INVESTIGATION OF *BETA LACTOGLOBULIN* GENE VARIATION IN SOUTH AFRICAN HOLSTEIN FRIESIAN COWS AND ITS ASSOCIATION WITH MILK PRODUCTION TRAITS

BY

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A MINI-DISSERTATION

Submitted in (partial) fulfilment of the requirements for the Degree of

Master of Science in Agriculture

In

Animal Production

In the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Agricultural and Environmental sciences)

at the

UNIVERSITY OF LIMPOPO, SOUTH AFRICA

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18 MARCH 2024

DECLARATION

I declare that this dissertation hereby submitted to the University of Limpopo for the Degree of Master of Science in Agriculture has not previously been submitted by me for a degree at this or any other University, that it is my own work in design and execution, and that all materials contained herein has been duly acknowledged.

Miss MAKAMU TC **DATE: 25 MARCH 2024**

ACKNOWLEDGEMENT

Am grateful to God who saw me through it all and gave me the strength to go on. I would like to express my special thanks of gratitude to my supervisors Prof TL Tyasi and Prof VG Mbazima for assisting me during this research preparation. I also want to acknowledge my mentor Mr K Mokoena who assisted me with this research.

I would like to also thank all my siblings and my parents (Makamu Samuel, Makamu Vimbai) who were always there for me physically, emotionally, financially, and spiritually. Special thanks go to my son Vutomi Blessing Jameson Phoku and his father Vutomi Sally Phoku who always gave me a reason to wake up and keep pushing. I would also like to thank the Tyasi research team for their support. I would like to acknowledge the farm manager of Limpopo dairy farm Mr Joubert Fourie for allowing me to use his farm, farm workers and animals in my research. I am truly grateful for the assistance.

The National Research Foundation (NRF) deserves special thanks for its financial support throughout the project. Finally, I'd want to express my gratitude to the University of Limpopo's Faculty of Science and Agriculture for providing me with the opportunity to further my education.

DEDICATION

This dissertation is dedicated to my mother Vimbai Makamu who endured all the suffering and abuse for her children's sake. I will make you proud and ensure that all you did for us was not in vain.

ABSTRACT

It is important to predict the change in one trait in response to selection for another, hence, the knowledge of the connection between traits is essential for the improvement of milk components and yield of dairy animals. Moreover, the genes also must be taken into consideration when selecting animals due to their potential in affecting milk production traits. The Beta-lactoglobulin gene (*β-LG*) was detected on bovine chromosome 11 and encodes the main whey protein. The genetic effect of *β-LG* gene on milk production traits and quality of milk in South African Holstein Friesian cows is poorly understood. This study was conducted to identify the genetic markers of *β-LG* gene that might be utilised as selection criterion during breeding to improve milk production traits of Holstein Friesian cows. A total of one hundred (n = 100) South African Holstein Friesian cows aged 2 to 3 years from the Limpopo dairy farm were used in this study. Pearson's correlation, student t-test analysis and DNA sequences were used for analysis. Milk samples (10 ml) were collected during morning milking using a measuring cup to represent the whole milking of each animal for detecting the milk constitution such as fat percentage (FP), protein percentage (PP), solid-not-fat (SNF), and lactose percentage (LP) using ultrasonic portable milk analyser (milko tester model- master mini). The data such as milk yield per day (MYD) and milk yield per 30 days (MY30D) were used for each used cow. Blood samples (5 ml) were collected from each animal underneath the tail using a butterfly needle and sent to Inqaba Biotechnology company for DNA extraction, amplification, and sequencing. The correlation results showed that MYD had a highly negative significant correlation ($p < 0.01$) with PP and SNF. The correlation findings also indicated that MY30D had a highly negative statistical correlation ($p < 0.01$) with FP and PP but was highly positive and significantly correlated ($p < 0.01$) with SNF. DNA sequencing findings indicated the presence of 5 different SNPs. Two of the SNPs were non-synonymous (T/C) and (C/T) at positions 5174 and 5251 of exon 4 of the *β-LG* gene which were named 5174T>C and 5251C>T, respectively. The other 3 SNPs were found in intron 3 (C/G), (G/A), and (T/C) at positions 5123, 4982, and 5099 and named 5123C>G, 4982G>A, and 5099T>C, respectively. Chi-square results demonstrated that the population used was under Hardy-Weinberg equilibrium (HWE) for SNP 5123C>G and had a dominant allele C. Dominant alleles for SNPs 5174T>C, 4982G>A, 5099T>C, and 5251C>T were T, G, T, and C, respectively. Marker-trait association results revealed that genotypes of SNPs 5174T>C, 5099T>C, and 4982G>A had no association with

MY30D, PP, and SNF, however, it had a significant association with MYD, LP, and FP. In addition, a significant association between SNP 5123C>G and LP, FP, MY30D, and SNF, but it showed no association with MYD and PP. SNP 5251C>T displayed no association between its genotypes and milk yield and PP, however, it showed an association with FP, SNF, and LP. In conclusion, correlation results suggest that protein and solid-not-fat might be used to improve milk yield of Holstein Friesian cows. DNA sequencing results suggest that 5123C>G, 4982G>A, 5174T>C, 5099T>C, and 5251C>T were the SNPs found in *β-LG* gene of South African Holstein Friesian cows used in this study. Marker-trait association results suggest that TT genotype for SNPs 5174T>C, and 5099T>C of *β-LG* gene might be used as a genetic marker when improving milk yield per day and lactose %, whereas genotype CT might be used to improve fat %. Genotype CC of SNP 5123C>G might be used to increase milk yield per 30 days and solid-not-fat, while CG be used to improve lactose % and fat %. The TC genotype for SNP 5251C>T might be used as a genetic marker to increase solid-not-fat and lactose %, whereas CC genotype can be used to improve fat %. The GG genotype for SNP 4982G>A of *β-LG* gene might be used as genetic marker when improving milk yield per day and lactose %, whereas AG genotype might be used to improve fat %. Further studies need to be conducted on the *β-LG* gene single nucleotide polymorphisms and their association with milk production traits with a large number of animals and more milk production traits of South African Holstein Friesian cows.

Keywords: Milk yield, Single nucleotide polymorphisms, Protein, Fat, Lactose, Solid-not-fat

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LIST OF ABBREVIATION

β-LG	*Beta-lactoglobulin* gene
MYD	Milk yield per day,
MY30D	Milk yield per 30 days
FP	Fat percentage
PP	Protein percentage
SNF	Solid-not-fat
LP	Lactose percentage
^{ns}	non-significant
EDTA	Ethylene diamine tetraacetic acid
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
µl	Microliter
°C	Degree Celsius
bp	Base pairs
NCBI	National Centre for Biotechnology Information
SPSS	Statistical Package for Social Sciences
SD	Standard deviation
CV	Coefficient of variance
SE	Standard error
Df	Degree of freedom

r	correlation coefficient
SNPs	single nucleotide polymorphisms
HWE	Hardy Weinberg equilibrium
x^2	chi-square test
H_0	Gene homozygosity
H_e	Gene heterozygosity
N_e	Effective Allele number
PIC	Polymorphism information
AREC	Animal research ethics committee
PI3	Parainfluenza-3
BVD	Bovine viral diarrhea
BRSV	Respiratory syncytial virus
L	Litres
M	Marker
T	Thymine
C	Cytosine
G	Guanine
A	Adenine
I	Isoleucine
V	Valine
G	Glycine
D	Aspartic acid

CHAPTER ONE

INTRODUCTION

1.1 Background

Two breeds of cattle, black animals from the Batavians (present-day Germany) and white animals from the Friesians (present-day Holland), were crossed to create Holstein Friesian in the Netherlands approximately 2000 years ago (Elischer, 2014). This crossbred animal is the most common and predominate exotic dairy cattle breed due to its ability to adapt a wide range climatic environments and with the world's known high milk production potential (Fanta, 2017). Milk production traits are important quantitative traits that have a tremendous influence on the dairy industry (Morkuniene et al., 2018). This makes the utilisation of molecular genetics technologies to recognize specific DNA markers that relate to milk production traits very important to make breeding program more effective through early selection of young animals as future breeding stock (Zaglool et al., 2016). Beta lactoglobulin (β-LG) gene is a major gene expressed in the milk of ruminants, and its polymorphism can serve as an informative molecular marker for milk production traits (Zakizadeh et al., 2012).

