GENETICS AND IMMUNITY OF INDIGENOUS CHICKEN IN KENYA

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A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT FOR THE REQUIREMENT OF THE AWARD OF THE DOCTOR OF PHILOSOPHY DEGREE IN ANIMAL SCIENCE OF EGERTON UNIVERSITY

EGERTON UNIVERSITY

OCTOBER 2018

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not, wholly or in part, been presented for the award of a degree in any University.

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KD111/13629/14

RECOMMENDATION

This thesis is the candidate's original work and has been prepared with our guidance and assistance; it has been submitted with our approval as the official university supervisors.

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DEDICATION

This work is dedicated to all stakeholders in indigenous chicken value chain, my family and supervisors for their unmatched support.

ACKNOWLEDGEMENT

I am very grateful to the Almighty God for the good health, mental stability, physical strength and gift of life during the whole PhD programme. The development of this thesis could not be possible without the support of a number of personalities and institutions. I wish to express my sincere appreciation to Egerton University for admission, registration and study leave. I am grateful to my supervisors Prof. Alexander Kahi, Dr. C. B. Wasike and Dr. R. S. Mwakubambanya. Special thanks to Prof. A. K. Kahi for having accepted me as his student, and tirelessly offering technical advise, guidance, professional supervision and inspiration. I have known and admired you, Prof. A. K. Kahi, from my days as an undergraduate student at Egerton University. Thanks to Dr. C. B. Wasike and Dr. R. S. Mwakubambanya for your devolution, technical and professional guidance to this work. Chris and Ramadhan (young in age but big in ideas) you were such a pillar to success of this work. Chris, I will always remember your sayings and I quote " this is our work make it unique and special". Ramadhan your unwavering visits to the laboratory and encouragement not only added value but built trust and authenticity to my work.

I am further thankful to Kenyan Agricultural and Livestock Research Organization (KALRO), Naivasha for support on chicken sample collection. This study was implemented as an activity of the InCIP and iLINOVA projects funded by the African Union and the European Union, respectively. I am indebted to the Centre of Excellence for Livestock Innovation and Business (CoELIB) at Egerton University for bench space, internet and stationery facilitations. Special thanks goes to Biosciences Eastern and Central Africa – International Livestock Research Institute Hub (BecA-ILRI Hub) for hosting molecular and metagenomics laboratory work. All these were supported by the BecA-ILRI Hub through the Africa Biosciences Challenge Fund (ABCF). I am grateful to Egerton University for the paid study leave and their contribution to my capacity development.

I am grateful to Dr. Roger Pelle, Dr. Wellington Ekaya, Ms Valerian Aloo, Ms Eunice Machuka, Ms Tina Kyalo, Ms Joyce Njuguna and Mr Dedan Githae for the support while at BecA-ILRI laboratory. Dr Pelle, you were such insightful in my write up and constructive criticism in the last two research chapters. Dr Ekaya and Ms Val, you made sure my stay was comfortable. Ms Machuka and Ms Kyalo, your molecular work experience was unmatched.

Joyce and Dedan, your inputs in bioinformatics were sincerely appreciated. To you all, your willingness to be of assistance even when you had too much work to do was exemplary.

I am grateful to my wife Faith and daughters Patrica and Eunice for always being so patient with me. It was difficult for you (and me too) that I was away from home for that long. I thank you for standing with me during those lonely moments and your encouragements. I am grateful to my brothers, sisters, nephews and nieces who have always stood with me this many years. Your prayers are always answered.

ABSTRACT

Indigenous chicken (IC) are the most common poultry in developing world, found in almost every homestead and constitute over 89% of total chicken populations in Kenya. Contributions of IC to economy include source of income, food security and nutrition. The IC productivity is compromised by diseases that contribute to over 50% of economic losses. This study was undertaken to contribute to improved productivity of indigenous chicken (IC) of Kenya through sustainable breeding for disease tolerance and enhanced immunity by searching for appropriate probiotics. The specific objectives of the study were: 1) to determine the sources of variation of Natural antibodies (Nabs) (IgG, IgM and IgA) binding keyhole limpet hemocyanin (KLH) amongst the IC, 2) to estimate the repeatability and variation of the ELISA assay (Nabs) with time within the IC, 3) to assess the diversity and population structure of IC using LEI0258 Marker, 4) to determine the effects of probiotic on IgM titre levels on IC 5) to determine composition and diversity of microbial populations in the chickens and 6) to identify potential bacterial species for use as probiotics for enhanced immunity. Blood was drawn from the wing vein of IC and plasma separated. Natural antibodies (IgM, IgA, IgG) titre values binding KLH were determined by indirect ELISA. One way ANOVA and Mixed model analyses were used to determine sources of Nab titre variation and estimate repeatability parameter. The IC genetic diversity and population structure was achieved by DNA extraction from blood and genotyping using the MHC linked LEI0258 marker and sequencing of subset of representative alleles. Polymorphism and population genetic parameters were determined using bioinformatic tools. Effect of commercial probiotics on IgM titre values was done by comparing treatment and control means using one factor ANOVA. Metagenomics employed usage of DNA from fecal samples and next generation sequencing. Qiime pipeline was used to call operational taxonomic units OTU) and for alpha and beta diversity analysis of microbial composition. The microbiome abundance between immune competency levels was compared using one factor ANOVA. The results showed presence and variation of Nabs amongst the IC. The variance estimate for chicken components were high and significant for IgM ($p=0.003$), IgG ($p=0.0001$) and IgA ($p=0.0001$).The repeatability of the ELISA assay to Nabs was high in all the immunoglobulin isotypes. Repeatability were 0.68, 0.99 and 0.99 for IgM, IgG and IgA respectively. The LEI0258 locus showed high diversity and presence of four gene pools among the IC. The locus observed high diversity as revealed by the average Shannon's information index of 2.768. The

mean overall observed heterozygosity and Polymorphic information Content (PIC) was 0.844 and 0.932 respectively for the total population sampled. The central population had the highest observed heterozygosity (0.878) while coastal had the lowest (0.792). Use of commercial probiotic did not have significant effect on IgM titre values of IC. The metagenomics revealed extensive microbial diversity. Candidate bacterial species differed significantly for immune response level. The study recommended routine management practices (e.g. vaccination) for disease control and evaluation of candidate bacterial species for enhanced immunity. Using genetic approaches to improve IC disease tolerance and the proposed bacterial candidates for probiotic will improve animal welfare, ensure friendly environment, has sustainable improvements and address consumer concerns about drug use and reduce the risk of food poisoning.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIL	Advanced Intercross Line
ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent assay
FDR	False Discovery Rate
GBLUP	Genomic Best Linear Unbiased Prediction
GWAS	Genome-wide association studies
HW	HardyWeinberg
IgA	Immunoglubulins A
IgG	Immunoglubulins G
IgM	Immunoglubulins M
INCIP	Indigenous Chicken Improvement Programme
KLH	Keyhole Limpet Hemocyanin
LD	Linkage disequilibrium
MHC	Major Histocompatibilty complex
Nabs	Natural Antibodies
NCD	New Castle Disease
NCV	New Castle Virus
NPDP	National Poultry Development Program
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCoA	Principal Coordinate analysis
QTL	Quantitative Trait loci
RNA	RibonucleicAcid
SNP	Single Nucleotide Polymorphism
SSR	Short Sequence Repeats

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Indigenous Chicken (IC) are reared by over 90% of households in rural areas. Chicken rearing is therefore one of the most viable activities used by rural households to improve their livelihood and mitigate socio-economic challenges such as malnutrition, poverty, ill health, unemployment and illiteracy (Okeno *et al.,* 2012). Productivity of IC varies due to genetic diversity and variation in the production environment (Dana *et al.,* 2010). Extensive system of production predominates IC production in rural areas. This system is characterized by scavenging, absence of elaborate disease management programmes, high rates of disease incidence, prevalence of predators, uncontrolled natural mating and, natural incubation and brooding of eggs and chicks, respectively. The semi intensive production system is also practiced to a lesser extent (Okeno *et al.,* 2012). Infestation of coccidian and other helminthes are prevalent in the extensive management system besides frequent Gumboro and Newcastle diseases outbreak, which inflict the major economic loss to farmers. These infections pose serious disease and production challenges to the growth and development of the IC industry (Lamont, 1998). Current strategies of disease management include prophylactic and curative medication. However, the plethora of different pathogens and continual emergence of drug resistant strains limit the effectiveness of both methods. The vaccine efficacy is sometimes compromised by evolution of the antigenic determinants of the pathogens in addition to infrastructural challenges. Use of drugs by farmers has become increasingly restricted due to risk of antibiotic resistance, allergies caused by carry-overs effects and policy regulations (Pinard *et al.,* 1998). These call for breeding of IC with enhanced immunity that can resist infection to help farmers save money and eradicate the consumers' food safety and insecurity concerns. Understanding of the immune response in IC is important in this regard.

Immune response is divided into innate and adaptive forms. Innate immunity is the first line of defense and plays an important role in preventing or combating infection (Parmentier *et al.,* 2004). Amongst the components of innate immunity are natural antibodies (Nab) which play an important role in innate immunity (Thompson-Crispi *et al.,* 2013). Natural antibodies have

been reported in non-immunized cattle (Thompson-Crispi *et al.,* 2013), humans (Madi *et al.,* 2012), fish (Kachamakova *et al.,* 2006) and poultry (Sun *et al.,* 2011). Natural antibodies are known to be present in living organism without prior exposure to antigen. To test natural antibodies, keyhole limphet hemocyanin is always used as the model antigen. This antigen is derived from a sea organism *Megathura cranulata* that none of the terrestrial animals had encountered before (Parmentier *et al.,* 2004). Natural antibodies are mostly poly-reactive and poly-specific, with low binding affinity (Cheng *et al.,* 2008) and are generally encoded by V genes in germline configuration (Lutz *et al.,* 2009). Evidence from various studies showed that Nabs are genetically controlled (Sun *et al.,* 2011). The Nab provides a barrier to infection by providing a pre-existing antibody reactivity. This allows the animals to recognize the invading pathogen prior to adaptive immunity (Parmentier *et al.,* 2004) and subsequently act as an adjuvant of specific immunity (Star *et al.,* 2007). The repertoire and levels of Nabs depend on factors such as the environment, genetic background and age (Khobondo *et al.,* 2012). Genetic background and its association with Nabs can be defined by use of molecular tools (Siwek *et al.,* 2010)

Molecular tools like genetic markers especially microsatellite markers have been used to determine the genetic background of indigenous chicken (Ngeno *et al.,* 2015). Microsatellite markers have been identified in chromosomal regions affecting immune traits in chickens (Siwek *et al.,* 2010). For instance, LEI0258 Marker located within the B region of MHC is associated with antibody responses to vaccination against Newcastle and Marek's diseases, resistance to worms and coccidiosis, body weight, egg production, survival, embryonic mortality, fertilization rate and hatchability (Fulton *et al.,* 2016). The diverse nature of this marker makes it highly polymorphic (Ngeno *et al.,* 2015) which could be associated with some helpful microbiome within gastrointestinal (GI) tract for enhanced immunity.

In immunity, gut microbiome as a whole helps the development of GI tract and educate birds' immune system. The GI tract of chicken is densely populated with microorganisms which interact with the host and ingested feed intensively and closely. The gut microbiota is a complex ecosystem that has a symbiotic relationship with its host. Their interactions affect the physiological, immunological and nutritional status of the host (Zhang *et al.,* 2010). The gut microbiome benefits the host by providing nutrients from otherwise poorly utilized dietary substrates and modulate the development and function of the digestive and immune system. As a

result, the risk of enteric diseases is significantly reduced thus assuring the functionality of the digestive system is not adversely affected (Crhanova *et al.,* 2011). There exist diversity in microbiome profile in animals GI tract which is affected by several factors among them host genetics (Zhao *et al.,* 2013). Methodologies employed in studying microbiome have evolved from early cultivation (Barnes *et al.,* 1972) to recent molecular technologies involving high throughput next generation sequencing (HT-NGS) and omics studies (Simon and Daniel, 2011). The HT-NGS targeting 16S rRNA genes have been extensively used to study microbial diversity in human and animal species (Caporaso et al., 2012). Healthy microbial populations in the GI tract are associated with enhanced animal performance, reflecting more efficient digestion (Cox and Dalloul, 2014). These groups of microbial populations could be fed to animals as probiotics to stimulate the immune system, making birds less vulnerable to diseases (Nicholson *et al.,* 2012) hence the need for this study.

1.2 Statement of the problem

Diseases cause losses of up to 50% of potential profit to farmers (Lamont, 2010). These losses accrue because of mortality, product condemnation, treatment costs, reduced production efficiency, and reduced consumer acceptance (Lamont, 2010). The free-range system of IC production predisposes the birds to high incidence of diseases and parasite load. On the other hand, intensified production system increases vulnerability of chickens to contagious diseases. Currently, vaccination and anti-microbial drugs are the main intervention strategies for these diseases used by a few semi intensive IC producing farmers in Kenya. However, the effectiveness of the vaccination is limited due to different pathogens and continual shift of pathogen epitopes. In addition, anti-microbial drug residues found in the chicken products posses a risk of antibiotic resistance and allergies to consumers as well as formulation of stringent international trade policy regulations. In Kenya, disease outbreaks and high parasite load are a common phenomenon. Despite this, there is limited research on the topic and no attempt on genetic improvement of IC targeting traits that influence health of the birds. In this study, genetic approaches were proposed and evaluated as potential strategies to circumvent the disease challenges encountered in IC farming. Moreover, advances of high throughput sequencing and microbiota metagenomics technology has impacted little on IC research, despite the potential to remedy the disease challenges. The gut microbiota is a complex ecosystem that affects physiological, immunological and nutritional status of the host. The microbiota is believed to

have a symbiotic relationship with its host. However, there is little information on the diversity and functions of indigenous chicken Gastro Intestinal tract microbiota, its impact on the host, and the interactions between them. It is unknown if the genetics of the IC kept under same management or controlled environment influences the composition of microbiota population/structure. The role of host genetics in shaping the microbiota is thus unclear and if novel probiotics exist in IC.

1.3. Objectives

1.3.1 Broad objective

The aim of the study was to contribute to improved productivity of IC through sustainable breeding for disease tolerance and enhanced immunity by using appropriate probiotics.

1.3.2 Specific objectives

The specific objectives were:

i. To determine the sources of variation of Nabs (IgG, IgM and IgA) binding keyhole limpet hemocyanin (KLH) amongst the indigenous chicken in Kenya.
ii. To estimate the repeatability and variation of the ELISA assay (Nabs) with time within the indigenous chicken of Kenya
iii. To estimate indigenous chicken diversity and population structure in Kenya using MHC linked LEI0258 Marker
iv. To determine the effects of probiotic on immunoglobulins M (IgM) titre levels on indigenous chicken of Kenya
v. To determine composition and diversity of microbial populations in the chickens of Kenya.
vi. To identify potential bacterial species for use as probiotics for enhanced immunity.

1.4 Research questions

i. What are the sources of variation of Nabs (IgG, IgM and IgA) binding keyhole limpet hemocyanin (KLH) amongst the indigenous chicken in Kenya?
ii. What are the estimates of repeatability and variation of the ELISA assay (Nabs) with time within the indigenous chicken?
iii. What is the population structure and diversity like of Kenyan IC using MHC linked LEI0258 marker?

iv. What is the effect of using probiotic on immunoglobulins M (IgM) on indigenous chicken of Kenya?

v. What is the extend of microbial composition and diversity in Kenyan IC?

vi. What are the potential bacterial species that can be used as probiotics for enhanced immunity in Kenyan IC?

1.5 Justification

Individual chicken have varied production potential and disease tolerance levels. The variability can be explored for breeding, management and conservation purposes. Genetic improvement is deemed sustainable because of additive effects of genes responsible for quantitative traits particularly disease tolerance. The determination of genetic variation, polymorphism and population structure is the foundation of defining breeding program for genetic improvement. In this study, diversity and gene pools were determined to infer genetic basis of the IC variation thus giving insights to improvements or better utilization. The choice of the MHC linked marker in determining polymorphism is essential in understanding the genetic basis of disease tolerance. The possibility of genetically improving chicken disease tolerance constitutes an attractive alternative for both the industry and consumers. The positive attributes for such endeavors include improved food security nationally, economic empowerment of women and the village poor as envisaged in Kenya's Vision 2030 policy. Genetic selection for enhanced immunity has been suggested as a safe and logical tool to reduce infectious diseases' problems in animals. This is poised to reduce or alleviate consumers' concerns over drug residues and improve the acceptability of IC products both locally and internationally. Alternative approaches of use of microbiome to enhance immunity is warranted.Thus the study aimed to comprehensively characterize the structure and diversity of the bacterial populations in fecal matter, explore the composition and their functions in relation to immune competency, thus, providing basic information for developing applicable probiotics.

1.6 Thesis outline

This thesis is organized in eight chapters. Chapter 1 introduces the overall background, rationale and objectives of the study. Chapter 2 highlights the relevance of poultry production in general and chicken domestication in Kenya. It provides an overview of the attempts made to improve IC productivity using genetics as a discipline. Genetic characterization of IC in Kenya is highlighted as well. The Chapter identifies the research gap and production challenges which

need urgent mitigations to realize disease tolerance for improved IC productivity. In Chapter 3 the study determined the presence of natural antibodies isotypes (IgA, IgM and IgG) against novel keyhole limpet hymocyanin (KLH) in indigenous chicken. The study further determined both genetic and non-genetic factors conferring observed variation in immunoglubulin isotypes. Factors influencing the variation are hence explained in their context. Chapter 4 reports the findings on random variability and repeatability of natural antibodies isotypes against KLH in IC populations using indirect ELISA. The Chapter further highlights the existence of variation of Nabs titre values of individual chicken of the same age and sex thus implicating genetic as a determinant. The genetic diversity of IC was assessed using highly polymorphic marker LEI0258 in Chapter 5. The locus attributes were defined, genetic relatedness among the three major genetic populations and gene pools circulating within Kenya determined. A new gene pool was observed to be present in the population.

In Chapter 6 the difference in IgM titre values between chicken fed probiotics at different levels and a control group are compared. The results suggest no significance difference between IC fed probiotics and the control group, however, the control group (no probiotics) had comperatively higher least square means of IgM instead. In the last study (Chapter 7), the microbiome diversity in fecal samples of chicken was determined. Illumina MiSeq platform was used to sequence the V4 hypervariable region of bacterial 16S rRNA gene and operational taxonomic units called using Qiime pipeline. The overall microbial diversity and composition are reported for the first time in Kenyan chicken. The Chapter also highlights potential bacterial candidates for use as probiotics for enhanced immunity. Lastly Chapter 8 gives the general discussion, conclusions and recommendations in relation to chicken immunity and metagenomics

CHAPTER TWO

LITERATURE REVIEW

THE PAST, PRESENT AND FUTURE GENETIC IMPROVEMENT OF INDIGENOUS CHICKEN OF KENYA

2.1 Abstract

Genetic improvement of farm animals encompasses both mating and selection for desired traits and indigenous chicken genetic resources are no exception. In Kenya, previous attempts to genetically improve indigenous chicken involved cross-breeding scheme utilizing cockerels and pullets of exotic breeds with local indigenous chicken. This scheme was complimented with farmer training on good management practices and vaccination for disease control. The scheme was partially successful with improved performance in the crossbreds that declined with subsequent generations. Failure of the programme to meet stakeholder's expectation led to its' termination. The current attempt through the Smallholder Indigenous Chicken Improvement programme has initiated a holistic and comprehensive approach to analyzing the entire indigenous chicken actors and avert the causes of previous failures. The programme has genetically and phenotypically characterized the chicken; established reference/base population collected from different ecotypes/counties, established the breeding goals and designed breeding programs that best suit the Kenyan stakeholders. The on-station research has reported variation on production traits, determined heritability estimate on growth. Current and ongoing research is focused on molecular characterization, selection for improved immune response, carcass quality, eggs production, growth and adaptation traits. The research is also concerned with conservation of these genetic resources.

2.2 Introduction

2.2.1 Domestication of chicken

Chicken are believed to be the most popular poultry species worldwide and are believed to have descended from the four species of the jungle fowl: the red jungle fowl (*Gallus gallus*), the grey jungle fowl (*Gallus sonnerati*), the Ceylon jungle fowl (*Gallus lafayettei*) and the green jungle fowl (*Gallus varius*) (Dana *et al.*, 2011). It is still an open question whether the modern chicken were domesticated from one or all of these species. Cumulative data from the geographic range of the species (Crawford, 1990), archaeological discoveries (West and Zhou, 1988), protein polymorphisms and morphological characteristics (Moiseyeva *et al.*, 2003), suggested that domestic chickens were derived from the red jungle fowl. The studies that analysed four species of genus *Gallus* (*G. gallus*, *G. varius*, *G. lafayettei* and *G. sonnerati*), three subspecies of *G. gallus* (*G. g. gallus*, *G. g. spadiceus* and *G. g. bankiva*), nine domestic breeds of chicken from South Asia, South East Asia, Japan and Europe, (Fumihito *et al*., 1994; Fumihito *et al*., 1996) on their 400 base pairs of mt DNA D-loop region, presented results which suggested that domestic chickens are derived from a single continental population of *G. g. gallus*. However, other subspecies of the red jungle fowl are believed to be modern ancestor of domestic chicken (Liu *et al*., 2006).

Genetic introgression of species in the genus *Gallus* has been demonstrated as well. This suggests that several species might have contributed to origin of modern chicken (Nishibori *et al.*, 2005). Eriksson *et al.*, (2008) examined the origins of skin colour variations in domestic chickens, revealed that although the white skin allele in modern chickens is derived from the red jungle fowl, the most likely origin of the yellow skin gene is the grey jungle fowl (*G. sonnerati*). Contrary to Crawford, (1990), (Liu *et al*., 2006) using mitochondrial DNA found evidence suggesting that origin of indigenous chicken was centered around South and South East Asia. This was in agreement with previous studies b (Fumihito *et al*., 1994; Fumihito *et al.*, 1996).

2.2.2 Introduction of chicken to Africa and Kenya

The recognized main wild ancestor of domestic chickens, the red junglefowl *Gallus gallus*, is endemic to sub-Himalayan northern India, southern China and Southeast Asia (Delacour 1977), where the putative centers of domestication of the species are present (Tixier-Boichard *et al.*, 2011). The route and dates by which chicken entered the African continent remain poorly understood. Terrestrial as well as maritime introductions likely brought chickens

to Africa (Mwacharo *et al.*, 2013). Archaeological and Historic evidence showed that the fourth to third millennia BC have been advanced as the period for the first arrival of domestic chickens in Africa (Chami, 2007). It is suggested that chickens were first introduced into Africa via Egypt from South-western Asia via the middle-east. According to Blench and Macdonald, (2000), archaeological evidence and other representations indicate occasional presence of chickens in Egypt in the period between 1425 and1123 B. During that time, the chickens were thought to have been kept for cockfighting sports. Around 650 BC and beyond there was change of chicken utility from recreational to cultural and spiritual usage; it is thought that chickens were used to tell the time of day and for sacrifices before planting or harvesting (Maina, 2000) and subsequently introduced as source of food. At around 500 BC, human migration to the South led to the spread of chickens to Central Africa (Magothe *et al.*, 2012). Increasing evidence support an early possible entry point of domestic species along the East African coast (Fuller *et al.*, 2011). The early Greco-Roman east-coast trade is hypothesized to have brought the Asiatic and game chickens along the Kenyan coast at around 100 AD (Blench and Macdonald, 2000) and eventually reaching Eastern Kenya probably at around 200 AD (Maina, 2000).The long span of period is thought to have caused the change of relative importance of chicken.

The modern world breeds of chickens can be grouped into four evolutionary lineages: (a) egg-type chickens of Mediterranean origin; (b) game chickens of Asiatic origin; (c) meat-type chickens of Asiatic origin and (d) true Bantams of various descents (Moiseyeva *et al.*, 2003). Trade with several empires may have introduced the Asiantic types into present Kenya, while migration from the north may possibly have brought more of the Mediterranean and the Bantams types. Selection, mutations and random drifts over time may have resulted in the modifications and subsequent development of the various chicken eco- and genotypes presently available in various climatic regions in Kenya. The primary reasons for domesticating animals in Africa were their cultural, ritual and social values. Their role as source of food came much later with the expansion of human population (Clutton-Brock, 1993). The conflict between archaeological findings to date on one hand and the apparently deep embedding of chicken in many African cultures, as well as the linguistic and ethnographic evidences on the other hand, suggest presence of chicken in Africa at much earlier dates (Williamson, 2000). Hence, it is possible that chicken were present in Africa well before the earliest date yet attested by archaeological findings.

2.3 Kenyan indigenous chicken genetic resources

2.3.1 Population, distribution and classification of indigenous chicken

The Kenyan chicken population is estimated to be 32 million, with approximately 80 percent being classified indigenous and 20 percent as commercial layers and broilers (KNBS, 2010). Other poultry types such as turkey, duck, pigeon, ostrich, guinea fowl and quail are becoming increasingly important. The indigenous chickens of Kenya consist of various unimproved, non-selected sub-populations of heterogeneous features such as plumage colour, comb and wattle types, earlobes and body sizes. They are evenly distributed across the country as presented in Table 2.1, with exception of Nairobi (which is small and mainly urban) and North Eastern (which is majorly an arid area with nomadic lifestyle) provinces.

Table 2.1 Population and distribution of indigenous chicken in Kenya (KNBS, 2010)

Geographical regions (provinces)	Population
Rift Valley	6,667,262
Nyanza	5,605,478
Western	4,144,351
Eastern	4,107,618
Central	3,039,786
Coast	1,599,696
North Eastern	422,899
Nairobi	279,397
Total	**25,866,487**

This distribution is attributed to rural human population, environmental conditions and availability of feed resources. Most of farmers in these regions practice crop production which is the major feed source for the indigenous chicken. Okeno *et al.*, (2012) observed that the number of indigenous chicken per household were high in crop production regions, but declined gradually from high agricultural potential regions to semi-arid and further in arid regions.

The Kenyan indigenous chicken as in most other developing countries in the tropics have not yet been classified to breeds, but commonly named according to regions of placements or ecotypes or phenotypic expression of major genes. For instance different ecotypes such as

Bondo, Bomet and Narok, Kakamega, West Pokot, Lamu and Taita-Taveta have been studied and named based on the regions where they are found in Kenya (Okeno *et al*., 2012). On the other hand the Kenyan indigenous chicken have been grouped as naked neck, frizzled-feathered, shanked feathered, normal feathered, dwarf, giant, rumples and crested head (Magothe *et al.*, 2010; Okeno *et al*., 2012) based on their phenotypic expression of major genes. The relationship between phenotypes and genotypes in chicken is well known as these phenotypes are a result of genes with major effects (Falconer, 1989). Although the exact population of both genotypes and ecotypes for the Kenyan indigenous chicken are not well documented, the study by Okeno *et al.*, (2011) could give an insight on the population structure for different genotypes and ecotypes. In that study, the populations of different indigenous chicken genotypes were sampled from six counties (Kakamega, Bondo, Bomet, Turkana, Narok and West Pokot) in different climatic conditions. In the pooled data, they demonstrated the population structure for different genotypes within the farmers' households as presented in Figure 2.1.

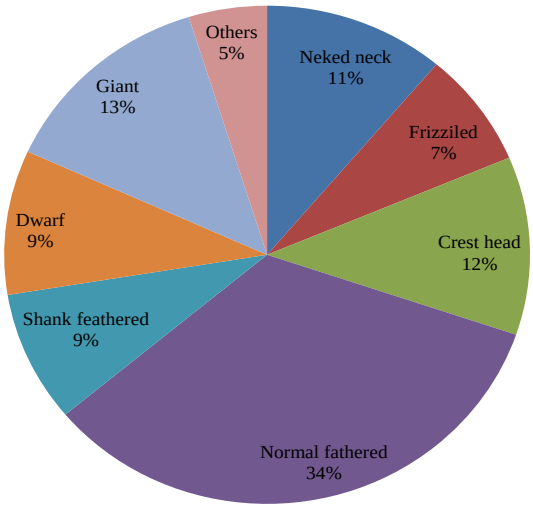

Figure 2.1 Population structure and distribution of different Kenyan indigenous chicken genotypes (Okeno *et al*., 2011).

