Genetic Polymorphisms of Milk Genes (β-Lactoglobulin and κ-Casien) in Palestinian Awwasi Sheep Breeds

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DEDICATION

To Whom Supported Me in Accomplishing This Success

My Advisor

My Dear Parents

My Brother

My Lovely Friends

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LISTOF ABBREVIATIONS

Name	Abbreviation
Tm	Melting Temperature
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
MAS	Marker Assisted Selection
RFLP	Restriction Fragment Length Polymorphism
β-LG	Beta lacto globulin
QTL	Quantitative trait loci
dH2O	Distilled water
DNA	Deoxyribonucleic acid
DNA SP	DNA Sequence Polymorphism
Dntp	Deoxyribo Nucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
EtOH	Ethanol
Bp	base pair
K3EDTA	Tripotassium Ethylene Diamine Tetra Acetic Acid
MgCl2	Magnesium Chloride
mg	Milligram
mL	Milliliter
mM	Milimolar
M	Molar
ng	Nanogram
PCR	Polymerase Chain Reaction
P	Page

Rpm	Rotations per minute
SDS	Sodium Dodecyl Sulfate
SNF	Solid Not Fat
K-CN	kappa-Casein Gene
TAE Buffer	Tris Acetate EDTA Buffer
µM	Micro Molar
PCBS	Palestinian Central Bureau of Statistics
SNF	Solid Not fat
J	Jericho
R	Ramallah
N	Nablus
H	Hebron
B	Bethlehem
J	Jenin
TMY	Total milk yield
SNP	Single nucleotide polymorphism
α- LA	α-lactalbumin
MOA	Ministry of Agriculture
PCLI	Palestinian Center for Livestock Improvement

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Abstract

Milk and dairy products from small ruminant species represent a significant part of the agricultural economy worldwide especially in marginal areas. Evidences showed that there is a strong association between milk protein polymorphisms and milk yield. So, it is necessary to make available in-depth information regarding genetic polymorphisms of milk proteins in sheep breeds in Palestine with consideration of the great genetic biodiversity of sheep breeds.This study was conducted to assess the polymorphisms of *κ*-casein (*κ-CN*) and *β*-lactoglobulin (*β-LG*) genes of the main breeds of Palestinian Awassi sheep (local and improved Awassi) and their effect on milk quality.

DNA was extracted from 54 sheep (23-local Awassi and 31- improved Awassi). The *κ-CN* and *ß-LG* were amplified using Polymerase Chain Reaction (PCR). Restriction Fragment Length Polymorphism (RFLP) technique was used to determine variations at the *ß-LG* loci, while for *K-CN* direct sequencing was performedby Sanger sequencing.

The amplified products for *ß-LG* and *K-CN* genes had the sizes of 471 bp and 670bp, respectively. Digestion of *β*-lactoglobulin gene using *RsaI*restriction enzyme revealed the presence of three genotypes (AA, AB, and BB). The results of *ß-LG* the sequence analysis of exon 2 revealed a point mutation (C→ T), a single base substitution T instead of C of the (X12817.1:c.112 T>C) locus in both local and improved Awassi which is

responsible for the amino acid exchange of histidine to tyrosine. The results of *κ-CN* sequence analysis showed the presence of three different patterns; CC, TC and TT. Sanger sequencing results revealed a single base pairsubstitution (C→ T) in codon no 56 of exon three, this mutation is asynonymous variant.

Statistical analysis showed that a significant effects of *β-LG* genotypes on fat contentand densityof milk in local but not improved Awassi;whereas significant effect of certain *κ- CN* genotype onSNF of milkin local Awassi only, while no effects of these genotypes on milk quality were significantly detected in improved Awassi.

Key words: Awassi Sheep, Improved Awassi, *β*-lactoglobulin, *K*-casein, PCR, SNP.

Chapter One

Introduction

Introduction

Sheep breeds in Palestine are mainly local Awassi, improved Awassi, east Friesian and Assaf. Awassi sheep are most common in the countries of the Middle East and arethe main sheep breed in both Palestine and Jordan (Talafha & Ababneh, 2011).In West Bank, they represent the major small ruminant species where they contribute a large source of meat and milk when compared to goats. According to Palestinian Center Bureau of Statistics (PCBS, 2013)the total number of sheep in West Bank reached to 730,894heads, where Awassi (local and improved) represents the major breed (68%), while Assaf and other breeds and crosses ranked secondly to Awassi (32%).

The fat-tailed Awassi sheep have remarkable advantages over other breeds as they possesses great adaptability to tropical environmental conditions, nutritional fluctuations anddisease resistance (Galal et al., 2008).Theintensiveselection within Awassi breed produced improved Awassi breed, which are well known to have the highest milk production after theEast Friesianbreed (Galalet al., 2008).

The milk industry in Palestine mainly depends on cow milk and to a lesser extent on sheep milk. It has strong interest in the raw milk quality, which is a prerequisitefor dairy products, especially from sheep that are almost

exclusively usedin yogurt- and cheese-making. In fact, in Palestine, milk is sold on volume basis; this makes it necessary to identify high-milk yielding sheep and cattle. On the other hand, in developed countries the industry pays good price for fat and protein concentrations in milk as these technically reflect the milk quality (Pambu et al., 2011). For this reason, there are high demands for sheep milk as it has distinctive characteristics and, as a consequence, specific productive destinations (Selvaggi et al., 2014).

The genetic improvement programs in livestock in order to enhance production and reproduction advantages were mainly depended on the selection within the population and this selection was depended mainly on phenotypic traits, and existing pedigree (Fleming et al., 2018). This type of selectionof breeding stock is based on observable phenotypes without knowing which genes are actually being selected. However, there are several problems associated with phenotypic selection including narrowing the genetic base of a population, and the approach can only be applied to traits that are easily measured. Moreover, in traits that are displayed only in adults, large number of animals is need to be raised for which the trait is recorded, so that a few can be chosen for breeding. Cost is very high in the case of progeny testing for milk yield and quality, as the test sires have to be raised and then the daughters themselves raised and bred before the trait can be measured and elite sires selected. This results into breeding programs that are expensive (Fleming et al.,2018). However, selection for

high milk quality and quantity production is not as easy as it looks as milkis a quantitative trait, means that controlled byseveral genes and the environment has influence on it. So, due to the environmental influences, the estimated breeding value from traditional selection methods alone is not a perfect to predict the genetic merit of an individual. In addition, milk production can only be measured in mature females; this makes it very difficult to analyze males influence. However, with the recent developments and advancements in DNA technology to identify genes which can be found on quantitative trait locus (QTL), many of these problems can be overcome because DNA can be obtained at any age and from any sex.

There are several genes found to be associated with milk quality and quantity, among these are Beta lactoglobulin and Kappa casein. The association of these genes with milk production and composition has encouraged scientists to use genetic polymorphism of these genes in molecular marker assisted selection (MAS) to improve milk productivity in farm animals (Raj., 2012).Selection efficiency, however, depends on allelic frequencies in the breeds of choice and on the effect of these polymorphisms on selected traits (e.g. dairy traits and technological properties of milk) (Karim et al., 2009).

Beta-lactoglobulin was selected as one of the candidate genes for this study for many reasons. Beta-lactoglobulin is one of the major whey proteins found in ruminant milk (Perez &Calvo et al., 1995). The gene encoding for beta-lactoglobulin has been found to be highly and specifically expressed in the mammary gland during lactation (Mercier &Vilotte et al., 1993).

Kappa caseinwasselected asanother candidate gene in this study for many reasons as well.*K-CN* plays an important role in the formation, stabilization and aggregation of the casein micelles thus altering the manufacturing properties and digestibility of milk (El-Shazly et al., 2013).

To our knowledge, the genetic of detection polymorphisms detection in *K-CN* and *β-LG* genes will be the first in Palestine and it will open the opportunities for the selection of sheep with high potential for milk quality and quantity.

1.1 Main Objective

The main objective of this study is the detection of genetic polymorphisms among *K-CN* and *ß-LG* genes of Palestinian Awassi sheep breed (local and Improved Awassi) and the assessment of the effects of these genetic polymorphisms on milk quality parameters.

1.2 Specific Objectives

- Identification of genotypes (genetic polymorphisms) and allele frequencies of *K-CN* and *ß-LG*genes in both local and improved Awassi breeds.
- Determination of milk composition in both Awassi and improved Awassi breeds.
- Assessment of the effect of *ß- LG* and *K-CN* genotypes on sheep milk quality.

Chapter Two

Literature Review

Literature Review

The selections of sheep breed by farmers and breeders in general, depend on economically important traits which are quantitative in nature like meat and milk production and composition. These traits are controlled by regions of the genome that contain one or more genes and called Quantitative Trait Loci (QTL) (Moradi et al., 2012).

Today, the development of applications of molecular genetics helps in the identification of the important QTL in livestock production (Dekkerset al., 2002).

The knowledge on the number, positions, and effects of all genes involved in such trait are essential for genetic improvement of farm animal's productivity (Dekkerset al.,2002). More information about these genes and their frequency, could be of great value in any future breeding programs for genetic improvement of livestock, especially sheep and goat.

The need to maintain and improve local genetic resources has been recognized as a priority, at the world level. Biodiversity studies showing a deep picture of the genetic variability of the available sheep breeds provide favorable opportunities for both genetic conservation programs as well as enhancing production efficiency by means of controlled and well-designed cross-breeding systems exploiting breed diversities, heterosis and breed complementarily (Ebrahim et al., 2017).

2.1 Sheep Population in Mediterranean region and in Palestine

In the east of Mediterranean, Awassi sheep is the most common breed; it is the main sheep breed in Iraq and Syria; whereas its native breed is found in Jordan (Awawdeh., 2011) Israel and Turkey (Galal et al.,2008). The Awassi breed has been described in different countries (Galal et al.,2008).

In Israel, within-breed selection, crossbreeding and gene introgression have contributed to the transition of the sheep industry from traditional extensive production using the native fat-tailed local Awassi breed to highly intensive production with the Assaf and Afec-Assaf sheep (Gootwine et al., 2011).Selection within the local Awassi sheep that began in the 1930s led to the formation of the improved Awassi dairy strain.

The average total milk yield (TMY) of improved Awassi ewes is 250-300 kg (Talafha, & Ababneh, 2011). This milk production is increased in Israel from 297 kg to over 500 kg / year (Gürsoy& Shaat, 2008), in Syria (128 kg to 335 kg) and in Turkey (67 to 152 kg) (Galal et al., 2008) by application of intensive selection breeding program.

In Palestine, Awassi sheep is the major small ruminantspeciesandit considered a larger source of meat and milk. It is famous by farmers as it is well - adapted to our harsh conditions like fluctuation in food supply and

high temperatures (Talafha, & Ababneh, 2011). According to MOA data, the average flock sheep size in the West Bank is small (Fig 1). It was found that most farmers in the West Bank have no genetic improvement skills.

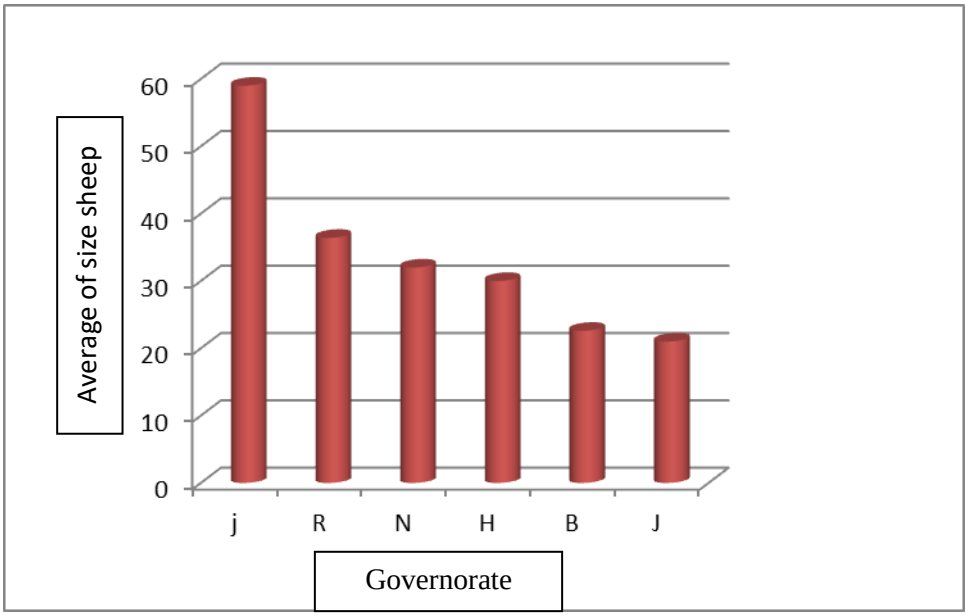

Figure 1: Average flock size of sheep in the West Bank. (j-Jericho, R- Ramallah- N-Nablus, H-Hebron, B- Bethlehem, J- Jenin). (PCBS, 2013)

2-2.Genetic Markers

A genetic marker can be defined as any stable and inherited variation that can be measured or detected by suitable method and can be used subsequently to detect the presence of specific genotype or phenotype (Alimet al., 2011).

