CHARACTERISATION OF LACTIC ACID BACTERIA ISOLATED FROM THE GUT OF GRASSCUTTER (*THRYONOMYS SWINDERIANUS*) IN THE COASTAL SAVANNAH ZONE OF GHANA

BY

MARTIN MAWULI AGBOVE

(10346163)

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DECLARATION

I hereby declare that this thesis which is submitted to the University of Ghana, for the award of Master of Philosophy in Animal Science degree, is the result of my own original work and that no part of it has been presented for another degree in this university or elsewhere. All assistance towards the production of this work and all the references contained herein have been duly credited.

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AGBOVE MARTIN MAWULI **DATE**

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PROF. BONIFACE B. KAYANG **DATE**

SUPERVISOR

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DR. JAMES E. FUTSE **DATE**

CO- SUPERVISOR

DEDICATION

I dedicate this work to the Almighty God to whom I owe my existence and also Mr. Nelson Mensah and Mrs. Charity Mensah for their unwavering love and support.

ABSTRACT

Intestinal microbiota is known to affect their host either beneficially or harmfully. Efforts have, therefore, been put into identifying and studying the microbial community in the gastrointestinal tract of livestock. Grasscutter is a promising microlivestock species with a short history of domestication, whose intestinal microflora is yet to be explored. Among the intestinal microflora, lactic acid bacteria confer probiotic benefits and are thus of special interest. This study was, therefore, conducted with the objective to isolate and characterise lactic acid bacteria from the gut of the grasscutter in Ghana. Fresh faecal samples were collected from a total of 26 grasscutters comprising nine domesticated grasscutters from the University of Ghana and 17 wild grasscutters from Mankessim and Gomoa Abontin in the Central Region of Ghana. The samples were cultured on MRS agar. DNA from 57 bacterial colonies was extracted and sequenced at the 16S rRNA gene to identify the bacteria at the species level using Basic Local Alignment Search Tool in the National Centre for Biotechnology Information database. Five genera comprising 15 species of lactic acid bacteria were identified with ≥99% similarity. These included *Lactobacillus fermentum* (n = 11), *L. salivarius* (n = 11), *L. ingluviei* (n = 9), *L. plantarum* (n= 7), *L. reuteri* (n = 2), *L. formosensis* (n = 1), *L. taiwanensis* (n = 1), *L. rhamnosus* (n= 1), *Pediococcus pentosaceus* (n = 5), *Enterococcus gallinarum* (n = 2), *E. hirae* (n = 2), *E. faecium* (n = 2), *Staphylococcus homini* (n = 2), *Weissella cibaria* (n = 1) and *W. paramesenteroides* (n = 1). All the five genera were isolated from the domesticated grasscutters while only two genera (*Lactobacillus* and *Pediococcus*) were isolated form wild grasscutters. The isolation of *L. ingluviei* is of interest since this species was originally isolated from birds and is associated with weight gain in mice. The bacteria identified by this study may be important in determining the intestinal health of the grasscutter and should be assessed for their potential as probiotics to improve grasscutter nutrition.

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LIST OF ACRONYMES AND ABBREVIATIONS

BHI	-	Brain heart infusion agar
BLAST	-	Basic Local and Aligned Search Tool
FAD	-	Flavin adenine dinucleotide
FMN	-	Flavin mononucleotide
GIT	-	Gastrointestinal tract
IECs	-	Intestinal epithelial cells
IgA	-	Immunoglobulin A
IgM	-	Immunoglobulin M
IFN	-	Interferon
IL	-	Interleukin
LAB	-	Lactic acid bacteria
MEGA	-	Molecular Evolutionary Genetic Analysis
MRS	-	de Man, Rogosa and Sharpe
NCBI	-	National Centre for Biotechnology Information
PAMPs	-	Pathogen-associated molecular patterns
PPRs	-	Pattern recognition receptors
TH cell	-	Helper T cell
TNF	-	Tumor necrosis factor
VRE	-	Vancomycin resistant enterococcus

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CHAPTER ONE

1.0 INTRODUCTION

The grasscutter (*Thryonomys swinderianus*) is a rodent also known as cane rat depending on the habitat it is found in. It is a wild herbivore related to the African brush-tailed porcupine as well as the guinea-pig, chinchilla and the capybara of South America (Asibey (a), 1974; Baptist & Mensah, 1986). In West Africa, the main habitat is grassland, hence the name grasscutter while in other parts of Africa particularly the southern part, it is associated with cane field, hence the name cane rat. It has an average weight of 3 kg for the female and 4.5 kg for the male and it is the next largest rodent after the crested porcupine (*Hystrix cristata*) (Eben, 2004).

Grasscutters are found to occur in Western, Central and Southern Africa (Adu *et al.*, 2017). In Ghana, domestication of this rodent started in the 1970s (Asibey, 1974a). The most preferred and consumed game by people living in rural areas in West Africa is the grasscutter (Asibey, 1974b). Its meat is said to resemble that of a piglet and is commercially preferred to other game (National Research Council, 1991). In some West African countries, particularly Ghana, grasscutter meat is a delicacy enjoyed by everyone regardless of religion or tribe. Several studies have therefore been conducted on the animal due to its delicacy, popularity, low fat level and high source of protein (FAO, 2012).

The promotion of grasscutter as microlivestock is expected to contribute significantly to reducing malnutrition which is prevalent in Ghana. Global Food Security Index (2012) found that food protein quality consumed in Ghana is low, which is about 0.205 g/kg. This is below the recommended 0.8 g/kg minimum daily protein requirements of animal origin (WHO, 2002). In 2015, Ghana produced about 150,751 metric tons of meat from traditional livestock which fell short of the required amount and therefore had to import an additional 80,338 metric tons (MoFA, 2016). It is therefore necessary to rear micro-livestock such as grasscutter to supplement the protein supply.

Rearing of grasscutter serves as a source of additional income for the breeder. Grasscutter rearing depends largely on peasant farmers who keep these animals in different forms of housing units. Being the most preferred (Martin, 1985) and the most expensive game meat in West Africa (Baptist and Mensah, 1986; Asibey and Addo, 2000), it has the potential to contribute to both local and export earnings in West African countries. Dependence of a nation on the wild grasscutter does not allow for sustainability and planned production for consumption (Yaro *et al*., 2012), hence the importance of domesticating and rearing them as microlivestock. Rearing of grasscutter can be easily managed since they depend on a lot of grass that are readily available especially during the rainy season. They do not compete with man for grain and other staple foods.

Grasscutters readily adapt to diets such as leguminous folder, roots, food crops, fruits and grasses (Eben, 2004). In the wild, grasscutters derive their nutrients from variety of feedstuff for production and maintenance of their body (Wogar, 2011). In captivity, a breeder must supply all the needed nutrients in the right proportions. However, the nutrient requirement of the animal is not well known. Among the major nutrients, protein is the one most likely to be deficient in the formulated feed of farm animals because of its diverse roles in the body of animals (McDonald *et al.*, 1996). Conventional protein sources such as fish meal and oil seed cakes (soya bean meal) are very expensive (Minson, 1997) and most farmers cannot afford it.

The unbalanced and low nutritional value of natural forage and feed fed to the grasscutter usually results in poor performance of the animal. Feeding plays an essential aspect in animal breeding because it results in good reproduction, health and growth in the animal (Zougou-Tovignon, 2005). All the activities of the grasscutter require energy and it is mainly obtained from nutrients in food.

Most food nutrients are made available to the grasscutter either by microbial or enzymatic activities in the gastrointestinal tract (Byanet *et al*., 2008). Despite the good attributes of the grasscutter, attempts made at its domestication have been marred by malnutrition, under-nutrition and high death rate (Yaro *et al*., 2012).

As a potential herbivorous animal for domestication, sufficient knowledge and understanding of the intestinal micro-flora may provide the much-needed information on how to formulate feed with a suitable probiotic. This will help increase the animal’s feed conversion ratio leading to increased productivity, increased litter size as well as reduced under-nourishment (Adu and Wallace, 2004) since the digestion and the absorption of nutrients are dependent on activities of normal microflora of the gastrointestinal tract (GIT) (Draser, 1989).

Normal microflora includes those microorganisms that colonize the GIT and benefit the host under the right conditions. A good balance of normal microflora plays an essential role in improving the animal's physiological functions and strengthening its immunity. The GIT in particular is the most heavily colonized region of the animal. The normal microflora of the grasscutter's GIT is constituted by 50% of faeces by dry weight (Yaro *et al.*, 2012). Research by Forchielli *et al*. (2005) indicated that the intestinal flora offers continuous stimulus to the lymphoid tissues of the host. GIT normal microflora, therefore, provides a general non-specific defense against infections. Some of these defenses include mechanisms such as competitive exclusion, availing some nutrients and production of substances harmful to pathogens. The digestion and the absorption of nutrients are dependent on a GIT's micro flora (Draser, 1989). Prominent among the microflora that promote the health and bioavailability of nutrient in the gut of the grasscutter are lactic acid bacteria (LAB).

LAB are a group of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of their main products of metabolism of carbohydrate (Ghanbari, 2009). Among the intestinal microflora, LAB confer probiotic benefits and are thus of special interest. Fuller (1989) defined probiotic to mean "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". LAB form part of an animal's GIT and play part in boosting the immune system against invading pathogens, availing some nutrients, increasing growth rate, increasing the rate of digestion and absorption of nutrients in the intestine and preventing the onset of some diseases (Florou-Paneri *et al.*, 2013).

Currently, in view of regulations restricting the use of antibiotics as feed additives, because of issues of generating antibiotic-resistant bacteria, there is a growing interest in employing probiotics as a suitable alternative in animal production. In this regard, LAB offer great promise for use as probiotics. However, it is important to first study the profile of LAB in livestock to identify which species of LAB to target for use as probiotic for a particular species of livestock. In Ghana, the grasscutters being reared have a relatively short history of domestication and not much is known about the microbial profile of their gut. In the face of nutritional and health challenges confronting grasscutter production, understanding their normal gut microflora could provide critical information that would be useful in improving the nutrition of this important microlivestock. This study was therefore conducted to fill this knowledge gap by profiling LAB isolated from the gut of the grasscutter in Ghana.

1.1 HYPOTHESIS

1. Lactic acid bacteria are present and can be isolated from the gut of grasscutter
2. Lactic acid bacteria profile in wild grasscutters differs from that in domesticated grasscutters.

1.2 PROBLEM STATEMENT

Unbalanced feeding, inadequate diet, high disease prevalence and high mortality constitute an enormous obstacle to large scale holding of livestock (Majiyagbe and Lamorde, 1997). During the dry season, severity of diseases depends on the nutritional status of the animal, when feed quality and quantity are low (Opara and Fagbemi, 2009). There is scarcity of information concerning the nutrition of the grasscutter. Inadequate understanding of the domesticated grasscutter's nutritional physiology can partially be blamed for the animal's poor reproductive performance (Adu *et al.*, 2017). For resourceful economic exploitation, caecal fermentation is inadequate to meet the glasscutter's nitrogen requirements (Adu *et al.*, 2017) and the amount of nutrient obtained from forage is insufficient to support the grasscutter's physiological functions (Adu 2003; Adu *et al.*, 2017). GIT microflora especially LAB can influence digestion and absorption of nutrient in the grasscutter. However, there is a dearth of information on the LAB profile in the gut of the grasscutter.