1.2 Problem statement

Holsteins are large animals with a characteristic of black and white or red and white colour pattern ranking first among the dairy breeds in average milk production per cow (Fanta, 2017). Milk is a vital component of food preparations for humans and a vital supply of nutrients for lactating calves (Zaglool et al., 2016). However, milk production traits such as milk yield, fat, protein, and lactose of cattle such as Holstein Friesian, Jersey, Sahiwal cattle breeds and Girolando cows requires genetic improvement (Zaglool et al., 2016; Badola et al., 2019; Benose et al., 2019). Selection of dairy cows has been based mostly on quantitative traits such as milk, fat, or protein yield, which are assumed to be controlled by multiple loci (Stipp et al., 2013). Therefore, genetic improvement of quantitative traits is relatively slow as productive traits can only be measured in one sex and is affected by numerous polygenes (Kusza et al., 2015). This lowers the accuracy of genetic evaluation of cows and the genetic progress per year (Singh et al., 2014). Several studies suggested that gene approach focusing on genetic variation between or within dairy cattle population is a helpful tool to identify genetic markers that might serve in marker-assisted selection during breeding to improve milk production traits (Chessa et al., 2013; Kishore et al., 2014; Shalla et al., 2014; Singh et al., 2015; Raschia et al., 2018).

1.3 Rationale

Once genes are established to be correlated to features of interest in animals, a viable alternative to the current approaches to trait selection is the use of polymorphic genes as detectable molecular markers (Zaglool et al., 2016). Archana (2013) reported that there is a significant enthusiasm for the use of molecular genetics technologies to recognize specific deoxyribonucleic acid (DNA) markers that relate to the economically important traits to make breeding program more effective through early selection of young animals as future breeding stock. According to a study that was conducted by Badola et al. (2019) the Beta lactoglobulin (β-LG) gene possesses genetic variants that are linked to Jersey, Holstein Friesian, and Sahiwal cattle breeds' milk production traits. Benose et al. (2019) investigated genetic association of variations in β-LG gene with milk traits of Girolando cows and did not find any significant correlation between β-LG gene and milk output. Zaglool et al. (2016) reported that Holstein Friesian cattle breed is polymorphic for β-LG gene producing three of genotypes with two alleles. However, Beta-lactoglobulin gene variation and how it relates to milk production traits in South African Holstein Friesian cows is not yet known. Hence, the current study will be helpful for enhancing milk production traits in Holstein Friesian cows through marker-assisted selection and outline a strategy to bypass the time-consuming, traditional ways of selecting Holstein Friesian cattle for milk production.

1.4 Aim

This study was aimed to discover the genetic markers of Beta-lactoglobulin gene that might be used as selection criterion during breeding to improve milk production traits of Holstein Friesian cows.

1.5 Objectives

The objectives of this study were to:

I. Determine the relationship between protein, fat, lactose, SNF, and milk yield traits in South African Holstein Friesian cows.

II. Identify the single nucleotide polymorphisms of *Beta-lactoglobulin* gene and its association with protein, fat, lactose, SNF, and milk yield in South African Holstein Friesian cows.

1.6 Hypotheses

I. There is no relationship between fat, lactose, protein, SNF, and milk yield in South African Holstein Friesian cows.

II. Single Nucleotide Polymorphisms of *Beta-lactoglobulin* gene have no association with protein, fat, lactose, SNF, and milk yield traits of South African Holstein Friesian cows.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

The goals of this chapter were to (1) offer an analysis of the reported characteristics of Holstein Friesian cows of South Africa, (2) explore the importance of Beta lactoglobulin gene (β-LG) and milk production traits, (3) emphasize genetic variations of β-LG gene by single nucleotide polymorphisms (SNPs) and their relationship with milk production traits of Holstein Friesian of South Africa. This review was structured as follows to fulfil the study objectives: (a) characterization of Holstein Friesian cows, (b) Beta lactoglobulin gene, (c) milk production traits, (d) SNPs of as genetic marker for milk production traits.

2.2. Characterization of Holstein cows

Holstein Friesland is a breed of dairy cattle that originated in the Dutch provinces of North Holland and Friesland, and Schleswig-Holstein in northern Germany (Wikipedia, 2022). They are large cattle with colour patterns of black and white or red and white, the original stock was black animals and white animals of the Batavians and Friesians (McGuffey and Hirley, 2011). The red and white coloring is a recessive gene that appears when both the dam and sire are carriers or exhibit the trait themselves (Elischer 2014). The Holstein breed is known for high milk production but has less butterfat and protein based on percentage in the milk, compared to other breeds (Guillou et al., 2019). Although other breeds have a higher percentage of fat, protein and solids, the very high milk production of the Holstein means that the total quantity of milk components is also superior (Fuquay et al., 2011). This breed is very adaptive to a wide range of environmental conditions (Singh et al., 2014). Holsteins have an unequalled genetically anchored achievement ability which has no biological ceiling with the average Holstein cow producing 9 900 kg of milk per 305 days lactation (Womack, 2020). The predominance of the Holstein Friesian can be largely attributed to the substantial genetic progress in milk production that has been made with the breed, with research evidence highlighting improved efficiency of conversion of feed into milk with increasing genetic merit for milk production (Fuquay et al., 2011).

Figure 2.1: Holstein cow. (www.stclairshetlandcattle.co.uk)

2.3. *Beta-lactoglobulin* gene (*β-LG*)

The phenotypic expression of milk production traits (e.g., milk yield and composition) are controlled by genes, which may or may not be transferred to the offspring (Zaglool et al., 2016). The genetic value of a trait indicates the likelihood that the genes responsible for that trait will be transferred to any offspring (Ren et al., 2011). Consequently, when dairy producers are selecting animals for breeding stock, they are typically more concerned with an animal's genetic value rather than its phenotypic value of a particular trait (Safronova et al., 2017). *β-LG* gene is the major whey protein in the milk of cattle, sheep, and dogs and it could have a role in metabolism of phosphate in the mammary gland and the transport of retinol and fatty acids in the gut (Kusza et al., 2015). It has been mapped on bovine chromosome 11, spans 4.7Kb which is arranged in seven small exons, six introns and encodes 162 amino acid residues (Zaglool et al., 2016).

2.4 Use of *Beta-Lactoglobulin* gene on milk production traits

βlg is the major whey protein gene associated with different concentrations of beta-lactoglobulin protein in cow's milk. It enables identical protein, long-chain fatty acid, and protein binding. This gene plays a significant role in the amount of milk yield, whey protein content, and fat content produced in milk (Jawasreh et al., 2019). Alim et al. (2015) indicated that *β-LG* gene can be used to improve milk yield, lactose, and fat, however, it could not be used to improve protein of Holstein cows. Milk yield, fat, and protein of Brown Swiss cattle breed was found to not be associated with *β-LG* gene Dogru (2015). Mir et al. 2014 reported that *β-LG* gene could not be used to improve milk yield of Sahiwal cattle breed. Whereas Morkuniene et al. 2018 stated that *β-LG* gene could be used to improve milk yield and protein of Holstein. The difference in the results of these studies might be due to the different breeds used.

2.5. Milk production traits

Milk is the whole, fresh, clean lacteal secretion obtained by the complete milking of one or more healthy cows. Milk solids components include fat, protein, lactose, and minerals, normal values for milk fat typically range from 3.7% (Holstein) to 4.9% (Jersey); milk protein ranges from +3.1 % (Holstein) to 3.8 % (Jersey). Lactose is usually 4.6–4.8 % for all breeds; minerals (ash) average 0.74 % (Grant and Kononoff, 2007). According to the study that was conducted by Kargo et al (2021), Danish Hosteiln yielded more milk, protein, and fat than Danish Red and Danish Jersey. Bobic et al. (2014) stated that Holstein cows had significantly ($p<0.001$) higher amount of milk per milking compared to the Simmental breed. Holstein Friesian produced more fat, protein, and lactose compared with Montbeliarde and Normande, However, it had similar fat, protein, and lactose with Norwegian Red (Walsh et al., 2008). Holstein Friesian cattle achieved the highest milk, fat, and protein yields in all environments, with New Zealand Jersey cattle producing the lowest milk, fat, and protein yields (Bryant et al., 2007). All the studies included are in line with each other, proving the superiority of the Holstein cattle breed.

Improving the efficiency of milk production and its constituents without increasing the size of the dairy herd is the foremost goal of the selection in dairy industry (Zaglool et al., 2016). Milk is an important source of essential nutrients for lactating calves and a key raw material for human food preparations (Reinhardt et al., 2012). Everywhere throughout the world, individuals satisfy around 13% of their protein requirement from milk and milk products. Milk proteins including casein and whey proteins have a crucial role in contributing to milk production traits (Kusza et al., 2015). The total milk protein composition unequivocally relies on the expression and secretion of individual proteins (Neamt et al., 2017).

2.6. Single nucleotide polymorphisms of *Beta-lactoglobulin* gene as genetic marker for milk production traits

A single nucleotide polymorphism (SNP) is a genomic variant at a single base position in the DNA (Zaglool et al., 2016). The candidate gene approach is a standout amongst the most vital ways to deal with quest for genetic markers related to production traits and investigating polymorphism of structural and protein coding genes (Oikonomou et al., 2011). Molecular technologies have been developed to recognize alleles and frequencies within milk protein genes, including specific polymerase chain reaction (PCR) sequences, restriction enzymes and single nucleotide polymorphism (Ren et al., 2011). Polymorphism of β-LG gene was first recognized in 1955 by Aschaffenberg and Drewry in cow's milk and a total of 15 alleles are known. Common alleles are A, B, C and D, with alleles A and B being the most incessant (Farrell et al., 2004). The bovine β-LG gene A allele differs from B allele by two amino acids substitution at positions 64 (aspartate → glycine) and 118 (valine → alanine) and has a higher β-LG gene protein concentration than allele B. It is likely that this difference in amount of β-LG gene protein is not caused by the amino acid substitutions, but instead by different levels of expression of the corresponding A and B alleles of the β-LG gene (Zaglool et al., 2016). While, BB genotype relates to higher casein and fat contents, which are ideal properties for cheese making additionally it might improve the quality of milk (Ren et al., 2011).

