4.2 Feed intake Kg DM/d of cows fed on basal diet supplemented with probiotics

Dietary Treatment	**DM intake (DMI ± SE (0.66))**
T1	9.533[a]
T2	9.567[a]
T3	11.000[b]
T4	10.933[b]
T5	11.011[b]
SEM	0.66
R^2	0.972
P-value	0.000[*]

[ab]Means in the same column with different superscripts differ significantly at P<0.05.

T1 Basal diet (control) ([*]) means significant

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of *Saccharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g *Saccharomyces cerevisiae* + 40 g *Lactobacillus plantarum*

Results of feed intake in **Table 4.2** showed that probiotic supplementation had significant effect (P<0.05) on feed intake. The highest feed intake was observed in T5 at 11.011±0.66 Kg DM/day. The lowest feed intake was observed in T1 at 9.533±0.66 KgDM/day. Feeds that had *Saccharomyces cerevisiae* had a significant increase in feed intake. There was no significant effect of adding a single strain of *Lactobacillus plantarum* as seen in Treatment 2.

The results of *in-vitro* dry matter digestibility of basal diet supplemented with probiotics are shown in **Table 4.3**.

Table 4.3 In-vitro dry matter digestibility of basal diet supplemented with different strains of probiotics

Treatment	GP AT 24 hr	GP AT 48 hr	a (ml)	b (ml)	a+b (ml)	c (fraction/hr)
T1	22.00^{a}	30.00^{ab}	0.87^{a}	34.88^{ab}	35.74^{ab}	0.040^{a}
T2	18.50^{a}	21.25^{a}	0.80^{a}	22.17^{a}	22.97^{a}	0.070^{b}
T3	27.00^{ab}	35.75^{bc}	0.93^{a}	38.50^{b}	$39. 42^{b}$	0.053^{ab}
T4	31.75^{b}	39.88^{bc}	2.66^{b}	$41. 26^{b}$	43.93^{b}	0.057^{ab}
T5	$34. 00^{b}$	43.75^{c}	2.54^{b}	$46. 12^{b}$	48.66^{b}	0.053^{ab}
R^2	0.803	0.810	0.814	0.809	0.811	0.774
SEM	2.034	2.706	0.288	2.778	2.983	0.004
P-Value	0.001	0.001	0.001	0.001	0.001	0.003

^{abc}Means in the same column with different superscripts are significantly different at P<0.05

T1 Basal diet (control)

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of *Saccharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g *Saccharomyces cerevisiae* + 40 g *Lactobacillus plantarum*

a Immediately soluble fraction; b Immediately degradable fraction; a+b Potential gas production; c Rate constant of gas production

Gas production at 24 hr was significantly affected (P<0.05). Treatment 5 had the highest gas production of 34. 00± 2.034mlat 24 hr. Treatment 2 had the lowest gas production of 18.500 ± 2.034 ml at 24 hr.

Gas production at 48 hr differed significantly (P<0.05) between treatments. Treatment 5 had the highest gas production of 43.75±2.706 ml at 48hr. Treatment 2 had the lowest gas production of 21.250±2.706ml at 48 hr.

Immediately soluble fraction (a) was significantly affected ($P<0.05$). Treatment 4 had the highest immediately soluble fraction of 2.66 ml. Treatment 2 had the lowest immediately soluble fraction of 0.80 ml.

Immediately Degradable fraction (b) was significantly affected ($P<0.05$) by the different treatments. Treatment 5 had the highest immediately degradable fraction of 46.12ml while treatment 2 had the lowest immediately degradable fraction of 22.17 ml.

Potential gas production (a+b) was significantly affected ($P<0.05$) by the different treatments. Treatment 5 had the highest potential gas production of 48.66 ml. Treatment 2 had the lowest potential gas production of 22.97 ml.

The rate constant of gas production was significantly affected ($P<0.05$) between treatments. Treatment 2 had the highest rate of constant gas production of 0.07 fraction/hr. Treatment 1 had the lowest rate of constant gas production of 0.04 fraction/hr.

Table 4.4 shows results of calculated Organic Matter Digestibility at 48 hr (OMD (%)), Metabolizable Energy (ME) and Short Chain Fatty Acids (SCFA).

Table 4.4 Calculated Organic Matter Digestibility, Metabolizable Energy and Short Chain Fatty Acids

TREATMENT	**OMD %**	**ME** (MJ/Kg DM)	**SCFA** (mMol/200mgDM)
T1	46.860^{ab}	5.890^{ab}	0.480^{a}
T2	38.773^{a}	4.517^{a}	0.407^{a}
T3	52.170^{bc}	6.793^{bc}	0.607^{ab}
T4	55.980^{bc}	7.440^{bc}	0.700^{b}
T5	59.560^{c}	8.050^{c}	0.750^{b}
R^2	0.810	0.809	0.806
SEM	2.499	0.425	0.045
P-VALUE	0.001	0.001	0.001

^{abc}Means in the same column with different superscripts are significantly different at $P<0.05$

T1 Basal diet (control)

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of S*accharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g of *Saccharomyces cerevisiae* + 40 g *Lactobaciluus plantarum*

Calculated OMD% was significant ($P<0.05$). T5 had the highest Organic Matter Digestibility of 59.56% while T2 had the lowest Organic Matter Digestibility of 38.77%.

Metabolizable Energy (ME) differed significantly ($P<0.05$) between treatments. Treatment 5 had the highest metabolizable energy of 8.050 ± 0.425 MJ/KgDM and Treatment 2 had the lowest metabolizable energy of 4.517±0.425 MJ/Kg DM.

Concentration of short chain fatty acid (SCFA) differed significantly ($P<0.05$) between treatments. The range of the Short Chain Fatty Acids ranged from 0.750±0.045 (mMol/200mg DM) to 0.407± 0.045(mMol/200mg DM) in Treatment 5 and Treatment 2 respectively. Treatment 5 had the highest concentration of SCFA, followed by Treatment 4, Treatment 3, Treatment 1 and Treatment 2 in that order.

For the variation in *in-vitro* dry matter digestibility (INVDM) the results showed that T5 had the highest INVDM while T2 had the lowest (**Figure 4.1**). *In-Vitro* Dry matter digestibility of all the treatments increased overtime though at different rates.

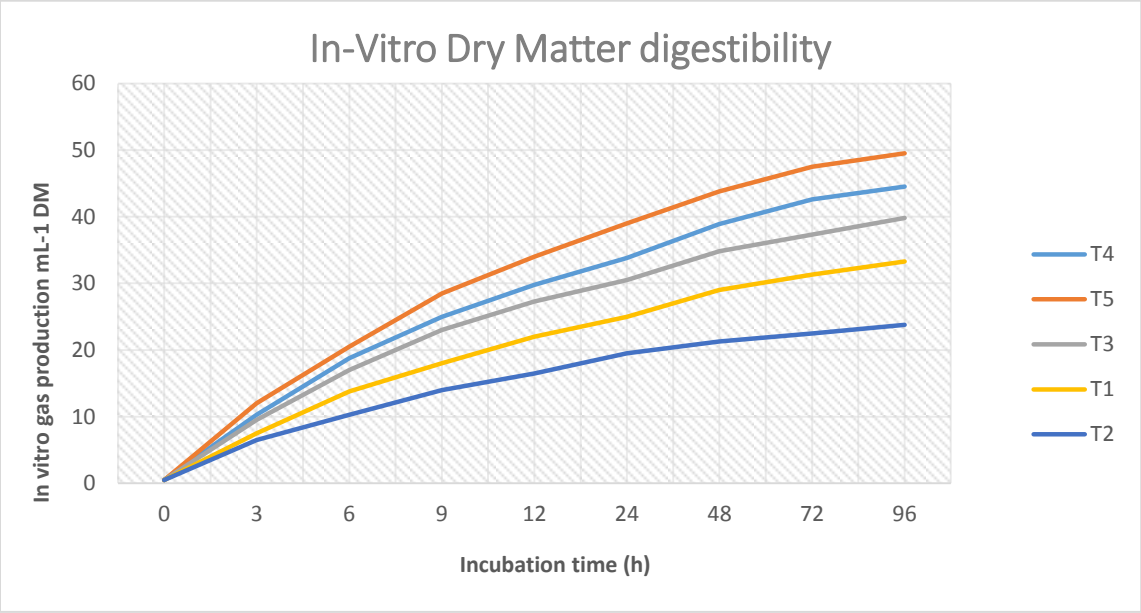

Figure 4.1 Dietary effects on *in-vitro* dry matter digestibility

T1 Basal diet (control

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of *Saccharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g *Saccharomyces cerevisiae* + 40 g *Lactobacillus plantarum*

Table 4.5shows results of volatile fatty acid analysis. Single and mixed strain probiotics had no significant effect (P> 0.05) on acetate production.

Table 4.5 Results of Volatile Fatty Acid analysis

Treatment	Acetate(ml)	Propionate(ml)	Butyrate (ml)
T1	68.440	56.365^{ab}	26.870^{ab}
T2	68.560	58.365^{ab}	27.665^{ab}
T3	57.345	48.825^{a}	22.845^{a}
T4	58.535	49.235^{a}	23.965^{a}
T5	86.130	70.935^{b}	33.610^{b}
P-VALUE	0.068^{NS}	0.042*	0.027*
SEM	7.775	5.326	2.223
R-square	0.779	0.82	0.851

^{abc}Means in the same column with different superscripts are significantly different at P<0.05

T1 Basal diet (control)

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of S*accharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g S*accharomyces cerevisiae* + 40 g *Lactobacillus plantarum*

Single and mixed strain probiotics had a significant effect (P<0.05) on propionate production. Treatment 5 had the highest propionate production of 70.935 ml. Treatment 3 had the lowest propionate production of 48.825 ml.

Single and mixed strain probiotics had a significant effect (P<0.05) on butyrate production. Treatment 5 had the highest butyrate production of 33.61 ml. Treatment 3 had the lowest butyrate production 22.845 ml.

Single and mixed strain probiotics had no significant effect (P<0.05) on acetate production.

Below are the standard curves of acetate, propionate and butyrate.

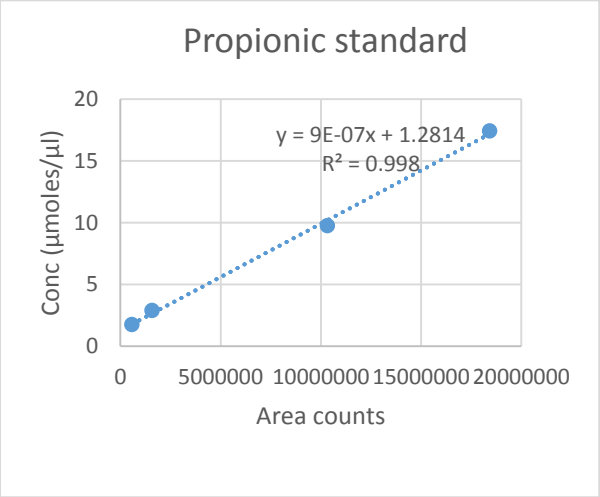

Figure 4.2 Volatile fatty acids individual standard curve for Propionic standard

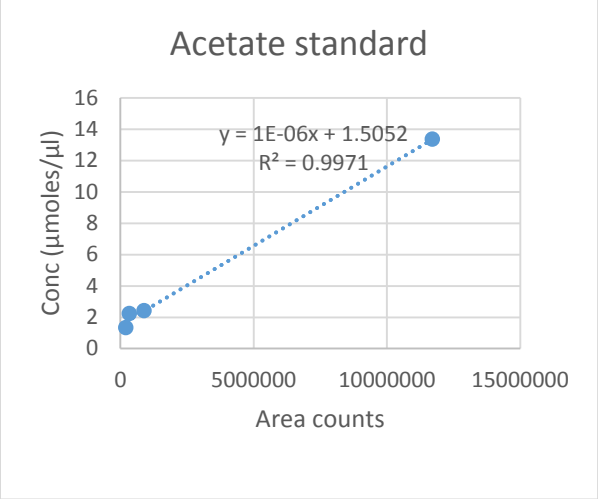

Figure 4.3 Volatile fatty acids individual standard curve for Acetate standard

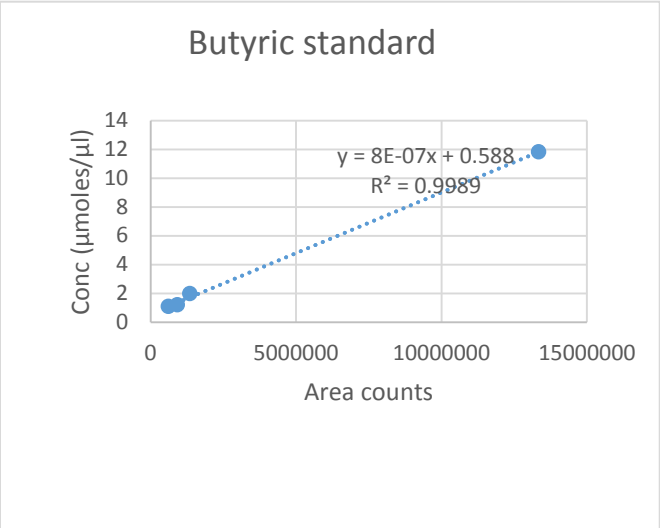

Figure 4.4 Volatile fatty acids individual standard curve for butyrate standard

4.2 Effect of single and mixed strain probiotics on methane gas emission of lactating dairy cows.

Table 4.6 shows results of methane emission.