Two types of genetic markers can be considered. First, markers that are sufficiently close to the trait gene on the chromosome such that, in most cases, alleles at the marker and the trait gene are inherited together. This type of marker is called a linked marker. At the population level, the alleles

at linked markers cannot be used to predict the phenotype until the association between alleles at the marker and alleles at the trait-gene is known (called ‘phase’). For phase determination, inheritance of the marker and trait gene has to be studied in a family. However, information on phase is only valid within that family and may change in subsequent generations through recombination (Raza et al., 2016).

The second type of genetic markers is a functional polymorphism in the gene that controls variation in the trait. These markers are called direct markers. Once the functional polymorphism is known, it is possible to predict the effect of particular alleles on trait phenotype in all animals of a population. Therefore, direct markers are more useful than linked markers for predicting the phenotypic variation of target traits within a population (Williams,2005).

2-3.Genetic Polymorphism

Genetic polymorphism can be defined as the occurrence of several phenotypic forms that are associated with alternative forms (alleles) of one gene in a population or among different populations. Genetic polymorphism arises from a mutation which ranged from a single nucleotide base change to variation in several hundred bases (Yahyaoui et al., 2003).

2-4. Molecular Markers Techniques for Polymorphism Detection

Molecular markers can be defined as markers revealing variationsat DNA level. Geneticists' tools and techniques to reveal polymorphisms at the DNA level are now considered the key players in animal genetics (Kučinskiene et al., 2005).

2-4-1. Restriction Fragment Length Polymorphisms (RFLP) and PCR-RFLP

RFLP techniques are used to visualize the differences in the DNA sequences using restriction enzymes (Madhumati et al, 2014).The principle of this method relies on the single base substitution that occurs within a restriction enzyme recognition site (Madhumati et al, 2014). Restriction fragment length polymorphism is detected when the single base mutations remove or create a new a restriction site (Teneva, 2009).

The DNA fragment of interest that potentially containing the polymorphism are first selectively amplified by PCR using specific primers followed by restriction and nuclease digestion. The resulting pattern is most conveniently visualized by agarose gel electrophoresis (Semagn et al., 2006). This technique has the ability to differentiate between homozygous and heterozygous genotypes for a specific trait (Othman et al., 2015).

2-4.2 Single Stranded Conformation Polymorphisms (SSCP).

Single-strand conformation polymorphism (SSCP) analysis is a post-PCR technique that can be used to screen for mutations that are not limited to a single hot spot but are randomly distributed throughout the exons. After the PCR amplification of the region of interest, the PCR products are denatured with heat and exposure to denaturing buffer and then subjected to polyacrylamide gel electrophoresis. If mutation is present within the amplified sequence, it will change the folding conformation of the sequence and its electrophoretic mobility. As a result, the wild-type and mutant sequences will migrate differently in the gel. PCR-SSCP analysis can be used as a screening tool for point mutations and small deletions and insertions. However, it cannot detect the precise nucleotide change. This requires the use of an additional technique, such as DNA sequencing (Kourkine et al., 2002).

The mobility of single-stranded DNA molecules is dependent on their structure and size. These structures are formed by base pairing between nucleotides within each strand and thus are determined by the DNA sequence of each strand.

Discrimination of DNA molecules differing by a single nucleotide is made possible by this method. PCR-coupled SSCP is technically simple and

effective in detecting polymorphisms in short DNA fragments of 100-300-bp.SSCP technique should be followed by DNA sequencing to detect the precise location of polymorphism (Othman et al., 2014).

2-4-3 Amplified Fragment Length Polymorphism (AFLP).

AFLP is a common combination of RFLP and PCR techniques (Vos et al, 1995). Genomic DNA is first digested with appropriate restriction enzymes. The resulted fragment is then legated to synthetic adaptors and amplified with specified primers which are complementary to a selective sequence of the adaptors. Separation is done by gel electrophoresis and visualized byautoradiography or fluorescence or silver staining depending on the reporter marker. AFLP is the effective, rapid and low-cost tool for detecting a large number of polymorphism that is highly reliable and reproducible and is able to be genotyped automatically (Raza et al, 2016).

2-4-4 Randomly Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA was described in 1990 (Williams et al., 1990). This technique is based on the use of short (usually 8- 10 bp), the segments of DNA that are amplified are random. The technique was developed independently by two different laboratories and called as RAPD. It used mainly for the detection ofpolymorphisms between and among organisms. It has an advantage that no prior information on DNA sequence is necessary (Kumari & Thakur, 2014).

2-4-5. Microsatellites

Microsatellites are small stretches of DNA sequences (1 to 10 nucleotides) that repeat in tandem at each locus. They are widely distributed throughout the genome (Selkoe & Toonen, 2006). Its difference in number and length allows it to be used as genetic markers which give a high degree of polymorphism. Specific primers are used to specify the length of each allele. Its high degree of polymorphism and their easy detection made the best choice by scientists (Selkoe& Toonen, 2006).To study the genetic variations among individuals and breeds.

2-4-6. Minisatellites

Minisatellites markers (more than 10 nucleotides) are based on certain hybridization probes for repetitive sequences (Jeffreys et al, 1985).Minisatellites patterns are highly polymorphic and widely used in the detection of polymorphism among and between species (Grover& Sharma, 2016)

2-4-7. Sequence Analysis

DNA sequencing is the process of determining the exact order of the nucleotide (A, T, C, and G) that make up the DNA(Alemayehu,& Getu, 2015). DNA sequencing is generally a vital tool in the analysis of gene

structure and expression. It also required for determination of any variation happened at the nucleotide level (Naqvi., 2007).

2.5 Sheep Milk Characteristics and Composition

In developed countries, milk is priced based on the composition rather than its quantity.

Milk with high protein and butterfat contents has a higher price than others. However, milk composition varies within and between breeds. Sheep milk contains relatively more protein, fat, and minerals than both cow and goat milk and human (Table 1). Sheep milk is richer in vitamins A, B, E, and C. It has higher minerals contentlike calcium, phosphorus, potassium and magnesium than cow milk (Ahmad et al.,2013). Also, it contains a higher proportion of short and medium chain fatty acids, with high health benefits (Ahmad et al., 2013).

Characterization of the raw milk is very important to allow cheese producers to optimize the cheese processing.

Table 1: Milk compositions from mammals

Species	Water %	Fat %	Protein %	Lactose %	Ash %	Non-fat solids %	Total solids %
Ewe	80.71	7.90	5.23	4.81	0.90	11.39	19.29
Goat	87.00	4.25	3.52	4.27	0.86	8.75	13.00
Cow	87.20	3.70	3.50	4.90	0.70	9.10	12.80
Human	87.43	3.75	1.65	4.98	0.21	8.82	12.57

Source: Harris and Frederick (2003).

2.6 Genetic Polymorphisms of Genes Associated with Milktraits.

In ruminant, whey proteins (*α-LA and β-LG*) and casein protein (αs1, β, αs2, and κ) constitute more than 95% of the milkproteins in goats, sheep, and cattle (Othman et al., 2013).The *β-LG* and *K-CN*are considered two of the most important milk proteins due to their crucial role in milk quality, coagulation process in cheese, butter and the formation, stabilization, and aggregation of the casein micelles (Albenzio& Santillo, 2011).

*B-LG*and *α-LA* are highly affected by the nutrition status of the animal and the functional properties of whey and its products (Albenzioet al., 2011).

The casein protein is the main components (76-86%) of total milk protein (Othman et al, 2013). This protein is highly affected by physicochemical, nutritional and technological properties of ruminant milk (Othman et al., 2013).

For dairy sheep, limited research on the genetic polymorphisms of caseins and whey proteins genes are available and most published researchers are from European countries; especially in the Mediterranean area. Few researches were published from Middle East area.

2.6.1 Beta Lactoglobulin

β-lactoglobulin is one of the most important whey proteins in sheep milkand contains 162 amino acids which, features a unique protein fraction profile (Moatsou et al., 2005).*B-LG* inruminant's milkis representing 60 to 65% oftotal whey protein in milk.Moreover, *β-LG* protein content depends on breed and lactation stage,in sheep and goat (Hejtmánková et al., 2012). Polymorphic genes, such as *β-LG*, were proven to be associated with traits of interest in sheep milk; therefore, similar genes can comprise entities of detectable molecular markers in a current selectionmethod for milk traits. The genetic polymorphism of *β-LG* gene was initially foundat the protein level in cattle (Aschaffenburg and Drewry, 1955) and later was examined in sheep breeds. After the detection of polymorphism in sheep, furtherstudies have demonstrated the *β-LG* gene polymorphic effect on milk yield, protein,fat, lactose content etc. (Kusza et al., 2015) as well as the impact of protein geneticvariants on technological properties of milk (Selvaggi et al., 2014). The associationof genetic polymorphisms with milk traits is especially important for rare indigenoussheep breeds. There is a lack of genetic information for important indigenous sheepbreeds around the world, i.e. allele and genotype frequencies, the goodness of fit toHWE, and importantly and there are unexplored associations between genetic polymorphismsin sheep genome with the milk traits.

Sheep*β-LG* gene has been found in chromosome 3 (El-Shazly et al., 2012) and its complete sequence consists of 7379 nucleotides arranged in seven small exons and six introns have been characterized (Kusza et al., 2015). It has three variants: A, B, and C (Elmaci et al., 2006). The A and B variants are most common in all breed, while C is rare and found in the Merino breed (Barillet et al., 2005). C genotype isa subtype of variant A with a single amino acid exchange at position 148 (Arg→Gln) (Erhardt, 1989), while, the genetic variant A differs from variant B in the amino acid sequence at position 20 (Tyr→His) (Gaye et al., 1986).

A significant association was found between the main variants A and B with milk production and fat and protein content (Dario et al., ,2008). AA and AB genotypes had higher milk yieldthan BB ewes (Nudda et al., 2003). A positiveassociation was found between AB genotype and fatand lactose percentages in sheep (Yousefi et al., 2013).

2.6.2 Kappa Casein

Casein is a milk protein secreted by mammary gland cells (Akers et al. 2016). Itconstitutes about 78-82% of ovine milk, and is divided into four main groups:αS1casien, αS2 casein, β-casein, and ƙ-casein (Khaizaran, & Al-Razem, 2014).Casein genes form a cluster in a 250-kb genomic DNA fragment, where αs1- is to β- followed by αs2- and *K-CN*very near(Pauciullo,& Erhardt, 2015).The performance of technological processes of cheese production depends on the structure of *K-CN*protein

(Bonfatti et al., 2010). *K-CN*plays an important role in the formation, stabilization and aggregation of the casein micelles thus altering the manufacturing properties and digestibility of milk (El-Shazly et al, 2013).The *K-CN* polymorphism is well described in cattle (Prinzenberg *et al.* 2008), goat (Jann *et al.* 2004) and sheep (Coral *et al.* 2010).

K-CN was found to be polymorphic in cattle with six variants characterized by Mir(2014) and two variants localized in the N-terminal region of the protein in goat (Law& Tziboula, 1993), but aremonomorphic in sheep (Yahyaoui et al., 2001).

*K-CN*is the least studied with respect to its effect on milk yield and composition. One positive association between polymorphism of *K-CN* gene and milk composition has been reported in goat (Bonanno *et al.* 2013).

The effect of *K-CN*variants on milk production traits has been extensively studied in different animals. For example, in cow's*K-CN*BB genotype contains higher proportions of fat, proteins and caseins than milk derived from *K-CN*AA genotype (Poulsen et al., 2017)). The B allele is significantly associated with higher casein andlowerwhey protein contents. The BB genotype has been associated with the production of milk with superior manufacturing properties e.g. shorter rennet coagulation time, formation of a firmer curd and higher cheese yield (Poulsen et al., 2017). This effect is associated with the milk casein micelles.