Singh et al. (2015), Safronova et al. (2017), Neamt et al. (2017), and Morkuniene et al. (2018) found that SNP of *β-LG* gene had the potential to be used as genetic marker for milk production traits of Frieswal, Black and White, Simmental, and Holstein cattle breeds respectively. However, Soyudal et al. (2019), Sahin Sermerci and Balcioglu (2022) indicated that *β-LG* gene had no potential to be used as genetic marker for milk production traits of Holstein cattle breed.

2.7. Conclusion

The ability of Holstein Friesian cows to produce high milk yield has been proven in literature. It has also been proven that milk production traits are controlled by genes and β-LG gene has been found to be one of those genes. Definition and importance of β-LG gene and milk production traits were outlined in this chapter, and it shows that β-LG gene can be used to improve milk production traits of Holstein cows. SNPs of β-LG gene has been recognized and linked with milk production traits. Prediction of the future performance of farm animals is the most rational point in animal breeding and animals of superior traits and phenotype should be selected to hasten genetic improvement. The use of polymorphic genes as genetic molecular markers is a promising surrogate for the current methods of selection once these genes are proven to be associated with traits of interest in animals.

CHAPTER THREE
METHODOLOGY AND ANALYTICAL PROCEDURES

3.1 Study area

The study was conducted at Limpopo Dairy Farm located in Louis Trichardt, Limpopo Province, South Africa. The ambient temperature around the Limpopo Dairy Farm ranges between 8°C and 21°C in winter and 19°C and 28°C in summer (Adesoy and Dondofema, 2021). Limpopo Dairy Farm lies at latitude 23°06'29.1"S and longitude 29°50'05.6"E.

3.2 Ethical approval

All procedures were performed following the standards and protocols set by the University of Limpopo Animal Research Ethics Committee (AREC) project number: AREC/28/2023: PG.

3.3 Experimental and animal management

A total number of 100 South African Holstein Friesian cows aged 2 to 3 years provided by the farmer were used in this study. The age of the cows was determined using the farm records. The cows at Limpopo dairy farm were kept under intensive production. Whereby, they were fed with mixed ration concentrate feed provided every morning after being measured to meet the animals' nutrient requirements. The leftover feed was cleared every morning before providing the cows with new feed. Clean water was also provided to the cows *ad-libitum*. The cows were kept in housing that had a roof and cemented floor with adjustable environmental temperature to achieve high milk production. The environmental temperature was adjusted using fans and water, when the temperature inside the housing was too high, the fans automatically switch on and small amount of water was automatically sprinkled on the animals. The housing was always kept clean by use of soap and water to prevent infections. Electronic ear tags were used as a method of identification, these ear tags recorded the performance of the animal, body temperature, feed consumed, daily activity of the animal, and the daily milk yield of the animal. Sick animals were isolated from the healthy animals to prevent them from infecting the healthy animals, this was done for 4 weeks the animals were monitored and treated. Automatic rotary milking parlour was the type of milking system used at Limpopo dairy farm. The animals were vaccinated as shown on Table 3.1 below.

Table 3.1: vaccination schedule for Holstein heifers

Age or time of administration	Disease	Type of vaccine or therapy
0-6 hours		Colostrum
6 weeks	Rhinotracheitis (IBR) virus, Parainfluenza-3 (PI3) virus, Bovine Viral Diarrhea (BVD) virus, Bovine Respiratory Syncytial Virus (BRSV), IBR-PI^3-BVD-BRSV Clostridial spp.	Modified live vaccine 7-way bacterin/toxoid
4-6 months	Brucellosis[a]	RB51
6 months	IBR-PI$_3$-BVD-BRSV	Modified live vaccine
	Clostridial spp.	7-way bacterin/toxoid
	Leptospirosis	5-way bacterin
40-60 days prior to calving	IBR-PI$_3$-BVD-BRSV	Killed vaccine
	Leptospirosis	5-way bacteria
	Calf scours:	Killed vaccine
	Rota and Corona virus	Bacterin/toxiod
	E. coli + Clostridium perfringens, type C & D	

Before milking there were workers who stood below the milking parlour with vetericyn utility spray and clean paper towels that were used to clean the teats of the cows and insert them inside the milking machine. This was done to prevent mastitis and milk contamination. The platform rotated very slowly, allowing cows to enter and exit the platform at regular intervals. Once the animal entered the milking parlour there was a sensor that identified the animal, and when the milking process started it was recorded as per how much milk yield that animal produced that day. This information was recorded and stayed in the daily farm records. Sick, injured, or pregnant animals were not included in this study because their condition might have a great influence on the performance of the animal. If included, they might lead to false results.

3.4 Research design

This study was observational research where the population in the study area was used in the observation without administering any treatment. Cross-sectional design was used with one replicate per cow. Data were gathered from numerous individuals at one time as part of a cross-sectional study, a sort of research design in which variables are not altered while being monitored (Setia, 2016).

3.5 Milk and blood sample collection

Milk yield of cows was taken from daily farm records. Venipuncture blood collection method was used in this study and the blood was collected in the morning before milking. The were taken to the milking parlor, where the blood samples (5ml) were taken from underneath the tail of each cow using a butterfly needle which draws blood from the animal straight to the sterilized vacutainer tube containing EDTA as anticoagulant. One hundred (n = 100) blood samples were collected by Limpopo Dairy Farm’s Veterinarian in the beginning of June 2023. The blood samples were then transported to the University of Limpopo laboratory in an ice box with gel cool packs and kept there inside the refrigerator at -20° C until they were sent to Inqaba Biotechnology company (525 Justice Mahommed St Muckleneuk, 200, Pretoria, South Africa). Inqaba Biotech came and collected the blood samples from the University of Limpopo, the samples were packaged in a cooler box with dry ice. One hundred milk samples (10 ml) were collected during morning milking using a measuring cup to represent the whole milking of each animal for detecting the milk constitution (fat percentage, protein percentage, SNF, and lactose percentage) by using ultrasonic portable milk analyzer (milko tester model- master mini) at Limpopo dairy farm. Milk samples were collected immediately after cleaning the cow’s teats for 2 minutes.

3.6 Amplification of *β-LG* gene by Polymerase Chain Reaction

Blood samples were sent to Inqaba Biotechnology company for DNA extraction and amplification by polymerase chain reaction (PCR). Amplification of *β-LG* gene covering intron 3, exon 4, and intron 5 was done using a pair of forward (5′GCC TCA GAC TCA GTG GTGA 3′) and reverse (5′ACC ACA CAG CTG GTC TCC 3′) primers. The primers were designed using primer 3.0 software and the published nucleotide sequence of the Bos taurus *β-LG* gene (GenBank Accession No X14710.1). The PCR reaction was carried out in 20 μl reactions consisting of 10 μl of NEB OneTaq 2X Master mix with standard buffer, 1 μl of genomic DNA (10-30ng/μl), 1 μl of each primer

(10 µM), and up to 7 µl of nuclease free water. Amplification was done under the following conditions: denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 1 minute, with a 10-minutes extension at 68°C as the last step and hold at 4°C. The integrity and molecular weight of the PCR amplicons were determined using 1% agarose gel (Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. PCR products were cleaned using ExoSAP Protocol as follows: 1. Exo/SAP master mix was prepared by adding Exonuclease I 20 U/ul 50 µl and Shrimp Alkaline Phosphatase 1 U/ul 200 µl to a 0.6 ml micro-centrifuge tube. 2. Amplified PCR product 10 µl and ExoSAP Mix (step 1) 2.5 µl mixtures were prepared. 3. Mixture was mixed well and incubated at 37°C for 15 min. 4. The reaction was stopped by heating the mixture at 80°C for 15 min.

3.8 DNA sequencing

PCR products of a region of introns 3 and 5 as well as exon 4 of the *β-LG* gene were sequenced at Inqaba Biotechnology to identify the single nucleotide polymorphism (SNPs). Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions (https://www.nimagen.com/products/Sequencing/CapillaryElectrophoresis/BrilliantDye-Terminator-Cycle-Sequencing-Kit/). Sequence alignment was performed using NCBI/ BLAST/ blastn suit. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053). The cleaned products were injected on an Applied Biosystems ABI 3500XL Genetic Analyser or Applied Biosystems ABI 3730XL Genetic Analyser with a 50cm array, using POP7. Sequence chromatogram analysis was performed using FinchTV analysis software. NCBI/BLAST site was used to perform sequence alignment.

