GENOTYPING OF BETA-CASEIN, KAPPA-CASEIN AND BETA-LACTOGLOBULIN GENES IN TURKISH NATIVE CATTLE BREEDS AND EFFORTS TO DELINEATE BCM-7 ON HUMAN PBMC

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ABSTRACT

GENOTYPING OF BETA-CASEIN, KAPPA-CASEIN AND BETA-LACTOGLOBULIN GENES IN TURKISH NATIVE CATTLE BREEDS AND EFFORTS TO DELINEATE BCM-7 ON HUMAN PBMC

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The main aim of this study is to determine genetic diversity of milk protein genes associated with milk traits, namely beta-casein, kappa-casein and beta-lactoglobulin, in native Turkish cattle breeds (Turkish Grey, Eastern Anatolian Red, Anatolian Black, and Southern Anatolian Red) and Turkish Holstein. Only 11% deviation from the Hardy-Weinberg equilibrium and insignificant Fis values for the populations were observed, indicating that samples are free of inbreeding.

B alleles of these genes, which are positively related with cheese yield and quality, seem to be relatively high in native Turkish breeds. Therefore, the results suggest that milk of Turkish native breeds is advantageous for producing high-quality and -yield cheese.

A1 allele of beta-casein, which releases a bioactive peptide called BCM-7 after successive gastrointestinal proteolytic digestions, has been claimed to have adverse health effects on humans. Another aim of this study is to develop a protocol and assess the potential detrimental effects of BCM-7 on human peripheral blood cells. Despite the fact that the results are inconclusive, the optimized experimental protocol will guide further researchers while judging the effect of BCM-7 on human health.

Even though A1 beta-casein, which has a low frequency in native Turkish breeds, and hence BCM-7 have no adverse health effects on humans, this probability should be enough to keep its frequency low in native cattle breeds. Bulls must be screened for A1 allele of beta-casein as well as E allele of kappa-casein, which is absent in native breeds and known to have detrimental effects on cheese quality.

Keywords: Beta-casein, Kappa-casein, Beta-lactoglobulin, Cheese quality/yield, BCM-7

ÖZ

BETA-KAZEİN, KAPPA-KAZEİN VE BETA-LAKTOGLOBULİN GENOTİPLERİNİN BELİRLENMESİ VE BKM-7'NİN PERİFERİK KAN TEK ÇEKİRDEKLİ İNSAN HÜCRELERİNDE TANIMLANMASINA YÖNELİK ÇALIŞMALAR

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Sunulan çalışmanın temel amacı yerli Türk sığır ırklarında (Bozırk, Doğu Anadolu Kırmızısı, Yerli Kara ve Güney Anadolu Kırmızısı) ve kültür ırkı olan Türk Siyah Beyaz Alaca'da süt özellikleri ile ilişkilendirilmiş olan beta-kazein, kappa-kazein ve beta-laktogobulin süt protein genlerinin genetik çeşitliliğini belirlemektir. Hardy-Weinberg dengesinden sadece %11'lik bir sapma ve populasyonlarda istatistiksel olarak önemsiz Fis değerlerinin gözlenmesi, örneklenen bireylerin soy içi üreme ile çiftleştirilmediklerini göstermektedir.

Bu genlerin, peynir verimi ve kalitesi ile pozitif bir ilişkisi olduğu gözlenmiş olan B alellerinin yerli Türk ırklarında göreceli olarak yüksek sıklıkta görülmektedir. Bu bilgi ışığında, bu araştırmada elde edilen sonuçlar, yerli Türk ırklarının kaliteli ve yüksek verimli peynir üretimi için uygun süt verdiklerini göstermektedir.

Birbirini izleyen gastrointestinal proteolitik parçalanmalar sonucunda bir biyoetken peptit olan BKM-7'nin ortaya çıkmasına sebep olan beta-kazein A1 alelinin, insan sağlığına kötü etkileri olabileceği iddia edilmektedir. Bu çalışmanın bir başka amacı ise, BKM-7'nin periferik kan tek çekirdekli insan hücreleri üzerindeki olası zararlı etkilerini belirlemek için bir protokol geliştirmek ve bu etkileri belirlemektir. Elde edilen sonuçların kesin olmamasına rağmen, optimize edilen deney protokolü BKM-7'nin insan sağlığı üzerindeki etkilerini araştıracak olan daha sonraki araştırıcılara rehber olacaktır.

Türk yerli ırklarında düşük frekansta bulunan A1 beta-kazein ve dolayısı ile BKM-7'nin insan sağlığı üzerinde olumsuz etkisi olmasa bile, böyle bir olasılığın varlığı A1 beta-kazein frekansını yerli sığır ırklarında düşük seviyelerde tutmak için yeterlidir. Suni dölleme için seçilecek boğalar, beta-kazein A1 aleli ve yerli ırklarda yok olan, peynir kalitesi üzerindeki zararlı etkisi olduğu bilinen kappa-kazein E aleli için bu mevcut çalışmada optimize edilmiş metotlarla taranmalıdır.

Anahtar Kelimeler: Beta-kazein, Kappa-kazein, Beta-laktoglobulin, Peynir kalitesi/ verimi, BKM-7

To my Grandfather;
Enver Gilanlıoğlu

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

AB	Anatolian Black
ACRS	Amplification Created Restriction Sites
APS	Ammonium Persulfate
BCM-7	Beta-casomorphin 7
BLG	Beta-lactoglobulin
bp	base pairs
BS	Brown Swiss
BSA	Bovine Serum Albumin
CFSE	Carboxyfluorescein diacetate succinimidyl ester
DGAT1	Diacylglycerol Acyltransferase1
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EAR	Eastern Anatolian Red
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
GH	Growth Hormone
HCB	Holstein Candidate Bulls
H-W	Hardy-Weinberg
IL	Interleukin
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium Chloride
NJ	Neighbour Joining

PBMC	Peripheral Blood Mononuclear Cells
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
PNPP	Para-nitrophenyl Phosphate
RFLP	Restriction Fragment Length Polymorphisms
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SA-AKP	Streptavidin-alkaline phosphatese
SAR	Southern Anatolian Red
SP	Scrambled Peptide
SSCP	Single-Strand Conformation Polymorphisms
TBE	Tris-Boric Acid-EDTA
TEMED	Tetramethylethylenediamine
TG	Turkish Grey
TH	Turkish Holstein
TNF	Tumor Necrosis Factor

CHAPTER 1

INTRODUCTION

1.1 Milk Proteins and Effect of Milk Protein Variants on Milk Traits

Studies on bovine milk protein genetic variation started over 50 years ago, by the identification of the bovine beta-lactoglobulin main variants (Aschaffenburg and Drewry 1957), and have intensively accelerated in recent years with the improvement of the experimental procedures and the contributions from the fields of molecular biology, genetics and biochemistry.

Bovine milk is composed of mainly water, proteins, fat, carbohydrates, lactose and minerals. Milk proteins are divided into two main groups; caseins (~80% of the milk proteins) and whey proteins. Casein is the insoluble fraction and is composed of four different caseins; alpha$_{s1}$-casein (CSN1S1), alpha$_{s2}$-casein (CSN1S2), beta-casein (CSN2) and kappa-casein (CSN3). Whey proteins make up the soluble fraction and they are composed of several different proteins, the most important of which are α-lactalbumin and beta-lactoglobulin (BLG) (Eigel *et al.*, 1984).

For all six major milk protein genes, there are autosomal and codominant alleles, which are called genetic variants. If those polymorphic sites in the corresponding genes causing amino acid changes in proteins are considered, genetic variants of milk proteins reported so far are as follows (Farrell *et al.*, 2004):

1) Alpha$_{s1}$-casein: A, B, C, D, E, F, G, H;
2) Alpha$_{s2}$-casein: A, B, C, D;

3) Beta-casein: A1, A2, A3, B, C, D, E, F, G, H1, H2, I;
4) Kappa-casein: A, B, C, E, F1, F2, G1, G2, H, I, J;
5) Beta-lactoglobulin: A, B, C, D, E, F, G, H, I, J, W;
6) Alpha-lactalbumin: A, B, C.

There are numerous studies on milk protein polymorphisms in different cattle breeds from different regions of the world (for instance; Lien *et al.*, 1999, Malik *et al.*, 2000, Strzalkowska *et al.*, 2002, Beja-Pereira *et al.*, 2002, Jann *et al.*, 2004, Ceriotti *et al.*, 2004, Ibeagha-Awemu E.M. and Erhardt 2005, Daniela and Vintila 2005, Rachagani *et al.*, 2008, Hallen *et al.*, 2008, Heck *et al.*, 2009). The main objectives of such studies can be summarized as follows: (1) to propose the evolutionary history of milk protein loci, (2) to confirm relationships between different species or breeds, (3) to observe variations that happen in time or space for particular animal populations, and (4) to clarify associations between genetic variants and milk traits (milk yield, milk protein content, milk fat content, cheese yield and quality), reproduction efficiency and adaptation capacity of the cattle. Moreover, in relation to milk trait properties, variants were also examined for their effects on cheese manufacturing (Formaggioni *et al.*, 1999).

The four casein genes are organized in tandem to form a casein locus of about 250 kb, located on bovine chromosome 6 (Threadgill and Womack 1990) and are closely linked (Ferretti *et al.*, 1990). The $alpha_{s1}$-casein, beta-casein, and $alpha_{s2}$-casein genes are the most closely linked ones and form an evolutionarily related family, whereas the kappa-casein gene is at least 70 kb away from them (Ferretti *et al.*, 1990). Hence, the genetic variant of kappa-casein might be in linkage equilibrium with another casein variant. One of the whey protein genes, BLG gene is on bovine chromosome 11.

In the current study, genetic variants of beta-casein, kappa-casein and beta-lactoglobulin (BLG) listed in Table 1.1, in four native Turkish cattle breeds (Turkish Grey, Eastern Anatolian Red, Anatolian Black and Southern Anatolian

Red), a non-native breed (Turkish Holstein) and independent subsample of Holstein breed; Holstein Candidate Bulls were studied.

Table 1.1. Milk protein genes and their variants examined in the present study.

Milk Protein Genes	Alleles	Exons	Nucleotide Substitutions	Amino Acid Position	Amino Acid Substitutions	References
Beta-casein	A2→A1	VII	C<u>C</u>T→C<u>A</u>T	67	Pro→His	Lien *et al.*, 1992
	A2→A3	VII	CA<u>C</u>→CA<u>A</u>	106	His→Gln	
	A2→B	VII	C<u>C</u>T→C<u>A</u>T	67	Pro→His	
			AG<u>C</u>→AG<u>G</u>	122	Ser→Arg	
Kappa-casein	A→B	IV	A<u>C</u>C→A<u>T</u>C	136	Thr→Ile	Malik *et al.*, 2000
			G<u>A</u>T→G<u>C</u>T	148	Asp→Ala	
	A→E	IV	<u>A</u>GC→<u>G</u>GC	155	Ser→Gly	
BLG	A→B	IV	G<u>A</u>T→G<u>G</u>T	64	Asp→Gly	Medrano and Aguilar-Cordova, 1990
			G<u>T</u>C→G<u>C</u>C	118	Val→Ala	

The main reason of focusing on such milk proteins and the genetic variants listed in Table 1.1 is that these variants are reported in literature to be related with milk yield, protein content, cheese yield/quality of bovine milk and that a wide range of frequencies of genetic variants are observed in cattle breeds around the world (Fitzgerald *et al.*, 1999). Yet, to our knowledge, there is only one DNA-based casein diversity study (Jann *et al.*, 2004) including only two native Turkish cattle breeds (Turkish Grey and Anatolian Black). It is believed that the data on frequencies of milk protein alleles will be useful in understanding the milk trait properties of Turkish breeds. Table 1.2 presents a summary from the literature on the effects of milk protein genes on milk traits.

Table 1.2. Summary of studies on the associations of beta-casein, kappa-casein and BLG loci on milk traits.

Loci	Alleles	Milk Traits			References
		Milk Yield	Casein Content	Cheese Yield/Quality	
Beta-casein	A2	ND	↑	ND	Kucerova *et al.*, 2006
	A2	↑	ND	ND	Matyukov, 1983, Bech and Kristiansen, l990
	A1	↑	ND	ND	Kucerova *et al.*, 2006
	A3	↑	ND	ND	Ng-Kwai-Hang, 1998
	B	ND	↑	↑	Marziali and Ng KwaiHang, 1986
	B	ND	↑	ND	Heck *et al.*, 2009
Kappa-casein	A	↑	ND	ND	Kucerova *et al.*, 2006
	A	↑	ND	ND	Strzalkowska *et al.*, 2002
	B	ND	ND	↑	Medrano and Anguilar-Cordova, 1990
	B	ND	↑	ND	Caroli *et al.*, 2004
	B	ND	↑	↑	Strzalkowska *et al.*, 2002
	B	ND	ND	↑	Ikonen *et al.*, 1997, Hallen *et al.*, 2007
	B	ND	↑	ND	Hallen *et al.*, 2008
	B	ND	↑	ND	Heck *et al.*, 2009
	E	ND	↓	↓	Ikonen *et al.*, 1997
	E	ND	ND	↓	Ikonen *et al.*, 1999
	E	ND	↓	ND	Hallen *et al.*, 2008
BLG	A	↑	ND	ND	Robitaille *et al.*, 2002
	A	-	-	-	Kucerova *et al.*, 2006
	A	↑	↑	ND	Strzalkowska 2002
	A	↑	ND	ND	Daniela and Vintila, 2005
	A	ND	↓	ND	Heck *et al.*, 2009
	B	ND	↑	ND	Robitaille *et al.*, 2002
	B	ND	↑	↑	Daniela and Vintila, 2005
	B	-	-	-	Kucerova *et al.*, 2006
	B	ND	↑	ND	Hallen *et al.*, 2008

Arrows indicate increase (↑), decrease (↓) and (-) indicates no association. ND: Not Determined.

Contradictory results in the literature on the effects of alleles of milk proteins are observed. This is because milk traits are not controlled by single genes and genetic make up of the breeds as well as their environments has effects on the relationship of milk protein allele and milk property or yield. This is another reason why every association must be tested in every breed. Yet, data (Table 1.2) suggests that in general A2 allele of beta-casein has been associated with a higher milk production (Lin *et al.*, 1986; Bech and Kristiansen, 1990) and the B variant with an increased protein content and better cheese-making properties as determined by higher casein concentration, faster milk coagulation and firmer curd (Marziali and Ng-Kwai-Hang, 1986). Beta-casein A3 allele is associated with higher milk production (Ng-Kwai-Hang 1998). Kappa-casein B allele have a favorable and significant effect on milk protein yield and BB of kappa-casein gene together seemed to increase the cheese yield approximately by 10%. Milk produced by beta-lactoglobulin BB genotype cows yield significantly more cheese than that of AA cows (Strzalkowska *et al.*, 2002; Kaminski 2004).

Among some other milk trait genes (growth hormone, prolactin, DGAT1) affecting properties of milk (e.g., fat content), especially kappa-casein and BLG gene variants have a determining role for casein concentration which has a positive effect on cheese yield (Heck *et al.*, 2009 and references therein) (see Table 1.2).

For the breeds; Turkish Grey, Eastern Anatolian Red, Anatolian Black, Southern Anatolian Red and Turkish Holstein examined in the current study based on the same individuals, results for three more milk trait related (growth hormone, prolactin, DGAT1) and four microsatellite (ETH10, ETH225, HEL5, ILSTS005) loci were available in the literature (Özkan 2005, Kepenek 2007, Özkan *et al.*, 2009). ln the present study, results of Kepenek's (2007) study was improved for prolactin and DGAT1 loci by increasing sample sizes. For one of these loci; growth hormone (GH) information from two polymorphic sites (*AluI* and *MspI*) was available. These sites were considered as two loci, hence total loci affecting the milk traits amounted to seven loci. Results obtained based on these loci will also be

examined in the present study. Table 1.3 summarizes the effects of mentioned genes on milk traits.

In summary, as can be seen from Table 1.2 and Table 1.3 protein content, cheese yield and quality is related with B and may be A2 alleles of beta-casein, B allele of kappa-casein, and B allele of BLG loci. E allele of kappa-casein is known to have an adverse effect on cheese quality. V allele of GH may also play role in higher cheese yield because it is reported as a factor increasing protein content of milk. On the other hand, A1 and A3 of beta-casein, A allele of kappa-casein and A allele of BLG loci seemed to be related with high milk yield. Furthermore, A alleles of DGAT1, and prolactin loci and L of GH-AluI and (+) of GH-MspI also seemed to be related with high milk yields. The main drawback of the present study is that genetic studies will not be directly correlated with the milk trait parameters of the Turkish native breeds because of the absence of such data.

Table 1.3. Summary of studies on the associations of growth hormone, DGAT1 and prolactin loci on milk traits.

Loci	Alleles	Milk Traits			References
		Milk Yield	Protein Content	Fat Content	
GH-AluI	L	↑	ND	ND	Lucy *et al.*, 1993
	V	ND	↑	↑	Sabour *et al.*, 1997
	V	ND	↑	↑	Zwierzchowski *et al.*, 2002
	V	ND	ND	↑	Hoj *et al.*, 1993, Lee *et al.*, 1994
GH-MspI	+	↑	ND	ND	Hoj *et al.*, 1993, Lee *et al.*, 1994
	+	↑	↑	↑	Yao *et al.*, 1996
	-	ND	ND	↑	Hoj *et al.*, 1993, Lee *et al.*, 1994
	-	ND	ND	↑	Falaki *et al.*, 1996
DGAT1	A	↑	ND	ND	Grisart *et al.,* 2002
	K	ND	ND	↑	Kaupe *et al.*, 2003, Grisart *et al.,* 2002
Prolactin	A	↑	ND	ND	Chung *et al.*, 1996 and Cherenk *et al.*, 1998 referred by Miceikiene *et al.*, 2006
	B	↓	ND	↑	Khatami *et al.*, 2005

Arrows indicate increase (↑) and decrease (↓).
ND: Not Determined.

1.2 A1, A2 Milk and Beta-casomorphin 7

Bovine beta-casein has 209 amino acids. Normally, cow's milk has so called A2 beta-casein with amino acid (aa) proline at its 67th position of beta-casein. As a result of a point mutation on exon VII of bovine beta-casein gene on sixth chromosome of bovine, a conversion from cytosine to adenine lead to replacement of proline (codon; CCT) by histidine (codon; CAT) at position 67 (Groves, 1969). This point mutation is not present in humans, apes, goat and sheep, so their milk only has A2-like beta-casein and is named as "A2 milk".

After observation of the new variant at 67th aa position, bovine beta-casein was classified into two groups as; A1 and A2 milk depending on the presence of this point mutation. However, A1 milk sometimes also described as "A1-like" milk harbors further variants as A1, B and all exhibiting **His^{67}** but varying in the rest of the protein. Similarly, A2 milk or "A2-like" milk includes A2 and A3 both exhibiting **Pro^{67}**. In summary A1 and A2 milk implies the following:

A1 milk (=A1-like milk) is: Beta-casein with A1, B and C alleles having the common variant **His^{67}** (-Tyr^{60}-Pro^{61}-Phe^{62}-Pro^{63}-Gly^{64}-Pro^{65}-Ile^{66}-**His^{67}**-).

A2 milk (=A2-like milk) is: Beta-casein with A2 and A3 alleles having the common variant **Pro^{67}** (-Tyr^{60}-Pro^{61}-Phe^{62}-Pro^{63}-Gly^{64}-Pro^{65}-Ile^{66}-**Pro^{67}**-).

Since A1 milk was observed only in bovine, many studies focused on characterizing beta-casein variability in bovine populations were carried out. The first evidence of genetic polymorphism in beta-casein came from the study done by Aschaffenburg (1961) based on milk samples from Jersey and Guernsey cows. In 1966, Peterson and Kopfler's study showed that beta-casein A allele could be separated into three additional alleles, now known as beta-casein A1, A2 and A3. A1 beta-casein was present only among cattle breeds of western world, all of their cattle belongs to taurine cattle; *Bos taurus*. Asian cattle belong to the zebu cattle;

Bos indicus and do not produce A1-like beta-casein. African cattle, although mainly *Bos taurus* also do not exhibit A1 beta-casein (Woodford, 2009).

It is believed that the point mutation differentiating A1-like beta-casein from A2-like beta-casein occurred about 5000 years ago in European taurine (*Bos taurus*) cattle probably somewhere close to Anatolia on the way to Europe from the domestication center (Loftus *et al.*, 1999 and Troy *et al.*, 2001). Therefore, it is expected to observe lower frequencies of A1 beta-casein in native Turkish cattle breeds compared to the European cattle breeds.

Milk protein polymorphisms may also have effects on human health besides their effects on milk traits. For instance; possible link between type-I diabetes and cow milk proteins has been realized in 1984 when incidence of type-I diabetes in children migrating from Samoan island to New Zealand was observed to be significantly increased. Researchers claimed that the reason could be the shift in the type of cow's milk that children consumed. Suspected culprit was the milk that they have consumed in New Zealand. Focus was particularly on beta-casein because it is a well known that beta-casein is an allergen (A2 Corporation Review). Additionally, the fact that there was only A2 milk in Samoan island and that 20% of cows in New Zealand was producing A1 milk lead researchers to examine association between occurrence of type-I diabetes in children and A1 milk. Epidemiological studies on this issue presented that consumption of A1 beta-casein has a correlation with occurrence of type-I diabetes (Elliott *et al.*, 1999; Thorsdottir *et al.*, 2000; McLachlan *et al.*, 2001; Laugesen ve Elliott, 2003; Birgisdottir *et al.*, 2002 ve Birgisdottir *et al.*, 2006). Furthermore, A1-milk also seemed to be associated with the ischaemic heart diseases (McLachlan *et al.*, 2001 and Laugesen and Elliott, 2003) based on epidemiological studies. The first comprehensive report suggesting beta-casein variant consumption as a risk factor of type I (insulin-dependent) diabetes mellitus and ischaemic heart disease in humans was published by a company called A2 Corporation in New Zealand (A2 Corporation Review).

Moreover, A1 milk has also been linked to symptoms of neurological disorders autism and schizophrenia in animal and human trials (Sun *et al.*, 1999 and Cade *et al.*, 2000) and also to sudden infant death syndrome (SIDS) in newborn babies (Sun *et al.*, 2003).

A1 beta-casein releases a bioactive peptide bovine beta-casomorphin 7 (BCM-7) after successive gastrointestinal proteolytic digestion by pepsin, pancreatic elastase and leucine amino peptidase as presented in Figure 1.1 (Jinsmaa and Yoshikawa, 1999). Elastase cleaves the peptide bond between isoleucine (Ile) and histidine (His), releasing the carboxyl terminus of BCM-7. Pepsin and leucine amino peptidase are required to release the amino terminus of this peptide. However, beta-casein variant A2 does not yield BCM-7 since it has proline at position 67 instead of histidine (Jinsmaa and Yoshikawa, 1999). BCM-7 is comprised of seven amino acids; Tyr-Pro-Phe-Pro-Gly-Pro-Ile (YPFPGPI) and is a peptide exhibiting a strong opioid activity (Kurek *et al.*, 1992, Gobbetti *et al.*, 2002). It is suggested that, since "Tyr-Pro-Phe" at the N-terminus of the bioactive peptide BCM-7 has opioid properties, it could act as a chemical with a morphine-like action in the body (Meisel and Bockelmann, 1999). On the other hand, the latter four amino acids (PGPI) of BCM-7 have a sequence homology with a residue 415–419 (PGPIP) of the pancreatic beta-cell-specific glucose transporter GLUT-2 molecule, and it is argued that possibly this molecular mimicry has a role in the observed increase of Type-I diabetes incidence of Samoan children in New Zealand (Inman *et al.*, 1993). Therefore, this sequence homology induced many studies performed by biochemistry, pharmacology, animal trials, immunology and human trials trying to reveal a relationship between A1 beta-casein and BCM-7 with type 1 diabetes (Woodford, 2009 and references therein). For instance, Monetini *et al.*'s (2001) study reported raised levels of beta-casein antibodies in bottle-fed infants compared to breast-fed infants and also observed significantly higher antibody levels to bovine beta-casein in children with type 1 diabetes compared to age matched healthy individuals. The results of the mentioned study postulated the possible immune response for beta-casein in sensitive type 1 diabetic infants for BCM-7.

However, the studies performed to date did not allow a conclusion on the role of neither BCM-7 (European Food Safety Authority Scientific Report, 2009) in development of type 1 diabetes nor A1 beta-casein as a causative factor in many Swinburn's report (2004) and Truswell's (2006) health problems. On the other hand, a recent book published by Woodford (2009), with the title: "Devil in the Milk: Illness, Health, and the Politics of A1 and A2 Milk", argues that A1 milk should not be consumed. To sum up, the topic is still controversial and researchers agree on that further research is needed to clarify the risk of A1 milk consumption.

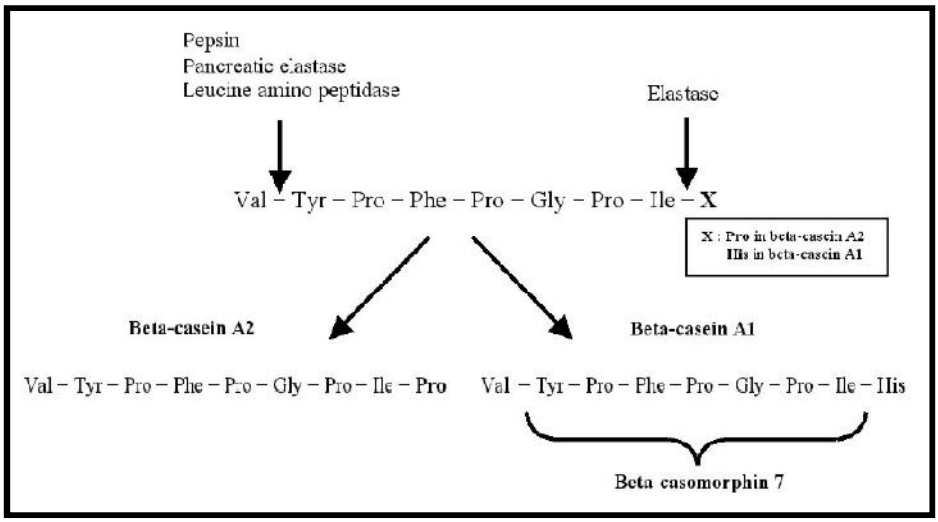


Figure 1.1. Release of beta-casomorphin 7 from beta-casein variant A1 but not from A2 (from Kaminski *et al.*, 2007).

Evaluating cytokine production in peripheral blood mononuclear cells (PBMC) after lipopolysaccharide (LPS) stimulation in diabetics and healthy subjects has been used to unravel the immune cellular responses in several studies (Foss *et al.*, 2007, Foss-Freitas *et al.*, 2008). Additionally in another study (Larocca *et al.*, 2007), cytokine determination was performed with the use of ELISA assay to reveal the effect of a peptide (vasoactive intestinal peptide) on LPS-induced PBMC cultures. Similar system (changes in cytokine levels released by PBMC upon treatment by BCM-7) to investigate the effect of BCM-7 on immune system, in diabetics was planned in the present study. In Dogan *et al.*'s (2006) study, blood serum concentrations of IL-1β, TNFα, IL-2 and IL-6 in type 1 diabetic children were investigated via ELISA assay and higher levels of IL-1β and TNFα and lower levels of IL-2 and IL-6 were detected in all stages of diabetes (Foss *et al.*, 2007, Foss-Freitas *et al.*, 2008). The latter two studies proposed the design of the preliminary experiments for evaluating the effects of BCM-7 in PBMC cultures of healthy and diabetic individuals in the current study.

In this study, the effect of BCM-7 on human health was assessed. However, the results are inconclusive. This is primarily due to the limited number of donors enrolled in this study. Nevertheless, an attempt to establish an experimental protocol was optimized for further researchers in relation to judge the effect of BCM-7 on human health.