They also demonstrated that although the population is dominated by normal-feathered birds, the other genotypes are well represented in all the counties. The genetic diversity of the Kenyan indigenous chicken could have been as a result of their dispersal from putative centres of domestication to different regions with diverse environmental conditions and people of different

cultural orientations (Mwacharo *et al.*, 2013). Other factors that may have played a role in the genetic differentiation include founder effects, natural and human selection, mutation and genetic drift (Dana, 2011). Although, both genetic, nutritional and disease resistance studies have been carried out on different genotypes (Magothe *et al.*, 2010) and ecotypes (Kaingu *et al*., 2010; Kingori *et al.*, 2010) of indigenous chicken and further studies are still ongoing under Indigenous Chicken Improvement Program (InCIP – http://incip.org/) under Kenyan conditions, their classification have not been published in the Domestic Animal Diversity Information System (DAD_IS) website (http://dad.fao.org/) except Naked-neck and frizzled-feathered.

2.3.2 Adaptation characteristics of different ecotype and genotypes of indigenous chicken

The indigenous chicken have a number of adaptive traits and genes such as naked-necks and frizzle feathers, black bones and meat, which have special utility in the hot and humid tropics (Horst, 1989). In Kenya for instance, the naked-neck, frizzle, dwarf and rump-less genotypes are mostly found in Western and Coastal regions which are characterized by warm and humid climatic conditions and Eastern and Northern parts which are hot and dry (Olwande *et al*., 2010; Okeno *et al.*, 2012). This could be explained by the fact that these genotypes are known to be tolerant to high ambient temperatures and other environmental stresses associated with such areas (Horst, 1989). In high altitude areas such as Mount Kenya and the highlands East and West of Rift Valley, which are characterized by cool and wet climatic conditions, normal-feathered, crested head, feathered shank and bearded genotypes are predominant (Njega, 2005). This could be explained by the fact that these genotypes have well-covered bodies with feathers which insulate and protect them against body heat loss. There is, however, ample evidence that some of these genotypes in most of these regions are endangered and on the verge of becoming extinct (Maina, 2000).

The expression of the genotypes in specific zones shows that there are a number of genes with major effects on the phenotype that seem to be of special interest for chicken keeping in smallholder systems (Sørensen, 2010). These adaptive genes can be split into three categories; feather reducing genes, genes that reduce body size and genes that control plumage colour (Sørensen, 2010). The feathered chickens/genotypes are predominant in cold climates, their body is well covered with feathers and this helps in insulation and protection against losing body heat. On the contrary, warm and hot climate is dominated by naked necks or frizzle feathers a feature that allows better heat dissipation from the skin. The naked-neck genotype is characterized by

featherless skin on the neck, on the breast and on ventral part of the thigh. This expression is caused by an incomplete dominant gene termed Na (Mérat, 1986). The homozygous dominant Na/Na chickens have no feathers on 30–40 percent of the body surface and the heterozygote Na/na chickens have no feather cover on 20–30 percent of the body surface. Moreover, the heterozygotes have a tuft of several dozen feathers on the front of the neck (Crawford, 1976). The frizzled genotype is caused by a single incomplete dominant gene F. In homozygotes, the shafts of all feathers are extremely recurved (i.e. fletched arrow feathers) and the barbs are curled. In the heterozygote, only the contour feathers are recurved.

These birds are not able to fly, and the feathers are easily broken off by crowding. The homozygotes, in particular, may look bare. There are modifying genes that make the extent of curling less extreme. Genotypes possessing the naked-neck and frizzle genes, either singly or in combination are associated with increased growth rates, superior body weights, better feed conversion, higher egg production and disease tolerance in tropical environments (Nishibori *et al.*, 2005; Magothe *et al*., 2010). These genotypes and Kuchi genotype (found in the coastal region of Kenya and Tanzania (Msoffe *et al.*, 2002) would be ideal for meat production in warm and humid areas. The Kuchi genotype has game chicken characteristics and would thus be better able to evade or fight off predators (Magothe *et al.*, 2012). The dwarf genotype has better feed efficiency and mass egg production. They have a lower feed intake due to reduced body size and produce more eggs and could thus be utilized for cross-breeding purposes thus employing mating as a breeding tool. Maina, (2000) characterized indigenous chicken based on morphology and feather colours, and reported wide variations in these features. They reported the crested genotype to be more distinct than others while the naked-neck and feathered shank were more genetically close to one another.

There exists many morphological clades of indigenous chicken (Okeno *et al.*, 2012) and evidenced adaptive, biological, reproductive and production traits variations (Kingori *et al.*, 2010). Despite these observed variations, molecular characterization using 30 microsatellite markers, concluded that the Kenyan indigenous chicken can be grouped into four genetic clades; Coastal, Central, Western and Northern Kenya groups (Figure 2.2) (Mwacharo *et al*., 2007). Recent studies by the same authors using 30 autosomal microsatellite markers on genomic DNA revealed two major gene pools/groups. They included Eastern (Kilifi, Taita-Taveta, Muranga, Kitui, Meru, Marsabit) and Western (Kisii,Nandi, HomaBay, Kakamega) Kenya (Mwacharo *et*

al., 2013).This study also revealed population admixture between the two gene pools (Figure 2.3) with east to west genetic cline of gene pool two. In tandem to that study, Ngeno *et al*. (in press), while studying genetic diversity and MHC region variability in indigenous chicken in Kenya, reported that the indigenous chicken host 46 polymorphic MHC marker LEI0258 alleles. The Cluster analysis in that study indicated a clear ecotype subdivision into two to three genetically distinct groups. Two main population clusters indicated by ΔK and PCoA are Lamu (one cluster) and populations from Kakamega, West Pokot, Turkana, Bomet, Narok and Siaya as a second cluster. An extra group (third cluster) was from Taita-Taveta. It is worth noting that although the two studies reported equal gene pool numbers, the sampling sites were different. Ngeno *et al.*, (2014) sampled from localities that were never reached by the cockerels exchange programme (Wainaina, 1994). Besides, Mwacharo *et al*., (2013) did not sample from Lamu a distinct cluster realized in Ngeno's study.

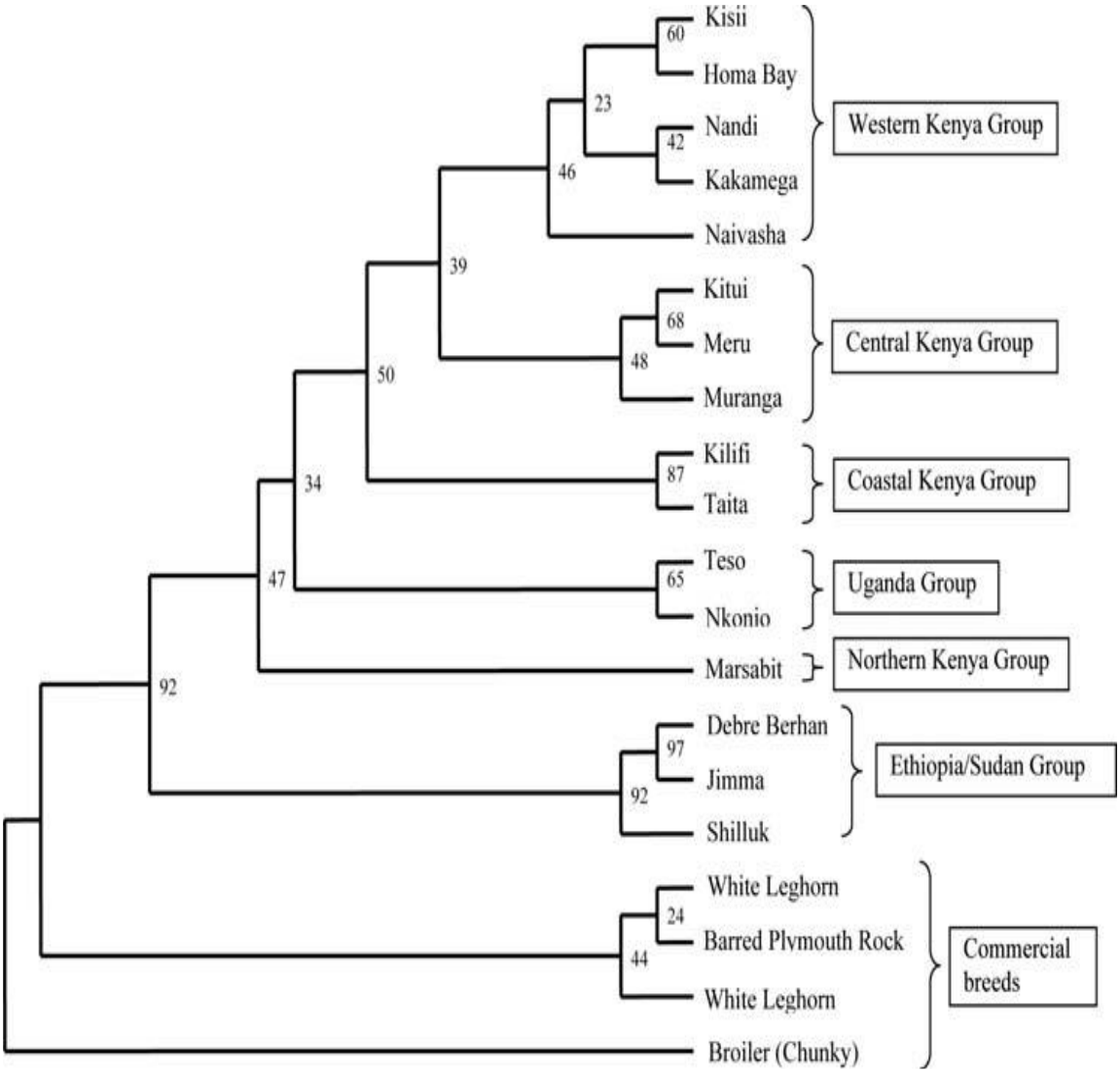

Figure 2.2 Dendrogram showing phylogenetic relationships within and between Kenyan and other indigenous chicken sampled from Ethiopia, Uganda and Sudan. The numbers at each interior node is the bootstrap value from 1000 re-samplings of loci with replacement (Mwacharo *et al.*, 2007).

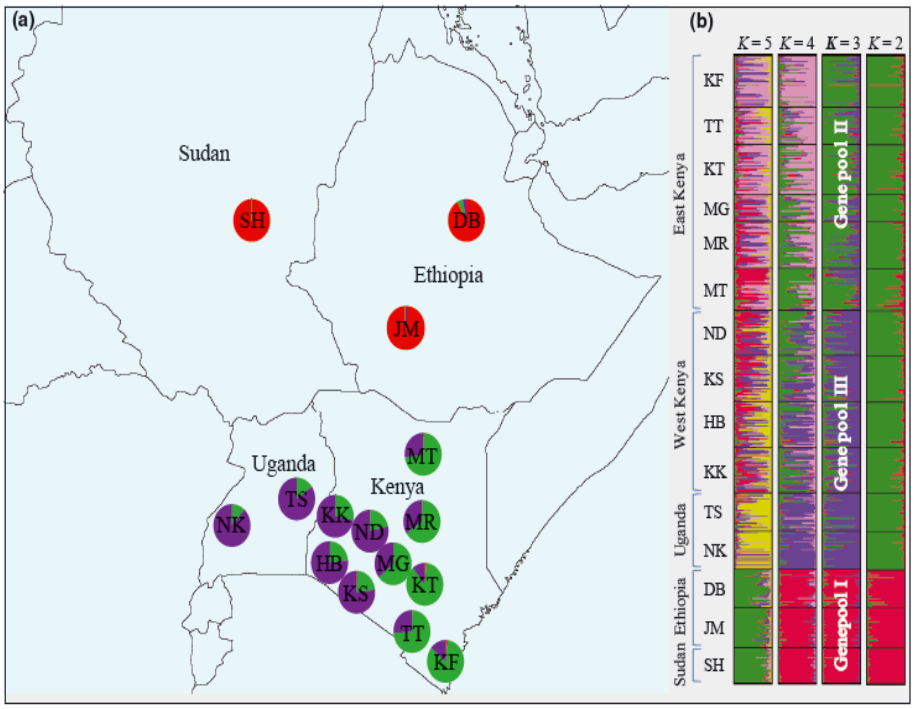

Figure 2.3 Geographic distribution of village chickens (Mwacharo *et al*., 2013).
The shaded area in each pie is proportional to the number of individuals in each population observed for each gene pool. (Population abbreviations: East of Kenya: KF, Kilifi; TT, Taita; KT, Kitui; MG, Murang'a; MR, Meru; MT, Marsabit; West of Kenya: KS, Kisii; ND, Nandi; HB, Homa Bay; KK, Kakamega; Ethiopia: DB, DebreBerhan; JM, Jimma; Sudan: SH, Shilluk; Uganda: TS, Teso; NK, Nkonjo). Colour codes: Red, Gene pool I; Green, Gene pool II: Purple, Gene pool III. (b) Bayesian analysis of population structure of East African village chickens. Individuals (represented by single vertical lines) are assigned to three distinct gene pools based on clustering result at K = 3. Color codes: Red, Gene pool I; Green, Gene pool II; Purple, Gene pool III (Mwacharo *et al*., 2013).

2.3.3 Contributions of indigenous chicken to rural households

Village chickens make substantial contributions to household food security throughout the developing world. Indigenous chicken serves as an investment and source of security for households in addition to their use as sources of meat and eggs for consumption and of income (Muchadeyi, *et al.*, 2007a ; Muchadeyi *et al.*, 2007b) Indigenous chicken are alternative source of animal protein due to the fact that they can be slaughtered and consumed as a single meal

hence do not require storage facilities. Their demand is ever increasing and thus marketing is easy thus providing source of income to resource-poor rural families (Bett *et al.*, 2012). They are also a means of investment in low-input farming systems in the tropics (Dana *et al.*, 2011; Okeno *et al.*, 2012). Besides rural households, these low-input, low output poultry-husbandry systems are an integral component of the livelihoods of most of peri-urban, and some urban households in most parts of the developing world (Magothe *et al*., 2012). A review by Guèye, (2003) indicated that an average family flock of five adult chickens (two males and three females) enabled women in Central Tanzania to generate an additional income equivalent to 10 percent of the average annual income. In the Niger Delta, family poultry husbandry contributes 35 percent of the income of household women, which represents about 25 percent of Nigerian minimum wage and 50 percent of the per capita income. Experiences in many other developing countries have shown that village poultry can be used as an effective means of empowering women and as a tool for poverty alleviation (Kitalyi, 1998). Besides economic considerations, the chickens are useful in a number of social, cultural and spiritual activities such as entertainments, gifts, funeral rights and spiritual cleansing (Njega, 2005). In some parts of the country, cock fighting is an exciting and popular entertainment for rural folk (Maina, 2000; Okeno *et al.*, 2011).Other uses include disposal of kitchen leftovers, manure production, being biological clocks for telling time of day especially in rural areas and a means of controlling insect.

2.3.4 Conservation and utilization of indigenous chicken in Kenya

Having recognized the enormous roles played by indigenous chicken to the resource-poor rural households, the Kenya Government through National Poultry Development Programme (NPDP); initiated InCIP in 1974 (Wainaina, 1994; Magothe *et al.*, 2012). The aim of the project was to increase the smallholder farmers' income and protein intake through commercialization of indigenous chicken production. The program was implemented by importation of exotic breeds such as Rhode Island Red, White Leghorn, Black Australop and Light Sussex which were to be crossed with indigenous chicken. The idea was that famers were to be supplied with exotic pullets and cockerels in exchange for indigenous chicken cocks. The program was also supported by management practices such as massive vaccination of indigenous chicken for Newcastle disease and training of farmers and field officers on management of the improved stock. Although, the resultant genotypes had improved productivity, the program was terminated in 1993 as the subsequent crosses were not adapted to the harsh environmental conditions among

other reasons (Magothe *et al.*, 2012). This unplanned and poorly organized cross-breeding strategy was also a danger to indigenous chicken genetic diversity and conservation.

Having learnt from mistakes done under NPDP (Magothe *et al*., 2012), the Kenya Government and World Bank initiated a second improvement program in 2006.This was introduced under the umbrella InCIP (http://incip.org/) which was a collaborative project between the Ministry of Livestock Development, Egerton University, and Kenya Agricultural Research Institute. The main objective of this program was to undertake comprehensive analysis of indigenous chicken actors for the purpose of understanding the sub-sector and build human capacity in different fields in sub-sector. Such undertaking was found to be critical as it would provide insight in to the existing situations in terms of strengths, weaknesses, dynamics and complexities, which are important in formulating solutions. Currently the indigenous chicken actors in Kenya has been analysed, (Bett *et al.*, 2012), the indigenous chicken sub-sector characterized, breeding objectives and management strategies defined and within indigenous chicken genotype and ecotype selection programme initiated (Ngeno, 2011; Magothe *et al.*, 2012; Okeno *et al.*, 2013).The InCIP programme activities are presently, mainly funded by European Union through African Union and have expanded its activities to other African countries such as Malawi in collaboration with Lilongwe University of Agriculture and Natural Resources. In Malawi the main aim of InCIP is to establishing local breeding programme for improvement and conservation of Malawian indigenous chicken which are also under threat due to indiscriminate cross-breeding with Black Australorp breed (Safalaoh, 2001). Previously, the Malawi Government introduced the Smallholder Poultry Improvement Programme (SPIP) in the 1950s with the objective to increase egg and meat production of indigenous chicken through cross-breeding with the Black Australorp (Safalaoh, 2001). Although the project has been running for more than 50 years, very few studies have been conducted to evaluate cross-breeding as a strategy to improve productivity of the indigenous chickens. The same scenario was realized in Kenyan case with no performance record taken to assess the magnitude, benefit and extend of genetic dilution of the indigenous chicken. In 2012, the Kenyan Government and stakeholders in the poultry sector formulated Poultry Development Bill 2012 (http://www.parliament.go.ke/plone/archive/archive-10th Parliament/bills). This bill has a lot of input emanating from InCIP activities and findings. Some of the key issues the bill addresses include characterization, conservation and development of indigenous poultry breeds, feed

resources and disease control, marketing and capacity building. When passed to law the bill will regulate indiscriminate cross-breeding and replacement of indigenous chicken with exotic which is not only a threat to genetic diversity, but could also lead to extinction of some of the uniquely adapted indigenous chicken genotypes and ecotypes.

Utilization of cross-breeding programme has been practiced and benefit noted (Safalaoh, 2001). However for its ultimate success a clear strategy has to be adhered to. Amongst them, is training of farmers on proper guidelines on how to conduct the cross-breeding. For instance because the exotic hens do not sit on their eggs, the farmers should be encouraged to buy exotic cocks which can then be crossed with local hens (Okeno *et al.*, 2013). Where local cocks are crossed with exotic hens, the fertilized eggs from the exotic hens should be placed under local brooding hens for natural incubation. Alternatively, the eggs can be placed, in artificial incubators if the facility is presented. Rather than leaving the task of cross-breeding to the farmers, InCIP could be encouraged to carry out the cross-breeding so that the farmers are actually sold the cross-breeds as a terminal product. This would require identification and selection of the local breeds with the best performance in terms of desired traits (Okeno *et al*., 2013). The cross-breeds could combine the characteristics of improved productivity from the exotic breeds and adaptability and stress tolerance to the local harsh conditions from the local chicken and should be able to survive in the prevailing harsh village conditions better than the exotic breeds.

2.3.5 Past attempts to genetically improve indigenous chickens of Kenya

The demand and popularity of indigenous chicken genetic resources has been increasing since time immemorial (Okeno *et al.*, 2012). Market trends in Kenya, see consumers shifting interest from the dominant hybrid layer eggs and broiler meat towards what is conceived as a healthier diet of free-range or indigenous eggs/chicken. According to Magothe *et al.*, (2012), from 1984 to 2004, indigenous chicken population increased by more than 75 percent and their egg and meat products increased by more than 34 and 79 percent, respectively. The observed increase was attributed to an increase in the human population and hence a corresponding demand for indigenous chicken products. In recent study to assess the demand and price dynamics of indigenous chicken products in Kenya (Bett *et al.*, 2011), there is evidence of escalating indigenous chicken products prices and the consumers' willingness to pay more as compared with other poultry products . The observed increase in demand of indigenous chicken

products by consumers has caused farmers, Research Institute, Universities, Government and Non-governmental organizations, to engage in attempts to improve their productivity. For instance, since 2006 in the forefront is the Smallholder InCIP at Egerton University that has established a breeding and multiplication centre and initiated a number of activities to improve the indigenous performance (www.incip.org). Previously, poor quality and inadequate feed resources, health care, marketing, housing and lack of breeding stock have been identified as constraints towards improved performance of indigenous chicken in developing countries (Gondwe and Wollny, 2007; Kingori *et al*., 2010; (Okeno *et al.*, 2011). To address nutritional constraints in Kenya, attempts have focused on models to supplement feeding at different times and stages of growth and production, determining protein and energy requirement levels (Chemjor, 1998; Kingori *et al*., 2010). Although, these interventions increased productivity under on-station (Kingori *et al.*, 2010), their adoption and sustainability under on-farm conditions was low due to high unavailability of feed resources, high cost of formulated feeds and low genetic potential of indigenous chicken for growth and egg production.

The past genetic improvement programs for increasing chicken productivity in developing countries mainly focused on use of imported temperate breeds (Dana *et al*., 2011). Many exotic breeds of chicken (White and brown Leghorns, Rhode Island Red, New Hampshire, Cornish, Australorp, Light Sussex etc.) were introduced over the years. The other approach to improve productivity of the village poultry production was based on use of cross-bred animals. This involved crossing of unselected local chicken to different levels of exotic blood. In Ethiopia for instance, evaluations of crossbred chicken at the DebreZeit Agricultural Research Centre indicated that 62.5 percent white leghorn crosses showed superior performance to the locals as well as pure white leghorns in terms of egg production (DZARC, (2007). In a cross-breeding programme at Assela, Brannang and Persson, (1990) also compared different York × local crosses. Their results indicated that egg production declined with increasing level of exotic inheritance (above 50 percent). Increasing the level of exotic blood also resulted in loss of broody behaviour, a trait of considerable economic value under village systems. Although the cross-breeding programs produced successful results under experiment stations almost all of them were discontinued decades ago for various reasons (Dana, 2011). In the 1970s and 1980s the Ministry of Agriculture in Kenya and Ethiopia, respectively, initiated a cockerel distribution scheme. This involved importation and distribution of cockerels to be used as breeding males in

villages. In Ethiopia, the scheme failed because farmers were unwilling to remove their local cocks and the exotic cocks failed to adapt in the village environments (Dana *et al*., 2011).

In Kenya, the genetic improvement was started through a cross-breeding scheme by the National Poultry Development Programme (NPDP); unlike Ethiopia, the programme utilized crossing between cockerels and pullets of exotic breeds with the local indigenous chicken. The initiative termed cockerel exchange programme started in 1976 initially in 12 districts and by 1980 another additional 9 districts were included. Later more districts were recruited into the programme and by 1993, 26 out of 54 districts were involved in the programme (FAO, 2007a; FAO, 2007b). A hybrid cock was exchanged for the local cock and then all the local cocks were killed. For the pullet exchange, a farmer was required to keep 10–15 pullets. Farmers' training on poultry management and vaccination for disease control was also undertaken as components. The programme witnessed improved performance in the crossbreds but declined with subsequent generations (Okeno *et al.,* 2012). Failure of the programme to meet stakeholders' expectation led to its termination in 1994 (FAO, 2007a; FAO, 2007b). The factors attributed to its failure included poor planning and understanding of the indigenous chicken sub-sector with respect to production environment, needs of actors (i.e. farmers, marketers and consumers), lack of clear breeding objectives and lack of sustainable breeding programme to supply constant pure-line breeding stock (Ndegwa *et al.,* 2012).

2.3.6 Current genetic improvement of Indigenous chicken in Kenya

Since 2006 another collaborative programme called the Smallholder InCIP was initiated with the view of holistic and comprehensive approach to analyzing the entire indigenous chicken production actors and avert the causes of previous failures. The InCIP mandate is to characterize the indigenous chicken production system, develop clear breeding objectives accounting for all players in the sectors, design appropriate selection and mating schemes for modern dissemination of superior genetic resources. It is therefore true that InCIP has in mind the fundamental questions common to a breeder namely; "which are the best indigenous chicken among and between the populations and how can they be genetically improved?"

In addressing these questions, InCIP initiated basic but prudent methodologies. First, eggs and live birds were collected from various Agro-ecological regions or ecotypes namely; Kakamega, Bondo, Narok, West Pokot, Bomet, Taita-Taveta, Lamu, Egerton and Mwingi. These ecotypes are currently on-station at Egerton University Poultry Research and Breeding (PRB)

facility for multiplication, performance recording and selection. It is upon the performance records that selection and mating are based. In line with the project objectives several studies have been conducted and findings disseminated. (Ngeno, 2011) reported variations on body weights among the ecotypes, and further estimated heritability and genetic correlations. A similar study by Magothe *et al*. (2010) showed that the naked-neck, frizzle and crested-head genotypes do influence body weights and growth patterns. Magothe *et al*. (2010) noted that the crested head genotype had a slower growth rate and were lighter compared with the normal-feather genotype when subjected to the same level of management. Growth rate and egg production being inversely correlated, the authors speculated that the crested-head genotype could be a superior egg producer. This hypothesis is currently being tested on-station at Egerton University. Several studies done outside Kenya on general or morphological characterization, production and reproduction performances of available indigenous chicken genotypes did not vary from Kenyan situation (Okeno *et al.*, 2010). In determining where to go, a study by Okeno *et al.* (2011) determined the selection criteria by farmers and traits of economic importance preferred by the farmers. In that study, the authors reported that farmers prefer selection based on growth rate, large body size, high egg production, hatchability and good mothering ability.

They also indicated that normal feathered, crest-head, naked-neck and giant (i.e. big body size) genotypes were the most predominant genotypes (Figure 1). To define breeding objectives among farmers, marketers and consumers, the study identified egg number, growth rate, body size, fertility, disease resistance, meat quality, egg size, egg shell colour, broodiness and mothering ability to be traits of economic importance (Okeno *et al*., 2012). However, all these traits cannot be included in the breeding objectives and some are negatively correlated, this therefore calls for critical decisions to be made on which trait to account for in the breeding objective. In a simulation study Okeno *et al*. (2013) demonstrated that improving IC for growth and therefore meat production would result to better genetic gains and economic returns. Based on this finding the selection for indigenous chicken at the PRB has been focused on growth.

2.3.7 Future genetic improvement strategies

The future genetic improvement practices will be done at PRB, Egerton University and are based on the previous findings of (Okeno *et al.*, 2013) and principles of genetic improvement of livestock. The InCIP noble course is anchored on Millennium Development Goals (UNDP, 2013) through Kenya Vision 2030 (www.vision2030.go.ke) for eradication of extreme poverty

and hunger and improved food security in Kenya respectively. In plan are a plethora of integrated quantitative and molecular research projects to be formulated and implemented. To improve disease resistance, InCIP intends to first determine genetic variation among the indigenous chicken ecotypes and genotypes using both innate (natural antibodies, compliment) and adaptive immunity (interleukins, specific antibodies after vaccine challenge).If variation exists, the birds will be divergently selected for low and high immune response for subsequent generations.

Individual performance will be recorded and pedigree data established. This will assist in estimating genetic parameters. Breeding for enhanced immunity is sustainable (Parmentier *et al.*, 2004; Okeno *et al.*, 2010), will save the farmer their money for antimicrobial drugs and eradicate the consumers' food safety concerns. Such study will give insight in the biological mechanism of immunity for genetic control of a plethora of ever increasing pathogens of birds and compliment vaccination strategy as a disease prevention measure (Parmentier *et al.*, 2004; Khobondo, 2012). Following Okeno *et al*. (2012), recommendation based on traits preferred by farmers, there is already on-station selection for improved production and reproduction traits for subsequent estimation of genetic parameters and variance components for the economically important traits. Such selection programs will cause change in input requirements, e.g. feed intake for maintenance and production requirements of the birds. Since feed costs account for over 70 percent of the cost of production (Kingori *et al*., 2010), InCIP considers avenues to reduce costs of production besides increasing revenues. This makes feed efficiency (a measure of how much saleable product is produced for each unit of feed consumed and can be expressed as either gross feed efficiency or net feed efficiency) a trait of economic importance that should be included in breeding objective alongside production traits due to its direct influence on production costs. The aim of incorporating feed efficiency study is to carry out an analysis of net feed efficiency within the indigenous chicken flock in Kenya. In the proposed study, variance components, genetic and phenotypic parameters for net feed efficiency traits and growth characteristics will be estimated and the genetic and phenotypic parameters obtained. The results from this study will provide information required for breeding programs aimed at improving efficiency in the production of indigenous chicken.