3.9 Statistical analysis

Statistical Package for Social Science (IBM SPSS, 2020) version 27.0 was used for data analysis. Pearson's correlation was used to achieve the first objective. The single nucleotide polymorphisms of *β-LG* gene on Holstein Friesian milk traits were identified using DNA sequencing. Hardy-Weinberg Equilibrium test software for Population Genetic Analysis was used to calculate genotypic and allelic frequencies. The Chi-square test was used to measure the genetic equilibrium at the population level for the Hardy-Weinberg theorem. Student t-test was performed for marker-trait association analysis. The following model was used:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where:

Y_{ij} = Phenotypic values of traits

μ = Population mean

G_i = Fixed effect of genotype

e_{ij} = Random residual error

CHAPTER FOUR

RESULTS

4.1 Descriptive statistics

Descriptive summary of milk production traits of Holstein Friesian is shown in Table 4.01. The highest mean value was that of milk yield per 30 days which was 997.92 L. Fat had the lowest mean value of 1.59%, the highest coefficient of variation of 32.33% whereas solid-not-fat had the lowest coefficient of variation of 5.55%.

Table 4.1: Descriptive statistics of measured traits

Traits	Mean	SD	SE	CV (%)
Milk yield/ day (L)	24.74	5.24	0.37	21.17
Milk yield/ 30 days (L)	997.92	146.03	10.33	14.63
Fat %	1.59	0.51	0.04	32.33
Protein %	2.85	0.32	0.02	11.27
Solid-not-fat %	8.48	0.47	0.03	5.55
Lactose %	4.95	0.31	0.02	6.36

SD - Standard deviation, SE - Standard error, CV - coefficient of variation, L - Litres

4.2 Correlation matrix

Phenotypic correlation between milk yield and milk constituents is presented in Table 4.02. There was no significant correlation ($p > 0.05$) between MYD and LP and FP. MYD showed a highly positive statistical significant correlation ($p < 0.01$) with MY30D. There was a highly statistically significant negative correlation ($p < 0.01$) between MYD with PP and SNF. MY30D showed highly statistically significant negative correlation ($p < 0.01$) with FP and PP. There was no significant correlation ($p > 0.05$) between MY30D and LP. MY30D showed a highly positive statistical significant correlation ($p < 0.01$) with SNF. There was a highly statistically significant negative correlation ($p < 0.01$) between FP and LP and SNF. There was also a highly statistical significant correlation ($p < 0.01$) between FP and PP. PP showed a highly statistical significant correlation ($p < 0.01$) with SNF and LP. There was a highly statistical significant correlation ($p < 0.01$) between SNF and LP.

Table 4.2: Phenotypic correlation between milk yield and milk constituents

Traits	MYD	MY30D	FP	PP	SNF	LP
MYD						
MY30D	0.71**					
FP	0.06^{ns}	-0.29**				
PP	-0.46**	-0.33**	0.22**			
SNF	-0.37**	0.22**	-0.25**	0.75**		
LP	-0.13^{ns}	-0.09^{ns}	-0.33**	0.37**	0.79**	

MYD - Milk yield per day, MY30D - Milk yield per 30 days, FP - Fat percentage, PP - Protein percentage, SNF – Solid-not-fat, LP - Lactose percentage, ^{ns} - Non-significant, * - Correlation is significant at 0.05 level, ** - Correlation is significant at a 0.01 level.

4.3 Amplified nucleotide sequence analysis

Figure 4.1 shows amplified PCR products of *β-LG* gene in Holstein Friesian cows used in this study. The amplicon size of 447bp was generated during the amplification.

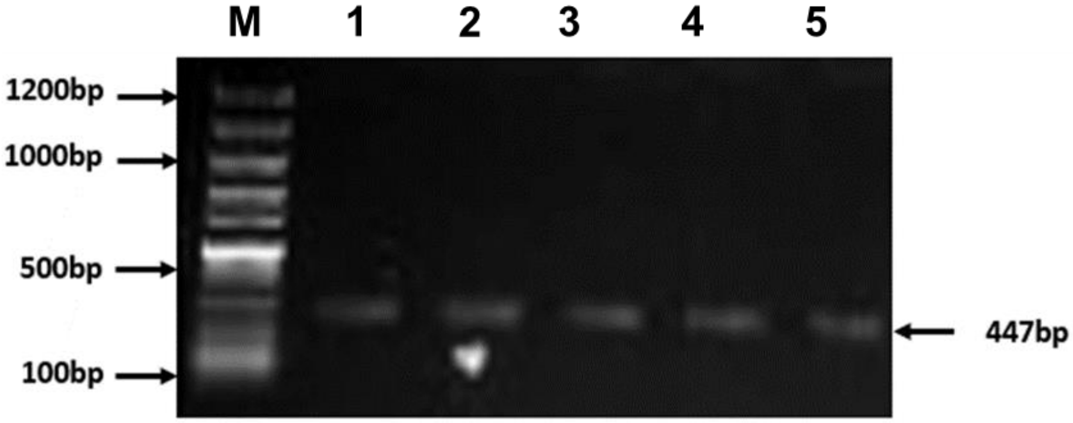
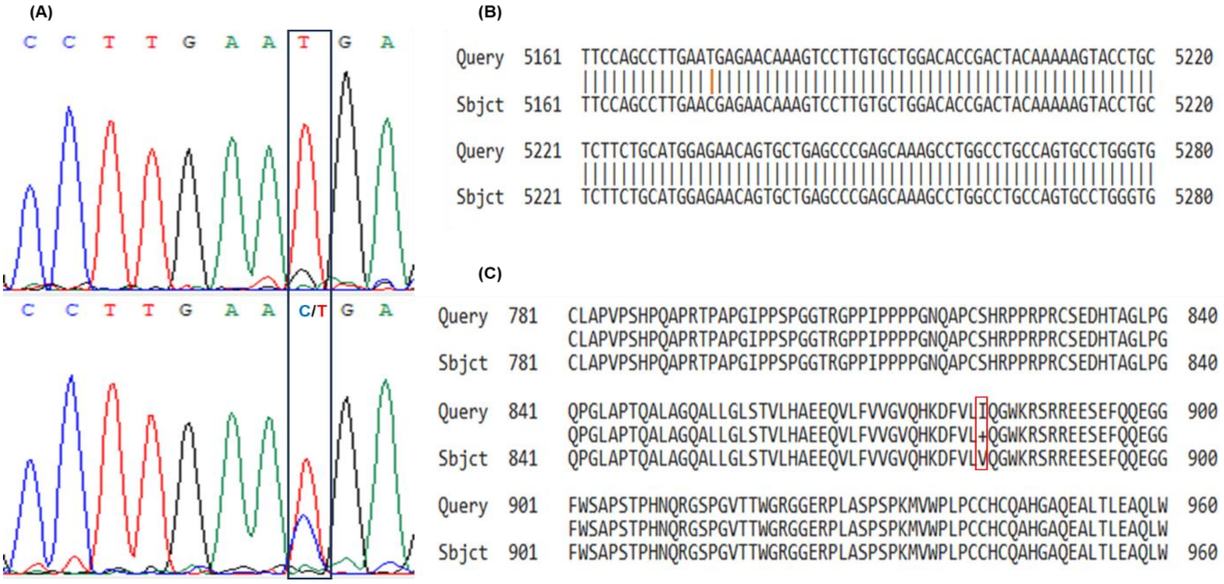


Figure 4.1: *β-LG* gene fragments amplicon. M, DL 1200 DNA marker (1200bp, 1000bp, 500bp, and 100bp, respectively); Lanes 1- 4, fragments of *β-LG* gene amplified.

4.4 Sequence analysis and alignments on 5174T>C of exon 4

The analysis and alignments of sequence on 5174T>C of exon 4 is shown on Figure 4.2. Chromas was used to analyse the sequence shown in Figure 4.2A. Intron 3, exon 4, and intron 5 were sequenced in the *β-LG*, 2 SNPs were detected on exon 4 whereas 3 were found on intron 3, and no SNP was found on intron 5. A polymorphism was detected with nucleotide transition from thymine (T) to cytosine (C) at position 5174 of exon 4 when compared with the *β-LG* gene *(*accession number: X14710.1). Blast was used to find the pairwise alignments of deoxyribonucleic acid (DNA) as highlighted in Figure 4.2B. The sequence alignment results showed 5174T>C as the SNP position (red line). Blast was used to determine the protein sequence alignment as indicated in Figure 4.2C. The results indicated non-synonymous SNP as highlighted with a red box. Amino acid change from isoleucine (I) to valine (v) at position 882 was found by comparing it with the *β-LG* gene (acc. no. Np_776354.2).