1.3 Computational Approaches

Since the present study interested in allele distributions of three milk protein loci in breeds from Turkey, it can be regarded as an example of population genetics study. One of the purposes of population genetics is to unravel the patterns of genetic variation in populations and then to understand the forces that shape the genetic variation (Marjoram and Tavare 2006). Therefore, in a population genetics study firstly, genetic variation must be recorded. After the development of protein electrophoresis method and its application in population genetics (Lewontin and

Hubby, 1966) great amount of data started to be accumulated and to be used in population genetic analyses. Furthermore, new genetic variation assaying methods such as RFLP (e.g. Jeffreys, 1979), DNA sequencing (Kreitman 1983) and employment of microsatellites (Jorde *et al.*, 2000) contributed even more to the data accumulation. Meanwhile, computational power was developing and this power was becoming accessible to researchers through personal computers. Moreover, computer programs, softwares, to deal with huge data sets were started to be written (e.g. Luikart and England, 1999). All these developments prompted the emergence of new statistical methods such as a clustering method which identifies the best number of basic components in a group of individuals from many breeds (Prichart, 2000).

In the present study, molecular genetic variability in three milk protein loci will be determined by studying part of their DNA with the help of RFLP and SSCP. At one instance DNA sequencing to confirm the results of RFLP and SSCP will be done. Allele frequencies will be obtained (Arlequin ver 3.11 by Excoffier *et al.*, 2006). Allele frequencies will be subjected to various analysis (Test of Goodness of fit to Hardy-Weinberg Equilibrium by Arlequin ver 3.11 and Analysis of F-statistics by FSTAT ver 2.9.3.2), Principal Component Analysis by NTSYSpc21 by Numerical Taxonomy and Multivariate Analysis System (Rohlf, 1993). Wealth of new era of computational power will enable us to obtain computationally heavy but accurate test results of Fisher's exact test (Arlequin ver 3.11 by Excoffier *et al.*, 2006) which substitutes ordinary χ^2 test (Daniel, 1999) related with Hardy–Weinberg equilibrium. In this study, deviations from Hardy–Weinberg equilibrium will not only be used to detect the presence of evolutionary forces because its statistical power is not high enough (Allendorf and Luikart, 2007), but will be mainly used to test the presence of inbreeding and to judge the accuracy of genotyping (Allendorf and Luikart, 2007). F-statistics (Wright 1931) describing homozygote deficiencies within total individuals of the breeds (Fit) and within each breed (Fis) and describing between breed differentiations (Fst) will be calculated (Hartl and Clark, 1997). Yet, their statistical significance which is again computationally very

intensive will be carried out with the help of a software program (FSTAT ver 2.9.3.2 by Goudet 2002). Between breed differentiations will be visualized on principal component analysis (PCA) which is a multivariate analysis taking into account the information obtained from all of the employed genes. With PCA on synthetic independent axes (composed by combinations of milk protein loci alleles) positions of the breeds can be displayed without any distortion (e.g. Loftus *et al.*, 1999). Availability of the PCA by a software (NTSYSpc21 by Rohlf, 1993) is again "part of modern computational approaches for analyzing molecular genetic variation data".

Milk proteins are known to play roles in milk traits (see for example Table 1.2 and Table 1.3). Therefore, they must be under selection since the domestication by man was started. While they are under heavy selection they must also be under random genetic drift because each new breed at a new geographic region must have gone through a bottleneck. Neutral alleles such as those of microsatellite alelles must be under genetic drift, only. In relation to the understanding of forces that shape the genetic variation within and among the populations (here breeds), results obtained by using milk protein loci will be compared with those obtained by microsatellites from the same individual's of the present study, which were available in the literature. The difference in their positions on principal components will tentatively indicate the difference between the effects of forces of selection-drift and drift only.

In relation to the assessment of BCM-7 effect in activation of immune system by PBMC, again softwares this time involving univariate analyses namely: "Paired Comparisons t-Test" and "Mann-Whitney U Test" found in MINITAB (Minitab statistical Software Release13,0 program) will be used.

1.4 Objectives of the Study

Objectives of the present study are as follows:

1) To optimize the conditions of DNA-based methods for determining genetic variants of bovine beta-casein, kappa-casein and beta-lactoglobulin (BLG) milk protein genes in cattle breeds, in the Population Genetics Laboratory of the Department of Biological Sciences, METU.

2) To investigate the genetic diversity of milk proteins in four native Turkish cattle breeds (Turkish Grey, Eastern Anatolian Red, Anatolian Black and Southern Anatolian Red), a non-native breed (Turkish Holstein) and an independent subsample of the Holstein breed (Holstein Candidate Bulls).

3) To see if the alleles of beta-casein, kappa-casein and BLG genes can be related with milk trait qualities of Turkish native breeds.

4) To contribute to the understanding of the genetic differentiation among the investigated cattle breeds based on the comparison of neutral marker results from the literature and results on milk traits related genes from both the literature and the current study.

5) To develop an experimental protocol and assess the potential detrimental effects of BCM-7 on human peripheral blood cells.

6) To propose, if possible, some strategies to be applied in breeding practices of native Turkish breeds.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cattle Samples

There are four native Turkish cattle breeds; Turkish Grey, Eastern Anatolian Red, Southern Anatolian Red and Anatolian Black. Figure 2.1 presents sampling sites of cattle populations in Turkey studied in the current research. Turkish Grey is distributed in Thrace and in other cities of Marmara Region and among native breeds it is the breed that was heavily affected by crossbreeding with non-native cattle breeds Brown Swiss and Holstein. Milk yield of Turkish Grey is 1200-1900 kg per lactation and fat content in milk is 4%. Eastern Anatolian Red is present in Eastern and Northeastern Anatolia, and was crossbred with non-native breeds Brown Swiss and Simmental partially. Average milk yield of Eastern Anatolian Red breed is 1000 kg per lactation and fat content in milk is 4.4%. Anatolian Black is widely distributed in Central and Northern Anatolia, it has the largest population among the native cattle breeds and it was crossbred with non-native breeds Brown Swiss, Holstein and Jersey. Milk yield of Anatolian Black is 400-1200 kg per lactation and fat content in milk is 5%. Southern Anatolian Red is widely present in the south of Toros Mountains in Mediterranean Region and in Southern Anatolia Region, therefore it is also a native breed of Syria, Lebanon, Iraq, Jordan and Israel. Southern Anatolian Red is divided into two groups; one is named as Native Southern Yellow with 600-700 kg milk yield per lactation and the other one is Kilis Cattle (also called as Southern Anatolian Red) with 1500-2500 kg milk yield per lactation and fat content in milk is 4.2% (Özkan, 2005).

Non-native cattle breed Turkish Holstein is widely distributed in Western Anatolia and Marmara Region. Holstein breed is one of the breeds with highest milk yield in the world; 3000-6000 kg milk yield per lactation. Therefore, Holstein breed was first brought to Turkey from America in 1958. Extensive breeding of Holstein in Turkey was accelerated after 1970s and this was well accepted by the breeders, so Holstein population studied in the current study is named as Turkish Holstein. Crossbreeding of native breeds with Turkish Holstein breed was carried out, therefore, number of cross-bred breeds increased dramatically in the last 20 years (Özkan, 2005). In addition to Turkish Holstein, a subsample of Holstein breed namely Holstein Candidate Bulls were also studied in the present study. Sperms of Holstein Candidate Bulls were used for artificial insemination of native breeds by Cattle Breeders Association of Turkey.

The sample size of each screened cattle population is presented in Table 2.1. Blood samples of five cattle populations were mostly collected from female individuals except from the Holstein Candidate Bulls.

2.2 Methods

Total genomic DNA from the blood samples was isolated in accordance with the phenol-chloroform-isoamylalcohol method (Sambrook *et al.*, 1989). Totally 255 DNA samples from four native Turkish cattle breeds (Turkish Grey, Eastern Anatolian Red, Anatolian Black and Southern Anatolian Red), a non-native cattle breed (Turkish Holstein) and an independent subsample of Holstein breed; Holstein Candidate Bulls were screened for beta-casein, kappa-casein and beta-lactoglobulin milk protein gene variants. DNA samples except the Holstein Candidate Bulls, were the ones previously isolated and used by Özkan (2005).

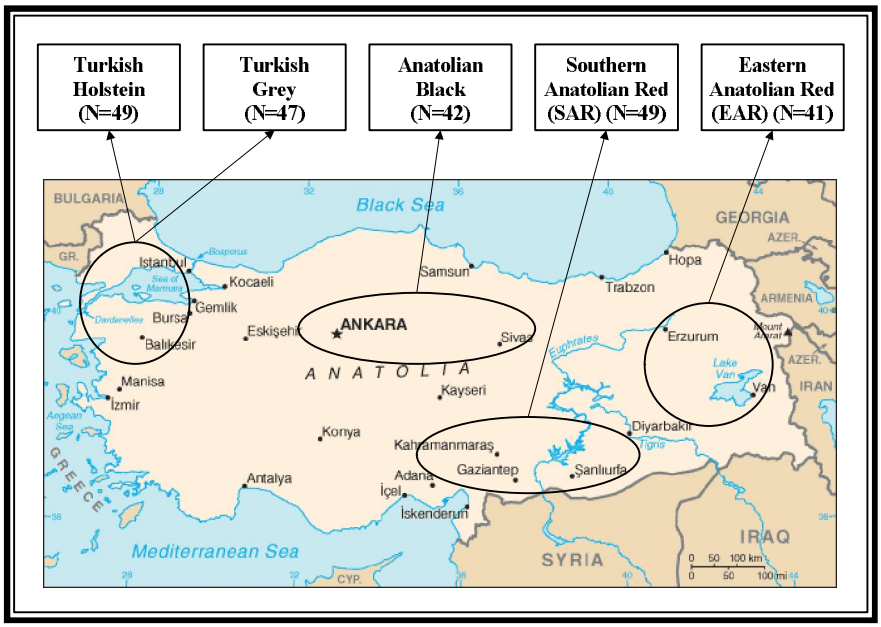


Figure 2.1. Map of Turkey with the locations of the studied populations and samples sizes (N) marked.

Table 2.1. Sample sizes of cattle populations used in the present study.

Cattle Populations	Sample Sizes (N)
Native Turkish Breeds	
Turkish Grey (TG)	47
Eastern Anatolian Red (EAR)	41
Anatolian Black (AB)	42
Southern Anatolian Red (SAR)	49
Non-native Populations	
Turkish Holstein (TH)	49
Holstein Candidate Bulls (HCB)	27

2.3 Determination of Bovine Beta-casein, Kappa-casein and Beta-lactoglobulin Gene Variants

2.3.1 Determination of Beta-casein Gene Variants

To determine beta-casein genotype of each individual, amplification created restriction sites (ACRS; Lien *et al.*, 1992) and single strand conformation polymorphism (SSCP; Barroso *ve ark.*, 1999a,b) methods were used simultaneously in this study. Both of these methods analyzed amplified part of exon VII of bovine beta-casein gene. Additionally, conformation of obtained results was performed by sequencing part of beta-casein gene (CASB) in selected individuals.

Amplification Created Restriction Sites (ACRS):

Polymorphism of beta-casein gene can be determined by amplification created restriction sites (ACRS) method which includes multiplex polymerase chain reaction (PCR) amplification as described in the study of Lien *et al.* (1992). The reason why ACRS is used instead of restriction fragment length polymorphisms (RFLP) is because there is no recognition site for any restriction enzyme at the polymorphic sites of different variants of beta-casein gene; therefore by ACRS method restriction site harboring the polymorphic site of the gene was created. Hence, different variants could be detected. Restriction sites (here three sites) are created by introduced mismatches in the primer sequences during beta-casein gene amplification. Thereby, introduced mismatches together with the existing variants might be restricted by different restriction enzymes. Upon restriction enzyme treatments, the sizes of the chopped DNA bands were examined by agarose gel electrophoresis and different alleles were observed.

In ACRS method, such primers with specified mismatches (underlined bases) at their 3' ends are used in the PCR amplification of beta-casein gene, CASB gene (Lien *et al.*, 1992):

CASB67 : 5'-CCTGCAGAATTCTAGTCTATCCCTTCCCTGGGCCCATCG-3'
CASB106: 5'-GTGAAGGAGGCTATGGCTCCTAAGTA-3'
CASB122: 5'-GAGTCGACTGCAGATTTTCAACATCAGTGAGAGTCAGGCCCTG-3'

There is a G-G mismatch at 3' end of CASB67 primer, there is a T-G mismatch at CASB106 primer and there is a C-A mismatch at CASB122 primer. These mismatches on primers depending on the following variable sites on the original sequence might have created recognition sites for the restriction enzymes *TaqI*, *RsaI* and *MvaI*, respectively. The restriction sites of the enzymes and underlined bases, which point the amplification created sites, were presented in Table 2.2.

Table 2.2. Restriction sites of *TaqI*, *RsaI* and *MvaI* enzymes and created bases.

Restriction Enzymes	Restriction Sites
TaqI	5'-T^C G A-3' 3'-A G C^T-5'
RsaI	5'-G T^A C-3' 3'-C A^T G-5'
MvaI	(T) 5'-C C^A G G-3' 3'-G G T^C C-5' (A)

Multiplex (for all three mentioned restriction sites) PCR amplification reaction volume was totally 50 µl and it contained 1X PCR buffer, 1.5 mM $MgCl_2$, 150 µM dNTP, 1.5 unit Taq DNA polymerase, 35 pmol CASB67 primer, 17 pmol CASB106 primer, 35 pmol CASB122 primer and 50-100 ng DNA. PCR reaction was performed by 40 cycles such that; 1 minute denaturation at 94°C, 1 minute annealing at 63°C and 1 minute extension at 72°C. Amplification products were visualized by 2% agarose gel electrophoresis and Ethidium Bromide (EtBr) staining. After the multiplex PCR amplification of the beta-casein gene by using three primers, 251 bp and 116 bp long DNA fragments of beta-casein (CASB) gene was observed as shown in Figure 2.2.

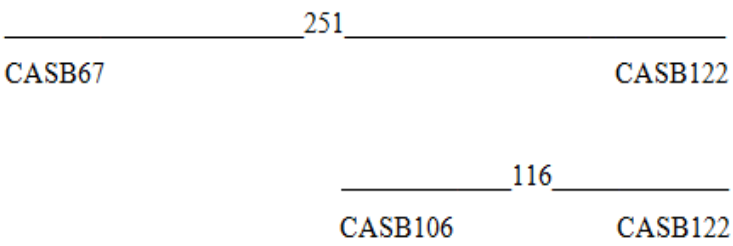


Figure 2.2. Two DNA fragments of beta-casein gene amplified by using three primers (CASB67, CASB106 and CASB122).

The amplified beta-casein gene fragments, which were presented in Figure 2.2, were then restricted with *TaqI*, *RsaI* and *MvaI* and genetic variants were determined depending on the DNA band sizes as presented in Table 2.3. Bands were observed by 4% agarose gel electrophoresis. *TaqI* enzyme restricts 251 bp long DNA fragment and this helps differentiation between A1 or A1-like alleles (A1 and B) and A2 or A2-like alleles (A2 and A3). *MvaI* enzyme also restricts 251 bp long DNA fragment and this enables identification of A1-like B allele. *RsaI* enzyme restricts 116 bp long DNA fragment and this restriction distinguishes A2-like A3 allele from other alleles.

Table 2.3. DNA band sizes (in base pairs) of beta-casein gene variants obtained after restriction with the following restriction enzymes according to Lien *et al.*, (1992).

Restriction Enzymes	Beta-casein Alleles			
	A1	**A2**	**A3**	**B**
TaqI	213+38 116	251 116	251 116	213+38 116
RsaI	251 161+90 90+26	251 161+90 90+26	251 161+90 116	251 161+90 90+26
MvaI	181+41+29 75+41	181+41+29 75+41	181+41+29 75+41	222+29 116

However, during the experiments, ACRS method enabled only the differentiation between A1-like beta-casein alleles (A1 and B) and A2-like beta-casein alleles (A2 and A3). While using ACRS method for further genotyping of beta-casein gene, especially in determining specific alleles (eg; B and A3), inconsistent enzyme restriction results were obtained. Amplification of beta-casein gene was also performed by amplifying 251 bp DNA fragment and 116 bp DNA fragment separately in order to overcome this inconsistency problem by eliminating any possible interference of simultaneously amplified two DNA fragments. Separately amplified beta-casein fragments were restricted with *RsaI, MvaI* and *TaqI* but again reliable determination of beta-casein alleles was not achieved. Restriction of beta-casein gene only by *TaqI* enzyme helped differentiation of individuals as having A1-like alleles and A2-like alleles. Therefore, individuals were genotyped for beta-casein gene as homozygote A1-like (A1A1-like), homozygote A2-like (A2A2-like) or heterozygote (A2A1-like) by using ACRS method. Additionally, single strand conformation polymorphisms (SSCP) method was used for further genotyping of beta-casein gene.

Single Strand Conformation Polymorphisms (SSCP):

Since ACRS method had limitations in determining exact beta-casein genotypes, in order to overcome this problem the genetic variants of beta-casein gene will also be determined by single strand conformation polymorphisms (SSCP) analysis. SSCP analysis is based on the assumption that subtle nucleic acid changes affect the migration of single-stranded DNA fragments and, therefore, result in visible mobility shifts across a nondenaturing polyacrylamide gel. Single base mutations can be detected because single base mutations may disrupt secondary structure of the single stranded DNA and leads to changes in mobility through the gel.

Samples were genotyped as A1-like beta-casein and A2-like beta-casein by using ACRS method and to determine exact beta-casein genotypes of individuals, SSCP analysis for detection of beta-casein variants was performed with minor modifications by using the studies of Barroso *et al.* (1999a,b). Firstly, amplification of beta-casein gene by polymerase chain reaction was done by using primers (Mf, Mr, Df, Bf and Br) selected from the study of Barroso *et al.* (1999a). The base sequences of mentioned primers are:

Mf : 5'-CCAGACACAGTCTCTAGTCTATCCC-3'
Mr : 5'-CAACATCAGTGAGAGTCAGGCTCTG-3'
Df : 5'-GATGAACTCCAGGATAAAATC-3'
Bf : 5'-CTAGTCTATCCCTTCCCTGG-3'
Br : 5'-AAGGTGCAGATTTTCAACAT-3'

The annealing positions of the primers used for SSCP analysis and the point mutations causing development of A1, A3 and B variants on Exon VII (from 5' to 3') of the bovine A2 beta-casein gene are presented in Figure 2.3 adapted from Barroso *et al.* (1999a).

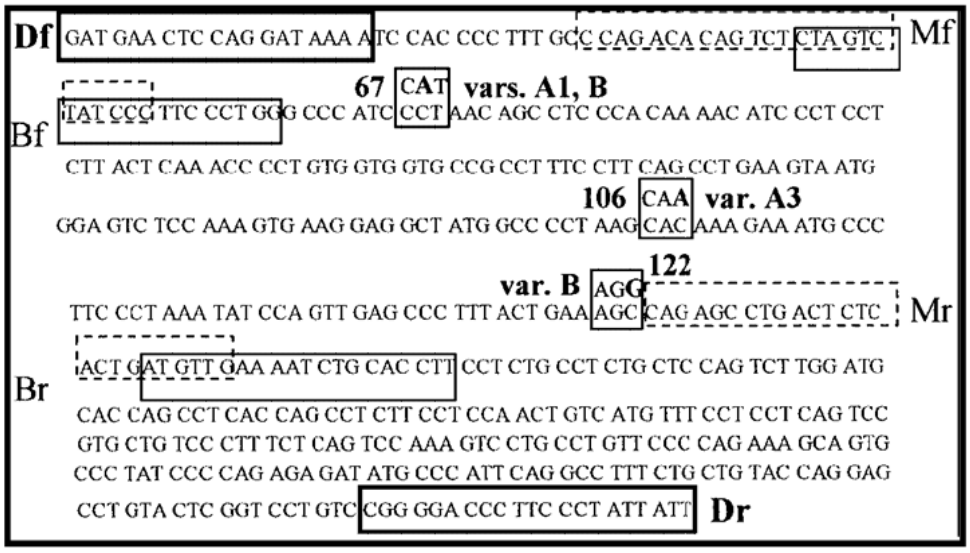


Figure 2.3. Nucleic acid sequence of Exon VII of the bovine A2 beta-casein gene. Target point mutations that differentiate alleles A1 (codon 67), A3 (codon 106) and B (codons 67 and 122) are indicated, as well as annealing sequences of the three primer pairs used; Df-Dr, Mf-Mr and Bf-Br (Figure adapted from Barroso *et al.*, 1999a).

PCR amplification reaction volume for SSCP method was totally 25 µl and it contained 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 1 unit Taq DNA polymerase, 400 nM primer and 50-100 ng DNA. PCR reaction was performed by 30 cycles such as; 30 seconds denaturation at 94°C, 45 seconds annealing at 54°C and 1 minute extension at 72°C. Firstly, individuals were differentiated by ACRS method (Lien *et al.*, 1992) as having A1-like beta-casein (A1 or B) and A2-like beta-casein (A2 or A3). Two individuals being homozygote A1A1-like beta-casein (possible genotypes: A1A1, A1B or BB), two individuals being heterozygote A2A1-like beta-casein (possible genotypes: A2A1, A3A1, A3B or A2B) and two individuals being homozygote A2A2-like beta-casein (possible genotypes: A2A2, A2A3 or A3A3) were chosen. In order to determine exact beta-casein genotypes of six mentioned individuals, three different primer pairs (Mf-Mr, Bf-Br and Df-Mr) from Barroso *et al.* (1999a) were used to amplify bovine beta-casein gene 7th exon fragments which are 233 bp, 234 bp and 265 bp long, respectively. Amplification

products were visualized by 2% agarose gel electrophoresis and ethidium bromide (EtBr) staining. After PCR amplification, 3 µl of PCR product was mixed with 10 µl denaturing loading buffer (0.05% xylene-cyanole, 0.05% bromophenol blue, 5.5 mM EDTA, pH 8.0, in deionized formamide) and the tube was heat denatured at 95 ºC for 5 minutes and snap chilled on ice.

In SSCP analysis, the content of the nondenaturing polyacrylamide gel and the gel running conditions affect the pattern of DNA bands formed, therefore, these are very important factors for the determination of genotypes. The nondenaturing polyacrylamide gel preparing and running conditions were used as the same as in the study of Barroso *et al.* (1999a) for the first trials. As a result of the DNA band patterns observed for pre-classified (as A1A1-like, A2A1-like and A2A2-like) individuals, it was concluded that using only Bf-Br primer pair was enough for beta-casein genotyping by SSCP analysis. Additionally, it was observed that purification of beta-casein PCR products with the help of a PCR product purification kit (GeneClean) before loading to the polyacrylamide gel supplied clearer DNA bands on polyacrylamide gels and therefore, more reliable genotyping. A lot of trials have been performed in order to optimize gel concentration, content and gel running conditions to be able to compare the DNA band patterns obtained in the current study with those of Barroso *et al.*, (1999a). The parameters modified to optimize SSCP method and selected example electrophoretograms of nondenaturing polyacrylamide gels obtained were presented in Appendix A.

After the optimization of the SSCP method in a way that it enabled determination of the desired beta-casein variants, the content of the nondenaturing polyacrylamide gel was as follows:

- 17% acrylamide concentration in polyacrylamide gel
- 99:1 ratio; acrylamide:bisacrylamide
- 0.5X Tris-Boric Acid-EDTA (TBE) Buffer Solution

- 1.0 µl/ml Tetramethylethylenediamine (TEMED)
- 4.5 µl/ml Ammonium persulfate (APS)

The polyacrylamide gel running conditions were optimized as run at 200 volts, 6 watts, with 40 milliamperes for almost 24 hours at 15 ºC. After the running of the gel, DNA bands were visualized by silver-staining method.

Sequencing of Bovine Beta-casein Gene:

After determining beta-casein genotypes of cattle individuals by using both ACRS and SSCP methods, the conformation of these results was performed by sequencing beta-casein gene of selected individuals. To search for the presence of new variants, especially the ones that had different band patterns in SSCP analysis, were sequenced.

PCR amplification reaction volume for bovine beta-casein sequencing was totally 50 µl and it contained 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 1 unit Taq DNA polymerase, 35 pmol from each of the following primers: CASB67 and CASBSEQ and 50-100 ng DNA. Nucleic acid sequences of the two primers used for the amplification of bovine beta-casein gene for sequencing as in Lien *et al.* (1992) are as follows:

CASB67 : 5'-CCTGCAGAATTCTAGTCTATCCCTTCCCTGGGCCCATCG-3'
CASBSEQ: 5'-TCTCTGGGGATAGGGCACTGCTTTCT-3'

Optimization of PCR reaction of beta-casein gene for sequencing was performed by several trials at three different annealing temperatures (64°C, 60.1°C and 54°C) and the result was visualized by 2% agarose gel electrophoresis and EtBr staining. Finally, PCR was performed totally by 40 cycles such as; 1 minute denaturation at 94°C, 1 minute annealing at 64°C and 1 minute extension at 72°C. After PCR amplification, amplified bovine beta-casein gene was purified by using PCR

product purification kit (GeneClean) and then purified PCR product was checked by 2% agarose gel electrophoresis with EtBr staining.

After purification of beta-casein gene amplification product, sequencing of beta-casein gene was performed by automatic DNA sequencing machine (ABI 310) present in a private gene research and biotechnology firm (called RefGen) at METU Technopolis.

2.3.2 Determination of Kappa-casein Gene Variants

Restriction Fragment Length Polymorphisms (RFLP):

The point mutations in exon IV of bovine kappa-casein gene determine three allelic variants A, B and E. These variants were distinguished by polymerase chain reaction (PCR) restriction fragment length polymorphisms (RFLP) method as presented by Soria *et al.*, (2003).

PCR amplification reaction volume for bovine kappa-casein gene was totally 50 µl and it contained 1X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP, 1 unit Taq DNA polymerase, 30 pmol from each primer and 50-100 ng DNA. Primers employed by Soria *et al.*, (2003) were used to amplify 935 bp long fragment of kappa-casein gene. Nucleic acid sequences of the primer pair used for the amplification of kappa-casein gene were as follows:

Kappa-casein Forward Primer : 5'-AGCGCTGTGAGAAAGATG-3'
Kappa-casein Reverse Primer : 5'-GTGCAACAACACTGGTAT-3'

PCR was performed totally with 32 cycles such as; 1 minute denaturation at 94°C, 1 minute annealing at 61°C and 1 minute extension at 72°C. Amplified bovine kappa-casein gene was checked by 2% agarose gel electrophoresis with EtBr staining.

After PCR amplification, 935 bp long kappa-casein gene was obtained and it was digested with *HindIII* and *HaeIII* restriction enzymes. Kappa-casein genotypes of individuals were determined by analyzing sizes of gathered kappa-casein gene fragments visualized by 4% agarose gel electrophoresis. Table 2.4 was from Soria *et al.*, (2003) and it presents the genotypes and their indicative sizes of expected kappa-casein gene fragments in base pairs after restriction with *HindIII* and *HaeIII* restriction enzymes.

2.3.3 Determination of Beta-lactoglobulin Gene Variants

Restriction Fragment Length Polymorphisms (RFLP):

The point mutations in exon IV of bovine beta-lactoglobulin (BLG) gene determine two allelic variants A and B. These variants were distinguished by polymerase chain reaction (PCR) restriction fragment length polymorphisms (RFLP) method as presented by Medrano and Aguilar-Cordova (1990).

Table 2.4. Fragment sizes corresponding to different kappa-casein genotypes after digestion of a 935 bp PCR product with two restriction enzymes (from Soria *et al.*, 2003).

Genotypes	Restriction Enzymes	
	HindIII	***HaeIII***
AA	935	641+294
AB	935+520+415	641+294
AE	935	641+496+294+145
BB	520+415	641+294
BE	935+520+415	641+496+294+145
EE	935	496+294+145

PCR amplification reaction volume for bovine BLG gene was totally 50 µl and it contained 1X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP, 1 unit Taq DNA polymerase, 30 pmol from each primer and 50-100 ng DNA. Primers employed by Medrano and Aguilar-Cordova (1990) were used to amplify 247 bp long fragment of BLG gene. Nucleic acid sequences of the primer pair used for the amplification of BLG gene were as follows:

BLG Forward Primer : 5'-TGTGCTGGACACCGACTACAAAAAG-3'
BLG Reverse Primer : 5'-GCTCCCGGTATATGACCACCCTCT-3'

PCR was performed totally with 32 cycles such as; 1 minute denaturation at 94°C, 1 minute annealing at 65°C and 1 minute extension at 72°C. Amplified bovine BLG gene was checked by 2% agarose gel electrophoresis with EtBr staining.