Achievement of these goal will employ basic breeding tools of mating and selection on-station. Pure-line ecotypes will be selected generation after generation based on economic traits

(Okeno *et al.,* 2012) at nucleus tier, multiplied at the second tier for ultimate crossbreeding/ mating at the commercial tier. The resulting hybrid will then be sold to farmers. It is with no doubt there are morphological and production variations amongst the different ecotypes and genotypes (Magothe *et al.*, 2010; Ngeno, 2011), this calls for molecular characterization using microsatellite and single nucleotide polymorphism (SNPs) to decipher the witnessed differences. InCIP will employ the safest conservation method by continuous breeding involving sufficient numbers of birds to avoid inbreeding and genetic drift (FAO, 2007a; FAO, 2007b). InCIP propose to train farmers to optimally utilize the indigenous chicken that match with their environment and production system. For instance, in hot and cold ecozones/regions, InCIP will advocate for rearing of adaptive genotypes that best suits the environment. Where management is enhanced, with high input availability, high producing meat and egg pure lines will be encouraged. This may be complimented with cross-breeding with exotic breeds. In addition, InCIP plans to use cryoconservation method. Cryoconservation of avian sperms, ova and embryo has been successful in France (Blesbois, 2007; Blesbois *et al.,* 2008). For traits with low heritability, InCIP will employ genome wide association studies to build a consensus prediction formula for genomic breeding value estimation in the nucleus scheme. This will lower generational interval and could be economical in long run.

2.4 Microbial diversity and metagenomics

2.4.1 Importance of microbial diversity and metagenomics

Microbial populations occur in every biological niche on earth, from the insect gut to the oceans of the world and in the sediment beneath them. They are the most bio diverse microorganisms on earth (Hugenholtz *et al.,* 1998; Bik *et al*., 2012). Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage or community (Torsvik *et al*., 1998). Microbial diversity refers unequivocally to biological diversity at three levels: within species (genetic), species number (species) and community (ecological) diversity (Harpole, 2010). The bio diversity analyses are important in several ways; 1) increase the knowledge of the diversity of genetic resources and understand the distribution of organisms, 2) increase the knowledge of the functional role of diversity, 3) identify differences in diversity associated with management disturbing, 4) understand the regulation of biodiversity and 5) understand the consequences of biodiversity (to what extent does ecosystem functioning and sustainability, depend on maintaining a specific level of diversity) (Zhao *et al.*, 2012). The new

generation of sequencing technology has proved to be uniquely suited to the application of biodiversity (Caporaso *et al*., 2012). This accumulation of sequence information has greatly expanded our appreciation of the dynamic nature of microbial populations and their impact on the environment and animal health through metagenomics. Metagenomics refers to the study of genomic DNA obtained from microorganisms that cannot be cultured in the laboratory. It is the functional and sequence-based analysis of the collective microbial genomes that are contained in an environmental sample (Zeyaullah *et al*., 2009). In metagenomics, the collective genome (metagenome or microbiome) of coexisting microbes – called microbial communities (Ghazanfar *et al*., 2010) is randomly sampled from the environment and subsequently sequenced (Schloss and Handelsman, 2003). Metagenomics has the potential to give a comprehensive view of the genetic diversity, species composition, evolution, and interactions with the environment of natural microbial communities (Simon and Daniel, 2011). This can be utilized to explore microbial diversity in several species of animals.

2.4.2 Intestinal microbiome of poultry

The GI tract of poultry (e.g., chicken, turkey, and duck) consists of esophagus, crop, proventriculus, gizzard, small intestines (duodenum, jejunum, and ileum), cecum, colon, and cloaca. Relative to body length, the poultry GI tract is much shorter than that of mammalian animals. As such, the digesta passes through the entire GI tract faster in poultry than in mammalians. Although diet and feeding can have an effect on passage rate, the average whole tract transit time is less than 3.5 h. (Hughes *et al.*, 2008). Retention time selects bacteria that can adhere to the mucosal layer and/or grow fast. However, the ceca have rather slow passage rate, are ideal habitats for a diverse microbiome that has considerable effect on host nutrition and health. The cecal microbiome is indeed the most studied intestinal microbiome of poultry. The cecum of both chickens and turkeys harbors a complex microbiome, which is almost exclusively composed of bacteria (Wei and Yu, 2013). In early years, *Peptostreptococcus, Propionibacterium, Eubacterium, Bacteroides,* and *Clostridium* were the major genera that were recovered from cecum by cultivation (Barnes *et al*., 1978). Temporal changes were also observed as chicken aged (Barnes *et al*., 1978). Sequencing of 16S rRNA genes by the Sanger sequencing technology and recently next-generation sequencing (NGS) technologies make it possible to comprehensively characterize the intestinal microbiome of poultry (Reid *et al*., 2011), and the sequence information has greatly expanded knowledge on the bacterial diversity present in the

intestinal tract (Wei and Yu, 2013). The new generation of sequencing technology, with its ability to sequence thousands of organisms in parallel, has proved to be uniquely suited to the environment and animal health (Caporaso *et al*., 2012). In metagenomics, the collective genome (metagenome or microbiome) of coexisting microbes – called microbial communities (Ghazanfar *et al*., 2010) is randomly sampled from the environment and subsequently sequenced (Schloss and Handelsman, 2003; Zeyaullah *et al*., 2009). By directly accessing the collective genome of co-occurring microbes, metagenomics has the potential to give a comprehensive view of the genetic diversity, species composition, evolution, and interactions with the environment of natural microbial communities (Simon and Daniel, 2011). Through phylogenetic and statistical analysis of 16S rRNA gene sequences recovered from intestinal microbiome of both chickens and turkeys. Using the NGS, phylogenetic and statistical analysis of 16S rRNA gene sequences recovered from intestinal microbiome of both chickens, 13 phyla of bacteria were found, but Firmicutes, Bacteroidetes, and Proteobacteria accounted for most (> 90%) of the intestinal bacteria of chickens and turkeys. More than 900 species-equivalent operational taxonomic units (OTUs, defined at 0.03 phylogenetic distance) were found in chicken, and these OTUs represent 117 established genera of bacteria. The most predominant genera found in both chicken and turkey were *Clostridium, Ruminococcus, Lactobacillus,* and *Bacteroides*.

2.4.3 Microbiome and immunity

Colonization with microorganisms in the poultry gut occurs immediately after hatch and microbial succession follows until eventual establishment of a complex and dynamic microbiome (Bristin *et al*., 2008). The inner surface of avian gut is coated with a gel-like mucus layer which is formed from mucin glycoprotein secreted by the goblet cells (Forder *et al*., 2012). This layer of mucin consists of an outer loose layer in which microorganisms can colonize and an inner compact layer which repels most bacteria (Hansson *et al*., 2010). The mucus layer prevents gut microorganisms from penetrating into the intestinal epithelium and serves as the first line of defense against infection (Bristin *et al*., 2008). Another important component of the innate immune system that functions in the avian gut is the antimicrobial peptides present on the intestinal epithelial surface (Bristin *et al*., 2008). In poultry, the most important and well-studied antimicrobial peptides are β-defensins. They are small cationic peptides produced by avian macrophages, heterophils, and epithelial cells, and they can kill various intestinal pathogens by disrupting cell membrane permeability, which leads to cell lysis (Derache *et al*., 2009).

Salmonella infection was reported to increase the expression of β-defensin genes in chicken, whereas administration of probiotics prior to *Salmonella* inoculation resulted in a decline in the gene expression of β-defensins (Bristin *et al*., 2008).

The interaction between gut microbiome and host innate immune system can leads to subsequent adaptive immune response. B cells and T cells, which elicit antibody-mediated and cell-mediated immune responses, respectively, are the two primary types of lymphocytes that are of fundamental importance in the adaptive immune system. In avian gut, B cells and T cells can be found in organized lymphoid tissues (cecal tonsils, Peyer's patches, and the bursa of Fabricius) and in more dispersed areas such as lamina propria and epithelium (Bar-Shira *et al*., 2003). It has been shown that manipulation of gut microbiome through administration of probiotics (beneficial microbes) can influence antibody-mediated immune response. Chicken receiving probiotics containing *L. acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecali*s showed enhanced systemic antibody response to sheep red blood cells (Haghighi *et al*., 2005). Serum IgG and IgM reactive to tetanus toxoid and *C. perfringens* α-toxin were increased in chickens fed the same probiotic product (Haghighi *et al*., 2006). However, it remains unclear how probiotics enhance antibody mediated immune response. It is speculated that probiotics can stimulate the production of Th2 cytokines (IL-4 and IL-10), which may subsequently enhance the immune response mediated by antibody (Haghighi *et al*., 2005). Cell-mediated immune response were found to be affected by gut microbiome. By using germ-free, conventional, and gnotobiotic chickens, Mwangi *et al*. (2010) demonstrated that enteric microbiome complexity had a dramatic influence on the gut T cell repertoire. Brisbin *et al.* (2012) reported that various *Lactobacillus* species had the capacity to induce differential cytokine expression in T cells of chicken cecal tonsils which could contribute to intestinal homeostasis. Feeding probiotics can therefore improve the utilization of proteins, beneficial microbial populations and suppress harmful bacterial growth in the digestive system, counteract adverse effect of antibiotics, nutrient synthesis, stimulate immune system, decreased diarrhoea and mortality. Further, it improve the feed intake, feed conversion ratio, body weight, lower cholesterol in blood, serum and meat, increase the tenderness and meat quality along with carcass yield (Mwangi *et al*., 2010).

2.5 Conclusions

There have been several attempts to improve indigenous chicken production and address constraints in their production in Kenya. These attempts had minimal success due to, among others, lack of a holistic approach in solving the constraints and dissemination of inappropriate technologies given the production circumstances and market dynamics. This review has highlighted the various past attempts, discussed current improvement strategies and suggested possible future directions to improve the indigenous chicken productivity which could improve the livelihood of the rural households in line with Kenya vision 2030. Current studies have suggested potential for improvement of indigenous chicken production in Kenya given the available genetic and physical resources and have recommended a holistic approach/strategy that increases productivity. Future research entails modern studies that will improve traits of economic importance using quantitative genetics and genomics. Besides, the current improvement programme entails conservation strategies and cross-breeding principles. The exploration and use of probiotics is posed to further compliment genetic improvement for enhanced productivity and immune response.

CHAPTER THREE

GENETIC AND NON-GENETIC SOURCES OF VARIATION IN NATURAL ANTIBODY TITRE VALUES AMONG INDIGENOUS CHICKEN

3.1 Abstract

Innate immunity plays significant role in combating diseases. Improvement of any trait including resistance to disease and infection requires identification of genetic and non-genetic sources of variation. This study aimed at deciphering the factors (both genetic and non-genetic) that confers variation in natural antibodies titre values in indigenous chicken of Kenya. The study was conducted at the Smallholder Indigenous Chicken Improvement Program Research Unit at Egerton University, Kenya. The meta data involving several factors (genotypes, ecotypes, cluster, sex, plate and breed) were analysed. The natural antibodies titre values were measured by indirect enzyme linked immunosorbent assay. Two sample t-test for means and variance were compared for breed (RIR and IC) and sex (male and female) using IgG, IgA and IgM natural antibody isotypes titre values as dependant variables. One factor linear model was used to determine source of variation. A mixed model fitting chicken as random variable was used as the final model. For the two sample t test, there was significant difference on means for breed and sex ($p=0.05$) for all immunoglobulins isotypes. The t test showed significant difference in variance of breed ($p=0.05$) but not sex. However, the indigenous chicken and male chicken had higher variance estimates. Breed effect was significant for IgA ($P=0.0323$) but not IgG and IgM. Sex was significant for IgG ($P=0.0279$) but not significant for IgA and IgM. Genotype and ecotype were not significant while plate was significant respectively for both isotypes. Cluster was significant for IgA but not IgM and IgG. The variance estimate for chicken components were high and significant for IgM ($p=0.003$), IgG ($p=0.0001$) and IgA ($p=0.0001$). The residual variance estimates were small and insignificant for all the three isotypes. The results implied that males had higher Nabs titre values for all isotypes and could be used in selection of male line. The big variance estimate within the IC imply genetic improvement in Nabs against plethora of pathogens could be achieved through selection and crossbreeding.

3.2 Introduction

Improvement of any trait including resistance to disease and infection requires that genetic and non-genetic sources of variation are identified. This is because non-genetic sources influence the expression of genetic potential for traits and provide a better understanding of biological or environmental mechanisms on performance (Mrode, 2014). Similarly, accounting for these factors help to unmask true differences between groups as well as reduce bias in performance evaluation. Several studies on production traits done on IC have been reviewed and genetic and environmental factors found to influence the traits. The evidence outlined in these reviews majored on productive traits and with scanty information on disease resistance and immune response (Chapter 2). The two branches of immunity (innate and specific) are important in combating diseases (Beutler, 2004). Innate immunity plays an important role in survival of organisms, although additionally acquired immunity is often required in vertebrates (Beutler, 2004). Natural antibodies (Nabs) are part of innate immunity, found in healthy unimmunized individuals and are an important part of the first line of defense in animals. They provide early resistance against plethora of infections (Ochsenbein and Zinkernagel, 2000) because of their polyspecificity and polyreactivity. Low levels of innate immunity (Nabs) may be related with enhanced disease susceptibility and high levels with disease resistance and high survival rate (Sun *et al.*, 2011) in commercial chicken breeds. In contrast, under intensive production system (modern housing systems) the relationship between Nab at early age and survival has been reported for commercial (elite) layer breeds (Star *et al.*, 2007; Sun *et al.*, 2011).

Recently, high Nab levels have been associated with low survival in IC breeds under confirnment in Ethiopia (Wondmeneh *et al.*, 2015). That finding suggested that Nabs have a positive effect on survival in adapted intensively reared chicken (exotic breeds) but that effect was reversed in IC that are not adapted to confinement. These findings on Nabs, however, did not determine factors causing variation in chicken. Studies using IC in Kenya using quantitative genetics methods highlighted differences in several production traits (Chapter 2). Molecular studies of diversity on the same population have grouped the IC into three phylogenetically distinct clusters (Ngeno *et al.*, 2014). It is not however known whether differences in Nabs titres exist between these clusters (Ngeno *et al*., 2014), genotype (Magothe *et al.*, 2012*)* and ecotypes (Okeno *et al.*, 2013) of IC. Therefore, the current study aimed at determining the sources of variation in Nabs titre values of IC.

3.3 Materials and Methods

3.3.1 Study site

The study was conducted at the Smallholder Indigenous Chicken Improvement Program Research Unit (INCIP-RU) at Egerton University, Njoro, Kenya. The University lies on 35°45'-35°46' E and 0°16' - 1°10' S, at an altitude of 1800 meters above sea level with an average annual rainfall of about 1000mm and mean temperature range between 17-22°C (Ayuya *et al.*, 2011) . The area lies in agro-ecological zone two of Kenya (Sombroek *et al.*, 1982).

3.3.2 Experimental population

The base population of chicken used in this study was established through collection of chicken and eggs from unselected, random mating populations of IC from the rural farmers of Kakamega, Bondo, West Pokot, Narok, TaitaTaveta, Lamu and Bomet counties in Kenya thus, called ecotypes. The counties were chosen because there had no introduction of exotic chicken before hence minimum genetic dilution of the IC were expected. From these chickens and eggs, a population of IC was established on station at INCIP-RU at Egerton University. The ecotypes were genotyped and the phylogenetic analysis clustered them into three distinct phylogenetic groups as described elsewhere (Ngeno *et al.*, 2014). Cluster one (K1) constituted chicken from the Western, North and South Rift Kenya (Kakamega, Bondo, West Pokot, Narok, and Bomet), cluster two (K2) constituted chicken from the Eastern region and cluster three (K3) constituted chicken from the coastal region in Kenya (Lamu, Taita Taveta) (Ngeno *et al.*, 2014). Cluster four (K4) constituted of Rhode Island Red (RIR) an exotic breed. Sex was determined by phenotypic appearance. Genotypes (naked-neck, normal feathered, frizzle feathered, Kuchi) were described based on phenotypic observation.

3.3.3 Management of experimental population

At hatching, each chick was wing-tagged and identified by the ecotype, cluster, genotype, sex and breed. Brooding was done from hatching to 8 weeks in deep litter brooders that were heated using infrared bulbs. The population density was 12 chicks/m^2. The chicks were fed rations with nutrient composition recommended for IC in confinement. The chicks were provided with a chick feed (20% CP and 2,950 kcal/kg) for 8 weeks after hatching. Twenty-two hours to 23 hours of lighting was provided during the first 3 days and 10 hours light afterwards for 8 weeks. Thereafter, natural lighting was used as the day length was more or less the same in Kenya during the study period. A infrared lamp of 250W was used to provide heat. The

temperature during the first 3 days was 28°C to 30°C and was reduced to 23°C in the 4th week. Afterwards, 23°C to 25°C was provided throughout the study period. The temperature was monitored using thermometer and ventilation was adjusted by opening the curtains. Thereafter the chicks were fed a grower ration (18% CP and 2,850 kcal/kg) from week 9 to 17. From 18 weeks onwards, the chickens were provided with routine *ad libitum* layers mash (16% CP and 2,750 kcal/kg) feed. Clean water was also provided ad libitum. Health management practices such as vaccination, deworming and disinfection were carried out procedurally. The chickens were kept under deep litter on concrete floor filled with wood shavings.. Standard density of 8 and 6 chickens/m^2 were used during the rearing and laying period, respectively. The chicken received routine vaccinations against Marek's disease (day 1), New Castle disease (NCD; week 3, 8, 18), infectious bursal disease (week 2, 7) and fowl typhoid (week 8).

3.3.4 Natural antibodies isotype assays

Blood samples (2 ml in EDTA) from 215 chickens of between 45 to 49 weeks were drawn from the wing vein of each chicken and plasma separated by centrifugation at 2000 rpm for 10 minutes. Isotype specific IgA, IgM and IgG antibody titers to keyhole limpet hemocyanin (KLH) in plasma from the chicken were determined by indirect enzyme-linked immunosorbent assay (ELISA). Keyhole limpet hemocyanin (KLH) is a high-molecular-weight protein antigen collected from the hemolymph of the sea mollusk (*Megathura crenulata*). It is a copper-containing high-molecular-weight protein, which is commonly used as a soluble model protein known to induce a T-helper 2 cell (Th2)-like response. This antigen has been used in several studies of chicken immunity (Minozzi *et al*., 2008) and QTL studies based on microsatellite markers (Siwek *et al*., 2003). Keyhole limpet hemocyanin is an antigen that birds have not encountered during their lifetime; therefore, it represents a novel antigen, suitable to measure primary immune responses. Briefly, 96 well plates were coated with 2µg/ml KLH (MP Biomedicals Inc., Aurora, OH) and incubated overnight at 4°C. The plates were washed five times with 100 µl using a washing/dilution buffer (phosphate buffered saline (PBS) containing 0.05% Tween) and incubated for 1.5 hours at 25°C with chicken plasma (in duplicate) diluted 1:10 in dilution buffer. The plates were washed five times to remove unbound plasma. To detect IgA, IgM and IgG antibodies binding to KLH, a secondary 1:20,000 diluted affinity purified goat anti-chicken IgM (Fc specific), conjugated with horseradish peroxidase (GACh/IgM (Fc) /PO) antibody, or 1:20,000 diluted whole rabbit anti-chicken IgG (heavy and light chains)

conjugated with horseradish peroxidase (RACh/IgG (H+L)/PO) antibody or 1:20,000 diluted affinity purified goat anti chicken IgA (Fc specific) conjugated with horseradish peroxidase (GACh/IgA (Fc) /PO) (Nordic Immunological Laboratories, Eindhoven, The Netherlands) was added and incubated for 1.5 hours at 25°C. The plates were washed five times again and 100µl substrate-buffer (containing aqua dest, 10% tetramethylbenzidine-buffer and 1.33% tetramethylbenzidine) per well was added and incubated for 10 minutes at room temperature in darkness. The reaction was stopped with 1.25M H_2SO_4. Absorbance levels were measured at 620 nm with a spectrophotometer (mrc Scientific Instrument-UT- 6100, Israel).

3.3.5 Statistical analysis

Means and variance analysis for sex and breed

Two sample t- test for means and variance were compared for breed (RIR and IC) and sex (Male and Female) using IgG, IgA and IgM Nabs titre values as dependant variables.

Factors influencing antibody titres values

A one way ANOVA was done to determine factors influencing variance was done using PROC GLM procedure of SAS 9.2 (SAS, 2009). Finally, a mixed model analysis of variance on antibody titre values traits was carried out to determine the factors that influence variation using PROC MIXED procedure of SAS 9.2 (SAS, 2009). The independent variables fitted in the model included 2 breeds, 2 sex, 7 ecotypes, 4 clusters, 4 genotypes and 3 plates. The identity of chicken was fitted as random. The mixed model used for the analyses is presented below:

$$Y_{ijklm} = \mu + S_i + G_j + C_k + B_l + Pm + ECn + A_k + e_{ijlkm} \quad \text{.....(1)}$$

where:

Y_{ijklm} = the IgA or IgG or IgM titre values of the m^{th} bird;

µ = overall mean;

S_i = effect of i^{th} sex (i = male, female);

G_j = effect of j^{th} genotype (j = normal feather, naked-neck, frizzled, kuchi);

C_k = effect of k^{th} cluster group (k = CL1, CL2, CL3, CL4);

$B_{l=}$ effect of l^{th} breed (l = IC, EX);

m = effect of m^{th} plate (m =1, 2, 3);

En = effect of n^{th} ecotype (Kakamega, Bondo, West Pokot, Narok, Bomet, Lamu, Taita taveta,)

A_{ki} = the random effect of the chicken and

e_{ijklm} = random error term.

3.4 Results

3.4.1 Comparison of mean and variance components between sex and breed

The difference in mean and variance estimates between sex (M/F) and breed (Exotic/ IC) was determined for all isotypes. In all the isotypes, males had significantly higher means than females ($P \leq 0.05$). For IgG isotype, there was significant difference in variance estimate ($P=0.0404$) for breed effect with the IC having relatively higher variance (Table 3.1). However, the variance estimates for sex and breed were not significant for IgM and IgA.

Table 3.1 The two sample T test for variance showing significant difference in variance for breed but not sex for IgG Isotype.

Group/Factor	Breed	Number	Mean	Variance	P-value (variance)
Breed	Exotic	21	2.3216	0.1501	0.0404
	Indigenous	194	2.1905	0.3318	
Sex	Female	128	2.1496	0.3308	0.1118
	Male	71	2.3405	0.2399	

3.4.2 Determinant of antibody titre values

The one way ANOVA for IgA, IgG and IgM revealed significant and insignificant effects. Breed effect was significant for IgA ($P=0.0323$) but not for IgG and IgM. Sex was significant for IgG ($P=0.0279$) but not significant for IgA and IgM. Genotype and ecotype were not significant while plate was significant respectively for both isotypes. Cluster was significant for IgA but not IgM and IgG. The mixed model analysis showed significant differences in chicken for all isotypes. The variance estimate for chicken (random variable) components were high and significant for IgM ($p=0.003$), IgG ($P=0.0001$) and IgA ($P=0.0001$). There was a big variance estimate among the chicken compared to residual variance. The residual variance was insignificant (Table 3.2).

Table 3.2 The variance estimates for chicken (genetic) and residual and their significance levels for IgA, IgG and IgM Nabs titre values. The number of stars shows level of significance

	Variance Estimates		
	IgM	IgG	IgA
Source of variation			
Chicken	0.01795***	0.2544****	0.1164****
Residual	0.00414	0.1068	0.0098

*** = p<0.001, **** = p<0.0001

3.5 Discussion

The study aimed at demystifying the variation of Nabs titre levels for three isotypes in IC in Kenya. Natural antibodies were found to be detectable in the IC population (Chapter 4), similar to reports of non-immunized cattle (Van Knegsel *et al*., 2007), humans (Ehrenstein and Notley, 2010) and poultry (Sun *et al.,* 2011). In mammals, the Nabs are mostly produced by CD5+ B cells in the peritoneal cavity and intestines but also CD5- B cells (Casali and Notkins, 1989) were described to produce Nab. The Nabs may arise independent of known antigenic stimulation. They are mostly poly-reactive, and poly-specific (Baccala *et al.*, 1989) with low binding affinity hence confer general immune competence (Casali and Notkins, 1989). Different isotypes of Nabs (IgM, IgA and IgG) have been reported to be present in many animals, which is in agreement with the findings of this study. The study also confirmed that the IgM isotype is the most abundant whereas IgG and IgA Nab have relatively higher titres as explained elsewhere (Chapter 4)

The breed difference for IgG (Table 3.1), cluster difference for IgA and within chicken difference for all isotypes (Table 3.2) observed in this study is consistent with other studies which showed that Nabs are genetically controlled (Sun *et al.,* 2011). The genetic clusters used in this study were initially determined by (Ngeno *et al*., 2014) using MHC linked markers. Two main population clusters were Lamu (cluster one) and populations from Kakamega, West Pokot, Turkana, Bomet, Narok and Siaya (cluster two). An extra group (cluster three) was from Taita-Taveta. This distinction was only evident with regard to IgA Nab titres isotype. The two sample

t- test for variance analysis of breed (Exotic/IC) explained significant breed differences for IgG isotype. In this analysis the IC had higher variance estimates than RIR implying that more genetic response can be achieved by selection within IC population. The large variance components conferred by IC in the mixed model showed individual variation within the chicken.

The high variance estimated among chickens suggested Nabs can be used for breeding for robustness that is a broad capacity to remain healthy across a wide range of challenges. A study using acquired immunity against Newcastle disease virus (NDV) involving local chickens from Tanzania (LL), Rhode Island Red derivative hybrids (EE), and their reciprocal crosses (LE and EL) showed immune response differences. The local ecotype had the highest mean antibody titres following primary and booster vaccination while the exotic breed had the lowest titres (Lwelamira and Katule, 2004). In another study, there was significant difference in Nab titres values on layers. The chickens originated from three different lines, which were either divergently bred for 29 generations for high (H line) or low (L line) primary (agglutinating) antibody responses at day 5 after primary intramuscular immunization with Sheep Red Blood cell (SRBC) at 37 days of age, or a random bred control (C line) (Parmentier *et al*., 2004).

Sexual dimorphism has been reported to influence other traits, for example weight at 12 weeks (Muasya *et al*., 2015) and growth. Breeding program in poultry is carried out in the nucleus stock (pedigree stock). In three to five generations, the genetic improvement is then forwarded through multipliers to commercial farmers. In this study, male chicken had higher Nab titre values compared to females for all isotypes. The results can be exploited in IC breeding where a terminal product is crossed between male and female lines. The male line can be used for Nabs traits for increased heterosis. For example, in breeding for meat producing chicken, male lines could be selected for Nab titre values, growth and conformation and crossed with female lines selected for reproductive characteristics.

The genotype of IC were reviewed and reported to affect productive traits (Chapter 2). Variations on body weights among the genotypes, and estimation of heritabilities and genetic correlations in IC in Kenya were reported (Magothe *et al.*, 2012). Furthermore a study on the same population showed that body weights and growth patterns are influenced by genotypes (the naked-neck, frizzle and crested-head) (Magothe *et al.*, 2012). The previous studies demonstrated that the crested-head genotype had a slower growth rate and were lighter compared to the normal-feather genotype when subjected to the same level of management. Furthermore,

genotypes (both exotic and indigenous) possessing the naked-neck and frizzle genes, either singly or in combination were associated with increased growth rates, superior body weights, better feed conversion, higher egg production and disease tolerance in tropical environments (Islam and Nishibori, 2009). The Kuchi genotype, found in Tanzania (Msoffe *et al.*, 2002) and similar to the Aseel genotype of Bangladesh (Bhuiyan *et al.*, 2005) had higher growth rate and would be ideal for meat production in warm and humid areas. This distinction has, however, no influence on Nabs titre values. The effect of the micro titre plate was significant in this analysis. This observation could be attributed to intrinsic value and properties of each ELISA plate. The effect of plate was observed in another study (Van der Klein *et al.*, 2015) where the plate effect was corrected for other (confounding) effects on the samples, such as sex, storage, and analyses effects. It is not yet established whether these effects could apply in this study and thus it is worth investigating further.