Table 4.6. Results from methane emission analysis**.**

TREATMENT	**MEAN ml**
T1	22487.76[b]
T2	27254.39[b]
T3	46428.46[c]
T4	73.27[a]
T5	68267.86[d]
P-Value	0.000[*]
R-Square	0.959
SEM	3353.229

[abcd]Means in the same column with different superscripts are significantly different at P<0.05

T1 Basal diet (control)

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of S*accharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g S*accharomyces cerevisiae* + 40 g *Lactobacillus plantarum*

Probiotic supplementation had a significant effect on methane emission. Highest methane emission was in Treatment 5 and lowest methane emission was in Treatment 4. Treatment 5 had 68267.861ml of methane emission. Treatment 4 had least methane emission of 73.265 ml.

4.3 Effect of single and mixed strain probiotics on milk yield and composition of lactating dairy cows

Table 4.7 shows results from milk yield and composition of dairy cows on basal diets supplemented with probiotics.

Table 4.7 Results of milk yield and composition analysis.

	Milk yield	**Milk composition**				
Treatments	**MSE**	**Protein**	**Fat**	**Solids-not fat**	**Lactose**	**Total solids**
T1	4.12[a]	3.056	3.917	8.370	4.608	12.287
T2	3.92[a]	3.080	3.889	8.352	4.646	12.212
T3	4.60[b]	3.086	3.776	8.451	4.652	12.494
T4	3.94 [a]	3.133	4.368	8.599	4.732	13.058
T5	3.80[a]	3.119	3.628	8.507	4.682	12.134
P-Value	0.007[*]	0.934[NS]	0.663 [NS]	0.816 [NS]	0.386 [NS]	O.122 [NS]
SEM	0.11	0.25	0.383	0.075	0.034	0.338

[ab]Means in the same column with different superscripts are significantly different at P<0.05

NS means not significant

T1 Basal diet (control)

T2 Basal diet + 40 g of *Lactobacillus plantarum*

T3 Basal diet + 40 g of *Saccharomyces cerevisiae*

T4 Basal diet + 20 g *Saccharomyces cerevisiae* + 20 g *Lactobacillus plantarum*

T5 Basal diet + 40 g *Saccharomyces cerevisiae* + 40 g *Lactobacillus plantarum*

Probiotics significantly (P<0.05) affected milk yield. T3 had the highest milk yield at 4.6 L and the. least was from T5 at 3.8 L. Results for milk compositions how that single and mixed strain probiotics had no significant effect (P>0.05) on% protein content. Probiotic supplementation had no significant effect (P>0.05) on butter fat content. Solids not-fat content of milk were not significantly affected (P>0.05) by single and or mixed strain probiotics. Single and mixed strain probiotics had no significant effect (P>0.05) on lactose and total solids.

CHAPTER FIVE

DISCUSSION

5.1 Effect of single and mixed strain probiotics on intake and digestibility of feed of lactating dairy cows

Probiotics for adult ruminants have mainly been selected to improve fiber digestion by rumen microorganisms. Such probiotics have positive effects on various digestive processes, especially cellulolysis and the synthesis of microbial protein (Uyeno *et al.,* 2015). Different strains of probiotics have different impacts on rumen fermentation.

The observed increased feed intake in diets supplemented with *Saccharomyces cerevisiae* (*S. cerevisiae*) was because *S. cerevisiae* provides soluble growth factors that stimulate the growth of cellulolytic bacteria and cellulose digestion (Callaway *et al*., 1997). Even though the mechanism of improved feed intake with *S. cerevisiae* has not been clearly defined, it is suggested that *S. cerevisiae* may cause a number of effects including increased pH, altered volatile fatty acid concentrations, increased number of cellulolytic bacteria, and increased rate or extent of ruminal fiber digestion (Chaucheyras-Durand *et al.,* 2008). On the basis of these previous results, it is proposed that *S. cerevisiae* may increase fiber digestion, which could increase rate of passage and therefore improve feed intake (Dann *et al*., 2000).

Results from S. *cerevisiae* and *Lactobacillus plantarum* (*L. plantarum*) mixed culture treatment diets showed higher cumulative gas production, rate gas constant, organic matter digestibility (OMD), short chain fatty acids (SCFA) and metabolizable energy (ME) because *L. plantarum* is a lactic acid producing bacteria hence lowering ruminal pH. This providedfavourable growth medium for yeast (*S. cerevisiae*) which in turn-controlled accumulation of lactic acid. The activity of the other microbial organisms in the rumen were enabled thus the observed better results in mixed strain probiotics. S. *cerevisiae* regulates the rumen pH and limits acidosis risk through regulating both of lactate producing and lactate utilizing bacteria. A less acidic ruminal environment has been shown to benefit the growth and fiber-degrading activities of cellulolytic microorganisms (Uyeno *et al*., 2015).

Increase in net gas production, volume of gas produced from insoluble fraction, and potential extent of gas production indicate an increase in the digestibility of substrates and activity of fibre-degrading microbes (Izuddin *et al.,* 2018). Improvement in OMD, ME can be explained by the improvement in major microbial population in the rumen.

S. cerevisiae as single strain increased fermentation characteristics at a higher rate than the single strain of *L. plantarum* which was below the level realized from the control. The low performance of *L. plantarum* probiotic could be attributed to it being a lactic acid producing bacteria. This lowered the rumen pH to levels that could not allow survival or cause death of other rumen microbiota that are not favoured by low pH media. Low rumen pH for prolonged periods tends to negatively affect feed intake, microbial metabolism and nutrient degradation (Chaucheyras-Durand *et al.,* 2012). Previous studies have reported the ability of lactic acid bacteria to survive in the rumen, change the parameters of *in-vitro* rumen fermentation and affect rumen microflora (Izuddin *et al*., 2018).

Continuous research with live *S. cerevisiae* supplementation has clearly established scientifically proven strategies for modifying and optimising microbial activities in the gastro-intestinal tract ecosystem and techniques for improving performance of ruminants. *S. cerevisiae* improves cellulolytic activities of rumen micro-organisms in such a way that they increase their total numbers, improve fibre digestion and reduce lactate accumulation (Beev *et al.*, 2007).

Addition of *S. cerevisiae* to the ruminant microflora improves animal production by promoting bacterial activity, reducing the amount of lactate (Santoso *et al.,* 2016).

The results from this study indicate that the addition of probiotics as mixed strains enhanced rumen fermentation further than when used as single strains. This aligns with reports from other working related study such as Santoso *et al.* (2015) that concluded that addition of mixed microbes in concentrate improved fermentation activity and *in vitro* nutrient digestibility.

5.2 Effect of single and mixed strain probiotics on methane emission of lactating dairy cows

Methane produced by ruminants is a major contribution to anthropogenic greenhouse gas inventories within the pastoral grazing systems. Enteric fermentation is a digestive process through which a community of microbes present in the forestomach of ruminants (the reticulo-rumen) break down fibrous plant material into nutrients that can be used by the animal for the production of high-value protein that include milk, meat and leather products. Hydrogen (H_2) and methyl-containing compounds generated as fermentation end products of this process are used by different groups of rumen methanogenic archaea to form CH_4, which is belched and exhaled from the lungs via respiration from the animal and released to the atmosphere (Doyle *et al.,* 2019). Direct ruminal intervention is a means to control ruminant methane emissions as methane producing archea known as methanogens are a distinct group of organisms which are

normal component of the rumen of the rumen microbial ecosystem. Hydrogen and carbon dioxide are the principle substrates used by rumen methanogens to produce methane and so compounds that directly inhibit activity of the methanogens are likely to reduce or eliminate methane production (Greening *et al.,* 2019).

Regarding the results of CH_4 emission intensity, Muñoz *et al.* (2016) reported that addition of yeast was followed by higher yield of CH_4/DMI and digestible organic matter intake. This agrees with high methane production in Treatment 3 (T3) and treatment 5 (T5) which were both supplemented with *S. cerevisiae*. As a natural feed additive, yeasts contribute to balance and stabilize rumen microbiota, to maintain a favorable pH and enhance the formation of fermentation end-products in the rumen, and to improve ammonia utilization by ruminal bacteria (Chaucheyras-Durand *et al.,* 2012). This effect might be dependent on dosage of yeasts, strain of yeasts, and diet composition.

Relative to *S. cerevisiae*, there is little information dealing with their potential effects on hydrogen transfer mechanisms and methanogenesis. One mechanism of action could be to shift hydrogen utilization from methanogenesis to reductive acetogenesis (Ungerfeld, 2015). This was seen with increased acetate production in dietary treatments supplemented with *Saccharomyces cerevisiae* though this does not translate into reduced methane production perhaps due to mixed culture of pre-existing rumen microbiota. *In vitro* studies have shown beneficial effects of feeding *S. cerevisiae* on growth and hydrogen utilization and acetate production by acetogenic bacteria isolated from a rumen of lambs, even in the presence of methanogens (Chaucheyras *et al*., 1995).

Lactic acid bacteria (LAB) are unable to initiate the metabolism of plant structural polysaccharides and are not regarded as major contributors to rumen fermentation (Doyle *et al.,* 2019). The low emission of methane in treatment two (T2) supplemented with *L. plantarum* could be due to the use of *L. plantarum* or their metabolites to shift the rumen fermentation so that there is a corresponding decrease in CH_4production, or use of *L.plantarum* or their metabolites to directly inhibit rumen methanogens and use of *L. plantarum* or their metabolites to inhibit specific rumen bacteria that produce H_2 or methyl-containing compounds that are the substrates for methanogenesis.

Addition of *L. plantarum* may have stimulated the growth of lactic utilizing bacteria leading to increased production of propionic acid and a subsequent decrease in the hydrogen

availability for methane production. This is confirmed by the presence of high volumes of propionate in treatment 2 (T2).

L. plantarum can reduce CH_4 production effectively though the effect is clearly strain dependent and it is not understood whether the *L. plantarum* or their metabolites affect the methanogens themselves, or whether they affect the other rumen microbes that produce substrates necessary for methanogenesis

In Treatment 4 (T4), both methane and propionate were minimal because the *S. cerevisiae* and *L. plantarum* used were in small quantities to effect any detectable change. A more advanced study needs to be done to determine why the methane output was extremely low compared to the other treatments.

Treatment 5 (T5) had the highest both methane and propionate production because of the combined effect of *L. plantarum* and *S. cerevisiae* which were available in sufficient amounts.

From the study it was noted that addition of *S. cerevisiae* increased methane production on ml/Kg Dm basis, perhaps partly from the increased DM intake. This agrees with results from Muñoz *et al.* (2016) that also reported that addition of *S. cerevisiae* was followed by higher yield of CH_4/Kg DMI and digestible organic matter intake.

5.3 Effect of single and mixed strain probiotics on milk yield and composition of lactating dairy cows

Results from dietary treatment T3 showed the highest milk yield. *Saccharomyces cerevisiae* improves the feed efficiency and milk yield as it is a source of naturally occurring B-vitamins and disaccharides enzymes which enhance digestion of fiber, protein, fats and minerals. Specifically, *S. cerevisiae* has capability to competitively inhibit pathogenic bacteria and to promote growth of beneficial bacteria.

S. cerevisiae also has a buffering effect in the rumen by mediating the sharp drop in rumen pH. This improves the use of lactate by ruminal microorganisms. It also removes oxygen from the surfaces of fresh feed to maintain metabolic activity in the rumen activity in the rumen and keep the rumen an anaerobic chamber (Mona *et al.,* 2015)

S. cerevisiae is known to increase milk production by stimulating bacterial growth which digests cellulose and hemicellulose as *Fibrobacter succinogens* and *Ruminococcus spp*. (Chaucheyras-Durand *et al.,* 2012). Increasing fiber digestion in the rumen may result in higher

consumption of organic matter and consequently increased milk production (Yang *et al*., 2014). Addition of *S. cerevisiae* to cows that feed on diets rich in fiber is a promising strategy to increase the digestibility and consequently the milk production. (Shabangu, 2014).