After PCR amplification, 247 bp long fragment of BLG gene was obtained and it was digested with *HaeIII* restriction enzyme. BLG genotypes of individuals were determined by analyzing sizes of gathered BLG gene fragments visualized by 4% agarose gel electrophoresis. BLG-A variant has 148 and 99 base pairs long fragments when digested with *HaeIII* where BLG-B variant has 74, 74 and 99 base pairs long fragments when digested with *HaeIII*. Table 2.5 summarizes the sizes of expected BLG gene fragments in base pairs after restriction with *HaeIII* restriction enzyme, for the specified BLG genotypes.

Table 2.5. Fragment sizes corresponding to different BLG genotypes after digestion of a 247 bp PCR product with *HaeIII* restriction enzyme.

Genotypes	Restriction Enzyme *HaeIII*
AA	148+99
AB	74+99+148
BB	74+99

2.4 Effect of Beta-casomorphin-7 (BCM-7) on Human Immune Response

Beta-casomorphin-7 (BCM-7) is a peptide of a string of seven amino acids released after the digestion of bovine milk containing A1 beta-casein by pepsin, pancreatic elastase and leucine amino peptidase. In this section of methods, as a preliminary study it was aimed to detect whether BCM-7 (with or without lipopolysaccharide (LPS) generates any immune response in humans based on activity changes of few interleukins (IL-1beta, IL-2, IL-4, IL-6, IL-10 and TNF-alpha). For this purpose the following procedure was carried out: (1) isolation of peripheral blood mononuclear cells (PBMC) from human whole blood, (2) stimulation of human PBMC by BCM-7, (3) Enzyme-linked immunosorbent assay (ELISA) for determination of cytokines and (4) Carboxyfluorescein diacetate succinimidyl ester (CFSE) assay steps in three experimental sets.

2.4.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC) from Human Whole Blood and Cell Count

Whole blood was collected in sterile EDTA (as anticoagulant) containing tubes at the METU Health Center from volunteer donors. Each donor was asked to sign a written consent form after being informed about the study (See Appendix B for details). Characteristics of individuals participated in the study were presented in

Table 2.6. In the second experimental set subject group was age-matched but not sex matched, however, in the third experimental set subject group was both age and sex-matched. Celiac disease is generally accepted as being an auto-immune disease where individuals react to a protein called gluten found in wheat, rye and barley. As a result of gluten digestion, a seven amino acid peptide called gliadorphin (Tyr-Pro-Gln-Pro-Gln-Pro-Phe**)** is released, and this peptide has amino acid sequence homology in four amino acid positions with BCM-7 (Tyr-Pro-Phe-Pro-Gly-Pro-IIe). Therefore, the effect of BCM-7 on immune response of celiac patients was also examined by limited number of samples.

Table 2.6. Characteristics of subjects involved in three experiment sets.

Experiment Set #	**Subjects**	**Coding**	**Volume of Whole Blood**
I	1 healthy individual (♀)	H	50 ml
II	2 healthy individuals (♀) + 2 type-1 diabetic patients (♂) + 2 celiac patients (♀)	H1, H2 + D1, D2 + C1, C2	10-15 ml
III	4 healthy individuals (2 ♀ and 2 ♂) + 6 type-1 diabetic patients (3 ♀ and 3 ♂)	H1-H4 + D1-D6	10-15 ml

PBMC, which involves T lymphocytes, B lymphocytes, monocytes, dendritic cells and natural killer cells, were separated from the whole blood by using Ficoll-Hypaque (Histopaque-1077) system which is a density gradient centrifugation technique. Whole blood was diluted 1:1 ratio with 1x phosphate buffered saline (PBS). Diluted blood was layered slowly onto the Ficoll (that had half volume of

blood) without mixing the two layers. Then, tubes were centrifuged at 400xg (1800 rpm) at room temperature (RT) for 30 minutes setting the break-off. After the centrifugation, 3 layers of blood cells were formed as illustrated in Figure 2.4.

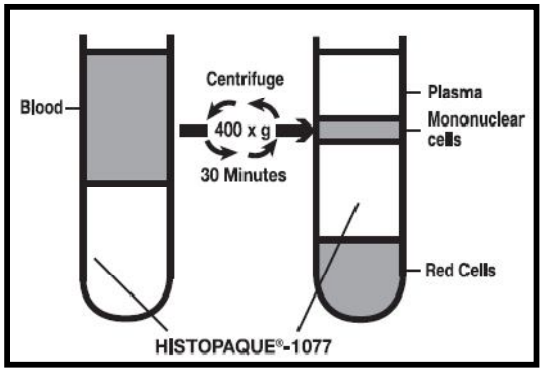


Figure 2.4. View of the layers formed after the centrifugation of histopaque and whole blood as a result of density gradient (from Sigma-Aldrich Histopaque-1077, Procedure No. 1077).

The cloudy layer was removed by a sterile Pasteur pipette and put into a new sterile falcon tube. Falcon tubes were then filled with 2% fetal bovine serum (FBS) supplemented regular 1x RPMI-1640 (Roswell Park Memorial Institute) medium without L-Glutamine and centrifuged for 10 minutes at 400xg (1800 rpm) at room temperature (RT) for washing. After the centrifugation, a cell pellet was formed and the supernatant was removed by aspiration. The pellet was resuspended in 15 ml, 2% regular RPMI-1640 medium and centrifuged for 10 minutes at 1800 rpm at RT. This washing process was repeated three times and the pellet was resuspended in 10 ml, 5% regular RPMI-1640 medium and isolated cells counted by using the hemocytometer. Cells that were suspended in 10 ml, 5% regular RPMI-1640 medium were diluted 10 fold and micropipetted into a hemocytometer as presented in Figure 2.5. Numbers of cells seen under the light microscope in the four chambers (A, B, C and D in Figure 2.5) were counted. Total number of cells

counted separately in the four chambers was divided into four and this obtained value was multiplied by 10^5 which gave the number of PBMC per milliliter (ml).

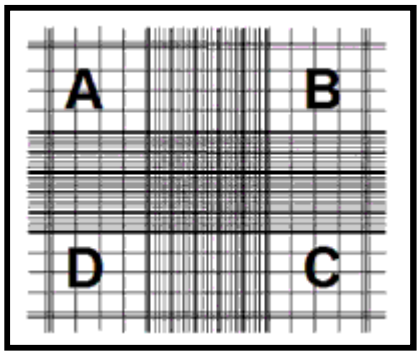


Figure 2.5. Illustration of a hemocytometer used for cell count; A, B, C and D indicated four chambers (from http://ink.primate.wisc.edu/~thomson/protocol.html, last visited on April 2009)

Number of isolated PBMC per ml from whole blood of each subject in three experimental sets is given in Table 2.7. Number of PBMC per ml was standardized to the same number of cells per ml in each experimental set by dilutions with 5% regular RPMI-1640 medium. Aliquots of PBMCs containing 1.6 millions of cells per 500 µl/well, 100,000 cells per 200 µl/well and 200,000 cells per 200 µl/well were used in cell cultures of experiment sets I, II and III, respectively.

Table 2.7. Number of PBMCs isolated from each subject.

Experiment Set #	Subjects	Number of PBMC/ml (x10^6)
I	H	11.000
II	H1	12.400
	H2	10.300
	D1	3.700
	D2	6.700
	C1	3.200
	C2	7.000
III	H1	2.900
	H2	3.250
	H3	2.250
	H4	2.725
	D1	3.050
	D2	1.475
	D3	3.900
	D4	2.375
	D5	3.300
	D6	1.200

2.4.2 Treatments of Human PBMC Cultures

PBMCs were stimulated with BCM-7 as the target peptide, with the scrambled peptide (SP) as the negative control, with lipopolysaccharide (LPS) and phytohemagglutinin (PHA) as the positive controls with concentrations indicated in Table 2.8. Amino acid sequences of BCM-7 and the scrambled peptide are given in Table 2.9.

Table 2.8. Stimulations applied to cell cultures of the three experiment sets (SP: scrambled peptide, BCM-7: beta-casomorphin-7, LPS: lipopolysaccharide, PHA: phytohemagglutinin; well volume=100 µl).

Experiment Set #	**I**	**II**	**III**
Subject	**H**	**H1, H2, D1, D2, C1, C2**	**H1-H4 D1-D6**
Stimulant			
Only RPMI medium	✓	✓	✓
SP (1 µg/ml)	✓		
SP (10 µg/ml)	✓		
SP (100 µg/ml)	✓	✓	
BCM-7 (1 µg/ml)	✓		
BCM-7 (10 µg/ml)	✓		
BCM-7 (100 µg/ml)	✓	✓	
PHA (0.12 µg/well)	✓		
PHA (0.48 µg/well)	✓		
PHA (1.92 µg/well)	✓		
LPS (0.1 µg/well)	✓	✓	✓
LPS (1 µg/well)	✓		
LPS (10 µg/well)	✓		
LPS+ SP (0.1 µg/well +100 µg/ml)		✓	
LPS+BCM-7 (0.1 µg/well +100 µg/ml)		✓	
LPS+SP (0.1 µg/well + 40 µg/ml)			✓
LPS+BCM-7 (0.1 µg/well + 40 µg/ml)			✓

Table 2.9. Amino acid sequences of BCM-7 and the scrambled peptide used for stimulation of PBMCs.

No. of Amino Acid	1	2	3	4	5	6	7
BCM-7	Tyr	Pro	Phe	Pro	Gly	Pro	IIe
Scrambled Peptide (SP)	Pro	Phe	IIe	Pro	Pro	Tyr	Gly

After culturing with 5% FBS supplemented regular RPMI-1640 medium with 100 units/ml penicillin-streptomycin and L-glutamine, and the peptide stimulations applied to PBMCs of each individual, the cells were incubated for 24 hours in 5% CO_2 incubator at 37 ºC. After 24 hours incubation, the cells were separated by centrifugation, the growth mediums, which contain cytokines produced by PBMCs, were collected in new 96-well plate and these supernatants were stored at -80 °C until needed.

2.4.3 Cytokine Enzyme-linked Immunosorbent Assay (ELISA)

Sandwich ELISA (Janeway *et al.*, 2005) was applied to detect concentrations of cytokines produced after the stimulations applied to PBMC in the first and third experimental sets. The principles and steps of sandwich ELISA were illustrated and explained in Figure 2.6.

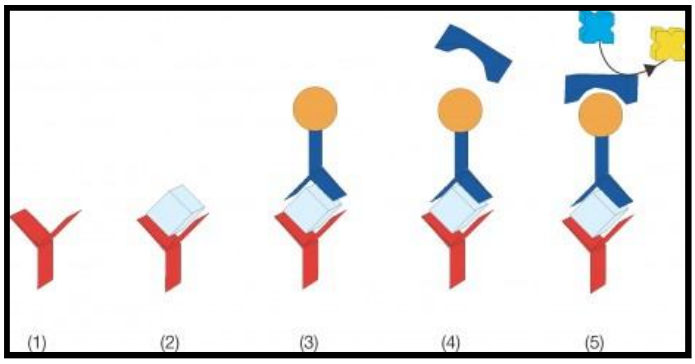


Figure 2.6. Stepwise representation of sandwich ELISA (From http://www.bendermedsystems.com/elisa--22, last visited on April 2009).

ELISA plate was designed that each stimulation for each individual were studied in duplicates or triplicates and ELISA plate was coated with human IL-6, IL-10 and/or TNF-alpha monoclonal antibody and incubated overnight at +4 °C (Figure 2.6; 1). Then, well surfaces of plates were blocked by adding 200 µl blocking buffer (bovine serum albumin) to each well and plates were incubated for 2 hours at room temperature. After blocking, wells of plates were washed five times lasting five minutes each with wash buffer (500 ml 10x PBS, 2.5 ml Tween20 and 4.5 l dH_2O), were rinsed with ddH_2O and were dried. Supernatants of cell cultures and serially diluted recombinant proteins of human IL-6 (1000 ng/ml) and human IL-10 (250 ng/ml) were added and incubated for 2 hours at room temperature or overnight at +4°C (Figure 2.6; 2). Plates were washed as previously described. For the detection of cytokine levels; biotinylated IL-6, IL-10 and/or TNF-alpha secondary detection antibodies were prepared in a T-cell buffer, with 1:1000 dilution, 50 µl added to the wells of plates and incubated for 2 hours at room temperature or overnight at +4°C (secondary detection antibody was bound to the antigen captured by the first antibody), followed by washing (Figure 2.6; 3). Streptavidin-alkaline phosphatese (SA-AKP) enzyme was prepared in T-cell buffer with 1:5000 dilution, 50 µl was added to the wells of plates and plates were incubated for 1 hour at room temperature or overnight at +4°C. SA-AKP was bound to the biotin conjugated detection antibody (Figure 2.6; 4). After washing

the plates; substrate para-nitrophenyl phosphate (pNPP), disodium salt was prepared and 50 µl was added to each well (Figure 2.6; 5). Coloured product was formed in proportion to the amount of antigen present in the sample. After the colour formation, in different intervals optical densities (ODs) were measured on an ELISA plate reader (BioTek, µQuant) at 405 nm. The reading was terminated for each plate when an S shaped recombinant cytokine standard curve was obtained. Concentrations of the cytokines present in the supernatants, which were produced by PBMCs, were determined by the 4-parameter standard curves generated by using recombinant proteins as mentioned above.

Sandwich ELISA was applied in the first and third experimental sets. In the first experimental set, concentrations of IL-6, IL-10 and TNF-alpha produced by PBMC were determined where in the third experimental set only IL-10 concentrations were detected.

Additionally, in the second experimental set, ELISA was performed by using Millipore's MILLIPLEX™ Human Cytokine kit which had fluorescent-coded beads known as microspheres at the bottom surface of each well of the plate. These microspheres were coated with six different specific capture antibodies such as; human IL-1beta, IL-2, IL-4, IL-6, IL-10 and TNF-alpha antibodies, hence production of these listed cytokines by PBMCs was examined by the kit.

The steps of multiplex ELISA were similar to the ELISA procedure mentioned above. Each stimulation of each individual was studied in duplicates. After the antigens in supernatants were captured by the beads, a biotinylated detection antibody was introduced. The reaction mixture was then incubated with Streptavidin-PE (phycoerythrin) conjugate, the reporter molecule, to complete the reaction on the surface of the each microsphere. The microspheres were allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excited PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors indentified each individual

microsphere and quantified the results of its bioassay based on fluorescent reporter signals. Signals were observed by using Luminex 100™ IS in Faculty of Medicine of Hacettepe University.

2.4.4 Carboxyfluorescein diacetate succinimidyl ester (CFSE) Assay

A fluorescent dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) experiment was performed to detect any T and B cell proliferation after the stimulations (SP, BCM-7 and PHA) applied to PBMCs by flow cytometry. The stable incorporation of CFSE into lymphocytes is a powerful tool to monitor lymphocyte migration *in vivo* and to quantify cell division both *in vivo* and *in vitro* (Parish and Warren, 2001).

Subjects (H1, H2, D1, D2, C1 and C2) that were examined by ELISA in the second experiment set were analyzed for any T and B cell proliferation by CFSE assay. Ten ml of dimethyl sulfoxide (DMSO) was added to 25 mg CFSE (5mM stock) and then CFSE working stock solution was prepared by adding 4 µl of CFSE into 20 ml PBS. Three million PBMCs from each individual were resuspended in 3 ml of warm PBS and then 3 ml of CFSE was added to each tube having PBMCs of each individual. Tubes were mixed and incubated at 37 °C for 10 minutes. Later, cold RPMI medium was added to the tubes to stop CFSE incorporation. They were incubated on ice for 5 minutes, were washed and resuspended in 1x 3 ml of warm RPMI medium. The CFSE labelled PBMCs of each individual were then stimulated with peptides. There were four stimulations for each individual:

i) Only medium, no stimulation
ii) Scrambled Peptide (SP; 100µg) stimulation
iii) Beta-casomorphin-7 (BCM-7; 100µg) stimulation
iv) Phytohemagglutinin (PHA; 10µg) stimulation; as a positive control. PHA is a lectin with mitogenic effect and promotes both T and B cell proliferation.

Stimulated PBMCs were cultured by incubating for 6 days in 5% CO_2 incubator at 37 ºC. CFSE assay proliferation results were studied by flow cytometer.

2.5 Statistical Analysis of Data

2.5.1 Within Group Variation

2.5.1.1 Calculation of Allele Frequencies, Heterozygosities, Polymorphic Information Contents and Evaluation of Hardy-Weinberg Equilibrium

Relative frequencies of the alleles of each locus in all of the five studied populations were calculated. Allele frequencies of the loci were calculated with the formula given below;

$$\text{Frequency of an allele (p)} = \frac{(2 \times \text{Homozygote \#}) + (\text{Heterozygote \#})}{2 \times \text{total number of individuals (N)}}$$

Heterozygosity which is one of the measures of the genetic variation in a population was calculated with the following formula;

$$\text{Observed Heterozygosity} = \frac{\text{Heterozygote \#}}{\text{Total number of individuals}}$$

Calculation of the allele frequencies of the alleles of each locus and the genotype frequencies in all of the five cattle breeds and the observed heterozygosities were

performed with the help of "Arlequin ver 3.11" package program (Excoffier *et al.*, 2006).

Polymorphic information content (PIC) values measure informativeness of a locus for detecting polymorphism within a population and for assessing the relevance of loci for linkage analysis. PIC values were calculated with the following formula;

$$PIC_i = 1-\sum_{j=1}^{n} Pij^2;$$

where PIC_i is the polymorphic information content of a marker i; Pij is the frequency of the jth allele for marker i and the summation extends over n alleles.

Testing Hardy-Weinberg (H-W) equilibrium in six cattle populations was performed by calculating the H-W expected genotype frequencies and comparing them with the observed ones. According to H-W equilibrium, the expected genotype frequencies were calculated as follows;

Expected frequency of homozygotes = p^2 and q^2

Expected frequency of heterozygotes = 2pq, where p + q = 1

The Hardy-Weinberg equilibrium was assessed by the Fisher's exact test to calculate the P value using the Markov-Chain Monte Carlo method (Guo and Thompson, 1992) provided by the Arlequin ver 3.11 package program (Excoffier *et al.*, 2006). The exact test of Hardy-Weinberg equilibrium was applied locus by locus, performing separate H-W equilibrium test for each locus.

2.5.1.2 Composite Genotypes

Beta-casein, kappa-casein, BLG composite genotypes was generated by combining all genotypes determined for individuals of three genetically similar native breeds; Eastern Anatolian Red, Southern Anatolian Red and Anatolian Black. The observed composite genotype frequencies were compared with the expected ones calculated under the assumption of independence of the loci. The differences of the frequency of observed composite genotypes and the frequency of expected ones in each cattle breed were calculated and any possible association among the studied loci were searched.

2.5.2 Among Group Variation

Fst Analysis

Fst is a measure of degree of genetic differentiation between subpopulations (here samples of breeds) and it can be estimated by the following formula:

Fst = (HT - HS) / HT (Nei and Kumar, 2000)

where; HT = average heterozygosity of the total population

HS = average expected heterozygosity in the subpopulations

Weir and Cockerham (1984)'s approach was used in the calculation of Fst related genetic distances and it was computed between two populations with the help of the "Arlequin ver 3.11" package program (Excoffier *et al.*, 2006). The Fst values for pairwise comparisons of cattle breeds included in this study were calculated. The data were permutated for 1000 times in order to test the significance of the pair-wise F_{ST} values. Fst dependent number of effective migrants per generation (N_em) was calculated with the following formula (Allendorf and Luikart, 2007):

$$N_em \text{ value} = 1/4\ [(1/Fst)-1)]$$

2.5.3 Neighbour Joining (NJ) Tree

Nei's (1978) D_A genetic distances, between the pairs of cattle breeds were calculated with the GENDIST program in the PHYLIP program package (Felsenstein, 1993). Neighbor-joining trees were produced by using the NEIGHBOR program, 1000 bootstrap replicates were generated by the SEQBOOT program and a consensus tree was built with the CONSENSE program as implemented in PHYLIP 3.6 (Felsenstein, 1993).

2.5.4 Principal Component Analysis (PCA)

To visualize genetic relationships between the samples of the breeds and to determine the relative positions of populations in 3 dimensional space, the principal component analysis (PCA) was performed with the help of a software program NTSYS; Numerical Taxonomy and Multivariate Analysis System (Rohlf, 1993).

In PCA from a mass of variables a set of independent compound axes are synthesized and relative positions of the populations are visualized in the space generated by these axes. In this method, the first axis will explain the highest variation of the all data that can be accounted by the compound axes; the second will explain the next highest variation and so on. Inspection of the weightings of the first few axes will show the contribution of variables to explain the variation between populations (Dytham, 2003). As a result, the relative importance of the variables for the discrimination of the populations depending on their milk traits was also studied with the means of the PCA.

2.5.5 Statistical Analysis of ELISA Results

Significance of differences of cytokine secretions (measured by ELISA analysis) in healthy individuals, diabetic and celiac patients after the LPS only, LPS and BCM-7 together and LPS and SP together stimulations in the second experimental set were performed by paired comparisons t-test by using MINITAB software. The paired comparisons (two treatments on the same individual) t-test for null hypothesis of equality, greater than and less than were tested. Moreover, means of concentrations (ng/ml) of IL-1beta, IL-6, IL-10 and TNF-alpha secreted after LPS stimulations in two healthy and two type 1 diabetic individuals were also compared.

In the third experimental set, paired comparisons t-test by MINITAB was also applied to test significance of differences of IL-10 secretions in PBMC cultures of healthy individuals and diabetic patients after the LPS only, LPS-BCM-7 together and LPS-SP together stimulations. The paired comparisons t-test under three different null hypotheses (means are equal, 1^{st} mean is greater than 2^{nd} and 1^{st} mean is less than 2^{nd}) were tested. Means of concentrations (ng/ml) of IL-10 secreted after LPS, LPS+BCM-7 and LPS+SP stimulations in four healthy and six type 1 diabetic individuals in the third experiment set were also compared.

Furthermore, Mann-Whitney U test, which is a non-parametric test, was used to test the significance of these individual based differences of individuals belonging to the two groups (healthy individuals and type-1 diabetic patients) by MINITAB software.

CHAPTER 3

RESULTS

3.1 Optimization of DNA-based Methods to Determine Bovine Beta-casein, Kappa-casein and Beta-lactoglobulin Gene Variants

3.1.1 Determination of Beta-casein Gene Variants

3.1.1.1 Results Obtained by Amplification Created Restriction Sites (ACRS) Method

Beta-casein gene amplification result of three individuals (No. 17, 28 and 58) from Eastern Anatolian Red breed is presented in Figure 3.1 as a representative of bovine beta-casein gene PCR product.

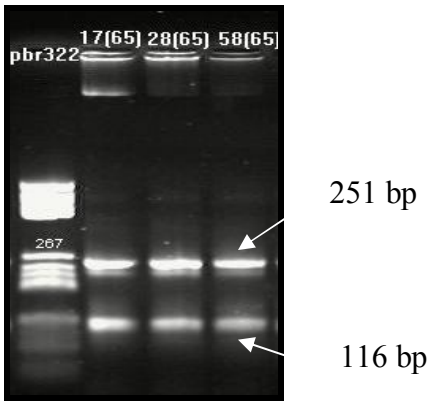


Figure 3.1. Amplification results of bovine beta-casein gene (251 bp and 116 bp) of three individuals on 2% agarose gel. Left lane displays the pBR322 DNA/HaeIII size marker. 17 (65), 28 (65), 58 (65) are the codes for samples.

Amplified beta-casein gene fragments were restricted by using *TaqI*, *MvaI* and *RsaI* restriction enzymes simultaneously in order to detect point mutations. The result of 4% agarose gel electrophoresis after the restriction of beta-casein gene fragments is presented in Figure 3.2. Sizes of restricted beta-casein DNA fragments were determined by using pBR322 DNA/HaeIII size marker. Therefore, theoretically genotype of an individual was going to be determined depending on the sizes of the DNA fragments as explained in Table 2.3. However practically, restriction of two beta-casein gene fragments (251 bp and 116 bp long) in the same reaction mixture led to conflicts in determining the genotypes of individuals. Additionally, low resolution of very small DNA fragments (41 bp and 26 bp), as can also be seen in the electrophoretogram in Figure 3.2, made it more difficult to determine genetic variants. Therefore, only ACRS method was used to differentiate samples as carrying "A2-like beta-casein" and "A1-like beta-casein". Figure 3.3 presents an example of 4% agarose gel electrophoresis image of beta-casein gene restricted with only *TaqI* enzyme.

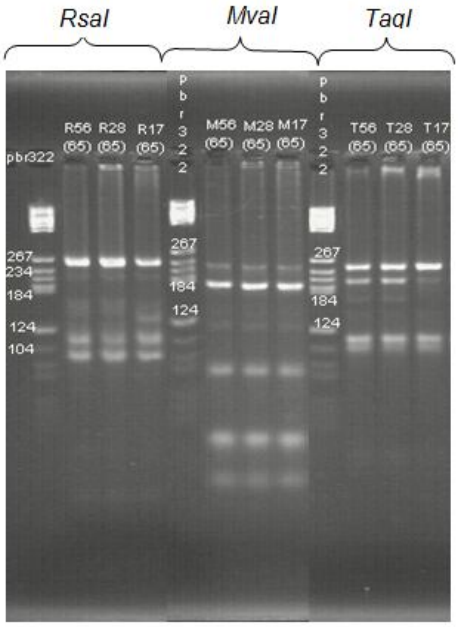


Figure 3.2. Agarose gel electrophoresis (4%) of beta-casein gene after enzyme (*RsaI, MvaI* and *TaqI*) digestions. Genotypes of the samples are determined as follows; the sample 17: A2A2 like beta-casein and samples 28 and 56: A2A1 like beta-casein. "pBR322" represents pBR322 DNA/HaeIII size marker.

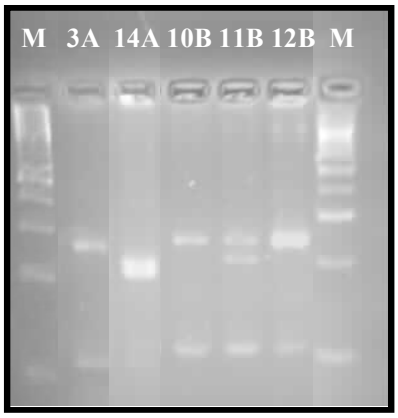


Figure 3.3. Electrophoretogram of beta-casein gene after restricted with *TaqI* restriction enzyme. "M" represents 100 bp DNA size marker, 3A, 14A, 10B, 11B and 12B indicate different samples. Genotypes of the samples on this gel are determined as follows: the sample 3A: A2A2-like, 14A: A1A1-like, 10B: A2A2-like, 11B: A2A1-like and 12B: A2A2-like beta-casein.

3.1.1.2 Results Obtained by Single-Strand Conformation Polymorphisms (SSCP) Method

SSCP analysis was applied to have a higher resolution and confidence in determining the beta-casein gene variants. In Figure 3.4, beta-casein gene amplification results of six individuals (1a, 1b, 2a, 2b, 3a, 3b) with Mf-Mr, Bf-Br and Df-Mr primer pairs (Barroso *et al.*, 1999a) is presented as 233 base pairs, 265 base pairs and 234 base pairs long DNA bands, respectively.

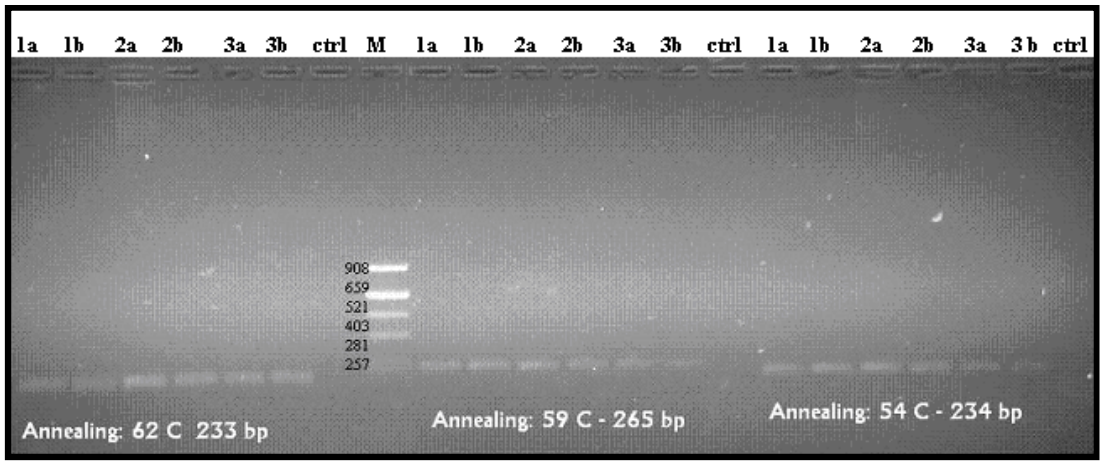


Figure 3.4. Electrophoretogram of beta-casein gene segments of six individuals (1a, 1b, 2a, 2b, 3a, 3b). Control (ctrl) and the pBR322 DNA/AluI size marker (M) lanes are displayed on the agarose gel.