3.6 Conclusions

The study confirmed significant variation in Nabs on the IC in Kenya. Breed and cluster (both genetic) were significantly different in IgA NAbs titre values binding KLH. The male IC had higher Nabs titres values for all isotypes and could be used in selection of male lines for breeding for high natural antibody titres. There was a large variance within the IC, it is proposed that substantial genetic improvement in Nabs against plethora of antigens could be achieved through both selection and crossbreeding. These strategies could alter allele frequencies of genes and markers linked to natural antibody titres.

CHAPTER FOUR

VARIATION AND REPEATABILITY OF NATURAL ANTIBODIES AGAINST KEYHOLE LIMPET HEMOCYANIN OF INDIGENOUS CHICKEN OF KENYA

4.1 Abstract

The immune system is designed to provide protection to the body by combating a plethora of pathogenic micro-organisms. Identifying animals with a natural ability to make superior immune responses reduces disease occurrences, increases farm profit and improves product quality as well as safety. Consequently, there is need to breed disease resistant animals that will eliminate the danger of currently used prevention strategies of drug prophylaxis and vaccination, which are unsafe and ineffective respectively. Many studies aimed at investigating the mechanisms involved in genetic resistance have been done but a standardized biological parameter indicative of disease resistance or susceptibility has remained elusive. The objectives of the study were to determine the presence and variation of IgA, IgG and IgM among indigenous chicken, and secondly, repeatability within the indigenous chicken over time of IgA, IgM and IgG natural antibody isotypes against Keyhole limpet hemocyanin. Blood samples from 24 indigenous chicken of the same age, genetic background and sex were collected four times within three weeks. IgA, IgM and IgG titre values were measured by indirect ELISA from the sera. A mixed model with repeated measures was performed to determine variation and repeatability. All the immunoglubulin isotypes binding KLH in chicken serum were recorded. There were significant differences between isotypes concentrations, with IgM and IgA reporting the lowest and highest titre values respectively. Repeatability were 0.68, 0.99 and 0.99 for IgM, IgG and IgA respectively. The isotypes were not only detectable and variable in serum of indigenous chicken but also consistently and repeatedly measurable in blood serum. IgM isotype showed high variation between indigenous chicken showing genetic influence. This finding may lay the basis for genetic improvement of immune response in the Kenyan indigenous chicken.

4.2 Introduction

Most of Nab binds pathogen-associated molecular patterns (PAMPs) that are conserved along different genera and this serve as targets for identification of microbes by the innate immune system (Kohler *et al.*, 2003). Important PAMPS are lipopolysaccharides (LPS) present on gram-negative entero-bacteria, such as escherichia *coli* or *Salmonella spp*.; lipoteichoic acid (LTA) present on gram-positive bacteria, such as *Staphylococcus aureus.* ; or peptidoglycan (PGN) present on gram-negative and gram-positive bacteria (Ploegaert *et al*., 2011). Besides the PAMPs, Nabs do bind many solubilized extracts of liver, kidneys, stomach, muscle, thymus, lungs, nuclear, tumors and red blood cells components prepared in the presence of Sodium dodecyl sulfate (SDS) (Madi *et al.*, 2012).

Natural antibodies can be categorized into two classes: the first class directed against self antigens, are called natural auto antibodies (Naab) or cryptic, hidden, masked, latent or silent Nab (Cheng and Chamley, 2008; Khobondo *et al.*, 2015). They inactivate cytokines, clear obsolete or damaged cells and metabolic waste and are believed to play part in surveillance or homeostasis maintenance (Lutz *et al.*, 2009). The second class is the overt Nabs, which are readily detected in unfractionated untreated normal sera. These Nabs bind antigen that an individual has never encountered before such as keyhole limpet hemocyanin (Bergstra *et al.*, 2010). The Keyhole limphet hemocyanin is a protein antigen from *Megathura cranulata*, a deep sea organism that is assumed no terrestrial animal has ever encountered before. Therefore any immune response against it will be innate in nature (Parmentier *et al.*, 2004). Nabs can thus be tested as a parameter for immune response and genetic variation estimation.

It is a prerequisite that, for a parameter of disease resistance to be acceptable in genetic studies, it should be variable to justify selection. Furthermore, there is no standard biological molecule so far to measure disease resistance or tolerance. Several disease parameters have been investigated to measure immunity and disease resistance. These include cytokines tumor necrosis factor (TNF) and interferon (IFN), cellular components (eg B, T and NK cells) and Reactive Oxygen Sulphite (ROS) production by phagocytic cells in blood and milk from healthy cows (Ploegaert *et al*., 2011). Other than variation, a trait should be repeatable to be sampled once under the same environmental. Repeatability of a parameter is important to assess whether a single test on single sample collection is enough for inferences or reproducible thus reducing the cost of doing repeated measures. The value of repeatability can be useful in estimating the upper

limit of heritability. Ploegaert *et al*. (2011) estimated repeatability of cytokines to be high, and significantly varied among cows. The study also found reasonable repeatability in the Nabs and milk components. Overt Nabs could be the antibodies of choice for exploring of disease resistance parameter and future association with diseases in IC and other animals. Therefore the objectives of this study were a) to determine the presence and variation of Nab isotypes IgA, IgG and IgM binding KLH in clinically healthy indigenous chicken (IC) and b) to estimate repeatability of IgA, IgG and IgM Nab titres binding KLH of IC sampled four times in three weeks. The research findings gave useful insight into genetic basis of Nabs and prospects of selective breeding for disease resistance or association in IC.

4.3 Materials and methods

4.3.1 Experimental population

The base population of chicken used in study were established as described in chapter 3 of section 3.3.2. From the established population, eggs were simultaneously incubated but separated according to ecotype within incubator. At hatching, each chick was wing tagged with an identification number. Brooding was from hatching to 6 weeks. Brooding of chicks from each ecotype was separated in deep litter brooders using infra-red electric bulbs. The population density was 12 birds/m^2. At the beginning of the 7th week, chicks were transferred to randomly selected deep litter rearing pens within the same house. Sex was determined by phenotypic appearance.

4.3.2 Feeding management

During the first five weeks the experimental birds were fed on chick mash, from the sixth to the 21st week they were fed on growers mash and thereafter layers mash. Feed and water were provided *ad libitum*. The feeding regime was the same across all the generations to account for diet effects.

4.3.3 Overt Natural antibodies isotype assays

Blood samples (2 ml in EDTA) from 24 IC for variation and repeatability studies was drawn from the wing vein of each bird and serum separated by centrifugation at 2000 revolution per minutes (2000rpm) for 10 minutes. Blood was sampled 4 times within 22 days (Days 1, 8, 15 and 22). These birds were all female, same age, genetic background and under same management. Isotype specific IgA, IgM and IgG antibody titers to keyhole limpet hemocyanin (KLH) in serum from the IC were determined by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, 96 well plates were coated with 2μg/ml KLH (MP Biomedicals Inc., Aurora, OH) and incubated overnight at 4°C. The plates were washed using a washing/dilution buffer (phosphate buffered saline (PBS) containing 0.05% Tween) and incubated for 1.5 hours at 25°C with IC serum diluted 1:10 in dilution fluid. The plates were washed using washing buffer to remove unbound serum. To detect IgA, IgM and IgG antibodies binding to KLH, a secondary 1:20,000 diluted affinity purified goat anti-chicken IgM (Fc specific), conjugated with horseradish peroxidase (GACh/IgM (Fc) /PO) antibody, or 1:20,000 diluted whole rabbit anti-chicken IgG (heavy and light chains) conjugated with horseradish peroxidase (RACh/IgG(H+L)/PO) antibody or 1:20,000 diluted affinity purified goat anti chicken IgA (Fc

specific) conjugated with horseradish peroxidase (GACh/IgA (Fc) /PO) (Nordic Immunological Laboratories, Eindhoven, The Netherlands) was added and incubated for 1.5 hours at 25°C. The plates were washed again and 100 μL substrate-buffer (containing aqua dest, 10% tetramethylbenzidine-buffer and 1.33% tetramethylbenzidine) per well was added and incubated for 10 minutes at room temperature. The reaction was stopped with 1.25 M H_2SO_4. Absorbance levels were measured with a spectrophotometer (mrc Scientific Instrument-UT- 6100, Israel) at 450 nm.

4.3.4 Statistical analysis

Descriptive statistics were used to explore the data and linear model in SAS (SAS Institute Inc., Cary;Version 9.1) used for initial analysis. Because Isotype of antibodies (IgA, IgG, IgM) were treated as different parameters, further analysis were performed using separate isotypes. Variation was estimated using the following mixed model;

$$Y_{ij} = \mu + time_i + IC_j + e_{ij} \qquad (2)$$

Where:

Y_{ij} = the Nab titre (IgM or IgG or IgA) of IC_j at time $_i$,

μ = the common mean.

Time = the fixed effect of time of measurement i (i = 1, 2, 3, 4).

IC = the random effect of the IC_j (j = 1...24; normal, independent and identically distributed (0 = σ^2IC) and

e_{ij} = the random residual (normal, independent and identically distributed).

Repeated measures analysis was performed using PROC MIXED of SAS. Covariance structure used was compound symmetry (αI= δ^2|i=j). Model assumptions regarding normality were evaluated by examining whether skewedness and kurtosis were close to 0 and whether a probability plot did not show deviation from a straight line.

Repeatability (r) along time and within IC was calculated as:

$$r = \frac{\sigma^2 IC}{\sigma^2 IC + \sigma^2 e} \qquad (3)$$

Where:

δ^2IC = the variance among IC and

δ^2e = the residual variance.

4.4 Results

4.4.1 Presence and variation of natural antibodies against KLH titres in Serum of IC

Natural antibodies binding KLH were detected for IgA, IgG and IgM isotypes in serum of the IC . The analysis of variance showed significant difference ($p<0.0001$) with type of isotypes and time of sampling the main sources of variation. IgM concentration/titre values had the highest mean vallue but with minimum variance and standard error of the mean. The IgG had the highest variance and standard error of the mean with moderate mean value (Table 4.1).

Table 4.1 The Means, variance and standard error of Isotype on the levels of Nabs titres of IC against KLH antigens.

Isotype				Range	
	Mean	SE	Variance	Minimum	Maximum
IgM	3.045	0.031	0.0234	2.59	3.46
IgG	1.575	0.110	0.2916	0.37	2.94
IgA	1.013	0.039	0.0369	0.63	1.53

4.4.2 Repeatability of natural antibodies titers

Natural antibodies (IgA, IgG and IgM) titers of IC were recorded at 4 time points to assess their repeatability. The estimated repeatability for IgA, IgG and IgM was 0.99, 0.68 and 0.99 respectively, with IgG isotype showing the highest variance (Table 4.2).

Table 4.2 Variation and repeatability of Natural IgA, IgM and IgG isotypes antibodies titers binding keyhole limpet hemocyanin in blood serum of IC.

Parameter	IgM	IgG	IgA
Overall variance	0.0180	0.3092	0.0383
Variance among IC	0.0123	0.3007	0.0379
Variance within IC	0.0057	0.0001	0.0004
Variation among IC (p values)	<0.0010	<0.0001	<0.6050
Repeatability	0.68	0.99	0.99

4.5 Discussion

The lack of effective control measures(management, vaccination and prophylaxis) for infectious diseases in livestock not only causes significant economic losses but may also endanger human health through zoonosis, compromise animal welfare and food security (Stear *et al.*, 2012). The use of genetic selection of animal for traits of resistance to infections has been presented as the "ultimate tool in sustainable disease control" (Chapter 2; Waller, 2006). Disease resistance and immune response is a complex trait polygenically expressed with several immune proteins. Due to this complexity, the most important and rate limiting challenge for disease genetic studies is likely to be obtaining suitable phenotypes (immune parameter). In goats and sheep for example, selection is based on phenotyping relevant traits such as zootechnical performance, Fecal Egg Count (FEC), and measures of anaemia and blood eosinophilia under conditions of either natural or experimental challenge (Mandonnet *et al.*, 2004).

In cattle, several immune parameters have been reported to be potentially related to susceptibility or resistance to various diseases (Thompson-Crispi *et al.*, 2013). These include soluble mediators like Nabs, cytokines, antimicrobial peptides and complement proteins, and cellular components like B, T and NK cells, y-T cells and granulocytes (Ploegaert *et al*., 2008). Therefore, choice of immune parameters for disease resistance study is critical (Boichard and Brochard, 2012). For example, specificity of acquired immunity is limiting to other pathogen except the one the animal has been primed with before. Therefore, natural antibodies could be promising since it is polyreactive and non specific despite being slow to combat pathogens.

In consistent with other studies (Ploegaert *et al*., 2011; Ujvari and Madsen, 2011), the current study reported the presence of Nabs isotypes IgA, IgG and IgM in IC' serum. This study

again confirms that the IgM Nabs isotype is the major isotype. In previous studies, IgM has accounted for most of the B cell repertoire in the fetus and neonate, and possibly play a major role in the development and physiology of the mammals B cell repertoire (Boes, 2000). Most Nabs of the IgM isotype class are present in vertebrates, but IgG and IgA Nabs are also present in higher vertebrates (Boes, 2000). The Nabs of IgM isotype are mostly produced by CD5+B cells in the peritoneal and intestinal cavity but also by CD5- B cells (Zhou and Notkins, 2004).

The other isotypes namely (IgA, IgE, IgD and IgG) do arise from the IgM class switching and this phenomenon could justify the higher titers of IgM compared to IgG and IgA in this study. The choice of Nab in this study is pegged on the fact that it is the natural arm of humoral immune response, polyreactive and nonspecific in nature (Parmentier *et al.*, 2004). For a parameter to be used as a potential measure of genetic disease resistance, it should be variable among individual animals. In genetics, lack of variability of a parameter shows that the animals are naturally the same with respect to immune parameter thus limiting selection (Chapter 2). In the current study Nabs binding KLH were found to be significantly variable among IC and could be exploited in selection for immunoglobulins titre values as a proxy for immune competence. Star *et al.*, (2007) reported Nabs binding KLH to be variable in layers, the same has been observed in other animals like cattle (Ploegaert *et al*., 2011) and also in this study in IC. The repertoire and levels of Nabs are dependent on several factors, amongst them is the environment (Kachamakova *et al.*, 2006), genetic background (Sun *et al.*, 2011) and age (Parmentier *et al.*, 2008; Ujvari and Madsen, 2011). Evidence from various studies showed Nabs to be genetically controlled (Gonzalez *et al.*, 1988; Sun *et al.*, 2011) and the same is true in the current study.

Repeatability of IgA, IgG and IgM titres was assessed in this study . The purpose was to ascertain whether a single test on one sample collection was enough for inferences thus reducing the cost of doing multiple measures. Alternatively repeatability could reflect how reproducible or similar a parameter (IgA, IgG or IgM) was under the same environment and conditions of experiment. This genetic parameter has been useful in estimating the upper limit of heritability. In deed the repeatability estimate for IgM (0.68), IgA (0.99) and IgG (0.99) were moderate to high. The lower repeatability of IgM experienced in this study was partially expected. The IgM is the primary isotype or precursor of other antibodies. It could therefore be a more stable isotype with time and in different or common environments. Thus a higher repeatability of IgM than other isotypes could be expected under this school of thought.

Alternatively, the IgM could represent the continuous presence of randomly produced antibodies that fit both exogenous and autologous plethora of antigens. The formation of antibody idiotypes is a random process through recombination or conversion; this could be true with IgM justifying the class switching role hence lower repeatability recorded. The low repeatability might be due to the high turnover that this isotype undergo within a very short period. It therefore means it is an isotype on transit and very unstable depending on environmental stimuli and antigen specificity. The genetic interpretation of the moderate repeatability estimates would imply a large variance between IC (genetic) as compared to variance within time (environment). This means that the genetic component plays an important role in the variations observed than non-genetic factors with regard to IgM antibody titre and repertoires.

The higher repeatability for IgG (0.99) and IgA (0.99) observed in this study is however justifiable. These higher estimates could imply lack of plasticity (i.e. stability) of this isotypes with time and in different or similar environments with respect to KLH (antigen in question), hence the low and high variances within the sampling period and between IC, respectively. The large variance estimate is due to IC (genetics) but not time (environment). It has to be kept in mind that this study is the first to determine repeatability of the anitibody titres in IC and comparison with other related studies is limited. The moderate to high repeatability observed in this study, affirms that a single measurement can be used to infer reference to Nabs titers of IC along time under same situations/environment with KLH specificity. Thus, the result can be reproduced under same management and conditions of the study and would likely be reproduced in the next generation under similar management. This eliminates the need to obtain several samples from the same IC over time. But for precision, the age of measurement or time of sampling should be indicated. The high repeatability found in this study, could help improve animal welfare especially on invasive blood sampling procedures and reduce cost especially to immunological assays.

In plasma of mature cows, however, repeatability of total Nabs against LPS, LTA and PGN was estimated to be 0.79, 0.80 and 0.93 respectively, and 0.60 for Nab binding KLH (Ploegaert *et al*., 2011). In milk, the repeatability estimates of nabs for LPS, LTA, PGN and KLH were 0.74, 0.81, 0.84 and 0.85 respectively (Ploegaert *et al*., 2011). In another study the repeatability estimates of daily milk, fat and protein yields in cows ranged from 0.63 to 0.83 at different stages of lactation (Vasconcelos *et al*., 2004). These estimates, although comparable to

those observed in this study, could not be used complimentarily. This is because chicken and cattle are genetically distinct species with different physiology. More so, the parameter of repeatability is different with respect to the samples, and secondary antibody used, the duration of experiment and experimental conditions. Generally, binding of serum Nabs to KLH, a model antigen that terrestrial animals have never encountered, might reflect immune competency. Therefore, well designed studies are required to test this hypothesis. The polygenic and nonspecific nature of Nabs in the host would probably deal with a plethora of epitopes in pathogens and may cooperate with other immune cells and compliment other immune proteins in combating diseases.

4.6 Conclusion

Natural antibodies isotypes IgA, IgG and IgM binding KLH were detected in blood serum of IC. The Nabs titers were variable and highly repeatable for IgA, IgG and IgM. Both isotypes can be used for further studies to explore association with disease resistance.

CHAPTER FIVE

POLYMORPHISM AND POPULATION STRUCTURE AT THE MICROSATELLITE LOCUS LEI0258 IN THREE MAJOR GENETIC GROUPS OF CHICKEN IN KENYA.

5.1 Abstract

There is need for sustainable genetic improvement for disease tolerance that can be achieved by selective breeding. Microsatellite markers can be exploited to determine immune competency and used in direct selection. In this study, microsatellite locus LEI0258 coded within the MHC region, was used to genotype 412 chicken representing three genetic groups of indigenous chicken in Kenya to determine geetic structure and relationships.. A total of 38 different alleles were detected in all the populations. The locus attributes showed high genetic diversity as revealed by the polymorphism information content (PIC=0.932) Shannon information and expected heterozygosity (H_e=0.915). Several indels and single nucleotide polymorphism were observed upstream and downstream of R13 and R12 repeat motifs respectively of the sequenced alleles. The highest unbiased Nei's genetic identity was between central and western populations. The highest effective number of migrants was between coastal and western populations. The most probable structure clustering of the populations was at dk = 4 with admixed genetic makeup. The information resulting from this study may be used to guide the design of further investigations for development of sustainable genetic improvement programmes focusing on improving immunity.

5.2 Introduction

Genetic control for disease tolerance is one of the most important targets for breeding schemes in the future (McElroy *et al.,* 2005). Immune-competence can be enhanced by using existing resources of genetic variation in chicken (Khobondo *et al.*, 2014) i.e. the gene variants (alleles) associated with disease tolerance (Ngeno *et al*., 2014b). However, the main obstacle in attaining this is inadequate knowledge of direct markers determining fitness and robustness in chickens. Microsatellite markers have been used in several studies including association studies. Microsatellites occur in the coding and noncoding regions of the genome; since they are under neutral selection, they are not influenced by environmental pressure. Due the co-dominant nature of microsatellites, they are highly informative and reproducible (Chazara *et al.*, 2013). The microsatellite LEI0258 marker located within the core area of the B region of the chicken Major Histocompatibility Complex (MHC) is highly polymorphic and can be correlated with serology. The MHC has abundant Single nucleotide polymorphism (SNPs) and indels, and is closely associated with host genetic resistance, immune response and susceptibility to a wide variety of viral, bacterial and parasitic diseases. (Fulton *et al.*, 2006). The LEI0258 marker is handy in characterisation work because it shows high polymorphism including a large number of alleles with a large range in allele sizes (Izadi *et al.*, 2011). The LEI0258 locus is the most variable in the MHC region, and is a useful marker in reflecting the variability of the region and therefore, the IC populations at large (Fulton *et al.*, 2006).

The marker has been used in genetic diversity studies using more than 1,600 chicken from more than 80 different populations to provide a picture of worldwide diversity and to categorize chicken MHC haplotypes (Chazara *et al.*, 2013). The LEI0258 marker is characterized by the repetition of two tandem and conserved short sequences of 12 bp and 13 bp and several sequence polymorphisms (indels and SNPs) in the flanking regions. It is the combination of the motifs and indels that determines the allele size and polymorphisms. This study was designed to decipher polymorphism, genetic structure, attributes and genetic relationships in three major population groups of chicken in Kenya using the LEI0258 microsatellite marker. The three populations have varied immune competency levels ranging from low, medium to high . The generated diversity was used to infer biological significance to the observed immune competence levels.

5.3 Materials and methods

5.3.1 Sample collection and total DNA extraction

A total of 412 blood samples were obtained from three genetic groups of Kenyan IC, including western group (n=169), central group (n=123) and coastal group (n=120). Total DNA was extracted using the DNeasy Blood and Tissue extraction kit (Qiagen, Germany Cat#69506) following the manufacturer's instructions. The total DNA was assessed for quantity and purity usingspectrophotometer (Nanodrop 1000 Spectrophotometer, ThermoFisher Scientific, Waltham, MA) and gel electrophoresis. The DNA samples were stored at -20°C prior to PCR amplification.

5.3.2 Genotyping

Highly polymorphic MHC-B microsatellite marker, LEI0258, was used to assess diversity in indigenous chickens (Hako Touko *et al.*, 2015). Fragment size was determined by capillary electrophoresis was used. The primer sequences used (GenBank accession number Z83781) for amplification of LEI0258 were: forward 5-CACGCAGCAGAACTTGGTAAGG 3 (length = 42 bp; GC content=47.6%; Tm=71.5°C) and reverse5-AGCTGTGCTCAGTCCTCAGTGC3 (length=39 bp; GC content=46.2%; annealing temperature 69.9°C). The LEI0258 forward primer was fluorescently labelled with NED. The PCR cocktail reaction comprised of 37.5 ng of total DNA, 0.15µM of each primer, 5 µl of 2X One Taq Premix (New England Biolabs, UK) and double distilled water to a final reaction volume of 10µl. The PCR program was conducted in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 94°C for 3 min, 30 cycles of 94°C for 45 sec, 63°C for 60 sec, 72°C for 60 sec, and a final extension at 72°C for 15 min, followed by holding at 10°C to infinity. A mixture of 12µl GeneScan 500 LIZ® Size Standard and 1000 µl of HIDI formamide were added to the PCR products at a ratio of 9:1 respectively. The mixture was then denatured at 95℃ for 3 min for capillary electrophoresis. The fragment (allele) sizes were calculated based on the internal size standards of electropherogram using the Gene Mapper version 4.1 and exported to Microsoft excel for processing as input files for statistical analyses.

5.3.3 Sequencing of selected samples

One hundred and thirteen representative alleles of LEI0258 fragments were sequenced by the Sanger method (Bioneer Corp, South Korea). Two samples were selected per allele. Homozygous individuals were selected first, followed by heterozygous samples for the rare and

other alleles not present in homozygous samples. The DNA sequence was obtained using primers that bind just outside of the LEI0258 binding region, as follows: forward (T7F): TAATACGACTCACTATAGGGCACGCAGCAGAACTTGGTAAGG; reverse (SP6R): ATTTAGGTGACACTATAGAGCTGTGCTCAGTCCTCAGTGC. The PCR was performed in a final volume of 25 µL with 5ng/µL of chicken genomic DNA, 12.5 µL of 2X OneTaq Premix (New England Biolabs, UK), 0.6 pmol/µL of each forward and reverse primers and 9.5 µL of nuclease free water to volume. The PCR cycles involved initial step at 94°C for 3min, followed by 35 cycles of 94°C for 30 sec, 63°C for 45 sec, 72°C for 45 sec, and a final extension step at 72°C for 10 min. The PCR products were resolved in 1.5% agarose gel and visualized under UV light on the GeneSyst software (InGenus, Syngene, USA). The PCR products for the homozygotes were purified using the QIAquick(R) PCR Purification Kit (QIAGEN). The PCR products for the heterozygotes were also purified after resolving of the two alleles on 1% agarose gels using the QIAquick(R) Gel Extraction Kit (QIAGEN). The purified samples sequenced by Bioneer Sequencing Service on the ABI 3730XL DNA Analyzer, using standard protocol. The entire LEI0258 was sequenced, including the repeat region and two flanking regions.

5.4 Data analysis

5.4.1 Genotyping data analysis

Attributes of the marker LEI0258 were determined by evaluating of Polymorphism Information Content (PIC), number of different alleles, number of effective alleles, Shannon's Information Index, observed heterozygosity, expected heterozygosity and fixation Index. The GeneMapper v4.1 (Applied Biosystems) was used to call the allele sizes of amplified fragments. Power Marker version 3.25 (Liu and Muse, 2005) was used to calculate Polymorphic Information Content (PIC). Genetic diversity was determined by calculating the observed and effective number of alleles (Na and Ne), observed heterozygosity (Ho) and expected heterozygosity (He) using GenAIEx v. 6.4 (Peakall and Smouse, 2006). Genetic distance matrix among the populations, principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA) were done using GenAIEx v 6.4. DARwin 6.0 (Perrier and Jacquemoud-Collet, 2006) was used to construct a cluster tree by estimating dissimilarity indices based on the binary data (simple allele matching). The genetic similarity matrix of the populations was calculated using Jaccard coefficient after which a dendrogram was generated using Unweighted Neighbor - Joining genetic disimilarity. The population structure was analysed using

STRUCTURE software and a Bayesian clustering approach (Pritchard *et al.*, 2000). The ‘‘admixture’’ option was used, with no linkage between loci and alleles. The ‘‘haploid’’ option was used to cater for the Hardy–Weinberg equilibrium hypothesis. Probabilities were estimated using a Markov chain Monte Carlo method (MCMC). Results were based on a burnin period of 10,000 iterations followed by 100,000 iterations of the MCMC chain. The MCMC chain was run 10 times for each of the 9 K options (1–20) tested. The optimal number of populations was established using the ad hoc statistic deltaK (ΔK) based on the rate of change in the log probability of data between successive K values (Evanno *et al.*, 2005). The method of Evanno enables the user to identify a single k value, out of a range of K values, which captures the uppermost level of structure (clumpak.tau.ac.il). This method was purposed by Evonno *et al.* (2005). The use ln(Pr(X|K) values in order to identify the k for which Pr(K=k) is highest (as described in STRUCTURE's manual, section 5.1) was employed. The optimal value k value was therefore chosen from CLUMPAK DeltaK graph (output).

5.4.2 Sequence polymorphisms and molecular mhylogenetic analysis

Complete sequences of LEI0258 were assessed for quality and assembled using CLC Main work bench software (Qiagen, Bioinformatics). Sequence alignment was done with clustalW in MEGA 6 software (Tamura *et al.*, 2013). Summary tables from Han *et al.* (2013) and Wang *et al.* (2014) were adopted to indicate the polymorphisms of all sequenced and aligned alleles. Phylogeny analysis were conducted in MEGA6 software (Tamura *et al.*, 2013). The evolutionary history was inferred by using the Maximum Likelihood algorithsm The bootstrap consensus tree inferred from 1000 replicates was used to infer the evolutionary history of the taxa analyzed (Nei and Kumar, 2000). Branches corresponding to partitions with in less than 50% bootstrap values were collapsed. The tree was obtained by applying the Neighbor-Joining algorithm to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach implemented in MEGA software.