Mixed and single strain probiotics had no effect on milk composition. These results were similar to those done by Boğa *et al*. (2007) who concluded that both *Lactobacillus spp.* alone when supplemented with *S. cerevisiae* did not affect milk yield and composition of lactating cows fed on basal diet with a ratio of 60:40 concentrate and alfalfa hay ratio.

Both *L. plantarum* and *S. cerevisiae* used either as single or mixed strain had no effect on milk composition possibly because in this work conditions, based on the action of probiotics, alteration in rumen microbial flora, changing patterns of rumen fermentation, increased passage rate of the nutrients in the intestine, increasing the digestibility of the diet, there wasn't suitable conditions for the performance of them. Among the various factors that affect the response of dairy cows supplemented with probiotic, stage of lactation, type of forage provided, the feeding strategy and forage to concentrate ratio of the diet are the most noticeable (Kassa, 2016). The other possible reason could be the small number of cows used in the experiment according to the experimental design.

The observation of no effect on milk yield and composition when Lactobacillus plantarum was used from Xu *et al.* (2017). Experiments where probiotics were added to the animal diet have shown discrepancies in animal performance. A number of different factors contribute to the discrepancies. One factor may that these experiments were performed by different laboratories and under different experimental conditions. Factors relating to the experiments were different, including probiotic mix preparation, the host-specific factors like age, physiological stage, health, feeding regime of the subject animals. Moreover, it is very likely that any beneficial effects seen are probiotic strain-specific (Forte *et al.*, 2016).

Generally, increase in milk production occurs in high producing dairy cows showing a better effect on the rumen, particularly in diets with high levels of concentrate and dry matter intake. In this study, the cows were of low production (around 5kgs of milk/cow/day) and received moderate amount of concentrate (3 kg of dairy meal per cow per day).

The data for *in vitro* gas production indicates a more favourable effect of the *Saccharomyces cerevisiae* compared with the *Lactobacilli plantarum* species as shown by both feed intake and *in vitro* gas production. The fact that these benefits were not reflected in milk

production can be traced to the use of cows of low milk production and the experimental design which should have incorporated some form of changeover of treatments or of adjustment by covariance for yields recorded prior to starting the experiment. The reason why the 16% improvement in feed intake was not reflected in increased milk production may be because of the low nutritive value of the diet, 70% of which was provided by Rhodes grass hay which was generally of relatively low quality.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

i. A combination of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* increased feed digestibility in lactating dairy cows. The single strain of *Saccharomyces cerevisiae* increased dry matter intake in lactating dairy cows.

ii. A combination of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* reduced methane emission when used in low quantities.

iii. Both *Lactobacillus plantarum* and *Saccharomyces cerevisiae* did not affect milk yield and composition of lactating cows fed with a diet based on 30:70 concentrate and Rhodes grass hay ratio.

Recommendations

i. Further studies are needed to be planned with different source, level of roughage and nutrient content in the diet with probiotic supplementation to compare the impact of probiotics addition on feed intake and digestibility.

ii. A study to understand if *Lactobacillus plantarum* or their metabolites affect the methanogens themselves, or whether they affect the other rumen microbes that produce substrates necessary for methanogenesis.

iii. *Lactobacillus plantarum* and *Saccharomyces cerevisiae* should be fed to high yielding early lactating stage of lactation to observe their impact on milk yield and composition.

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APPENDIX

Appendix A. Nacosti Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

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RESEARCH LICENSE

This is to Certify that Miss. Kembabazi Brendah of Egerton University, has been licensed to conduct research in Nakuru on the

Topic: IMPACT OF SINGLE AND MIXED STRAIN PROBIOTICS ON FEED INTAKE, DIGESTABILITY, MILK YIELD AND

METHANE EMISSION OF LACTATING DAIRY COWS for the period ending:

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Appendix B. Publications and Confrences

International Journal of Veterinary Sciences and Animal Husbandry 2021; 6(6): 20-25

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Impact of probiotics on volatile fatty acid production and methane emission of lactating dairy cows

Brendah Kembabazi, Perminus K Migwi, James O Ondiek and Nicholas Kibitok

Abstract
The significant job of probiotics in the weight control plans of ruminants is to balance rumen digestion which improves supplement usage proficiency and creature execution. Probiotics might update the ability of colonizing the gastrointestinal tract. Methane, a by-product of rumen fermentation is considered to affect herbivores as it brings about gross energy misfortune to the climate. Additionally, methane gas radiating from enteric fermentation in ruminants is a significant supporter of ozone depleting substance outflow that essentially prompts an unnatural weather change which is a significant danger to economical domesticated animals' creation universally. An experiment to study the impact of single and mixed strain probiotics on methane discharge in dairy cows was carried out. Gas produced during *in-vitro* gas production was siphoned from each sample and taken for rumen methane analysis using a GC-flame ionization detection (FID) gas chromatography. Data were exposed to analysis of variance utilizing General Direct Model and mean partition done utilizing Tukey's (HSD) test at 0.05 huge level. Consequences of rumen methane discharge showed that probiotic supplementation significantly affected methane gas emanation. Methane emission differed between 68, 267.861 ml (Treatment 5) and 73.265 ml (T4). A blend of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* diminished rumen methane discharge when utilized in balance in dairy cows and accordingly highlighting a synergistic impact between the two microorganisms.

Keywords: *Lactobacillus plantarum*, *Saccharomyces cerevisiae* methane emission, digestibility

Effect of single or mixed strain probiotics on milk yield of dairy cows

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Abstract

An experiment was conducted to determine the effect of single and mixed strain probiotics on milk yield of dairy cows. Treatments were CTL: basal diet, LP40: basal diet + 40g of *Lactobacillus plantarum*, SC40: basal diet + 40g of *Saccharomyces cerevisiae*, SC20LP20: basal diet + 20g of *Lactobacillus plantarum* + 20g of *Saccharomyces cerevisiae* and SC40LP40: basal diet + 40g of *Lactobacillus plantarum* + 40g of *Saccharomyces cerevisiae*. The basal diet consisted of 70% Rhodes grass hay and 30% dairy meal. Fifteen dairy cows in their early and mid-lactation stage had 14 days of adaptation and 21 days of data collection for milk yield and composition.

The combination of *Lactobacilli plantarum* and *Saccharomyces cereviciae* and greater DM intakes by the cows.

Key-words: *in-vitro digestibility, Lactobacillus plantarum, Saccharomyces cerevisiae*

Conference presentations: Resource Speaker 6th International Livestock Biotechnology Symposium

Department of Agriculture
PHILIPPINE CARABAO CENTER
CERTIFIED:ISO 9001 | ISO 14001 | OHSAS 18001

February 23, 2022

Dr. BRENDAH KEMBABAZI
Agricultural Inspector
Ministry of Agriculture,
Animal Industry, and Fisheries
Entebbe, Uganda

Dear **Dr. Brendah**:

The Livestock Biotechnology Center would like to thank you for your generous support to the recently concluded 6th International Livestock Biotechnology Symposium via webinar last February 22, 2022. Your presentation on "Impact of Probiotics on Volatile Fatty Acid Production and Methane Emission of Lactating Dairy Cows" was very informative and valuable. It was an honor for us to have you as one of our resource speakers.

Once again, please accept our sincerest gratitude and appreciation.

Very yours truly,

CLARO N. MINGALA
Chief, Livestock Biotechnology Center
OIC Deputy Executive Director on Production and Research

Appendix C. Statistic Output

Feed Intake Data Output

/method=sstype(3)

/intercept=include

/posthoc=treatment (tukeyduncan)

/emmeans=tables(treatment) compare adj(lsd)

/criteria=alpha(.05)

/design=treatment breed lactation treatment*breed treatment*lactation breed*lactation treatment*breed*lactation.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1.00	T1	9
	2.00	T2	9
	3.00	T3	9
	4.00	T4	9
	5.00	T5	9
BREED	1	FRIESIAN	21
	2	GUARNSEY	24
LACTATION	1	EARLY	24
	2	MID	21

Tests of Between-Subjects Effects

Dependent Variable: FEED INTAKE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	41.043[a]	14	2.932	74.957	.000
Intercept	4398.307	1	4398.307	112456.723	.000
TREATMENT	31.045	4	7.761	198.441	.000
BREED	6.814	1	6.814	174.233	.000
LACTATION	.008	1	.008	.210	.650
TREATMENT * BREED	8.762	3	2.921	74.676	.000
TREATMENT * LACTATION	12.461	3	4.154	106.203	.000
BREED * LACTATION	.000	0	.	.	.
TREATMENT * BREED * LACTATION	.000	0	.	.	.
Error	1.173	30	.039		
Total	4917.740	45			
Corrected Total	42.216	44			

a. R Squared = .972 (Adjusted R Squared = .959)

Estimated Marginal Means

TREATMENT

Estimates

Dependent Variable: FEEDINTAKE

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	9.533[a]	.066	9.399	9.668
T2	9.567[a]	.066	9.432	9.701
T3	11.000[a]	.066	10.865	11.135
T4	10.933[a]	.066	10.799	11.068
T5	11.011[a]	.066	10.876	11.146

a. Based on modified population marginal mean.

Pairwise Comparisons

Dependent Variable: FEEDINTAKE

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[d]	95% Confidence Interval for Difference[d]	
					Lower Bound	Upper Bound
T1	T2	-.033[a,b]	.093	.723	-.224	.157
	T3	-1.467[a,b,*]	.093	.000	-1.657	-1.276
	T4	-1.400[a,b,*]	.093	.000	-1.590	-1.210
	T5	-1.478[a,b,*]	.093	.000	-1.668	-1.287
T2	T1	.033[a,b]	.093	.723	-.157	.224
	T3	-1.433[a,b,*]	.093	.000	-1.624	-1.243
	T4	-1.367[a,b,*]	.093	.000	-1.557	-1.176
	T5	-1.444[a,b,*]	.093	.000	-1.635	-1.254
T3	T1	1.467[a,b,*]	.093	.000	1.276	1.657
	T2	1.433[a,b,*]	.093	.000	1.243	1.624
	T4	.067[a,b]	.093	.480	-.124	.257
	T5	-.011[a,b]	.093	.906	-.202	.179
T4	T1	1.400[a,b,*]	.093	.000	1.210	1.590
	T2	1.367[a,b,*]	.093	.000	1.176	1.557
	T3	-.067[a,b]	.093	.480	-.257	.124
	T5	-.078[a,b]	.093	.411	-.268	.113
T5	T1	1.478[a,b,*]	.093	.000	1.287	1.668
	T2	1.444[a,b,*]	.093	.000	1.254	1.635
	T3	.011[a,b]	.093	.906	-.179	.202
	T4	.078[a,b]	.093	.411	-.113	.268

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. An estimate of the modified population marginal mean (I).

b. An estimate of the modified population marginal mean (J).

d. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: FEEDINTAKE

	Sum of Squares	Df	Mean Square	F	Sig.
Contrast	22.168	4	5.542	141.696	.000
Error	1.173	30	.039		

The F tests the effect of TREATMENT. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: FEEDINTAKE

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	-.03	.093	.996	-.30	.24
		T3	-1.47*	.093	.000	-1.74	-1.20
		T4	-1.40*	.093	.000	-1.67	-1.13
		T5	-1.48*	.093	.000	-1.75	-1.21
	T2	T1	.03	.093	.996	-.24	.30
		T3	-1.43*	.093	.000	-1.70	-1.16
		T4	-1.37*	.093	.000	-1.64	-1.10
		T5	-1.44*	.093	.000	-1.71	-1.17
	T3	T1	1.47*	.093	.000	1.20	1.74

		T2	1.43*	.093	.000	1.16	1.70
		T4	.07	.093	.951	-.20	.34
		T5	-.01	.093	1.000	-.28	.26
	T4	T1	1.40*	.093	.000	1.13	1.67
		T2	1.37*	.093	.000	1.10	1.64
		T3	-.07	.093	.951	-.34	.20
		T5	-.08	.093	.918	-.35	.19
	T5	T1	1.48*	.093	.000	1.21	1.75
		T2	1.44*	.093	.000	1.17	1.71
		T3	.01	.093	1.000	-.26	.28
		T4	.08	.093	.918	-.19	.35

Based on observed means.