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Query  5161  TTCCAGCCTTGAATGAGAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGC  5220
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Sbjct  5161  TTCCAGCCTTGAACGAGAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGC  5220

Query  5221  TCTTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAAGCCTGGCCTGCCAGTGCCTGGGTG  5280
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Sbjct  5221  TCTTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAAGCCTGGCCTGCCAGTGCCTGGGTG  5280
```

```
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             CLAPVPSHPQAPRTPAPGIPPSPGGTRGPPIPPPPGNQAPCSHRPPRPRCSEDHTAGLPG
Sbjct  781   CLAPVPSHPQAPRTPAPGIPPSPGGTRGPPIPPPPGNQAPCSHRPPRPRCSEDHTAGLPG  840

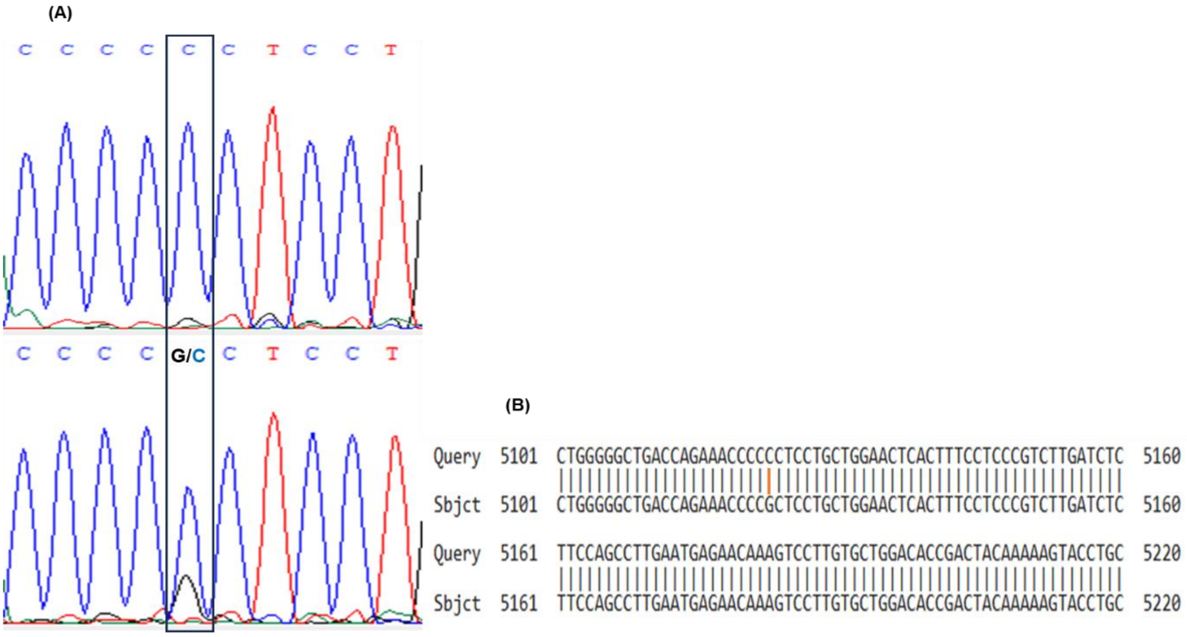
Query  841   QPGLAPTQALAGQALLGLSTVLHAEEQVLFVVGVQHKDFVLIQGWKRSRREESEFQQEGG  900
             QPGLAPTQALAGQALLGLSTVLHAEEQVLFVVGVQHKDFVL+QGWKRSRREESEFQQEGG
Sbjct  841   QPGLAPTQALAGQALLGLSTVLHAEEQVLFVVGVQHKDFVLVQGWKRSRREESEFQQEGG  900

Query  901   FWSAPSTPHNQRGSPGVTTWGRGGERPLASPSPKMVWPLPCCHCQAHGAQEALTLEAQLW  960
             FWSAPSTPHNQRGSPGVTTWGRGGERPLASPSPKMVWPLPCCHCQAHGAQEALTLEAQLW
Sbjct  901   FWSAPSTPHNQRGSPGVTTWGRGGERPLASPSPKMVWPLPCCHCQAHGAQEALTLEAQLW  960
```

Figure 4.2: Sequence analysis and alignments for SNP 5174T>C of *β-LG* gene in Holstein Friesian cows. **A** Nucleotide sequence analysis showing the SNP site on exon 4. **B** Nucleotide sequence alignment with line indicating the SNP and its location. **C** Protein sequence alignment with red box indicating the protein change and its position.

4.5 Sequence analysis and alignments on 5123C>G of intron 3

The analysis and alignments of sequence on 5123C>G of intron 3 is shown on Figure 4.3. Chromas was used to analyse the sequence shown in Figure 4.3A. A polymorphism was found with nucleotide transition from cytosine (C) to guanine (G) at position 5123 of intron 3 when compared with the *β-LG* gene (accession number: X14710.1). Blast was used to find the pairwise alignments of DNA as indicated in Figure 4.3B. The sequence alignment results showed 5123C>G as the location of the SNP as highlighted in red line.



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Query  5101  CTGGGGCTGACCAGAAACCCCCCTCCTGCTGGAACTCACTTTCCTCCCGTCTTGATCTC  5160
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Sbjct  5101  CTGGGGCTGACCAGAAACCCCCGCTCCTGCTGGAACTCACTTTCCTCCCGTCTTGATCTC  5160

Query  5161  TTCCAGCCTTGAATGAGAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGC  5220
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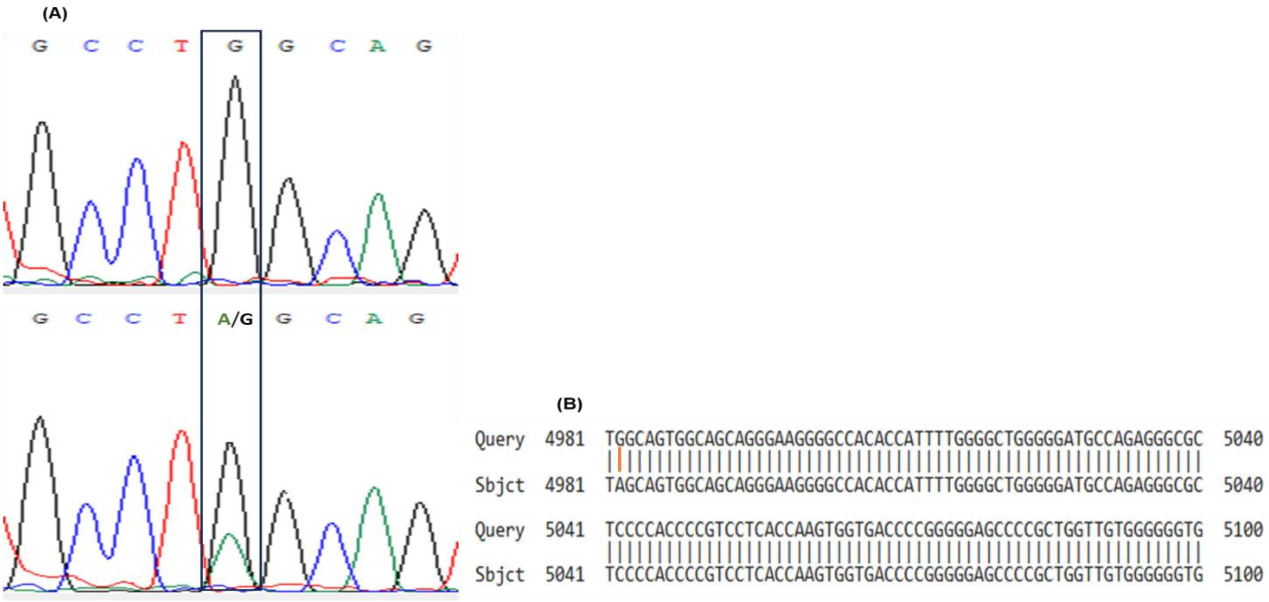
Sbjct  5161  TTCCAGCCTTGAATGAGAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGC  5220
```

Figure 4.3: Sequence analysis and alignments for SNP 5123C>G of *β-LG* gene in Holstein Friesian cows. **A** Nucleotide sequence analysis showing the SNP site on intron 3. **B** Nucleotide sequence alignment with red line indicating SNP and its location.

4.6 Sequence analysis and alignments on 4982G>A of intron 3

Sequence analysis and alignments of gene on 4982G>A of intron 3 is shown on Figure 4.4. Chromas was used to analyse the sequence shown in Figure 4.4A. Nucleotide transition from guanine (G) to adenine (A) polymorphism was detected at position 4982 of intron 3 when compared with the *β-LG* gene (accession number: X14710.1). Blast was used to find the pairwise alignments of DNA as indicated in Figure 4.4B. The sequence alignment results showed 4982G>A as the position of the SNP as highlighted with the red line.