5.5 Results

5.5.1 Attributes of LEI0258 marker

The LEI0258 marker revealed 38 alleles with a mean of 27.3 alleles across the three population groups (Table 5.1). In particular, central, coastal and western populations had 21, 28 and 33 alleles respectively. In all of the samples 38 different alleles were observed. There were fewer numbers of effective alleles (8.2) in central population as compared to coastal (14.7) and western (15.4) populations. The average number of effective alleles was 12.8 for the combined populations. There was higher expected than observed heterozygosity frequencies of for coastal (0.932 versus 0.792) and western (0.935 versus 0.864) populations respectively. The Shannon's information index was highest in western population (2.972) and lowest (2.386) in central population. The locus showed high diversity as revealed by the average Shannon's Information Index of 2.768. The fixation index (F) was positive for western and coastal populations but not for central population (Table 5,1). The mean overall observed heterozygosity and Polymorphic information Content (PIC) was 0.844 and 0.932 respectively for the total population sampled. The central population had the highest observed heterozygosity (0.878) while coastal had the lowest (0.792). The PIC was highest in the coastal population (0.936) and lowest in the central population (0.867). The western population had observed heterozygosity and PIC values of 0.864 and 0.932 respectively (Table 5.1).

Table 5.1 Polymorphism of LEI0258 analyzed in 412 samples of three population groups of indigenous chicken from Kenya

Pop	**Locus**	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**PIC**	**IP**	**F**
Central	LEI0258	123	21	8.1778	2.386	0.878	0.877	0.867	1	-0.001
Coastal		120	28	14.769	2.946	0.792	0.932	0.936	20	0.151
Western		169	33	15.489	2.972	0.864	0.935	0.932	9	0.076
All	**Mean**	**137.33**	**27.3**	**12.812**	**2.768**	**0.844**	**0.915**	**0.932**	**-**	**0.075**
	SE	15.857	3.48	2.3263	0.191	0.027	0.019	0.019	-	0.044

Note: Na = Number of different alleles, Ne = Number of effective alleles, I = Shannon's information index, Ho = Observed heterozygosity, He = Expected heterozygosity, PIC = Polymorphism information content, IP = Individual with private allele, F = Fixation index.

5.5.2 Distribution of private alleles

Thirty (30) chicken had 11 private alleles distributed among all the populations. One (1) individual from central population had one private allele. Twenty (20) and nine (9) individuals from coastal and western population groups, respectively had each private allele (Table 5.1).A total of 13 private alleles were detected. The central population had one (1) private allele, coastal population had four (4) while western population had eight (8) private alleles. The coastal population had the highest cumulative private alleles frequencies while the central population had the lowest average frequency.

5.5.3 Genetic relatedness among the populations

Genetic identity and distance

A large Nei's unbiased genetic distance of 0.736 was observed between central and coastal populations. The distance between coastal and western populations was 0.281 while central and western populations had the shortest genetic distance (0.151). The highest unbiased Nei genetic identity of 0.860 was observed between central and western genetic groups while the lowest (0.479) was between central and coastal populations (Table 5.2).

Table 5.2 Pairwise population matrix of unbiased Nei genetic distance and unbiased Nei genetic identity values

Pop1	Pop2	UNei GD	UNei ID	Fst	Nm
Central	Coastal	0.736	0.479	0.029	8.488
Central	Western	0.151	0.860	0.011	22.899
Coastal	Western	0.281	0.755	0.010	24.920

Note: Pop1 = population 1, Pop2 = population 2, UNei GD = Unbiased Nei genetic distance, UNei ID = Unbiased Nei genetic identity values, Nm = effective number of migrants, Fst = Fixation index.

Fixation index (Fst) was used to assess genetic/population differentiation. Genetic differentiation was 0.029 between central and coastal, 0.011 between central and western, and 0.010 between coastal and western population groups. The central and coastal population groups

had the lowest effective number of migrants (8.488), while coastal and western had the highest number (25) (Table 5.2). For all the population groups, the F_{ST} was positive indicative of random mating thus maintained heterozygosity. The inbreeding relative to foundation stock was (F_{IT} = 0.023), contemporary inbreeding (F_{IS} =0.077) and effective alleles was 11.21.

Principal coordinate analysis (PCoA)

The PCoA showed that the first 2 axes explained 22.50% and 20.59% of the variation among the three population groups respectively. The samples were grouped into three clusters (Fig 5.1). The samples from the coastal and western populations occured in all the three clusters. The majority of the samples from central populations were however grouped together in one cluster. This can be ascribed to the fact that the central population group is a closed one undergoing intense selection under controlled mating.

The PCoA analysis provided an alternative way to view the genetic similarity and diversity among and within the populations.

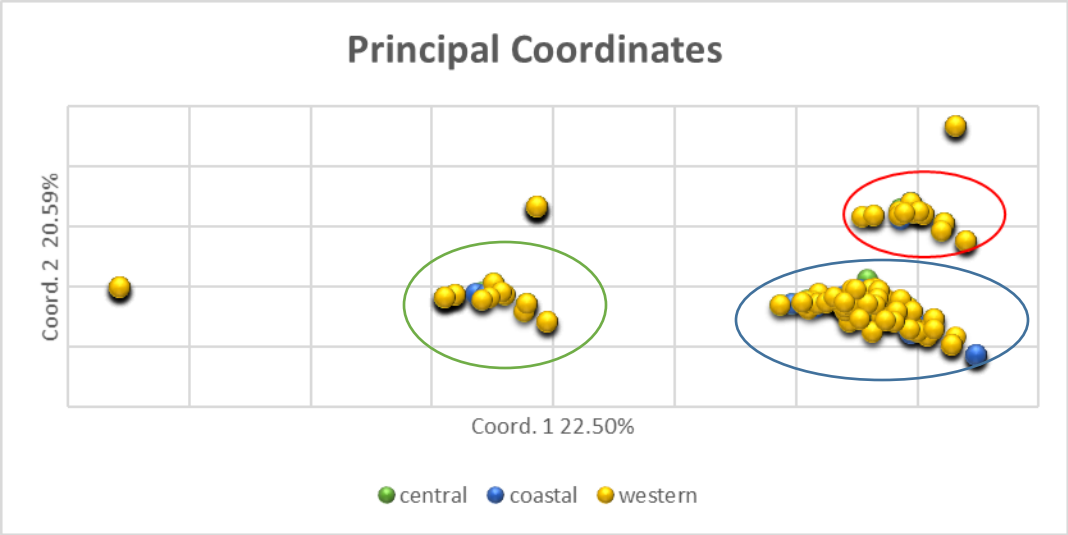

Figure 5.1 Principal coordinate analysis of the chicken samples from central, coastal and western populations

Analysis of molecular variance

The analysis of molecular variance (AMOVA) showed that there was a significant variation among populations (p=0.001). About 95% of the variation was observed within population while the remaining 5% was observed among population (Table 5.3).

Table 5.3 Summary of the analysis of the molecular variance

Summary AMOVA Table

Source	df	SS	MS	Estimated Variance	%	P value
Among Pops	2	15.324	7.662	0.049	5%	0.001
Within Pops	409	406.846	0.995	0.995	95%	
Total	411	422.170		1.044	100%	

Cluster analysis

The samples were clustered into four distinct clusters with admixture of the populations (Figure 5.2a). The largest was cluster I consisted of 8 sub clusters (1-8). The second largest cluster had 7 sub-cluster (a-g). The third cluster had 5 sub-cluster while the forth cluster had 2 sub-cluster. In all the clusters there were representative samples from all the three populations. The phylogenetic tree in Figure 2a shows bootstrap (100) value at the inner node indicating the shared similarity between the different samples in the specified bootstrap replications. The phylogenetic relationship constructed selected sequenced alleles is represented in Figure 5.2b. The sequences were clustered into five main clusters with observed admixture (every population representative was found in each cluster). The tree in similarity to allele tree (Figure 5.2a) showed the existing relationships among the chicken population with admixture sequences.

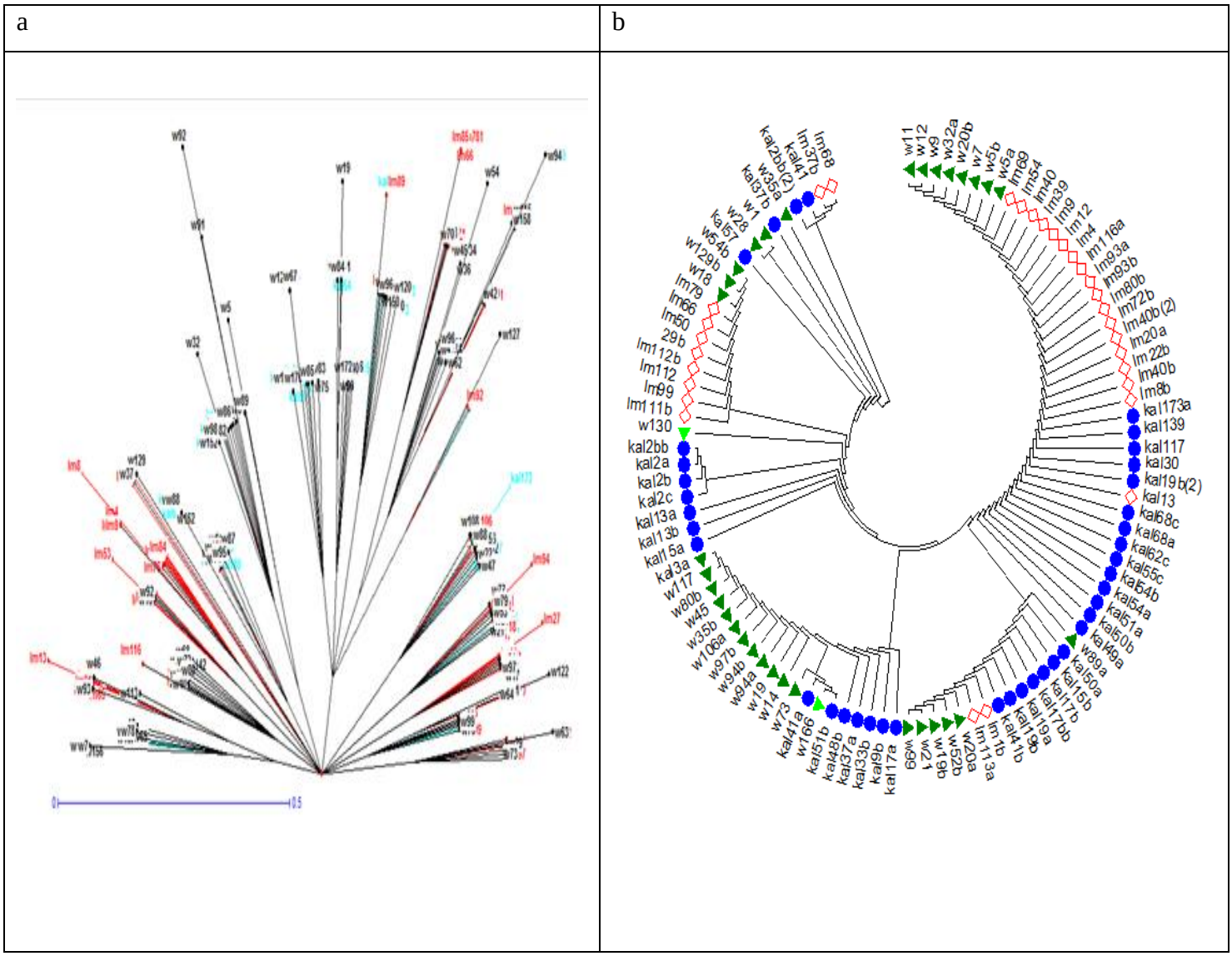

Figure 5.2 (a) A radiation neighbour-joining dendrogram constructed from allele-sharing distances among 412 individual chicken from three Kenyan chicken population **(b)** Phylogenetic tree showing genetic relationships of selected samples of chicken from 101 sequenced alleles

The structure analysis revealed four gene pools with evidenced population admixture as well (Figure 5.3). The western population had nearly equal contribution to the four gene pools at 25%. Gene pool 1 was dominant within central population while gene pool 4 was dominant in coastal population. Gene pool 3 was equally distributed among the three populations (Figure 5.4).

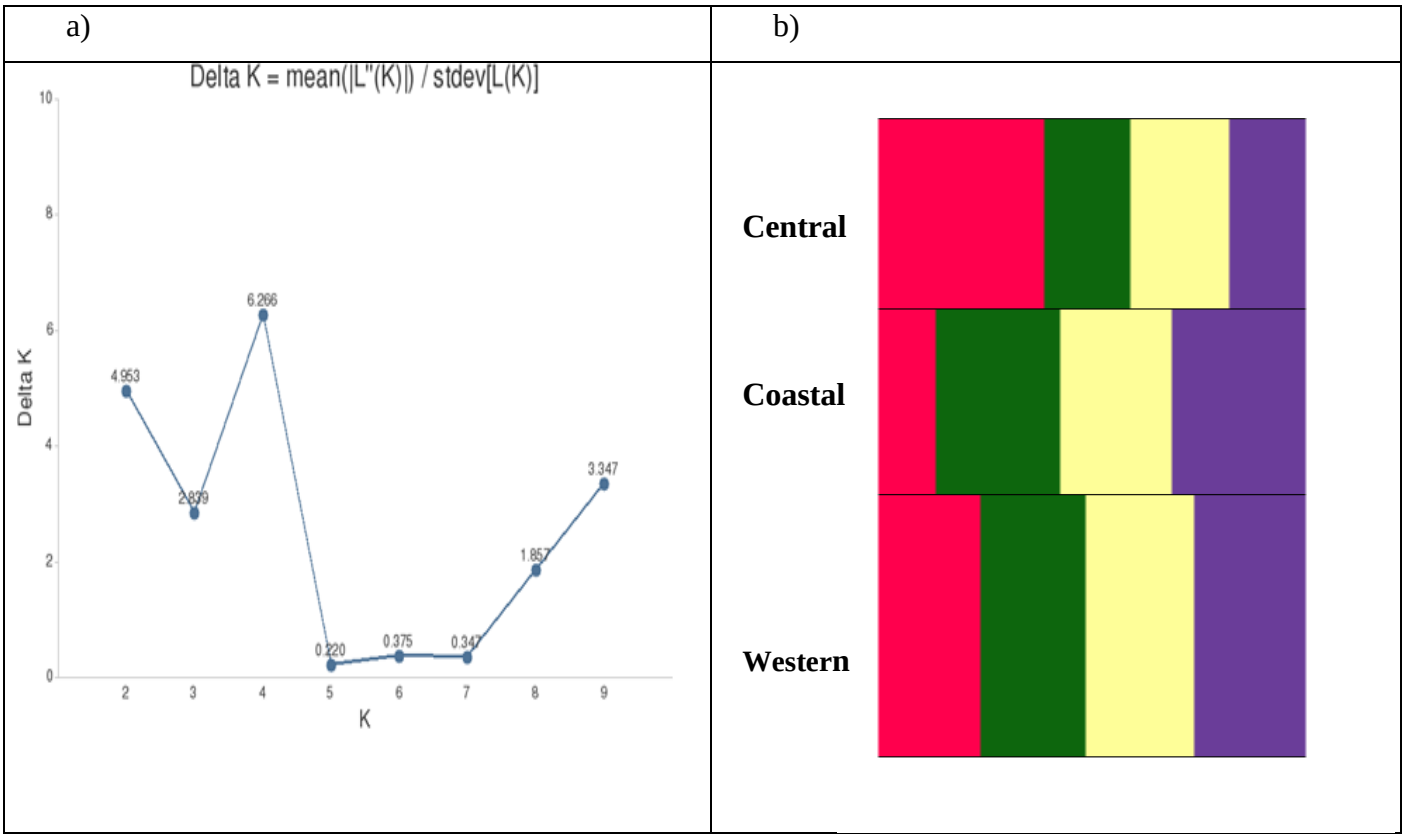

Figure 5.3 Structure clustering of the three chicken population obtained for a) k = 4 as the best Delta K and b) gene pools with admixture

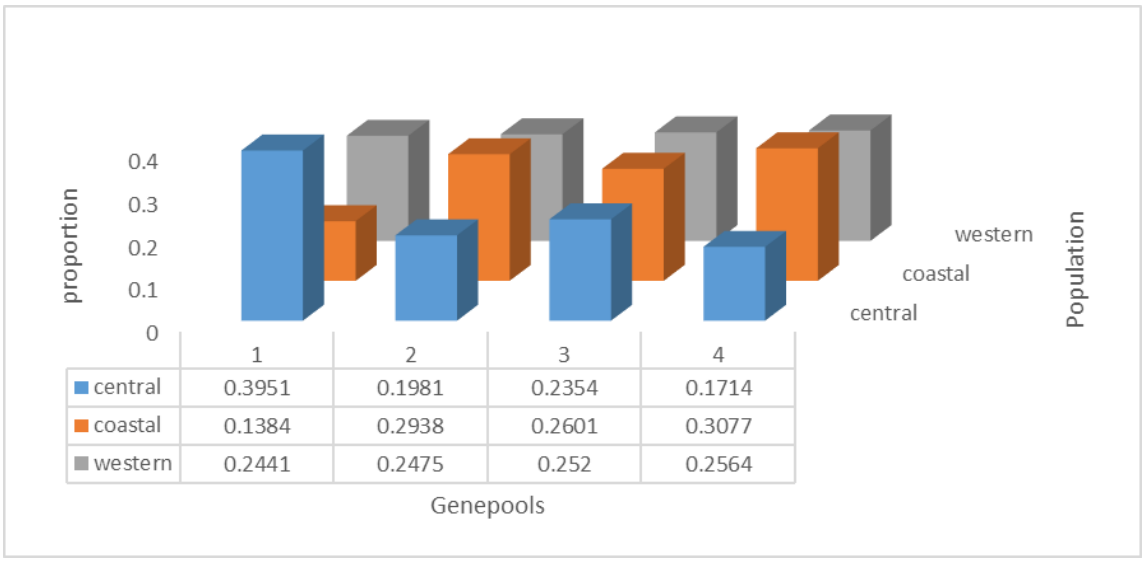

Figure 5.4 Distribution of the four gene pools among central, coastal and western populations

5.5.4 Polymorphisms identified within the LEI0258 alleles

Sequence information, repeat regions and two flanking regions from a subset of alleles per population were analyzed. The sequences revealed repetitions, conserved short motifs of 12bp (R12: TTCCTTCTTTCT) and 13 bp (R13:ATGTCTTCTTTCT) and several sequence polymorphisms in the flanking regions (small insertions and deletions (indels) and SNPs). The R13 repeat motif ranged from 1 to 28 with some samples lacking the repeat. All the sequenced samples had the R12 motif which ranged from 3 to 27. However, some samples had sequence insertions within the R12 and R13 motifs. Furthermore, allele sequencing result ascertained the presence of single nucleotide polymorphisms (SNPs) and indels in the flanking regions of LEI0258 locus. The flanking regions consisted of the upstream and the downstream of the repeat region R13 and R12 respectively. The upstream region was from −81 to −1, and the downstream region from 1 to 73, excluding the R13 and R12 motifs. The Upstream of the R13 motifs exhibited several SNPs at position -2bp, -9bp, -10bp, -13bp, -14bp and indels at position (-23 to -22bp), -24bp, (34 to -32bp) . Downstream R12 motifs several insertions and deletions were exhibited at positions 16bp, 21bp, 45bp, (17-18bp). There was 8bp large deletion of ATTTTGAG found at position 22 to 29bp including the last R12 repeat (Table 5.4).

Table 5.4 Polymorphisms identified by the LEI0258 alleles in Kenyan indigenous chicken population

Chicken population	Fragment length (bp, by genotyping)	Consensus size (bp, by sequencing)	Position (upstream)					Position (downstream)	GenBank number
			-22-23	**-2**			**5**	**22-29**	
			TT	**C**	**R13**	**R12**	**T**	**ATTTTGAG/△**	
Ref	-	552	-	-	14	6	-	-	DQ239521.1
Lm29	242	238	-	-	1	7	-	△	
Lm79	264	205	-	-	1	4	-	△	
Lm99	218	204	-	-	1	5	-	△	
Lm111	308	204	-	-	1	13	-	△	
Lm80	325	321	-	-	0	13	-	-	
W45	264	431	-	-	16	5	-	-	
W20	264	263	-	-	1	8	-	-	
W94	450	423	-	-	1	19	-	-	
W117	266	431	-	-	16	6	-	-	
Kal2	450	261	-	-	1	8	-	-	
Kal41	250	309	-	-	1	12	-	-	
Kal173	301	552	-	-	28	3	-	-	
Kal51	250	309	-	-	1	16	-	-	

Note: Δ Defined deletion compared with the reference sequence; - Consistent with the reference sequence

5.6 Discussion

5.6.1 Locus diversity and attributes of LEI0258 Marker

The genetic diversity of a population can be measured by genotype and allelic heterozygosity, proportion of polymorphic loci and by observed and expected heterozygosity (Nei, 1973). There was high diversity for marker LEI0258 as observed in the absolute and effective number of alleles (Table 5.1). This study identified 38 different alleles, 8 short of alleles observed in a previous study (Ngeno *et al*., 2014b). In one study, 26 LEI0258 alleles ranging in size from 182 to 552 bp were identified in North American and European layer-type chickens (Fulton *et al.*, 2006). In Tanzanian, 22 and 23 alleles were identified in two local chicken ecotypes (Lwelamira *et al.*, 2008), 19 alleles in local chickens of Vietnam (Schou *et al*., 2007), and 15 alleles in local Caipira chickens of Brazil (Lima-Rosa *et al.*, 2005). Chazara *et al.*, (2013) identified 50 alleles in 80 populations while Ngeno *et al*. (2014b) identified 46 different alleles in Kenyan populations. In this study, many of the identified alleles were shared among different chicken populations suggestive of common ancestry and population admixture as revealed in cluster and structure analyses. Some alleles were private and specific to populations. Variation in the number of private alleles between the populations may be due to different origins of the populations, selection history and immune pressure exerted by different environments on these populations. Diverse alleles in the studied populations could be as a result of hybridized origin of Asian and European (Ngeno *et al*., 2014a).

The total number of alleles, PIC and Shannon's information index reported in this study indicated high diversity in LEI0258 in Kenyan IC. This further suggests that this locus is very informative for genetic diversity, structure and relationship analyses of Kenyan IC populations. This is also an indication that Kenyan IC possess a wide genetic base that allows for adaptation to a wide variety of agro-ecological, production environments and disease pressure. The PIC assesses the diversity of a gene or DNA segment in a population illuminating the evolutionary pressure on the allele and the mutation the locus might have undergone over a time period (Van Marle-Koster and Nel, 2000). The PIC value will be almost zero if there is no allelic variation and it can reach a max of 1.0 if a genotype has only new alleles which are rare phenomenon.

In general, the high PIC value of this marker/locus revealed the extent of genetic variation in its alleles. This can be exploited for accurate characterization of the variability and association studies done for selection of desirable alleles for association studies. The higher PIC

value seen in coastal genetic population as compared to other populations implies some alleles were constricted within this group. This was justified by the high number of individual (20) with private alleles and higher frequency of private alleles in this group. Normally variation in the numbers of private alleles between the ecotypes may be due to different origins and adaptation to different production environments of the populations. Allele sharing among populations is an indication of being subjected to similar directional selection or due to the effect of high rate of gene flow. A scenario revealed by shared genepools in structure analysis and effective number of migrants.

The polymorphism or allelic variation evidenced in this locus was created by the presence of different number of R13 and R12 motifs, indels and SNPs in the sequenced alleles. The two main VNTR of the R13 and R12 were consistent with previous reports (Chazara *et al.*, 2013; Fulton *et al*., 2006). The variability of R12 ranged from 3 to 27 depicting different polymorphs in allele sizes. The upper limit of 27 repeat motifs were reported in Chinese indigenous chickens (Han *et al.*, 2013). The R13 motif was found at a frequency of only one in most of the samples, as observed by (Wang *et al.*, 2014). A few sequenced samples however had multiple motifs of R13 mimicking previous findings (Chazara *et al.*, 2013). The largest deletion of 8bp (ATTTGAG) was found in the downstream sequence of R12 motif. This deletion agreed with previous findings (Wang *et al*., 2014) but did not conform to the sequence ATTTGAGG detected by (Fulton *et al.*, 2006).

Two related parameters, heterozygosity (He and Ho) and the number of alleles harbored by each population, were used to indicate genetic variability. The western population had the highest genetic variability through high number of alleles (33) while central has the lowest (21). The central population is currently undergoing artificial selection and are maintained as stable improved local chicken. Theoretically, this population should have the lowest heterozygosity as observed in this study. There was a higher expected than observed heterozygosity for coastal and western populations, respectively suggesting subdivision of these populations. On the contrary, the central population had higher observed than expected heterozygosity implying departure from random mating and preference of specific alleles. Higher observed heterozygosity within central population implies few alleles are dominant to the other and the dominant genotype is observed and segregating more in the individual chicken. This is justified by few dominating alleles (308, 312 and 250) hence less number of effective alleles. Overall, this result might also

indicate that free range chicken from western and coastal populations have more variability in the MHC region than that of the intensively selected central population chicken. This observation might have resulted from mating between relatives.

The F_{IS} for the population ranged from low positive to negative values. Relatively low positive F_{IS} values indicated low level of inbreeding, maintained genetic variation within the chicken population. All the F_{IS} values were below 5%, suggesting the chicken populations were not endangered (Ramadan *et al.*, 2012). Alternatively, the fixation index (F) was positive for western and coastal populations but not for central population implicating non-random segregation of alleles within central populations. F_{IS}, is usually positive, but if negative, it indicates systematic avoidance of consanguine mating within the subdivisions as witnessed in central subpopulation. The negative F_{IS} could means excess heterozygosity. The positive values in coastal and western populations showed random mating as these populations are known to be in free range production systems (Okeno *et al.*, 2012).

5.6.2 Genetic relatedness

The high Nei genetic distance between central and other regions would imply central gene pool could be different to others. Indeed central population contributed most (roughly 40%) of gene pool 1. Alternatively, it might imply less identity by descent among the three population groups. This is justified by high contribution (roughly 40%) of genepool 1 of this population and the highest Fst witnessed between central and other populations indicative of central population differentiation from others. Regarding the interpretation of F_{ST}, it has been suggested that a value lying in the range 0 – 0.05 indicates little genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation; and values above 0.25, very great genetic differentiation (Nei, 1986). The F_{ST} of < 0.05 (0.010 – 0.029) realized between the populations are considered reasonably low and might imply that structuring between populations was weak. Alternatively, the low relative F_{ST} would imply low genetic drift, this was manifested in high heterozygosity values. However, we have to note that this study was based only on one marker with a small chromosomal region. Considering the effective number of migrants between the populations, the low F_{ST} was intuitively justified with high gene flow due to exchange of IC among the population groups. Despite the high number of effective number of migrants between western and central or coastal populations, the lower F_{ST} would imply less fitness rendering low reproductive success. More so, high genetic identity between coastal and

western could be attributed to cultural practices of people from western part of Kenya that could facilitated genetic exchange between the population groups.

There is high exchange of IC of these chicken population groups through movement of people carrying live birds from coastal to western and vice versa. People from western Kenyan are known to be chicken mongers. Similar selection criterion probably led to high genetic relatedness. Chicken lovers prefer fast growing birds a characteristic exhibited in coastal chicken population hence their preference by western people. More so, the high genetic identity observed between central and western populations was expected. This is because the on station breeding program utilizes cocks from western populations hence the high genetic identity and low genetic distance. Therefore the central populations sold to farmers originated from hybridization between exotic and western IC. In general, IC is a portable food resource, particularly well adapted to harsh environmental and management conditions, that has been extensively used in human migrations, easily disposed as gifts, cultural respects and in rituals, and commercial trade, therefore leading to extensive genetic exchanges.

5.6.3 Principal coordinate analysis (PCoA), cluster and structure analysis

The samples were grouped into three clusters (Figure 5.1) with admixture for all the populations in each cluster. However, the central population was majorly clustered together ascribing to the fact that it is a closed population undergoing intense selection under controlled mating. The dendrogram analyses from allele and sequence data showed an intermixing of the chicken populations. From these analyses, all the three populations did not divide into clearly distinct groups an indication of genetic introgression over time. The structure analysis revealed four gene pools with evidenced population admixture indicating genetic intermixing. . However, the findings of this study on population structure (four gene pools) is in contrast to two gene pools observed in previous studies (Mwacharo *et al.*, 2013 ; Ngeno *et al*., 2014b) . This discrepancy could be attributed to central population that is unique to this study. This population had never been included in any other studies before. The additional gene pool brought about by this population suggests it might be a new population introduced.