The error term is Mean Square(Error) = .039.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

FEED INTAKE

			Subset	
	TREATMENT	N	1	2
Tukey HSD[a,b]	T1	9	9.53	
	T2	9	9.57	
	T4	9		10.93
	T3	9		11.00
	T5	9		11.01
	Sig.		.996	.918
Duncan[a,b]	T1	9	9.53	
	T2	9	9.57	
	T4	9		10.93
	T3	9		11.00

T5	9		11.01
Sig.		.723	.439

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .039.

a. Uses Harmonic Mean Sample Size = 9.000.

b. Alpha = .05.

DATA OUTPUT FOR GAS PRODUCTION AT 24 HOURS

```
UNIANOVA X BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
  /CRITERIA=ALPHA(.05)
  /DESIGN=TREATMENT.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: X

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	506.250[a]	4	126.562	10.196	.001

Intercept	10733.437	1	10733.437	864.728	.000
TREATMENT	506.250	4	126.563	10.196	.001
Error	124.125	10	12.413		
Total	11363.813	15			
Corrected Total	630.375	14			

a. R Squared = .803 (Adjusted R Squared = .724)

Estimated Marginal Means

Dependent Variable: X

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	22.000	2.034	17.468	26.532
T2	18.500	2.034	13.968	23.032
T3	27.500	2.034	22.968	32.032
T4	31.750	2.034	27.218	36.282
T5	34.000	2.034	29.468	38.532

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: X

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	3.5000	2.87663	.743	-5.9672	12.9672
		T3	-5.5000	2.87663	.370	-14.9672	3.9672
		T4	-9.7500*	2.87663	.043	-19.2172	-.2828
		T5	-12.0000*	2.87663	.013	-21.4672	-2.5328
	T2	T1	-3.5000	2.87663	.743	-12.9672	5.9672
		T3	-9.0000	2.87663	.064	-18.4672	.4672

		T4	-13.2500*	2.87663	.007	-22.7172	-3.7828
		T5	-15.5000*	2.87663	.002	-24.9672	-6.0328
	T3	T1	5.5000	2.87663	.370	-3.9672	14.9672
		T2	9.0000	2.87663	.064	-.4672	18.4672
		T4	-4.2500	2.87663	.597	-13.7172	5.2172
		T5	-6.5000	2.87663	.234	-15.9672	2.9672
	T4	T1	9.7500*	2.87663	.043	.2828	19.2172
		T2	13.2500*	2.87663	.007	3.7828	22.7172
		T3	4.2500	2.87663	.597	-5.2172	13.7172
		T5	-2.2500	2.87663	.930	-11.7172	7.2172
	T5	T1	12.0000*	2.87663	.013	2.5328	21.4672
		T2	15.5000*	2.87663	.002	6.0328	24.9672
		T3	6.5000	2.87663	.234	-2.9672	15.9672
		T4	2.2500	2.87663	.930	-7.2172	11.7172
LSD	T1	T2	3.5000	2.87663	.252	-2.9095	9.9095
		T3	-5.5000	2.87663	.085	-11.9095	.9095
		T4	-9.7500*	2.87663	.007	-16.1595	-3.3405
		T5	-12.0000*	2.87663	.002	-18.4095	-5.5905
	T2	T1	-3.5000	2.87663	.252	-9.9095	2.9095
		T3	-9.0000*	2.87663	.011	-15.4095	-2.5905
		T4	-13.2500*	2.87663	.001	-19.6595	-6.8405
		T5	-15.5000*	2.87663	.000	-21.9095	-9.0905
	T3	T1	5.5000	2.87663	.085	-.9095	11.9095
		T2	9.0000*	2.87663	.011	2.5905	15.4095
		T4	-4.2500	2.87663	.170	-10.6595	2.1595
		T5	-6.5000*	2.87663	.047	-12.9095	-.0905
	T4	T1	9.7500*	2.87663	.007	3.3405	16.1595
		T2	13.2500*	2.87663	.001	6.8405	19.6595

		T3	4.2500	2.87663	.170	-2.1595	10.6595
		T5	-2.2500	2.87663	.452	-8.6595	4.1595
	T5	T1	12.0000*	2.87663	.002	5.5905	18.4095
		T2	15.5000*	2.87663	.000	9.0905	21.9095
		T3	6.5000*	2.87663	.047	.0905	12.9095
		T4	2.2500	2.87663	.452	-4.1595	8.6595

Based on observed means.

The error term is Mean Square(Error) = 12.413.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

X

	TREATMENT	N	Subset		
			1	2	3
Tukey HSD[a,b]	T2	3	18.5000		
	T1	3	22.0000		
	T3	3	27.5000	27.5000	
	T4	3		31.7500	
	T5	3		34.0000	
	Sig.		.064	.234	
Duncan[a,b]	T2	3	18.5000		
	T1	3	22.0000	22.0000	
	T3	3		27.5000	27.5000
	T4	3			31.7500
	T5	3			34.0000
	Sig.		.252	.085	.056

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 12.413.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

GAS PRODUCTION OUT AT 48 HOURS

```
UNIANOVA Y BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
  /CRITERIA=ALPHA(.05)
  /DESIGN=TREATMENT.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	933.433[a]	4	233.358	10.624	.001
Intercept	17468.076	1	17468.076	795.246	.000
TREATMENT	933.433	4	233.358	10.624	.001
Error	219.656	10	21.966		
Total	18621.164	15			
Corrected Total	1153.089	14			

a. R Squared = .810 (Adjusted R Squared = .733)

Estimated Marginal Means

TREATMENT

Dependent Variable:

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	30.000	2.706	23.971	36.029
T2	21.250	2.706	15.221	27.279
T3	35.750	2.706	29.721	41.779
T4	39.877	2.706	33.848	45.906
T5	43.750	2.706	37.721	49.779

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable:

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	8.7500	3.82672	.226	-3.8440	21.3440
		T3	-5.7500	3.82672	.583	-18.3440	6.8440
		T4	-9.8767	3.82672	.148	-22.4707	2.7174
		T5	-13.7500*	3.82672	.031	-26.3440	-1.1560
	T2	T1	-8.7500	3.82672	.226	-21.3440	3.8440
		T3	-14.5000*	3.82672	.023	-27.0940	-1.9060
		T4	-18.6267*	3.82672	.005	-31.2207	-6.0326
		T5	-22.5000*	3.82672	.001	-35.0940	-9.9060
	T3	T1	5.7500	3.82672	.583	-6.8440	18.3440

		T2	14.5000*	3.82672	.023	1.9060	27.0940
		T4	-4.1267	3.82672	.813	-16.7207	8.4674
		T5	-8.0000	3.82672	.295	-20.5940	4.5940
	T4	T1	9.8767	3.82672	.148	-2.7174	22.4707
		T2	18.6267*	3.82672	.005	6.0326	31.2207
		T3	4.1267	3.82672	.813	-8.4674	16.7207
		T5	-3.8733	3.82672	.844	-16.4674	8.7207
	T5	T1	13.7500*	3.82672	.031	1.1560	26.3440
		T2	22.5000*	3.82672	.001	9.9060	35.0940
		T3	8.0000	3.82672	.295	-4.5940	20.5940
		T4	3.8733	3.82672	.844	-8.7207	16.4674
LSD	T1	T2	8.7500*	3.82672	.045	.2235	17.2765
		T3	-5.7500	3.82672	.164	-14.2765	2.7765
		T4	-9.8767*	3.82672	.027	-18.4031	-1.3502
		T5	-13.7500*	3.82672	.005	-22.2765	-5.2235
	T2	T1	-8.7500*	3.82672	.045	-17.2765	-.2235
		T3	-14.5000*	3.82672	.004	-23.0265	-5.9735
		T4	-18.6267*	3.82672	.001	-27.1531	-10.1002
		T5	-22.5000*	3.82672	.000	-31.0265	-13.9735
	T3	T1	5.7500	3.82672	.164	-2.7765	14.2765
		T2	14.5000*	3.82672	.004	5.9735	23.0265
		T4	-4.1267	3.82672	.306	-12.6531	4.3998
		T5	-8.0000	3.82672	.063	-16.5265	.5265
	T4	T1	9.8767*	3.82672	.027	1.3502	18.4031
		T2	18.6267*	3.82672	.001	10.1002	27.1531
		T3	4.1267	3.82672	.306	-4.3998	12.6531
		T5	-3.8733	3.82672	.335	-12.3998	4.6531

T5	T1	13.7500*	3.82672	.005	5.2235	22.2765
	T2	22.5000*	3.82672	.000	13.9735	31.0265
	T3	8.0000	3.82672	.063	-.5265	16.5265
	T4	3.8733	3.82672	.335	-4.6531	12.3998

Based on observed means.

The error term is Mean Square(Error) = 21.966.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

	TREATMENT	N	Subset		
			1	2	3
Tukey HSD[a,b]	T2	3	21.2500		
	T1	3	30.0000	30.0000	
	T3	3		35.7500	35.7500
	T4	3		39.8767	39.8767
	T5	3			43.7500
	Sig.		.226	.148	.295
Duncan[a,b]	T2	3	21.2500		
	T1	3		30.0000	
	T3	3		35.7500	35.7500
	T4	3			39.8767
	T5	3			43.7500
	Sig.		1.000	.164	.073

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 21.966.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUTPUT FOR IMMEDIATELY SOLUBLE FRACTION (a)

```
UNIANOVA A BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
  /CRITERIA=ALPHA(.05)
  /DESIGN=TREATMENT.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: a

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10.882[a]	4	2.721	10.958	.001
Intercept	36.535	1	36.535	147.153	.000
TREATMENT	10.882	4	2.721	10.958	.001
Error	2.483	10	.248		
Total	49.901	15			
Corrected Total	13.365	14			

a. R Squared = .814 (Adjusted R Squared = .740)

Estimated Marginal Means

TREATMENT

Dependent Variable: a

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	.870	.288	.229	1.511
T2	.803	.288	.162	1.444
T3	.927	.288	.286	1.568
T4	2.663	.288	2.022	3.304
T5	2.540	.288	1.899	3.181

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable:

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	.0667	.40684	1.000	-1.2723	1.4056
		T3	-.0567	.40684	1.000	-1.3956	1.2823
		T4	-1.7933*	.40684	.009	-3.1323	-.4544
		T5	-1.6700*	.40684	.014	-3.0089	-.3311
	T2	T1	-.0667	.40684	1.000	-1.4056	1.2723
		T3	-.1233	.40684	.998	-1.4623	1.2156
		T4	-1.8600*	.40684	.007	-3.1989	-.5211
		T5	-1.7367*	.40684	.011	-3.0756	-.3977
	T3	T1	.0567	.40684	1.000	-1.2823	1.3956
		T2	.1233	.40684	.998	-1.2156	1.4623
		T4	-1.7367*	.40684	.011	-3.0756	-.3977
		T5	-1.6133*	.40684	.018	-2.9523	-.2744

	T4	T1	1.7933*	.40684	.009	.4544	3.1323
		T2	1.8600*	.40684	.007	.5211	3.1989
		T3	1.7367*	.40684	.011	.3977	3.0756
		T5	.1233	.40684	.998	-1.2156	1.4623
	T5	T1	1.6700*	.40684	.014	.3311	3.0089
		T2	1.7367*	.40684	.011	.3977	3.0756
		T3	1.6133*	.40684	.018	.2744	2.9523
		T4	-.1233	.40684	.998	-1.4623	1.2156
LSD	T1	T2	.0667	.40684	.873	-.8398	.9732
		T3	-.0567	.40684	.892	-.9632	.8498
		T4	-1.7933*	.40684	.001	-2.6998	-.8868
		T5	-1.6700*	.40684	.002	-2.5765	-.7635
	T2	T1	-.0667	.40684	.873	-.9732	.8398
		T3	-.1233	.40684	.768	-1.0298	.7832
		T4	-1.8600*	.40684	.001	-2.7665	-.9535
		T5	-1.7367*	.40684	.002	-2.6432	-.8302
	T3	T1	.0567	.40684	.892	-.8498	.9632
		T2	.1233	.40684	.768	-.7832	1.0298
		T4	-1.7367*	.40684	.002	-2.6432	-.8302
		T5	-1.6133*	.40684	.003	-2.5198	-.7068
	T4	T1	1.7933*	.40684	.001	.8868	2.6998
		T2	1.8600*	.40684	.001	.9535	2.7665
		T3	1.7367*	.40684	.002	.8302	2.6432
		T5	.1233	.40684	.768	-.7832	1.0298
	T5	T1	1.6700*	.40684	.002	.7635	2.5765
		T2	1.7367*	.40684	.002	.8302	2.6432
		T3	1.6133*	.40684	.003	.7068	2.5198

T4	-.1233	.40684	.768	-1.0298	.7832

Based on observed means.

The error term is Mean Square(Error) = .248.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

A

	TREATMENT	N	Subset	
			1	2
Tukey HSD[a,b]	T2	3	.8033	
	T1	3	.8700	
	T3	3	.9267	
	T5	3		2.5400
	T4	3		2.6633
	Sig.		.998	.998
Duncan[a,b]	T2	3	.8033	
	T1	3	.8700	
	T3	3	.9267	
	T5	3		2.5400
	T4	3		2.6633
	Sig.		.778	.768

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .248.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUTPUT FOR IMMEDIATELY DEGARDABLEFRACTION (b)