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Query  4981  TGGCAGTGGCAGCAGGGAAGGGGCCACACCATTTTGGGGCTGGGGGATGCCAGAGGGCGC  5040
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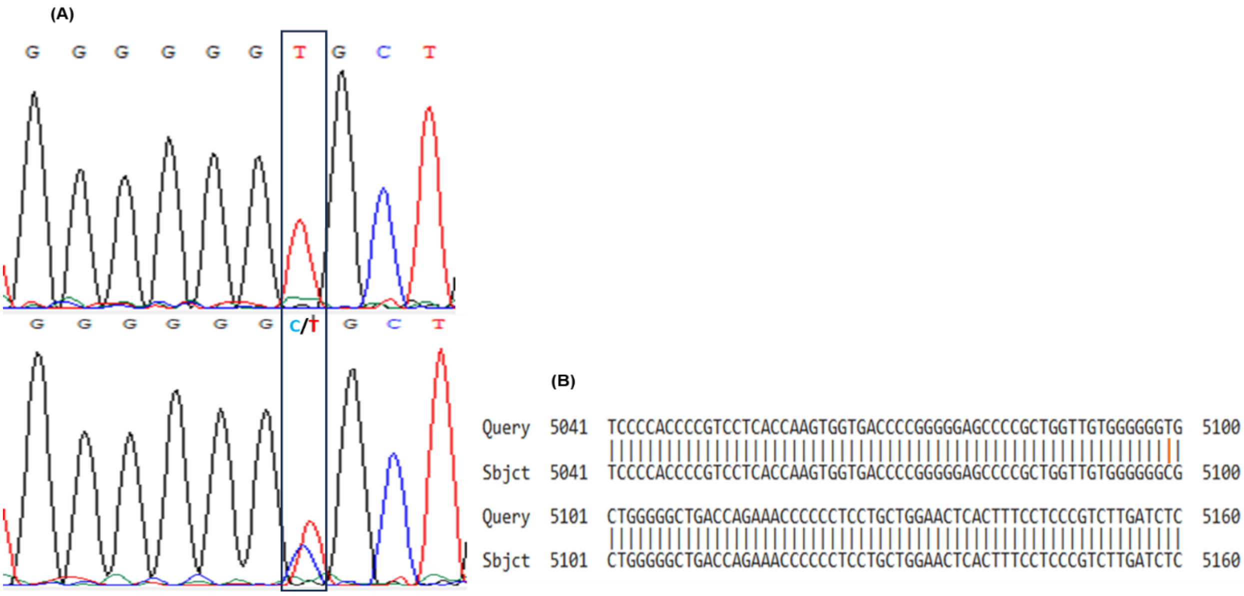
Sbjct  4981  TAGCAGTGGCAGCAGGGAAGGGGCCACACCATTTTGGGGCTGGGGGATGCCAGAGGGCGC  5040

Query  5041  TCCCCACCCCGTCCTCACCAAGTGGTGACCCCGGGGGAGCCCCGCTGGTTGTGGGGGGTG  5100
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5041  TCCCCACCCCGTCCTCACCAAGTGGTGACCCCGGGGGAGCCCCGCTGGTTGTGGGGGGTG  5100
```

Figure 4.4: Sequence analysis and alignments for SNP 4982G>A of *β-LG* gene in Holstein Friesian cows. **A** Nucleotide sequence analysis showing the SNP site on intron 3. **B** Nucleotide sequence alignment with red line showing the SNP position.

4.7 Sequence analysis and alignments on 5099T>C of intron 3

Gene sequence analysis and alignments on 5099T>C of intron 3 is shown on Figure 4.5. Chromas was used to analyse the sequence shown in Figure 4.5A. A polymorphism was detected with nucleotide transition from thymine (T) to cytosine (C) at position 5099 of intron 3 when compared with the *β-LG* gene (accession number: X14710.1). Blast was used to find the pairwise alignments of DNA as indicated in Figure 4.5B. The sequence alignment results showed 5099T>C as the SNP location as highlighted in the red line.



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Query  5041  TCCCCACCCCGTCCTCACCAAGTGGTGACCCCGGGGGAGCCCCGCTGGTTGTGGGGGGTG  5100
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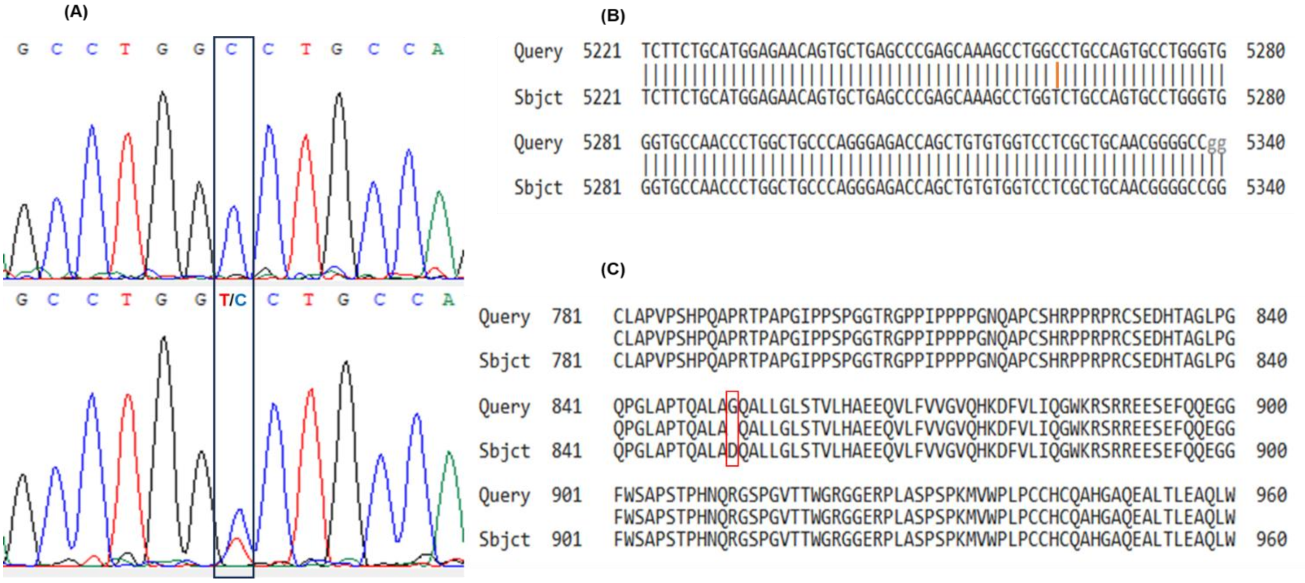
Sbjct  5041  TCCCCACCCCGTCCTCACCAAGTGGTGACCCCGGGGGAGCCCCGCTGGTTGTGGGGGGCG  5100

Query  5101  CTGGGGGCTGACCAGAAACCCCCCTCCTGCTGGAACTCACTTTCCTCCCGTCTTGATCTC  5160
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5101  CTGGGGGCTGACCAGAAACCCCCCTCCTGCTGGAACTCACTTTCCTCCCGTCTTGATCTC  5160
```

Figure 4.5: Sequence analysis and alignments for SNP 5099T>C of *β-LG* gene in Holstein Friesian cows. **A** Nucleotide sequence analysis showing the SNP site on intron 3. **B** Nucleotide sequence alignment with red line indicating the SNP and its location.

4.8 Sequence analysis and alignments on 5251C>T of exon 4

The analysis and alignments of sequence on 5251C>T of exon 4 is shown on Figure 4.6. Chromas was used to analyse the sequence shown in Figure 4.6A. Polymorphism was detected with nucleotide transition from cytosine (C) to thymine (T) at position 5251 of exon 4 when compared with the *β-LG* gene (accession number: X14710.1). Blast was used to find the pairwise alignments of DNA as highlighted in Figure 4.6B. The sequence alignment results showed 5251C>T as the SNP position (red line). Blast was used to determine the protein sequence alignment as indicated in Figure 4.6C. The results indicated nonsynonymous SNP as highlighted in red box. Change in amino acid from glycine (G) to aspartic acid (D) at position 852 was detected by comparing it with the *β-LG* gene (acc. no. Np_776354.2).