1.3 JUSTIFICATION OF THE STUDY

Diseases, malnutrition and mortality decrease production and cause huge losses to the farmer. The nutritional physiology of the domesticated grasscutter is inadequately understood and the introduction of probiotics may boost the immune system. Many studies have been conducted with respect to other farm animals such as poultry, pig, cattle, horse and rabbit related to the gut microbiota and nutrition and health. LAB have been used to promote the health and growth of these animals to minimise loss to the farmer. Information on LAB in the gut of grasscutter is currently unavailable, so this study will provide information on the LAB profile which would be useful in formulating probiotic in promoting the health of the grasscutter.

1.4 OBJECTIVES

The objectives of this study were:

- To isolate and identify lactic acid bacteria found in the gut of the grasscutter
- To determine if there are different profiles of lactic acid bacteria in wild and domesticated grasscutters.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE DIGESTIVE SYSTEM AND DIGESTION IN THE GRASSCUTTER

The main purpose of the digestive system is to provide for the breakdown of undigested food and absorption of nutrients needed for biological functions such as growth, reproduction, maintenance and lactation. The grasscutter is a monogastric herbivore (Skinner and Smithers, 1990), which means it feeds mostly on grasses but also on feed eaten by monogastric animals such as man, chickens and pigs. The digestive system of the grasscutter starts from the buccal cavity and ends at the rectum (Figure 1). It is a unique system which has an enlarged caecum which forms part of the large intestine (Akinnusi *et al.*, 2009). It has a mean weight and length of about 142.3 ± 11.94 g and 289.5 ± 21.30 cm, respectively, and accounts for about 14% of the total body weight of the grasscutter (Byanet *et al.*, 2008).

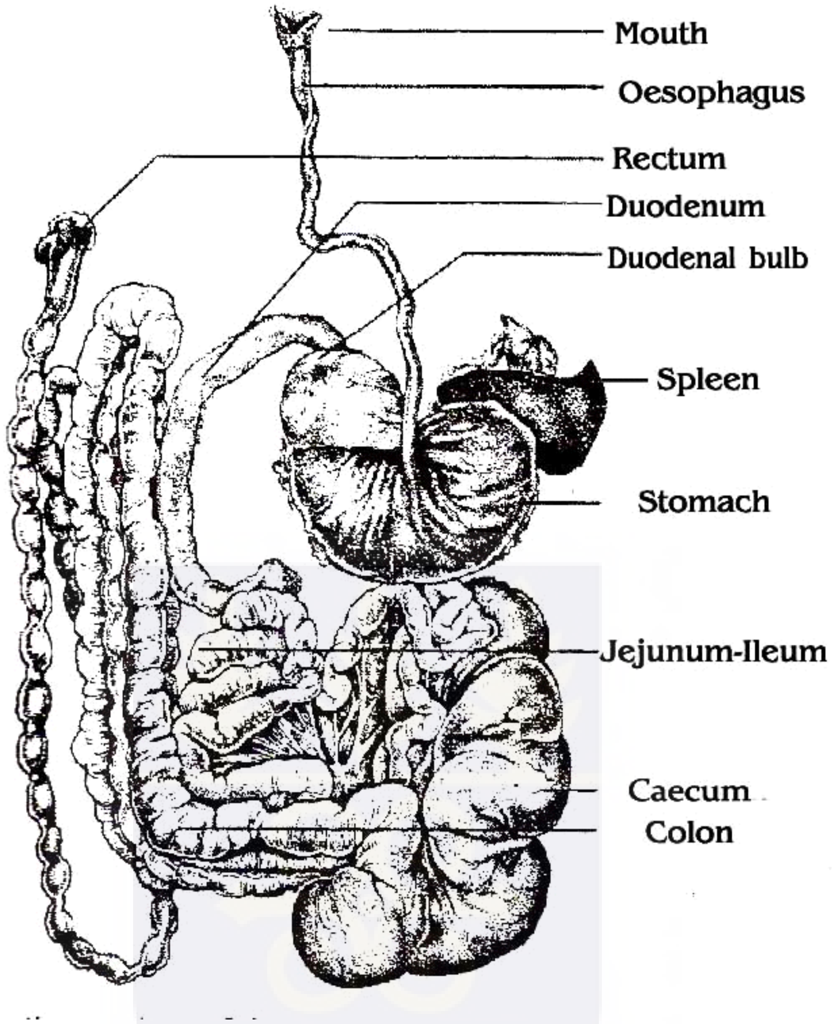

Figure 1: The digestive system of a grasscutter (Schrage and Yewadan, 1999)

2.1.1 The Mouth

The mouth of the grasscutter contains the teeth, tongue and the salivary glands. The dentition of the grasscutter is specific with 10 teeth, with a dental formula of I $^{1}/_{1}$, C $^{0}/_{0}$, PM $^{1}/_{1}$, M $^{3}/_{3}$ (Akinola *et al.*, 2015). Each tooth has its specific function in terms of digestion: the incisor for cutting of grass or feed and the molar and premolars for grinding. The incisor, which is mostly yellow, strong and sharp, is also very brittle.

The development of the molar can be used, to some extent, to predict the age of the grasscutter (Rosevear, 1969).

The tongue, a muscular organ, is also located in the buccal cavity (Merwe, 2000). In the mouth is the salivary gland that secretes saliva for softening and lubricating of food for easy digestion (Rosevear, 1969). The saliva also releases amylase which helps to begin the digestion of starch.

2.1.2 The Stomach

The stomach of the grasscutter which comprises cardiac, body and pyloric regions has a mean length of about 9.79 ± 0.83 cm. It also constitutes about 3% of the total length of the GIT. The stomach is a simple compartment which acts both as an organ for digestion and storage of feed. It is relatively small in comparison with animal's body size and has thin walls. The stomach which has one chamber is considered as a simple stomach and looks like an inverted 'J', appearing in the form of a curved sack (Byanet *et al.*, 2008).

Thorough mixing of feed with hydrochloric acid changes the pH of stomach contest from alkaline to acidic (Byanet *et al.*, 2008). The hydrochloric acid in the stomach activates pepsinogens, converting them to pepsins. The end products of protein digestion are mainly polypeptides and a few amino acids (Rudolf and Stromberg, 1976). Emptying of the stomach contents into the duodenum is controlled by osmotic sensors in the duodenum (Schrage and Yewadan, 1999).

2.1.3 The Small Intestine

The small intestine of the grasscutter consists of three segments: duodenum, jejunum and ileum. According to Byanet *et al.* (2008), the duodenum, the first segment of the small intestine, appears smooth and starts from the pylorus and ends at the origin of the jejenum. The jejenum is located at the abdominal floor and lies between the urinary bladder caudally and the stomach cranially. It is also seen to be coiled or convoluted and long but progressively stops coiling near the end. The ileum also appears smooth and curved only where it joins the large intestine.

The partially digested feed leaving the stomach enters the small intestine where it is mixed with secretions from the duodenum, liver and pancreas (Olusanya and Olowo, 1988). Majority of digestion and absorption of feed occurs in the small intestine (Schrage and Yewadan, 1999).

Bile, stored in the gall bladder passes down to the duodenum through the bile duct where it activates pancreatic lipase and emulsifies fats. The pancreas produces enzymes (amylase, protease and lipase) that break down the protein part of food into amino acids. The end products of fat (fatty acids and glycerol) and carbohydrate (glucose) are absorbed via the intestinal walls into the bloodstream (Schrage and Yewadan, 1999).

2.1.4 The Large Intestine

The large intestine consists of three segments, namely, caecum, colon and rectum. The caecum is a coma-shaped, blind ended sac slightly longer than the stomach and is the largest organ in the abdominal cavity (Byanet *et al.*, 2008). The caecum of the grasscutter occupies about 60% of the abdominal cavity and harbors microbial organisms for efficient fermentation and utilisation of fibrous diets. The caecum retains and stirs digesta well and supports microbial fermentation (Schrage and Yewadan, 1999). It appears as a branch from the junction of the small intestine and the colon. It is the major site for the holding of food residues that serves as the primary substrate for microbial fermentation.

The colon is the longest segment of the gastrointestinal tract with a wide lumen and it contains the faecal balls. The rectum is relatively short and straight and terminates at an enlarged region, the anal orifice (Schrage and Yewadan, 1999).

The microbes in the caecum are responsible for efficient utilisation and fermentation of diets of high fibre (Byanet *et al*., 2008). Microbes in the caecum synthesise B vitamins such as thiamine, B_6 and B_{12} which pass out with the soft faeces and become accessible to the grasscutter via the process of coprophagy. The end products of fibre fermentation by the microorganisms in the caecum are volatile fatty acids consisting mainly of propionate, butyrate and acetate (Hume, 1997).

2.2 COPROPHAGY

Coprophagy is a feeding behavior in animals enabling them to re-ingest their own excreted faeces. It has been observed in horses (Schurg *et al*. 1977) and capybaras (Mendes *et al*. 2000). The grasscutter is considered as a coprophagous animal since it also re-ingests its caecal digester (Sakaguchi, 1992). Coprophagy does not only happen in herbivores and omnivores but also in carnivores. The benefits of coprophagy are much larger for hindgut fermenters than foregut fermenters (Alexander, 1993). During resting periods, coprophagous animals produce two types of faeces; soft (which is re-ingested) and hard (which is not re-ingested). Coprophagy is the most effective strategy to utilise dietary nitrogen (Chilcott and Hume 1985). It serves as a source of amino acids, minerals, vitamins, and other nutrients that are excreted with faeces and not effectively utilised in the gastrointestinal tract (Barnes and Fiala 1957). The re-ingested feed usually contains higher amount of protein than in the fed diet (Sakaguchi, 1992)

2.3 NUTRITION OF THE GRASSCUTTER

According to Onyeanusi *et al*. (2001), there is a relationship between the local environment, productive life, husbandry techniques and health status. Domestication (housing) deprives the grasscutter of its unrestricted and unlimited free access to habitat, food items and minerals of their choice. Simply, grasscutter is a herbivore that requires feed supplementation in captivity to thrive better. In captivity, their walking distance is limited thereby reducing the level of exercise in search for food. They also know the right time to relocate to a more favourable habitat during a bad season to avoid diseases and pest infestation in the wild (Opara, 2010).

The grasscutter is a rodent that feeds mainly on grass species that are thick stemmed. While the rabbit for example feeds mainly on leaves, the grasscutter, in contrast, feeds mainly on stems thereby wasting the leaves especially during the dry season when it's difficult to get access to grass (Schrage and Yewandan, 1999). Mensah and Okeyo, (2005) stated that grasscutter irrespective of the type of forage prefer to feed in the order: stalk, bark of twigs and leaves. Unfortunately, this practice by the grasscutter leads to wastage of forage and wearing out of the teeth of the animal (Adu, 2005).