After the amplification of desired beta-casein DNA fragments, SSCP analysis was applied to these six individuals (1a, 1b, 2a, 2b, 3a, 3b) and result of 17% polyacrylamide nondenaturing gel containing 5% glycerol is presented in Figure 3.5. It was emphasized by Barroso *et al.*, (1999a) that using only 234 bp long beta-casein gene fragment would be enough to genotype beta-casein gene. This was also tested in the present study and it was observed that analysis of 234 bp long beta-casein fragment enabled detection of beta-casein B and A3 gene variants on SSCP polyacrylamide gel, as shown in Figure 3.6 and Figure 3.7, respectively. Five samples (3A, 14A, 10B, 11B and 12B) in Figure 3.6 were genotyped by ACRS method as "A2 and/or A1 like" in parallel to what was observed in Figure 3.3. Further beta-casein genotyping was performed by analyzing DNA bands as visualized in Figure 3.6. Based on these bands, exact beta-casein genotypes of these samples were determined as follows: 3A: A2A2, 14A: A1A1, 10B: A2A2, 11B: A2B and 12B: A2A2.

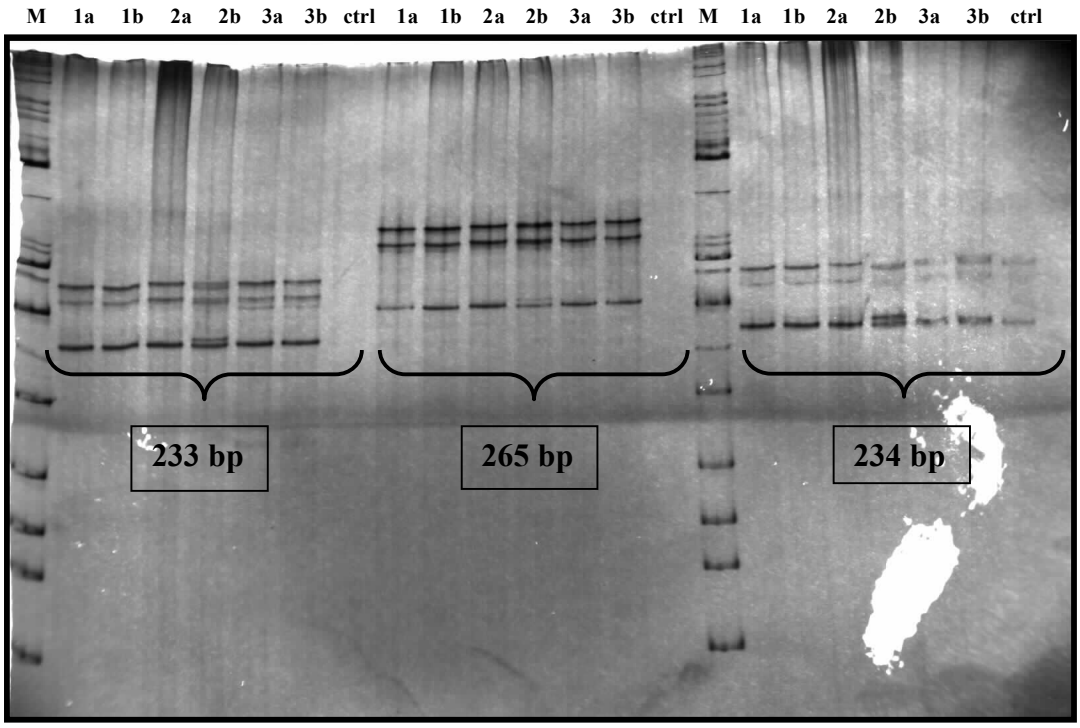
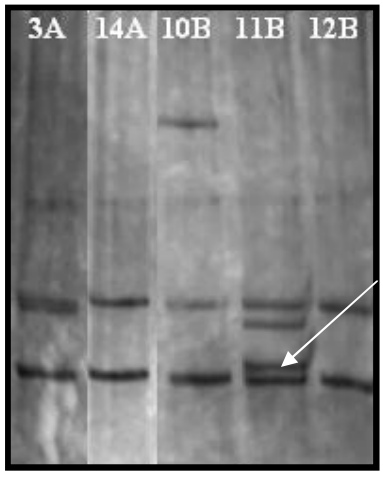
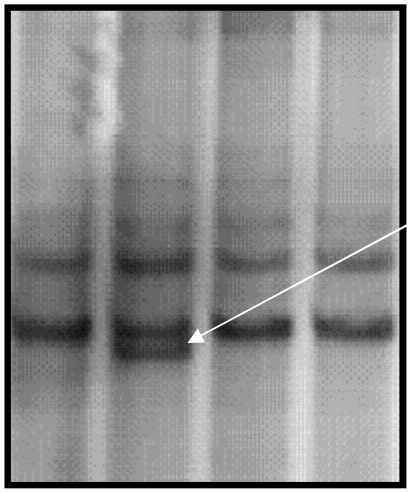


Figure 3.5. PCR-SSCP of beta-casein gene on 17% polyacrylamide nondenaturing gel containing 5% glycerol. Marker (M) and control (ctrl) lanes are displayed on the image.



Defined as allele "B" by Barroso *et al.* (1999a)

Figure 3.6. Polyacrylamide gel (17%, 99:1 polyacrylamide:bisacrylamide, without glycerol) by SSCP method.



Defined as allele "A3" by Barroso *et al.* (1999a)

Figure 3.7. Polyacrylamide gel (17%, 99:1 polyacrylamide:bisacrylamide, without glycerol) by SSCP method.

3.1.1.3 Results Obtained by Sequencing of Beta-casein Gene

Joint use of ACRS and SSCP methods enabled us to genotype the beta-casein gene. Yet, to confirm the results beta-casein gene sequencing was performed in selected samples. Sequencing, also helped to identify further differences between the individuals, hence new alleles within the region of the sequence. Beta-casein sequencing was performed by using primers given by Lien *et al.*, (1992) and PCR amplification optimization efforts (with and without BSA, 64 °C, 60.1 °C and 54 °C annealing temperatures) were conducted on 2% agarose gel electrophoresis that was presented in Figure 3.8. Figure 3.9 shows 2% agarose gel electrohporesis of the purified beta-casein gene amplifications.

After sequencing by ABI automatic sequencing machine, peaks were obtained in 4 different colors representing 4 bases (A, G, C, T). Sequencing results of two samples (14A and 11B) were presented in Figure 3.10 where polymorphic sites (codon 67, codon 106 and codon 122) of beta-casein gene were colored in light blue and peaks of a heterozygote were pointed by arrows. In Figure 3.10.a sample 14A was genotyped as A1A1 where in Figure 3.10.b sample 11B was genotyped as

A2B. The presence of both cytosine and adenine at codon 67 and the presence of both cytosine and guanine at codon 122 of sample 11B revealed that this individual is a heterozygote individual for beta-casein gene.

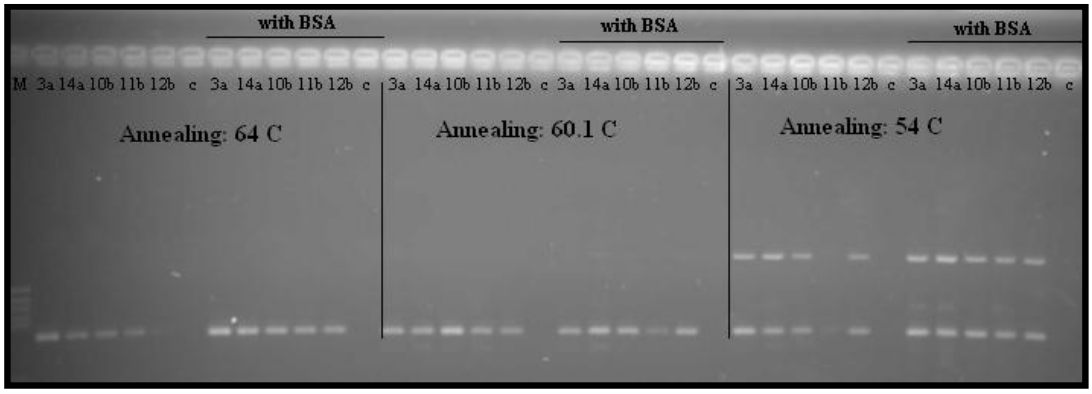


Figure 3.8. Electrophoretogram of amplification results of beta-casein gene with different optimizations with or without bovine serum albumin (BSA).

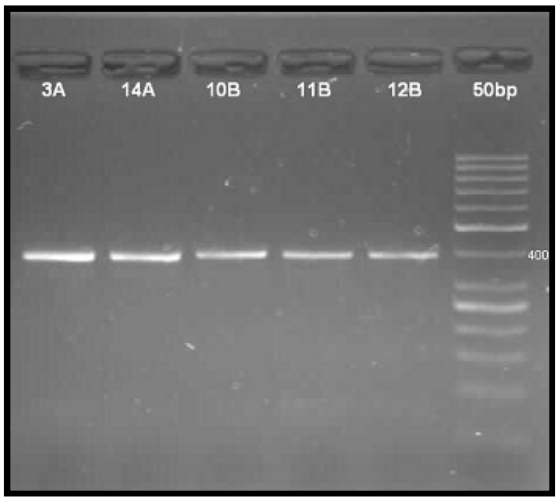
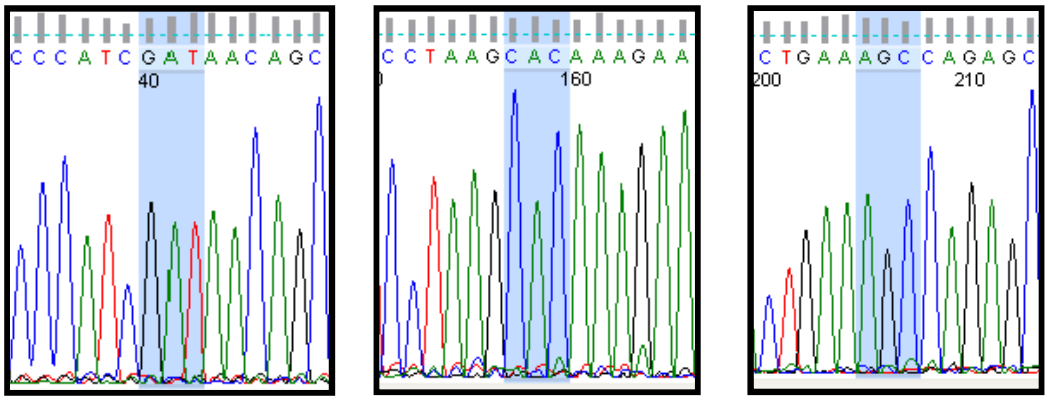


Figure 3.9. Electrophoretogram of purified beta-casein gene amplification. 3A, 14A, 10B, 11B, 12B are codes for samples and 50 bp is DNA size marker.

a) 14A

67th codon: **106th codon:** **122nd codon:**



b) 11B

67th codon: **106th codon:** **122nd codon:**

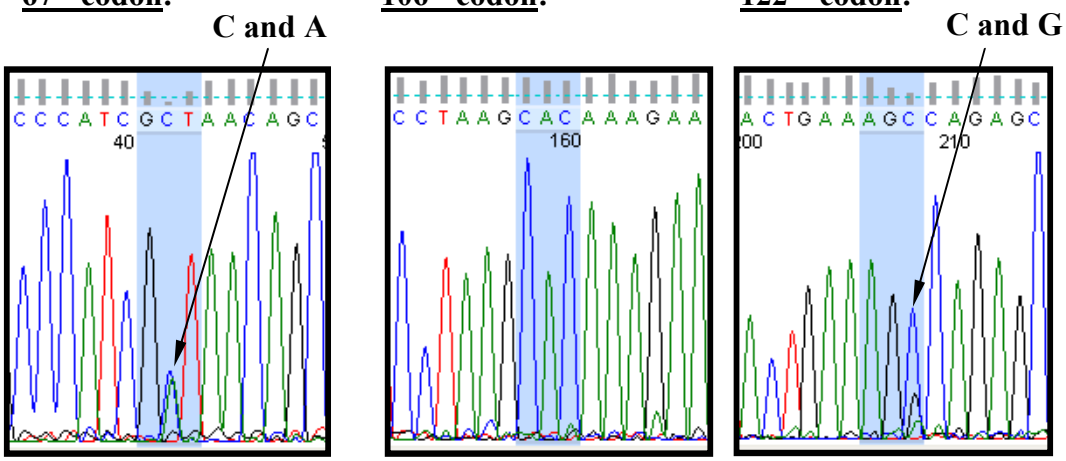


Figure 3.10. Sequencing results of 14A **(a)** and 11B (**b)** individuals for beta-casein gene; genotypes were determined as A1A1 and A2B, respectively.

Determination of beta-casein genotypes by analyzing point mutations at codons 67, 106 and 122 of chosen sequenced samples (n=11) was presented in Table 3.1. For the comparative purpose, beta-casein dependent genotype results obtained by sequencing, ACRS and SSCP methods, and overall results were also presented in Table 3.1.

Table 3.1. Genotypes of 11 samples based on sequencing, ACRS and SSCP methods. Letters (A1, A2, A3 and B) indicate alternative genotypes of the studied individuals.

Sequenced Sample	67th Codon [A1&B]	106th Codon [A3]	122nd Codon [B]	ACRS Result	SSCP Result	Overall Result
1A	GCT/GAT Het† A1, A2, A3, B	CAC A1, A2, B	AGC/AGG Het A1, A2, A3, B	A2A1-like	+B	**A2B**
2A	GCT/GAT Het A1, A2, A3, B	CAC A1, A2, B	AGC/AGG Het A1, A2, A3, B	A2A1-like	+B	**A2B**
3A	GCT A2, A3	CAC A1, A2, B	AGC A1, A2, A3	A2A2-like	-	**A2A2**
8A	GCT A2, A3	CAC A1, A2, B	AGC A1, A2, A3	A2A2-like	-	**A2A2**
10A	GCT A2, A3	CAC A1, A2, B	AGC A1, A2, A3	A2A2-like	-	**A2A2**
11A	GCT/GAT Het A1, A2, A3, B	CAC A1, A2, B	AGC A1, A2, A3	A2A1-like	-	**A2A1**
12A	GCT/GAT Het A1, A2, A3, B	CAC A1, A2, B	AGC A1, A2, A3	A2A1-like	-	**A2A1**
14A	GAT A1, B	CAC A1, A2, B	AGC A1, A2, A3	A1A1-like	-	**A1A1**
10B	GCT A2, A3	CAC A1, A2, B	AGC A1, A2, A3	A2A2-like	-	**A2A2**
11B	GCT/GAT Het A1, A2, A3, B	CAC A1, A2, B	AGC/AGG Het A1, A2, A3, B	A2A1-like	+B	**A2B**
12B	GCT A2, A3	CAC A1, A2, B	AGC A1, A2, A3	A2A2-like	-	**A2A2**

†Het: Heterozygote sample

Instances (at samples 1A, 2A and 11B) when ACRS and SSCP methods both provide information for the beta-casein genotype determination of the individuals were presented in Table 3.1. For example for the first individual (sample 1A) unless ACRS is carried out A2 allele could not be detected, similarly B allele could be detected by the SSCP method. Therefore, both methods (ACRS and SSCP) must be applied together when there is not any chance for sequencing. Furthermore, results of both methods were in conformity (as also checked by sequencing results), hence true genotypes were determined more reliably than the one that would be obtained by any method alone.

3.1.2 Determination of Kappa-casein Gene Variants by Restriction Fragment Length Polymorphisms (RFLP) Method

Kappa-casein gene amplification and digestion with restriction enzymes (*HindIII* and *HaeIII*) results are presented in electrophoretograms in Figure 3.11 and Figure 3.12, respectively.

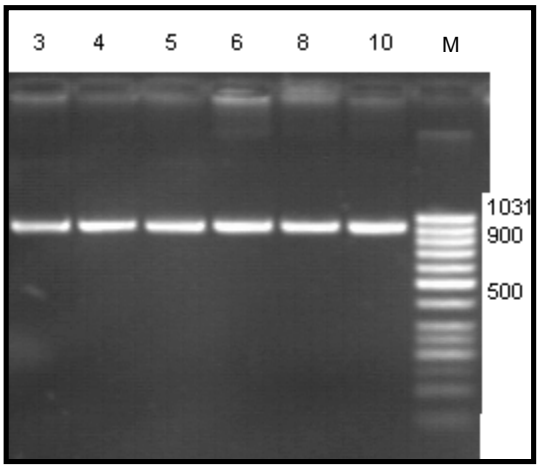


Figure 3.11. Amplification results of kappa-casein gene (935 bp) on 2% agarose gel. "M" represents Mass Ruler^{TM} DNA ladder, low range size marker.

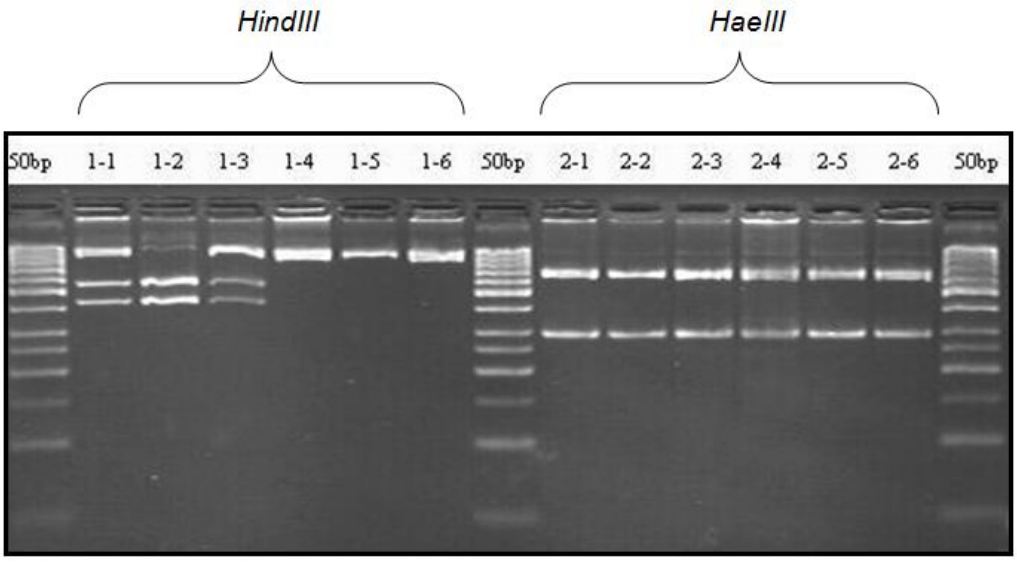


Figure 3.12. DNA fragments of amplified kappa-casein gene after enzyme (*HindIII* and *HaeIII*) digestions on 4% agarose gel. Three "50bp" labelled lanes indicate 50 bp DNA size markers.

Electrophoretogram in Figure 3.12 shows that samples coded with numbers 1-1 and 1-3 have AB; 1-2 has BB and 1-4, 1-5 and 1-6 have AA genotypes for kappa-casein gene.

3.1.3 Determination of Beta-lactoglobulin Gene Variants by Restriction Fragment Length Polymorphisms (RFLP) Method

Beta-lactoglobulin gene amplification and digestion with *HaeIII* enzyme results are presented in electrophoretograms in Figures 3.13 and Figure 3.14.

Figure 3.13. Amplification results of beta-lactoglobulin gene (247 bp) on 2% agarose gel. "pbr" represents pBR322 DNA/HaeIII size marker.

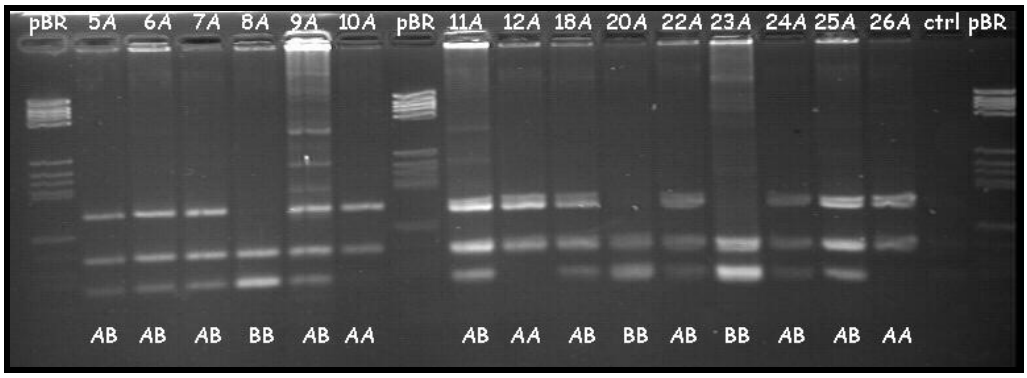


Figure 3.14. Electrophoretogram of *HaeIII* digested PCR product generated by amplification of genomic DNA using beta-lactoglobulin specific primers. Genotype of each sample is indicated on the 4% agarose gel as AA, AB and BB. pBR322 DNA/HaeIII size marker and negative control (ctrl) lanes are also shown on the electrophoretogram.

3.2 Statistical Analysis

3.2.1 Within Group Variations

3.2.1.1 Genotype and Allele Frequencies

Two hundred and fifty five (255) animals from four native Turkish cattle breeds (Turkish Grey, Eastern Anatolian Red, Anatolian Black, and Southern Anatolian Red), a non-native breed (Turkish Holstein) and independent subsample of Holstein breed: Holstein Candidate Bulls were genotyped for beta-casein, kappa-casein and beta-lactoglobulin (BLG) genes, genotype and allele frequencies were estimated and presented in Table 3.2, Table 3.3 and Table 3.4, respectively. Genotypes of each individual for each loci (beta-casein, kappa-casein and BLG) were presented in Appendix C.

The highest allele frequency for A1-like (A1 allele and B allele) beta-casein (0.546), which is presumably associated with adverse health effects in humans, was observed in non-native cattle population: Turkish Holstein. Among the native Turkish cattle populations, the highest A1-like beta-casein frequency (0.455) was observed in Turkish Grey which is the geographically closest population to Europe. The lowest A1-like beta-casein frequency (0.120) was observed in Eastern Anatolian Red native Turkish cattle population, which is located in Eastern and Northeastern Anatolia (Table 3.2).

Table 3.2. Beta-casein genotype and allele frequencies in six cattle populations (sample size of each population is given in parentheses)

Populations	Beta-casein Genotype and Allele Frequencies								
	A2A2	A2A3 or A3A3	A2A1	A2B	A1A1	A1B or BB	A2	A1	B
Turkish Grey (34)	0.324	0	0.382	0.059	0.235	0	0.544	0.426	0.029
Eastern Anatolian Red (25)	0.760	0	0.160	0.080	0	0.074†	0.880	0.080	0.040
Anatolian Black (16)	0.625	0	0.125	0.188	0.062	0	0.781	0.125	0.094
Southern Anatolian Red (30)	0.534	0	0.233	0.233	0	0	0.766	0.117	0.117
Turkish Holstein (22)	0.091	0.043‡	0.682	0.045	0.182	0	0.454	0.523	0.023
Holstein Candidate Bulls (18)	0.444	0	0.556	0	0	0	0.722	0.278	0

†Despite the employment of two methods (ACRS and SSCP) still there are ambiguities in differentiating A1B genotype from BB genotype. Since these were observed in only 2 individuals, they were not included in the frequency calculations.
‡Despite the employment of two methods (ACRS and SSCP) still there are ambiguities in differentiating A2A3 genotype from A3A3 genotype. Since this was observed in only 1 individual, it was not included in the frequency calculations.

Kappa-casein A allele (presumably associated with high milk yield) was predominantly observed in the studied cattle populations. Yet, in Turkish breeds it is not as high as it is in Turkish Holstein and Holstein candidate bulls. A third kappa-casein E allele (presumably associated with poorest milk coagulation properties and has negative effects on milk production parameters and protein quality) was only observed in Holstein Candidate Bulls with a low frequency of 0.0556 (Table 3.3).

The highest frequency for BLG A allele (presumably associated with high milk yield) was observed in non-native cattle populations (Holstein Candidate Bulls and Turkish Holstein, 0.5455 and 0.4694, respectively) and in native population Turkish Grey (0.5213). BLG B allele (presumably associated with high fat and casein content and is more desirable for cheese making) was observed with the highest frequency in native populations Southern Anatolian Red (0.8125) and Eastern Anatolian Red (0.7805) (Table 3.4).

Beta-casein A1-like alleles (A1 allele and B allele) which may have unfavorable health effects on humans (for further references see Woodford, 2009) are present in lower frequencies in native cattle populations compared to non-native populations. Native cattle populations with higher frequencies of BLG B allele, are more desirable for milk production traits (Heck *et al.*, 2009 and references therein). Additionally, native cattle populations do not harbor kappa-casein E allele which presumably has negative effects on milk production parameters and protein quality (Ikonen *et al.*, 1997).

Table 3.3. Kappa-casein genotype and allele frequencies in six cattle populations (sample size of each population is given in parentheses)

Populations	Kappa-casein Genotype and Allele Frequencies						
	AA	AB	BB	AE	Allele A	Allele B	Allele E
Turkish Grey (47)	0.5106	0.3829	0.1064	0.0000	0.7021	0.2978	0.0000
Eastern Anatolian Red (41)	0.5122	0.2927	0.1951	0.0000	0.6585	0.3415	0.0000
Anatolian Black (42)	0.4286	0.4524	0.1190	0.0000	0.6548	0.3452	0.0000
Southern Anatolian Red (48)	0.4375	0.4375	0.125	0.0000	0.6563	0.3437	0.0000
Turkish Holstein (49)	0.6531	0.3061	0.0408	0.0000	0.8061	0.1939	0.0000
Holstein Candidate Bulls (27)	0.5926	0.2963	0.0000	0.1111	0.7963	0.1481	0.0556

Table 3.4 Beta-lactoglobulin genotype and allele frequencies in six cattle populations (sample size of each population is given in parentheses)

Populations	BLG Genotype and Allele Frequencies				
	AA	AB	BB	Allele A	Allele B
Turkish Grey (47)	0.2766	0.4894	0.2340	0.5213	0.4787
Eastern Anatolian Red (41)	0.0244	0.3902	0.5854	0.2195	0.7805
Anatolian Black (42)	0.2381	0.3095	0.4524	0.3929	0.6071
Southern Anatolian Red (48)	0.0417	0.2917	0.6667	0.1875	0.8125
Turkish Holstein (49)	0.2449	0.4489	0.3061	0.4694	0.5306
Holstein Candidate Bulls (22)	0.2727	0.5455	0.1818	0.5455	0.4545

3.2.1.2 Heterozygosities, Polymorphic Information Content and Test for Hardy-Weinberg Equilibrium

The observed and expected heterozygosities which are parameters indicating the genetic variability of a population, p-values (calculated by Exact test in Arlequin software) for Hardy-Weinberg equilibrium, number of observed alleles and the polymorphic information content (PIC) values for beta-casein, kappa-casein and BLG genes are presented in Table 3.5, Table 3.6 and Table 3.7, respectively. Two out of 18 tests for Hardy-Weinberg equilibrium (for each gene and population separately) rejected the null hypothesis of Hardy-Weinberg equilibrium at a 5% probability level. One of these deviations is at kappa-casein gene in Eastern Anatolian Red and the other is at BLG gene in Anatolian Black.

Table 3.5. Beta-casein gene Hardy-Weinberg Equilibrium p-values, observed (H_o) and expected (H_e) heterozygosities and polymorphic information content (PIC) estimates for six cattle populations (sample sizes are given in parentheses).