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Query  5221  TCTTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAAGCCTGGCCTGCCAGTGCCTGGGTG  5280
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Sbjct  5221  TCTTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAGTGCCTGGGTG  5280

Query  5281  GGTGCCAACCCTGGCTGCCCAGGGAGACCAGCTGTGTGGTCCTCGCTGCAACGGGGCCgg  5340
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Sbjct  5281  GGTGCCAACCCTGGCTGCCCAGGGAGACCAGCTGTGTGGTCCTCGCTGCAACGGGGCCGG  5340
```

```
Query  781   CLAPVPSHPQAPRTPAPGIPPSPGGTRGPPIPPPPGNQAPCSHRPPRPRCSEDHTAGLPG  840
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Sbjct  781   CLAPVPSHPQAPRTPAPGIPPSPGGTRGPPIPPPPGNQAPCSHRPPRPRCSEDHTAGLPG  840

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Sbjct  841   QPGLAPTQALADQALLGLSTVLHAEEQVLFVVGVQHKDFVLIQGWKRSRREESEFQQEGG  900

Query  901   FWSAPSTPHNQRGSPGVTTWGRGGERPLASPSPKMVWPLPCCHCQAHGAQEALTLEAQLW  960
             FWSAPSTPHNQRGSPGVTTWGRGGERPLASPSPKMVWPLPCCHCQAHGAQEALTLEAQLW
Sbjct  901   FWSAPSTPHNQRGSPGVTTWGRGGERPLASPSPKMVWPLPCCHCQAHGAQEALTLEAQLW  960
```

Figure 4.6: Sequence analysis and alignments for SNP 5251C>T of *β-LG* gene in Holstein Friesian cows. **A** Nucleotide sequence analysis showing the SNP site on exon 4. **B** Nucleotide sequence alignment with red line showing SNP and its position **C** Protein sequence alignment with red line indication protein change and its position.

4.9 Genotypic and allelic frequencies

Table 4.3 shows the allelic and genotypic frequencies of *β-LG* gene locus in the Holstein Friesian population. The chi-square test was used to determine the significant differences in allelic and genotypic frequencies of Holstein Friesian cows. Two alleles and two genotypes (homozygous and heterozygous) were noted for each SNP. For SNPs 5174T>C, 5123C>G, 4982G>A, 5099T>C, and 5251C>T, allelic frequencies of T, C, G, T, and C were higher than that of C, G, A, C, and T respectively. Genotypic frequencies of CT, CC, AG, CT, and TC were higher than the genotypic frequencies of TT, GC, GG, TT, and CC for SNPs 5174T>C, 5123C>G, 4982G>A, 5099T>C, and 5251C>T respectively. The Chi-square (X^2) test for 5123C>G showed that genotypic and allelic frequencies were not significantly different from the expectations of Hardy-Weinberg ($X^2 = 1.23$). The results indicate that genotypic and allelic frequencies of population remain constant from generation to generation. However, 5174T>C, 4982G>A, 5099T>C, and 5251C>T SNPs were tested and demonstrated remarkable genetic disequilibrium between alleles ($P>0.05$). The results indicate that genotypic and allelic frequencies of population changes from generation to generation.

Table 4.3: Genotypic and allelic frequencies at the single nucleotide polymorphisms locus of *β-LG* gene in Holstein Friesian cows

SNPs	Genotype	Number of animals	Genotypic frequency	Allele	Allele frequency	χ^2
5174T>C	TT	20	0.2	T	0.6	44.44*
	CT	80	0.8	C	0.4	
5123C>G	CC	80	0.8	C	0.9	1.23^{ns}
	GC	20	0.2	G	0.1	
4982G>A	GG	20	0.2	G	0.6	44.44*
	AG	80	0.8	A	0.4	
5099T>C	TT	20	0.2	T	0.6	44.44*
	CT	80	0.8	C	0.4	
5251C>T	CC	40	0.4	C	0.7	18.37*
	TC	60	0.6	T	0.3	

SNPs - Single nucleotide polymorphisms, χ^2- Chi-square, * - Statistically significant ($p < 0.05$) when the data were analysed using a Pearson’s goodness-of-fit chi-square test (degree of freedom = 1), ^{ns} - non-significant ($p < 0.05$).

4.10 Polymorphism information analysis

Polymorphism information analysis and genetic diversity of the population as shown on Table 4.4 was achieved by Hardy Weinberg Equilibrium Test. The gene homozygosity was higher than the gene heterozygosity for SNPs 5174T>C, 5123C>G, 4982G>A, 5099T>C, and 5251C>T with effective allele number (N_e) of 1.92, 1.22, 1.92, 1.92, and 1.72 respectively. Polymorphisms information content (*PIC*) indicated that there were high polymorphisms within the Holstein Friesian population for SNPs 5174T>C, 4982G>A, 5099T>C, and 5251C>T. However, it showed that there were moderate polymorphisms within the Holstein Friesian population for SNP 5123C>G.

Table 4.4: Polymorphism information analysis of *β-LG* gene of Holstein Friesian cows

SNPs	**Gene homozygosity *(H_0)***	**Gene heterozygosity *(H_e)***	**Effective allele number *(N_e)***	**Polymorphism information content *(PIC)***
5174T>C	0.52	0.48	1.92	0.36
5123C>G	0.82	0.18	1.22	0.16
4982G>A	0.52	0.48	1.92	0.36
5099T>C	0.52	0.48	1.92	0.36
5251C>T	0.58	0.42	1.72	0.33

SNPs - Single nucleotide polymorphisms

4.11 Association analysis of *β-LG* gene with milk production traits

Association between marker-traits are displayed in Table 4.5. Student T-test was used to examine the association between identified genetic markers and measured traits. The results indicated that genotypes of SNPs 5174T>C, 4982G>A, and 5099T>C were not significantly different for the MY30D, PP, and SNF traits ($p>0.05$), however, they had significant difference amongst MYD, FP, and LP traits ($p < 0.05$). 5123C>G showed significant difference between CC and CG genotypes for MY30D, FP, SNF, and LP ($p < 0.05$), this SNP showed non-significant difference between CC and CG genotypes with MYD and PP ($p>0.05$). There was a significant difference between CC and CT genotypes with FP, SNF, and LP for SNP 5251C>T ($p<0.05$), however, CC and CT genotypes were not significantly different from MYD, MY30D, and PP ($p>0.05$).

Table 4.5: Association of the polymorphism of *β-LG* gene with milk production traits of Holstein Friesian cows

SNPs	**Genotype**	**MYD (L) (Mean± SE)**	**MY30D (L) (Mean± SE)**	**FP (%) (Mean± SE)**	**PP (%) (Mean± SE)**	**SNF (%) (Mean± SE)**	**LP (%) (Mean± SE)**
5174T>C	TT	30.20±0.00^{a}	1099.00±0.00	1.16±0.00^{b}	2.85±0.00	8.54±0.00	5.10±0.00^{a}
	CT	25.39±0.76^{b}	1040.00±17.93	1.64±0.06^{a}	2.92±0.04	8.39±0.06	4.88±0.03^{b}
5123C>G	CC	26.91±0.79	1083.75±16.30^{a}	1.31±0.14^{b}	2.91±0.35	8.59±0.41^{a}	4.06±0.01^{b}
	CG	24.09±0.00	924.00±0.00^{b}	2.49±0.00^{a}	2.89±0.00	7.74±0.00^{b}	4.38±0.00^{a}
4982G>A	GG	30.20±0.00^{a}	1099.00±0.00	1.16±0.00^{b}	2.85±0.00	8.54±0.00	5.10±0.00^{a}
	AG	25.39±0.76^{b}	1040.00±17.93	1.64±0.06^{a}	2.92±0.04	8.39±0.06	4.88±0.03^{b}
5099T>C	TT	30.20±0.00^{a}	1099.00±0.00	1.16±0.00^{b}	2.85±0.00	8.54±0.00	5.10±0.00^{a}
	CT	25.39±0.76^{b}	1040.00±17.93	1.64±0.06^{a}	2.92±0.04	8.39±0.06	4.88±0.03^{b}
5251C>T	CC	27.15±0.49	1011.50±14.01	1.83±0.11^{a}	2.87±0.00	8.14±0.06^{b}	4.74±0.06^{b}
	TC	25.82±1.01	1078.67±21.74	1.36±0.01^{b}	2.93±0.05	8.61±0.05^{a}	5.04±0.01^{a}

MYD - Milk yield per day, MY30D - Milk yield per 30 days, FP - Fat percentage, PP - Protein percentage, SNF – Solid-not-fat, LP - Lactose percentage, ^{ns} - Non-significant, SE - Standard error, L - Litres, SNPs - Single nucleotide polymorphisms, ^{a,b} - Different superscript on the same row shows significant difference ($p < 0.05$).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The main goal in dairy cattle breeding is to find economically effective way of improving milk production traits (Hristov et al., 2011). Since the discovery of *β-LG* gene alleles in cattle, genetic polymorphism in milk protein genes has raised great interest in animal breeding and dairy industry due to its relationship with milk production traits (Alim et al., 2015). It is essential to predict the change in one trait in response to selection for another, thus, the knowledge of the relationship between traits is necessary for the improvement of fat, protein, SNF, lactose and milk yield of dairy animals (El-Moghazy et al., 2015).

Hence, the first objective of this study used Pearson's correlation to determine the relationship between milk yield and milk constituents in Holstein Friesian cows in South Africa. The findings showed a negative relationship between milk yield per day with protein % and SNF. Milk yield per 30 days was negatively correlated with fat % and protein %, but it was positively correlated with SNF. Milk yield had no relationship with lactose %, and milk yield per day had no statistical relationship with fat %.The findings of the current study agree with the study conducted by El-Moghazy et al. (2015) who discovered that SNF was positively and highly correlated with milk yield of Egyptian Buffaloes, however, this study also disagrees with the same study that found that fat, protein, and lactose were positively correlated with milk yield. The findings of the study conducted by Alphonsus and Essien, (2011) who stated that SNF, fat, and protein were not significantly correlated with total milk yield in Bunaji and Friesian ×Bunaji cows of Nigeria disagreed with the findings of the current study. The current study agrees with the study that was done by Yoon et al. (2004) who stated that milk yield was highly and negatively correlated with fat and protein of Holstein cows in Korea. The difference between the current study and other studies might be due to the use of different species, breed, and environment. The result of this study implies that decreasing protein % and SNF increases milk yield per day, furthermore, fat % and lactose % does not affect milk yield per day. An increase in SNF increases milk yield per 30 days, whereas increasing fat and protein leads to a decrease in milk yield per 30 days. Lactose % does not have any effect on milk yield per 30 days. The genetic improvement of quantitative traits is relatively slow as productive traits can only be measured in one sex and is affected by numerous polygenes (Kusza *et al*., 2015).