Grasscutters prefer grasses with high moisture content and soluble carbohydrate (Agbelusi, 1997). They also like stems and grasses like sugar cane (Adu, 1995). They can also be fed with kitchen left-overs (Addo, 1997), fruits, different crops and nuts (Fitzinger, 1995). Some grasses that are particularly consumed by the grasscutter during both wet and dry season have been identified. These include *Pennisetum purpureum* (elephant grass), *Saccharum officianarium* (sugar cane), *Zea mays* (maize), *Sorghum vulgare* (guinea corn), *Oryza sativa* (rice) and *Andropogon gayanus* (Gamba grass) (Ebenebe, 2005). Henry and Njume (2008) reported that the highest live weight and good carcass quality were attained when the animals were fed 2000 KcalME/kg of energy from a combination of elephant grass (*Pennisetum purpureum*) and other preferred grasses. In the humid tropics, the optimum amount of energy required for a growing grasscutter is about 2200 KcalME/kg (Henry and Njume, 2008).

2.4 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are a group of microorganisms that form part of an animal's GIT and play part in boosting the immune system against invading pathogens and availing some nutrients, increasing growth rate, increasing the rate of digestion and absorption of nutrients in the intestine and prevention of the onset of some diseases (Florou-Paneri *et al.*, 2013). They comprise an ecologically diverse group of microorganisms united by formation of lactic acid as the primary metabolite of sugar metabolism. These bacteria utilise sugars by either homo- or hetero-fermentative pathways (Fugelsang and Edwards, 2007). The homo-fermentative species produce lactic acid (≤ 85%) as the sole end product, while the hetero-fermentative species produce lactic acid, carbon dioxide and ethanol or acetate. Many genera of bacteria produce lactic acid as primary or secondary fermentation products but typical LAB are those of the order Lactobacillales (Hutkins, 2006).

LAB are mostly very sensitive to environmental factors such as heat, temperature and acid (Heller, 2001) and must be able to withstand some physiological and functional conditions by counteracting the action of harmful microorganisms, producing antimicrobial substances, stimulating the immune response, colonising the lumen of the GIT and adhering to the intestinal epithelium successfully (Soccol *et al*., 2010).

LAB have been chosen as probiotic to replace antibiotic in the feed of animals and humans (Florou-Paneri *et al.*, 2013). Major genera of Lactic acid bacteria include: *Enterococcus, Lactosphaera, Lactobacillus, Oenococcus, Lactococcus, Carnobacterium, Leuconostoc, Pediococcus, Vagococcus, Streptococcus, Tetragenococcus, Weissella, Aerococcus, Melissococcus and Microbacterium* (Djadouni and Kihal, 2012).

2.5 NICHE-SPECIFIC ADAPTATION OF LAB

Soil and plants are considered as the first niche of LAB and afterwards the gut of animals (Morelli *et al*., 2012). Health essential microorganisms that colonize the mammalian gut are about 100 trillion (Hooper and Macpherson, 2010). The movement of microorganisms from soil and plants to the intestine of animals has three strategies of adaptation: resistance to host defense mechanisms, adhesion to intestinal walls and fermentation of some substances in the gut. Bile salts and low pH affects the membrane lipid composition (Klaenhammer *et al*., 2005). Extracellular Lipopolysaccharides play an important role in the resistance to host defense but the mechanism is unclear (Morelli *et al*., 2012).

Due to the increased level of production of intestinal mucins, some bacteria are able to inhibit the adherence of pathogenic bacteria to the intestinal epithelial surfaces (Moal and Servin, 2006). Some LAB species (eg. *L. plantarum*) species also increase mucins mRNA levels thereby inhibiting pathogenic bacteria such as *Escherichia coli* from cell attachment (Mack *et al*., 2003). LAB possessing genes to degrade sugar and carbohydrate have the advantage to multiply in the gut since there is access to sugar and carbohydrate (Morelli *et al*., 2012).

The digestive system is an open ecosystem and the microorganisms consumed with food and water have the potential to easily colonise the hindgut and influence fermentation. If microorganisms can attach themselves to the lining of the intestine or multiply at a faster rate compared to the rate of flow of the digesta, then they can become permanent residents. A host's diet, stress level, environment and drug administered affects the microbial species found in the digestive gut. The growth of lactic acid bacteria in the gut is encouraged by the lactose content of the mammalian milk. In young maturing animals, strict anaerobic microorganisms slowly outgrow the lactic acid bacteria in the GIT as feed becomes more solid and the lumen enlarges. Lactic acid bacteria inhibit the growth of other microorganisms and are recognised as desirable and therefore given as feed supplement (probiotic) to promote health.

2.6 LACTIC ACID BACTERIA AS SOURCE OF FUNCTIONAL NUTRIENTS

2.6.1 Lactic Acid Bacteria and Vitamins

Vitamins are essential micronutrients that are required by every organism for metabolism. They are also organic compounds that are obtained from diets either because an organism cannot produce them in sufficient quantities or because they do not have the necessary enzymes to synthesis them. Humans and animals cannot synthesise most of these vitamins, although the gut LAB can produce some vitamins (folate, vitamin K_2, thiamine, vitamin B_{12} and riboflavin). The gut microbiota has also been considered as a source of water soluble vitamins (LeBlanc *et al*, 2011) and they play an important role in metabolism especially the cellular metabolism of carbohydrate (thiamine), fats (pyridoxine and riboflavin) and proteins (LeBlanc *et al*., 2010). To produce a novel food with high nutritional value and/or health stimulating properties, inclusion of LAB is a good approach (LeBlanc *et al*, 2011).

2.6.1.1 Riboflavin

Riboflavin or vitamin B_2 is essential in cellular metabolism and also being a water-soluble vitamin is produced by many microorganisms and plants. It is also a precursor of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), with both acting as electron carriers in oxidation–reduction reactions as well as functioning as coenzymes for many of the FMN or FAD-dependent enzymes called flavo-proteins (Burgess *et al*., 2006).

The riboflavin synthesis genes in LAB form a single operon in the order of the genes: “riboflavin-specific deaminase and reductase (*ribG*), riboflavin synthase alpha subunit (*ribB*), a bifunctional enzyme which also catalyzes the formation of 3, 4-dihydroxy-2-butanone 4-phosphate from ribulose 5-phosphate (*ribA*), and riboflavin synthase beta subunit (*ribH*)” (Wels *et al*. 2006). In the coenzymes, FAD and FMN, the biologically active forms of riboflavin can be seen (Burgess *et al*. 2009). For each species or strain of LAB, the genetic information of vitamin B_2 biosynthesis is specific (Kleerebezem *et al*. 2003). Instead of the normal level, some bacteria and fungi over-produce riboflavin (Perkins *et al*. 1999).

2.6.1.2 Folate

Folates are involved in many metabolic pathways such as conversion of amino acids, biosynthesis of RNA and DNA (LeBlanc *et al*., 2007), methylation and repair, synthesis of nucleotides and vitamins (LeBlanc *et al*. 2010). Due to its antioxidant ability, they are able to protect the genome by avoiding free radical hack of DNA (LeBlanc *et al*., 2007). Dietary folate is important since the mammalian cells cannot synthesise it (Eitenmiller and Landen, 1999).

Streptococcus spp., Lactobacillus spp. (*L. lactis, L. bulgaricus and L. plantarum*) and *Enterococcus spp.* are species of LAB that have the ability to produce folate, although not all *Lactobacillus spp* have the ability to produce folate (O'Connor *et al*., 2005). Animals cannot produce folate and must acquire it exogenously to prevent its deficiencies. Some dietary sources of folate are leafy, green vegetables and dairy products (Finglas *et al*. 2003). When folate-producing probiotic strains are administered orally, they may present a more resourceful protection against cancer and inflammation, by presenting folate to colonic-rectal cells (Pompei *et al*., 2007). In pigs, folate is synthesised by bacteria and absorbed by across large intestine and integrated into the kidneys and liver (Asrar and O'Connor, 2005).

2.6.1.3 Vitamin B_{12} Or Cyanocobalamin

Vitamin B_{12} is required for the metabolism of amino acids, carbohydrates, fatty acids and nucleic acids (Quesada-Chanto *et al*., 1994). Vitamin B_{12} does not exist naturally but is produced naturally. Anaerobic microorganisms are able to produce vitamins exclusively but plants, animals and fungi are unable to produce cyanocobalamin (Smith *et al*., 2007).

2.7 LACTIC ACID BACTERIA AS PRODUCERS OF ANTI-MICROBIAL AGENT

Antimicrobial agents produced by lactic acid bacteria through carbohydrate fermentation include low molecular weight proteins (Vandenberg, 1993), organic acids, hydrogen peroxide and carbon dioxide (CO_2) (Naidu *et al*., 1999). Yeast, Gram-negative bacteria and molds are inhibited by Diacetyl (2.3-butanedione). Some Gram-positive and Gram-negative bacteria are inhibited through the lowering of the intestinal pH by the production organic acids such as volatile fatty acids and lactic acids (Naidu *et al*., 1999), propionate and acetate (Ouwehand and Vesterlund, 2004). Carbon dioxide is able to interfere with cell membranes (Lindgren and Dobrogosz, 1990). Bacteriocins are antimicrobial peptides produced as defense against narrowly linked strains (Klaenhammer, 1993) and have numerous modes of action (Cleveland *et al*., 2001).

To classify antimicrobial agents, their mode of action is used and there are usually four groups; inhibition of protein synthesis, inhibition of metabolism, disruption of cell wall synthesis and prevention of nucleic acid synthesis (Tenover, 2006). The different types of antimicrobial agents produced by lactic acid bacteria are bacteriocins, reutericyclin, reuterin, diacetyl, carbon dioxide, hydrogen peroxide and organic acids.

2.7.1 Bacteriocins

Bacteriocins are either bactericidal proteins or peptides that are synthesised ribosomally (Balla *et al*., 2000). Species of microorganisms closely related to bacteriocin producing strains of LAB are most affected by the bactericidal activities (Rajaram *et al*., 2010). Bacteriocins target bacterial membrane and exhibit hydrophobic properties (Savadogo *et al*., 2006). Bacteriocins produced by LAB are differentiated from antibiotics because they are synthesised ribosomally with narrow killing spectrum whiles antibiotics are produced as secondary metabolite by some bacteria or fungi with broad spectrum anti-microbial activity.

Bacteriocins inhibit a number of other bacteria (Ogunbanwo *et al*., 2003) and their production is influenced by pH, temperature and source of nutrient (Todorov and Dicks, 2004). Although antimicrobial peptides possess a narrower inhibition spectrum compared to antibiotics (Morency *et al*., 2001), bacteriocins produced by LAB have been reported to perforate the outer membrane of Gram-negative bacteria and also in combination with other environmental factors, such as organic acid, detergents and low temperature they are able to encourage the inactivation of Gram-negative bacteria (Elliason and Tatini, 1999).