Loci	Populations	No. of alleles	Hardy-Weinberg Equilibrium (p-values)	H_o †	H_e	PIC values
Beta-casein gene	**Turkish Grey (34)**	3	0.231 NS	0.441	0.530	0.514
	Eastern Anatolian Red (25)	3	1.000 NS	0.240	0.222	0.217
	Anatolian Black (16)	3	0.304 NS	0.313	0.379	0.366
	Southern Anatolian Red (30)	3	0.798 NS	0.467	0.390	0.386
	Turkish Holstein (22)	3	0.086 NS	0.727	0.527	0.520
	Holstein Candidate Bulls (18)	2	0.245 NS	0.556	0.408	0.401

†Observed heterozygosities are the same as given in Table 3.2.
NS: Not significant.

PIC values, which measure informativeness of a locus for detecting polymorphism within a population and for assessing the relevance of loci for linkage analysis, were estimated as predominantly close to and around 0.5 for the three loci (beta-casein, kappa-casein, BLG). The maximum PIC value of a locus with two alleles is 0.5; clearly, loci with greater numbers of alleles tend to have higher PIC values and thus are more informative. The highest PIC values (0.5198 and 0.5137) for beta-casein loci were observed in Turkish Holstein and Turkish Grey populations where the lowest estimated PIC value (0.2176) for beta-casein loci was observed in Eastern Anatolian Red population. Turkish Holstein and Turkish Grey populations also have highest PIC values (0.4981 and 0.4991) for BLG loci.

Table 3.6. Kappa-casein gene Hardy-Weinberg Equilibrium p-values, observed (H_o) and expected (H_e) heterozygosities and polymorphic information content (PIC) estimates for six cattle populations (sample sizes are given in parentheses).

Loci	Populations	No. of alleles	Hardy-Weinberg Equilibrium (p-values)	H_o[†]	H_e	PIC values
Kappa-casein gene	**Turkish Grey (47)**	2	0.505^{NS}	0.383	0.423	**0.418**
	Eastern Anatolian Red (41)	2	0.034*	0.293	0.457	**0.450**
	Anatolian Black (42)	2	1.000^{NS}	0.452	0.458	**0.452**
	Southern Anatolian Red (48)	2	1.000^{NS}	0.438	0.456	**0.451**
	Turkish Holstein (49)	2	1.000^{NS}	0.306	0.316	**0.313**
	Holstein Candidate Bulls (27)	3	0.309^{NS}	0.296	0.346	**0.341**

[†]Observed heterozygosities are the same as given in Table 3.3.
NS: Not significant, *$p<0.05$.

Therefore, beta-casein and BLG loci would provide a considerable contribution to a genome scan with on average 50% of the meiosis expected to be informative in the mentioned populations. Additionally, it can be concluded that instead of beta-casein loci, kappa-casein loci with a PIC value of 0.4498 is a more informative genetic marker for linkage analysis in Eastern Anatolian Red population.

Table 3.7. Beta-lactoglobulin genotype Hardy-Weinberg Equilibrium p-values, observed (H_o) and expected (H_e) heterozygosities and polymorphic information content (PIC) estimates for six cattle populations (sample sizes are given in parentheses).

Loci	Populations	No. of alleles	Hardy-Weinberg Equilibrium (p-values)	H_o †	H_e	PIC values
BLG gene	**Turkish Grey (47)**	2	1.000 ^{NS}	0.489	0.505	0.499
	Eastern Anatolian Red (41)	2	0.652 ^{NS}	0.390	0.346	0.343
	Anatolian Black (42)	2	0.025*	0.310	0.485	0.477
	Southern Anatolian Red (48)	2	0.654^{NS}	0.292	0.308	0.305
	Turkish Holstein (49)	2	0.564 ^{NS}	0.449	0.504	0.498
	Holstein Candidate Bulls (22)	2	1.000 ^{NS}	0.546	0.506	0.496

†Observed heterozygosities are the same as given in Table 3.4.
NS: Not significant, *p<0.05.

3.2.1.3 Composite Genotypes

To reveal any possible association among the studied loci, composite genotypes were considered and their observed frequencies were compared with the expected ones calculated as presented in Table 3.8.

Table 3.8. Observed (O) and expected (E) frequencies of the β-κ–BLG composite genotypes and their differences in descending order in EAR, SAR, and AB (N=71).

Composite Genotypes			N		Frequency		
β	**κ**	**BLG**	**O**	**E**	**O**	**E**	**Difference**
A2A1	AB	AB	3	1.5	0.042	0.022	0.021
A2A2	AA	AB	9	7.6	0.127	0.107	0.020
A2B	BB	BB	2	0.8	0.028	0.011	0.017
A2A2	BB	BB	4	2.9	0.056	0.041	0.015
A2B	BB	AA	1	0.1	0.014	0.002	0.012
A2A1	AA	AB	3	2.2	0.042	0.031	0.011
A1A1	AB	BB	1	0.2	0.014	0.003	0.011
A2B	AA	BB	4	3.6	0.056	0.051	0.005
A2A2	AB	AA	2	1.6	0.028	0.023	0.005
A2A1	AA	AA	1	0.7	0.014	0.009	0.005
A2B	AA	AA	1	0.6	0.014	0.009	0.005
A2A1	AB	BB	3	2.7	0.042	0.039	0.004
A2A2	AA	AA	2	2.3	0.028	0.033	-0.004
A2A2	AB	AB	5	5.3	0.070	0.075	-0.005
A2B	AB	AB	1	1.4	0.014	0.020	-0.006
A2A2	AB	BB	9	9.5	0.127	0.134	-0.007
A2A2	AA	BB	13	13.5	0.183	0.191	-0.008
A2B	AB	BB	2	2.5	0.028	0.036	-0.008
A2A2	BB	AB	1	1.6	0.014	0.023	-0.009
A2A1	AA	BB	3	3.9	0.042	0.055	-0.013
A2B	AA	AB	1	2.0	0.014	0.029	-0.014

3.2.2 F-statistics Analysis

Distribution of genetic diversity within and between populations was estimated by means of Wright's (1978) fixation indexes; F (Fit), θ (Fst) and f (Fis) for each locus. Weir and Cockerham's (1984) estimation method was used by employing FSTAT program (version 2.9.3.2) (Goudet 2002). Results of F-statistics analysis for six cattle populations and for only four native cattle populations were presented in Table 3.9 and Table 3.10, respectively. The global deficit of heterozygotes across populations (Fit) amounted to 9.8% across six cattle populations and to 14.7% across four native cattle populations. The overall genetic differentiation among populations (Fst) was moderate (7.5% and 6.7%) in both cases. It can be concluded that, beta-casein gene with highest Fst values (0.118 and 0.110) contributed the most in the genetic differentiation among populations and kappa-casein gene with the lowest Fst values (0.015 and -0.010) contributed the least.

Table 3.9. Results of F-statistics analysis for each of three milk trait loci across six cattle populations calculated by FSTAT program

Loci	F (Fit)	θ (Fst)	f (Fis)
Beta-casein	0.035	0.118	-0.094
Kappa-casein	0.096	0.015	0.082
BLG	0.160	0.085	0.082
Overall Loci (Multi-locus)	0.098	0.075	0.024

Note: "Bootstrapping over loci", could not be performed because total number of loci and number of alleles were low.

Table 3.10. Results of F-statistics analysis for each of three milk trait loci across four native cattle populations calculated by FSTAT program

Loci	F (Fit)	θ (Fst)	f (Fis)
Beta-casein	0.139	0.110	0.033
Kappa-casein	0.113	-0.010	0.122
BLG	0.187	0.099	0.097
Overall Loci (Multi-locus)	0.147	0.067	0.086

Note: "Bootstrapping over loci", could not be performed because total number of loci and number of alleles were low.

Within-population inbreeding estimates (Fis) and their significance per population and loci, calculated with the same software, were presented in Table 3.11. None of the Fis values are significant. Hence there is not significant deficiency of heterozygotes in any population for any of the three loci. p-values for Fis values were presented in Appendix D.

Table 3.11. Within-population inbreeding estimates (Fis) values for each milk protein locus and per population (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein, HCB: Holstein Candidate Bulls).

Loci	TG	EAR	AB	SAR	TH	HCB
Beta-casein	0.168	-0.083	0.176	-0.196	-0.380	-0.360
Kappa-casein	0.095	0.360	0.011	0.041	0.031	-0.177
BLG	0.030	-0.127	0.362	0.053	0.109	-0.077
All	0.099	0.100	0.187	-0.036	-0.101	-0.196

Estimates of Fst between each population pair and their significances, based on the three gene polymorphisms were presented in Table 3.12. p-values were obtained after 15000 permutations and indicative adjusted nominal level (5%) for determination of the significance levels of multiple comparisons is 0.003333. Significant pairwise differentiation (Fst) occurred between western cattle populations (TG, TH and HCB) and eastern cattle populations (EAR and SAR). However, Anatolian Black as a central Anatolian population has no significant pairwise Fst value with any of the other populations, indicating no genetic differentiation from any other cattle populations. Also, there was no significant difference between Eastern Anatolian Red and Southern Anatolian Red. Similarly no significant difference between Turkish Grey, Turkish Holstein and Holstein Candidate Bulls was observed.

Table 3.12. Pairwise estimates of Fst values calculated by beta-casein, kappa-casein and BLG genes of six cattle populations by using FSTAT software program (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein, HCB: Holstein Candidate Bulls)

	TG	**EAR**	**AB**	**SAR**	**TH**	**HCB**
TG	-					
EAR	0.1306***	-				
AB	0.0468 ^{NS}	0.0149 ^{NS}	-			
SAR	0.1168***	-0.0032^{NS}	0.0186 ^{NS}	-		
TH	0.0021^{NS}	0.1793***	0.0961^{NS}	0.1521***	-	
HCB	0.0144^{NS}	0.1182*	0.0338^{NS}	0.1196***	0.0396^{NS}	-

NS: Not significant, *p<0.05, ***p<0.001

Results for pairwise Fst values can be summarized and visualized as:

TG, TH, HCB, AB, EAR, SAR

Here jointly underlined breeds are not significantly different from each other.

3.2.3 Between Group Variations

3.2.3.1 Nei's DA Genetic Distances and Neighbour Joining (NJ) Tree

Nei's (1978) D_A genetic distance values of paired cattle populations were presented in Table 3.13. The highest genetic distances were calculated between Eastern Anatolian Red and Turkish Holstein (0.1579), Southern Anatolian Red and Turkish Holstein (0.1431) and among the native Turkish breeds between Eastern Anatolian Red and Turkish Grey (0.1352). The lowest genetic distance values were estimated between Southern Anatolian Red-Eastern Anatolian Red (0.0052) and Turkish Grey-Turkish Holstein (0.0099) pairs.

Table 3.13. Nei's (1978) D_A genetic distances calculated with beta-casein, kappa-casein and BLG genes of six populations by GENDIST program in PHYLIP (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein, HCB: Holstein Candidate Bulls).

	TG	EAR	AB	SAR	TH	HCB
TG	-					
EAR	0.1352	-				
AB	0.0731	0.0175	-			
SAR	0.1288	0.0052	0.0233	-		
TH	0.0099	0.1579	0.1021	0.1431	-	
HCB	0.0336	0.0925	0.0424	0.1040	0.0441	-

Neighbour joining (NJ) tree constructed with Nei's (1978) genetic distances and bootstrap values are shown in Figure 3.15. NJ tree topology is consistent with the results displayed by Fst values: two groups composed of eastern (EAR and SAR) and western (TG, TH, HCB) cattle populations can be identified and Anatolian Black is observed to be on a branch in the middle of the tree.

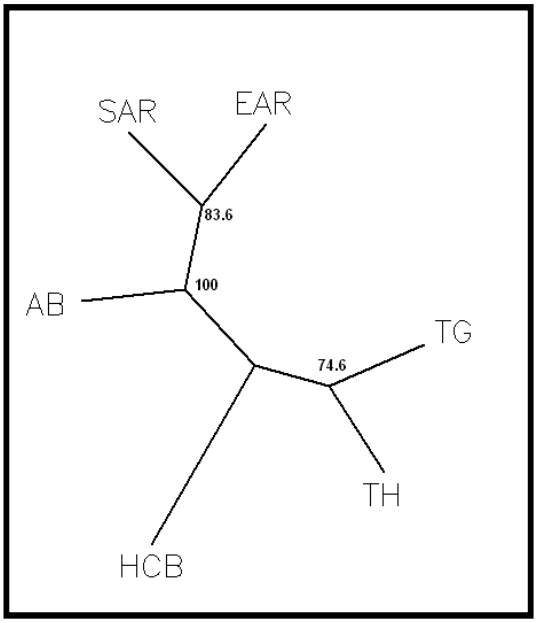


Figure 3.15. NJ tree constructed with beta-casein, kappa-casein and BLG gene polymorphisms by using package programs in PHYLIP, bootstraps are in percentages (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein, HCB: Holstein Candidate Bulls).

3.2.3.2 Principal Component Analysis (PCA)

In order to reveal the major patterns of genetic variation based on allele frequencies, three dimensional PCA of cattle populations based on their genetic relatedness was computed by NTSYSpc-2.1 package program (Rohlf, 1993) and

the results were presented in Figure 3.16 and Figure 3.17. They were constructed by allele frequencies of three milk trait genes; beta-casein, kappa-casein and BLG gene.

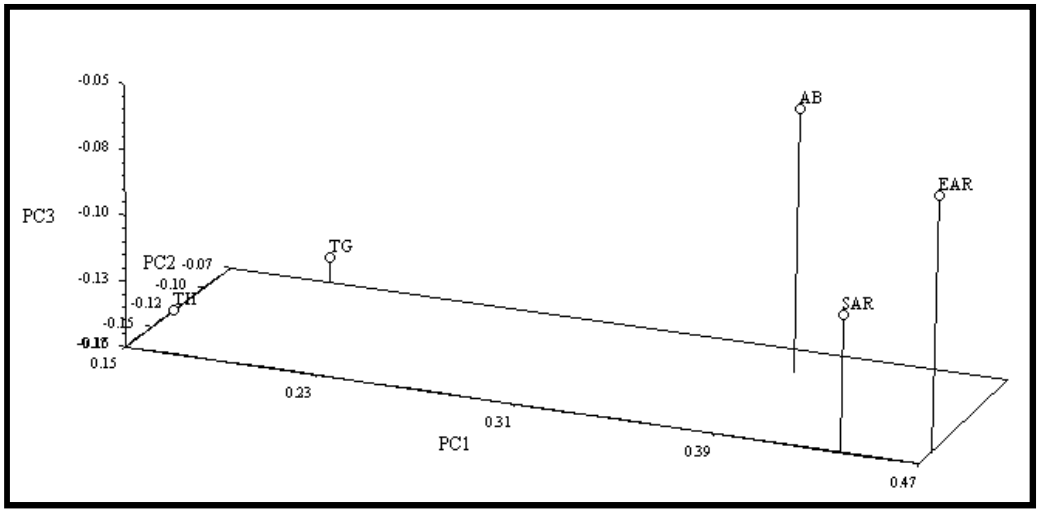


Figure 3.16. PCA analysis based on allele frequencies of three milk protein genes in five cattle populations by using NTSYSpc software program (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein).

The first principal component (PC1) of PCA in Figure 3.16 accounts for 80.38% of the total variation of allele frequencies, the second principal component (PC2) accounts for 11.02% of the total genetic variation and the third principal component (PC3) accounts for 6.82% of the total genetic variation. Therefore, three axes of PCA explain totally 98.23% of the genetic variation of allele frequencies.

The equations of three principal components presenting the weights of each marker in contributing to the differentiation of populations in the given axes are as follows:

PC1 = 0.983 Beta-casein-A2 - 0.990 Beta-casein-A1 + 0.892 Kappa-casein-B + 0.877 BLG-B

Since weights of all loci are high, all loci are contributing to the separation of populations on the first principal component (PC1) but beta-casein A1 allele has the highest weight on this axis.

PC2 = 0.019 Beta-casein-A2 - 0.008 Beta-casein-A1 + 0.419 Kappa-casein-B - 0.457 BLG-B

On the second axis (PC2), breeds are differentiated mainly by their kappa-casein B and BLG B alleles.

PC3 = 0.169 Beta-casein-A2 - 0.115 Beta-casein-A1 - 0.170 Kappa-casein-B - 0.147 BLG-B

On the third axis (PC3), differentiation of the breeds was mainly by beta-casein A2 and kappa-casein B allele.

On three dimensional graph in Figure 3.16, once more the genetic similarity of Southern Anatolian Red (SAR) and Eastern Anatolian Red (EAR), intermediate position of Anatolian Black (AB), distinctness of Turkish Grey (TG) from other native populations and differentiation of Turkish Holstein (TH) from native populations but the least from Turkish Grey (TG) can be observed. Furthermore, by examining the weights of the coefficients of the principal components it can be said that allele frequency differences of all three loci contributed to the discrimination between the cattle populations (first principal component). Kappa-casein-A and BLG-A discriminated Turkish Grey (TG) from the other populations (second

principal component), whereas, mostly, beta-casein-B frequencies differentiated Turkish Holstein (TH) from the others yet Turkish Holstein (TH) was closest to Turkish Grey (TG) in this respect.

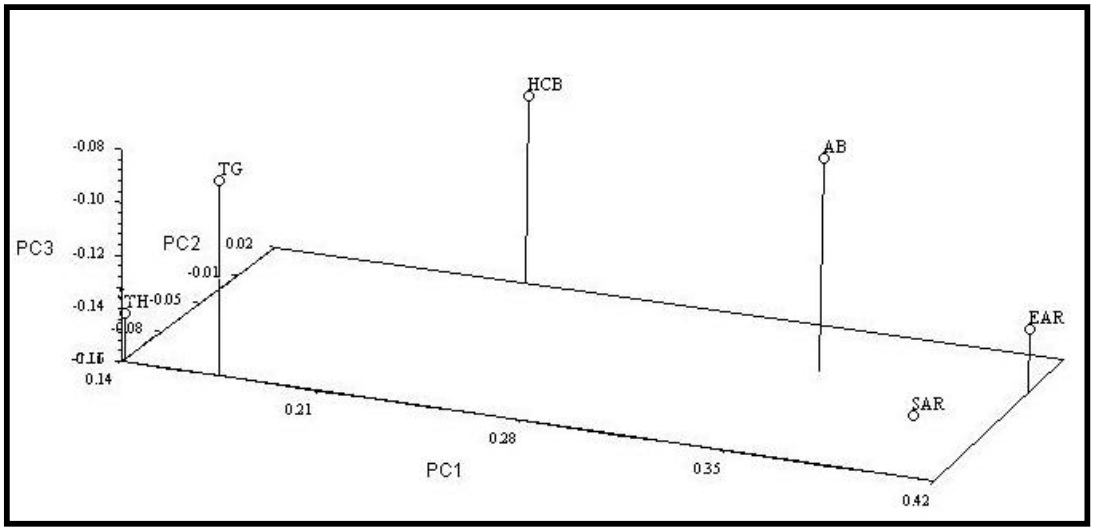


Figure 3.17. PCA analysis based on allele frequencies of three milk protein genes in six cattle populations by using NTSYSpc software program (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein, HCB: Holstein Candidate Bulls).

Moreover, three dimensional PCA in Figure 3.17 incorporated HCB population to the analysis and the position of the populations is similar to the one in Figure 3.16 with an intermediate position of HCB. The first principal component (PC1) of PCA in Figure 3.17 accounts for 78.02% of the total variation of allele frequencies, the second principal component (PC2) accounts for 15.74% of the total genetic variation and the third principal component (PC3) accounts for 5.92% of the total genetic variation. Therefore, three axes of PCA explain totally 99.68% of the genetic variation of allele frequencies.

As a summary, it is observed that there is no inbreeding in any of the six cattle populations (deviations from Hardy-Weinberg equilibrium are minor and few, furthermore Fis values are not significant) depending on three milk trait genes (beta-casein, kappa-casein, BLG) analyzed. Beta-casein gene with the highest Fst value (0.118) contributed the most in differentiating the populations. Additionally, beta-casein gene has the highest PIC values (0.5198 for Turkish Holstein and 0.5137 for Turkish Grey) among the other genes indicating higher capacity to detect linkage disequilibriums. In parallel to these observations, PCA analysis also revealed that beta-casein gene alleles play the most important role in breed differentiation on the first and the third axes where the first axis accounts the highest amount of the explained variance of the data.

3.3 Preliminary Results of Some *In Vitro* Experiments Testing the Human Immune Response to BCM-7

3.3.1 Determination of Cytokine Release

Preliminary results of the effects of BCM-7 on human PBMC (Peripheral Blood Mononuclear Cells) were obtained by using ELISA test exploiting target cytokine antibodies. ELISA was performed in three different experimental sets. The amount of secretion of three target cytokines; IL-6, IL-10 and TNFα after stimulations of first experimental set were presented in Figure 3.18, Figure 3.19 and Figure 3.20, respectively. These secretion results lead us to the point that even at a high concentration of BCM-7 (100 μg/ml) it has no effect on cytokine secretion when it was used alone as a stimulant. However, it can be clearly seen that LPS and PHA as positive controls lead to secretion of IL-6, IL-10 and TNFα cytokines by PBMC cultures.

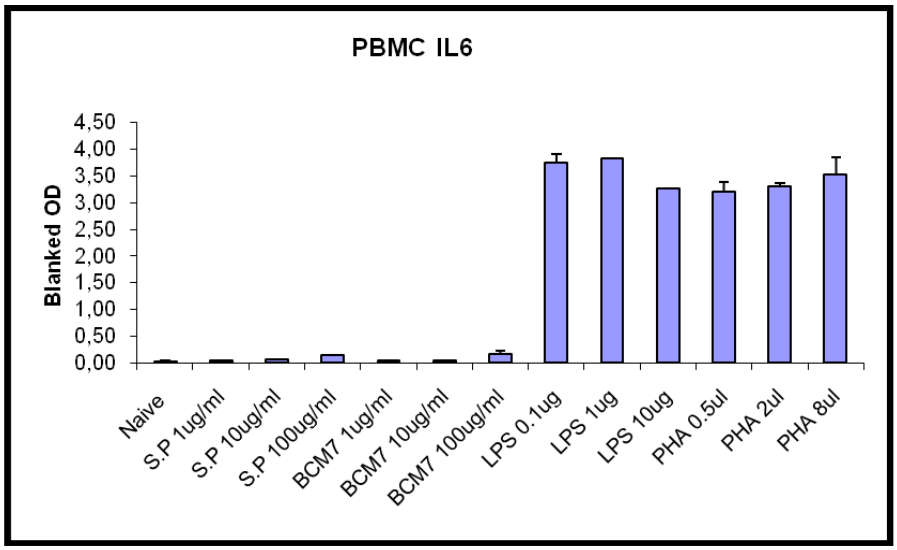


Figure 3.18. IL-6 secretion results after different stimulations (Naive: no stimulation, only medium, S.P: scrambled peptide, BCM-7: beta-casomorphin 7, LPS: lipopolysaccharide, PHA: phytohemagglutinin) in one non-diabetic human PBMC culture.

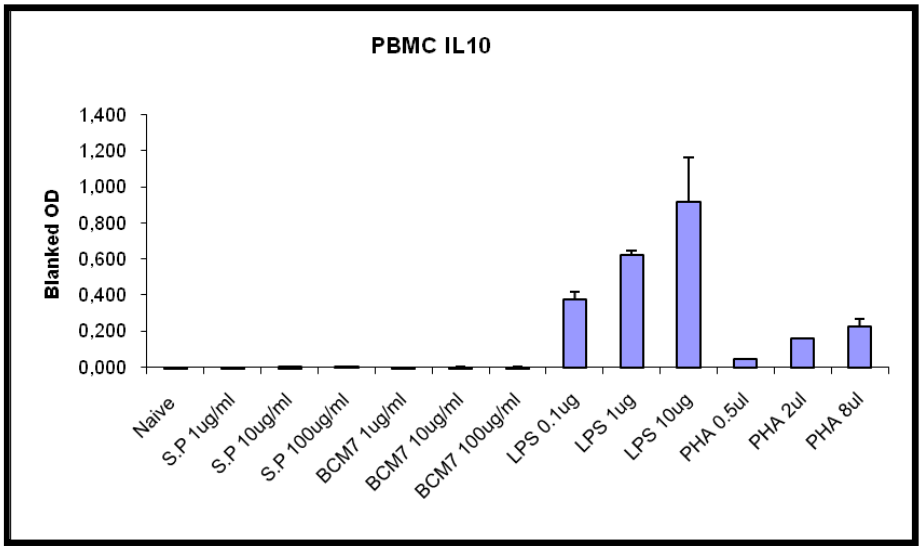


Figure 3.19. IL-10 secretion results after different stimulations in one non-diabetic human PBMC culture. Symbols and treatments are as given in the legend of Figure 3.18.

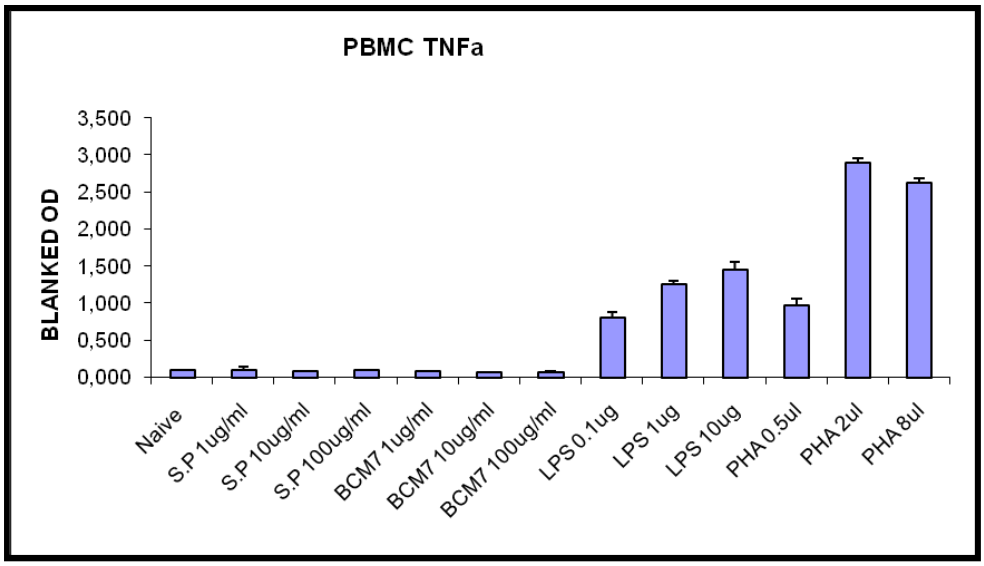


Figure 3.20. TNFα secretion results after different stimulations in one non-diabetic human PBMC culture. Symbols and treatments are as given in the legend of Figure 3.18.

In the second experimental set, amount of secretion of six target cytokines; IL-1β, IL-2, IL-4, IL-6, IL-10 and TNFα were tested with the help of an ELISA kit in six human PBMCs (from two healthy individuals, two diabetic and two celiac patients). With the help of the results of previous experiment, concentrations of peptides to be applied were finalized and it was concluded BCM-7 to be used together with an adjuvant; LPS as a stimulant (BCM-7+LPS) on PBMCs. However, no IL-2 and IL-4 cytokine secretion was observed and secretions of IL-1β, IL-6, IL-10 and TNFα cytokines after specific stimulations in each PBMC were presented in Figure 3.21 to Figure 3.28.