Chapter Three

Material and Methods

3. Materials and Methods

3.1 Milk Sample Collection and Analysis.

In total, 54 milk samples were collected from 31improved Awassi sheep located in Beit Quad Station – Beit Qad – Jenin and from 23 localAwassi sheepfrom Nablus. The samples were transported in the ice-cooled box to the laboratory and stored at -20°C for further analysis milk composition (total protein, total fat, lactose, total solids and nonfat solid contents) was determined using Milk Analyzer (Milk scope –Model JulieC8-110-250V system).

3.2 Genotyping

3.2.1 Blood sample collection

The study involved a total of 54 sheep belonging to Palestinian local and Improved Awassi. The collected blood used for genotyping of*K-CN* and*β-LG* (Table 2).

All samples were collected with the help of a staff member of Palestinian Center for Livestock Improvement (PCLI). Blood samples were collected in a 5 ml EDTA tubes, from the jugular vein. Samples were stored at +4 °C during transportation and then placed at +4 °C and processed next day for DNA extraction.

Table 2: Collection of blood samples from sheep breeds and studied genes used in the study

Breeds	Total samples collected	Location	*K-CN*	*β-LG*
Local Awassi	23	Nablus	23	23
Improved Awassi	31	Beit- Qad – Jenin	31	31

3.2.2 DNA extraction

DNA was extracted from 100 µl of blood, using a commercial kit (Qiagen, USA) following the manufacturer's protocol. The quality and quantity of DNA weredetermined using NanoDrop (2000, Thermo fisher, USA) spectrophotometer and by electrophoresis on 1.5 % agarose gel.

3.2.3 Primer design for the amplification of the *ß-LG, K-CN*genes.

Primers were designed according to available sheep gene sequences in the gene bank. We used the BLAST N program to ensure the specificity of forward and reverse primers for the two genes (*β-LG, K-CN*) as shown in Table 3.

Table 3: PCR primers sequences used in the present study with the expected PCR product size

Gene	**Primer sequence**	**Annealing temperature °C**	**Expected Product size (bp)**
β-LG	F 5'- CCCAAGATCCAAATGTTGCT -3'R 5'- CGCCGGGTACCAGTAAACTC -3'	56.5	471
K-CN	F 5'- CTGGGTTCACTATTCCCAATG-3' R 5'- TTGCTCATTTACCTGCGTTG -3'	59	670

3.2.4 PCR amplification of ß-LG.

The DNA amplification of the *β-LG* gene was achieved by PCR technique using forward primer: 5´- CCCAAGATCCAAATGTTGCT-3´ and reverse primer: 5´- CGCCGGGTACCAGTAAACTC-3´. The PCR amplification reaction solution was performed in a total volume of 25 µl containing 2 µl (200 ng) DNA, 4.5 µl nuclease free water, 12.5 µl of GoTaq® Green Master Mix (2x), 2 µl $MgCl_2$ and 2 µl (0.8 µM) of each primer. The PCR cycling condition was a preliminary denaturation at 95 ºC for 5 min, followed by 35 cycles, each cycle includeddenaturing at 95 ºC for 30 seconds, annealing at 56.5 ºC for 40 seconds, and extension at 72 ºC for 40 seconds followed by10 min at 72 ºC as a final extension. The PCR reactions were performed on a LifePro Thermal Cycler. The PCR products were visualized by resolving on 1.5% agarose containing ethidium bromide in parallel with 100-bp DNA ladder. Gel electrophoresis was carried out at a constant voltage of 120 V for 45 min.

3.2.5 Restriction fragment length polymorphism for ß-LG gene

The 471bpPCR amplified productof *β-LG* was digested by the RsaI restriction enzyme in a digestion reaction consisting of 10 µl PCR product, 2 µl of 10 x buffer, and 2 µl of the restriction enzyme in a final volume of 32 µl.

The reaction mixtures were incubated for 3 hours at 37°C in temperature - controlled waterbath and the digested products were separated by electrophoresis on 3% agarose gel stained with ethidium bromide and the bands were scored manually under UV light.

3.2.6 Statistical analysis

Allele frequency and polymorphism under Hardy-Weinberg equilibrium (HWE) were analyzed using Cervus software package (Marshall et al., 1998). In order to evaluate the main effects of genotype and their interactions on the milk traits, we used General Linear Model (SAS Institute, 2008. SAS User Guide. GLM Procedure. Cary, NC, USA).

3.2.7 PCR product sequencing of *ß-LG* gene

The PCR amplified products were sequenced by Sanger method using the same primers of amplification. The sequencing was done by Macrogen Incorporation (Seoul, Korea).The results were visualized with FINICH TV program.

3.3. PCR amplification of *K-CN*gene

The DNA amplification of the *K-CN* gene was achieved by PCR technique using forward primer: 5- CTGGGTTCACTATTCCCAATG - 3´and reverse primer 5´ - TTGCTCATTTACCTGCGTTG - 3´. The PCR amplification reaction solution was performed in the total volume of 25 µl

containing 2 µl (200 ng) DNA, 4.5 µl nuclease free water, 12.5 µl of GoTaq® Green Master Mix (2x), 2 µlMgCl2 and 2 µl (0.8 µM) of each primer. The PCR cycling condition was a preliminary denaturizing at 95 ºC for 5 min, followed by 40 cycle, each cycle included denaturation at 95 ºC for 30 seconds, annealing at 59 ºC for 30 seconds, and extension at 72 ºC for 40 second followed by 10 min at 72 ºC as a final extension. The PCR product was separated on 1.5% agarose containing ethidium bromide in parallel with 100- bp DNA ladder. Gel electrophoresis was carried out at a constant voltage of 120 V for 45 min.

3.3.1 Cleaning of PCR products

After gel electrophoresis step was finished and PCR product bands were seen on the gel. PCR product "clean-up" protocol was done which is an important step to each PCR products to chew up excess primers and remove excess unincorporated dNTPs, enzymes, salts and small fragments from these products. Also, this procedure was used to purify the amplified fragments from agarose gel after electrophoresis or from the PCR reaction. So, Antarctic Phosphatase and Exonuclease I enzymes were used to purify the products from the PCR reaction. Antarctic Phosphatase enzyme is responsible for removing the leftover nucleotides. While the role of Exonuclease I enzyme is to degrade the remaining primers (Table4), and the program used shown in table 5 and (Appendix4).

Table 4: Master Mix of enzymes with 5 µl of PCR products per 7 µl of total volume

Reagents	**Volumein µl**
Antaractic Phosphatase	0.25
Exonuclease I	0.25
Nuclease free H2O	1.5
PCR Products	5
Total Volume	7

Table 5: PCR clean program

Temp©	**Time(min)**
37	30
80	20
4	8

3.3.2 Direct sequencing of purified PCR Products

After PCR products were purified, the next step was DNA sequencing. Sanger method of DNA sequencing was done depending on Big Dye terminator. The Big Dye™Terminators V1.1 Cycle Sequencing Reaction Kit (Applied Biosystems) includes ddNTPs (dideoxynucleotides) that inhibit chain-elongation by DNA polymerase. Samples were run on ABI 3130XL Genetic Analyzer (Applied Biosystem) at Bethlehem University. The results werevisualized with FINICH TV program (Table 6).

Table 6: Standard Sanger sequencing reaction mix per 16µl total volume

Reagents	**Volume in µl**
Big Dye	0.4
46X Buffer	0.75
5X Buffer	1.5
Forward Primer	0.5
PCR Product	1.5
Nuclease Free H2O	10.35
Total Volume	16

Table 7: Sequencing PCR Reaction Program:

Step	Temperature (°C)	Time
1	96	1min
2	96	10sec
3	50	5sec
4	60	4min
5	4	10min
	Steps 2 to 4 were repeated 25 times	

Chapter Four

Results

4. Results

4.1. Beta lactoglobulin gene

The polymorphism of tested whey protein gene β-lactoglobulin was identified by PCR-RFLP technique. This technique helps to identify the nucleotide polymorphism by digestion of PCR product using specific restriction enzyme. The PCR amplified a fragment with 471-bp in size (**Fig.2**).

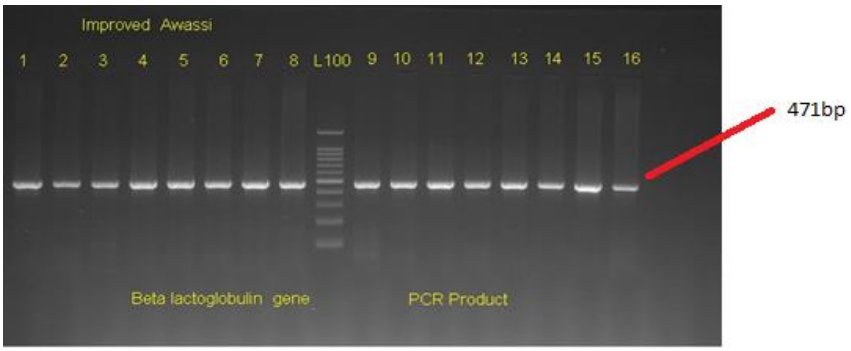

Fig.2: Ethidium bromide-stained gel of PCR products representing amplification of *β-LG* gene in sheep.
L: 100-bp ladder,
Lanes 1-16: 471-bp amplified fragments of *β-LG* gene.

The digestion of PCR fragments (471-bp) in size with *RsaI* enzyme revealed the presence of two alleles A and B in the Awassi sheep populations under study. Three genotypes were observed (**Fig. 3**); the homozygous AA with two bands at 286- and 185-bp, the heterozygous AB genotype with four bands at 286-, 220-, 185- and 66-bp and the homozygous BB genotype with three bands at 220-, 185- and 66-bp.

Of fifty-four tested animals; fifteen animals had AA genotype (27.78%), thirty-five animals had AB genotype (64.81%) whereas only four animals

had BB genotype (7.41%). In both Awassi breeds; AB genotype was recorded at the highest frequency compared with other two genotypes. Improved Awassi sheep had the highest frequency of AB genotype (67.74%) with the lowest frequency of BB genotype (3.23%). In local sheep; AB genotype frequency was lower than that in improved but still with the highest frequency in this breed; it was 60.87% while AA genotype frequency was 26.09%. Although the frequency of BB genotype in local breed was the lowest (13.04%) but it is still with the higher frequency than that in improved Awassi (3.23%).

In all tested animals, allele A frequency (60.19%) was higher than allele B (39.81%). The results showed that alleles A and B were present with different frequencies in both tested Awassi sheep. Improved Awassi breed possessed the highest frequency of allele A (62.90%) than that in local breed (56.52%) whereas the allele B frequency ranged from 37.10% in improved to 43.48% in local Awassi lines (**Table 7**).

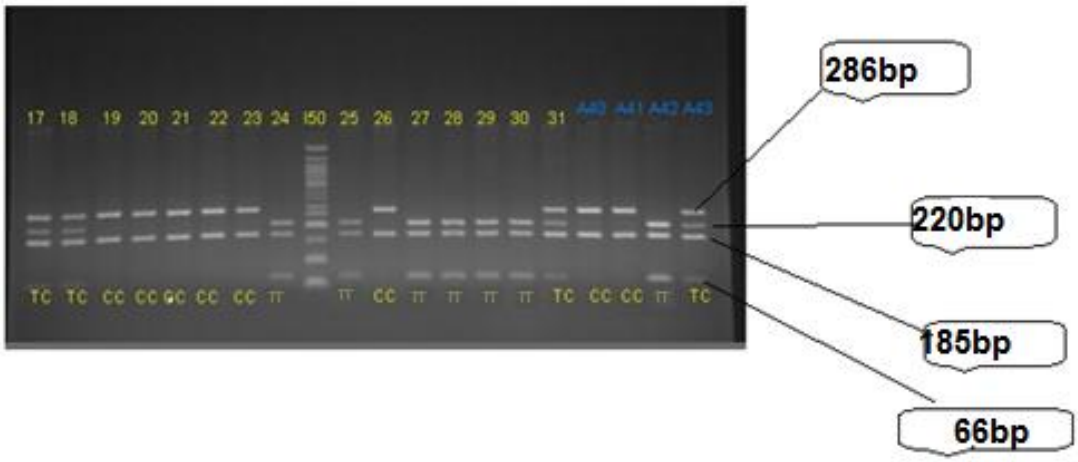

Fig. 3: PCR-RFLP results for *β -LG* gene using *RsaI* restriction enzyme on 3% agarose gel.
The homozygous AA with two bands at 286- and 185-bp.
The heterozygous AB genotype with four bands at 286-, 220-, 185- and 66-bp.
The homozygous BB genotype with three bands at 220-, 185- and 66-bp.
3;'4lp

Table 8: Genotype and allele frequencies of *β-LG* gene in bothAwassi tested breeds

Awassi breeds	**No. of Animal**	**Genotype Frequencies**						**Allele Frequencies**	
		AA		**AB**		**BB**		**A**	**B**
		No. of animal	Freq. %	No. of animal	Freq. %	No. of animals	Freq. %	Freq. %	Freq. %
Local	23	6	26.09	14	60.87	3	13.04	56.52	43.48
Improved	31	9	29.03	21	67.74	1	3.23	62.90	37.10
Total	54	15	27.78	35	64.81	4	7.41	60.19	39.81

Hardy-Weinberg equilibrium test returned the following observed and expected genotypes in our study (Table 9).