5.6.4 Genetic diversity of LEI0258 marker inference to biological significance

There was high diversity for LEI0258 marker as observed in the number of alleles. The observed polymorphism of marker LEI0258 locus might explain the plasticity of the chicken in coping with diseases challenges (Farmers personal communications; (Ngeno *et al*., 2014b)

conferring the highest selective advantage of B-heterozygosity for survival and immune competency. This locus has a considerable size diversity than expected for a simple sequence repeat in various literatures. It is worth noting that western population had the highest number of alleles and effective allele numbers. The western chicken population consists of indigenous chicken (IC) with minimal exotic chicken gene pool; this might imply that IC are more robust in combating diseases compared to the other populations. Several LEI0258 alleles were shared among the populations suggesting that they have been subjected to similar directional selection or due to recombination effect. Alternatively, this sharing of alleles might suggest identity by decent or shared binding site to antigenic determinants. There was high heterozygous genotype of the 38 LEI0258 alleles observed.

The heterozygosity of the MHC region may not be closely related to the heterozygosity of the rest of the genome (Van Marle-Koster and Nel, 2000), but may be more related to the environment under which the populations were developed as well as their selection history . (Pinard-Van Der Laan, 2002). The central populations were developed under more protected (against diseases) and controlled environments, and were under intensive selection for many generations, whereas western and coastal populations were developed under more open and exposed (to disease vectors) environments. Noncommercial free-run and free-range chickens (western and coastal populations) are likely to have been exposed to more parasite and disease vectors hence the many alleles and heterozygotes exhibited by these populations. The majority of genotypes that were created by 38 alleles found in this study were heterozygotes. A higher frequency of heterozygous individuals at the LEI0258 marker suggests a higher antigen diversity being presented to T cells to mediate disease tolerance (Chazara *et al.*, 2013). The high degree of heterozygosity observed could probably be immune plasticity mechanism to combat relatively high antigenic diversity prevailing in the environments in which these chickens have evolved. The frequency of heterozygosity at the MHC is also expected to be higher in out-bred populations exposed to all kinds of infectious agents with differing selection pressure as observed in this study.

The selection pressure might contribute to the difference of observed immune competency level in these populations. The western population has a reputation of being hardy and resistant to disease vectors, the coastal are moderate while the central population is the most susceptible (Farmer's observation). Based on the qualitative observation, we can generalize that

the central population seemed to have less genetic variability in the MHC region than that of the other populations. The results of this study led to the hypothesis that western population constituting of less diluted gene pool may have better general disease resistance compared with that of other population. For example, there was reported high prevalence of parasites in this population despite satisfactory performance (Kaingu *et al*., 2010). However, more association and challenge studies need to be conducted to test this hypothesis.

5.7 Conclusion

The LEI0258 is highly polymorphic and diverse in its repertoire. This helps in recognizing different epitopes during antigen processing and presentation. The locus showed four gene pools in Kenyan populations of chicken, a deviation from two observed in other studies. There was an additional gene pool circulating in Kenyan indigenous chicken.

CHAPTER SIX

THE EFFECTS OF DIETARY PROBIOTICS ON NATURAL IgM ANTIBODY TITRES OF KENYAN INDIGENOUS CHICKEN

6.1 Abstract

This study was conducted to investigate the effects of probiotic supplementation on serum natural IgM levels binding keyhole limpet hemocyanin in indigenous chicken (IC). One hundred and fifty two months old chicken raised under low input-output system were sourced from small scale indigenous chicken farmers from Nyakach and Emineng sub counties of Kisumu and Baringo, Kenya respectively. The IC were of mixed sex, randomly divided into five treatment groups of 25 birds each. The treatments were 5 ml of Molaplus dissolved into 250, 500, 1000, 1500 and 2000 ml of drinking water. The birds were raised into group cages, and fed commercial grower mash for two months during the study period. A window of 14 days was left for immune stabilization; blood was then drawn from the wing vein and serum separated immediately. Levels of IgM binding was assayed using an indirect ELISA technique. IgM binding KLH was found but dietary probiotic supplementations did not significantly affect levels of IgM binding KLH in the serum. Probiotic supplementation in diet did not further enhance KLH binding IgM in IC reared under village production system.

6.2 Introduction

The indigenous chicken (IC) industry has become an important economic activity in Kenya and many countries. In large-scale rearing facilities, where chicken are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of these diseases have led in recent decades to a substantial increase in the veterinary drug usage. The usage of these chemicals as a preventive measure has been questioned, there exist extensive documentation of the evolution of pathogens resistant to chemicals. The possibility of alternatives to drug usage may be the use of probiotics and/ or breeding for disease resistance (Khobondo *et al*., 2015b). Probiotics are being considered to fill this gap and already some farmers are using them in preference to antibiotics (Nava *et al.,* 2005). Probiotic, meaning 'for life' in Greek, are defined as ' live microbial feed supplement, which beneficially affects the host animal by improving intestinal balance' (Fuller, 2001). The species currently being used in probiotic preparations are varied and many. These are mostly *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus salivarius, Lactobacillus plantarum, Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis, Bifidobacterium spp.* and *Escherichia coli* (Kabir, 2009). Some other probiotics are microscopic fungi such as strains of yeast belonging to *Saccharomyces cerevisiae* (Fuller, 2001). Probiotics may be composed of one or a combination of many strains.

Probiotics are used to help maintain a healthy microbial balance within the intestine to promote gut integrity and prevent enteric disease (Cox and Dalloul, 2014). This is accomplished through three main mechanisms: competitive exclusion, bacterial antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). The manipulation of gut microbiota via the administration of probiotics influences the development of the immune response (McCracken and Gaskins, 1999). It has been shown that probiotics stimulate different subsets of immune system cells to produce cytokines, which in turn modulate the immune response (Lammers *et al*., 2003) and activate other cells. Amongst the subset of B cells, B-1 cells constitute the predominant subset of B cells in mammals (Islam *et al.,* 2004). While B-2 cells produce the majority of circulating specific antigen induced antibodies possessing high binding affinities. The antibodies secreted by B-1 cells called natural antibodies, typically have low binding affinities and broad specificities (Parmentier *et al.,* 2004). Natural antibodies are usually

produced without prior exposure to antigens (Khobondo *et al.*, 2015a; Khobondo *et al.*, 2015b). Foreign antigens like lipopolysaccharides (LPS), lipoteichoic acid (LTA), keyhole limpet hemocyanin, and bovine serum albumin (BSA) (Higgins *et al.*, 2007) that bind natural antibodies in the sera of unimmunized chickens have been found. In higher organisms including chicken, natural antibodies may be of isotype IgM, IgG, or IgA, but IgM is the most predominant isotype. In mammals, B-1 cells are responsible for production of natural IgM antibodies in serum (Khobondo *et al.,* 2015a) but in chicken B1 cells have not been defined yet. Natural IgM antibodies possess a wide range of activities, including regulation of immune response, induction of specific IgG antibodies, and protection against bacterial and viral infections (Nava *et al*., 2005).

Natural antibodies in the chicken bind to antigens in a specific manner and the affinity of these interactions increases with age, suggesting a role for external stimuli. These roles can be exploited for breeding for disease resistance. Due to consumer concerns on the use of antibiotics as growth promoters and prophylaxis in poultry diets, investigations evaluating the potential of dietary probiotics as substitutes for antibiotics do receive high priority and IC should not be exemptions. This is because, the IC are predominantly raised under extensive production system (Chapter 2). This expose the IC to various environmental challenges and disease causing pathogens hence the rationale of this study. Earlier, it was shown that probiotics stimulate natural antibodies in poultry (Haghighi *et al.*, 2006). This experiment was carried out to determine possible effects of dietary probiotics on serum natural IgM levels in IC. The findings could be used to prevent disease burden or compliment breeding for disease resistance.

6.3 Materials and Methods

6.3.1 Source of birds, feeding regime and management

A feeding trial was conducted using one hundred and fifty (150) indigenous chicken sourced at two months of age from free range small scale farmers. The farmers were from Nyakach and Emening of Kisumu and Baringo counties, Kenya respectively. The trial was done in a randomized complete design with control. There were 25 birds per treatment. The dietary treatments and water were offered ad libitum. To constitute the treatments, the probiotic were added into drinking water by giving a specific concentration of 5 ml of Molaplus microbes (Molaplus LTD, Kenya) in different volumes (250, 500, 1000, 1500, 2000 ml) of the respective water once a day at 0900 hours. The control had no Molaplus microbes added in drinking water. The Molaplus is a complex solution of various beneficial micro-organisms which are found naturally and are used in food manufacturing. When used in poultry production, they avail, chelated minerals, anti-oxidant, enzymes, vitamins, organic acids, lactic bacteria, yeast and phototropic bacteria (Molaplus.com). The experiment was done for a period of two months (60 days), thereafter IC were left for two weeks (14 days) for immunological stabilization, then blood sample was taken for natural antibody assay.

6.3.2 Natural antibodies (IgM) measurement

Blood samples (~2 ml in EDTA) from 150 IC was drawn from the wing vein of each bird and serum was separated immediately as described in section 3.3.2. Isotype specific IgM antibody titers to keyhole limpet hemocyanin (KLH) in serum from the IC was determined by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, 96 well plates were coated with 2 μg/ml KLH (MP Biomedicals Inc., Aurora, OH) and incubated overnight at 4^0C. Following washing with deionized water, the plates were incubated for 1.5 hours at 25^0C with IC serum diluted 1:10 with dilution fluid (phosphate buffered saline (PBS) containing 0.5% horse serum and 0.05% Tween). Unbound serum was removed through washing. To detect IgM antibodies binding to KLH a 1:20,000 diluted affinity purified goat anti-chicken IgM (Fc specific), conjugated with horseradish peroxidase (GACh/IgM (Fc)/PO) antibody (Nordic Immunological Laboratories, Eindhoven, The Netherlands) was added and incubated for 1.5 hours at 25^0C. After incubation with the conjugate and subsequent washing, 100 μl substrate-buffer (containing aquadest, 10% tetramethylbenzidine-buffer and 1.33% tetramethylbenzidine) per well was added and incubated for 10 minutes at room temperature. The reaction was stopped with 1.25M H_2SO_4.

Absorbance levels per sample was measured with a spectrophotometer (mrc Scientific Instrument-UT- 6100, Israel) at a wavelength of 450 nm.

6.3.3 Statistical analysis

The data (antibody titre values) was analysed by using one way ANOVA using Proc GLM of SAS (SAS Institute, 2009).

The following model was used;

$$Y_{ij} = \mu + PR_i + e_{ij} \quad \text{.....(4)}$$

Where:

Y_{ij} = the IgM absorbance level (titre value),

μ = the overall mean,

PR_i = the fixed effect of treatment (i=2),

$e_{ij...}$= the residual error.

6.4 Results

6.4.1 Presence of natural IgM antibodies binding KLH in serum of IC and effects of dietary probiotics

Natural IgM antibodies binding KLH were detected in IC serum but, there was no significant difference between the IC fed probiotics and the IC not fed probiotics (control) ($P > 0.05$). Neither was there significant difference in the titres of KLH binding IgM at 14 days after treatments ($P > 0.05$) and between the treatments (different inclusion levels of probiotic concentration). However, the control group (no probiotic fed) had higher Least Square Means (LS means) than the birds fed probiotics of 1.753 and 1.730 respectively (Table 6.1).

Table 6.1 The LS means and standard error (SE) of the dietary treatment of IgM titres binding KLH.

Isotype	IgM		
Parameter	**LS means**	**Std error**	**P value**
Control	1.75	0.121	
Probiotic	1.73	0.065	0.873

LS means = least square means; S.e = standard error

6.5 Discussion

In accordance with previous findings, the present study revealed that serum antibodies (IgM binding KLH) are present in unimmunized IC. Since the IC likely did not encounter before, will not encounter KLH and cross reactivity with other antigen is unknown, this IgM antibodies binding KLH can be regarded as natural antibodies. Natural IgM antibodies isotype are amongst the innate immunity (Vani *et al*., 2008). Innate immunity as the first line of defense plays an important role in preventing or combating infection (Ehrenstein and Notley, 2010). Natural antibodies have been detected in non-immunized cattle (Van Knegsel *et al.*, 2007), humans (Ehrenstein and Notley, 2010), rats, rabbits, fish, snakes and poultry (Sun *et al.*, 2011). In mammals, Nabs are mostly produced by CD5+ B cells in the peritoneal cavity and intestines but also CD5- B cells (Casali and Notkins, 1989) were described to produce Nab. Natural antibodies may arise independently of known antigenic stimulation. They are mostly poly-reactive, and poly-specific (Baccala *et al.*, 1989) with low binding affinity, and are generally encoded by the un-mutated V genes in germ line configuration (Khobondo *et al*., 2015b) Evidence from various studies show that they are genetically controlled and inherited (Sun *et al.*, 2011). Most of Nab are of the IgM isotype class in lower vertebrates, fetus and neonates, but IgG and IgA Nab are also present as well in higher vertebrates (Boes, 2000). Production of Nabs may also be induced by contact with non-pathogenic microbes, food, intestinal flora, probiotics and self-antigens (Quintana and Cohen, 2004).

Most Nab bind pathogen-associated molecular patterns (PAMP), e.g. lipopolysaccharide, lipoteichoic acid or peptidoglycan conserved along different genera and these serve as targets for identification of microbes by the innate immune system (Parmentier *et al* 2004). The levels of Nabs are likely dependent on several factors, amongst them the environment (Kachamakova *et al.*, 2006), genetic background (Ardia *et al.*, 2011) and age (Berghof *et al.*, 2010). Despite the plethora of data demonstrating the positive effects of probiotics on immune function (Haghighi *et al.*, 2006), this study and some others have reported no significant enhancements due to probiotic supplementation (Rahimi *et al.*, 2011). It has to be kept in mind, however, that titres of IgM to KLH were measured 14 days after dietary treatment, so short term temporary effects could not be found. It is clear from the present study and other published research that responses to probiotic supplementation are inconsistent. Numerous investigations were done on possible factors that could influence the responses to these additives. For example, in broilers possible

causes of variation in response to probiotic supplementation could be attributed to differences between strains, hybrids, age, sex, plane of nutrition, nutrient composition of the diet, microbial population of gastrointestinal tract, levels of inclusion of probiotics in the diet, duration of supplementation or other environmental conditions (Midilli *et al.*, 2008). In this study involving IC, administration of probiotics did not significantly enhance serum IgM antibodies reactive to KLH. In this case, probiotic treatment resulted in the reduction of the mean reactive IgM antibodies in serum of the IC. These discrepancies could be due to a variety of factors including, but not limited to, strain(s) of bacteria utilized, composition and viability of the probiotic, preparation method, dosage and application method, frequency of application, overall diet, drug interactions and condition of the animal (Huang *et al*., 2004). The experimental design, source of birds and early stage production system could be the cause of discrepancies in result of this study. It is worth noting that the IC used here were naturally hatched and brood, and the rearing was free range as well. This management system from incubation, hatching to the time they were sourced (2 months old) might have exposed them to these commensal microbes from the feces and environment. The chicken could have received a complete gut flora from the mother's faeces and would confer immune response similar to probiotic microbes. The shell microbial contamination during incubation and hatching could also play a role. The free range extensive system of IC production may have predominantly exposed the IC to plethora of microbes, infested the gut with several microflora and consequently influenced Nab levels. These results are in agreement with the results of other studies in which probiotics (Huang *et al.*, 2004) or prebiotics (Franklin *et al.*, 2002) or combinations of probiotics and prebiotics (Midilli *et al*., 2008) were used in different animal species.

Several studies reported the role of probiotic in augmenting immune response (Cox and Dalloul, 2014). There is evidence that probiotics stimulate production of natural antibodies (Haghighi *et al*., 2006) and different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of immune responses (Maassen *et al.*, 2000). It was found recently Nab levels in elite improved breeds reflect different physiological health status (in this case enhanced survival) as opposed to IC kept in confinement (in which Nab levels may signal stress status (Wondmeneh *et al.*, 2015). Thus enhancement or decrease of Nab in birds may mimic sensitivity to stress or changing (dietary) conditions, indirectly reflecting the animals, condition to respond. In IC, conditions due to husbandry may have been such that

probiotics could not further enhance or decrease immune sensitivity. The induction of immune response and the preimmune antibody repertoire is a subject of debate. It is possible that resident dendritic cells (DCs) in the lamina propria, which directly sample the intestinal lumen and engulf commensal bacteria, could play a role (Yaman *et al*, 2006). DCs express a repertoire of Toll-like receptors (TLRs) (Kabir *et al.*, 2005), and binding of structural components of commensal bacteria or probiotics to TLRs expressed on the surface of DCs may lead to activation and maturation of these cells (Apata, 2008). Upon activation, DCs process and present antigens to other cells thereby promoting the activation and differentiation of different subsets of immune system cells, leading to the production of Th2 cytokines, such as interleukin 4 (IL-4), IL-10, and transforming growth factors, that are important for antibody production and isotype switching (Apata, 2008). The former may have the implications on the Nabs level witnessed in this study. Most microbes used in probiotic may have shared PAMPs with the microbes already ingested by the chicken in free range production systems.

6.6 Conclusion

This study provides no evidence that the administration of probiotics to IC diets caused any significant changes in systemic natural IgM antibody concentration. Although non-significant reduction results were observed for the examined parameter, further studies are needed to understand the effects of these additives and to clarify the effect on the immune status of IC. More comprehensive experimental designs examining performance and humoral immunities especially natural IgG and IgA and levels of adaptive IgA, IgG and IgM responses, age of exposure and production systems should be conducted.

CHAPTER SEVEN

MICROBIAL DIVERSITY AND POTENTIAL BACTERIA CANDIDATES FOR IMMUNE ENHANCEMENT IN CHICKEN

7.1 Abstract

Gut microbiome affected by different genetic makeup of chicken may be partially responsible for the difference in productivity traits and susceptibility to diseases. We investigated the difference in gut microbiome among chickens of seven genetic backgrounds. For each genetic group, fecal samples were collected from 5 individuals and pooled together by weight. Genomic DNA was extracted from those samples and Illumina sequencing of the V4 hypervariable region of bacterial 16S rRNA gene amplicons was performed. A Qiime pipeline was used to call operational taxonomic units. Alpha and beta diversity indices were calculated for genetic groups. A phylotype-based analysis was performed on the sequence data (abundance data) and significant differences in microbiome abundance among genetic groups and immune competency levels were tested ($p \leq 0.05$) at phylum, class, order, family and species level. The results showed great microbiome diversity in the samples. The microbiome relative abundance differed among genetic groups and immune competency levels. Some taxa showed significant difference in microbial abundance between immune competency levels. At phylum and family level only *Elusimicrobia* and *Bacillaceae* were significantly different respectively. Order and species levels showed more taxa being significantly different. Further studies on these phylotypes may help to find non-antibiotic alternatives to enhance productivity and protect chickens from diseases.

7.2 Introduction

The gastrointestinal (GI) tract of chicken is densely populated with microorganisms which interact with the host and ingested feed intensively and closely. Their interactions affect the physiological, immunological and nutritional status of the host (Zhang *et al*., 2010). The gut microbiome benefits the host by providing nutrients from otherwise poorly utilized dietary substrates and modulate the development and function of the digestive and immune system. Consequently, the host provides a permissive habitat and nutrients for bacterial colonization and growth (Snel *et al.*, 2002). With respect to nutrition, members of gut microbiome function as interface between the host and feed ingested, thus hydrolyzing and then fermenting indigestible carbohydrates, producing short chain fatty acids (SCFAs) which serve as extra energy and carbon sources to be utilized by the host (Hooper *et al.*, 2002). A comprehensive understanding of these interactions has helped develop new dietary or managerial interventions that had enhanced bird growth and maximized host feed utilization. In immunity, gut microbiome as a whole helps the development of GI tract and educate birds' immune system and reduces risk of enteric diseases, assuring the functionality of the digestive system (Crhanova *et al.*, 2011). Several factors; diet, age and host genetics may affect gut microbiome composition and diversity. The role of host genetics on shaping this vital gut microbiome is now clear (Zhao *et al.*, 2013). The genotype of the host may affect its microbiota composition either directly through secretions into the gut, control of gut motility and modification of epithelial cell surfaces, or indirectly, through food and lifestyle preferences. These effects are likely to be small, and detecting them will require well controlled effects other than those of the host genotype.

There has been rapid progress in methodologies employed in studying microbiome from early cultivation to next generation sequencing. The earliest reported were by using culture-dependent methods (Barnes *et al.*, 1972) to recent molecular technologies involving high throughput next generation sequencing (HT-NGS) and omics studies (Simon and Daniel, 2011). The HT-NGS targeting on 16S rRNA genes have been extensively used to study microbial diversity in human and animal species (Caporaso *et al*., 2011). The 16S gene occurs in all living organisms, with the notable exception of viruses and represents more than 80% of total bacterial RNA (Caporaso *et al*., 2011). Next-generation sequencing (NGS) technologies make it possible to comprehensively characterize the intestinal microbiome of poultry, and the sequence information has greatly expanded our knowledge on the bacterial diversity present in the

intestinal tract of chickens and turkeys (Wei *et al.*, 2013). Through phylogenetic and statistical analysis of 16S rRNA gene sequences recovered from intestinal microbiome of both chickens and turkeys, a global bacterial census was created for poultry intestinal microbiome (Wei *et al.*, 2013). The exploration of these microbiome diversity to increase productivity has been successful in chicken and ruminants (Taff *et al.*, 2016). Highly productive chickens have been developed by selection for elite genetic traits; it is possible that in the future, gains in productivity and health outcomes could be influenced by selection of elite GI tract microbiota (Stanley *et al.*, 2014).

Therefore, studies on the composition and functions of gut microbiota in chicken of distinct immune competency levels are significant for the improvement of immune response and health status. This strategy is poised to alleviate use of antibiotics for growth promotion and disease prevention. Consumer interest in free-range and organic poultry is growing due to concern in use of antibiotics and residue associated with their use. Furthermore, the meat of the free range chickens had more protein than the indoor chickens (Castellini *et al.*, 2008). The chicken population used in this study are well named local breeds in Kenya with characterized production system (Khobondo *et al.*, 2015). This study aimed to comprehessively characterize the structure and diversity of the bacterial populations in fecal matter, explore the composition and their functions in relation to immune competency. We further demonstrated that absence or presence of certain bacteria might be correlated with immune competency, thus, providing base information for developing applicable probiotics.

7.3 Materials and methods

7.3.1 Animals and sample collection

Fecal samples (droppings on ground) were collected from 35 chicken of seven groups (Kuchi Egerton, Kuchi Maseno, indigenous western, Rhode Island Red, Kroiler, improved KALRO kienyeji white and black genotypes). In addition, 41 individual Kuchi Maseno chicken of the same age and under similar management had their feces sampled. To make the genetic pool, the fecal contents of 5 individual birds of same genetic group were pooled together to reduce inter individual variation as reported elsewhere (Zhou *et al.,* 2007). The fecal material was wrapped in foil in Eppendorf tube and immersed in cold ice. Subsequently it was transported cold (in dry ice) to the laboratory. Samples were stored at −80°C until processed. The chickens had no history of gut infectious disease or antimicrobial administration in the preceding 2 months and were fed an antibiotic-free diet formulated to meet their nutritional requirement.

7.3.2 Pre-processing and cell lysis.

First, freeze stored fecal samples were transferred in a pre-chilled 2 ml microcentrifuge tube and resuspended into 1% PBS buffer in 1:1 ratio to homogenize. Since, all the samples contain both microbial cells and extracellular matrix like, mucin or undigested food particles, it was important to detach the microbes for adequate access of buffering agents and enzymes, used for spheroplast formation. With this aim, 100 glass beads (2.5 mm) were added and vortexed continuously for 1 min or until the sample was thoroughly homogenized. 300 mg of zirconia beads were added to the suspension and cell lysis done by mechanical disruption using SpeedMill PLUS bead beater (Analytical Jena, Germany) for 10 (ten) minutes.

7.3.3 DNA extraction quantification and quality check

Total DNA from the fecal samples was extracted using ZR Soil Microbe DNA MiniPrep^{TM} (Cat#6001) (ZYMO RESEARCH, Irvine, 17062 Murphy Ave. Irvine, U.S.A) following the manufacture's recommendation but with cell disruption modification. The total DNA was quantified and purity assessed via 260/280 and 260/230 absorbance ratios, usingthe Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Genomic DNA quality and integrity was checked on 0.8% agarose gel at 5v/cm. Samples were stored at -20°C until PCR amplification.

7.3.4 PCR amplification of 16S ribosomal RNA

Bacterial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using single-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences under the following parameters: 95°C(3:00)+[95°C(0:45)+50°C(1:00)+72°C(1:30)] × 35 cycles +72°C(10:00) +4°C(Hold). The following 515fB PCR barcoded forward Primer Sequence 5′-AATGATACGGCGACCACCGAGATCTACACGCTXXXXXXXXXXXXTATGGTAATT GT GTGYCAGCMGCCGCGGTAA- 3', and 806rB PCR Reverse primer- 5′ CAAGCAGAAGACGGCATACGAGATAGTCAGTCAGCCGGACTACNVGGGTWTCTAAT - 3' were used. The amplicons were purified by GeneJet PCR Purification kit (GeneJet, K#0701) (Thermo Fisher Scientific, Waltham, MA).

7.3.5 Quantification and quality check of purified 16s rRNA amplicons for sequencing.

DNA amplicon concentrations were determined fluorometrically using Qubit dsDNA BR assay (Life Technologies, Carlsbad CA) and purity was assessed via 260/280 and 260/230 absorbance ratios using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The amplicons were then pooled by concentration to 40ng/ul per sample before pooling. The quality check of pooled amplicon was done using Agilent Technologies on a tape station. Amplicons were sequenced using the Illumina MiSeq platform and V2 chemistry with 2×250 bp paired-end reads, as previously described by Caporaso *et al*. (2011). Samples returning greater than 10,000 reads were deemed to have successful amplification.

7.3.6 Quality check for sequence data

Informatics analysis, assembly, binning, and annotation of DNA sequences was performed as follows. Contiguous DNA sequences were assembled using USEARCH and UCLUST scripts (Edgar, 2010). Culling of sequence was done if a) it was found to be shorter than 150bp after trimming, b) average phred score was less than 25, c) it containedambiguous bases, d) homopolymer run exceeded 6, or e) there were mismatches in primers.

7.3.7 Taxonomy classification and statistical analysis

Qiime v1.8 (Caporaso *et al*., 2010) software pipeline was used to perform de novo and reference-based chimera detection and removal, and remaining contiguous sequences were assigned to operational taxonomic units (OTUs) via de novo OTU clustering and a criterion of 97% nucleotide identity. Taxonomy was assigned to select OTUs using BLAST against the Greengenes database (Release 13.8, http://greengenes.secondgenome.com/) of 16S rRNA sequences and taxonomy (DeSantis *et al.*, 2006). Ace, Chao, Simpson index were calculated using summary single command in QIIME. For specificity, alpha diversity indices were analyzed statistically using Past3 software (Hammer *et al.*, 2008). One way analysis of variance (ANOVA) and test of significant differences between immune competency level means were determined using Tukeys test in SAS. Beta diversity of Principal component and principal coordinate analyses were performed using OTU relative abundance data via the Bray–Curtis algorithm of Past3 software (Hammer *et al.*, 2008).

7.4 Results

7.4.1 Topographical differences in 16S sequence richness and diversity in pooled and individual samples

A total of 5,188,316 and 3,609,076 sequence reads were obtained before and after quality check respectively with a median length of 253 base pairs (bp) (V4 ~ 151–253 bp). Rarefaction curves generated from the OTUs suggested that moderate to high sampling coverage (~97%) was achieved. Based on our results, distinctive differences in fecal microbiota richness and diversity between pooled and individual samples were observed. This was shown based on the rarefaction curves (Figure 7.1) where individual samples had significantly greater richness, diversity and variation. In spite of this high sampling coverage, rarefaction curves also showed at least some samples did not have that many sequences (lines not extending to the end of x – axis). Most of such samples were pooled samples (Figure 7.1). This showed a clear indication of the differences in sequence coverage between pooled and individual samples. It is worth noting that alpha diversity measurements only reflect overall diversity, but not significant differences of relative abundance among species or groups. This necessitated the statistical test undertaken later.