```
UNIANOVA B BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
```

/POSTHOC=TREATMENT(TUKEY DUNCAN LSD)

/EMMEANS=TABLES(TREATMENT)

/CRITERIA=ALPHA(.05)

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: b

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	981.377[a]	4	245.344	10.596	.001
Intercept	20077.299	1	20077.299	867.127	.000
TREATMENT	981.377	4	245.344	10.596	.001
Error	231.538	10	23.154		
Total	21290.214	15			
Corrected Total	1212.915	14			

a. R Squared = .809 (Adjusted R Squared = .733)

Estimated Marginal Means

TREATMENT

Dependent Variable: B

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound

T1	34.880	2.778	28.690	41.070
T2	22.170	2.778	15.980	28.360
T3	38.497	2.778	32.307	44.687
T4	41.260	2.778	35.070	47.450
T5	46.120	2.778	39.930	52.310

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: B

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	12.7100	3.92885	.055	-.2202	25.6402
		T3	-3.6167	3.92885	.883	-16.5468	9.3135
		T4	-6.3800	3.92885	.516	-19.3102	6.5502
		T5	-11.2400	3.92885	.097	-24.1702	1.6902
	T2	T1	-12.7100	3.92885	.055	-25.6402	.2202
		T3	-16.3267*	3.92885	.013	-29.2568	-3.3965
		T4	-19.0900*	3.92885	.005	-32.0202	-6.1598
		T5	-23.9500*	3.92885	.001	-36.8802	-11.0198
	T3	T1	3.6167	3.92885	.883	-9.3135	16.5468
		T2	16.3267*	3.92885	.013	3.3965	29.2568
		T4	-2.7633	3.92885	.951	-15.6935	10.1668
		T5	-7.6233	3.92885	.358	-20.5535	5.3068
	T4	T1	6.3800	3.92885	.516	-6.5502	19.3102
		T2	19.0900*	3.92885	.005	6.1598	32.0202
		T3	2.7633	3.92885	.951	-10.1668	15.6935

		T5	-4.8600	3.92885	.732	-17.7902	8.0702
	T5	T1	11.2400	3.92885	.097	-1.6902	24.1702
		T2	23.9500*	3.92885	.001	11.0198	36.8802
		T3	7.6233	3.92885	.358	-5.3068	20.5535
		T4	4.8600	3.92885	.732	-8.0702	17.7902
LSD	T1	T2	12.7100*	3.92885	.009	3.9560	21.4640
		T3	-3.6167	3.92885	.379	-12.3707	5.1374
		T4	-6.3800	3.92885	.135	-15.1340	2.3740
		T5	-11.2400*	3.92885	.017	-19.9940	-2.4860
	T2	T1	-12.7100*	3.92885	.009	-21.4640	-3.9560
		T3	-16.3267*	3.92885	.002	-25.0807	-7.5726
		T4	-19.0900*	3.92885	.001	-27.8440	-10.3360
		T5	-23.9500*	3.92885	.000	-32.7040	-15.1960
	T3	T1	3.6167	3.92885	.379	-5.1374	12.3707
		T2	16.3267*	3.92885	.002	7.5726	25.0807
		T4	-2.7633	3.92885	.498	-11.5174	5.9907
		T5	-7.6233	3.92885	.081	-16.3774	1.1307
	T4	T1	6.3800	3.92885	.135	-2.3740	15.1340
		T2	19.0900*	3.92885	.001	10.3360	27.8440
		T3	2.7633	3.92885	.498	-5.9907	11.5174
		T5	-4.8600	3.92885	.244	-13.6140	3.8940
	T5	T1	11.2400*	3.92885	.017	2.4860	19.9940
		T2	23.9500*	3.92885	.000	15.1960	32.7040
		T3	7.6233	3.92885	.081	-1.1307	16.3774
		T4	4.8600	3.92885	.244	-3.8940	13.6140

Based on observed means.

The error term is Mean Square(Error) = 23.154.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

B

	TREATMENT	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b]	T2	3	22.1700		
	T1	3	34.8800	34.8800	
	T3	3		38.4967	
	T4	3		41.2600	
	T5	3		46.1200	
	Sig.		.055	.097	
Duncan[a,b]	T2	3	22.1700		
	T1	3		34.8800	
	T3	3		38.4967	38.4967
	T4	3		41.2600	41.2600
	T5	3			46.1200
	Sig.		1.000	.152	.093

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 23.154.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUT PUT FOR POTENTIAL GAS PRODUCTION (a+b)

```
UNIANOVA a+b BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
```

/CRITERIA=ALPHA(.05)

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: a+b

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1145.027[a]	4	286.257	10.724	.001
Intercept	21825.234	1	21825.234	817.620	.000
TREATMENT	1145.027	4	286.257	10.724	.001
Error	266.936	10	26.694		
Total	23237.197	15			
Corrected Total	1411.963	14			

a. R Squared = .811 (Adjusted R Squared = .735)

Estimated Marginal Means

TREATMENT

Dependent Variable: a+b

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	35.743	2.983	29.097	42.390
T2	22.970	2.983	16.324	29.616
T3	39.423	2.983	32.777	46.070

T4	43.927	2.983	37.280	50.573
T5	48.660	2.983	42.014	55.306

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: a+b

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	12.7733	4.21850	.075	-1.1101	26.6568
		T3	-3.6800	4.21850	.901	-17.5634	10.2034
		T4	-8.1833	4.21850	.358	-22.0668	5.7001
		T5	-12.9167	4.21850	.071	-26.8001	.9668
	T2	T1	-12.7733	4.21850	.075	-26.6568	1.1101
		T3	-16.4533*	4.21850	.019	-30.3368	-2.5699
		T4	-20.9567*	4.21850	.004	-34.8401	-7.0732
		T5	-25.6900*	4.21850	.001	-39.5734	-11.8066
	T3	T1	3.6800	4.21850	.901	-10.2034	17.5634
		T2	16.4533*	4.21850	.019	2.5699	30.3368
		T4	-4.5033	4.21850	.819	-18.3868	9.3801
		T5	-9.2367	4.21850	.258	-23.1201	4.6468
	T4	T1	8.1833	4.21850	.358	-5.7001	22.0668
		T2	20.9567*	4.21850	.004	7.0732	34.8401
		T3	4.5033	4.21850	.819	-9.3801	18.3868
		T5	-4.7333	4.21850	.792	-18.6168	9.1501
	T5	T1	12.9167	4.21850	.071	-.9668	26.8001
		T2	25.6900*	4.21850	.001	11.8066	39.5734
		T3	9.2367	4.21850	.258	-4.6468	23.1201

		T4	4.7333	4.21850	.792	-9.1501	18.6168
LSD	T1	T2	12.7733*	4.21850	.013	3.3739	22.1727
		T3	-3.6800	4.21850	.403	-13.0794	5.7194
		T4	-8.1833	4.21850	.081	-17.5827	1.2161
		T5	-12.9167*	4.21850	.012	-22.3161	-3.5173
	T2	T1	-12.7733*	4.21850	.013	-22.1727	-3.3739
		T3	-16.4533*	4.21850	.003	-25.8527	-7.0539
		T4	-20.9567*	4.21850	.001	-30.3561	-11.5573
		T5	-25.6900*	4.21850	.000	-35.0894	-16.2906
	T3	T1	3.6800	4.21850	.403	-5.7194	13.0794
		T2	16.4533*	4.21850	.003	7.0539	25.8527
		T4	-4.5033	4.21850	.311	-13.9027	4.8961
		T5	-9.2367	4.21850	.053	-18.6361	.1627
	T4	T1	8.1833	4.21850	.081	-1.2161	17.5827
		T2	20.9567*	4.21850	.001	11.5573	30.3561
		T3	4.5033	4.21850	.311	-4.8961	13.9027
		T5	-4.7333	4.21850	.288	-14.1327	4.6661
	T5	T1	12.9167*	4.21850	.012	3.5173	22.3161
		T2	25.6900*	4.21850	.000	16.2906	35.0894
		T3	9.2367	4.21850	.053	-.1627	18.6361
		T4	4.7333	4.21850	.288	-4.6661	14.1327

Based on observed means.

The error term is Mean Square(Error) = 26.694.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

a+b

	TREATMENT	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b]	T2	3	22.9700		
	T1	3	35.7433	35.7433	
	T3	3		39.4233	
	T4	3		43.9267	
	T5	3		48.6600	
	Sig.		.075	.071	
Duncan[a,b]	T2	3	22.9700		
	T1	3		35.7433	
	T3	3		39.4233	39.4233
	T4	3		43.9267	43.9267
	T5	3			48.6600
	Sig.		1.000	.093	.063

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 26.694.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUTPUT FOR RATE CONSTANT OF GAS PRODUCTION (c)

```
UNIANOVA C BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
  /CRITERIA=ALPHA(.05)
```

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: C

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.001[a]	4	.000	8.583	.003
Intercept	.045	1	.045	1120.667	.000
TREATMENT	.001	4	.000	8.583	.003
Error	.000	10	4.000E-5		
Total	.047	15			
Corrected Total	.002	14			

a. R Squared = .774 (Adjusted R Squared = .684)

Estimated Marginal Means

TREATMENT

Dependent Variable: C

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	.040	.004	.032	.048
T2	.070	.004	.062	.078
T3	.053	.004	.045	.061

T4	.057	.004	.049	.065
T5	.053	.004	.045	.061

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: C

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	-.0300*	.00516	.001	-.0470	-.0130
		T3	-.0133	.00516	.148	-.0303	.0037
		T4	-.0167	.00516	.055	-.0337	.0003
		T5	-.0133	.00516	.148	-.0303	.0037
	T2	T1	.0300*	.00516	.001	.0130	.0470
		T3	.0167	.00516	.055	-.0003	.0337
		T4	.0133	.00516	.148	-.0037	.0303
		T5	.0167	.00516	.055	-.0003	.0337
	T3	T1	.0133	.00516	.148	-.0037	.0303
		T2	-.0167	.00516	.055	-.0337	.0003
		T4	-.0033	.00516	.964	-.0203	.0137
		T5	.0000	.00516	1.000	-.0170	.0170
	T4	T1	.0167	.00516	.055	-.0003	.0337
		T2	-.0133	.00516	.148	-.0303	.0037
		T3	.0033	.00516	.964	-.0137	.0203
		T5	.0033	.00516	.964	-.0137	.0203
	T5	T1	.0133	.00516	.148	-.0037	.0303
		T2	-.0167	.00516	.055	-.0337	.0003

		T3	.0000	.00516	1.000	-.0170	.0170
		T4	-.0033	.00516	.964	-.0203	.0137
LSD	T1	T2	-.0300*	.00516	.000	-.0415	-.0185
		T3	-.0133*	.00516	.027	-.0248	-.0018
		T4	-.0167*	.00516	.009	-.0282	-.0052
		T5	-.0133*	.00516	.027	-.0248	-.0018
	T2	T1	.0300*	.00516	.000	.0185	.0415
		T3	.0167*	.00516	.009	.0052	.0282
		T4	.0133*	.00516	.027	.0018	.0248
		T5	.0167*	.00516	.009	.0052	.0282
	T3	T1	.0133*	.00516	.027	.0018	.0248
		T2	-.0167*	.00516	.009	-.0282	-.0052
		T4	-.0033	.00516	.533	-.0148	.0082
		T5	.0000	.00516	1.000	-.0115	.0115
	T4	T1	.0167*	.00516	.009	.0052	.0282
		T2	-.0133*	.00516	.027	-.0248	-.0018
		T3	.0033	.00516	.533	-.0082	.0148
		T5	.0033	.00516	.533	-.0082	.0148
	T5	T1	.0133*	.00516	.027	.0018	.0248
		T2	-.0167*	.00516	.009	-.0282	-.0052
		T3	.0000	.00516	1.000	-.0115	.0115
		T4	-.0033	.00516	.533	-.0148	.0082

Based on observed means.

The error term is Mean Square(Error) = 4.00E-005.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

C

	TREATMENT	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b]	T1	3	.0400		
	T3	3	.0533	.0533	
	T5	3	.0533	.0533	
	T4	3	.0567	.0567	
	T2	3		.0700	
	Sig.		.055	.055	
Duncan[a,b]	T1	3	.0400		
	T3	3		.0533	
	T5	3		.0533	
	T4	3		.0567	
	T2	3			.0700
	Sig.		1.000	.552	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 4.00E-005.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUTPUT FOR ORGANIC MATTER DIGESTABILITY (OMD)