Table 9: Expected Heterozygosity Observed Heterozygosity and Chi square value and HWE value obtained for β-LG gene

Expected Heterozygosity	Observed Heterozygosity	HWE	Chi square value	P-value	PIC
0.4837	0.6481	5.0	5.3244	0.0211	0.3644

4.2. Sanger sequencing result of the exon 2 in *β-LG* gene.

Sanger sequencing was done to detect the single nucleotide polymorphism (SNP) which is responsible for the polymorphism of this gene. Sequencing results revealed that there is a single base substitution: T instead of C at position 1617-bp of the (X12817.1: c.112T>C; **chr3: 3,574,794**) locus in both local and improved Awassi (**Figs. 4, 5 and 6)**. It is a missense mutation (*rs430610497*) convert amino acid no 36 from Histidine to Tyrosinfrom (CAC) to (TAC)). .

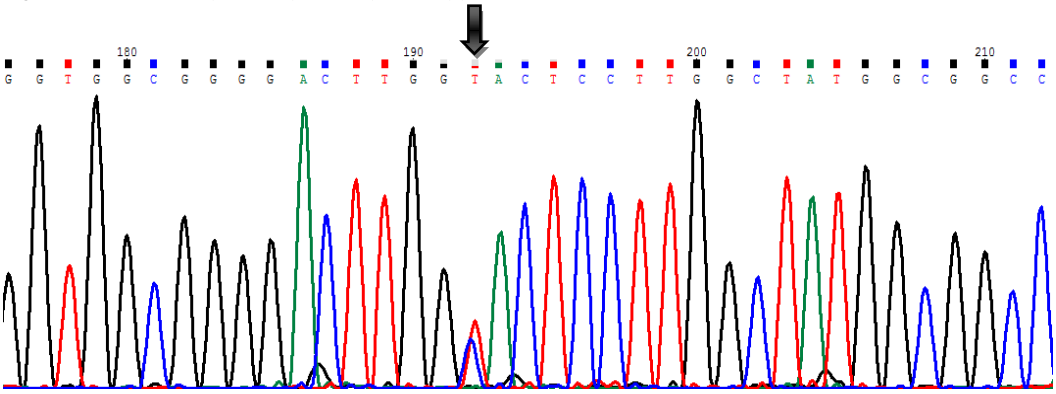

Fig. 4: The heterozygous TC of *B-LG* gene detected in Awassi sheep breed. (Hetro)

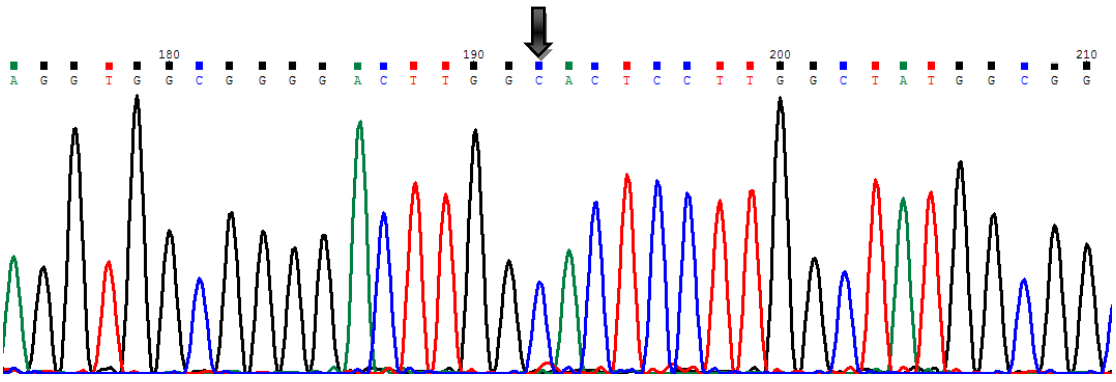

Fig. 5: The homozygous CC of *B-LG* gene detected in Awassi sheep breed.**(Wild type C)**

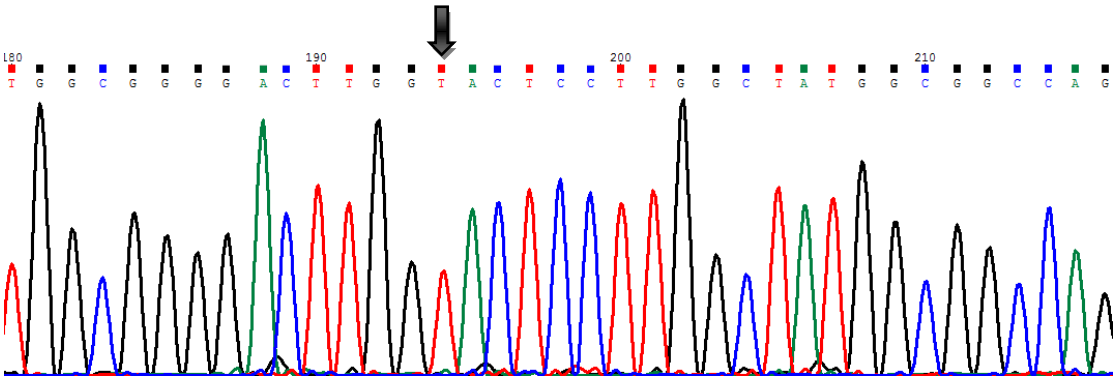

Fig. 6: The homozygous TT of *B-LG* gene detected in Awassi sheep strains. (Mutant)

4.3 Kappa Casein gene

The polymorphism of this gene was detected using Sanger sequence technique. A 670-bp fragment was amplified by polymerase chain reaction (**Fig. 7**). Sequencing results recorded the presence of three different patterns; CC, TC and TT in the fifty-four tested sheep animals. The sequence analysis showed a single nucleotide polymorphism (SNP) C (**Fig. 8**) → T (**Fig. 9**). This mutation synonymous variant - A sequence variant where there is no resulting change to the encoded amino acid. The results showed that the presence of pattern III (CC) inonly one local Awassi sheep (4.35%) whereas the pattern I (TT) was found in high frequencies in tested animals. The highest (83.87%) frequency was recorded in improved Awassi for patterns TT with the absence of pattern CC. The frequencies of patterns TC and TT in local Awassi were 39.13 and 56.52, respectively (**Table 10**).

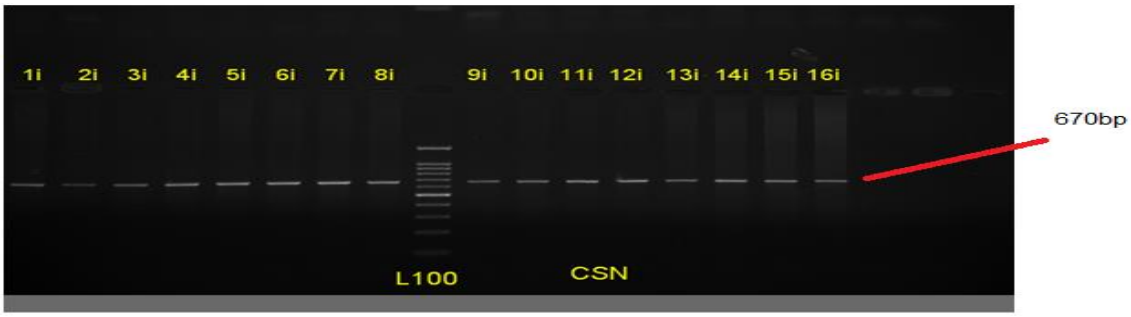

Fig. 7: Ethidium bromide-stained gel of PCR products representing amplification of *K-CN* gene in sheep.
L: 100-bp ladder,
Lanes 1-16: 670-bp amplified fragments of *K-CN* gene.

Table (10): The Genotypic pattern of K-CN gene in both local and improved Awassi breeds.

Breeds	No. of animal	Pattern frequencies					
		Pattern I (TT)		Pattern II(CT)		Pattern III(CC)	
		No. of animal	Freq.	No. of animal	Freq.	No. of animal	Freq.
Local	23	13	56.5%	9	39.13%	1	4.35%
Improved	31	26	83.87%	5	16.13%	0	0
Total	54	39	140.37 %	14	55.26%	1	4.35%

The sequence analysis is represented in Fig 8,9 and 10. The results showed a single nucleotide polymorphism (SNP) (C → T). This mutation is asynonymous variant: A sequence variant where there is no change to the encoded amino acid.

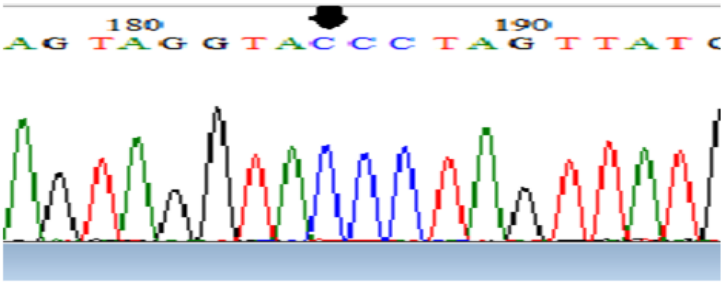

Fig. 8:.The C nucleotide in pattern CC in Awassi sheep. (Wild type C)

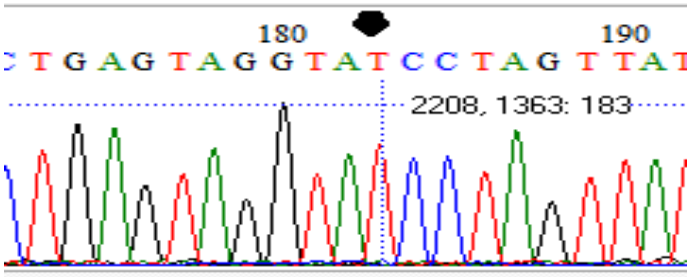

Fig. 9: The T nucleotide in pattern TT in Improved Awassi sheep. (Mutant)

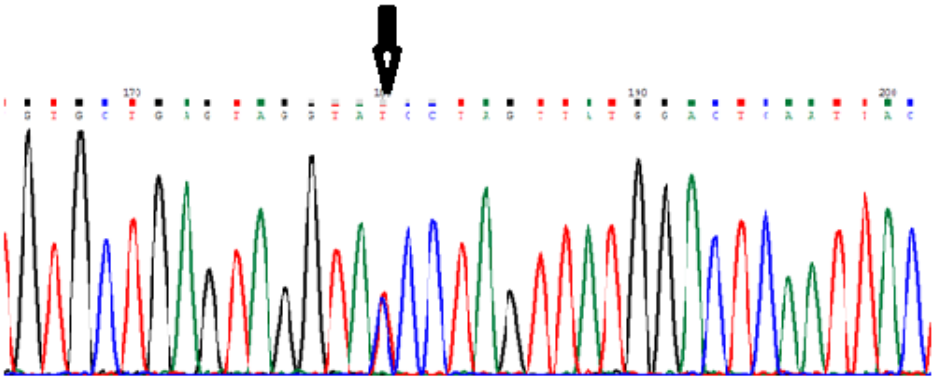

Fig. 10: The T nucleotide in pattern TC in Improved Awassi sheep. (Heterozygous)

4.4 Effect of the genotypes of β-lactoglobulin and K-casin gene on milk components in local and improved Awassi sheep breeds.

4.4.1 Local Awassi

In this study, the associations between *β-LG* genotype and milk composition were examined as shown in (**Table11).**We observed greater Fat2 (Fat ratio in milk analysis at the end of the season) percentage for genotype **AA**compared to genotype**AB and BB**. Also, *β-LG*genotype AA was associated with the higher Density2 (Density ratio in milk analysis at the end of the season) compared to genotype**BB** (Table 12).The other milk traits remained unaffected. Conclusively, associations were examined between *β-LG* polymorphisms and milk composition but there were no significant differences with milk components, except for fat and density percentage.