There are three major classes of bacteriocins, with a fourth class generally not accepted (Ouwehand & Vesterlund, 2004):

- ✓ Class I (lantibiotics) – elongated, small cationic peptides (Zendo and Sonomoto, 2011).
- ✓ Class II – small heat-stable proteins which are subdivided into IIa (pediocin-like bacteriocins) and IIb (two peptide bacteriocins) (Zendo and Sonomoto, 2011).
- ✓ Class III (helveticin) (Dobson *et al*., 2007)
- ✓ Class IV (Complex bacteriocins) – protein with lipid and/or carbohydrate (Ouwehand and Vesterlund, 2004).

2.7.2 Organic Acids

Lactic acid is produced during homo-fermentative and hetero-fermentative pathways. Propionic and acetic acid which are also produced during hetero-fermentative pathway have broad spectrum inhibition on microorganisms such as yeast, bacteria and mould (Ouwehand and Vesterlund, 2004). Lactic acid makes membranes permeable and reduces pH so as to make lactic and propionic acid more effective by dissociating in neutral cytoplasm, releasing anions and protons thereby acting as antimicrobial agent (Alakomi *et al*., 2000).

2.7.3 Hydrogen Peroxide

Hydrogen peroxide generated by LAB hunt oxygen thereby creating an anaerobic environment hostile for some microorganisms (Ouwehand & Vesterlund, 2004). Hydrogen peroxide is able to inhibit the growth of Gram-positive bacteria whereas killing Gram-negative bacteria (Blom & Mörtvedt, 1991). It has a resilient oxidising effect on membrane lipids, bacterial cell and cell proteins sulfhydryl groups (Lindgren & Dobrogosz, 1990). Blocking of glycolysis is the main antimicrobial effect of hydrogen peroxide (Carlsson *et al*., 1983).

2.7.4 Carbon Dioxide

The production of carbon-dioxide during hetero-fermentative pathway usually creates a micro-aerophilic environment. Gram-positive bacteria are less sensitive to carbon-dioxide compared to Gram-negative bacteria (Devlieghere and Debevre, 2000).

2.7.5 Diacetyl

Lactobacillus and *Pediococus* produced diacetyl is not only responsible for butters aroma and flavour but also has antimicrobial function. Diacetyl interferes with arginine utilisation by Gram-negative bacteria. It is also more active against yeast, mould and Gram-negative bacteria compared with Gram-positive bacteria (Marelize, 2008).

2.7.6 Reuterin

In an anaerobic environment and the presence of glucose, glyceraldehydes or glycerol, a low molecular-weight antimicrobial substance, reuterin is produced by *Lactobacillus reuteri. L. reuteri* produces reuterin when in contact with target cells such as bacteria, viruses, protozoa and viruses (Axelsson *et al*., 1989). Reuterin inhibits sulfhydryl enzymes and DNA synthesis (Dobrogosz *et al*., 1989).

2.7.7 Reutericyclin

Another low molecular weight antimicrobial substance with high hydrophobicity and negative charge produced by *Lactobacillus reuteri* is reutericyclin (Höltzel *et al*., 2000). Gram-positive bacteria but not Gram-negative bacteria are sensitive to reutericyclin (Gänzle *et al*., 2000). It is able to form pores in target cells membranes (Gänzle and Vogel, 2003).

2.8 MECHANISMS FOR SURVIVAL OF LAB

LAB have several and various effects on the host. Some LAB like *Bifidobacterium longum* colonize and become part of the gut microbiota while other transient LAB such as *Lactobacillus casei* indirectly exert their effect by transformation or manipulating the existing microbial community as they pass through (Ohland and Macnaughton, 2010). To be able to stimulate the immune system, LAB must be able to resist the enzymes in the oral cavity (amylases and lysozyme), small intestines mucus and pancreatic juice and low pH in the stomach (Ouwehand and Salminen 1998). Some of the major mechanisms of action of LAB on the host are barrier function, production of antimicrobial substances, competitive exclusion of pathogens and immunomodulation.

2.8.1 Barrier Functions

Functions of many components of the epithelial barrier of the immune system are influenced by probiotics either by increasing mucin production or decreasing apoptosis of intestinal cells. Cytokine-induced apoptosis in intestinal epithelial cell by *Lactobacillus rhamnosus* leads to inhibition of tumor necrosis factor (TNF) (Yan and Polk, 2006). In *in-vitro Lactobacillus* species, there is an increased mucin expression in intestinal epithelial cells thus blocking the adherence and invasion of pathogenic *E. coli.* (Mattar *et al.*, 2002). Inflammation and apoptosis of the lining of the intestinal epithelial cells has also been prevented by *Lactobacillus rhamnosus* (Gaudier *et al.*, 2005) as well as enhancing mucosal regeneration (Caballero-Franco *et al.*, 2007). Probiotics also increase mucus production by controlling the cytoskeletal and tight junction protein phosphorylation (Sherman *et al.*, 2005).

2.8.2 Competition For Adherence

For bacteria, a pre-requisite for colonisation of the intestinal mucosa is adhesion and is important for interaction between the host and LAB (Perdigon *et al.*, 2002). Adhesion of LAB to the intestinal mucosa is also important for its antagonism against pathogens and modulation of immune system (Hirano *et al.*, 2003). LAB display surface proteins, that interact with mucous and epithelial cells. Intestinal epithelial cells (IECs) secrete complex glycoprotein mixture called mucin, a main component of mucous, which helps prevent the adhesion of pathogenic bacteria (Neutra and Forstner, 1987).

Mucous gel contains salts, immunoglobulins, lipids, and free proteins (Ouwehand *et al.*, 2002). Several *Lactobacillus* proteins encourage mucous adhesion (Van Tassell and Miller, 2011) and the bacteria show surface adhesins that intervene in its attachment to the mucous layer (Buck *et al.*, 2005). The attachment procedure is usually intervened by proteins, although saccharide moieties and lipoteichoic acids also play some part (Vélez *et al.*, 2007).

2.8.3 Competitive Exclusion Of Pathogens

Different species of bacteria use different mechanisms to exclude or suppress the growth of other species. These include elimination bacterial receptor sites, creating antagonistic micro-ecology, secretion and/or production of antimicrobial substances and depletion of essential nutrients (Rolfe, 1991). Bacteria communicate with each other and their immediate environment through chemical signaling molecules known as auto-inducers. This phenomenon is called quorum sensing (Miller and Bassler, 2001). Quorum sensing facilitates the regulation of traits of enteric microbes that permit their successful colonisation and/or infection in their host (Kendall and Sperandio, 2007). For example, *Lactobacillus acidophilus* produces a molecule that interacts with transcription of *E. coli* O157 gene or hampers the quorum sensing signaling involved in colonization thereby opposing bacterial toxicity (Medellin-Peña *et al.*, 2007).

2.8.4 Immunomodulation

In terms of diseases, LAB play an important role by influencing the innate and adaptive immune systems. LAB affect different types of lymphocytes (NK cells and T-cells), the dendritic cells, the epithelial cells and the monocytes/macrophages directly or secondarily (Ng *et al.*, 2009). Neoplastic host cells can be eliminated by this action of probiotics (Soccol *et al.*, 2010). The effect of probiotics on B-lymphocyte and antibody production is an increased IgA secretion and increased response to vaccination (Ng *et al.*, 2009).

The innate immune system responds to structures called pathogen-associated molecular patterns (PAMPs) presented by most pathogens (Gómez-Llorente *et al.*, 2010). Pathogens trigger pattern recognition receptors (PPRs), which bind PAMPs as a form of primary response (Lebeer *et al.*, 2010). *Lactobacillus casei, L. rhamnosus and L. Plantarum* are some strains of LAB that are able to stimulate effectively phagocytic cells than other bacteria since they are able to adhere more suitably to the gut mucosa (Schiffrin *et al*. 1997). Some strains of LAB are able to increase the intestinal inflammatory response while others induced specific secretory immunity (Perdigon *et al*. 2002).

2.9 LAB AS PROBIOTIC AND ITS EFFECT ON ANIMAL HEALTH

In agriculture, most of the probiotic products used contain lactic acid bacteria, including *Pediococcus spp., Lactobacillus spp., Enterococcus spp., Streptococcus spp.*, and yeast strains such as *Saccharomyces cerevisiae* and *Kluyveromyces* spp. Probiotics have various therapeutic applications but the health benefits exerted by each probiotic is strain-specific. No specific strain of probiotic can therefore serve all purposes (Figueroa-Gonzalez *et al.*, 2011). In animal nutrition, direct fed microbes help to keep a healthy intestinal microflora. The microflora helps to improve the health status and the performance of the animal as well as inhibit the growth of pathogens such as *Campylobacter* and *Salmonella*. With the right conditions provided by the probiotics, meat and meat products can be considered as safe for production (Vila *et al*., 2010).

Probiotics modulate pathogen induced inflammation through toll-like receptor-regulated signaling pathways as well as enhancing the innate immunity (Vanderpool *et al*., 2008). Vaginally born infants harboring *Bifidobacterium* species and *Bacteroides fragilis* tend to have more immunoglobulin (Ig) M-secreting and IgA-secreting cells circulating (Martín *et al*., 2003).

LAB are able to synthesise vitamins, antioxidants, bacteriocins and enzymes (Knorr, 1998) and these properties enable them provide an important mechanism for the detoxification of invading foreign materials as well as metabolism (Salminen, 1990). Certain strains of LAB translocate across the intestinal mucosa at regular interval without causing infection but rather enhancing the systemic immune response have been reported (Cross, 2002).

The immune system secretes an antibody isotype known as secretory IgA (sIgA) during intestinal antibody secretion. Sub-epithelial plasma cells produce most IgA (Cerutti and Rescigno, 2008). Epithelial cells express polymeric Ig (pIgR) specific receptors which are vital for the careful transportation of immunoglobulins to the gut lumen. The immunoglobulin classes IgG and IgM are also present in the secretions but are in low amounts. Of all the immunoglobulins, IgA is the most resistant to the action of proteolytic enzymes in gastrointestinal secretions (Brandtzaeg, 1984). IgA-antigen complexes do not trigger the complement with inflammatory outcomes.

2.10 BENEFITS OF PROBIOTICS

In the livestock production industry, there are many potential uses for probiotics and some of these include;

I. Growth promotion
II. Protein sparing effect
III. Nutrient bioavailability and synthesis

2.10.1 Growth Promotion

Carbohydrates can be broken down by probiotics into saccharides making them available for absorption through the digestive system. In some farm animals, *Bifidofacteria* spp. and *Lactobacillus* spp. increase weight gain and decrease mortality. In swine, piglets given feed supplemented with *Bacillus* experience lower mortality rate and improved weight gain compared to those fed *Bacillus* un-supplemented feed and antibiotic supplemented feed (Chang *et al*., 2001).

2.10.2 Protein Sparing Effect

Probiotic such as *Lactobacillus* basically uses carbohydrate as its growth medium while most pathogens use protein. However, through competitive exclusion, higher percentage of protein is made available for host’s digestive system to absorb nutrients (Chang *et al*., 2001).

2.10.3 Nutrient Synthesis And Bioavailability

Certain amino acids are synthesised by probiotic bacteria which are directly assimilated e.g. certain strains of *Lactobacillus plantarum* synthesise lysine. Probiotic bacteria also help with the production and stimulation of B-Vitamins such as niacin, pantothenic acid, riboflavin, B_{12} and B_6 all of which are biocatalysts in food metabolism and fighting stress (FAO/WHO, 2005).