Selecting and breeding animals with interesting genotypes is very important for genetic improvement of dairy cattle (Alim et al., 2015). Hence, the second objective of this study was to identify the single nucleotide polymorphisms of *β-LG* gene and its association with milk production traits of South African Holstein Friesian cows. Findings of the current study revealed 2 non-synonymous SNPs (T/C) and (C/T) at positions 5174 and 5251 of exon 4 of the *β-LG* gene which were named 5174T>C and 5251C>T, respectively. This study also noted 3 other SNPs in intron 3 (C/G), (G/A), and (T/C) on positions 5123, 4982, and 5099 which were named 5123C>G, 4982G>A, and 5099T>C respectively. A single nucleotide polymorphism (1810C>T) in exon 3 in *β-LG* gene of Chinese Holstein cows was discovered by Alim et al. (2015) who conducted a study on DNA polymorphisms in the *β-lactoglobulin* gene associated with milk production traits in Holstein dairy cattle in China. Mancini et al. (2013) found a SNP (C>A) at position 968 of upstream gene variant of *β-LG* gene on Italian Brown cattle in Italy. Yang et al. (2012) investigated polymorphism in the exon 4 of *β-LG* gene variant B precursor and its association with milk traits and protein structure in Chinese Holstein and identified 3 nonsynonymous SNPs (5239C>A, 5240A>C, 5305C>T), meaning that the three SNPs caused amino acid change (Aspartic acid to Glutamic acid, Threonine to Proline and Alanine to Valine) respectively. Disagreement could be due to differential expression of genes which influences animal's production traits. The results of this study suggest that SNPs 5174T>C and 5251C>T causes an amino acid change from isoleucine to valine and glycine to aspartic acid respectively, which influences protein structure and function. Chi-square results of the current study demonstrated that the population used was under Hardy-Weinberg equilibrium (HWE) for SNP 5123C>G. However, it was not under HWE for SNPs 5174T>C, 4982G>A, 5099T>C, and 5251C>T. The findings of the study conducted by Alim et al. (2015) indicated that chi-square test for SNP 1810C>T showed all genotypic frequencies in the population to be in Hardy-Weinberg equilibrium indicating that selection pressure on this site in the population was not too powerful. Yang et al. (2012) reported that after chi-square test the 3 SNPs (5239C>A, 5240A>C, 5305C>T) were not under Hardy-Weinberg equilibrium (HWE). The current study suggest that the studied population is under HWE implying that the genotypic and allelic frequency for SNP 5123C>G of *β-LG* gene on Holstein Friesian cows does not change from generation to generation. However, the studied population was not under HWE for SNPs

5174T>C, 4982G>A, 5099T>C, and 5251C>T, implying that for these SNPs genotypic and allelic frequency changes from generation to generation.

Marker trait association findings for SNPs 5174T>C and 5099T>C showed that there was no statistical relationship between genotype TT and CT with milk yield per 30 days, protein %, and SNF. Genotype TT had the highest impact on milk yield per day and lactose %, whereas genotype CT had the greatest impact on fat %. For SNP 4982G>A genotype GG and AG had no statistical effect on milk yield per 30 days, protein %, and SNF. Genotype GG had the highest impact on milk yield per day and lactose %, whereas genotype AG had the greatest impact on fat %. Genotype CC and CG of SNP 5123C>G had no relationship with milk yield per day and protein %. CG had the highest effect on lactose % and fat %, whereas CC showed a greater impact on milk yield per 30 days and SNF. Marker trait association results for SNP 5251C>T indicated no significant relationship between genotype CC and CT with milk yield and protein %. However, CC showed great impact on fat %, while TC appeared to have highest impact on SNF and lactose %. Association of *β-LG* gene polymorphism with milk yield, fat, and protein in Holstein Friesian cattle in Egypt was investigated by Zaglool et al. (2016), who discovered 3 genotypes (AA, AB, and BB) and found AA had more milk yield and protein %, while BB genotype recorded higher fat %, the results are not in line with the ones of the current study. The current study for SNPs 5123C>G on milk yield and 5251C>T on fat % is in agreement with the study conducted by Hristov et al. (2011) who found 2 genotypes AA and AB of *β-LG* gene in Bulgarian Black Pied cattle, that revealed BB genotype to have the highest effect on milk yield and fat %. For SNPs 5124T>C, 4982G>A, and 5099T>C on SNF, and SNP 5251C>T on milk yield, the current study agrees with that of Tolenkhomba et al. (2014) that revealed two genotypes AB and BB that had no significant effect on milk yield and SNF of Sahiwal cattle breeds of India. The results of the current study are in contradiction with the ones of the study that was conducted by Dogru (2015) who investigated *β-lactoglobulin* genetic variants in Brown-Swiss dairy cattle and their association with milk yield and quality traits in Turkey and found no significant association between different genotypes (AA, AB, and BB) of *β-LG* gene and milk production traits. The difference in this study might be to different environmental conditions and breeds used.

TT genotype for SNPs 5174T>C, and 5099T>C of *β-LG* gene might be used as genetic marker when improving milk yield per day and lactose %, whereas genotype CT might be used to improve fat %. Genotype CC of SNPs 5123C>G might be used to increase milk yield per 30 days and SNF, while CG be used to improve lactose % and fat %. TC for SNP 5251C>T might be used as a genetic marker to increase SNF and lactose%, whereas CC be used to improve fat%. GG genotype for SNP 4982G>A of *β-LG* gene might be used as genetic marker when improving milk yield per day and lactose %, whereas genotype AG might be used to improve fat %.

5.2 Conclusion

The findings of this study conclude that protein and soli-not-fat has the potential to be used when improving milk yield of Holstein Friesian cows. Genotypic and allelic frequency for SNP 5123C>G of *β-LG* gene on Holstein Friesian cows does not change from generation to generation. However, genotypic and allelic frequencies change from generation to generation for SNPs 5174T>C, 4982G>A, 5099T>C, and 5251C>T of *β-LG* gene on Holstein Friesian cows. DNA analysis revealed 2 non-synonymous SNPs (5174T>C, 5251C>T) on exon 4 of *β-LG* gene of Holstein Friesian cows, that caused a change in amino acids Isoleucine to valine and glycine to aspartic acid respectively which led to a change in protein structure and function.

It was noted that genotypes TT and CT were found to have association with milk yield per day, lactose %, and fat % on SNPs 5174T>C and 5099T>C, with genotype TT contributing more on milk yield per day and lactose %, and genotype CT contributing more on fat %. Milk yield per day, lactose %, and fat % were associated with genotypes GG and AG of SNP 4982G>A, with GG having the higher effect on milk yield per day and lactose %, while AG had great impact on fat%. CC and CG genotypes of SNP 5123C>G were associated with milk yield per 30 days, SNF, lactose % and fat %, with CC contributing more on milk yield per 30 days and SNF, whereas CG affected lactose % and fat % more. There was an association between CC and TC genotypes of SNP 5251C>T and fat %, SNF, and lactose %, with CC having high impact on fat % and TC having high effect on SNF and lactose %.

5.3 Recommendations

The findings of this study suggest that farmers could use SNF and protein % to improve milk yield of Holstein Friesian cows. The study further recommends more research on a broader scale, using different milk production traits and/or breeds. Holstein Friesian with genotype TT for SNPs 5174T>C and 5099T>C may be selected for the improvement of milk yield per day and lactose %, and those with genotype CT may be selected for improvement of fat%. For SNP 4982G>A animals with genotype GG may be selected for enhancement of milk yield per day and lactose %, and those with genotype AG may be selected for improvement of fat%. Animals with genotype TC for SNP 5251C>T may be considered for breeding of cows that produces milk with high SNF and lactose %, whereas those with genotype CC may be considered for improvement of fat %. Cattle with CG genotype for SNP 5123C>G may be considered for selection of improved lactose % and fat %, whereas those with CC genotype may be selected for improved milk yield per 30 days and SNF. More studies need to be conducted on *Beta lactoglobulin* gene polymorphisms and their association with milk production traits using larger sample sizes and more milk production traits.

CHAPTER SIX

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