The SNP of *K-CN* has become a popular genetic marker tool commonly used for genetic characterization and identification of possible linkage associations between *K-CN* gene and milk performance traits. We also in this study examinedthe associations between *K-CN* genotype and milk composition as shown in Table 13, we observed asignificant effect of *K-CN* genotype **TT compared to genotype- TC,** TT genotype was associated with higher SNF compared to genotype **TC.** Other genotypes of *K-CN* gene

showed no associations with other milk components as shown (Table 11).

See appendices 11, 12 and 13 for more details.

Table11: Genotype effect of β-lactoglobulin and K-Casein on milk composition in Local Awassi sheep breeds.

Variable	N	Mean	Std Dev	KCN F Value	Pr> F	BLG F Value	Pr> F
Fat1	23	8.993	2.043	0.79	0.4675	0.65	0.5336
SNF1	23	10.425	0.812	4.02	0.036	0.84	0.4465
Density1	23	30.854	3.956	1.82	0.191	1.65	0.2202
Protein1	23	3.8234	0.295	2.2	0.1393	0.87	0.4361
Lactose1	23	5.7408	0.468	2.24	0.1356	1.17	0.334
Solids1	23	0.7182	0.114	1.56	0.2365	1.04	0.3754
Fat2	23	6.8252	1.335	0.63	0.5463	4.66	0.0234
SNF2	23	7.6943	0.948	0.56	0.5782	0.09	0.9125
Density2	23	24.9	2.69	0.3	0.7469	2.62	0.01
Protein2	23	3.2765	0.182	0.24	0.7919	0.88	0.4319
Lactose2	23	4.814	0.466	1.8	0.1935	0.06	0.9946
Solids2	23	0.7283	0.113	1.57	0.2347	1.02	0.342

Table (12): Significant differences of β-lactoglobulin genotypes on Fat percentage and density fat in final stage in local Awassi sheep breed.

Variable	Comparisons significant at the 0.05 level are indicated by ***.				
	BLG Comparison	Difference Between Means	95% Confidence Limits		
Fat2	**AA – AB**	1.4300	0.2153	2.6447	***
Fat2	**AA – BB**	2.1800	0.4198	3.9402	***
Density2	**AA – BB**	2.1800	0.4198	3.9402	***

Table (13): Significant differences of K-casein genotypes on solid Non Fat in first stage in local Awassi sheep breed

Variable	Comparisons significant at the 0.05 level are indicated by ***.				
	KCN Comparison	Difference Between Means	95% Confidence Limits		
SNF1	TT – TC	0.7963	0.1369	1.4558	***

4.4.2 Improved Awassi

In this study the associations between *β-LG and K-CN* genotype and milk composition were examined as shown in (**Table14**).Furthermore, statistical analysis within breed has shown that, for the studied the genotypes,there were nosignificant effects onmilk composition.

Table14: Genotype effect of β-lactoglobulin and K-Casin on milk composition in improved Awassi sheep breeds.

Variable	N	Mean	Std Dev	KCN-F Value	Pr> F	BLG F Value	Pr> F
Fat1	31	6.053	1.783	3.35	0.0781	1.8	0.1854
SNF1	31	9.184	0.659	1.07	0.3099	1.02	0.3758
Density1	31	28.808	2.551	0.73	0.4007	1.06	0.3597
Protein1	31	3.371	0.266	0.31	0.5811	1.15	0.3323
Lactose1	31	5.049	0.334	0.73	0.4007	0.03	0.9692
Solids1	31	0.711	0.053	0.01	0.926	0.95	0.399
Fat2	31	5.099	0.705	2.98	0.0955	0.71	0.5001
SNF2	31	7.279	0.743	0.03	0.8728	0.03	0.9723
Density2	31	24.678	4.924	1.64	0.2108	1.3	0.289
Protein2	31	3.086	0.45378	0.3	0.5857	1.3	0.2884
Lactose2	31	4.377	0.4335	0	0.9266	2.39	0.1103
Solids2	31	0.712	0.055	0.04	0.9361	1.02	0.3998

4.4.3Milk composition for the local and improved Awassi

The mean differences between different parameters of milk composition betweenlocal and improved Awassi are shown in Table 15.Statistical analysis ($P<0.05$) to configure the significant differences between the means of different milk parameters has shown that there were a significant difference between local and improved Awassi of all tested parameters in stage 1 milk while there were only significant difference between fat 2 and lactose 2 in stage 2 milk between both breeds as shown in Table 16.

Table 15A: Mean differencesbetween milk composition in localAwassi on first and final season.

Variable	N	Mean	StdDev
Fat1	23	8.99348	2.04327
SNF1	23	10.4252	0.81201
Density1	23	30.8543	3.95624
Protein1	23	3.82348	0.29569
Lactose1	23	5.74087	0.4681
Solids1	23	0.85826	0.07272
Fat2	23	6.82522	1.33556
SNF2	23	7.69435	0.94897
Density2	23	24.9	2.69022
Protein2	23	3.27652	0.18297
Lactose2	23	4.81391	0.46639
Solids2	23	0.71826	0.1144

Table 15B: Mean differencesbetween milk composition in improved Awassi on first and final season.

Variable	N	Mean	StdDev
Fat1	31	6.05387	1.7832
SNF1	31	9.18484	0.65942
Density1	31	28.8084	2.55184
Protein1	31	3.37194	0.26649
Lactose1	31	5.04968	0.33459
Solids1	31	0.76226	0.07428
Fat2	31	5.09903	0.70498
SNF2	31	7.27903	0.74335
Density2	31	24.6787	4.92458
Protein2	31	3.08645	0.45378
Lactose2	31	4.37742	0.4335
Solids2	31	0.71194	0.05313

Table 16: Breed effect between local (A) and improved (B) Awassi on milk traits

	Means with the same letter are not significantly different.			
Variable	**t Grouping**	**Mean**	**N**	**Breed**
Fat1	A	8.9935	23	Awassi
	B	6.0539	31	Imprv
SNF1	A	10.425	23	Awassi
	B	9.1848	31	Imprv
Density1	A	30.854	23	Awassi
	B	28.808	31	Imprv
Protein1	A	3.8235	23	Awassi
	B	3.3719	31	Imprv
Lactose1	A	5.7409	23	Awassi
	B	5.0497	31	Imprv
Solids1	A	0.8583	23	Awassi
	B	0.7623	31	Imprv
Fat2	A	6.8252	23	Awassi
	B	5.099	31	Imprv
SNF2	A	7.6943	23	Awassi
	A	7.279	31	Imprv
Density2	A	24.9	23	Awassi
	A	24.679	31	Imprv
Protein2	A	3.2765	23	Awassi
	A	3.0865	31	Imprv
Lactose2	A	4.8139	23	Awassi
	B	4.3774	31	Imprv
Solids2	A	0.7183	23	Awassi
	A	0.7119	31	Imprv

Chapter Five

Discussion

5. Discussion

Dairy sheep is considered as one of the most important livestock like dairy cow. Sheep milk has a unique richness and has a special high nutritional value in human health (Haenlein, 2006). The sheep milk is an excellent raw material for the milk processing industry especially in cheese production (Boyazoglu& Morand-Fehr, 2001). Therefore, the target of the sheep breeding is currently shifted from meat to milk and wool (Gürsoy et al., 2006).

In Palestine, there aremainlytwo sheep breeds; local and improved Awassi and Assaf. In these days, the main focus of farmers and animal breeders is local Awassi for its adaptation to our local and harsh environment. Recently, MOAis paying attention to include local Awassi in the breeding program withthe main goal of increasing milk quantity and quality.

Some investigation proved that sheep genetic polymorphisms affect physicochemical properties of milk (El-Shazlyet al., 2012). The relationships between milk protein polymorphism and milk composition, quality, and production traits are highly investigated in sheep (Pecka-Kiełbet al., 2018).

5.1 Genetic Polymorphismsof *β-LG*

Beta-Lactoglobulin is a major whey protein in the milk of ruminants representing 60 to 65% of total whey protein in milk. Ovine*β-LG* gene has been assigned to chromosome 3 and its complete sequence consists

of 7379 nucleotides arranged in seven small exons and six introns (El-Shazly et al., 2012).

In our study, PCR-RFLP results identified three genotypes: AA, AB and BB in both breeds. The AB genotypehas appeared with high frequencies in both improved (67.74%) and local (60.87%) Awassi Sheep. The genotype AA has appeared with moderate frequencies, fewer than AB genotype and greater than BB genotype (Table7). The frequency of allele A is more frequent than allele B in both Awassi sheep breeds (Table7).

Our results are in conformity with other findings, where the allele A is more frequent than allele B inEgyptian sheep breeds; Barki, Rahmani and Ossimi (Othman et al., 2013);Noami, Sawakni, Harryand Nagdi(El-Shazly et al.,2012);in Turkish sheep breeds:Kıvırcık, Gökçeada, and Sakız (Elmaci et al., 2006); Karagouniko breed (Triantaphyllopoulos etal.,2017);Racka and East Friesian sheep (Kawecka and Radko., 2011).However, other reserachers(Nanekarani et al., 2010; Mohammadi et al. 2006, Mele et al. 2007, Dario et al. 2008, Michalcova & Krupova 2009, and Corral et al. 2010)reported that allele A is dominant in different sheep breeds.

In the contrary, the frequency of dominantB allele been reported in Awassi and other breeds (Kusza et al., 2015) and Chios breed (Triantaphyllopoulos etal.,2017). Moreover, (Yousefi et al., 2013; Staiger et al., 2010; Arora et al., 2010, Baranyi et al. 2010) reported that allele B is dominant in different

sheep breeds. The prevalence of B allele is mainly encountered insheep breeds from India, with its ancestral origin (Selvaggi et al., 2015).

According to Rozbicka-Wieczorek et al. (2015), the high frequency of B allelehas been also observed in British dairy sheep, Hungarian Merinos, Racka and Polish Lowland breeds.

No evidencewas found of C allele in our study, which is considered a rare variant detected only infew breeds such as Merinoland, Lacha, Carranzana, Spanish Merino, Serra da Estrela, White and Black Merino, originated from the Spanish Merinos (Selvaggi et al., 2015).

Sequencing analysis of *B-LG*detected a substitution from Cytosine (allele C) to Thymine (allele T) at the nucleotide no. 192, which leads to amino acid exchange from histidine (CAC) to tyrosine (TAT), The mutation genomic no is chr3:3,574,794according to GenBank: X12817.1.It is missense mutation convert amino acid no 36 from Histidine to Tyrosin from (CAC) to (TAC).

Kolde &Braunitzer, (1983) found, the allelic variants A and B are present in all breeds and differ by a tyrosine →histidine substitution in position 20; *β-LGA* has tyrosine whereas *β-LG B* has histidine. These two amino acid variants are corresponding to a single nucleotide substitution (T→C) in the *β-LG* gene. In another research, the rare variant *β-LG C* is a subtype of ovine *β-LG A* with a single exchange of amino acid Arginine→Glutamic acid at position 148 was detected (Picarielloet al., 2012**)**.

5.2 Genetic Polymorphism of *K-CN*Gene

Casein is a milk protein secreted by mammary gland cells (Akers et al.,2016). It constitutes about 78-82% of ovine milk protein, and is divided into four main groups: αS1casien, αS2 casein, β-casein, and K-casein (Abbas et al., 2011).*K-CN*plays an important role in the formation, stabilization and aggregation of the casein micelles thus altering the manufacturing properties and digestibility of milk (El-Shazly et al., 2013).

Although *K-CN* is widely polymorphic in cattle with six variants characterized by (Atamer et al., 2017) and in goat with two variants localized to the N-terminal region of the protein (Vithalet al.,2011), it is considered to be monomorphic in sheep (Othman et al, 2015).

Sequence results of a 670 bp fragment of *K-CN* recorded the presence of three different patterns: CC, TC and TT in fifty-four tested sheep animals**.** The sequence analysis showed a single nucleotide polymorphism (SNP) (C→ T) **chr6:85,316,422** in codon no 56 of exon three.This mutation is a synonymous variant - *A sequence variant where there is no resulting change to the encoded amino acid*.

In our study, the resultsshowed the presence of pattern III (CC) inonly one local Awassi sheep whereas the pattern I (TT) was found in high frequencies. The highest (83.8%) frequencies were recorded in improved Awassi for patterns TT and absence of CC. The frequencies of the three

recorded patterns were 4.3%, 39% and 56.5% for patterns CC, TC and TT, respectively in local Awassi.