2.11 IMPORTANCE OF PROBIOTICS TO WEANERS

Weaning is an important period for young animals where they experience stress after being separated from their mother to join other mates in a different environment with different nutritional adaptations. The stress tolerance level of young animals is low, which results in high cortisol level in systemic circulation, hence immune-suppression leading to high sensitivity to enteric diseases (Kanitz *et al.*, 2004). Systemic immunological defense is highly affected through deteriorated digestion and absorption of nutrients (O’Hara and Shanahan, 2006).

Integration of antibiotics in young animals feed was the strategy used to prevent or control pre-weaning and post-weaning enteric diseases (Berge, 1996). However, the request from FAO to exclude antibiotics from animal feed presents a challenge to farmers and researchers to find a less harmful alternative means of prophylaxis. In light of this, probiotics have been found as the replacement for antibiotic and has advantages such as stimulating the immune system, promoting growth by competing with harmful gut flora and increasing resistance to infectious diseases (O'Hara *et al*., 2006). Animals such as rabbit develop several health problems that affect their fattening period. Administration of probiotics during this period increases growth performance, reduces mortality and/or morbidity (Trocino *et al*., 2005).

2.12 SELECTION CRITERIA OF GUT LAB

To select a probiotic LAB strain, the first step is to determine the taxonomic classification, which may indicate the origin, physiology and habitat of the strain. LAB are associated with nutrient rich habitat such as plant material and various food products (Morelli, 2007).

One of the most important selecting criteria is the ability to adhere to the intestinal mucosa because adhesion to the intestinal mucosa is considered to be necessary for colonisation. The primary selection and screening of probiotics includes testing of the following significant criteria: "phenotype and genotype stability, including plasmid stability; carbohydrate and protein utilisation patterns; acid and bile tolerance and survival and growth; intestinal epithelial adhesion properties; production of antimicrobial substances; antibiotic resistance patterns; ability to inhibit known pathogens, spoilage organisms, or both; and immunogenicity" (Wedajo, 2015).

The host to be fed the probiotic must be immuno-tolerant meanwhile the probiotic strain must also act as an adjuvant and stimulate the host's immune system against pathogenic micro-organisms. The probiotic strain to be selected must not be harmful to the host, thus it must not; be pathogenic in nature and/or cause mutagenic/carcinogenic or allergic reaction (Desai, 2008).

As stated earlier, the LAB probiotic strain to be selected must be able to resist gastric and bile acid, adhere to the GIT epithelial lining, colonise the GIT, produce antimicrobial substances and modulate immune resistance.

2.12.1 Resistance To Gastric Acid

The selected probiotic strain must first endure its transit through the stomach before reaching the intestinal tract (Henriksson *et al*., 1999). In the stomach, gastric acid is produced as a basic form of defense against ingested microorganisms. However, survival of lactic acid bacteria strains in the gastric juice is an indication of the ability of strains to endure passage through the stomach (Draser *et al*., 1969). Irrespective of the host's health status and pH level, LAB is able to survive, adhere to the epithelial lining and colonise the surface. This suggests that isolated strains can successfully transit the host's stomach, reach the intestinal environment and function effectively (Thornton, 1996).

2.12.2 Bile Acid Resistance

Another important criterion to consider when evaluating the potential of lactic acid bacteria as an effective probiotic is their ability to resist the bile acid effect (Lee and Salminen, 1995). In the liver, bile acids are synthesised from cholesterol and are secreted in the conjugated form from the gall bladder in the duodenum. Through the activities of microorganisms, these acids undergo chemical transformation (dehydrogenation, deglucuronidation, deconjugation, dehydroxylation) in the colon (Shimada *et al.*, 1969). Bile acids (conjugated and deconjugated) show antibacterial activities which inhibit *Escherichia coli* strains, *Enterococcus sp*. and *Klebsiella sp*.in vitro (Lewis and Gorbach, 1972; Stewart *et al*., 1986). The deconjugated compared to the conjugated form is more inhibitory, with gram negative bacteria less sensitive compared to gram positive (Floch *et al*., 1972).

2.12.3 Anti-Bacterial Activities

Most of the metabolic substances stimulated by lactic acid bacterial (hydrogen peroxide, organic acids, diacetyl and fatty acids) exhibit antimicrobial activities (Ouwehand, 1998). Bacteriocins and proteinaceous substances having specific inhibitory activities are the most studied (McAuliffe *et al*., 1998). The only purified bacteriocin recommended for use in products is nisin produced by some *Lactobacillus lactis* subsp. *lactis* strains (Dodd and Gasson, 1994).

2.13 LAB AS VACCINE VECTORS

Live attenuated vaccines have been developed to deliver vaccine constituents to induce a specific immunity at mucosal level (Medina and Guzman, 2001). There is however a disadvantage to using attenuated vaccine due to possibility of reversion to the original virulence strain leading to disease especially in immune-compromised individuals. To overcome this hindrance, LAB have been introduced as an alternative for use as live attenuated vaccines. Some species of LAB especially *bifidobacterium* and *lactobacilli* found in the GIT exert range of health promoting benefits for improving the intestinal homeostasis, the modulation of an immune response and the competitive exclusion of intestinal pathogens (Marutpong and Baltasar, 2014). The availability of genetic information (plasmid, prophages, insertion element, and transposons) and tools have made it easy to convert LAB into vaccine (Shareck *et al.*, 2004).

A study by Jennings *et al*. (1998) showed that an ideal mucosal vaccine should stimulate cell-mediated immunity and specific humoral response, be stable and non-toxic, stimulate a lasting protection after a single-dose during infancy, and encourage contact between an antigen and the immune system. Other work has shown the prospects of lactic acid bacteria as antigen presenters appropriate for mucosal administration. For instance, Mercenier (1999) recorded no toxic or pathogenic activity when *Lactococcus lactis* was administered orally and subcutaneously to mice. It is also interesting to note that certain species of lactobacilli may be able to promote the immunogenicity of heterologous antigens because of their intrinsic adjuvant capabilities (Pouwels, 1996).

To develop LAB as live vaccines, they should be genetically transformed. Recombinant proteins (antigen) production can be attained by three different methods: surface-bonded, extracellularly and intracellularly. Choosing any one of these methods is very important because the organization, size, molecular weight and nature may affect both the humoral and cellular immune responses. Studies have shown that recombinant *Lactococcus lactis* strains were able to elicit humoral response against a bacterial antigen (tetanus toxin) (Wells *et al.*, 1995).

Good and efficient vectors have been successfully developed to secrete and express heterologous fusion proteins. Constitutive promoter and an inducible system are the forces that drive the expression of the heterologous proteins in the vector while signal sequences drive the secretion known to be functional in several LAB (Agren *et al.*, 1999).

2.14 ANTIBIOTIC RESISTANCE OF LACTIC ACID BACTERIA

In the health care industry, antibiotic is the major medicine used to fight bacterial infections but since bacteria are highly adaptable organisms, they are able to develop resistance to antibiotics (Mathur and Singh, 2005). According to Hughes and Datta (1983), antibiotic resistance rarely occurs in bacteria until the introduction of antibiotic for the treatment of diseases. There is a development of resistance to each new antimicrobial compound soon after its introduction (Levy, 1997) and the problem is magnified by the misuse and overuse of the antibiotic and the likelihood of bacteria to transmit resistance determinants horizontally (Levy, 1992). Transfer of resistance genes to pathogenic bacteria is the main threat related to these bacteria. The main route of transmission of antibiotic resistant bacteria between human and animal population is through the food chain (Marshall and Levy, 2011).

In humans, un-treated fermented meat and dairy products before consumption are the main transport means of resistant bacteria with direct association between the animal native microflora and the human GIT. Although most LAB food are considered Generally Regarded As Safe (GRAS), health risk due to the transmission of antibiotic resistance genes to the GIT resident microflora bacteria has not been fully studied (Mathur and Singh, 2005). The two main factors responsible for the resistance are selective pressure from antibiotic use and the presence of resistance gene either at species or genus level (Levy, 1992). The resistance developed by the bacteria is either intrinsic (natural or inherent) or acquired.

An opportunistic pathogenic bacterium known to develop resistance is enterococcus, with limited reports on Lactococci and Lactobacilli. Vancomycin resistant enterococcus (VRE) has been known as a cause of infections and there is a possibility of cross-resistance when used with avoparcin as growth promoter in livestock feed (Pavia *et al.*, 2000). *Lactobacilli*, *Leuconostoc* and *Pediococci* spp. are known to have high natural resistance to vancomycin which is useful in separating them from other Gram-positive bacteria (Simpson *et al.*, 1988).

2.15 CHARACTERISATION OF LACTIC ACID BACTERIA IN RODENTS AND OTHER ANIMALS

Rodents are important mammalian groups that are able to inhabit any environment successfully in the world. They form more than 42% of the world's known mammalian population (Macdonald, 2001). Rodents are known mostly as pests although they play an important role in the field of research. Mice and rats have been used mostly as models in research works. One of these research fields includes microbiology where lactic acid bacteria have been isolated from their digestive system. For instance, Takahashi *et al.* (1993) showed that a mouse fed with *L. acidophilus* or *B. longum* induced serum antibody but not tolerance in them.

Lactobacillus has been isolated from livestock as a dominant species. Wang *et al.* (2014) isolated *Lactobacillus* as a dominant species from the gastrointestinal tract of a chicken. *L. fermentum* and *L. salivarius* were also isolated as dominant LAB species from the gastrointestinal tract of chicken (Kobierecka *et al.*, 2017, Wang *et al.*, 2014). *L. salivarius* has also been isolated as dominant species from chicken (Wang *et al.*, 2014) and horse (Rafat *at al.*, 2005).

Mahrous *et al.*, (2011) showed that out of four *Lactobacillus* species groups, three increased weight in mice whiles one group decreased weight in mice. The weight gain is an indication of the protective effect of the strains used (*L. acidophilus*). *L. plantarum* is able to tolerate bile salt, grow at low pH, exhibit antimicrobial activity and antibiotic resistance (Xanthopoulos *et al.*, 2000) and develop on MRS agar anaerobically for 48 hrs at 37 °C (Galanis *et al.*, 2015). *L. plantarum* has been widely studied for its numerous application and wide adaptation capacity due to its response to high concentration of lactic acid (Pieterse *et al.*, 2005), low pH and ethanol (De Angelis *et al.*, 2004), bile (Bron *et al.*, 2006) and heat shock (De Angelis *et al.*, 2004).

Enterococcus faecium group has species such as *E. faecium, E. duran, E. porcinus, E. villorum E. hirae* and *E. mundtii* (Klien, 2003). They are able to grow at temperatures ranging from 5 to 50°C. Under aerobic conditions on brain heart infusion (BHI) agar, at a temperature range of 6.5°C to 47.8 °C (Van den Berghe *et al.*, 2006). They are also able to survive heating at 60°C for 30 min. *Enterococcus spp* can be distinguished from streptococcus by their ability to grow at 10°C in 6.5% (w/v) sodium chloride (Doming *et al.*, 2003). They are able to grow at pH range of 4.6-9.9, with 7.5 as optimum (Van den Berghe *et al.*, 2006).