```
UNIANOVA OMD BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
  /CRITERIA=ALPHA(.05)
```

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: OMD

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	796.575[a]	4	199.144	10.631	.001
Intercept	38509.707	1	38509.707	2055.719	.000
TREATMENT	796.575	4	199.144	10.631	.001
Error	187.330	10	18.733		
Total	39493.611	15			
Corrected Total	983.905	14			

a. R Squared = .810 (Adjusted R Squared = .733)

Estimated Marginal Means

TREATMENT

Dependent Variable: OMD

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	46.860	2.499	41.292	52.428
T2	38.773	2.499	33.206	44.341

T3	52.170	2.499	46.602	57.738
T4	55.980	2.499	50.412	61.548
T5	59.560	2.499	53.992	65.128

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: OMD

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	8.0867	3.53393	.225	-3.5438	19.7171
		T3	-5.3100	3.53393	.583	-16.9404	6.3204
		T4	-9.1200	3.53393	.148	-20.7504	2.5104
		T5	-12.7000*	3.53393	.031	-24.3304	-1.0696
	T2	T1	-8.0867	3.53393	.225	-19.7171	3.5438
		T3	-13.3967*	3.53393	.023	-25.0271	-1.7662
		T4	-17.2067*	3.53393	.005	-28.8371	-5.5762
		T5	-20.7867*	3.53393	.001	-32.4171	-9.1562
	T3	T1	5.3100	3.53393	.583	-6.3204	16.9404
		T2	13.3967*	3.53393	.023	1.7662	25.0271
		T4	-3.8100	3.53393	.814	-15.4404	7.8204
		T5	-7.3900	3.53393	.295	-19.0204	4.2404
	T4	T1	9.1200	3.53393	.148	-2.5104	20.7504
		T2	17.2067*	3.53393	.005	5.5762	28.8371
		T3	3.8100	3.53393	.814	-7.8204	15.4404
		T5	-3.5800	3.53393	.844	-15.2104	8.0504
	T5	T1	12.7000*	3.53393	.031	1.0696	24.3304

		T2	20.7867*	3.53393	.001	9.1562	32.4171
		T3	7.3900	3.53393	.295	-4.2404	19.0204
		T4	3.5800	3.53393	.844	-8.0504	15.2104
LSD	T1	T2	8.0867*	3.53393	.045	.2126	15.9607
		T3	-5.3100	3.53393	.164	-13.1841	2.5641
		T4	-9.1200*	3.53393	.027	-16.9941	-1.2459
		T5	-12.7000*	3.53393	.005	-20.5741	-4.8259
	T2	T1	-8.0867*	3.53393	.045	-15.9607	-.2126
		T3	-13.3967*	3.53393	.004	-21.2707	-5.5226
		T4	-17.2067*	3.53393	.001	-25.0807	-9.3326
		T5	-20.7867*	3.53393	.000	-28.6607	-12.9126
	T3	T1	5.3100	3.53393	.164	-2.5641	13.1841
		T2	13.3967*	3.53393	.004	5.5226	21.2707
		T4	-3.8100	3.53393	.306	-11.6841	4.0641
		T5	-7.3900	3.53393	.063	-15.2641	.4841
	T4	T1	9.1200*	3.53393	.027	1.2459	16.9941
		T2	17.2067*	3.53393	.001	9.3326	25.0807
		T3	3.8100	3.53393	.306	-4.0641	11.6841
		T5	-3.5800	3.53393	.335	-11.4541	4.2941
	T5	T1	12.7000*	3.53393	.005	4.8259	20.5741
		T2	20.7867*	3.53393	.000	12.9126	28.6607
		T3	7.3900	3.53393	.063	-.4841	15.2641
		T4	3.5800	3.53393	.335	-4.2941	11.4541

Based on observed means.

The error term is Mean Square(Error) = 18.733.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

OMD

	TREATMENT	N	Subset 1	2	3
Tukey HSD[a,b]	T2	3	38.7733		
	T1	3	46.8600	46.8600	
	T3	3		52.1700	52.1700
	T4	3		55.9800	55.9800
	T5	3			59.5600
	Sig.		.225	.148	.295
Duncan[a,b]	T2	3	38.7733		
	T1	3		46.8600	
	T3	3		52.1700	52.1700
	T4	3			55.9800
	T5	3			59.5600
	Sig.		1.000	.164	.073

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 18.733.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUTPUT FOR METEBOLISABLE ENERGY (ME)

```
UNIANOVA ME BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
  /CRITERIA=ALPHA(.05)
  /DESIGN=TREATMENT.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: ME

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	23.012[a]	4	5.753	10.612	.001
Intercept	641.182	1	641.182	1182.745	.000
TREATMENT	23.012	4	5.753	10.612	.001
Error	5.421	10	.542		
Total	669.615	15			
Corrected Total	28.433	14			

a. R Squared = .809 (Adjusted R Squared = .733)

Estimated Marginal Means

TREATMENT

Dependent Variable: ME

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	5.890	.425	4.943	6.837
T2	4.517	.425	3.569	5.464
T3	6.793	.425	5.846	7.741
T4	7.440	.425	6.493	8.387
T5	8.050	.425	7.103	8.997

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: ME

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	1.3733	.60117	.226	-.6052	3.3518
		T3	-.9033	.60117	.583	-2.8818	1.0752
		T4	-1.5500	.60117	.148	-3.5285	.4285
		T5	-2.1600*	.60117	.031	-4.1385	-.1815
	T2	T1	-1.3733	.60117	.226	-3.3518	.6052
		T3	-2.2767*	.60117	.023	-4.2552	-.2982
		T4	-2.9233*	.60117	.005	-4.9018	-.9448
		T5	-3.5333*	.60117	.001	-5.5118	-1.5548
	T3	T1	.9033	.60117	.583	-1.0752	2.8818
		T2	2.2767*	.60117	.023	.2982	4.2552
		T4	-.6467	.60117	.815	-2.6252	1.3318
		T5	-1.2567	.60117	.295	-3.2352	.7218

	T4	T1	1.5500	.60117	.148	-.4285	3.5285
		T2	2.9233*	.60117	.005	.9448	4.9018
		T3	.6467	.60117	.815	-1.3318	2.6252
		T5	-.6100	.60117	.843	-2.5885	1.3685
	T5	T1	2.1600*	.60117	.031	.1815	4.1385
		T2	3.5333*	.60117	.001	1.5548	5.5118
		T3	1.2567	.60117	.295	-.7218	3.2352
		T4	.6100	.60117	.843	-1.3685	2.5885
LSD	T1	T2	1.3733*	.60117	.045	.0338	2.7128
		T3	-.9033	.60117	.164	-2.2428	.4362
		T4	-1.5500*	.60117	.027	-2.8895	-.2105
		T5	-2.1600*	.60117	.005	-3.4995	-.8205
	T2	T1	-1.3733*	.60117	.045	-2.7128	-.0338
		T3	-2.2767*	.60117	.004	-3.6162	-.9372
		T4	-2.9233*	.60117	.001	-4.2628	-1.5838
		T5	-3.5333*	.60117	.000	-4.8728	-2.1938
	T3	T1	.9033	.60117	.164	-.4362	2.2428
		T2	2.2767*	.60117	.004	.9372	3.6162
		T4	-.6467	.60117	.307	-1.9862	.6928
		T5	-1.2567	.60117	.063	-2.5962	.0828
	T4	T1	1.5500*	.60117	.027	.2105	2.8895
		T2	2.9233*	.60117	.001	1.5838	4.2628
		T3	.6467	.60117	.307	-.6928	1.9862
		T5	-.6100	.60117	.334	-1.9495	.7295
	T5	T1	2.1600*	.60117	.005	.8205	3.4995
		T2	3.5333*	.60117	.000	2.1938	4.8728
		T3	1.2567	.60117	.063	-.0828	2.5962
		T4	.6100	.60117	.334	-.7295	1.9495

Based on observed means.

The error term is Mean Square(Error) = .542.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

ME

	TREATMENT	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b]	T2	3	4.5167		
	T1	3	5.8900	5.8900	
	T3	3		6.7933	6.7933
	T4	3		7.4400	7.4400
	T5	3			8.0500
	Sig.		.226	.148	.295
Duncan[a,b]	T2	3	4.5167		
	T1	3		5.8900	
	T3	3		6.7933	6.7933
	T4	3			7.4400
	T5	3			8.0500
	Sig.		1.000	.164	.073

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .542.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC DATA OUT PUT FOR SHORT CHAIN FATTY ACIDS(SCFA)

```
UNIANOVA SCFA BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATMENT)
```

/CRITERIA=ALPHA(.05)

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1	T1	3
	2	T2	3
	3	T3	3
	4	T4	3
	5	T5	3

Tests of Between-Subjects Effects

Dependent Variable: SCFA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.251[a]	4	.063	10.368	.001
Intercept	5.198	1	5.198	858.688	.000
TREATMENT	.251	4	.063	10.368	.001
Error	.061	10	.006		
Total	5.510	15			
Corrected Total	.312	14			

a. R Squared = .806 (Adjusted R Squared = .728)

Estimated Marginal Means

TREATMENT

Dependent Variable: SCFA

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
T1	.480	.045	.380	.580
T2	.407	.045	.307	.507
T3	.607	.045	.507	.707
T4	.700	.045	.600	.800
T5	.750	.045	.650	.850

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: SCFA

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	.0733	.06353	.776	-.1357	.2824
		T3	-.1267	.06353	.334	-.3357	.0824
		T4	-.2200*	.06353	.038	-.4291	-.0109
		T5	-.2700*	.06353	.011	-.4791	-.0609
	T2	T1	-.0733	.06353	.776	-.2824	.1357
		T3	-.2000	.06353	.062	-.4091	.0091
		T4	-.2933*	.06353	.007	-.5024	-.0843
		T5	-.3433*	.06353	.002	-.5524	-.1343
	T3	T1	.1267	.06353	.334	-.0824	.3357
		T2	.2000	.06353	.062	-.0091	.4091

		T4	-.0933	.06353	.602	-.3024	.1157
		T5	-.1433	.06353	.236	-.3524	.0657
	T4	T1	.2200*	.06353	.038	.0109	.4291
		T2	.2933*	.06353	.007	.0843	.5024
		T3	.0933	.06353	.602	-.1157	.3024
		T5	-.0500	.06353	.929	-.2591	.1591
	T5	T1	.2700*	.06353	.011	.0609	.4791
		T2	.3433*	.06353	.002	.1343	.5524
		T3	.1433	.06353	.236	-.0657	.3524
		T4	.0500	.06353	.929	-.1591	.2591
LSD	T1	T2	.0733	.06353	.275	-.0682	.2149
		T3	-.1267	.06353	.074	-.2682	.0149
		T4	-.2200*	.06353	.006	-.3615	-.0785
		T5	-.2700*	.06353	.002	-.4115	-.1285
	T2	T1	-.0733	.06353	.275	-.2149	.0682
		T3	-.2000*	.06353	.010	-.3415	-.0585
		T4	-.2933*	.06353	.001	-.4349	-.1518
		T5	-.3433*	.06353	.000	-.4849	-.2018
	T3	T1	.1267	.06353	.074	-.0149	.2682
		T2	.2000*	.06353	.010	.0585	.3415
		T4	-.0933	.06353	.173	-.2349	.0482
		T5	-.1433*	.06353	.048	-.2849	-.0018
	T4	T1	.2200*	.06353	.006	.0785	.3615
		T2	.2933*	.06353	.001	.1518	.4349
		T3	.0933	.06353	.173	-.0482	.2349
		T5	-.0500	.06353	.449	-.1915	.0915
	T5	T1	.2700*	.06353	.002	.1285	.4115
		T2	.3433*	.06353	.000	.2018	.4849

	T3	.1433*	.06353	.048	.0018	.2849
	T4	.0500	.06353	.449	-.0915	.1915

Based on observed means.

The error term is Mean Square(Error) = .006.