It is obvious from the results of this study, thedominance of pattern T with the very low frequency of C, it is considered to be monomorphic in sheep.

Also, Othman et al (2013) reported the monomorphism of *K*-casein in Egyptian sheep breeds “Rahmani, Barki and Ossimi". Also Harry and Nagdi (El-Shazly et al., 2012), Ossimi sheep (Othman et al., 2014), in Czech Sumava and Valachian breeds (Sztankoova *et al*., 2011), Churra da Terra Quente (Selvaggi et al., 2014), sheep India (Singh et al., 2015) reported the monomorphic in K-casin, Giambra *et al.*,2014 for monomorphism locus in different sheep breeds.

On contrast to the above reported results, the sequence analysis of K-casein in our sheep animals showed the dominance of pattern T with the very low frequency of C. The same result was recorded by Yousefi et al. (2013) in Zel breed. Also Ceriotti*et al.* (2004) , Azari *et al.* (2011), Feligini *et al.* (2005), Staiger *et al.*, (*2010*), Di Gregor et al.(2017), and Giambra et al.,2014)reported thepolymorphic.

5.3 Effect of Beta lactoglobulin and Kappa Casin Genotypes on Milk Composition

5.3.1 Local Awassi

β-lactoglobulin is one of the most important whey proteins in sheep milk which, features a unique protein fraction profile (Moatsou et al., 2005). Beta-lactoglobulin is a major whey protein in the milk of ruminants representing 60 to 65% of total whey protein in milk. Moreover, *β*-lactoglobulin protein content depends on breed and lactation stage, in sheep and goat (Hejtmánková et al., 2012). The polymorphic genes, such as *β-LG*, were proven to be associated with traits of interest in sheep milk; therefore, similar genes can comprise entities of detectable molecular markers in a current selection method for milk traits.

Polymorphisms of a *β-LG* gene may be helpful as informative molecular markers for milk composition as well as for rheological properties of milk.

The association of *β-LG* polymorphism with milk yield and composition has been reported in cows (Ng-Kwai-Hang et al, 1988), sheep (El-Shazly et al., 2012) and Indian goats (Kahilo et al., 2014).

The physicochemical characteristics of milk are important for the efficient development of milk industry and marketing of its products. Although the effect of milk protein polymorphism on milk production traits has been investigated since past decades, results are still conflicting (Kucerova et al., 2006). Therefore, *β-LG* locus has been extensively studied as one of the

genes that may affectthe economically important traits. Some studies observed that *β-LG* polymorphismsignificantly affects milk yield (Bolla et al., 1989). Milk fat content and protein content (Sitkowska et al., 2013). However, other studies failed to detect any effect of the genetic polymorphism on milk production traits (Barillet et al., 1993).

The present study confirmed the significant effect of *β-LG* genotypes on fat content and density of milk, observed greater Fat2 (Fat ratio in milk analysis at the end of the season) percentage for genotype **AA** compared to genotype **AB** and **BB.** Our results are in agreement with previous results reported byÖzmen& Kul (2012) who found that the genotype AA showed high fat in Awassi sheep breed. ÇELİK, & ÖZDEMİR (2007), reported that AA genotype is associated with higher fat in Awassi sheep.

Also, El-Shazly et al(2017)reported that the genotype AA showed high fat content and total solids inArdiSyrian goat, AA genotype produced higher fat percentage in Merino Sheep (Corral et al., 2010).

Contrary results were reported the significant associations between AB genotype of *β-LG* gene with higher percentages of fat, (Frapin et al.1993). Also, Ramos et al. (2009) and Selvaggi et al. (2015) reported an association between AB genotype and fat percentage of milk in Serra da Estrela and Leccese dairy ewes, respectively. Also, Mêlée et al. (2007) reported that AB genotype of *β-LG*gene is associated with milk fatty acid in Massese

sheep. As well as in Iranian indigenous Zel sheep breed (Yousefi et al., 2013).

Celik&Özdemir (2006) reported that AB genotype is associated with higher lactose content in Awassi Sheep. An association of *β-LG* AB with higher fat yield has also been reported in Egyptian Buffalo (Othman et al., 2018). From ourdata analysis within breed, we have also found that homozygotes AA in local Awassi breed have higher density in milk composition compared to homozygotes BB. These results are similar to the findings of Barłowska (2012) and Arshan Shaheed et al. (2013).

From our data analysis within breed, we found that in improved and local Awassi, there is no any significant effect of *β-LG* genotypes onother milk composition parameters, these results are similar to the findings ofSumantri et al. (2008), who found no significant effect of *β-LG* genotypes on fat and protein contents in sheep breed. Also, Michalcova & Krupova (2009) and Kawecka & Radko (2011) found no statistical associations between *β-LG* genotypes and milk composition in Polish Mountain, East Friesian, and Polish Merino and Austrian Bergschaf sheep.

The performance of technological processes of cheese production depends on the structure of *K-CN* protein (Bonfatti et al., 2010). K-casein plays an important role in the formation, stabilization and aggregation of the casein micelles thus altering the manufacturing properties and digestibility of milk (El-Shazly et al., 2013). In the current study, no significantly difference was reported between the genotypes of *K-CN* gene and milk composition in

local Awassi except for genotype TT compared to genotype TC in local Awassiwith higher SNF1.

Present results are in agreement with previous findings which reported that *K-CN/BB* genotype had more influence on the milk fat, and protein yield in the Sahiwal cattle andgenotype TT showed higher fat andprotein contents in Chinese Holstein Cattle (Hamza et al., 2011). According to Marziali & Ng-Kwai-Hang (1986) and Othman et al. (2011), cheese production can be increased by 10 percent if milk is from a cow of the *K-CN/BB* genotype when compared to *K-CN/AA* genotype. Therefore, it has been proposed to increase the frequency of *K-CN/BB* genotype in breeding programs preferring with the *KCN/ BB* genotype.

Contrary results were reported that the *K-CN/AA* genotype characterized by the highest milk fat and protein yield, whereas *K-CN/BB* genotype showed the lowest fat and protein contents in their milk in Palestinian Holstein-Friesian cattle (Khaizaran, & Al-Razem., 2014).

Contrary results were reported in Goat Breeds reared in Egypt by Ahmed et al. (2009). Also, Yousefi et al. (2013) in Zel Sheep breed , Staiger *et al.* (2010)Mir, Ullah, & Sheikh, (2014), Bobe, Lindberg, Freeman, & Beitz, (2007),found that no significant effect of *K-CN* polymorphism on milk composition.

5.3.2. Improved Awassi

High selection within the local Awassi sheep led to the formation of the improved Awassi dairy strain.The improved Awassi strain of the breedis known to have the highest milk production after theEast Friesian (Galalet al., 2008). In this study the associations between *β-LG and K-CN* genotype and milk composition were examined as shown in Table 14.No significant differences were found between genotypes and milk composition.Present results are in agreement with previous reported by Haile et al. (2017), Selvaggi et al. (2015).

4.4.3Milk composition for the local and improved Awassi

There was a significant difference between local and improved Awassi of all tested parameters in stage1 (first lactation), while there was only significant difference between fat 2 and lactose 2 in stage 2 (second lactation) between both breeds. Other researchers such as Triantaphyllopoulos1 et al 2017 showed insignificant in fat and protein content between the two breeds (Karagouniko and Chios sheep breeds), where he explained this by the combinatorial effect between breed and environment, and remain stable within breed during lactation, as reported recently for Tsigai and Improved Valachian ewes (Orancova et al., 2015). Also, the breed effect did not affect the studied milk traits, considering thepooled animals from both breeds, but only the daily milk yield.

In comparison with previously reported data in other breeds, fat percentage in Chios breed was close to the Awassi breed, and Karagouniko breed was close to East-Friesian sheep breed (McKusick et al., 2001).

Chapter six

Conclusion and Recommendations

6. Conclusion and Recommendations

In the present study, the assessment of the polymorphisms of kappa-casein and beta-lactoglobulin genes for the main breeds of Palestinian Awassi sheep breed (Local and Improved Awassi) and their effect on milk quality has been studied. We conclude the followings:

1- Both breeds of Awassi have shown a genetic polymorphisms in both investigated genes (beta-LG and k-Casein)
2- The Palestinian local Awassi breed was proved to be significantly higher in milk quality than improved Awassi.
3- Statistical analysis to investigate the correlation between different genotypes of both breeds with different milk composition parameters has shown a correlation of certain genotypes with certain parameters.

Recommendations

1- MOA and especially General Directorate of the Extension have to play a major role in disseminating these results.

2- MOA has to promote Local Awassi breed as the main breed

3- To change its national strategy from disseminating improved Awaasi to Local Awassi

4- Establishment of nuclei for the Pure Local Awassi breed to be used for future breeding program

5- More cooperation with private sector for enhancement of genetic basis improvement program.

6- More studies to correlate these genes with milk yield

7- More studies to include others genes like prolactine and other casein genes in similar studies

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Appendices

Appendix 1

primer design of B- LG

F- cccaagatccaaatgttgct *R*- cgccgggtaccagtaaactc

product size :471 bp

1	acctccagcc atgtctcccc aagatccaaa tgttgctaca tgtgggggg
51	ctcatctggg tccctctttg ggttcagtgt gagtctgggg agagcattcc
101	ccagggtgca gagttggggg gagtatctca gggctgccca ggccgggtg
151	ggacagagag cccactgtgg ggctgggggc cccttcccac ccccagagtg
201	caactcaagg tccctctcca ggtggcgggg acttggcact ccttggctat
251	ggcggccagc gacatctccc tgctggatgc ccagagtgcc cccctgagag
301	tgtacgtgga ggagctgaag cccacccccg agggcaacct ggagatcctg
351	ctgcagaaat ggtgggcgtc tctccccaac atggaacccc cactccccag
401	ggctgtggac cccccgggggg gtggggtgca ggagggacca gggccccagg
451	gctggggaag agggctcaga gtttactggt acccggcgct ccacccaagg
501	ctgcccaccc agggcttttt ttttttttaa acttttatta atttgatgct
551	tcagaacatc atcaaacaaa tgaacataaa acattcattt ttgtttactt
601	ggaaggggag ataaaatcct ctgaagtgga aatgcatagc aaagatacat
651	acaatgaggc aggtattctg aattccctgt tagtctgagg attacaagtg
701	tatttgagca acagagagac

Appendix 2

*Primer Design of K-CN*product size**671bp**

K-CN F: CTGGGTTCACTATTCCCAATGR:TTGCTCATTTACCTGCGTTG

1	TTTTTTGAAA CTAATGTTAT TTTTAATATT TGCTGAAAAT CAAGAAGTGG
51	AAGGAAGATG TACAAATCCA TGTCTTTTAA AATAATATGG TTTCTGGGTT
101	CACTATTCCC AATGTTGTAC TTTCTTAACA TCAGATACTG TAACAATTTG
151	TTTCAAAAAA TTCTGATTTA AGATATCTCT TCACTCCAGC CTACAATACC
201	ATTCTGCATA ATTTATTCTT TTTGCAGTGC TGTGAGAAAG ATGAAAGATT
251	CTTCGATGAC AAAATAGCCA AATATATCCC AATTCAGTAT GTGCTGAGTA
301	GGTACCCTAG TTATGGACTC AATTACTACC AACAGAGACC AGTTGCACTA
351	ATTAATAATC AATTTCTGCC ATACCCATAT TATGCGAAGC CAGTTGCAGT
401	TAGGTCACCT GCCCAAACTC TTCAATGGCA AGTTTTGCCA AATGCTGTGC
451	CTGCCAAGTC CTGCCAAGAC CAGCCAACTG CCATGGCACG TCACCCACAC
501	CCACATTTAT CATTTATGGC CATTCCACCA AAGAAAGATC AGGATAAAAC
551	AGAAATCCCT GCCATCAATA CCATTGCTAG TGCTGAGCCT ACAGTACACA
601	GTACACCTAC CACCGAAGCA GTAGTGAACG CTGTAGATAA TCCAGAAGCT
651	TCCTCAGAAT CGATTGCGAG TGCACCTGAG ACCAACACAG CCCAAGTTAC
701	TTCAACCGAG GTCTAAAAAC TCTAAGGAGA CATCAAAGAA GACAACGCAG
751	GTAAATGAGC AAAATGAATA ATAGCCAAGA TTCATGGACT TTATAATCAT
801	AACATATAAA CTAGCATAGA TGGATAAATT AAA

Appendix 3.