A study by Adeniyi *et al.* (2015) using cow faeces showed that *E. hirea* was isolated in high numbers but *E. faecium* in small numbers. Devriese *et al.* 1987 also isolated different *Enterococcus spp.* in large numbers from different livestock species. *Enterococcus* species, especially *E. faecalis* and *E. faecium*, have been studied and known to produce a variety of bacteriocin agents against many pathogenic bacteria (Ogier and Serror, 2008). *W. confusa and W. cibaria* have been isolated from human as opportunistic bacteria (Bjorkroth *et al.*, 2002; Fusco *et al.*, 2015), *W. cibaria* possess anti-inflammatory, antifungal, anti-cancer, antibacterial and immune boosting ability (Kwak *et al*. 2014).

In a study by Garcia *et al.* (2016), isolated *L. fermentum* tolerated pH of 3 and grew in the presence of 2% bile salt. It also produced hydrogen peroxide and lactic acid and was able to inhibit IL-8 production induced by *H. pylori*. *L. fermentum* was also found to maximise digestibility of crude protein in piglets (Yu *et al.*, 2008), improve intestinal immunity in neonatal pigs (Liu *et al.*, 2014), as well as improve feed conversion and weight gain, reduce the incidence of diarrhoea and improve meat quality in grower-finisher pigs (Suo *et al.*, 2012). *L. fermentum* has been found to dominate during the intermediate and final stages of the fermentation (Kogno *et al.*, 2017).

L. ingluviei has only been isolated in pigeons (Baele *et al*., 2003) and ostriches (Khan *et al.*, 2007). They have been recently shown to be responsible for weight gain in ducks and chicks (Angelakis and Raoult, 2010) and mice (Angelakis, 2012).

W. confusa and *W. cibaria* have the potential to produce ammonia (NH3) from arginine but differ in sugar acidification. Both also tested negative for xylose and galactose fermentation but positive for arabinose (Bjorkroth *et al*., 2002). *Weissella* species are also commonly found in habitats associated with the human or animal body (Nistal *et al*., 2012). *Weissella spp* were observed to occur at higher levels in mid-gut of lab-reared populations of the European corn borer than in the field population (Belda *et al*., 2011).

Pediococcus pentosaceus has been shown to reduce fatty liver and obesity (Zhao *et al.*, 2012) and inflammation (Bengmark, 2009) in animals. It has also been employed as food preservative due to its ability to produce antimicrobial agents (Martino *et al.*, 2013). *Staphylococcus* spp. have been isolated from poultry litter in small numbers (Vadari *et al.*, 2005). *Staphylococcus hominis* has been isolated as bacteriocin-producing agent against *Staphylococcus aureus* (Sung *et al.*, 2010).

Genetic and molecular information on most of the above mentioned lactic acid bacteria are made available by molecular and biotechnological techniques such as DNA extraction, PCR amplification and sequencing.

2.16 SOME MOLECULAR TECHNIQUES USED IN DNA ANALYSIS

DNA extraction, polymerase chain reaction and sequencing are molecular biology techniques widely used in research, diagnostics and biotechnology industry. Professionals of health and biological sciences and biotechnology are challenged with techniques such as sample collection and extraction of DNA, evaluation by spectrophotometry and agarose gel electrophoresis, polymerase chain reaction (PCR) amplification of the DNA as well as sequencing (Lázaro-Silva *et al.*, 2015). Some important applications of some of these techniques are stated.

DNA is the genetic material of organisms hence DNA variations will reflect the genetic difference between individuals. The procedure starts with sample collection and DNA extraction. DNA extraction is essential to biotechnology (Alberts *et al.*, 2012). DNA extraction is the starting point for various applications, ranging from research to therapeutic and routine diagnostic decision making. Extraction and purification are important for determining the unique characteristics of DNA including its size, function and shape. It is also important for studying the genetic causes of diseases and the development of drugs and diagnostics. Another essential use is for sequencing genomes, carrying out forensic science, determining paternity and detecting bacteria and viruses (www.whatisbiotechnology.org).

Polymerase chain reaction is now a common and often crucial technique used in research and clinical laboratories for a wide variety of applications. Some of the important applications of PCR are: rapid amplification of minute fragments of DNA which has made it possible for techniques such as northern and southern blot hybridization to work, even when the amount of DNA material available is small (Mohini and Deshpande, 2011). PCR has made it possible to analyse cells and tissues in different stages to check for the expression of a specific gene. PCR helps techniques like DNA sequencing by using segments of DNA from areas of interest that can easily be amplified to study genetic mutation. Other applications of DNA amplification include: gene cloning, diagnosis and monitoring of hereditary diseases, construction of DNA-based phylogeneis, amplification of ancient DNA and gene mutagenesis (Maheaswari *et al.*, 2016).

DNA sequencing is a process that determines the precise order of nucleotides within a DNA molecule. DNA sequencing is the most efficient way to classify RNA or proteins. This technique may be used to determine larger genetic regions and sequence of individual genes (Alberts *et al.*, 2012). It allows for the identification of changes in genes associated diseases and phenotypes. DNA sequencing also helps to study how different organisms are related and how they evolve and also determine which types of microbes that are available in a microbiome (Mandal *et al.*, 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

A total of 26 fresh fecal samples were collected from domesticated and wild grasscutters. The domesticated grasscutters (n = 9) were sampled from the grasscutter facility at the Department of Animal Science, University of Ghana (n = 4) and Legon Staff village (n = 5). The wild grasscutters (n = 17) were sampled from Mankessim and Gomoa Abontin in the Central Region of Ghana. Samples were collected using sterile tweezers into sterile tubes containing De Man, Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company, Sparks, USA) and transported as soon as possible to the laboratory. The collected samples were cultured overnight in an incubator at 37˚C. The fecal samples were then carefully removed from the MRS broth to get a broth with the microbes. The resultant MRS broth in tubes was kept in a plastic bag together with an AnaeroPack-Anaero gas generator (Mitsubishi Gas Chemical CO., INC., Japan) and stored at 4˚C.

3.2 ISOLATION OF LAB

Samples were homogenized and cultured on MRS agar using the streak method. MRS agar plates were then incubated anaerobically using the AnaeroPack-Anaero gas generator at 37˚C for 24 hrs. Dominant and discrete colonies were selected (based on morphological characteristics such as color, size of colony, roughness or smoothness and transparency) and transferred onto a new MRS agar and incubated at 37˚C for 24 hrs. Sub-culturing was further carried out to obtain homogeneous colonies. The homogeneous colonies were then collected into a sterilized skimmed milk and stored at -80˚C until use.

3.3 DNA EXTRACTION

For each of the homogenised colony, a sterilised loop was used to collect the colony into 200 µl of a 0.5% triton-X extraction buffer. The resultant solution was vortexed and kept at -20˚C overnight. Samples were removed, thawed and placed in a bead beater for about 20 sec at a speed of about 5000 rpm. The samples were then centrifuged at 13,000 rpm for 5 mins at a temperature of 4˚C. 30 µl of each resulting supernatant was pipetted into a 96 well plate and stored at -20˚C until use.

3.4 PCR AMPLIFICATION

Amplification of V3 region of 16S rRNA gene from LAB was carried out by using primers 27F (5'- AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5'- GGT TAC CTT GTT ACG ACT 3') (Lane, 1991). The PCR amplification was performed in a 50 µL reaction mixture, containing 0.3 µL of each primer, 2.4 µL of dNTP, 7.5 µL 10×buffer, 3.35 µL distilled water, 0.15 µL Taq DNA polymerase and 1 µL template DNA. PCR amplification was performed on Takara thermal cycler (Takara bio-medical Tokyo, Japan) with denaturing at 94 °C for

10 sec followed by 35 cycles with denaturing at 94 °C for 10 sec, extension at 55 °C for 30 sec, elongation at 72 °C for 30 sec.

3.5 PCR PRODUCT PURIFICATION AND SEQUENCE REACTION

The PCR product was topped up with distilled water to 100 µL and mixed thoroughly with 500 µL of binding buffer and transferred into spin columns. The PCR product was then centrifuged at 14,000 rpm for 1 min. 500 µL of washing buffer was added and centrifuged at 14,000 rpm for 1 min. 200 µL of washing buffer was added again and centrifuged at 14,000 rpm for three mins. Spin columns containing the PCR products were transferred into new 1.5 ml tubes and the flowthrough discarded. 50 µL of distilled water was added and centrifuged at 14,000 rpm for one minute to elute the purified PCR product. The spin columns were discarded and the purified PCR product stored at 4 °C.

Sequence reaction was performed in a 15 µL reaction mixture, containing 3 µL Big Dye, 3 µL of buffer, 1.5 µL of each primer and 7.5 µL of purified PCR product. Cycling was carried out in a Takara thermal cycler (Takara bio-medical Tokyo, Japan) with denaturing at 96°C for 10 sec followed by 25 cycles of denaturing at 96 °C for 10 sec, extension at 50 °C for 5 sec, and elongation at 72 °C for 1 min.

3.6 ETHANOL PRECIPITATION AND SEQUENCING

The sequence reaction product was purified by ethanol precipitation in a 100 µL reaction mixture containing 3 µL of NaOAc (3M), 24.5 µL of distilled water, 62.5 µL of EtOH (99.5%) and 10 µL of sample. The mixture was vortexed and kept at room temperature for 15 mins and later centrifuged at 3,100 x g for 20 mins to allow the DNA to precipitate and the supernatant discarded. 200 µL of 70% ethanol was then added to the plate and mixed by inverting the plate slowly five times to wash the DNA. The plate was again centrifuged at 3,100 x g for 5 mins, following which the supernatant was carefully discarded. The plate, now containing the clean precipitated DNA, was wrapped with tissue and cellophane without the covers and centrifuged upside down at 800 x g to dry the DNA. Subsequently, 4 µL HIDI formamide was added to each sample to re-suspend the DNA. Next, 10 µL HIDI formamide was pipetted into sequencing plate and 1 µL of each dissolved DNA sample was added. The plate was then incubated on a heat block at 95°C for 2 mins and quickly transferred onto cold block for 5 mins. The samples were finally electrophoresed on ABI prism 3100 DNA sequencer (Applied Biosystem Division, Foster City, CA, USA).

3.7 SEQUENCE ANALYSIS

The 16S rRNA sequences were edited to obtain to the required length of about 500-600 bp using Finch TV (www.geospiza.com) and Mega 7 (Kumar *et al.*, 2015) software. The Basic Local Alignment Search Tool

(BLAST) was used to align sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI) for species assignment. Strains selected showed ≥99% similarity with 16s rRNA genes in the NCBI GenBank.