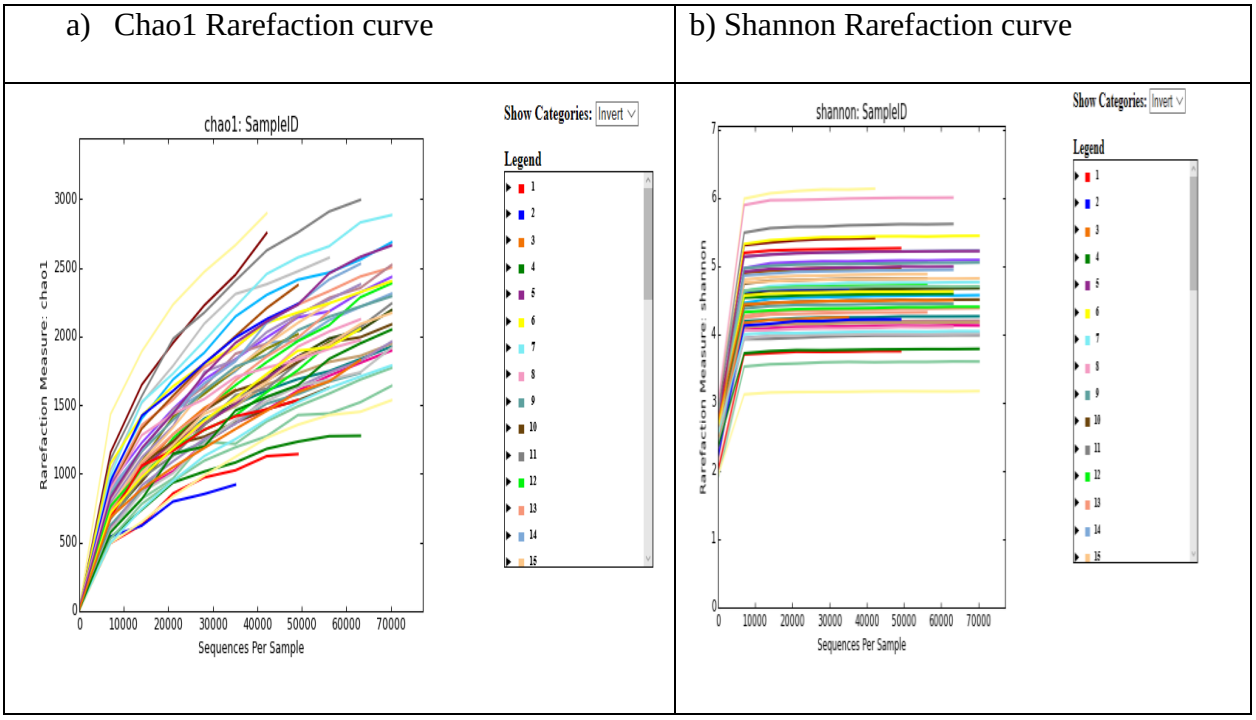

Figure 7.1 a) Chao1 **b)** Shannon rarefaction curves showing sampling coverage

7.4.2 Principle Coordinate analysis (PCoA)

The PCoA analysis did not show distinct clustering of samples instead were they spread all over suggesting the independence of each individual chicken on relative microbiome abundance (Figure 7.2).

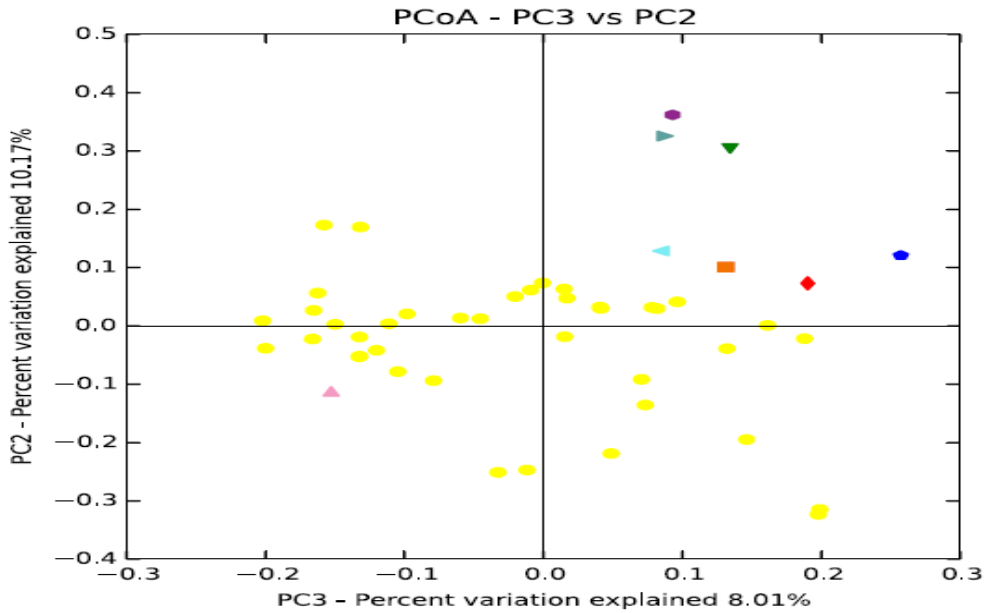

Figure 7.2 PCA plot showing the grouping of 7 pooled and 41 individual chickens based on the structure of their gut microbiome

The observed PCoA results and diversity indices warranted the splitting of relative abundance data into genetic groups and individual chicken for downstream analysis.

7.4.3 Global diversity of microbiome of all samples

The microbial diversity and compositional distribution pattern of different taxonomic classification including kingdom, phylum, class, order, family, genus and species level are presented in table 7.1. At kingdom level prokaryotes (archaea and bacteria) were detected.

Phylum: In total 26 phyla were observed, 24 were known bacterial phyla while 2 were unassigned. *Firmicutes* and *Proteobacteria* were the most abundant phyla at 65% and 27%, respectively. Other predominant microbiome included *Actinobacteria* and *Bacteroidetes* at 6% and 2% respectively. *Chlamydiae, Lentisphaerae* and *Fusobacteria* were found to be less abundant (less than 1%) in this study at this level.

Class: A total of 50 different classes were detected. *Bacilli* (61%) was the most abundant class among the genetic groups. *Gammaproteobacteria* (10.2%), *Actinobacteria* (10.8%), *Clostridia* (10.8%), *Bacteroidia* (3.4%) and *Betaproteobacteria* (2%) were among the predominant classes with over 3% presence (Table 7.1)**.** Other less predominant classes identified were *Lentisphaeria, Anaerolineae, Acidimicrobiia, Fusobacteriia, Rubrobacteria* and *Pedosphaerae.* However, *Bacilli and Gammaproteobacteria* sequences were overrepresented hence did not fall under 95% confidence eclipse.

Table 7.1 Global microbial composition and diversity in GIT of Chicken

Taxa	**Microbial composition**	**Most Abundant**
Kingdom	2	
Phylum	26	Firmicutes (74.8%); Proteobacteria (10.8%); Actinobacteria (10.8 %); Bacteroidetes (3.2%); Unassigned Others (0.3%).
Class	50	Bacilli (66.1%); Gammaproteobacteria (10.2%); Actinobacteria (10.8%); Clostridia (10.8%);Bacteriodia (3.4%)
Order	80	Lactobacillales (51.4%); Pseudomonadales (4.6%); Bacillales(11.1%); Enterobacteriales (5.5%); Clostridiales (8.7%),
Family	107	Enterococcaceae (26.4 %), Lactobacillaceae (7.9%), Enterobacteriaceae (5.5%), Lachnospiraceae (4.7%) Leuconostocaceae (6.1%)
Genus	343	Weissela (3.5%), Lactobacillus (7.8%), Enterococcacus (26.2%),Actinobacter (2.4%), corynebacterium (3.7%)Staphylococcus (8.4%) and Brevibacterium (3.4%).
Species	193	*Staphylococcus sciuri* (38%), *Jeotgalicoccus psychrophilus* (13%), *Lactobacillus brevis* (9%), *Brachybacterium conglomeratum* (6.7%), *Pseudomonas stutzeri* (5.4%), *Enterococcus cecorum* (5.4%), *Acinetobacter lwoffii* (3.8%), *Lactobacillus reuteri* (3.7%), *Lactobacillus agilis* (2.5%)

Order: Eighty taxa at the order level were detected of which Lactobacillales (51.4%), Pseudomonadales (4.6%), Bacillales (12%), Turicibacterales (8%), Enterobacteriales (5.5%), Actinomycetales (6%), Clostridiales (8.7%), Burkholderiales (2%) and Bacteroidales (1%) constituted orders with more than 1% relative abundance. The rest had relative abundance of less than 1%. Of the 80 taxa only three orders (Bacillates, Actinomycetales and Lactobacillates) were not within 95% confidence level.

Species: The relative abundance of species are shown in figure 7.3. The most abundant species were *Staphylococcus sciuri, Jeotgalicoccus psychrophilus* and *Lactobacillus brevis* at 38%, 13% and 9% respectively. Others that accounted for more than 1% relative abundance were *Brachybacterium conglomeratum* (6.7%), *Pseudomonas stutzeri* (5.4%), *Enterococcus cecorum* (5.4%), *Acinetobacter lwoffii* (3.8%), *Lactobacillus reuteri* (3.7%), *Lactobacillus agilis* (2.5%), *Gallibacterium genomosp* (2.1%), *Lysinibacillus boronitolerans* (2.0%), *Bacteroide fragilis* at (1.6%), *Bacteroides barnesiae*(1.4%) and *Sphingobacterium faecium* (1%). As shown in the figures 7.1 these species constituted over 96% of the total relative abundance. However, of all the 62 identified known species, *Staphylococcus sciuri, Jeotgalicoccus psychrophilus, Lactobacillus brevis* and *Pseudomonas stutzeri* were the only species that were not distributed within 95% confidence eclipse.

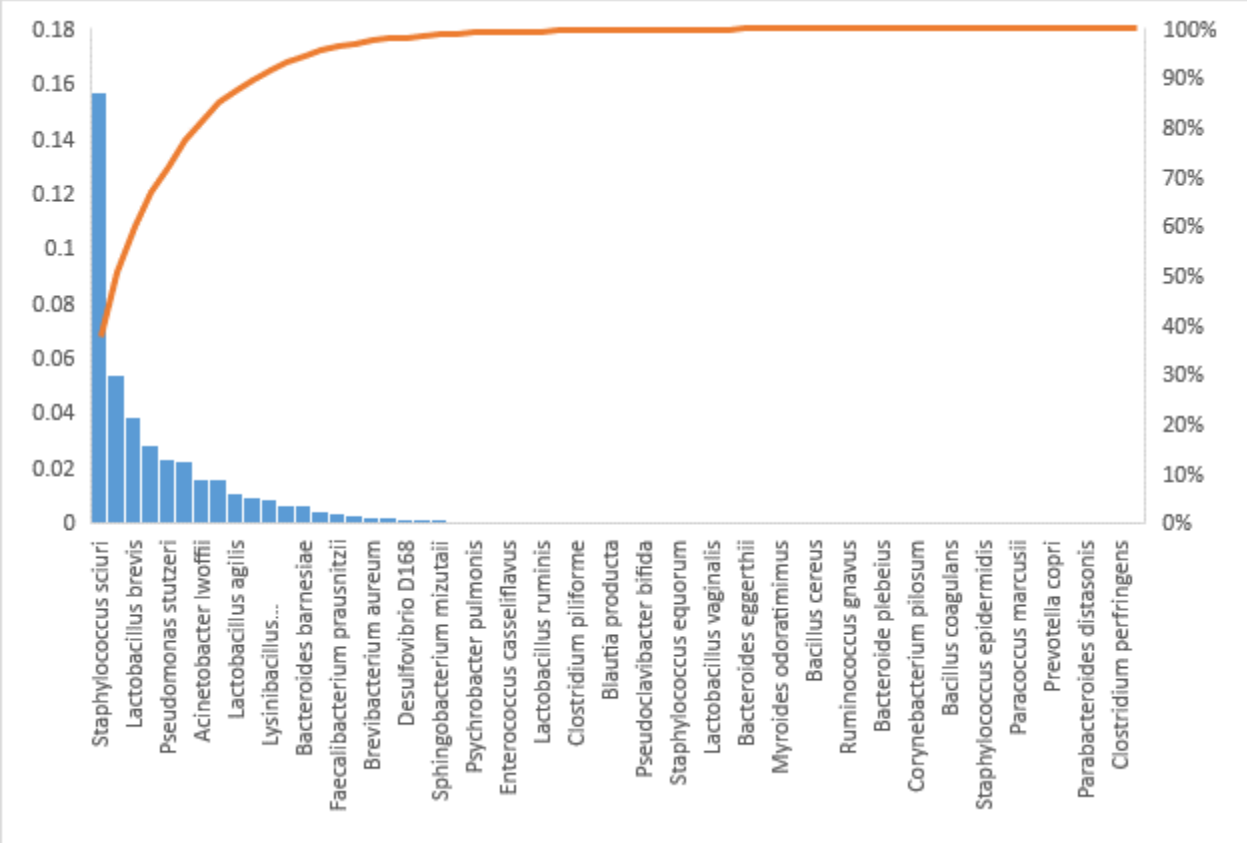

Figure 7.3 The relative abundance of OTUs at species level

7.4.4 Identification of OTUs with differential relative abundance between immune competency groups

The alpha (Figure 7.1) and beta diversity (figure 7.2) analyses did reveal subtle differences in gut microbiome among the genetic groups. Therefore, one way ANOVA fiting immune levels (resistant, moderate resistant and susceptible) as independent variable was employed to test for significant differences at different taxa. At phylum level only *Elusimicrobia* was significantly different, while *Coriobacteriia, Flavobacteriia, Chloroplast, Clostridia, Epsilonproteobacteria* and *RF3* were significantly different at class level. At Order level several taxa including *Coriobacteriales, Flavobacteriales, Streptophyta, Turicibacterales, Clostridiales, MBA08, Pirellulales, Rhodospirillales, Campylobacterales, Enterobacteriales, Xanthomonadales* and *ML615J-28* showed significant difference. Only six species level phylotypes, namely *Staphylococcus equorum, Rothia aeria, Bacteroides fragilis, Bacteroides plebeius, Blautia producta* and *Janthinobacterium lividum* showed significant variation in immune competence levels.

7. 5 Discussion

The microbial diversity and compositional distribution pattern of different taxonomic classification including kingdom, phylum, class, order, family, genus and species level was determined. At kingdom level prokaryotes (archaea and bacteria) were detected. This is consistent with the 16S protocol that was used in this study which is designed to amplify bacteria and archaea using paired-end 16S community sequencing on the Illumina platform. More so, using the universal primers 515F-806R which target the V4 region of the 16S SSU rRNA could have contributed to more detection of bacteria (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/). At phylum level several taxa were observed while 2 were unassigned. The two unassigned phyla indicated our limited knowledge on bacterial community residing in chicken GI tract. The unassigned OTUs at phylum level represents a collection of all sequences that cannot be classified at deeper taxonomy ranks (i.e. class) or otherwise. In this study such OTUs were not used for subsequent analysis and further reference was restricted because it is difficult to interpret result emanating from them.

Firmicutes and *Proteobacteria* were the most abundant at all populations indicative of high sequences obtained from this taxonomic level. This observation is consistent with previous report (Wei *et al.*, 2013) despite difference in the type of samples and region of 16S rRNA sequenced**.** On the contrary, our findings differed from previous results which reported *Proteobacteria* was the most dominant followed by *Firmicutes* and *Bacteroidetes* in fecal samples of broiler growers (Singh *et al*., 2012). It should be noted that direct comparison of OTUs and taxonomic composition between reported studies and present study may not be accurate due to differences in approaches, region sequenced, concepts of study and genetic background of the chicken used. Several other factors such as environment, treatment, feed additive, antibiotic, age, horizontal gene transfer, hygiene level, diet, type of chicken, geography and climate may also affect the chicken gut microbiota (Danzeisen *et al.*, 2011). These difficulties in comparison are aggregated by different approaches to sample analysis with some studies looking at results from individual samples and others on pooled samples (Yeoman *et al*., 2012). In this study, other predominant microbiome included *Actinobacteria* and *Bacteroidetes*. Overall, this was in line with pyrosequencing analysis that detected the same dominant species within a sample as those detected by clone libraries (Orcutt *et al.*, 2009).

Firmicutes, Bacteroides, Proteobacteria and *Actinobacteria* are therefore by consensus, the most abundant phyla observed in chicken studies irrespective of temporal and spatial sites. The predominant taxa of *Firmicutes, Bacteroides and Proteobacteria* might be influenced majorly by diet than genetics. These taxa are believed to accelerate fermentation of otherwise indigestible carbohydrates, thereby leading to increased acetate production and subsequently higher energy absorption (Zhang *et al.,* 2009). 7.4.4

Identification of OTUs with differential relative abundance between immune competency groups did reveal subtle differences in gut microbiome among the genetic groups. It is possible that genetic background only affected a small number of OTUs which did not cause major clustering on the PCoA plot. The observation or analysis at species level could be explored for possible usage of probiotics to enhance immunity. Probiotics are defined by the WHO/FAO as: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Many commercial products use multi-strain probiotics, although the benefits of using more than one strain or species in a single product has not been clearly established (Zhao *et al*., 2013). A review indicated 9 selection criteria for probiotics: 1) health benefit on the host; 2) viability upon intake; 3) survival during passage; 4) adherence to the gut epithelium; 5) antagonism against pathogens; 6) stabilization of microbiota composition; 7) animal origin; 8) stability to bile, acid, enzyme, oxygen; and 9) safety (includes being nonpathogenic, non-toxic, non-allergic, non-mutagenic) (Sharma *et al.*, 2014).

The literature search and review of the significantly different species found none to be among the probiotics used in animals and humans (FAO/WHO, 2001). Therefore, our finding is the first to propose their usage on chicken. On the basis of safety, *Rothia aeria* (Michon *et al.*, 2010), *Bacteroides fragilis* (Nagy *et al.*, 2011) and *Staphylococcus equorum* were found to be pathogenic and excluded for further consideration. S. equorum wasfound in relevant human clinical materials (Alcaráz *et al.*, 2003). This species was originally isolated from healthy horses (Schleifer and Kilpper-Balz, 1984), and later isolates were obtained from the milk of a cow with mastitis and from healthy goats (Meugnier *et al*., 1996).*Bacteroides plebeius*, *Blautia producta* and *Janthinobacterium lividum* were found to be non pathogenic. *Bacteroides plebeius* was found as normal flora of Japenese gut microbiota (Hehemann *et al.*, 2010). It plays an essential role in the catabolism of dietary fibers using carbohydrate active enzymes (CAZymes). The dietary fibre are the part of plant material in the diet that are not metabolized in the upper

digestive tract. , Many monogastric genome does not encode it adequately (Hehemann *et al.*, 2010). Besides, it has been reported in protecting the host against pathogens (Rakoff-Nahoum *et al.*, 2004). *Janthinobacterium lividum* and *Blautia producta* have been reported to have antifungal effects (Rebollar *et al.*, 2016). *B. producta* is a commensal anaerobic bacteria in the gut that provide a key defense mechanism by inhibiting the growth of potentially pathogenic bacteria. One possible mechanism for maintaining pathogen GI tract colonization resistance involves commensal anaerobe induction of mucosal immune effectors (e.g. antimicrobial peptides, AMP) that directly kill the pathogen.

Manipulation of these mucosal immune effectors can not only decrease GI tract colonization with potentially pathogenic bacteria but also decrease the risk of invasive infection (Brandl *et al*., 2008). *B. producta* induce greater colonic expression of the transcription factor Hypoxia inducible factor one alpha (HIF -1α), an important regulator of mammalian innate immunity, and the cathelicidin antimicrobial peptide LL -37 with antifungal activity, as compared with other commensal bacteria. Furthermore, *B. producta* produce small chain fatty acids (SCFAs) that signal through the G protein-coupled receptor GPR43 (Arpaia *et al*., 2013) and can induce host immune responses, including induction of regulatory T (Treg) cells (Smith *et al*., 2013) and antimicrobial peptides (AMPs) (Schauber *et al*., 2003). Therefore, literature suggest immune enhancement by probiotics could be cross-talk between innate and acquired immunity (Cox and Dalloul, 2014). It is possible that resident dendritic cells (DCs) in the lamina propria, which directly sample the intestinal lumen and engulf commensal bacteria, could play a role (Yaman *et al*., 2006). DCs express a repertoire of Toll-like receptors (TLRs) (Kabir *et al.*, 2005), and binding of structural components of commensal bacteria or probiotics to TLRs expressed on the surface of DCs may lead to activation and maturation of these cells (Apata, 2008). Upon activation, DCs process and present antigens to other cells thereby promoting the activation and differentiation of different subsets of immune system cells, leading to the production of Th2 cytokines, such as interleukin 4 (IL-4), IL-10, and transforming growth factor, that are important for antibody production and isotype switching (Apata, 2008).

7.6 Conclusion

There exist extensive microbial composition and diversity within GI tract of IC. A few groups (phylum, class, order, family and species) of bacteria were found to be significantly difference in relative abundance among different immune competency level. These phylotypes

may be partially responsible for the differences in productivity and disease resistance seen in chickens of different immune competency levels.

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 Indigenous chicken genetic resources variability

Local or indigenous chicken, among chicken species, show the highest rate of variation of population types. For the local chicken characterized so far in Nigeria, Botswana, Kenya, Malawi, Sudan, Ethiopia etc. a great variation is observed in morphological characteristics and production parameters (Chapter 2). With respect to body weight three variants have been distinguished i.e. dwarf, normal and heavy body weights. Plumage pigmentation is varied but mainly tends towards blackish and brownish colours showing extended and pied colourations. Plumage distribution is mainly normal while special forms such as naked neck, frizzle and silkiness appear sporadically. The comb is mostly single but rose, pea, walnut, duplex and crest are also present. The shank and skin are also frequently pigmented showing green, grey and blue variants. In addition, melanin deposition in skin, meat, internal organs and bones are also encountered in some genotypes (FAO, 2010). Some of these variants are due to the presence of major morphological marker genes which increases the adaptability of these breeds to tropical climatic environments. For example, the comb type indicates that the bird have been favoured by hot climatic conditions whereby large comb, such as single comb allows for efficient heat regulation (Apuno *et al.,* 2011). Scientists have conducted a series of experiments utilizing the indigenous chicken of many countries and several major genes were identified. These genes can be split into three categories; feather reducing genes, genes that reduce body size and genes that control plumage colour (FAO, 2010). The genes were associated with ecological areas. The feathered chickens/genotypes are predominant in cold climates; their body is well covered with feathers to help in insulation and protection against losing body heat. The warm and hot climates are dominated by naked necks and frizzle feathers, expression caused by incomplete dominant genes Na and F respectively. This feature allows better heat dissipation. The naked-neck genotype is characterized by featherless skin on the neck, on the breast and on ventral part of the thigh (Chapter 2).

Recent advances in molecular genetics and genomics has given more insight to these indigenous chickens. Major histocompatibility complex (MHC)-linked markers, LEI0258 and

MCW0371 found 46 alleles (194-550 bp) and 10 alleles (198-207 bp), respectively for Kenyan indigenous chicken (Ngeno *et al.,* 2014). Molecular characterization of indigenous chicken in Ethiopia, Uganda, Sudan and Kenya using 30 microsatellite markers, found that the sampled indigenous chicken can be grouped into six genetic clades thus minimizing the morphological variants (Mwacharo *et al*., 2007). However, another study by the same author using 30 autosomal microsatellite markers on genomic DNA revealed three major gene pools/groups (Mwacharo *et al.,* 2013). That study also revealed population admixture between the three gene pools with east to west genetic cline of gene pool two. Clustering analysis by Ngeno *et al.,* (2014) indicated a clear ecotype subdivision into two to three genetically distinct groups for MHC linked microsatellites and two groups for non MHC markers. This finding confirms that these IC ecotypes host many and highly diverse MHC and non MHC markers showing genetic variability. Cameroon native chicken population was clustered in three distinct groups according to agro-ecological zones (ecotypes) and their genetic make-up for LEI0258 and MCW0371 loci. Their findings concluded that the genetic diversity of the Cameroon native chicken MHC-B is very rich and potential resource for the development of new serological reagent adapted to chicken outbred populations (Hako Touko *et al.,* 2015).

The sequencing of the different animal genome made large amounts of genetic markers available in the form of single nucleotide polymorphisms (SNPs). This massive increase in marker numbers, allowed diversity studies and routine genome-wide association studies (GWAS) to be performed in many animal populations (Meuwissen *et al.,* 2001). For instance, Horro and Jarso indigenous chickens of Ethiopia were genotyped with a 620K SNP array. A multidimensional scaling analysis showed that the two populations were genetically distinct (Psifidi *et al.,* 2014) thus confirming diversity. This diversity mentioned above is the basis of this study using microsatellite and sequence data.

8.2 Sources of variation and repeatability of Nabs among indigenous chicken

Variation in a trait is the basis of animal breeding. Therefore, determination of a potential parameter for improvement requires that the parameter should be variable among individual animals. Lack of such variability show that the animals are homogenous thus limiting potential for selection. In this thesis, disease tolerance through immune competence was considered. Infectious diseases are of major importance to livestock breeders due to cost, potential zoonotic threats, animal welfare issues, and threats arising from breakdown of currently used diseases'

control strategies (Pinard *et al*., 1989). In the tropics, for example, predominant diseases vary between production systems. Endemic infectious diseases are also problematic because traditional disease control strategies are failing. In such cases, alternative or complementary sustainable control strategies such as breeding programs to increase host resistance to infection (or disease) are required. For these reasons, disease resistance is now one of the major targets of genetic studies in livestock aimed at conferring heritable ability to the animal to evade or withstand infection as a result of enhanced innate immunity (Parmentier *et al*., 2004). Innate immunity as the first line of defense plays an important role in preventing or combating infections (Baccala *et al.*, 1989). Amongst the components of innate immunity are natural antibodies (Nab) of the humoral arm which play significant role because of its non-specificity and polyreactivity (Elluru *et al.*, 2008). Natural antibodies might therefore be robust hence its significance as immune response trait.

This study explored presence and variation of Nabs titre levels for three isotypes (IgA, IgG and IgM) in IC in Kenya (Chapter 3 and Chapter 4). Natural antibodies were found to be detectable in the IC population (Chapter 4), similar to findings elsewhere (Van Knegsel *et al.*, 2007; Ehrenstein and Notley, 2010; Sun *et al.*, 2011). This study found that the IgM Nabs isotype was the major isotype (Chapter 4). Immunoglobulin M has been found to be predominant in B cell repertoire in the fetus and neonate, and possibly play a major role in the development and physiology of the mammals B cell repertoire (Boes, 2000). The IgM isotype are produced by CD5B cells in the peritoneal and intestinal cavity (Zhou and Notkins, 2004).The other isotypes namely IgA, IgE, IgD and IgG do arise from IgM class switching and this phenomenon could justify the higher titers of IgM as compared to IgG and IgA in this study.

In genetics, variability of a parameter show that the animals are naturally dissimilar with respect to trait parameter thus warranting selection (Ploegaert *et al.*, 2011). In Chapters 3 and 4 of this study, Nabs were found to be significantly variable among IC and could be exploited in selection for immunoglobulins titre values, a proxy for immune tolerance. This variability is consistent with findings in layers (Star *et al.*, 2007) and cattle (Ploegaert *et al*., 2011). The repertoire and levels of Nabs are dependent on several factors, amongst them is the environment (Kachamakova *et al.*, 2006), genetic background (Sun *et al.*, 2011) and age (Parmentier *et al.*, 2008; Ujvari and Madsen, 2011). In this study (Chapter 3) evidence of significant variation were observed specific to immunoglobulins isotypes. For example, the breed and sex difference for

IgG, cluster difference for IgA and within chicken difference for all isotypes (IgA, IgG and IgM). This observation is consistent with other studies which showed that Nabs are genetically controlled (Sun *et al.*, 2011). In comparison, the IC had higher variance estimates than RIR implying higher genetic gain can be achieved by selection within the IC population. The male chicken had higher Nabs titre values compared to females for all isotypes. Therefore, sex dimorphism can be exploited in IC breeding where the males can be used for Nabs traits for increased heterosis. The large variance components conferred by IC in the mixed model showed individual variation within the chicken. This high variance estimated among chickens suggested Nabs can be used for breeding for robustness that is a broad capacity to remain healthy across a wide range of challenges. The non genetic factor that significantly conferred significant difference in Nabs variation was microtitre plate. The effect of the micro titre plate could be attributed to intrinsic value and properties of each ELISA plate. The effect of plate had been observed elsewhere (Berghof *et al.*, 2015). Other than variation, repeatability is an important parameter in animal breeding.