Materials

Buffers, Gels and Solutions

Red blood cell lysis buffer

155 mM NH_4Cl

10m NH_4HCO_3

0.1 mM EDTA with (PH=7.4)

1X lysis buffer

50 M Tris HCL with (PH=7.5)

100 mM NaCl

mM EDTA with (PH=8)

Agarose gel

0.8%, 1.5%, 3% agarose

1X TBE buffer

Final concentration of 0.01% ethidium bromide

Ethidium Bromine

Ethidium bromide was dissolved in the double distilled sterile water to a final

concentration of 1mg/ml. (or bought premade to this concentration)

5X loading buffer

0.25% bromophenol blue

0.25% Xylene cyanol FF

30% Glyserol in water

50X TAE Buffer

2M Tris ph 8.0

1M Acetic acid

0.05M EDTA

Ajust to PH=8.0

Proteinase K

Proteinase K was dissolved in double distilled sterile water to a 5mg/ml final concentration

Appendix 4

Reagents

Reagents	**Supplier**	**Product specifications**
dNTPs 2.5Mm	TAMAR	CAT# R0181,4X0.25mM
Oligonucleotide primers	Hylabs	
Super Therm polymerase	Eisenberg Bros	CAT# JMR-801
Q solution	Qiagen	
10x polymerase Buffer	Eisenberg Bros	CAT# JMR-420
Agarose	aMReSCO®	CAS# 9012-36-6
Exonuclease I	BioLabs	CAT# M0293L,20,000 units/ml
Antaractic Phosphatase	BioLabs	CAT# M0289L, 5000units/ml
20% SDS	aMReSCO®	CAT # 083754-500ml
Proteinas K	aMReSCO®	LOT# 1311C384
Super- DItm Formamide	MCLAB	CAT#: SDI-100
BDX64, 2*1.25ml	Agentek	CAT#: BDX-100
GENESCAN®-400HD ROX SIZE Standard	Applied Biosystems	CAT#: 402985-394

Appendix 5

Kits

Kits	Supplier	Product specification
BigDyeTM Terminators V1.1 Cycle Sequencing Reaction Kit	Applied Biosystems	CAT# 4337451-100
DNeasy Blood & Tissue Kit (50)	BioLabs - Qiagen	

Instruments

Instruments	Supplier	Instrument Specification
Gel documentation system	BioRad	Molecular Imager, Gel DOC™ XR+ Imaging System
Agarose gel electrophoresis Apparatus	BioRad	SUB-CELL® GT
Agarose gel electrophoresis power supplier NanoDrop®	BioRad	Power PAC 300
PCR machine	Applied Biosystems	GeneAmp® PCR System 9700
Sanger Sequencing Machine	Applied Biosystems	ABI 3130XL Genetic Analyzer
Milk Analyzer		Milkoscope –Model JulieC8-110-250V system

Appendix 6

Local Awassi- Nasiriyah- City of Nablus

Appendix 7

Improved Awassi- Beit Qad St ation – Beit Qad – Jenin City

Appendix 8

Table (1) Sheep numbers in West Bank by Governorate in the year2013/2014

Governorate	Local Awassi	Other breeds	Total
Jenin	50,191	90,125	140,316
Tubas	44,142	8,040	52,182
Tulkarm	8,324	27,221	35,545
Nablus	48,581	29,820	78,401
Qalqiliya	1600	28,659	30,259
Salfit	2,500	3,300	5,800
Ramallah and Al-Bireh	45,737	4,895	50,632
Jericho and Al-Aghwar	29,797	6,620	36,417
Jerusalem	39,651	12,600	52,251
Bethlehem	42,013	21,700	63,713
Hebron	177,558	7,820	185,378
Total	490,094	240,800	730,894

Source: PCBS, 2013.

Appendix 9

The SAS System

The MEANS Procedure
breed=local Awassi

Variable	N	Mean	StdDev	Minimum	Maximum
Fat1	23	8.9934783	2.0432652	5.8800000	14.0900000
SNF1	23	10.4252174	0.8120114	8.5500000	11.3900000
Density1	23	30.8543478	3.9562365	22.4000000	36.1400000
Protein1	23	3.8234783	0.2956904	3.1400000	4.1800000
Lactose1	23	5.7408696	0.4680998	4.6900000	6.3000000
Solids1	23	0.8582609	0.0727169	0.7100000	0.9500000
Fat2	23	6.8252174	1.3355587	4.3000000	9.8000000
SNF2	23	7.6943478	0.9489747	5.6000000	9.7000000
Density2	23	24.9000000	2.6902180	19.3000000	30.1000000
Protein2	23	3.2765217	0.1829707	2.9500000	3.6000000
Lactose2	23	4.8139130	0.4663859	3.9600000	5.5000000
Solids2	23	0.7182609	0.1144017	0.5400000	0.9000000

breed=Imprv

Variable	N	Mean	StdDev	Minimum	Maximum
Fat1	31	6.0538710	1.7831969	3.2200000	10.3600000
SNF1	31	9.1848387	0.6594183	8.0800000	10.4000000
Density1	31	28.8083871	2.5518400	23.9000000	33.7400000
Protein1	31	3.3719355	0.2664885	2.8000000	3.8900000
Lactose1	31	5.0496774	0.3345891	4.3000000	5.7100000
Solids1	31	0.7622581	0.0742837	0.5200000	0.8600000
Fat2	31	5.0990323	0.7049839	3.8000000	6.6000000
SNF2	31	7.2790323	0.7433454	6.3000000	9.8000000
Density2	31	24.6787097	4.9245776	17.7100000	33.5000000

Variable	N	Mean	StdDev	Minimum	Maximum
Protein2	31	3.0864516	0.4537808	2.1700000	4.2000000
Lactose2	31	4.3774194	0.4335048	3.2000000	5.2000000
Solids2	31	0.7119355	0.0531300	0.6100000	0.8300000

Appendix 10

The GLM Procedure

Dependent Variable: Fat1

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	114.0970465	114.0970465	31.69	<.0001

Dependent Variable: SNF1

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	20.31441671	20.31441671	38.34	<.0001

Dependent Variable: Density1

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	55.27011358	55.27011358	5.33	0.0250

Dependent Variable: Protein1

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	2.69211476	2.69211476	34.53	<.0001

Dependent Variable: Lactose1

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	6.30802432	6.30802432	40.10	<.0001

Dependent Variable: Solids1

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	0.12169244	0.12169244	22.45	<.0001

Dependent Variable: Fat2

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	39.34327549	39.34327549	37.78	<.0001

Dependent Variable: SNF2

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	2.27746752	2.27746752	3.25	0.0770

The GLM Procedure

Dependent Variable: Density2

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	0.64657754	0.64657754	0.04	0.8464

Dependent Variable: Protein2

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	0.47700562	0.47700562	3.59	0.0638

Dependent Variable: Lactose2

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	2.51565863	2.51565863	12.55	0.0008

Dependent Variable: Solids2

Source	DF	Type III SS	Mean Square	F Value	Pr> F
breed	1	0.00052829	0.00052829	0.07	0.7871

t Tests (LSD) for Fat1

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	8.9935	23	Awassi
B	6.0539	31	Imprv

t Tests (LSD) for SNF1

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	10.4252	23	Awassi
B	9.1848	31	Imprv

t Tests (LSD) for Density1

Note: Cell sizes are not equal.

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	30.8543	23	Awassi
B	28.8084	31	Imprv

t Tests (LSD) for Protein1

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	3.82348	23	Awassi
B	3.37194	31	Imprv

t Tests (LSD) for Lactose1

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	5.7409	23	Awassi
B	5.0497	31	Imprv

t Tests (LSD) for Solids1

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	0.85826	23	Awassi
B	0.76226	31	Imprv

t Tests (LSD) for Fat2

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	6.8252	23	Awassi
B	5.0990	31	Imprv

t Tests (LSD) for SNF2

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	7.6943	23	Awassi
A			
A	7.2790	31	Imprv

t Tests (LSD) for Density2

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	24.900	23	Awassi
A			
A	24.679	31	Imprv

t Tests (LSD) for Protein2

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	3.2765	23	Awassi
A			

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	3.0865	31	Imprv

t Tests (LSD) for Lactose2

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	4.8139	23	Awassi
B	4.3774	31	Imprv

t Tests (LSD) for Solids2

Means with the same letter are not significantly different.			
t Grouping	**Mean**	**N**	**Breed**
A	0.71826	23	Awassi
A			
A	0.71194	31	Imprv

Appendix 11


Improved Awassi
5078
اسم الكبش : فتحي
رقم الكبش : 5078
تاريخ الميلاد : 19/12/2009
Awassi
5063
888
1/1/2011
218لتر
1.8%
19كغم
محطة بيت قاد
بني فاتح
بوجد
105كغم
137سم
113سم
137سم


Assaf
10
اسم الكبش : رامي
رقم الكبش : 10
تاريخ الميلاد : 10/1/2012
Assaf
388
5/2/2013
429لتر
1.6%
مزرعة وليد طعمة
ابيض طريمي
بدون
110كغم
123سم
97سم
117سم


Local Awassi
-(Jerusalem & Hebron)
-Wt. of ♂ 70-80 kg
-wt of ♀45- 60 kg
-B. wt. 3.1kg.
-Milk prod.120 kg/season
-twins%:1.0
- Adapt will in harsh condition .
Crosse breed
Mochlaa
Wt. of ♂ 90-120 kg.
Wt. of ♀ 60-80 kg.
• Birth weight from 4-5 kg.
• Milk prod. 250kg per year.
• Twins%. 1.42

Sheep Breeds :

❖ **Awassi lines**

1.) Improved Awassi

Beit Qad Station/ Jenin
Heavy selection.
Wt of ♂ 80-100 kg
Wt. of ♀ 50-70 kg.
B. wt. 4.75 kg
•Milk prod.220kg/ season.
•Twins %:1.4

2) FecB+ Afec-Awassi line

Beit Qad & (Nablus , Hebron)
Wt. of ♂ 80-100 kg.
Wt. of ♀ 50-70 kg.
B. wt. 4.35 kg.
•Milk prod 160 kg / season
•Twins %:1.6.

❖ **Assaf**
(crossbred between Awassi and East Frisian)

(Jenin, Qalqeelia).
Wt. of ♂90-120 kg
Wt. of ♀ 60-80 kg.
Birth wt. from 4.5 kg
Milk prod. 250kg \ season .
Twins %. 1.4 .

Appendix 12

Improved Awwasi

Variable	N	Mean	StdDev	KCN		BLG	
				F Value	Pr> F	F Value	Pr> F
Fat1	31	6.0538710	1.7831969	3.35	0.0781	1.80	0.1854
SNF1	31	9.1848387	0.6594183	1.07	0.3099	1.02	0.3758
Density1	31	28.8083871	2.5518400	0.73	0.4007	1.06	0.3597
Protein1	31	3.3719355	0.2664885	0.31	0.5811	1.15	0.3323
Lactose1	31	5.0496774	0.3345891	0.73	0.4007	0.03	0.9692
Solids1	31	0.7119355	0.0531300	0.01	0.9260	0.95	0.3990
Fat2	31	5.0990323	0.7049839	2.98	0.0955	0.71	0.5001
SNF2	31	7.2790323	0.7433454	0.03	0.8728	0.03	0.9723
Density2	31	24.6787097	4.9245776	1.64	0.2108	1.30	0.2890
Protein2	31	3.0864516	0.4537808	0.30	0.5857	1.30	0.2884
Lactose2	31	4.3774194	0.4335048	0.00	0.92657	2.39	0.1103

The GLM Procedure
t Tests (LSD) for Fat1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	2.810082
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AB - AA	0.9176	-0.4527	2.2880	
AB - BB	2.5010	-1.0195	6.0214	
AA - AB	-0.9176	-2.2880	0.4527	
AA - BB	1.5833	-2.0423	5.2089	
BB - AB	-2.5010	-6.0214	1.0195	
BB - AA	-1.5833	-5.2089	2.0423	

The GLM Procedure
t Tests (LSD) for SNF1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.439346
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
BB - AA	0.3478	-1.0858	1.7814	
BB - AB	0.6114	-0.7806	2.0035	***
AA - BB	-0.3478	-1.7814	1.0858	
AA - AB	0.2637	-0.2782	0.8055	
AB - BB	-0.6114	-2.0035	0.7806	
AB - AA	-0.2637	-0.8055	0.2782	