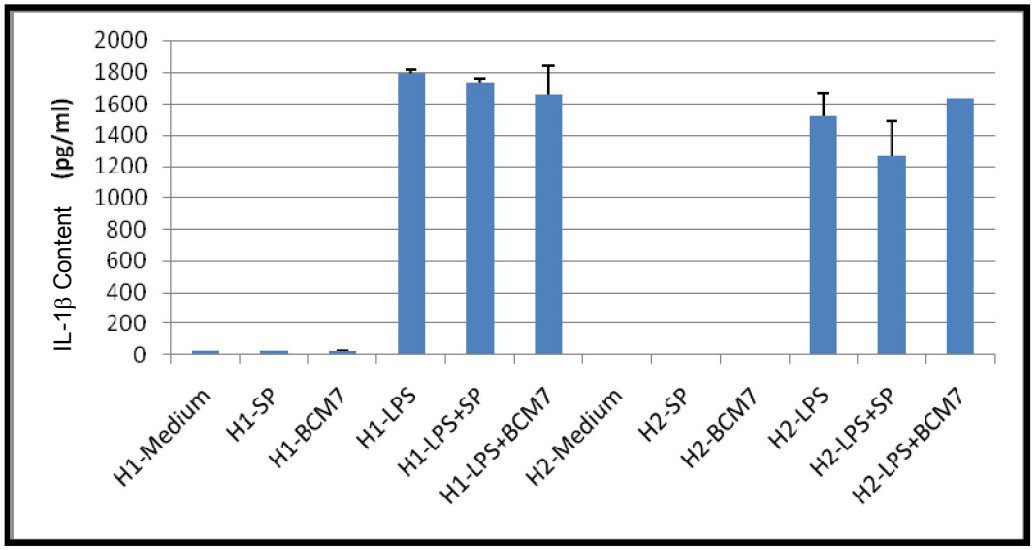


Figure 3.21. IL-1β secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two healthy (H1 and H2) human PBMC cultures.

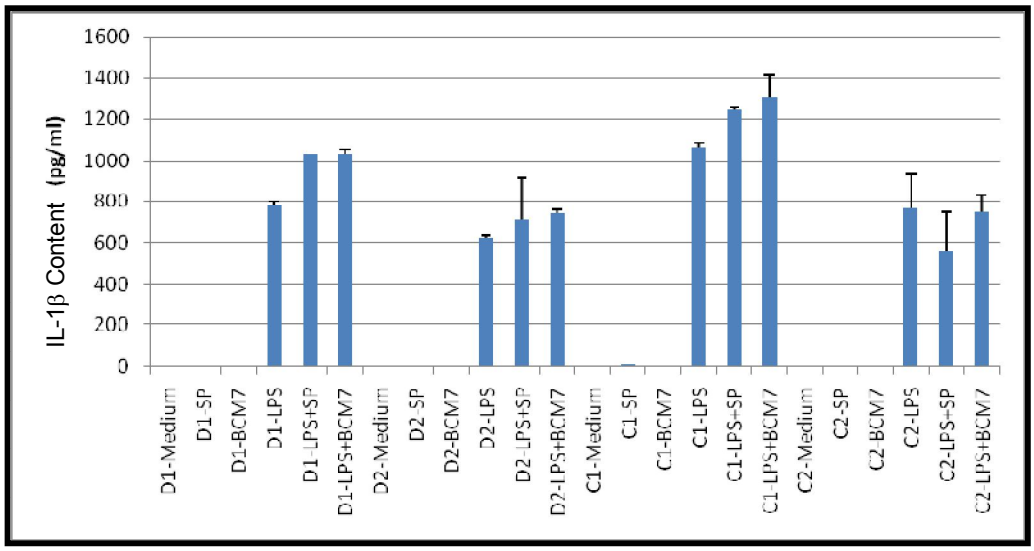


Figure 3.22. IL-1β secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two type-I diabetic (D1 and D2) and two celiac (C1 and C2) human PBMC cultures.

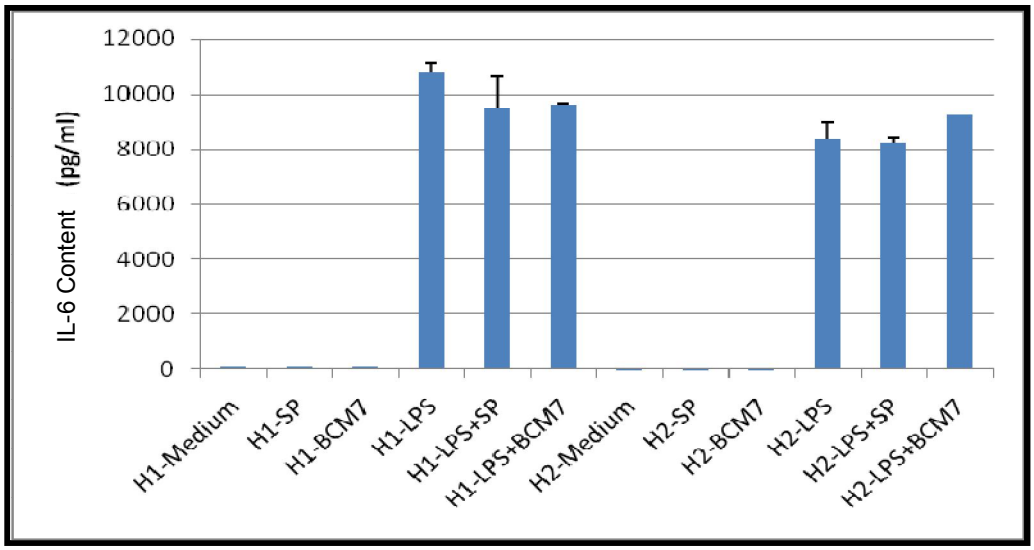


Figure 3.23. IL-6 secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two healthy (H1 and H2) human PBMC cultures.

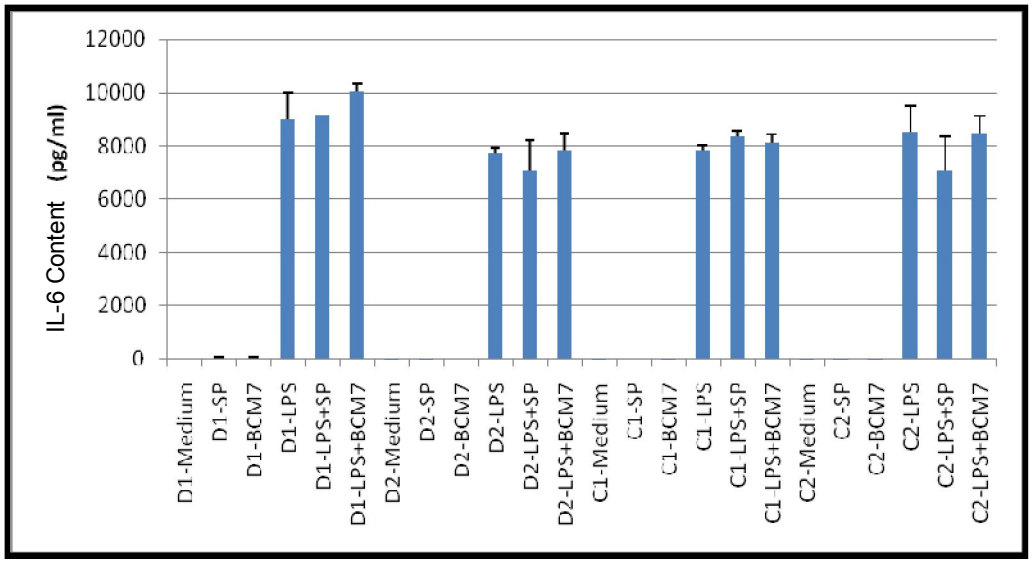


Figure 3.24. IL-6 secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two type-I diabetic (D1 and D2) and two celiac (C1 and C2) human PBMC cultures.

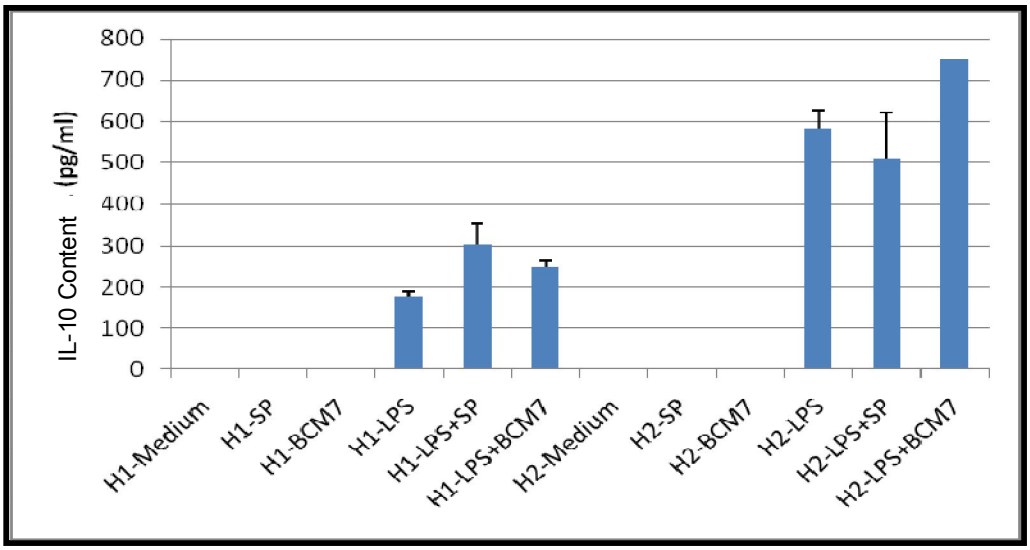


Figure 3.25. IL-10 secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two healthy (H1 and H2) human PBMC cultures.

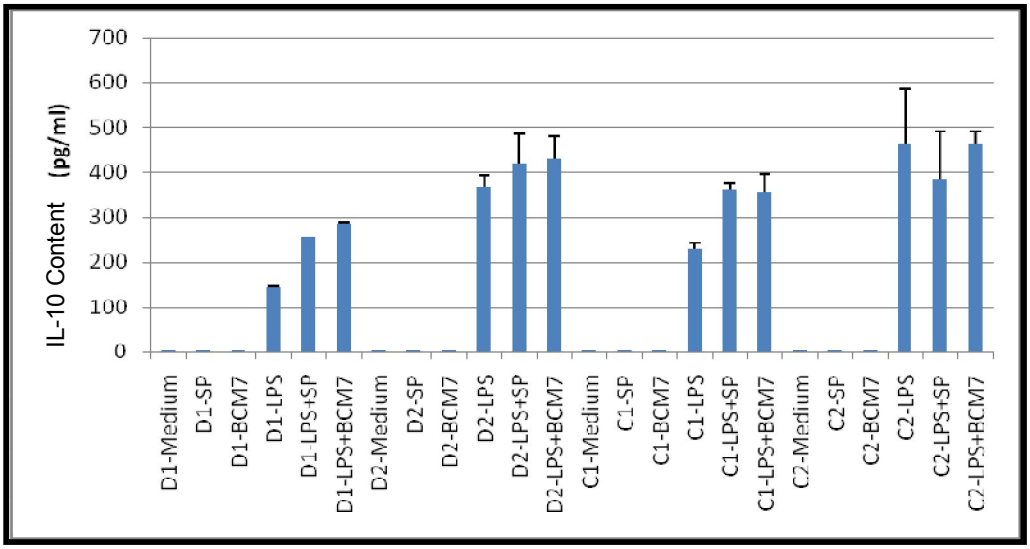


Figure 3.26. IL-10 secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two type-I diabetic (D1 and D2) and two celiac (C1 and C2) human PBMC cultures.

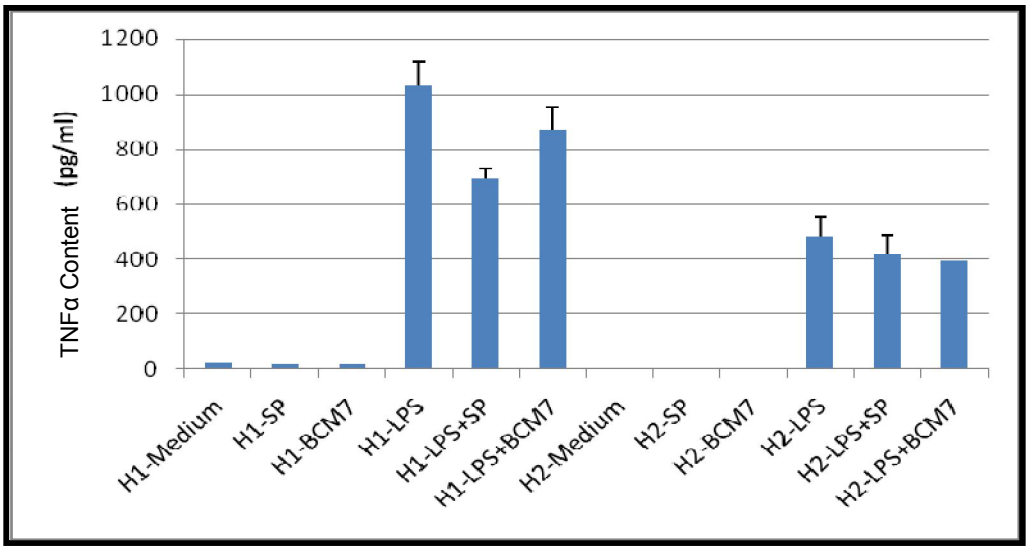


Figure 3.27. TNFα secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two healthy (H1 and H2) human PBMC cultures.

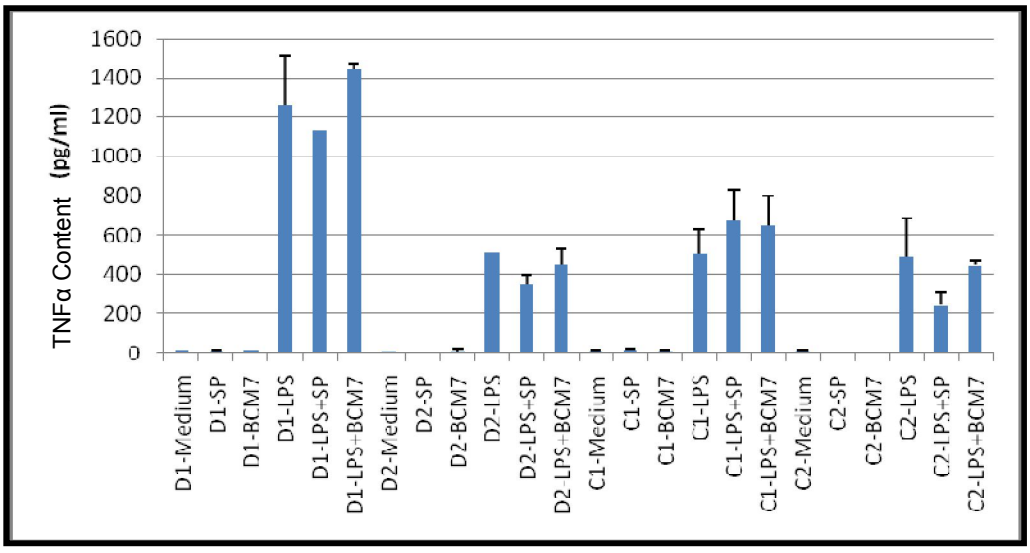


Figure 3.28. TNFα secretion results after different stimulations (Medium: no stimulation, only medium, SP: scrambled peptide, BCM7: beta-casomorphin 7, LPS: lipopolysaccharide) in two type-I diabetic (D1 and D2) and two celiac (C1 and C2) human PBMC cultures.

Paired comparisons t-test was used to test significance of differences of cytokine secretions in healthy individuals, diabetic and celiac patients after the LPS only, LPS and BCM-7 together and LPS and SP together stimulations. The results of paired comparisons (two treatments on the same individual) t-test for null hypothesis of equality, greater than and less than were presented in Table 3.14, Table 3.15 and Table 3.16, respectively.

Table 3.14. Significance of differences in cytokine secretions after stimulations in each group (two healthy individuals; H, two diabetics; D and two celiac; C) tested by paired comparisons t-test in MINITAB program with 95% confidence. The null hypothesis is "the means are equal" and the alternative hypothesis is "the means are not equal".

Tested Equality Comparisons	**p-values**			
	IL-1β	**IL-6**	**IL-10**	**TNFα**
H-LPS=H-LPS+BCM7	0.963^{NS}	0.894^{NS}	0.243^{NS}	0.175^{NS}
H-LPS=H-LPS+SP	0.365^{NS}	0.419^{NS}	0.834^{NS}	0.375^{NS}
H-LPS+SP=H-LPS+BCM7	0.628^{NS}	0.427^{NS}	0.651^{NS}	0.584^{NS}
D-LPS=D-LPS+BCM7	0.236^{NS}	0.452^{NS}	0.240^{NS}	0.737^{NS}
D-LPS=D-LPS+SP	0.309^{NS}	0.673^{NS}	0.225^{NS}	0.079^{NS}
D-LPS+SP=D-LPS+BCM7	0.510^{NS}	0.043*	0.296^{NS}	0.313^{NS}
C-LPS=C-LPS+BCM7	0.531^{NS}	0.565^{NS}	0.490^{NS}	0.688^{NS}
C-LPS=C-LPS+SP	0.948^{NS}	0.721^{NS}	0.850^{NS}	0.874^{NS}
C-LPS+SP=C-LPS+BCM7	0.284^{NS}	0.613^{NS}	0.530^{NS}	0.574^{NS}

NS: Not significant, *$p<0.05$

Table 3.15. Significance of differences in cytokine secretions after stimulations in each group (two healthy individuals; H, two diabetics; D and two celiacs; C) tested by paired comparisons t-test in MINITAB program with 95% confidence. The null hypothesis is "the first mean is equal and greater than the second mean" and the alternative hypothesis is the opposite.

Tested Comparisons	p-values			
	IL-1β	**IL-6**	**IL-10**	**TNFα**
H-LPS ≥ H-LPS+BCM7	0.518^{NS}	0.553^{NS}	0.121^{NS}	0.912^{NS}
H-LPS ≥ H-LPS+SP	0.818^{NS}	0.791^{NS}	0.417^{NS}	0.813^{NS}
H-LPS+SP ≥ H-LPS+BCM7	0.314^{NS}	0.214^{NS}	0.325^{NS}	0.292^{NS}
D-LPS ≥ D-LPS+BCM7	0.118^{NS}	0.226^{NS}	0.120^{NS}	0.368^{NS}
D-LPS ≥ D-LPS+SP	0.155^{NS}	0.663^{NS}	0.112^{NS}	0.960^{NS}
D-LPS+SP ≥ D-LPS+BCM7	0.255^{NS}	0.021*	0.148^{NS}	0.157^{NS}
C-LPS ≥ C-LPS+BCM7	0.265^{NS}	0.282^{NS}	0.245^{NS}	0.344^{NS}
C-LPS ≥ C-LPS+SP	0.526^{NS}	0.640^{NS}	0.425^{NS}	0.563^{NS}
C-LPS+SP ≥ C-LPS+BCM7	0.142^{NS}	0.306^{NS}	0.265^{NS}	0.287^{NS}

NS: Not significant, *$p<0.05$

Table 3.16. Significance of differences in cytokine secretions after stimulations in each group (two healthy individuals; H, two diabetics; D and two celiacs; C) tested by paired comparisons t-test in MINITAB program with 95% confidence. The null hypothesis is "the first mean is equal and less than the second mean" and the alternative hypothesis is the opposite.

Tested Comparisons	p-values			
	IL-1β	**IL-6**	**IL-10**	**TNFα**
H-LPS ≤ H-LPS+BCM7	0.482^{NS}	0.447^{NS}	0.879^{NS}	0.088^{NS}
H-LPS ≤ H-LPS+SP	0.182^{NS}	0.209^{NS}	0.583^{NS}	0.187^{NS}
H-LPS+SP ≤ H-LPS+BCM7	0.686^{NS}	0.786^{NS}	0.675^{NS}	0.708^{NS}
D-LPS ≤ D-LPS+BCM7	0.882^{NS}	0.774^{NS}	0.880^{NS}	0.632^{NS}
D-LPS ≤ D-LPS+SP	0.845^{NS}	0.337^{NS}	0.888^{NS}	0.040*
D-LPS+SP ≤ D-LPS+BCM7	0.745^{NS}	0.979^{NS}	0.852^{NS}	0.843^{NS}
C-LPS ≤ C-LPS+BCM7	0.735^{NS}	0.718^{NS}	0.755^{NS}	0.656^{NS}
C-LPS ≤ C-LPS+SP	0.474^{NS}	0.360^{NS}	0.575^{NS}	0.437^{NS}
C-LPS+SP ≤ C-LPS+BCM7	0.858^{NS}	0.694^{NS}	0.735^{NS}	0.713^{NS}

NS: Not significant, *$p<0.05$

Analyses presented in Tables 3.14 – Tables 3.16 revealed that LPS+BCM-7 treatments were not significantly different than those of LPS only or LPS neither in healthy individuals, nor in diabetic or celiac patients.

Means of concentrations (ng/ml) of IL-1β, IL-6, IL-10 and TNFα secreted after LPS stimulations in two healthy and two type 1 diabetic individuals in the second experiment set were presented in Table 3.17. It is observed that IL-1β, IL-6 and IL-

10 secretions were lower in type 1 diabetics, where TNFα secretion was higher in type 1 diabetics compared to healthy individuals.

Table 3.17. Mean cytokine concentrations (ng/ml) after LPS stimulations in two healthy and two type 1 diabetic patients.

Individuals	Mean Cytokine Concentrations (ng/ml)			
	IL-1β	**IL-6**	**IL-10**	**TNFα**
Healthy	1.656	9.578	0.378	0.757
Diabetic	0.704	8.355	0.257	0.889

Because in a similar experiment (Foss *et al.*, 2007) on human PBMC, IL-10 secretion was higher in diabetics compared to healthy individuals after LPS stimulation, it was decided to focus on IL-10 secretion. Moroever, although there was not any significant IL-10 secretion result, based on increase in IL-10 secretion in diabetic subjects D1 and D2 shown in Figure 3.26, IL-10 secretion was tested further with higher number of subjects. In the third experimental set, amount of secretion of only IL-10 in four healthy individuals (two females and two males), in contrast to those in diabetics (three female diabetic patients and three male diabetic patients) were presented in Figure 3.29, Figure 3.30 and Figure 3.31, respectively.

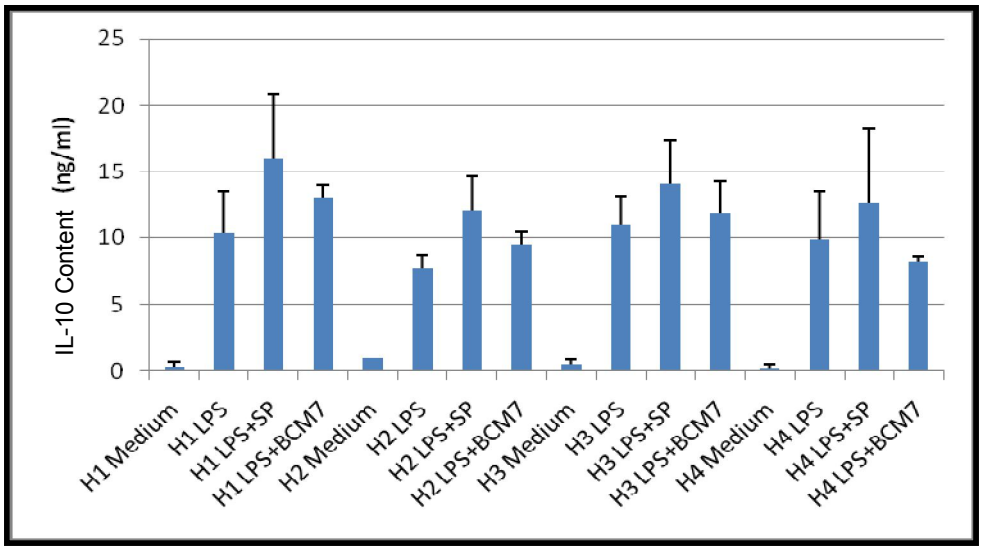


Figure 3.29. IL-10 secretion results after specific stimulations (Medium: no stimulation, only medium, LPS: lipopolysaccharide, SP: scrambled peptide, BCM7: beta-casomorphin 7) in healthy individuals (H1 and H2: females; H3 and H4: males).

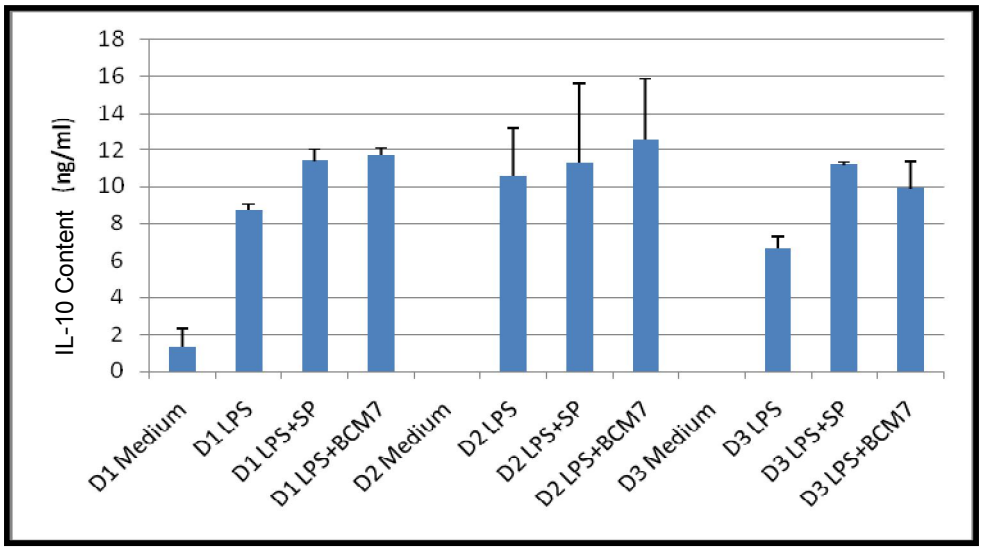


Figure 3.30. IL-10 secretion results after specific stimulations (Medium: no stimulation, only medium, LPS: lipopolysaccharide, SP: scrambled peptide, BCM7: beta-casomorphin 7) in female type-I diabetic patients (D1, D2 and D3).

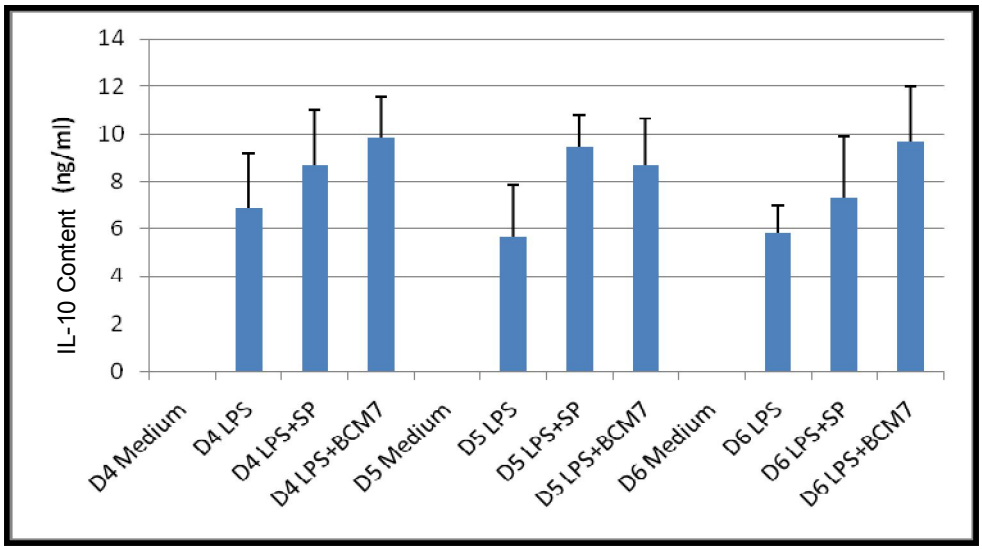


Figure 3.31. IL-10 secretion results after specific stimulations (Medium: no stimulation, only medium, LPS: lipopolysaccharide, SP: scrambled peptide, BCM7: beta-casomorphin 7) in male type-I diabetic patients (D4, D5 and D6).

Paired comparisons t-test was applied to test significance of differences of IL-10 secretions in PBMC cultures of healthy individuals and diabetic patients after the LPS only, LPS-BCM-7 together and LPS-SP together stimulations. The results of paired comparisons t-test under three different null hypotheses (means are equal, 1st mean is greater than 2nd and 1st mean is less than 2nd) were presented in Table 3.18. Since sample sizes of healthy and diabetic groups were increased slightly, results of this experimental set perhaps can be considered as more reliable than those of the previous experimental set. It was observed from Table 3.18 that for healthy individuals H-LPS+BCM7 stimulation is equal to H-LPS, and H-LPS+SP stimulation is significantly more than H-LPS+BCM7 stimulation. However, the highest significance was observed in comparison of differences of IL-10 secretion in diabetic patients; in both D-LPS+BCM-7 and D-LPS+SP stimulated IL-10 secretion (D-LPS+BCM-7 more so). However, their D-LPS+BCM-7 and D-LPS+SP stimulation effects are not significantly different than each other.