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

SCFA

			Subset		
	TREATMENT	N	1	2	3
Tukey HSD[a,b]	T2	3	.4067		
	T1	3	.4800		
	T3	3	.6067	.6067	
	T4	3		.7000	
	T5	3		.7500	
	Sig.		.062	.236	
Duncan[a,b]	T2	3	.4067		
	T1	3	.4800	.4800	
	T3	3		.6067	.6067
	T4	3			.7000
	T5	3			.7500
	Sig.		.275	.074	.056

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .006.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

STATISTIC OUTPUT FOR PROPIONATE

```
UNIANOVA PROPIONATE BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
```

/SAVE=SEPRED

/POSTHOC=TREATMENT(TUKEY DUNCAN LSD)

/CRITERIA=ALPHA(0.05)

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1.000	T1	2
	2.000	T2	2
	3.000	T3	2
	4.000	T4	2
	5.000	T5	2

Tests of Between-Subjects Effects

Dependent Variable: PROPIONATE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1064.247[a]	4	266.062	4.402	.068
Intercept	45971.112	1	45971.112	760.545	.000
TREATMENT	1064.247	4	266.062	4.402	.068
Error	302.225	5	60.445		
Total	47337.583	10			
Corrected Total	1366.471	9			

a. R Squared = .779 (Adjusted R Squared = .602)

Post Hoc Tests
TREATMENT

Multiple Comparisons

Dependent Variable: PROPIONATE

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	T1	T2	-.120000	7.7746344	1.000	-31.307983	31.067983
		T3	11.095000	7.7746344	.639	-20.092983	42.282983
		T4	9.905000	7.7746344	.717	-21.282983	41.092983
		T5	-17.690000	7.7746344	.287	-48.877983	13.497983
	T2	T1	.120000	7.7746344	1.000	-31.067983	31.307983
		T3	11.215000	7.7746344	.631	-19.972983	42.402983
		T4	10.025000	7.7746344	.709	-21.162983	41.212983
		T5	-17.570000	7.7746344	.292	-48.757983	13.617983
	T3	T1	-11.095000	7.7746344	.639	-42.282983	20.092983
		T2	-11.215000	7.7746344	.631	-42.402983	19.972983
		T4	-1.190000	7.7746344	1.000	-32.377983	29.997983

		T5	-28.785000	7.7746344	.067	-59.972983	2.402983
	T4	T1	-9.905000	7.7746344	.717	-41.092983	21.282983
		T2	-10.025000	7.7746344	.709	-41.212983	21.162983
		T3	1.190000	7.7746344	1.000	-29.997983	32.377983
		T5	-27.595000	7.7746344	.078	-58.782983	3.592983
	T5	T1	17.690000	7.7746344	.287	-13.497983	48.877983
		T2	17.570000	7.7746344	.292	-13.617983	48.757983
		T3	28.785000	7.7746344	.067	-2.402983	59.972983
		T4	27.595000	7.7746344	.078	-3.592983	58.782983
LSD	T1	T2	-.120000	7.7746344	.988	-20.105334	19.865334
		T3	11.095000	7.7746344	.213	-8.890334	31.080334
		T4	9.905000	7.7746344	.259	-10.080334	29.890334
		T5	-17.690000	7.7746344	.072	-37.675334	2.295334
	T2	T1	.120000	7.7746344	.988	-19.865334	20.105334
		T3	11.215000	7.7746344	.209	-8.770334	31.200334

	T4	10.025000	7.7746344	.254	-9.960334	30.010334
	T5	-17.570000	7.7746344	.073	-37.555334	2.415334
T3	T1	-11.095000	7.7746344	.213	-31.080334	8.890334
	T2	-11.215000	7.7746344	.209	-31.200334	8.770334
	T4	-1.190000	7.7746344	.884	-21.175334	18.795334
	T5	-28.785000*	7.7746344	.014	-48.770334	-8.799666
T4	T1	-9.905000	7.7746344	.259	-29.890334	10.080334
	T2	-10.025000	7.7746344	.254	-30.010334	9.960334
	T3	1.190000	7.7746344	.884	-18.795334	21.175334
	T5	-27.595000*	7.7746344	.016	-47.580334	-7.609666
T5	T1	17.690000	7.7746344	.072	-2.295334	37.675334
	T2	17.570000	7.7746344	.073	-2.415334	37.555334
	T3	28.785000*	7.7746344	.014	8.799666	48.770334
	T4	27.595000*	7.7746344	.016	7.609666	47.580334

Based on observed means.
The error term is Mean Square(Error) = 60.445.

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

PROPIONATE

	TREATMENT	N	Subset 1	Subset 2
Tukey HSD[a,b]	T3	2	57.345000	
	T4	2	58.535000	
	T1	2	68.440000	
	T2	2	68.560000	
	T5	2	86.130000	
	Sig.		.067	
Duncan[a,b]	T3	2	57.345000	
	T4	2	58.535000	
	T1	2	68.440000	68.440000
	T2	2	68.560000	68.560000
	T5	2		86.130000
	Sig.		.222	.078

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 60.445.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = 0.05.

STATISTIC OUTPUT FOR BUTYRATE

```
UNIANOVA BUTYRATE BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=SEPRED
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /CRITERIA=ALPHA(0.05)
```

/DESIGN=TREATMENT.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1.000	T1	2
	2.000	T2	2
	3.000	T3	2
	4.000	T4	2
	5.000	T5	2

Tests of Between-Subjects Effects

Dependent Variable: BUTYRATE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	141.252[a]	4	35.313	7.147	.027
Intercept	7285.141	1	7285.141	1474.394	.000
TREATMENT	141.252	4	35.313	7.147	.027
Error	24.706	5	4.941		
Total	7451.098	10			
Corrected Total	165.958	9			

a. R Squared = .851 (Adjusted R Squared = .732)

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: BUTYRATE

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound

Tukey HSD	T1	T2	-.795000	2.22286 08	.995	-9.712017	8.122017
		T3	4.025000	2.22286 08	.457	-4.892017	12.942017
		T4	2.905000	2.22286 08	.700	-6.012017	11.822017
		T5	-6.740000	2.22286 08	.131	-15.657017	2.177017
	T2	T1	.795000	2.22286 08	.995	-8.122017	9.712017
		T3	4.820000	2.22286 08	.321	-4.097017	13.737017
		T4	3.700000	2.22286 08	.523	-5.217017	12.617017
		T5	-5.945000	2.22286 08	.190	-14.862017	2.972017
	T3	T1	-4.025000	2.22286 08	.457	-12.942017	4.892017
		T2	-4.820000	2.22286 08	.321	-13.737017	4.097017
		T4	-1.120000	2.22286 08	.983	-10.037017	7.797017
		T5	-10.765000*	2.22286 08	.024	-19.682017	-1.847983
	T4	T1	-2.905000	2.22286 08	.700	-11.822017	6.012017
		T2	-3.700000	2.22286 08	.523	-12.617017	5.217017
		T3	1.120000	2.22286 08	.983	-7.797017	10.037017

		T5	-9.645000*	2.22286 08	.037	-18.562017	-.727983
	T5	T1	6.740000	2.22286 08	.131	-2.177017	15.657017
		T2	5.945000	2.22286 08	.190	-2.972017	14.862017
		T3	10.765000*	2.22286 08	.024	1.847983	19.682017
		T4	9.645000*	2.22286 08	.037	.727983	18.562017
LSD	T1	T2	-.795000	2.22286 08	.735	-6.509046	4.919046
		T3	4.025000	2.22286 08	.130	-1.689046	9.739046
		T4	2.905000	2.22286 08	.248	-2.809046	8.619046
		T5	-6.740000*	2.22286 08	.029	-12.454046	-1.025954
	T2	T1	.795000	2.22286 08	.735	-4.919046	6.509046
		T3	4.820000	2.22286 08	.082	-.894046	10.534046
		T4	3.700000	2.22286 08	.157	-2.014046	9.414046
		T5	-5.945000*	2.22286 08	.044	-11.659046	-.230954
	T3	T1	-4.025000	2.22286 08	.130	-9.739046	1.689046
		T2	-4.820000	2.22286 08	.082	-10.534046	.894046

	T4	-1.120000	2.2228608	.636	-6.834046	4.594046
	T5	-10.765000*	2.2228608	.005	-16.479046	-5.050954
T4	T1	-2.905000	2.2228608	.248	-8.619046	2.809046
	T2	-3.700000	2.2228608	.157	-9.414046	2.014046
	T3	1.120000	2.2228608	.636	-4.594046	6.834046
	T5	-9.645000*	2.2228608	.007	-15.359046	-3.930954
T5	T1	6.740000*	2.2228608	.029	1.025954	12.454046
	T2	5.945000*	2.2228608	.044	.230954	11.659046
	T3	10.765000*	2.2228608	.005	5.050954	16.479046
	T4	9.645000*	2.2228608	.007	3.930954	15.359046

Based on observed means.

The error term is Mean Square(Error) = 4.941.

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

BUTYRATE

	TREATMENT	N	Subset	
			1	2
Tukey HSD[a,b]	T3	2	22.845000	
	T4	2	23.965000	
	T1	2	26.870000	26.870000
	T2	2	27.665000	27.665000
	T5	2		33.610000
	Sig.		.321	.131
Duncan[a,b]	T3	2	22.845000	
	T4	2	23.965000	
	T1	2	26.870000	
	T2	2	27.665000	
	T5	2		33.610000
	Sig.		.092	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 4.941.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = 0.05.

STATISTIC OUTPUT FOR ACETATE

```
UNIANOVA ACETATE BY TREATMENT
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /SAVE=SEPRED
  /POSTHOC=TREATMENT(TUKEY DUNCAN LSD)
  /CRITERIA=ALPHA(0.05)
  /DESIGN=TREATMENT.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATMENT	1.000	T1	2
	2.000	T2	2
	3.000	T3	2
	4.000	T4	2
	5.000	T5	2

Tests of Between-Subjects Effects

Dependent Variable: ACETATE

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	1064.247[a]	4	266.062	4.402	.068
Intercept	45971.112	1	45971.112	760.545	.000
TREATMENT	1064.247	4	266.062	4.402	.068
Error	302.225	5	60.445		
Total	47337.583	10			
Corrected Total	1366.471	9			

a. R Squared = .779 (Adjusted R Squared = .602)

Post Hoc Tests

TREATMENT

Multiple Comparisons

Dependent Variable: ACETATE

	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound

Tukey HSD	T1	T2	-.120000	7.77463 44	1.000	-31.307983	31.067983
		T3	11.095000	7.77463 44	.639	-20.092983	42.282983
		T4	9.905000	7.77463 44	.717	-21.282983	41.092983
		T5	-17.690000	7.77463 44	.287	-48.877983	13.497983
	T2	T1	.120000	7.77463 44	1.000	-31.067983	31.307983
		T3	11.215000	7.77463 44	.631	-19.972983	42.402983
		T4	10.025000	7.77463 44	.709	-21.162983	41.212983
		T5	-17.570000	7.77463 44	.292	-48.757983	13.617983
	T3	T1	-11.095000	7.77463 44	.639	-42.282983	20.092983
		T2	-11.215000	7.77463 44	.631	-42.402983	19.972983
		T4	-1.190000	7.77463 44	1.000	-32.377983	29.997983
		T5	-28.785000	7.77463 44	.067	-59.972983	2.402983
	T4	T1	-9.905000	7.77463 44	.717	-41.092983	21.282983
		T2	-10.025000	7.77463 44	.709	-41.212983	21.162983
		T3	1.190000	7.77463 44	1.000	-29.997983	32.377983

		T5	-27.595000	7.7746344	.078	-58.782983	3.592983
	T5	T1	17.690000	7.7746344	.287	-13.497983	48.877983
		T2	17.570000	7.7746344	.292	-13.617983	48.757983
		T3	28.785000	7.7746344	.067	-2.402983	59.972983
		T4	27.595000	7.7746344	.078	-3.592983	58.782983
LSD	T1	T2	-.120000	7.7746344	.988	-20.105334	19.865334
		T3	11.095000	7.7746344	.213	-8.890334	31.080334
		T4	9.905000	7.7746344	.259	-10.080334	29.890334
		T5	-17.690000	7.7746344	.072	-37.675334	2.295334
	T2	T1	.120000	7.7746344	.988	-19.865334	20.105334
		T3	11.215000	7.7746344	.209	-8.770334	31.200334
		T4	10.025000	7.7746344	.254	-9.960334	30.010334
		T5	-17.570000	7.7746344	.073	-37.555334	2.415334
	T3	T1	-11.095000	7.7746344	.213	-31.080334	8.890334
		T2	-11.215000	7.7746344	.209	-31.200334	8.770334

	T4	-1.190000	7.7746344	.884	-21.175334	18.795334
	T5	-28.785000*	7.7746344	.014	-48.770334	-8.799666
T4	T1	-9.905000	7.7746344	.259	-29.890334	10.080334
	T2	-10.025000	7.7746344	.254	-30.010334	9.960334
	T3	1.190000	7.7746344	.884	-18.795334	21.175334
	T5	-27.595000*	7.7746344	.016	-47.580334	-7.609666
T5	T1	17.690000	7.7746344	.072	-2.295334	37.675334
	T2	17.570000	7.7746344	.073	-2.415334	37.555334
	T3	28.785000*	7.7746344	.014	8.799666	48.770334
	T4	27.595000*	7.7746344	.016	7.609666	47.580334

Based on observed means.

The error term is Mean Square(Error) = 60.445.

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

ACETATE

			Subset	
	TREATMENT	N	1	2
Tukey HSD[a,b]	T3	2	57.345000	

	T4	2	58.535000	
	T1	2	68.440000	
	T2	2	68.560000	
	T5	2	86.130000	
	Sig.		.067	
Duncan[a,b]	T3	2	57.345000	
	T4	2	58.535000	
	T1	2	68.440000	68.440000
	T2	2	68.560000	68.560000
	T5	2		86.130000
	Sig.		.222	.078

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 60.445.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = 0.05.

STATISTIC OUTPUT FOR METHANE