CHAPTER FOUR

4.0 RESULTS

4.1 MOLECULAR IDENTIFICATION OF LAB ISOLATES

Fifty-seven isolates were cultured from 26 grasscutter samples and were identified to belong to the following five genera: *Lactobacillus*, *Weissella*, *Pediococcus*, *Enterococcus* and *Staphylococcus* (Table 1). The domesticated grasscutters had 18 isolates of LAB which belonged to the five genera above while the wild grasscutter had 39 isolates of LAB which belonged to just two of the five genera namely *Lactobacillus and Pediococcus*. The aligned sequences were identified to the species level (Table 1). All the five genera with their species isolated included, *Lactobacillus fermentum* (n = 11), *L. salivarius* (n = 11), *L. ingluviei* (n = 9), *L. plantarum* (n = 7), *L. reuteri* (n = 2), *L. formosensis* (n = 1), *L. taiwanensis* (n = 1), *L. rhamnosus* (n = 1), *Pediococcus pentosaceus* (n = 5), *Enterococcus gallinarum* (n = 2), *E. hirae* (n = 2), *E. faecium* (n = 2), *Staphylococcus homini* (n = 2), *Weissella cibaria* (n = 1) and *W. paramesenteroides* (n = 1).

Table 1: Basic Local Alignment Search Tool Result of Lactic Acid Bacteria Isolated from the Gut of Domesticated and Wild Grasscutters

Source of Animal	Sample ID	Colony ID	Sequence length (bp)	BLAST % identity	E-value	Species
Wild	28 TS	1	675	99	0	*Lactobacillus ingluviei*
Domesticated	13 TS	3	698	100	0	*Weissella cibaria*
		5	796	99	0	*Lactobacillus salivarius*
Wild	30 TS	7	680	99	0	*Lactobacillus ingluviei*
Wild	31 TS	8	641	100	0	*Lactobacillus fermentum*
		9	776	100	0	*Lactobacillus salivarius*
		10	588	99	0	*Lactobacillus ingluviei*
Wild	34 TS	12	565	100	0	*Lactobacillus fermentum*
		13	699	100	0	*Lactobacillus fermentum*
Wild	29 TS	15	686	100	0	*Lactobacillus plantarum*
		16	606	100	0	*Pediococcus pentosaceus*
		17	809	99	0	*Lactobacillus plantarum*
Wild	32 TS	18	579	100	0	*Lactobacillus ingluviei*
		19	576	100	0	*Lactobacillus ingluviei*

Table 1 (Continued)

Source of Animal	Sample ID	Colony ID	Sequence length (bp)	BLAST % identity	E-value	Species
Wild	33 TS	20	580	100	0	*Lactobacillus fermentum*
		21	671	100	0	*Lactobacillus fermentum*
		22	576	100	0	*Lactobacillus fermentum*
Wild	43 TS	23	696	99	0	*Lactobacillus fermentum*
		24	704	99	0	*Pediococcus pentosaceus*
		25	680	99	0	*Lactobacillus fermentum*
Wild	41 TS	27	695	100	0	*Lactobacillus plantarum*
		28	690	99	0	*Lactobacillus plantarum*
Wild	42 TS	29	677	99	0	*Pediococcus pentosaceus*
Wild	36 TS	30	677	99	0	*Lactobacillus ingluviei*
		32	678	99	0	*Lactobacillus ingluviei*
		33	791	100	0	*Lactobacillus salivarius*
Wild	35 TS	34	671	100	0	*Lactobacillus fermentum*
		35	695	99	0	*Lactobacillus formosensis*

Table 1 (Continued)

Source of Animal	Sample ID	Colony ID	Sequence length (bp)	BLAST % identity	E-value	Species
Wild	37 TS	37	786	99	0	*Lactobacillus salivarius*
		38	642	99	0	*Lactobacillus reuteri*
		39	778	99	0	*Lactobacillus salivarius*
Wild	38 TS	40	778	100	0	*Lactobacillus salivarius*
		41	666	100	0	*Lactobacillus salivarius*
		42	756	99	0	*Lactobacillus ingluviei*
Wild	39 TS	43	666	99	0	*Lactobacillus fermentum*
		44	753	100	0	*Pediococcus pentosaceus*
		45	752	99	0	*Lactobacillus fermentum*
Wild	44 TS	46	749	100	0	*Lactobacillus salivarius*
		47	657	99	0	*Lactobacillus salivarius*
		48	681	99	0	*Lactobacillus ingluviei*
Domesticated	02 TS	49	741	100	0	*Staphylococcus hominis*
		51	800	100	0	*Weissella paramesenteroides*
Domesticated	03 TS	52	691	100	0	*Staphylococcus hominis*
		53	733	99	0	*Enterococcus gallinarum*
Domesticated	01 TS	56	692	100	0	*Lactobacillus salivarius*
		57	777	100	0	*Lactobacillus reuteri*

Table 1 (Continued)

Source of Animal	**Sample ID**	**Colony ID**	**Sequence length (bp)**	**BLAST % identity**	**E-value**	**Species**
Domesticated	07 TS	58	668	99	0	*Lactobacillus salivarius*
		59	605	99	0	*Lactobacillus plantarum*
Wild	40 TS	60	642	99	0	*Lactobacillus taiwanensis*
Domesticated	08 TS	61	670	99	0	*Lactobacillus plantarum*
		63	592	99	0	*Lactobacillus plantarum*
Domesticated	09 TS	64	753	100	0	*Pediococcus pentosaceus*
		65	742	100	0	*Enterococcus faecium*
Domesticated	10 TS	66	711	99	0	*Enterococcus hirae*
Domesticated	04 TS	67	728	99	0	*Enterococcus faecium*
		68	566	100	0	*Enterococcus gallinarum*
		69	669	99	0	*Lactobacillus rhamnosus*

4.2 CULTIVABLE LACTIC ACID BACTERIA FROM THE GUT OF THE GRASSCUTTER

The cultivable LAB from the gut of grasscutter that were successfully isolated are presented in Table 2. The 57 LAB isolates belonged to five genera and consisted of 15 species. The genus *Lactobacillus* had eight species, the genus *Weissella* had two species, the genus *Pediococcus* had one species, the genus *Enterococcus* had three species and the genus *Staphylococcus* had one species (Table 2).

Table 2: Cultivable Lactic Acid Bacteria from the Gut of the Grasscutter

Genus	Species
Lactobacillus	*L. fermentum* *L. salivarius* *L. ingluviei* *L. plantarum* *L. formosensis* *L. reuteri* *L. taiwanensis* *L. rhamnosus*
Weissella	*W. cibaria* *W. paramesenteroides*
Pediococcus	*P. pentosaceus*
Enterococcus	*E. gallinarum* *E. hirae* *E. faecium*
Staphylococcus	*S. hominis*

Species that were found to be common and/or specific to domesticated and wild grasscutters are presented in Table 3. Four LAB species were isolated from both the wild and domesticated grasscutters. These were

L. salivarius, L. reuteri, L. plantarum and *P.pentosaceus.* Four LAB species were also found to belong specifically to the wild grasscutter. These included *L. ingluviei, L. taiwanensis, L. formosensis and L. fermentum.* However, seven LAB species were found exclusively in the domesticated grasscutter. These were *L. rhamnosus, W. cibaria, W. paramesenteroides, S. hominis, E. faecium, E. gallinarum* and *E. hirae.* There was therefore greater diversity in the domesticated grasscutter than the wild grasscutter.

Table 3: Species of Lactic Acid Bacteria Common and/or Specific to Domesticated and Wild Grasscutter

Species of LAB	Origin*	
	Domesticated	Wild
Lactobacillus salivarius	√	√
Lactobacillus reuteri	√	√
Lactobacillus plantarum	√	√
Pediococcus pentosaceus	√	√
Lactobacillus ingluviei	x	√
Lactobacillus taiwanensis	x	√
Lactobacillus formosensis	x	√
Lactobacillus fermentum	x	√
Lactobacillus rhamnosus	√	x
Weissella cibaria	√	x
Weissella paramesenteroides	√	x
Staphylococcus hominis	√	x
Enterococcus faecium	√	x
Enterococcus gallinarum	√	x
Enterococcus hirae	√	x

*: √ = isolated, x = not isolated

Table 4 shows the frequency of LAB isolated from all the grasscutters (domesticated and wild). *Lactobacillus fermentum* and *L. salivarius* had the highest number of isolates (11 each) which represented 19.2% of the total LAB isolates respectively, with *L. ingluviei* isolated as the third most dominant isolate and representing 15.7% of the total isolates. *L. plantarum* was the fourth most dominant isolate and also represented 12.3%. *L. formosensis, L. taiwanensis, L. rhamnosus, Weissella cibaria, W. paramesenteroides* and *Enterococcus hirae* had the least number of isolates (1 each) which represented 1.8% each (Table 4).

Table 4: Frequency of Lactic Acid Bacteria Species Isolated

Genus	Species of LAB	Frequency	Percentage (%)
Lactobacillus	*L. fermentum*	11	19.2
	L. salivarius	11	19.2
	L. ingluviei	9	15.7
	L. plantarum	7	12.3
	L. reuteri	2	3.5
	L. formosensis	1	1.8
	L. taiwanensis	1	1.8
	L. rhamnosus	1	1.8
Weissella	*W. cibaria*	1	1.8
	W. paramesenteroides	1	1.8
Pediococcus	*P. pentosaceus*	5	8.8
Enterococcus	*E. Faecium*	2	3.5
	E. gallinarum	2	3.5
	E. hirae	1	1.8
Staphylococcus	*S. hominis*	2	3.5

4.3 NUMBER OF LAB ISOLATED FROM WILD AND DOMESTICATED GRASSCUTTER

The domesticated grasscutter had all the five genera of LAB that were isolated (Figure 2) while the wild grasscutter had only two genera (Figure 3). For the domesticated grasscutter, the five genera represented by 18 isolates included *Lactobacillus* (n = 8), *Weissella* (n = 2), *Enterococcus* (n = 5), *Pediococcus* (n = 1) and *Staphylococcus* (n = 2) (Figure 2) but the wild grasscutter which had two genera and were represented by 39 isolates included *Lactobacillus* (n = 39) *and Pediococcus* (n = 4) (Figure 3).