Table 3.18. Significance of differences in cytokine secretions after stimulations in each group (four healthy individuals; H and six diabetics; D) tested by paired comparisons t-test in MINITAB program with 95% confidence.

Tested Comparisons	p-values for IL-10		
	Equality	**≥**	**≤**
H-LPS / H-LPS+BCM7	0.390^{NS}	0.195^{NS}	0.805^{NS}
H-LPS / H-LPS+SP	0.010*	0.005**	0.995^{NS}
H-LPS+SP / H-LPS+BCM7	0.008**	0.996^{NS}	0.004**
D-LPS / D-LPS+BCM7	0.000***	0.000***	1.000^{NS}
D-LPS / D-LPS+SP	0.008**	0.004**	0.996^{NS}
D-LPS+SP / D-LPS+BCM7	0.398^{NS}	0.199^{NS}	0.801^{NS}

NS: not significant, *p<0.05, **p<0.01, ***p<0.001

Additionally, IL-10 secretion differences of individuals belonging to two groups (healthy individuals and diabetics) were compared by a non-parametric test; Mann-Whitney U Test. Results of the test were presented in Table 3.19. It was observed that there were significant IL-10 secretion differences in two groups: When the tested differences were between (LPS+BCM7) and LPS, and (LPS+SP) and LPS stimulations. It can also be concluded that (LPS+BCM7) and LPS IL-10 secretion difference is greater in diabetics than in healthy individuals.

Table 3.19. Significance of comparison of differences for IL-10 secretion in the third experimental set in healthy individuals; H with respect to diabetics; D tested by Mann-Whitney test by MINITAB with 95% confidence. L: Only LPS, LB: LPS+BCM-7, LS: LPS+SP.

Tested Hypothesis	IL-10 p-values
H(LB-L) = D(LB-L)	0.0252*
H(LS-L) = D(LS-L)	NS
H(LB-LS) = D(LB-LS)	0.0142*
H(LB-L) ≤ D(LB-L)	NS
H(LS-L) ≤ D(LS-L)	NS
H(LB-LS) ≤ D(LB-LS)	NS
H(LB-L) ≥ D(LB-L)	0.0126*
H(LS-L) ≥ D(LS-L)	NS
H(LB-LS) ≥ D(LB-LS)	0.0071**

NS: Not significant, *p<0.05, **p<0.01

In diabetics (6 people) difference between LPS+BCM-7 (LB) and only LPS (L) stimulations was significantly different (p=0.0252) than that of healthy individuals (4 people) and was more than that of healthy individuals. Similarly, the difference between LPS+BCM7 (LB) and LPS+SP (LS) was not equal (p=0.0142) for the diabetics and healthy individuals and it was more in diabetics compared to healthy individuals. Hence, it seems that LPS+BCM-7 (LB) related IL-10 secretion was more prominent than both LPS (L) and LPS+SP (L+SP) in diabetics compared to those of healthy individuals as if on the average diabetic patients are more sensitive to BCM-7 compared to scrambled peptide when they are exposed to these peptides together with LPS.

Means of concentrations (ng/ml) of IL-10 secreted after LPS, LPS+BCM-7 and LPS+SP stimulations in four healthy and six type 1 diabetic individuals in the third

experiment set were presented in Table 3.20. It is clear that IL-10 secretion is lower in diabetics compared to healthy individuals after LPS, LPS+SP and LPS+BCM-7 stimulations. Table 3.20 also presents mean IL-10 concentration differences of LPS+SP and LPS+BCM-7 stimulations from LPS stimulation. It can be concluded that the magnitude of increase in IL-10 secretion induced by LPS+SP in healthy individuals and by LPS+BCM-7 in diabetics is similar.

Table 3.20. Mean IL-10 concentrations (ng/ml) in four healthy and six type 1 diabetic individuals and differences of means from means of LPS stimulation

Stimulations	Mean IL-10 Concentrations (ng/ml)		Mean IL-10 Concentration Differences from LPS	
	Healthy	Diabetic	Healthy	Diabetic
LPS	9.73±1.45	7.37±1.92	0.000	0.000
LPS+SP	13.70±1.74	9.88±1.70	3.977	2.517
LPS+BCM7	10.67±2.19	10.41±1.43	0.942	3.041

3.3.2 Determination of T and B Cell Proliferation

CFSE (carboxyfluorescein diacetate succinimidyl ester) assay was employed to check the presence of T and B cell proliferation after specific stimulations in PBMCs of six individuals (two healthy individuals, two diabetic and two celiac patients) from the second experimental set. Diagrams of CFSE results of each individual obtained from the experiment carried out by flow cytometry were presented in Appendix E.

The stable incorporation of CFSE dye into lymphocytes helps to monitor lymphocyte migration *in vivo* and to quantitatively analyze cell division both *in*

vivo and *in vitro*. On the output diagrams, four quadrates are indicated as UL (lower left), UR (upper right), LL (lower left) and LR (lower right) and the percentages of cells present in each quadrate are indicated in Appendix E. The x-axis of the diagram shows SSC-H (side scatter) and the y-axis of the diagram shows FL (forward scatter for green fluorescence). SSC-H axis indicates the sizes of the cells and FL axis indicates the intensity of dye. As cells proliferate, which means as they divide, they decrease in size and intensity of the incorporated dye diminishes. Therefore, lowest values for SSC-H and FL axes indicate cell proliferation and cells in the LL (lower left) quadrate are proliferated cells. Therefore, the percentage of cells in the LL (lower left) quadrate of the diagram gives the information for the amount of cell proliferation. The percentages of cells in the LL (lower left) for PBMC of six individuals after four stimulations were summarized in Table 3.21. In all of the CFSE results, cell proliferation was only observed after phytohaemagglutinin (PHA) stimulations.

Table 3.21. Percentages of total cells (proliferated cells) present in lower left quadrate of flow cytometry results after four stimulations (medium, scrambled peptide, BCM-7, PHA) in PBMC of six individuals.

Individuals	**Stimulations**			
	Medium	**Scrambled Peptide**	**BCM-7**	**PHA**
Healthy 1	0.99%	0.85%	0.98%	10.81%
Healthy 2	1.55%	1.16%	1.17%	16.90%
Diabetic 1	1.20%	0.85%	1.07%	66.19%
Diabetic 2	1.54%	1.27%	1.09%	24.33%
Celiac 1	3.15%	2.51%	2.29%	8.17%
Celiac 2	1.66%	1.28%	1.51%	4.46%

CHAPTER 4

DISCUSSION

Animal milk is a very important source of protein and microelements and continues to be the major constituent of the young and adult human diet. Many animals, such as cows, goat, sheep, buffaloes, camels and mares, are exploited as the basis of commercial milk production in various parts of the world in order to produce milk for human consumption. In many parts of the world, cow is of overwhelming importance in milk production. While about ~85% of current annual production of milk in Western countries is from bovine, 11, 2, and 2% is from buffalo, caprine, and ovine, respectively (Smit, 2003).

Total annual milk production in Turkey is about 10 billion liters and Turkey is one of the top 15 milk producing countries of the world (Figure 4.1). While about 90% of current annual production of milk in Turkey is from bovine, 8, 2 and 0.4% is from ovine, caprine and buffalo, respectively. In the last 15 years, the percentage of bovine milk production has increased dramatically and the percentages of ovine, caprine and buffalo decreased (Table 4.1). Total annual milk production in Turkey has ranged from 9.5 to 10.6 million tons in years 1990, 1995, 2000 and 2004 as presented in Table 4.1.

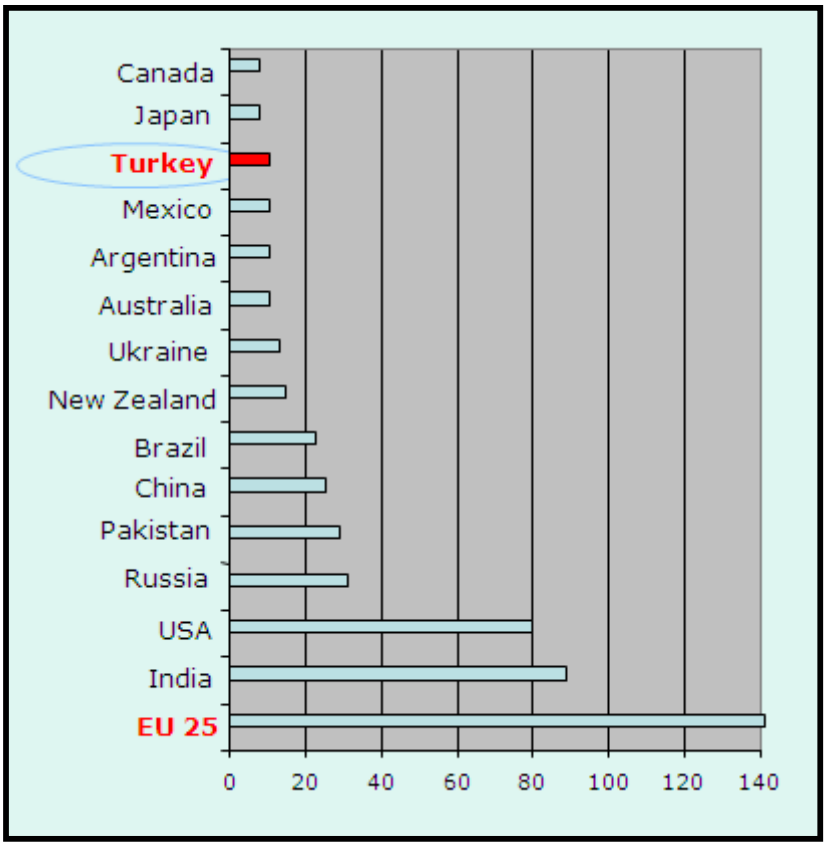


Figure 4.1. Milk production of countries between years 2000 and 2005 (million tons) (Faostat, ZMP).

Table 4.1. Milk production in Turkey (tons and percentages) (Turkish Statistical Institute).

Year	Bovine		Buffalo		Ovine		Caprine		Total
	Ton	%	Ton	%	Ton	%	Ton	%	Ton
1990	7.960. 600	82.89	174.200	1.81	1.145.000	11.92	323.700	3.37	9.603.500
1995	9.275.300	87.55	114.500	1.08	934.500	8.82	269.700	2.55	10.594.000
2000	8.732.000	89.19	67.300	0.69	774.400	7.91	216.300	2.21	9.790.000
2004	9.609.300	90.01	39.300	0.37	771.700	7.23	255.500	2.39	10.675.800

The major constituent of milk is water (~85%), but according to species milk contains varying quantities of lipids, proteins and carbohydrates, which are synthesized in the mammary gland (Özhan *et al.*, 2001). A composition of cow's milk was presented in Table 4.2. These data represent average values obtained from various researches and may not reflect the average value of any individual animal, breed, herd, geographic region, and so forth.

Table 4.2. Percent chemical composition of cow's milk. The data represent average values obtained from various researches (adapted from Kindstedt, 2005).

Constituent	Cow (Bovine)
Fat	3.7
Total Protein	3.4
Casein	2.6
Lactose	4.8
Ash (Minerals)	0.7
Total Solids	12.7

Values of annual milk production and consumption per individual in Turkey, EU and USA are given in Table 4.3. Annual milk consumption per individual in Turkey is 138 kg, it is almost one third of the consumption in EU (313 kg), and half of that of USA (265 kg).

Table 4.3. Annual milk production and consumption values in EU (25), USA and Turkey (MARA, Turkey).

	Milk Production (1000 tons)		Milk Consumption (individual/kg)	
	2005	**2013**	**2005**	**2013**
EU (25)	143.900	145.500	313	311
USA	80.055	88.906	265	267
Turkey	10.000	23.000	138	250

Since as a protein source the milk is very important in diet, consumption of milk must be increased in Turkey. Although milk yield of Holstein breed is approximately 6000-6500 kg/lactation, it is 938.92 kg/lactation-1875.36 kg/lactation among the native breeds (Notification about the Registration of Breed and Lineage Information for Native Animals; downloaded from http://www.tarimsal.com/yerliirklar/yerliirklar.htm, last visited on July 2009).

Native Turkish cattle breeds have a special status among all of the cattle breeds. As was previously stated, Middle Eastern breeds including Southern Anatolian Red (SAR) and Eastern Anatolian Red (EAR) were accepted as the breeds from the main domestication center of taurin cattle (Loftus *et al.*, 1999, Troy *et al.*, 2001, Bruford *et al.*, 2003). All the cattle in the Middle Eastern region were found to have high genetic variability indicating that this region is representing "genetic hot spot" (Freeman *et al.*, 2006) for the cattle. High genetic variability within the native breeds close to the domestication centers in turn is believed to harbor genetic information yet to be employed in resisting to harsh environmental conditions (e.g. poor feed), to diseases or adaptability to the draught to be faced in the coming years. Hence, the breeds from the region must have a high priority to be conserved (Bruford *et al.*, 2003).

However, in 1970s to increase the milk production, genetic make up of the native breeds were tried to be modified such that the higher yield of milk will be obtained. For this purpose, native breeds were started to be hybridized by economically important breeds: Holstein, Jersey, Brown Swiss and Simmental (Kumlu, 2000). Indeed those hybrid (cross-bred breed) individuals revealed higher milk yield than those of native breeds (Figure 4.2). However, the danger of losing native breeds (Kumlu, 2000) with invaluable traits was realized and the practice of hybridization between economically important and native Turkish breeds was said to be stopped in late 1980s (Kumlu, 2000). Imported pure breeds with their high milk yield (for instance, Holstein) were established and native breeds were continuing to be hybridized by economically important cattle breeds in Turkey (personal communication). Comparative milk productions of different breeds can be seen in Figure 4.2.

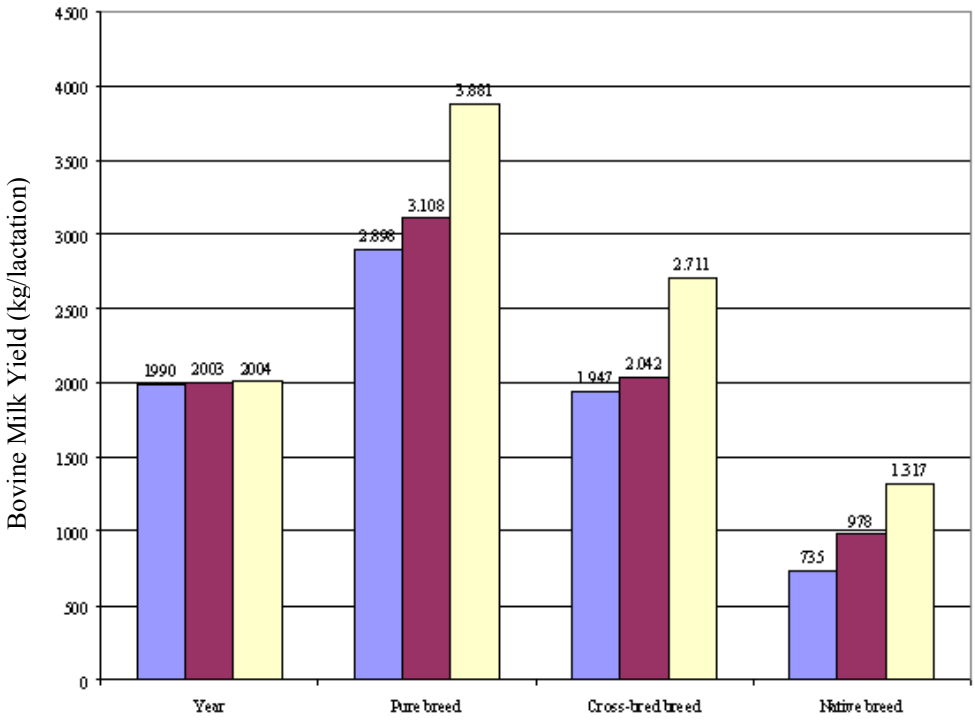


Figure 4.2. Bovine milk yield in Turkey in 1990, 2003 and 2004 (kg/lactation) (Turkish Statistical Institute).

Milk is consumed in different ways. It is consumed mostly as cheese (67.4%) and yoghurt (22.5%) in Turkey (Table 4.4).

Table 4.4. Annual milk and milk products consumption in Turkey per individual (kg/individual/year) (Ministry of Agriculture and Rural Affairs (MARA), Turkey).

	2002	**2003**	**2004**	**2005**	**2006**
Drinking Milk	21.00	21.00	21.00	21.00	20.96
Cheese	89.00	93.00	92.00	91.69	93.55
Yoghurt/Cream†	32.54	38.88	32.42	31.60	31.04
Butter	17.83	18.20	16.03	18.03	19.11
Milk Powder	2.51	3.41	2.81	2.71	2.60
Ice cream	1.01	0.90	1.00	1.02	1.02
Total Milk*	132.09	143.95	136.77	136.20	138.14

*All milk and milk products including drinking milk.
†"Kaymak" in Turkish.

Unfortunately, there are few studies relating the yoghurt making properties with the genetics of milk composition.

On the other hand, as was stated by Bobe *et al.* (1999) that the protein composition of bovine milk, which has an effect on cheese manufacturing, is predominantly determined by genetic factors. Hence, milk protein polymorphisms have been studied intensively to understand the genetic relationship between alleles of milk protein loci and milk protein composition and concentration (Hallen *et al.*, 2008, Heck *et al.*, 2009) which are affecting quality of cheese and milk yield. Such information can be employed during cattle breeding activities with the intention of increasing the proportion of cows producing milk with improved values, such as

higher levels of casein that will ultimately enhance the yield and processing properties of milk and its products (Wedholm *et al.*, 2006, Hallen *et al.*, 2008, Heck *et al.*, 2009). A large collection of research exists on the properties of milk from bovine (e.g. Mayer *et al.*, 1997, Ceriotti *et al.*, 2004, Hallen *et al.*, 2008).

Milk composition traits are not controlled by single genes. Therefore, association of an allele of a locus with a trait depends on the genetic background of the individual and genetic make up of the breed. Therefore, for instance, to measure the effect of an allele on the protein concentration of milk in a breed, genotypes are related with the phenotypes i.e. protein concentration in milk of the individuals. In the present study, genotypes of three milk protein loci (beta-casein, kappa-casein and BLG) were examined in native Turkish breeds. Unfortunately, data for phenotypes of individuals were not available for native breeds. Still results of the present study will be evaluated together with the literature covering data of both genotypes and phenotypes. General observations will be assumed to be the rule and some conclusions about the milk protein related properties of native breeds will be drawn.

Since milk yields of native Turkish breeds are relatively low, their genetic properties with respect to milk products can be screened and capitalized for higher economic revenue.

To begin with, the results of the studied loci were discussed. Then, the results of the present study were integrated to the results of larger set of loci for which the data was collected from the very same individuals of the present study. Furthermore, results belonging to milk production traits (loci under selection) were also compared with those obtained by the neutral loci. Finally, preliminary results of experiments in relation to assessing the effects of A1 milk were discussed.

4.1 Beta-casein, Kappa-casein and BLG Genes

In the present study, DNA-based genotyping of four native Turkish cattle breeds (Turkish Grey, Eastern Anatolian Red, Anatolian Black, and Southern Anatolian Red), a non-native breed (Turkish Holstein) and independent subsample of Holstein breed; Holstein Candidate Bulls was performed for three milk trait genes (beta-casein, kappa-casein and BLG).

4.1.1 Determination of Beta-casein Gene Variants

In the present study, DNA-based methods for determining genetic variants of bovine beta-casein, kappa-casein and BLG milk trait genes were optimized in our laboratory. Problem in beta-casein genotyping method (inconsistent results obtained by using three restriction enzymes) was overcome by using two methods (ACRS and SSCP) together. This way results were double checked for most of the time and hence confidence in the results was increased. In a recent literature, the problem in beta-casein genotyping was solved by using allele-specific PCR method with primers designed for A1 and A2 beta-casein genes (Keating *et al.*, 2008). That method cannot resolve the components of "A1 like" as A1 allele and B allele. Similarly, A2 allele and A3 allele cannot be differentiated as being "A2 like" alleles. However, as will be emphasized, beta-casein A2 allele is found to be related with good cheese making property. Hence, resolution of "A2 like" alleles is necessary. Additionally, there may be more beta-casein alleles in native cattle breeds and allele-specific PCR method does not help to diagnose new alleles.

4.1.2 Genotype and Allele Frequencies

One of the concerns of population genetic studies is that the samples of the breeds might be collected within a very narrow region and therefore individuals were close relatives of each other. Samples represented with close relatives in turn cannot be used to infer the genetic properties of the breeds. Another, concern is the

correct typing of the genotypes. In both of the cases signal as the deviations from Hardy-Weinberg equilibrium might be seen. As an example for the latter case the following can be given: In genotyping of Alu loci, heterozygotes could not be identified and they were mistyped as homozygotes. As a result, excess observation of homozygotes caused a significant deviation in Hardy-Weinberg equilibrium. Yet, this deviation was observed in many populations but of their particular locus: angiotension I-converting enzyme (ACE) gene. With the help of hot start method (Ueda *et al.*, 1996), heterozygous individuals were identified for ACE insertion (by improving the stringency of primer annealing) and genotyping was corrected. In the present study, out of 18 tests of deviations from Hardy-Weinberg equilibrium there were only 2 (at kappa-casein gene in Eastern Anatolian Red and at BLG gene in Anatolian Black), slightly significant ($p<0.05$) deviations. In both of the cases, there was a shortage of heterozygous individuals. Since level of deviation was low and 5% of the tests (2/18) were expected to exhibit deviation by chance it can be assumed that these deviations (11 %) are not indicating close genetic relatedness or inbreeding in the samples of the breeds. Absence of inbreeding was confirmed once more by the insignificant Fis values of the populations (Table 3.10). Furthermore, because these breeds were not exhibiting deviation in one particular locus, it can be concluded that there was no methodological error in genotyping the individuals.

In Jann *et al.'s* (2004) study, DNA-based genotyping of alpha-casein, beta-casein and kappa-casein in two native Turkish breeds (Turkish Grey and Anatolian Black) were performed. Comparison of beta-casein allele frequencies in two common breeds was presented in Table 4.5 and it is observed that the highest frequencies are for the same beta-casein allele (A2).

Table 4.5. Comparison of beta-casein allele frequencies of common populations (Turkish Grey and Anatolian Black) in the present study and Jann *et al.*'s (2004) study.

Alleles	Turkish Grey		Anatolian Black	
	Present Study N=34	Jann *et al.*, 2004 N=50	Present Study N=16	Jann *et al.*, 2004 N=50
A2	0.544	0.500	0.781	0.790
A1	0.426	0.240	0.125	0.040
B	0.029	0.220	0.094	0.110
C[†]	-	0.05	-	0.040
I[‡]	-	-	-	0.010

C[†]: A1 like beta-casein allele.
I[‡]: A2 like beta-casein allele.

However, A1 allele frequencies of the two studies were very different from each other. A1 seemed to be higher at the expense of allele B in the present study. These frequency differences were expected partly as a sampling effect from the breeds. Furthermore, probably breeds were sampled at different times; it is a well known fact that just because of the random genetic drift allele frequencies change over the time (Caroli *et al.*, 2004 and Heck *et al.*, 2009).

Among the three loci examined beta-casein gene was of importance because as well as its possible relation with milk production traits (milk yield, protein and fat content in milk), A1 allele was thought to be positively related with adverse health effects (for review see Woodford, 2009). Among the screened breeds the frequency of A1 allele was observed to be high in Red breed of Denmark and in Ayrshire breeds of New Zealand, Finland, United Kingdom and USA. Additionally, A1 frequency is also high in Red-and-White, Black-and-White and Holstein Friesen breeds (Table 4.6 from Kaminski *et al.*, 2007).

Table 4.6. The occurrence of beta-casein gene variants in various breeds and countries; HF indicates Holstein Friesen and data sorted by increasing A1 allele frequency (from Kaminski *et al.*, 2007).

Breed	Country	No. of animals	Frequency of beta-casein alleles			References
			B	A1	A2	
Guernsey	USA	100		0.010		Swaissgood 1992
	USA	3861	0.010–0.020	0.010–0.060	0.880–0.970	Enennam et al. 1991
Jersey	Germany	43	0.186	0.093	0.721	Ehrmann et al. 1997
	Denmark	157	0.350	0.070	0.580–0.650	Bech et al. 1990
	New Zealand	1328	–	0.123	0.591	Winkelman and Wickham 1997
	USA	387	0.290–0.370	0.090–0.220	0.490–0.540	Eenennam et al. 1991
Brown Swedish	Germany	232	0.170	0.108	0.705	Ehrmann et al. 1997
	USA	282	0.100–0.180	0.140–0.150	0.660–0.720	Swaissgood 1992
	USA	259	0.100–0.180	0.140–0.180	0.660–0.720	Eenennam et al. 1991
Simmental	Croatia	621	0.150	0.190	0.630	Curik et al. 1997
	Germany	229	–	0.343	0.566	Ehrmann et al. 1997
HF	USA	526	0.010–0.060	0.310–0.660	0.240–0.620	Swaissgood 1992
	USA	6000	0.010–0.040	0.310–0.490	0.490–0.620	Eenennam et al. 1991
	Hungary	768	0.107	0.418	0.470	Baranyi et al. 1997
	Germany	229	0.026	0.472	0.496	Ehrmann et al. 1997
	Poland	143	–	0.402	0.598	Kamiński et al. 2006a
	New Zealand	3761	–	0.465	0.510	Winkelman et al. 1997
	Norway	306	0.010	0.400	0.490	Lien et al. 1993
Black-and-White	Denmark	223	0.030–0.080	0.550	0.390	Bech et al. 1990
Red-and-White	Sweden	394	0.008	0.460	0.531	Lunden et al. 1997
	Germany	179	0.020	0.573	0.366	Ehrmann et al. 1997
Ayrshire	New Zealand	37	–	0.432	0.527	Winkelman and Wickham 1997
	Finland	686	0.001	0.509	0.490	Ikonen 1997
	United Kingdom	29	0–0.003	0.600	0.400	Swaissgood 1992
	USA	45	0	0.720	0.280	Swaissgood 1992
Red	Denmark	169	0.044–0.060	0.710	0.230	Bech et al. 1990

It is highly likely that samples of the present study were collected more recently than that of Jann *et al.*'s (2004). However, since A1 generally is claimed to be associated with the adverse health effects (for instance; Woodford, 2009) and it is known to be the allele observed in economically important cattle breeds for instance Holstein breed (Table 4.6), results of the present study may indicate the fact that A1 allele frequency is increasing may be through the unrecorded hybridizations between the native breeds and for example with Holstein. Among the Turkish native breeds Turkish Grey has the highest A1 allele frequency: 0.426 (Table 3.2) and it is comparable with that of Holstein frequencies (Table 4.6). The question of "Is it because it is highly related with Holstein or because of a random

genetic drift or selection for high milk yield?" will be tackled in relation to the evaluations of microsatellite data in this chapter of the thesis.

Results of the present study revealed that frequencies of B alleles of the present breeds (0.029-0.117) are higher than all high milk yielding breeds except than that of Jersey and Brown Swedish (Table 4.6). Paralleling to these observations they are also higher than those of populations of Holstein in Turkey (Table 3.2). Upper limit of A2 allele frequency range of native breeds (0.544-0.880) is suggesting that similarity of A2 allele is more to that of Jersey and Brown Swedish among the economic breeds introduced to Turkey (Holstein, Jersey, Brown Swiss and Simmental).

Comparison of kappa-casein allele frequencies was presented in Table 4.7. In Jann *et al.'s* (2004) study four alleles of kappa-casein gene were observed for each population, where in the present study it is two (A and B) for each population with the predominance of A allele. The reason for this discrepancy is the employment of different methods (RFLP in the present study and SSCP in Jann *et al.'s* 2004 study). Since RFLP method presented in Soria *et al.*'s (2003) cannot differentiate between kappa-casein A, A1 and H alleles, no comparisons can be made for the two studies depending on this variant. However, frequency of A in the present study was higher than that of Jann *et al.*'s (2004), perhaps, partly because it harbors frequencies of A1 and H. It can be stated that observed frequencies for kappa-casein B allele are similar in the two studies.