```
UNIANOVA Methane BY Treatment
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=Treatment(TUKEY DUNCAN LSD)
  /CRITERIA=ALPHA(0.05)
  /DESIGN=Treatment.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Treatment	T1	Treatment 1	3

	T2	Treatment 2	3
	T3	Treatment 3	3
	T4	Treatment 4	3
	T5	Treatment 5	3

Tests of Between-Subjects Effects

Dependent Variable: Methane

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	7955360708.074[a]	4	1988840177.018	58.959	.000
Intercept	16238465584.556	1	16238465584.556	481.390	.000
Treatment	7955360708.074	4	1988840177.018	58.959	.000
Error	337324322.155	10	33732432.215		
Total	24531150614.784	15			
Corrected Total	8292685030.228	14			

a. R Squared = .959 (Adjusted R Squared = .943)

Post Hoc Tests

Treatment

Multiple Comparisons

Dependent Variable: Methane

	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Treatment 1	Treatment 2	-4766.6390	4742.18179	.847	-20373.5488	10840.2708
		Treatment 3	-23940.7000*	4742.18179	.004	-39547.6098	-8333.7902
		Treatment 4	22414.4900*	4742.18179	.006	6807.5802	38021.3998
		Treatment 5	-45780.1060*	4742.18179	.000	-61387.0158	-30173.1962
	Treatment 2	Treatment 1	4766.6390	4742.18179	.847	-10840.2708	20373.5488
		Treatment 3	-19174.0610*	4742.18179	.016	-34780.9708	-3567.1512
		Treatment 4	27181.1290*	4742.18179	.001	11574.2192	42788.0388
		Treatment 5	-41013.4670*	4742.18179	.000	-56620.3768	-25406.5572
	Treatment 3	Treatment 1	23940.7000*	4742.18179	.004	8333.7902	39547.6098

		Treatment 2	19174.0610*	4742.18179	.016	3567.1512	34780.9708
		Treatment 4	46355.1900*	4742.18179	.000	30748.2802	61962.0998
		Treatment 5	-21839.4060*	4742.18179	.007	-37446.3158	-6232.4962
	Treatment 4	Treatment 1	-22414.4900*	4742.18179	.006	-38021.3998	-6807.5802
		Treatment 2	-27181.1290*	4742.18179	.001	-42788.0388	-11574.2192
		Treatment 3	-46355.1900*	4742.18179	.000	-61962.0998	-30748.2802
		Treatment 5	-68194.5960*	4742.18179	.000	-83801.5058	-52587.6862
	Treatment 5	Treatment 1	45780.1060*	4742.18179	.000	30173.1962	61387.0158
		Treatment 2	41013.4670*	4742.18179	.000	25406.5572	56620.3768
		Treatment 3	21839.4060*	4742.18179	.007	6232.4962	37446.3158
		Treatment 4	68194.5960*	4742.18179	.000	52587.6862	83801.5058
LSD	Treatment 1	Treatment 2	-4766.6390	4742.18179	.339	-15332.8785	5799.6005

	Treatment 3	-23940.7000*	4742.18179	.001	-34506.9395	-13374.4605
	Treatment 4	22414.4900*	4742.18179	.001	11848.2505	32980.7295
	Treatment 5	-45780.1060*	4742.18179	.000	-56346.3455	-35213.8665
Treatment 2	Treatment 1	4766.6390	4742.18179	.339	-5799.6005	15332.8785
	Treatment 3	-19174.0610*	4742.18179	.002	-29740.3005	-8607.8215
	Treatment 4	27181.1290*	4742.18179	.000	16614.8895	37747.3685
	Treatment 5	-41013.4670*	4742.18179	.000	-51579.7065	-30447.2275
Treatment 3	Treatment 1	23940.7000*	4742.18179	.001	13374.4605	34506.9395
	Treatment 2	19174.0610*	4742.18179	.002	8607.8215	29740.3005
	Treatment 4	46355.1900*	4742.18179	.000	35788.9505	56921.4295
	Treatment 5	-21839.4060*	4742.18179	.001	-32405.6455	-11273.1665
Treatment 4	Treatment 1	-22414.4900*	4742.18179	.001	-32980.7295	-11848.2505

		Treatment 2	-27181.1290*	4742.18179	.000	-37747.3685	-16614.8895
		Treatment 3	-46355.1900*	4742.18179	.000	-56921.4295	-35788.9505
		Treatment 5	-68194.5960*	4742.18179	.000	-78760.8355	-57628.3565
	Treatment 5	Treatment 1	45780.1060*	4742.18179	.000	35213.8665	56346.3455
		Treatment 2	41013.4670*	4742.18179	.000	30447.2275	51579.7065
		Treatment 3	21839.4060*	4742.18179	.001	11273.1665	32405.6455
		Treatment 4	68194.5960*	4742.18179	.000	57628.3565	78760.8355

Based on observed means.

The error term is Mean Square(Error) = 33732432.215.

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Methane

			Subset			
	Treatment	N	1	2	3	4
Tukey HSD[a,b]	Treatment 4	3	73.2650			
	Treatment 1	3		22487.7550		
	Treatment 2	3		27254.3940		
	Treatment 3	3			46428.4550	

	Treatment 5	3				68267.8610
	Sig.		1.000	.847	1.000	1.000
Duncan[a,b]	Treatment 4	3	73.2650			
	Treatment 1	3		22487.7550		
	Treatment 2	3		27254.3940		
	Treatment 3	3			46428.4550	
	Treatment 5	3				68267.8610
	Sig.		1.000	.339	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 33732432.215.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = 0.05.

STATISTIC OUTPUT FOR MILK YIELD

Tests of Between-Subjects Effects

Dependent Variable: MILK_YIELD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	16.584[a]	15	1.106	10.168	.000
Intercept	649.197	1	649.197	5970.417	.000
LACTATION	1.992	1	1.992	18.319	.000
TREATMENT	2.676	4	.669	6.153	.001
BREED	.288	1	.288	2.648	.115
LACTATION * TREATMENT	5.148	4	1.287	11.837	.000
LACTATION * BREED	.402	1	.402	3.698	.064
TREATMENT * BREED	1.686	4	.421	3.875	.012

LACTATION * TREATMENT * BREED	.000	0	.	.	.
Error	3.153	29	.109		
Total	768.010	45			
Corrected Total	19.738	44			

a. R Squared = .840 (Adjusted R Squared = .758)

Estimates

Dependent Variable: MILK_YIELD

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1	4.122[a]	.110	3.897	4.347
2	3.922[a]	.110	3.697	4.147
3	4.600[a]	.110	4.375	4.825
4	3.783	.121	3.535	4.032
5	3.800[a]	.110	3.575	4.025

a. Based on modified population marginal mean.

Pairwise Comparisons

Dependent Variable: MILK_YIELD

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[d]	95% Confidence Interval for Difference[d]	
					Lower Bound	Upper Bound
1	2	.200[a,b]	.155	.208	-.118	.518
	3	-.478[a,b,*]	.155	.005	-.796	-.160
	4	.339[a,*]	.164	.047	.004	.674
	5	.322[a,b,*]	.155	.047	.004	.640
2	1	-.200[a,b]	.155	.208	-.518	.118
	3	-.678[a,b,*]	.155	.000	-.996	-.360

	4	.139[a]	.164	.403	-.196	.474
	5	.122[a,b]	.155	.438	-.196	.440
3	1	.478[a,b,*]	.155	.005	.160	.796
	2	.678[a,b,*]	.155	.000	.360	.996
	4	.817[a,*]	.164	.000	.482	1.152
	5	.800[a,b,*]	.155	.000	.482	1.118
4	1	-.339[b,*]	.164	.047	-.674	-.004
	2	-.139[b]	.164	.403	-.474	.196
	3	-.817[b,*]	.164	.000	-1.152	-.482
	5	-.017[b]	.164	.920	-.352	.318
5	1	-.322[a,b,*]	.155	.047	-.640	-.004
	2	-.122[a,b]	.155	.438	-.440	.196
	3	-.800[a,b,*]	.155	.000	-1.118	-.482
	4	.017[a]	.164	.920	-.318	.352

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. An estimate of the modified population marginal mean (I).

b. An estimate of the modified population marginal mean (J).

d. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: MILK_YIELD

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	4.003	4	1.001	9.203	.000
Error	3.153	29	.109		

The F tests the effect of TREATMENT. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

STATISTIC OUTPUT FOR MILK COMPOSITION

A) Protein

SPSS RESULTS FOR PROTEINS

Tests of Between-Subjects Effects

Dependent Variable: PROTEINS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.599[a]	18	.033	5.706	.000
Intercept	.135	1	.135	23.159	.000
BREED	4.494E-5	1	4.494E-5	.008	.931
TREATMENT	.005	4	.001	.204	.934
STAGE_LACT	.002	1	.002	.312	.581
TREATMENT * STAGE_LACT	.050	4	.013	2.157	.102
FI	.000	1	.000	.077	.783
BREED * TREATMENT	.041	4	.010	1.770	.165
BREED * STAGE_LACT	.015	1	.015	2.496	.126
BREED * TREATMENT * STAGE_LACT	.000	0	.	.	.
PARITY	.039	1	.039	6.743	.015
AP	.007	1	.007	1.167	.290
Error	.152	26	.006		
Total	431.714	45			
Corrected Total	.751	44			

a. R Squared = .798 (Adjusted R Squared = .658)

Estimates

Dependent Variable: PROTEINS

TREATMENT	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
TRT 1	3.055[a,b]	.068	2.914	3.196
TRT 2	3.129[a,b]	.065	2.996	3.262
TRT 3	3.104[a,b]	.054	2.993	3.215
TRT 4	3.111[a]	.053	3.002	3.219
TRT 5	3.073[a,b]	.058	2.954	3.192

A) FATS

```
UNIANOVA FATS BY TREATEMENT BREED LACTATION
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=TREATEMENT(TUKEY DUNCAN LSD)
  /EMMEANS=TABLES(TREATEMENT) COMPARE ADJ(LSD)
  /CRITERIA=ALPHA(.05)
  /DESIGN=TREATEMENT BREED BREED*TREATEMENT LACTATION
LACTATION*TREATEMENT BREED*LACTATION*TREATEMENT.
```

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
TREATEMENT	1.00	1	9
	2.00	2	9
	3.00	3	9
	4.00	4	9
	5.00	5	9
BREED	1.00	FRIESIAN	21
	2.00	GUARNSEY	24
LACTATION	1	EARLY	24
	2	MID	21

Tests of Between-Subjects Effects

Dependent Variable: FATS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.807[a]	14	1.058	.799	.663
Intercept	626.637	1	626.637	473.603	.000

Multiple Comparisons

Dependent Variable: TOTAL_SOLIDS

	(I) TREATEMENT	(J) TREATEMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Inte	
						Lower Bound	Upp Bour
Tukey HSD	1	2	-.0422	.47754	1.000	-1.4274	1.
		3	.0744	.47754	1.000	-1.3107	1.
		4	-.7711	.47754	.500	-2.1563	.
		5	.1522	.47754	.998	-1.2329	1.
	2	1	.0422	.47754	1.000	-1.3429	1.
		3	.1167	.47754	.999	-1.2685	1.
		4	-.7289	.47754	.554	-2.1141	.