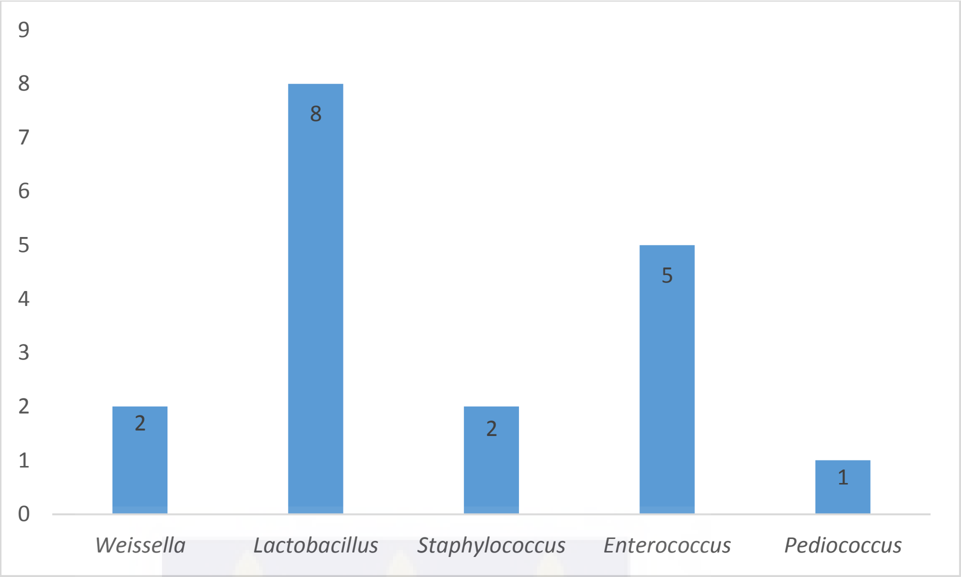

Figure 2: Number of LAB Species Isolated from Domesticated Grasscutter

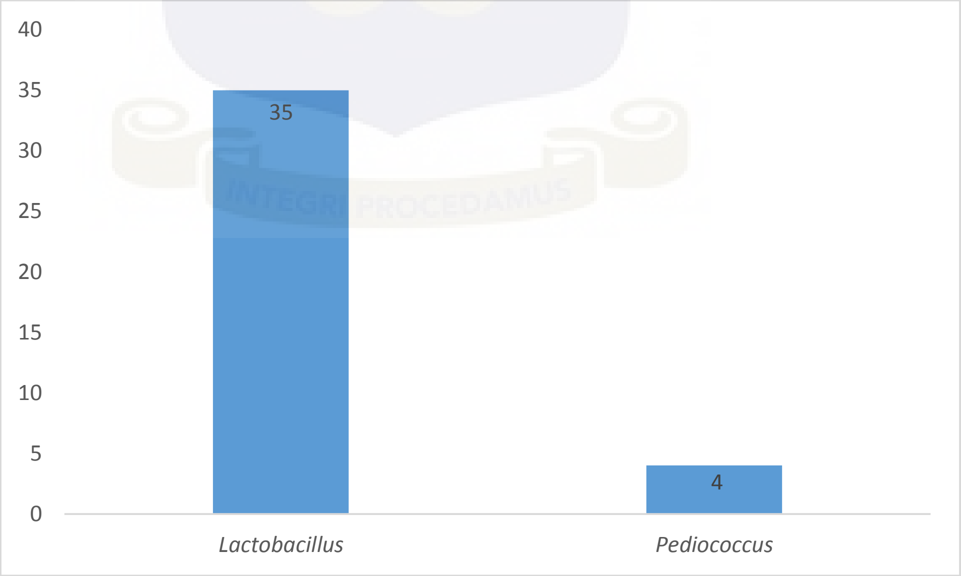

Figure 3: Number of LAB species Isolated from Wild Grasscutter

CHAPTER FIVE

5.0 DISCUSSION

In this study, all the 57 isolates (Table 1) belonged to the genera *Lactobacillus, Weissella, Pediococcus, Enterococcus and Staphylococcus*. There were 15 individual species isolated; *L. ingluviei, L. fermentum, L. salivarius, L. plantarum, L. formosensis, L. reuteri, L. taiwanensis, L. rhamnosus, W. cibaria, W. paramesenteroides, P. pentosaceus, E. gallinarum, E. hirae, E. faecium and Staphylococcus hominis* (Table 2). Within the isolated LAB genera, *Lactobacillus* had the highest proportion of bacteria in all the samples, followed by *Enterococcus* and *Pediococcus*. *Staphylococcus* and *Wiessella* had the lowest proportion (Table 4).

Lactobacillus represented 75.4% (Table 4) of the overall LAB isolates, a finding which is similar to the findings of Wang *et al*., (2014) for the isolation of LAB from the gastrointestinal tract of a chicken. The genus *Lactobacillus* (43 isolates) was represented by eight species: *L. ingluviei, L. fermentum, L. salivarius, L. plantarum, L. formosensis, L. reuteri, L. taiwanensis and L. rhamnosus*.

L. fermentum and *L. salivarius* had the highest number of isolates which represented 19.2% of the overall LAB isolates (Table 4) respectively and this result is comparable to the findings of Kobierecka *et al.* (2017) pertaining to study of LAB isolated from the chicken intestinal system. *L. fermentum* maximised digestibility of crude protein in piglets (Yu *et al*., 2008). *L. fermentum* had also increased average daily weight, improved intestinal immunity in neonatal pigs (Liu *et al.*, 2014), improved feed conversion and weight gain, reduce the incidence of diarrhoea and improved meat quality in grower-finishing pigs (Suo *et al.*, 2012). It was not surprising to find *L. fermentum* as one of the most dominant lactic acid bacteria in this study since they dominate during the intermediate and final stages of the fermentation (Kogno *et al.*, 2017).

It was not surprising *L. salivarius* was isolated as one of the dominant species (19.2%) of the total LAB isolates (Table 4) since it is fast growing and a bacteriocin producer. From this study, *L. salivarius* had been isolated as dominant species which is similar to the results from the study of LAB been isolated as dominant species from chicken (Wang *et al.*, 2014) and horse (Rafat *at al.*, 2005). *L. salivarius* had been known to develop at temperature of 37°C. *L. salivarius* has been observed to enhance the breakdown of fiber during fermentation (Yang *et al.*, 2006) and also reduce glucosinolate and crude fibre (Aljuobori *et al.*, 2014).

In the present study, *Lactobacillus ingluviei* was isolated as the third most dominant LAB representing 15.7% (Table 4) of the total LAB isolates in the wild grassscutter, indicating it as one of the main lactic acid microbiota of the wild grasscutter. *L. ingluviei* may therefore be one the main lactic acid microbiota in the wild grassscutter. *L. ingluviei* has only previously been isolated in birds, specifically in pigeons (Baele *et al.*, 2003) and ostriches (Khan *et al.*, 2007) but not mammals. Quite recently, *L. ingluviei* has been shown to be responsible for weight gain in ducks and chicks (Angelakis and Raoult, 2010) as well as in mice (Angelakis *et al.*, 2012). Therefore, *L. ingluviei* would be a lactic acid bacterial species of interest since this is the first isolation from a mammalian species.

L. plantarum represented 12.2% of the total LAB isolates (Table 4). This species has been widely studied for its numerous application and wide adaptation capacity with most research focused on its response to high concentration of lactic acid (Pieterse *et al.*, 2005), low pH and ethanol (De Angelis *et al.*, 2004), bile (Bron *et al.*, 2006) and heat shock (De Angelis *et al.*, 2004).

The least number of *Lactobacillus* species isolated were *L. reuteri, L. taiwanensis, L. rhamnosus* and *L. formosensis. L. reuteri* which represented 3.5% of the total LAB isolates while *L. taiwanensis, L. rhamnosus* and *L. formosensis* each represented 1.8% of the total LAB isolates (Table 4).

The genus *Enterococcus* had five isolates represented by three species: *E. Faecium, E. gallinarum and E. hirea*, representing 8.8% of the total LAB isolates (Table 5). This result is in contrast with 50% for *E. hirea* isolated from cow feaces by Adeniyi *et al.* (2015) and the 22% reported by Devriese *et al.* (1987) with respect to the isolation of LAB from the intestines of different farm animals. *Enterococcus* species, especially *E. faecalis* and *E. faecium*, have been studied and are known to produce a variety of bacteriocins against many pathogenic bacteria (Ogier and Serror, 2008)

The genus *Pediococcus* also had five isolates represented by *P. pentosaceus*. This which accounted for 8.8 % of the total LAB isolates in this study (Table 4). *P. pentosaceus* has been shown to reduce fatty liver and obesity (Zhao *et al.*, 2012) and inflammation (Bengmark, 2009) in animals. It has also been employed as food preservative due to its ability to produce antimicrobial agents (Martino *et al.*, 2013).

The genus *Staphylococcus* was represented by *Staphylococcus hominis*, which accounted for 3.5 % of the total LAB isolates (Table 4). *Staphylococcus hominis* is part of the normal microflora of the skin of humans and sometimes animals. Although it is one of the least isolated species, its presence in the grasscutter is perhaps an indication of the level of contact between the grasscutters and humans. *S. hominis* has been reported to produce bacteriocin, an antibacterial agent against pathogenic *S. aureus* (Sung *et al.*, 2010). Its presence in domesticated grasscutters could therefore have beneficial health implications.

The genus *Weissella* was represented by *W. cibaria* and *W. paramesenteroides*. They constituted 3.5% (Table 4) of the total LAB isolates. Belda *et al*. (2011) showed that *Weissella* occurred at higher levels in mid-gut of lab-reared populations of the European corn borer than in the field population. *Weissella* species are also commonly found in habitats associated with the human or animal body (Nistal *et* *al*., 2012) and so it was not surprising to isolate them from domestic but not wild grasscutters.

Although the number of samples collected was not even for both domesticated (n = 9) and the wild (n = 17) grasscutters, the number of LAB genera isolated from the domesticated grasscutter (n = 5) (Figure 2) dwarfed that of the wild grasscutter (n = 2) (Figure 3). The genera *Lactobacillus*, *Weissella*, *Enterococcus*, *Staphylococcus* and *Pediococcus* were isolated from the domesticated grasscutter (Figure 2) while only *Lactobacillus* and *Pediococcus* were isolated from the wild grasscutter (Figure 3). The difference in the profile can probably be attributed to the fact that domesticated grasscutters are fed with different feed with different microbial compositions and also exposed to human contact, hence the higher number of LAB genera while the wild grasscutters select their own feed mainly grass, hence the lower number of LAB genera. The stress conditions (health and diet) of the animals were unknown at the time of the sample collection and these factors usually affect the lactic acid microbiota negatively. Majority of the species isolated from both domesticated grasscutters and wild grasscutters were from the genera *Lactobacillus*.

Although the points of sample collection were also far apart, there are some LAB species that were common to both the domesticated and wild grasscutter, notably, *L. salivarius*, *L. reuteri*, *L. plantarum* and *Pediococcus pentosaceus*. Four species were specific to domesticated grasscutter while seven other species were specific to the wild grasscutter (Table 3).

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

In conclusion, sequence analysis showed that a wide range of LAB species are available for isolation and characterisation in the gut of the grasscutter. The domesticated grasscutter had more diversity of LAB compared to the wild grasscutter. The difference in diversity could be attributed to the exposure of domesticated grasscutters to different types of feed and to human contact. Isolation of *Lactobacillus ingluviei* in this study is of great significance since it has previously been isolated from pigeons and ostriches only, and so this marks the first case of isolation in mammals.

6.2 RECOMMENDATIONS

Based on the findings from this study, the following recommendations can be made:

Further characterisation studies on LAB in grasscutter should be carried out using larger sample size covering a wider geographical location.

There is the need to investigate the importance of *Lactobacillus ingluviei* in domesticated and wild grasscutters.

There is also the need to investigate the use of specific LAB as probiotics to enhance digestibility in grasscutters under farming conditions.

Furthermore, characterisation of the different LAB profiles should be studied in relation to age and sex groups in grasscutter.

Finally, the significance of the different LAB species isolated from wild and domesticated grasscutters should be studied.

CHAPTER SEVEN

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