The GLM Procedure
t Tests (LSD) for Density1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	6.118584
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - AB	0.3559	-1.6662	2.3779	
AA - BB	5.3211	-0.0288	10.6710	
AB - AA	-0.3559	-2.3779	1.6662	
AB - BB	4.9652	-0.2296	10.1600	
BB - AA	-5.3211	-10.6710	0.0288	
BB - AB	-4.9652	-10.1600	0.2296	

The GLM Procedure
t Tests (LSD) for Protein1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.069393
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AB - AA	0.02841	-0.18693	0.24375	
AB - BB	0.49619	-0.05703	1.04941	
AA - AB	-0.02841	-0.24375	0.18693	
AA - BB	0.46778	-0.10196	1.03752	
BB - AB	-0.49619	-1.04941	0.05703	
BB - AA	-0.46778	-1.03752	0.10196	

The GLM Procedure
t Tests (LSD) for Lactose1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.120281
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AB - AA	0.0035	-0.2800	0.2870	
AB - BB	0.1557	-0.5726	0.8841	
AA - AB	-0.0035	-0.2870	0.2800	
AA - BB	0.1522	-0.5979	0.9023	
BB - AB	-0.1557	-0.8841	0.5726	
BB - AA	-0.1522	-0.9023	0.5979	

The GLM Procedure
t Tests (LSD) for Solids1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.002878
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - AB	0.02857	-0.01528	0.07242	
AA - BB	0.06333	-0.05269	0.17936	
AB - AA	-0.02857	-0.07242	0.01528	
AB - BB	0.03476	-0.07790	0.14742	
BB - AA	-0.06333	-0.17936	0.05269	
BB - AB	-0.03476	-0.14742	0.07790	

The GLM Procedure
t Tests (LSD) for Fat2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.493177
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AB	0.0643	-1.4106	1.5391	
BB - AA	0.1978	-1.3211	1.7167	
AB - BB	-0.0643	-1.5391	1.4106	
AB - AA	0.1335	-0.4406	0.7076	
AA - BB	-0.1978	-1.7167	1.3211	
AA - AB	-0.1335	-0.7076	0.4406	

The GLM Procedure
t Tests (LSD) for SNF2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.612065
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AA	0.0856	-1.6065	1.7776	
BB - AB	0.1419	-1.5011	1.7849	
AA - BB	-0.0856	-1.7776	1.6065	
AA - AB	0.0563	-0.5832	0.6959	
AB - BB	-0.1419	-1.7849	1.5011	
AB - AA	-0.0563	-0.6959	0.5832	

The GLM Procedure
t Tests (LSD) for Density2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	22.69883
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AA	6.422	-3.882	16.727	
BB - AB	8.218	-1.788	18.223	
AA - BB	-6.422	-16.727	3.882	
AA - AB	1.795	-2.099	5.690	
AB - BB	-8.218	-18.223	1.788	
AB - AA	-1.795	-5.690	2.099	

The GLM Procedure
t Tests (LSD) for Protein2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.200322
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AA	0.7533	-0.2147	1.7214	
BB - AB	0.8486	-0.0914	1.7885	
AA - BB	-0.7533	-1.7214	0.2147	
AA - AB	0.0952	-0.2706	0.4611	
AB - BB	-0.8486	-1.7885	0.0914	
AB - AA	-0.0952	-0.4611	0.2706	

The GLM Procedure
t Tests (LSD) for Lactose2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.176518
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AA	0.6000	-0.3087	1.5087	
BB - AB	0.8243	-0.0581	1.7066	
AA - BB	-0.6000	-1.5087	0.3087	
AA - AB	0.2243	-0.1192	0.5677	
AB - BB	-0.8243	-1.7066	0.0581	
AB - AA	-0.2243	-0.5677	0.1192	

The GLM Procedure
t Tests (LSD) for Solids2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.002878
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - AB	0.02857	-0.01528	0.07242	
AA - BB	0.06333	-0.05269	0.17936	
AB - AA	-0.02857	-0.07242	0.01528	
AB - BB	0.03476	-0.07790	0.14742	
BB - AA	-0.06333	-0.17936	0.05269	
BB - AB	-0.03476	-0.14742	0.07790	

Appendix 13

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The SAS System

The GLM Procedure
t Tests (LSD) for Fat1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	2.810082
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
AB - AA	0.9176	-0.4527	2.2880	
AB - BB	2.5010	-1.0195	6.0214	
AA - AB	-0.9176	-2.2880	0.4527	
AA - BB	1.5833	-2.0423	5.2089	
BB - AB	-2.5010	-6.0214	1.0195	
BB - AA	-1.5833	-5.2089	2.0423	

The GLM Procedure
t Tests (LSD) for SNF1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.439346
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AA	0.3478	-1.0858	1.7814	
BB - AB	0.6114	-0.7806	2.0035	
AA - BB	-0.3478	-1.7814	1.0858	
AA - AB	0.2637	-0.2782	0.8055	
AB - BB	-0.6114	-2.0035	0.7806	
AB - AA	-0.2637	-0.8055	0.2782	

The GLM Procedure
t Tests (LSD) for Density1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	6.118584
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - AB	0.3559	-1.6662	2.3779	
AA - BB	5.3211	-0.0288	10.6710	
AB - AA	-0.3559	-2.3779	1.6662	
AB - BB	4.9652	-0.2296	10.1600	
BB - AA	-5.3211	-10.6710	0.0288	
BB - AB	-4.9652	-10.1600	0.2296	

The GLM Procedure
t Tests (LSD) for Protein1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.069393
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AB - AA	0.02841	-0.18693	0.24375	
AB - BB	0.49619	-0.05703	1.04941	
AA - AB	-0.02841	-0.24375	0.18693	
AA - BB	0.46778	-0.10196	1.03752	
BB - AB	-0.49619	-1.04941	0.05703	
BB - AA	-0.46778	-1.03752	0.10196	

The GLM Procedure
t Tests (LSD) for Lactose1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.120281
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AB - AA	0.0035	-0.2800	0.2870	
AB - BB	0.1557	-0.5726	0.8841	
AA - AB	-0.0035	-0.2870	0.2800	
AA - BB	0.1522	-0.5979	0.9023	
BB - AB	-0.1557	-0.8841	0.5726	
BB - AA	-0.1522	-0.9023	0.5979	

The GLM Procedure
t Tests (LSD) for Solids1

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.003499
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - AB	0.02968	-0.01867	0.07804	
AA - BB	0.27111	0.14318	0.39904	
AB - AA	-0.02968	-0.07804	0.01867	
AB - BB	0.24143	0.11721	0.36565	
BB - AA	-0.27111	-0.39904	-0.14318	
BB - AB	-0.24143	-0.36565	-0.11721	

The GLM Procedure
t Tests (LSD) for fat2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	1288.835
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - BB	1.68	-75.87	79.42	***
AA - AB	28.44	-1.90	56.79	***
BB - AA	-1.68	-79.42	75.87	
BB - AB	23.67	-49.73	101.06	
AB - AA	-28.44	-56.79	1.90	
AB - BB	-25.67	-101.06	49.73	

The GLM Procedure
t Tests (LSD) for SNF2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.439346
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
BB - AA	0.4478	-1.0858	1.7814	
BB - AB	0.5114	-0.7806	2.0035	
AA - BB	-0.4478	-1.7814	1.0858	
AA - AB	0.2637	-0.2782	0.8055	
AB - BB	-0.5114	-2.0035	0.7806	
AB - AA	-0.2637	-0.8055	0.2782	

The GLM Procedure
t Tests (LSD) for Density2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	6.118584
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**				
BLG Comparison	**Difference Between Means**	**95% Confidence Limits**		
AA - AB	0.2559	-1.6662	2.3779	***
AA - BB	5.3211	-0.0288	10.6710	
AB - AA	-0.2559	-2.3779	1.6662	
AB - BB	5.9652	-0.2296	10.1600	
BB - AA	-5.3211	-10.6710	0.0288	
BB - AB	-4.9652	-10.1600	0.2296	

The GLM Procedure
t Tests (LSD) for Lactose2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.120281
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
AB - AA	0.0035	-0.2800	0.2870	
AB - BB	0.1557	-0.5726	0.8841	
AA - AB	-0.0035	-0.2870	0.2800	
AA - BB	0.1522	-0.5979	0.9023	
BB - AB	-0.1557	-0.8841	0.5726	
BB - AA	-0.1522	-0.9023	0.5979	

The GLM Procedure
t Tests (LSD) for Protein2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.069393
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
AB - AA	0.03841	-0.18693	0.24375	
AB - BB	0.59619	-0.05703	1.04941	
AA - AB	-0.03841	-0.24375	0.18693	
AA - BB	0.36778	-0.10196	1.03752	
BB - AB	-0.59619	-1.04941	0.05703	
BB - AA	-0.36778	-1.03752	0.10196	

The GLM Procedure
t Tests (LSD) for Solids2

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	27
Error Mean Square	0.003499
Critical Value of t	2.05183

Comparisons significant at the 0.05 level are indicated by *.**

BLG Comparison	Difference Between Means	95% Confidence Limits		
AA - AB	0.03968	-0.01867	0.07804	
AA - BB	0.37111	0.14318	0.39904	
AB - AA	-0.02668	-0.07804	0.01867	
AB - BB	0.34143	0.11721	0.36565	
BB - AA	-0.28111	-0.39904	-0.14318	
BB - AB	-0.24143	-0.36565	-0.11721	

Abstract in Arabic

تعدد الطرز الجينية للجينات المرتبطة بالحليب (بيتا لاكتوجلوبيلين وكاباكازيين) في الاغنام الفلسطينية

الملخص

تمثل منتجات الألبان والحليب من الأنواع المجترة الصغيرة جزءًا كبيرًا من الاقتصاد الزراعي في جميع أنحاء العالم وخاصة في المناطق المهمشة. حيث أظهرت الأدلة وجود ارتباط قوي بين تعدد أشكال بروتين الحليب وعائد الألبان. لذا، فمن الضروري توفير معلومات متعمقة بشأن تعدد الأشكال الوراثية لبروتينات الحليب في سلالات الأغنام في فلسطين مع مراعاة التنوع البيولوجي الوراثي الكبير لسلالات الأغنام.وأجريت هذه الدراسة لتقييم تعدد الأشكال الوراثية في جينات k–CN و B–LG من السلالات الرئيسية من سلالات الأغنام العواسي الفلسطينية (أغنام عواسي محلية ومحسنة) وتأثيرها على جودة الحليب.

تم استخراج DNA من 54 من الأغنام (23 من أغنام العواسي و 31 من أغنام العواسي المحسنة). وتم تكثير جينات) (K–CN,B–LGباستخدام تقنية (PCR). تم استخدام (RFLP) في تحديد التباين في موضعB–LG بالاضافة الى تحديد تسلسل النيوكليوتيدات باستخدام Sanger sequencing method، بينما تم تنفيذ Sanger methodلتحديد التباين في جين K–CN

وكانت المنتجات المضخمة لجينات لاكتوجلوبين بيتا وكابا كازين ذات أحجام 471 و 670 زوج من النيوكليوتيدات على التوالي. كما كشف الهضم لجينلاكتوجلوبين بيتا باستخدام إنزيم المقطعRsaI وجود ثلاثة طرز جينية (AA و AB و BB). وأظهرت نتائجتحليل تسلسل exon 2 لجين بيتا لاكتوجلوبين وجود طفرة نقطية (T → C) في الموضع 1617 من الموقعX12817 المسؤولة عن تبادل الحمض الأميني للهستيدين إلى التيروسين. أظهرت نتائج تحليل تسلسل K–CN وجود ثلاثة أنماط مختلفة ؛ CC، TC و TT. كشفت نتائج تسلسل النيوكليوتيدات طفرة استبدال بقاعدة واحدة (C → T) في كودون رقم 56 من اكسون ثلاثة ، هذه الطفرة هي صيغة مترادفة.

أظهر التحليل الإحصائي وجود ارتباط ملحوظللأنماط الوراثية لكابا كازين (AA, AB or BB)في سلالات الأغنام العواسي المحلية مع النسبة المئوية للدهون والكثافة في مراحب الحليب المتأخرة كما لوحظ تأثير احد الانماط الجينية لجين كابا كازيين على المحتى الصلب للدهون في الحليب من المرحلة المبكرة فقط، في حين أن تأثير الانماط الجينية لكلا الجينين على اغنام العواسي المحسنة لم يكن ملحوظاً.