Repeatability of IgA, IgG and IgM titres is reported in Chapter 4 with the purpose to ascertain whether one test on a sample collection was enough for inferences or how reproducible the same parameter (IgA, IgG or IgM) were under the same environment and conditions of experiment. The repeatability estimate reported for IgM, IgA and IgG were moderate to high in that order. The high repeatability for IgG and IgA was justifiable. This higher estimate could imply lack of plasticity (i.e stability) of these isotypes with time and in different or same environment, hence the low and high variances within the sampling period and between IC respectively. The large variance estimate was conferred by the IC (genetic) but not time period (environment). This high repeatability for both isotypes implied a single measurement can be used to infer reference to Nabs titers of IC along time under same situations/environment.. This eliminates the need to take several samples from the same IC over time. In comparison, in plasma of mature cows, repeatability of total Nabs was 0.60 for Nab binding KLH (Ploegaert *et al*., 2011) which was lower than our findings.

8.3 Polymorphisms and population structure at the microsatellite locus LEI0258 in three major genetic groups of chicken in Kenya

8.3.1 Locus diversity and attributes of LEI0258 Marker

The genetic diversity of a population can be measured by heterozygosity, number of alleles, allele richness and , proportion of polymorphic loci (Nei, 1973). This study identified 38 alleles (Chapter 5), 8 short of those observed by Ngeno *et al.* (2014) using the same population. The polymorphism of marker LEI0258 might explain the plasticity of the chicken in coping with diseases challenges (Farmers personal communications; Ngeno *et al.*, 2014) conferring the highest selective advantage of MHC-heterozygosity for survival and immune competency. In all the populations, the majority of genotypes were heterozygous. The heterozygosity of the MHC region may be related to the environment under which the populations were developed as well as their selection history (Van Marle-Koster and Nel, 2000). For instance, the previously imported central Kenyan populations locally called KALRO improved (www.dominant.cz) were developed under more protected (against diseases) and controlled environments, and were under intensive selection for many generations, whereas western and coastal populations were developed under more open and exposed (to disease vectors) environments (Personal observations). This observation is consistent with report on outbred lines of chickens that were more resistant to experimental infection with coccidiosis than inbred lines (Pinard-Van Der Laan *et al.*, 1998). In this regard, free-range chickens (western and coastal populations) are likely to have been exposed to more parasite and disease vectors hence the many alleles and heterozygotes exhibited by these populations. This could be helpful in antigen presentation to mediate disease tolerance (Chazara *et al.*, 2013). The selection pressure might contribute to the difference of observed immune competency level in the three populations.

In this study, many of the identified alleles were shared among different chicken populations suggestive of common ancestry and population admixture. Alternatively, this might imply the populations were subjected to similar directional selection. or due to recombination Diverse alleles in the studied population could be a result of hybridized origin of Asian and European breeds (Ngeno *et al.*, 2014). The population genetic parameters (total number of alleles, PIC and Shannon's Information Index) reported in this study is suggestive of high genetic diversity, structure and relationship of Kenyan IC populations. In general, the high PIC value of this marker/locus revealed the extent of genetic variation of its alleles. This can be exploited for

association studies with with productive or adaptive traits. The genetic diversity is further confirmed by sequencing of specific alleles representative of the population. Using the sequence data, the polymorphs were observed by the presence of different numbers of R13 and R12 repeat motifs, indels and SNPs. The two main variable nucleotide tandem repeats (VNTR) of the R13 and R12 were consistent with previous reports (Chazara *et al.*, 2013; Fulton *et al.*, 2006). The R13 motif was found once in most of the samples, observation similar to study by Wang *et al.* (2014). However, a few sequenced samples had multiple motifs of R13 mimicking previous findings (Chazara *et al.*, 2013).

8.3.2 Genetic relatedness

This study compared genetic relatedness among the three populations. There was high Nei genetic distance between central and other regions, implying that central gene pool could be different from the others or simply have less identity by descent between it and other populations. This was justified by high contribution (roughly 40%) of genepool 1 to this population. Conversely, there was high genetic identify between coastal and western population. This is suggestive of higher identity by decent which could be attributed to cultural practices of people from western part of Kenya that could have facilitated genetic exchange between the populations. Similar selection criterion probably led to high genetic relatedness as well. Chicken lovers like fast growing birds a characteristic exhibited in coastal chicken population hence their preference by people from western Kenya. There was high genetic relatedness between western and central populations too, this could be due to hybridization among central and western indigenous chicken. In general, it is worth noting shared genepools among the population implying genetic intermixing among the populations. This could be because, IC is a portable food resource, particularly well adapted to harsh environmental and management conditions, that has been extensively associated with human migrations. The IC are also easily disposed as gifts, used in cultural respects and in rituals, and commercial trade, therefore leading to extensive genetic exchanges as witnessed in the populations.

8.3.3 Principal coordinate analysis (PCoA), cluster and structure analysis

The principal coordinate analysis (PCoA) grouped the samples into three clusters. Admixture was observed for all the populations as witnessed in dendrogram analyses from allele and sequence data. This population admixture indicates movements of people, been subjected to genetic introgression or intermixing over time. The structure analysis revealed four gene pools

with evidenced population admixture. These genetic groups could be associated with geographical features, socio-economic isolation of populations as well as their ancestors or may relate to chicken origin in Kenya. These ancestors might have an impact on the diverse gene pool realized. However, the findings of this study on population structure (four gene pools) is in contrast to two gene pools observed in previous studies (Mwacharo *et al*., 2013; Ngeno *et al.*, 2014). This discrepancy could be attributed to central population that is was not sampled in the previous studies (unique to this study).

8.4 Effects of probiotics, microbial diversity and potential bacterial candidates for use as probiotics

The rampant use of drugs as a preventive measure against diseases has been questioned in poultry industry. There exists extensive documentation of the evolution of pathogens resistant to drugs. Therefore, possibility of alternatives to drug use may be breeding for disease resistance and/or use of probiotics. Probiotics are used to help maintain a healthy microbial balance within the GI tract to promote gut integrity and prevent enteric disease (Cox and Dalloul, 2014).

This is accomplished through three main mechanisms: competitive exclusion, bacterial antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). This study evaluated the effect of using a commercial probiotic on serum immunoglobulins M (Chapter 6). This study reported no significant enhancement of IgM due to probiotic supplementation. This was consistent with previous findings (Rahimi *et al.*, 2011). However, this contradicts data demonstrating the positive effects of probiotics on immune performance (Haghighi *et al.*, 2006). In this regard, it is clear from the present study and other published research that responses to probiotic supplementation are inconsistent.

The discrepancy of the findings could be due to differences between microbial strains and combinations, age, sex, plane of nutrition, nutrient composition of the diet, microbial population of gastrointestinal tract, levels of inclusion of probiotics in the diet, duration of supplementation or other environmental conditions (Midilli *et al.*, 2008). In this study, it was hypothesized the that bacterial species utilized was to be the main source of variation in the results. This led to another experiment (Chapter 7) to determine microbial composition in IC population and find potential bacterial species for immune enhancement. This was augmented by the fact that several studies reported the role of probiotic in augmenting immune response (Cox and Dalloul, 2014).

There is evidence suggesting that probiotics stimulate production of natural antibodies (Haghighi *et al.,* 2006) and different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune responses (Maassen *et al.*, 2000). Determination of bacterial species to stimulate immune response was carried out (Chapter 7), first microbial composition was determined in chicken fecal materials and comparison of microbiome abundance done among three immune competency levels of chicken. The microbial diversity and distribution pattern of different taxonomic classification including kingdom, phylum, class, order, family, genus and species level were observed. At kingdom level archaea and bacteria were detected. At phylum level, a total of 26 phyla were observed, of which 24 were known bacterial phyla while two were unassigned.

The unassigned OTUs at phylum level represents a collection of all sequences that could not be classified at deeper taxonomy ranks (i.e. class) or otherwise. *Firmicutes* and *Proteobacteria* were the most abundant phyla, an observation consistent with previous reports (Wei *et al.*, 2013) but contrary to Singh *et al.* (2012) who reported *Proteobacteria* was the most dominant followed by *Firmicutes* and *Bacteroidetes* in broilers. Other predorminant microbiome included *Actinobacteria* and *Bacteroidetes* at 6% and 2%, respectively. This finding is consistent with pyrosequencing analysis and clone libraries that detected the same dominant species within a sample (Orcutt *et al.*, 2009). The predorminant taxa of *firmicutes, bacteroides and proteobacteria* might be influenced majorly by diet than genetics. These taxa are believed to accelerate fermentation of otherwise indigestible carbohydrates, thereby leading to increased acetate production and subsequently higher energy absorption (Zhang *et al.*, 2009). There was observed microbiome abundance difference in the subsequent taxa up to species level. Species level is the highest basic determined point for selection of probiotics hence the further comparison for immune competency.

The analysis found S*taphylococcus equorum, Rothia aeria, Bacteroides fragilis, Bacteroides plebeius, Blautia producta* and *Janthinobacterium lividum* showed significant variation between immune competence levels. The six species were reviewed based on selection criteria for probiotics (FAO/WHO, 2001) and as outlined by Sharma *et al.* (2014). On the basis of safety, *Rothia aeria* (Michon *et al.*, 2010), *Bacteroides fragilis* (Nagy *et al.*, 2011) and *Staphylococcus equorum* were excluded for further consideration while *Bacteroides plebeius,*

Blautia producta and *Janthinobacterium lividum* were found to be non-pathogenic and proposed for further consideration as probiotics in chicken trials.

8.5 Implication of genetic approaches and microbiome diversity to improve IC disease tolerance and enhanced immunity

The breeding objective for IC in Kenya has been defined. The breeding objective is to develop chicken with better production (egg number, growth rate, and body size), reproduction (fertility and mothering ability) and adaptability (disease resistance/tolerance) (Okeno *et al.*, 2013). There is similarity in breeding of IC in Kenya and Ethiopia (Dana *et al.*, 2010). The feasibility of such breeding objective could be elusive or challenging due to antagonistic relationship between the different breeding objective traits. For example, there is negative genetic correlation between egg traits (egg number) and growth traits (growth rate and live body weight). For example, in Ethiopian Horro IC, the genetic correlation between cumulative egg number at 24 weeks and bodyweight at 16 weeks is -0.12 (Wondmeneh *et al.*, 2014). The antagonistic relationship between traits is increased between production and adaptation traits. For example, the genetic correlation between egg number and the antibody response against Newcastle disease was negative, but low, -0.04 (Okeno *et al.*, 2011). This could pose serious challenges to disease resistance and hence rationale for incorporating disease tolerance to breeding objective of IC. Therefore, within trait lines selection (egg number or growth rate or immune response) may be implemented, then the selected lines crossed to produce dual purpose IC. The prospect for dual purpose breeding objective was proposed by Okeno *et al.* (2011), this would produce IC that can provide the farmer with eggs and meat under the scavenging environments. However, the dual purpose IC proposed do not cater for disease tolerance/ resistant, yet disease tolerance was among the highly ranked economic traits (Okeno *et al.*, 2011; Chapter 2).

Diseases are a major setback in the success of chicken farming (Lamont, 2010) warranting the need to genetically improve diseases tolerance of IC. Genetic improvement is additive, permanent and transmittable to the progeny. Boosting disease tolerance can replace the use of antibiotics and could lead to higher economic returns, because several market niches such as organic market can be exploited. However, identification and collection of the appropriate phenotypes for disease tolerance have been elusive (Chapter 1) with several phenotypes tested with low success (Ploegaert *et al.*, 2011). This study proposed the use of Nabs as alternative

(Chapter 1). Natural antibodies are defined as antigen binding antibodies present in individuals without a (known) previous exposure to this antigen (Baumgarth *et al.,* 2005). Nab play an essential role in both the innate and adaptive immunity (Panda and Ding, 2015). Nab are part of the first line of defense against all types of pathogens (e.g. viruses, bacteria, parasites) in a wide variety of species (Van Altena *et al.,*2016). One of the described working mechanisms of Nab is enhancement of antibody responses (Ochsenbein and Zinkernagel 2000). Due to low antibody affinity and polyspecificity, Nab might provide a non-antigen-dependent protection to diseases (Berghof *et al.,* 2018).

The Natural antibodies isotypes (IgA, IgG and IgM) titre values were found to be significantly different in IC (Chapter 3) and had high repeatability (Chapter 4). This variability can be explored for selective breeding for immune tolerance. Previous studies showed that lower mortality of layer chickens was associated to higher Nab levels binding KLH (Wondmeneh *et al.,* 2015) . The higher Nab levels might explain the ability of IC to survive disease pressure and managed stress for generations without proper nutrition and vaccination in village production system (Udo, 1997). In addition, Nab are heritable (Berghof *et al.,* 2015), therefore, selective breeding could be a feasible strategy in disease tolerance improvement in IC as proposed in this study (Chapter 4). Selection for high and low Nab chicken is ongoing at Wageningen University, after two generations of selection, average IgT Nab titer difference between the High line and the Low line was one titer point. Meaning that the High line has on average around twice as many KLH-binding IgT Nab at 16 weeks of age compared to the Low line (Berghof *et al.,* 2018). This suggests that higher Nab levels have stronger humoral immune response for Th2-stimulating antigens with potentially a better memory formation compared to lower Nab levels (Berghof *et al.,* 2018). In this study, the variation in Nab was dependent on sex with cocks having higher titre values for all Nab isotypes (Chapter 3). Just like other traits, sexual dimorphism was reported to influence weight and growth rate at 12 weeks of age in IC (Muasya *et al.,* 2015) as well. The feasilibilty of producing dual purpose IC and incorporating disease tolerance could be achieved by selecting for growth rate and Nabs in male lines and egg production in female lines. Different breeds of chickens showed variation in expressing the genes related to immunity and in Nabs titre values (Chapter 3; Redmond *et al.,* 2009).

Breed composition and genetic architecture within and among breeds is defined by allelic composition, hence the polymorphism witnessed in this study (Chapter 5). Analysis of IC

revealed 38 highly diverse MHC-linked LEI0258 alleles. It is worth noting that western population (consisting of indigenous chicken with minimal exotic chicken gene pool) had the highest number of alleles and effective allele numbers compared to KALRO improved chicken. This could imply that IC are more robust in combating diseases compared to the other populations. This observation could limit the KALRO improved chicken when it comes to disease tolerance. The importance of some MHC LEI0258 in immunity have been reported to correlate either positively or negatively. The MHC-LEI0258 allele 205 and 307 were correlated with primary antibody responses against Newcastle disease (Lwelamira *et al.,* 2008). The LEI0258 allele 194 and 349 has been found by Fulton *et al.* (2006) to host MHC haplotype B11 which is known to confer resistance to Marek's disease virus (Wakenell *et al.,* 1996) All these alleles were detected in IC (Ngeno *et al.,* 2014; Chapter 5). Selection of preferred alleles and increasing their frequencies to fixation is possible (Lamont, 1998). To boost disease tolerance of IC, the MHC alleles that are linked to high Nab should be incorporated in the breeding objective either directly or indirectly. To exploit the allelic diversity, the contribution of the identified MHC alleles to Nabs and their relation with other genes need to be ascertained to guide which alleles to select (Ngeno *et al.,* 2014).

The polymorphism of marker LEI0258 might imply the plasticity of IC in coping with diseases challenges (Ngeno *et al.,* 2014). This may in turn confer the highest selective advantage of MHC heterozygosity for survival, immune competency and cross talk between innate and adaptive immunity (Berghof *et al.,* 2018). Genetic improvement of productive traits and Nabs discussed above can only be achieved on-station where data collection can be easily achieved. However, the improved IC developed will be reared on farm under scavenging environment. Therefore, the production of IC at farm level may be compromised by genotype by environment interaction (GxE).

Genotype by environment interaction manifest as genotype re-ranking in different environments and varying genetic variances (Lynch and Walsh, 1998). Re-ranking of the genotypes reverses the superiority of genotype making genetic improvement unrealized under productive environment (Mulder, 2007). Further to re–ranking, the effect of GxE exist when the genetic correlation is lower than 0.61 (Mulder *et al.,* 2006). In breeding program for IC, re-ranking of the genotypes can be challenging due to the differences between the on-station breeding and the village scavenging production environments. Besides, in Kenya, seven agro-

ecological zones exist with varied climatic conditions and GxE may occur. Therefore, developing a single genotype that fits well to diverse environments across all the agro-ecological zones may be a challenge, hence agro-ecological zone-specific breeding programs may need to be established (Ngeno *et al.*, 2014) or different IC genotypes be exploited to adapt the specific agro-ecological environmental conditions (Chapter 2). Alternatively, manipulating the environmental condition such as nutrition can address the effect of GxE interaction in a breeding goal. For IC, GxE can be reduced by manipulating the scavenging production environment encouraging farmers to adopt the semi-scavenging condition where feed supplementation is done.

Supplementation is necessary because the free range scavenged feeds can be scarce and seasonal, and therefore may not support high egg production as well as faster growth of the improved chicken. The phenomenon of GxE is well documented on production traits (Mulder, 2007) and limited reports on Nabs are available. The GxE may or may not exist on Nabs because Nabs' paratopes are polyreactive and directed to similar structures of damage associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP) (Zelenay and Sousa, 2013). The two molecular patterns have similar functionality and are well-known to influence antibody levels in chickens (Parmentier *et al.*, 2004). Most of Nab bind DAMP and PAMP that are conserved along different genera and these serve as targets for identification of microbes by the innate immune system (Parmentier *et al*., 2004). Genetic improvement is a continuous process that may be realized after some generations, therefore alternative to enhance immunity were sorted by testing commercial probiotic on IgM Nab isotype titre value of IC.

Several studies have reported positive effects of probiotics on production and immune performance of chicken (Haghighi *et al.*, 2006). However, this study (Chapter 7) and some others have reported contrary results (Rahimi *et al.*, 2011). Probiotic treatment resulted in the reduction of the mean reactive IgM antibodies in serum of the IC (Chapter 7). Strain(s) of bacteria utilized, composition and viability of the probiotic, preparation method, dosage, and application method, frequency of application, overall diet, drug interactions, and condition of the animal (Huang *et al.*, 2004) could be the cause of discrepancy in results. This result of negative IgM titre values on IC indicated not all probiotics are efficient for IC, therefore genetic approaches might provide permanent solution to disease tolerance in IC.

8.6 General conclusions

This study has provided vital information on insight of the IC subsector in Kenya in regards to genetic diversity, natural antibodies and metagenomics. In this study

i. Natural antibodies isotypes IgA, IgG and IgM binding KLH were detected in blood serum of IC.

ii. The Nabs titers were variable within the IC and highly repeatable for IgA, IgG and IgM.

iii. The LEI0258 locus was found to be highly diverse in its repertoire. The locus showed four gene pools in Kenyan populations of chicken.

iv. Administration of MOLAPLUS to IC diets showed no significant difference in systemic natural IgM antibody concentration.

v. The microbiome profiling showed diverse composition and diversity of micro organismon in GIT of chicken.

vi. Potential bacterial species were identified for usage as probiotics for enhanced immunity in IC.

8.7 Recommendations

8.7.1 Recommendations based on result

i. Indigenous chicken have more alleles within LEI0258 locus which makes them more diseases tolerant, however for effective immunity routine management should be practised

ii. Not all probiotics are efficient for indigenous chicken

iii. Kalro improved indigenous chicken is not purely indigenous, their management should be enhanced for efficient productivity.

8.7.2 Areas for further research

i. Further research on associatiation of the MHC alleles (polymorphs) with immune parameters, disease tolerance and production traits should to be done.

ii. Determination of *Bacteroides plebeius, Blautia producta* and *Janthinobacterium lividum* to enhance immunity in chicken is also required

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APPENDIX

Appendix 1:Publications in peer reviewed journals from this thesis

1. **Khobondo, J.O**., Mwakubambanya, R., Wasike, C.B., Kahi, A.K. (2017). Genetic and non-genetic sources of variation in natural antibodies titre values among indigenous chicken. American Journal of Research Communication, 5(7): 30-45} www.usa-journals.com, ISSN: 2325-4076.

2. **Khobondo, J.O**., Mwakubambanya, R., Wasike, C.B., Kahi, A.K. (2016). Variation and Repeatability of Natural Antibodies Against Keyhole Limpet Hemocyanin of Indigenous Chicken of Kenya. Genomics and Applied Biology, 2016, Vol. 7, No. 1.

3. **Khobondo, J. O.**, Ogore, P. B., Atela, J. A., Onjoro, P. S., Ondiek, J. O and Kahi, A. K. (2015). The effects of dietary probiotics on natural IgM antibody titres of Kenyan indigenous chicken. Livestock Research for Rural Development. Volume 27, Article #230.

4. **Khobondo, J.O.**, Okeno, T.O., Lihare, G.O.,Wasike, C.B and Kahi A.K. (2014). The past, present and future genetic improvement of indigenous chicken of Kenya. Animal Genetic Resources, 55, 125–135. © Food and Agriculture Organization of the United Nations,

Appendix 2:Conference presentations

1. **Khobondo, J.O**., Kahi, A and Roger, P. (2017). Polymorphisms at the Microsatellite Locus LEI0258 in Three Genetic Groups of Indigenous Chicken in Kenya. Proceedings of Tropentag, September 20-22, 2017, Bonn, Germany.

2. **Khobondo J.O**., Mwakubambanya, R., Wasike, C.B and Kahi, A.K. (2015). Natural antibodies against keyhole limpet hemocyanin in indigenous chicken: variability among and repeatability within Indigenous Chicken sampled within three weeks. Proceedings of 2rd International conference on Biodiversity for food and nutrition, November 2015, Safari park hotel, Nairobi.

3. **Khobondo J.O** and Kahi A.K. (2015). Exploitation of variation of indigenous chicken genetic resources in Kenya. Proceedings of the 9TH Egerton University International conference, March 2015, Egerton University, Kenya.

Appendix 3: SAS syntaxes for statistical analyses

SAS program for one way ANOVA

```
PROC GLM DATA = abs;
CLASS pla;
MODEL abs = pla ;
LSMEANS pla / P PDIFF TDIFF STDERR ADJUST=TUKEY;
CONTRAST ‘pla1,pla2 : pla3’ pla 1 1 –3;
CONTRAST ‘pla1 : pla2’ pla 1 -3;
RUN;
```

SAS program for repeated measures

```
PROC MIXED DATA=abs;
CLASS time;
MODEL abs =  time IC / ;
REPEATED / TYPE=CS SUB= time;
LSMEANS time / DIFF;
RUN;
```

Appendix 4: Qiime Workflow

1. Pairing the reads

commands

```
interactive –w taurus
cp /var/scratch/joyce/scripts/usearch7 .
cp /var/scratch/joyce/scripts/1.2_fastq_mergepairs2.sh .
sh 1.2_fastq_mergepairs2.sh
```

2.Quality control of the data

commands

```
cp /var/scratch/joyce/scripts/1.3_fastq_stats.sh .
sh 1.3_fastq_stats.sh
```

3.Filtering for good quality

commands

cp /var/scratch/joyce/scripts/1.4_fastq_filter.sh .

sh 1.4_fastq_filter.sh

4.Validating the metadata

commands

module load qiime

validate_mapping_file.py -m metadata.txt -o validate_meta

5.Adding Qiime labels

commands

cp /var/scratch/joyce/scripts/1.6_add_qiime_labels.sh .

sh 1.6_add_qiime_labels.sh

5.Chimera Removal

commands

cp /var/scratch/joyce/scripts/rdp_gold.fa .

identify_chimeric_seqs.py -i labeled/combined_seqs.fna -m usearch61 -o chimeras -r rdp_gold.fa

filter_fasta.py -f labeled/combined_seqs.fna -o combined_seqs_NonChimera.fna -s chimeras/chimeras.txt -n

7. Picking OTUs

commands

interactive –w taurus

scp combined_seqs_NonChimera.fna qiime@192.168.5.46:/home/qiime/user_name

pick_open_reference_otus.py -i combined_seqs_NonChimera.fna -r /home/qiime/joyce/gg_13_8_otus/rep_set/99_otus.fasta -o otus

8.Downstream analysis of the Biom file

command

biom summarize-table -i otu_table_mc2_w_tax.biom -o otu_table_stats.txt

command

biom convert -i otu_table_mc2_w_tax.biom -o otu_table.txt --to-tsv --header-key taxonomy

9.Summarize and plot by taxonomy

command

summarize_taxa.py -i otu_table_mc2_w_tax.biom -L 2 -o phylum/

plot_taxa_summary.py -i phylum/otu_table_L2.txt -l phylum -c pie,bar,area -o phylum_charts/

try doing the same for class and order level view the output with a text editor

10.Aplha diversity

command

echo "alpha_diversity:metrics shannon,PD_whole_tree,chao1,observed_species" > alpha_params.txt

alpha_rarefaction.py -i otu_table_mc2_w_tax.biom -m metadata.txt –o alpha_diversity/ -p alpha_params.txt -t rep_set.tre

11.Beta Diversity and PCoA

command

beta_diversity.py -i CSS_normalized_otu_table.biom -m bray_curtis,unweighted_unifrac -t rep_set.tre -o beta_div

command

principal_coordinates.py -i ./beta_div/bray_curtis_CSS_normalized_otu_table.txt -o ./beta_div_coords.txt

command

make_2d_plots.py -i beta_div_coords.txt -m metadata.txt -o 2d_plots

12.Normalizing the OTU Table

command

normalize_table.py -i otu_table.biom -a CSS -o CSS_normalized_otu_table.biom

13.Identifying differentially abundant OTUs

command

module load R

R

source("https://bioconductor.org/biocLite.R")

biocLite("DESeq2")

differential_abundance.py -i otu_table_mc2_w_tax.biom -o diff_otus.txt -m metadata.txt -a DESeq2_nbinom -c Env -x Rock -y Soil –d

Appendix 5: SAS outputs

Significant difference effect of plate on IgA titre values

The SAS System
The GLM Procedure

Dependent Variable: abs abs

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1.48744705	0.74372353	5.84	0.0034
Error	213	27.10310657	0.12724463		
Corrected Total	215	28.59055363			

Least Squares Means for Effect pla
t for H0: LSMean(i)=LSMean(j) / Pr > |t|

Dependent Variable: abs

i/j	1	2	3
1		-3.36063	-2.31101
		0.0026	0.0564
2	3.360629		1.659596
	0.0026		0.2233
3	2.311008	-1.6596	
	0.0564	0.2233	

Results output for repeated measures of IgM isotype

Model Information

Data Set	WORK.IGM
Dependent Variable	abs
Covariance Structure	Compound Symmetry
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
time	4	S1 S2 S3 S4

Dimensions

Covariance Parameters	2
Columns in X	5
Columns in Z	0
Subjects	96
Max Obs Per Subject	1
Observations Used	96
Observations Not Used	0
Total Observations	96

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-95.61364861	
1	1	-95.61364861	0.00000000

Convergence criteria met

Covariance Parameter Estimates

Cov Parm	Estimate
CS	0.0123
Residual	0.0057

The SAS System
The Mixed Procedure

Fit Statistics

-2 Res Log Likelihood	-95.6
AIC (smaller is better)	-91.6
AICC (smaller is better)	-91.5
BIC (smaller is better)	-86.5

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
time	3	91	10.52	0.00001
ic	1	91	4.17	0.00001

Least Squares Means

Effect	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	S1	3.1523	0.02741	92	114.99	<.0001
time	S2	2.9850	0.02741	92	108.88	<.0001
time	S3	2.9602	0.02741	92	107.98	<.0001
time	S4	3.0826	0.02741	92	112.44	<.0001

Result output for repeated measures of IgG isotype

Model Information

Data Set	WORK.IGG
Dependent Variable	abs
Covariance Structure	Compound Symmetry
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
time	4	S1 S2 S3 S4

Dimensions

Covariance Parameters	2
Columns in X	6
Columns in Z	0
Subjects	96
Max Obs Per Subject	1
Observations Used	96
Observations Not Used	0
Total Observations	96

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	177.74206947	
1	1	177.74206947	0.00000000

Convergence criteria met

Covariance Parameter Estimates

Cov Parm	Estimate
CS	0.300700
Residual	0.000061

The SAS System
The Mixed Procedure

Fit Statistics

-2 Res Log Likelihood	177.7
AIC (smaller is better)	181.7
AICC (smaller is better)	181.9
BIC (smaller is better)	186.9

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
time	3	91	0.93	0.00001
ic	1	91	1.17	0.00001

Least Squares Means

Effect	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	S1	1.7050	0.1103	91	15.46	<.0001
time	S2	1.4452	0.1103	91	13.11	<.0001
time	S3	1.5873	0.1103	91	14.40	<.0001
time	S4	1.5650	0.1103	91	14.19	<.0001