Table 4.7. Comparison of kappa-casein allele frequencies of common populations (Turkish Grey and Anatolian Black) in the present study and Jann *et al.*'s (2004) study.

Alleles	Turkish Grey		Anatolian Black	
	Present Study N=47	**Jann *et al.*, 2004 N=50**	**Present Study N=42**	**Jann *et al.*, 2004 N=50**
A	0.7021[†]	0.41	0.6548[‡]	0.24
A1	-	-	-	0.03
B	0.2978	0.24	0.3452	0.41
E	-	0.02	-	-
H	-	0.33	-	0.33

[†]Frequency of A + A1 + H alleles.
[‡] Frequency of A + A1 + H alleles.

Frequency distribution of kappa-casein alleles observed in different European cattle populations were summarized in Table 4.8. Kappa-casein B allele frequencies ranged from 0.2978 to 0.3452 in Turkish native breeds, on the contrary B allele had lower frequencies (0.1939 and 0.1481) in Turkish Holstein populations (see Table 3.3).

Table 4.8. Occurrence of kappa-casein gene variants in various breeds and countries.

Allele Frequencies				Breed (N)	References
A	B	E	H		
0.610†	0.080	0.310	-	Finnish Ayrshire (FAy)	Ikonen *et al.*, 2000
0.770†	0.230	-	-	Polish Black-and-White (102)	Strzalkowska *et al.*, 2002
0.481†	0.519	0.000	-	Reggiana (Italian) (435)	Caroli *et al.*, 2004
0.725†	0.172	0.103	-	Italian Friesian (311)	Caroli *et al.*, 2004
0.347†	0.650	0.000	-	Italian Brown (392)	Caroli *et al.*, 2004
0.290-0.830	0.130-0.710	0.050-0.320	-	Five breeds from UK	Jann *et al.*, 2004
0.240-0.410	0.240-0.410	0.000-0.020	0.330	Two breeds from Turkey	Jann *et al.*, 2004
0.200-0.550	0.380-0.630	0.010	0.010-0.190	Five breeds from Spain	Jann *et al.*, 2004
0.590-0.600	0.400	0.01	-	Two breeds from Germany	Jann *et al.*, 2004
0.720-0.820	0.190-0.280	-	-	Two breeds from Belgium	Jann *et al.*, 2004
0.530	0.390	0.080	0.010	One breed from Czech Republic	Jann *et al.*, 2004
0.420	0.580	-	-	One breed from France	Jann *et al.*, 2004
0.160-0.450	0.450-0.840	-	0.060-0.080	Four breeds from Italy	Jann *et al.*, 2004
0.430-0.630	0.460-0.130	-	0.110-0.250	Two breeds from Croatia	Jann *et al.*, 2004
0.310	0.690	-	-	One breed from Nigeria	Jann *et al.*, 2004
0.600	0.330	-	0.050	One breed from Poland	Jann *et al.*, 2004
0.598†	0.378	0.024	-	Czech Fleckvieh (440)	Kucerova *et al.*, 2006
0.770†	0.170	0.060	-	Swedish Red and Swedish Holstein (116)	Hallen *et al.*, 2008
0.599†	0.309	0.092	-	Dutch Holstein-Friesian (1912)	Heck *et al.*, 2009

†Frequency of A + H alleles.

Kappa-casein B allele frequencies of Turkish native breeds are close to those of Croatia, Czech, Polish, Belgium and Friesian breeds of European breeds. This allele was found to be one of the most well known allele related with cheese quality. Indeed, breeds of countries famous with their cheeses (Italy, France, Germany, UK) seemed to exhibit relatively high frequencies of this allele. It seems that a management policy to increase frequency of this allele might be considered to have a better cheese quality in Turkey. Among the breeds Anatolian Black, Southern Anatolian Red and Eastern Anatolian Red are the ones who have equally high B allele frequencies. May be management can be applied in one of these. Another point which is noteworthy is that E ("bad for cheese") allele of this locus is completely absent among the native breeds. However as it can be seen from Table 3.3, E allele is present in Holstein Bulls and the milk from Holstein cows in Turkey is used to make cheese. At least milk for cheese making might be manipulated such that it is E allele free. Furthermore, effect of E allele in yoghurt quality might be another question of concern. If allele is deteriorating yoghurt quality, perhaps total elimination of it from the bulls can be considered. Furthermore, measures must be taken such That native breeds must not be contaminated by E allele.

Frequency distribution of BLG alleles in different European cattle populations were presented in Table 4.9. In Eastern Anatolian Red, Anatolian Black and Southern Anatolian Red breeds BLG B allele had 0.6071-0.8125 as the allelic range (see Table 3.5). These values are higher than most of the BLG B allele frequencies presented in Table 4.9. However, BLG B allele frequencies are similar to those of zebu cattle in Brazil BLG-B allele with a frequency range of 0.559-0.955 (Silva and Lama, 1997) and 0.43-0.83 (Kemenes *et al.*, 1999) and to those of zebu cattle in India with frequency values of 0.83 and 0.61 for Indian Sahiwal and Indian Tharparkar cattle populations, respectively (Rachagani *et al.*, 2006). These observations, as was already stated (Özkan *et al.*, 2009), could be the sign of genetic contribution of zebu cattle to native Turkish cattle which were originally taurine. Since the highest two frequencies were observed in two eastern breeds:

SAR (0.8125) and EAR (0.7805) and since zebu cattle is the cattle of Indus Valley the latter interpretation was supported.

Beta-lactoglobulin is one of the whey proteins (beta-lactoglobulin and alpha-lactalbumin). Therefore, it plays an important role in cottage cheese, “lor” and “çökelek” making. Since these products are free of caseins and low in lactose, even lactose intolerent people and people allergic to caseins can consume these products. Especially, “çökelek” is widely consumed in Turkey. Perhaps, high B allele of BLG is preferred, selected allele for healthy protein to be consumed as a kind of cheese, over the ages in Anatolia.

Cheese production is increased in 2008 and 2009 in the world; therefore, there is less demand on the world market. Decrease in world-wide cheese exports which went up until 2007, decreased by 7.8% in 2008 and 12.3% in the first three months of 2009. The EU is particularly hit by this development as the largest cheese exporter (EU dairy market situation report, 2009). However, under this economic environment Turkish feta or (kasar) cheese, with free or low A1 content may provide comparative advantage in the cheese market and may provide important economic revenue.

Table 4.9. Occurrence of BLG gene variants in various breeds and countries.

Allele Frequencies		Breed (N)	References
A	B		
0.280	0.720	Finnish Ayrshire (FAy)	Ikonen *et al.*, 2000
0.370	0.630	Polish Black-and-White (102)	Strzalkowska 2002
0.558	0.442	Reggiana (Italian) (435)	Caroli *et al.*, 2004
0.436	0.564	Italian Friesian (311)	Caroli *et al.*, 2004
0.347	0.653	Italian Brown (392)	Caroli *et al.*, 2004
0.511	0.489	Czech Fleckvieh (440)	Kucerova *et al.*, 2006
0.380	0.620	Swedish Red and Swedish Holstein (116)	Hallen *et al.*, 2008
0.583	0.417	Dutch Holstein-Friesian (1912)	Heck *et al.*, 2009

4.2 Similarities among the Breeds in Turkey

Eastern Anatolian Red, Anatolian Black and Southern Anatolian Red have similar frequencies than those of in Turkish Grey, Turkish Holstein and Holstein Candidate Bulls (Table 3.3, Table 3.4 and Table 3.5). To see the similarities based on three milk loci, beta-casein, kappa-casein and BLG loci were considered together and analyses were carried out.

Turkish Holstein and Holstein Candidate Bulls were generally different from the native breeds based on D_A distances, pairwise Fst values, positioning on neighbour joining tree and also on principal component analysis (Holstein Candidate Bulls was not included). Again based on the same measures and methods, among the native breeds Eastern Anatolian Red and Southern Anatolian Red were genetically the most similar ones with respect to beta-casein, kappa-casein and BLG loci. Anatolian Black joined to this pair as can be seen on neighbour joining tree (Figure 3.15) and principal component analysis (Figure 3.16) Turkish Grey was the most

distinct one from the three native breeds (Eastern Anatolian Red, Anatolian Black and Southern Anatolian Red).

4.3 Composite Genotypes

To reveal any possible association among the studied loci, composite genotypes were considered and their observed frequencies were compared with the expected ones calculated under the assumption of independence of the loci. Although among the three loci examined, two of them (beta-casein and kappa-casein) were linked they were found to be in linkage equilibrium by the previous study. Therefore, any association between some alleles of genes can be considered as the selection for the composite genotype which is possibly related with the milk production or composition trait. Alternatively, linkage between the alleles of loci, driven by random genetic drift within the populations can cause deviations from expectations in composite genotype frequencies. However, if similar linkage disequilibriums for the composite genotypes were detected in three neighboring breeds it may indicate that selection for special trait composition was favored in the regions of these breed.

Unfortunately, because of the many possible combinations of the genotypes, the frequencies of observed composite genotypes are generally low or even null per breed. In that case associations cannot be subjected to significance test (Heck *et al.*, 2009)Therefore, similar breeds (Eastern Anatolian Red, Southern Anatolian Red and Anatolian Black) with respect to three milk loci were pooled, total number of observations to be decomposed into composites reached to 71. Even then significance test could not be carried out because there were many expected values which were less than 5, and excpected frequencies less than 5 are not allowed to be used in goodness of fit tests (Daniel, 1999, Heck *et al.*, 2009). However, it can be seen that the most deviant beta-casein, kappa-casein, BLG composite genotypes from the expectations were; A2A1-AB-AB, A2A2-AA-AB, A2B-BB-BB and A2A2-BB-BB and as a summary: A2- -B-B. This composite

genotype with respect to beta-casein, kappa-casein and BLG loci, and the genotype especially with respect to the last two loci indicated that milk of Turkish cattle breeds (AB, SAR and EAR) seemed to be capitalized on good quality/high cheese yield. Perhaps it can further be improved by increasing B allele frequency of kappa-casein gene. In cattle management practices when native breeds are hybridized by non native ones, native breeds may loose their relatively good cheese quality. Furthermore, entrance of kappa casein E allele to native breed gene pool must be avoided since it has a negative effect on cheese quality (Ikonen *et al.*, 1997, Ikonen *et al.*, 1999, Hallen *et al.*, 2008).

4.4 Eleven Loci from the Same Individuals of the Present Study

Data of the present study was considered together with the previous data collected from the very same individuals of the present study which was available in the literature (Ozkan, E., 2005, Kepenek, E.S., 2007, Ozkan *et al.*, 2009) and for the combined data (cd), statistical analyses were performed again. However, data for Holstein Candidate Bulls were largely missing therefore they were excluded from the further analysis. In these analyses, purpose was to see the differentiation of the breeds based on a higher number of milk trait loci. Furthermore, in this part of the study, results based on microsatellites (neutral loci) were available (Ozkan, E., 2005) and they were compared with those of the milk traits to have a better understanding of differentiation mechanisms of breeds.

In relation to milk trait loci growth hormone (GH), DGAT1 and prolactin none of them were found to be related with cheese quality; however GH AluI (V) allele and GH MspI (+) allele seemed to be related with milk protein content (Table 1.3). Milk protein content is generally found to be associated with cheese yield and quality (Table 1.2). Therefore, this locus may also be related with cheese quality or cheese yield. As well as these alleles K allele of DGAT1 and B allele of prolactin genes were found to be related with fat content of the milk. When the frequencies of the above mentioned alleles were examined together with those of other breeds,

presented as Appendix F tables (GH, DGAT1, prolactin), it is seen that all of them have intermediate values in the reported ranges.

Population based Fis values for each breed and milk loci and each breed and microsatellite loci were given in Table 4.10 and Table 4.11, respectively. Out of 35 tests 5 of (14%) them (4 of them being slightly ($p<0.05$) were significant, indicating that there is a low degree of inbreeding or misgenotyping.

Table 4.10. Within-population inbreeding estimates (Fis) values for each seven milk protein loci and per population (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein). Significances for larger Fis values than the observed were indicated and p-values for Fis values are presented in Appendix D.

Loci	TG	EAR	AB	SAR	TH
Beta-casein	0.185	-0.083	0.176	-0.196	-0.380
Kappa-casein	0.095	0.360*	0.011	0.041	0.031
BLG	0.030	-0.127	0.362*	0.053	0.109
GH-MspI	-0.206	-0.231	0.041	-0.127	-0.057
GH-AluI	0.202	0.371*	-0.326	-0.005	0.263
Prolactin	-0.410	-0.500	-0.156	0.072	0.184
DGAT1	0.305	-0.269	0.644***	-0.194	0.493*
All	0.025	-0.083	0.106*	-0.049	0.079

*$p<0.05$, ***$p<0.001$

Table 4.11. Within-population inbreeding estimates (Fis) values for each of the four microsatellite loci and per population (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein). Significances larger Fis values than the observed were indicated and p-values for Fis values were presented in Appendix D.

Loci	TG	EAR	AB	SAR	TH
ETH10	0.012	0.088	0.090	0.103	0.003
ETH225	0.122	-0.012	0.150*	0.071	-0.036
HEL5	0.172*	0.134	-0.004	0.099	-0.001
ILSTS005	0.125	0.235	0.081	0.037	-0.022
All	0.107	0.099	0.080	0.082	-0.014

*p<0.05

Fst statistics for milk trait loci and microsattelite loci in the presence and absence of Turkish Holstein were separately calculated and were given in Tables 4.12 and Table 4.13, respectively.

Table 4.12. Fst values for each of seven milk traits related loci across four Turkish native cattle breeds and across five cattle breeds (four Turkish native + one non-native Turkish Holstein breed).

Loci	θ (Fst)	
	Native Breeds	**Native Breeds+TH**
Beta-casein	0.102	0.134
Kappa-casein	-0.010	0.009
BLG	0.099	0.085
GH-MspI	-0.001	0.019
GH-AluI	0.022	0.016
Prolactin	0.020	0.073
DGAT1	0.008	0.071
Overall Loci (Multi-locus)	0.036	0.062
Bootstrapping over Loci (95% confidence interval)	0.004 - 0.070	0.030 - 0.094
Bootstrapping over Loci (99% confidence interval)	-0.001 - 0.080	0.022 - 0.103

Table 4.13. Fst values for each of four microsatellite loci across four Turkish native cattle breeds and across five cattle breeds (four Turkish native + one non-native Turkish Holstein breed).

Loci	θ (Fst)	
	Native Breeds	**Native Breeds+TH**
ETH10	0.017	0.047
ETH225	0.038	0.041
HEL5	0.020	0.093
ILSTS005	0.006	0.043
Overall Loci (Multi-locus)	0.021	0.058

Note: "Bootstrapping over loci", could not be performed because total number of loci and number of alleles were low.

Results for Fst can be summarized as follows:
Fst for milk loci in the presence of Turkish Holstein (+TH) is 0.062 and it is significant ($p<0.01$), in the absence of TH (-TH) it is 0.036 and it is less but still significant ($p<0.05$). In both cases beta casein locus seems to contribute the differentiation between the breeds. For the microsattelites Fst values are 0.058 with and 0.021 without Holstein and because of the low number of loci used their significance could not be obtained. ETH locus seems to be the major contributor of the microsatellite based differentiation measured by Fst.

Apparently, among the native breeds, selection on the milk protein loci caused a slightly excess differentiation between the breeds (Fst=3.6%), because differentiation based on neutral alleles was half of that of milk proteins (Fst=2.1%). Presence of Holstein almost doubles these numbers that is, in analyses, differentiation among the native breeds is relatively low in the absence of Turkish Holstein breed.

Level of differentiation is really low among the native breeds as can be seen by the comparison with reported values from the literature. Microsatellite based Fst value of 0.112 was estimated in seven European cattle breeds (MacHugh *et al.*,1998), 0.107 was calculated in North European breeds (Kantanen *et al.*, 2000), 0.090 was found in Swiss breeds (Schmid *et al.*, 1999), 0.068 was estimated in South European Beef cattle breeds (Jordana *et al.*, 2003), 0.060 was calculated in twelve African cattle breeds (nine zebu and three taurine breeds) (Ibeagha-Awemu and Erhardt 2005) and 0.035 was found in Belgian breeds (Mommens *et al.*, 1999). The 0.021 Fst value for the overall 4 microsatellite loci in the present study is considerable lower than that of Belgian breeds (0.035) which is the minimum of the reported values. This low differentiation from the center of domestication (Zeder, 2008) seemed to be the general phenomena as it was also observe for sheep (Koban *et al.* unpublished manuscript) and goat (Cañon *et al.*, 2006).

In the following Table 4.14 pairwise Fst estimates between the breeds based on seven milk traits related loci were given.

Table 4.14. Pairwise estimates of Fst values calculated by seven milk protein loci of five cattle breeds by using FSTAT software program (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein).

Breeds	**TG**	**EAR**	**AB**	**SAR**	**TH**
TG	-				
EAR	0.0594*	-			
AB	0.0303^{NS}	0.0180^{NS}	-		
SAR	0.0582*	-0.0008^{NS}	0.0246^{NS}	-	
TH	0.0354^{NS}	0.1403**	0.1097**	0.1334 **	-

NS: Not significant, *p<0.05, **p<0.01

As can be seen from Table 4.14, EAR and SAR are almost identical to each other (Fst is 0). AB is not significantly different from this pair TG is significantly ($p<0.05$) different from EAR and SAR but not from AB. TH is significantly different from EAR, SAR and AB but not from TH. All these relationships can be visualized with the help of positions of breeds on PCA space (Figure 4.3.B). Total amount of genetic variation accounted by this graph is 96.40%. On this graph PCA analysis based on 3 milk protein loci (beta-casein, kappa-casein and BLG) were also presented for ease of comparison. As it is seen genetic similarities of the breeds are the same in both cases (Figure 4.3.a, and Figure 4.3.b).

When pairwise Fst estimates between the breeds, now based on microsatellite loci was considered (Table 4.15). Again EAR and SAR were not significantly different from each other, but now AB was significantly different than both of them. Yet

differentiation from EAR was somewhat less significant ($p<0.05$), than that of SAR ($p<0.01$). TG and TH were significantly different from each other and from all of the other breeds ($p<0.01$).

Table 4.15. Pairwise estimates of Fst (below diagonal) and N_em (above diagonal) values calculated by four microsatellite loci of five cattle breeds by using FSTAT software program (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein).

Breeds	TG	EAR	AB	SAR	TH
TG	-	13.0479	11.5425	9.1840	1.9411
EAR	0.0188**	-	14.5429	18.4067	2.0186
AB	0.0212**	0.0169*	-	8.6152	2.4733
SAR	0.0265**	0.0134^{NS}	0.0282**	-	2.1890
TH	0.1141**	0.1102**	0.0918**	0.1025**	-

NS: Not significant, *p<0.05, **p<0.01

Fst dependent gene flow (N_em) estimates between each population pair and their significances calculated based on four loci were presented in Table 4.15. p-values were obtained after 10000 permutations and indicative adjusted nominal level (5%) for multiple comparisons was 0.005. N_em is the number of effective migrants per generation under the island model and neutrality assumption; therefore N_em values were only calculated for 4 microsatellite loci since they are the neutral markers. Actually, as stated in a study on genetic diversity based on 44 microsatellite loci in Swiss sheep breeds, not all of the microsatellites were neutral (Glowatzki-Mullis *et al.,* 2009). In the present study, neutrality of all 4 loci was confirmed with the help of Arlequin package program (Excoffier *et al.*, 2006). The highest N_em value (18.41) was observed between EAR and SAR breeds, indicating high rate of genetic flow between the populations. The lowest N_em values were estimated

between non-native breed TH and all of the native Turkish breeds, indicating minimal genetic flow between TH and the native Turkish cattle breeds.

4.5 Principal Component Analysis (PCA)

Three dimensional visualization of principal component analysis constructed with allele frequencies of three (beta-casein, kappa-casein) and seven milk traits related genes (beta-casein, kappa-casein, BLG, GH-AluI, GH-MspI, prolactin and DGAT1) and four microsatellite loci (ETH10, ETH225, HEL5, ILSTS005) in different combinations of six cattle breeds (TG, EAR, AB, SAR, TH, BS) and results were presented in Figure 4.3.A- Figure 4.3.C.

Figure 4.3.C displays the genetic relatedness of breeds as revealed by microsatellite loci. Here it is seen despite the similar pattern of genetic relatedness observed based on milk protein loci TG is now significantly different than TH. Another economically important breed: Brown Swiss (BS) was added to the analysis because its data was available (Özkan, 2005). Comparing Figure 4.3.A with Figure 4.3.B it can be said that apparent similarity between TG and TH in milk protein loci is not due to the general similarity of these two breeds. Probably resemblance in milk protein loci is acquired from another breed through hybridization, may be through Brown Swiss. In Figure 4.3.C Brown Swiss seems to be relatively close to TG and there is a known history of hybridization between these two breeds (Özkan, 2005). Or, due to random genetic drift just by chance with respect to milk proteins TG converged to TH, especially with respect to A1 allele of beta casein gene (Table 3.2).

The first principal component (PC1) of PCA in Figure 4.3A accounts for 87.76% of total genetic variation, the second principal component (PC2) 9.62% and the third principal component (PC3) 2.30%. Therefore, three axes of PCA explain totally 99.68% of genetic variation.

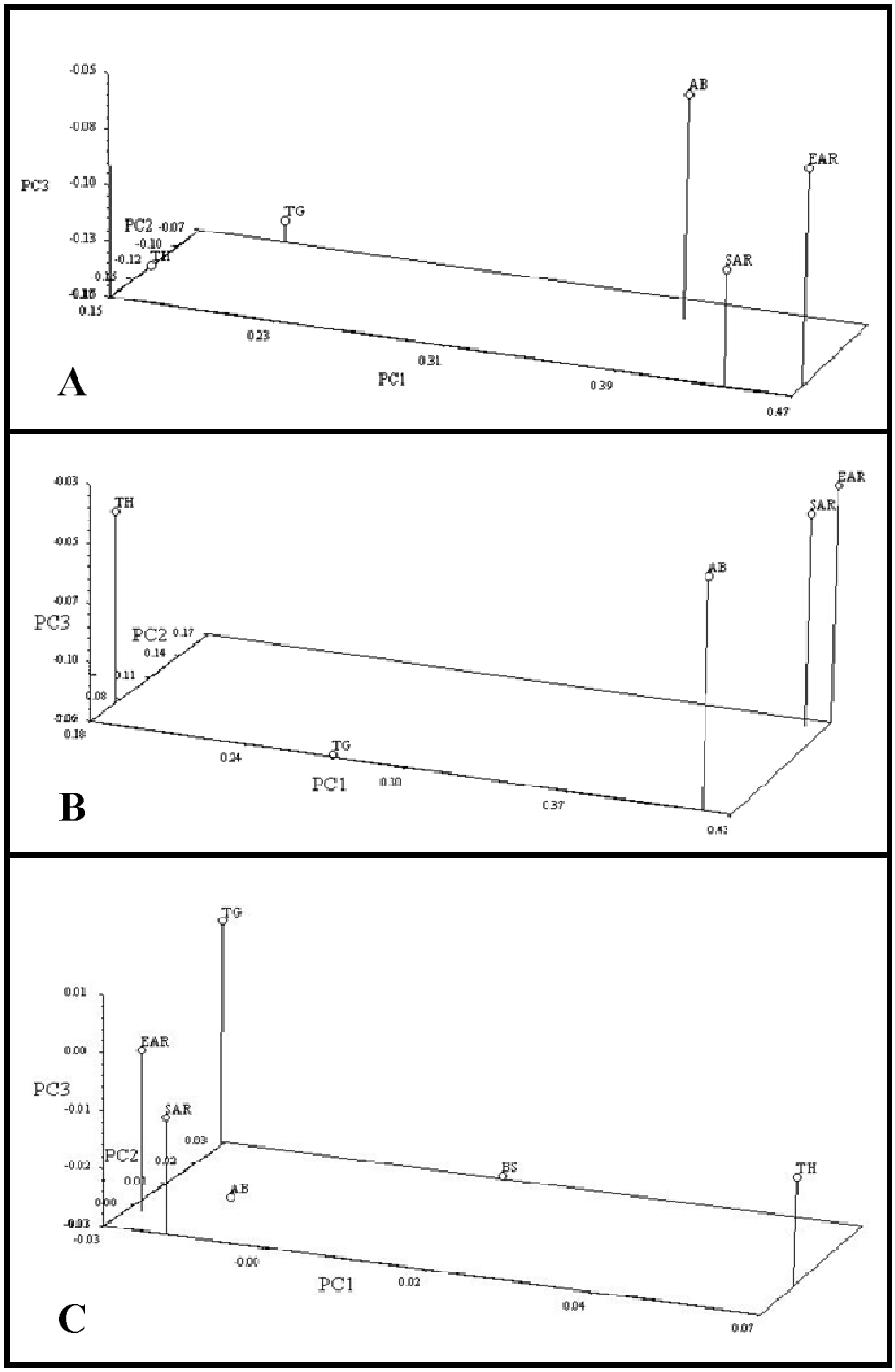


Figure 4.3. PCA performed with allele frequencies of (A) three milk traits related genes in five cattle breeds (TG: Turkish Grey, EAR: Eastern Anatolian Red, AB: Anatolian Black, SAR: Southern Anatolian Red, TH: Turkish Holstein), (B) seven milk traits related genes in five cattle breeds (TG, EAR, AB, SAR, TH), (C) 4 microsatellite loci in six cattle breeds (TG, EAR, AB, SAR, TH, BS: Brown Swiss) by using NTSYSpc software program.

The first principal component (PC1) in Figure 4.3B accounts for 70.52% of total genetic variation, the second principal component (PC2) 17.17% and the third principal component (PC3) 8.71%. Therefore, three axes of PCA cover totally 96.40% of genetic variation.

The first principal component (PC1) of PCA in Figure 4.3C accounts for 38.79% of total genetic variation, the second principal component (PC2) 33.35% and the third principal component (PC3) 24.42%. Therefore, three axes of PCA explain totally 96.56% of genetic variation.

4.6 BCM-7 Effect in Diabetics: No conclusion, yet

In the present study, preliminary experiments were carried out for the potential use of peripheral blood mononuclear cell (PBMC) culture in evaluating the effects of BCM-7 in healthy, celiac and mainly in diabetic individuals for future experiments. In relation to the aetiology of type-I diabetes different mechanisms are suggested genetic and environmental (for instance enteroviruses) (Conrad *et al.*, 1997).

First of all it can be anticipated that even if BCM-7 is related with the development of type-I diabetes its effect need not be seen in all of the patients. Similarly, there is a genetic diversity among the healthy individuals (control subjects) and some of them might be susceptible to BCM-7 effect yet free of Type-I diabetes at least for the time being. These arguments suggests that to detect the affect of BCM-7, unless it is completely associated with the health state of the individuals, high number ($n>30$) of both healthy and diabetic individuals must be considered in experiments.

In the present study, three experiment sets were carried out, in the first one the main goal was to optimize the concentrations of the antigens (scrambled peptide and BCM-7), and also the positive controls (lipopolysaccharide; LPS and phytohaemagglutinin; PHA) in ELISA. Secretion of three target cytokines (IL-6, IL-10 and TNFα) was tested in one healthy individual. The reasons of choosing these particular cytokines were as follows: activation of IL-6 would indicate that innate immunity system is activated because it is secreted by macrophages; IL-10, in general is expected to suppress the activation of macrophages and production of cytokines; TNFα, would indicate pro-inflammatory response. Furthermore, although for different treatments in the literature, IL-10, IL-6 and TNFα were also examined in diabetics (Foss *et al.*, 2007; Foss-Freitas *et al.*, 2008). Finally, antibodies of these cytokines were available in the laboratory. In the literature, IL-10, IL-6, TNFα was also examined in diabetics. The results of the first set of experiments indicated that no cytokine secretion was present except in LPS and PHA (positive control) stimulations based on this healthy individual.

Therefore, stimulations were optimized and LPS was used together with scrambled peptide and BCM-7 as an adjuvant for these antigens in the second experimental set. Six target cytokines were tested in six subjects (two healthy individuals, two diabetic and two celiac patients) in the second experiment. Functions of the cytokines that were used in this set were presented in Table 4.16.

Celiac patients cannot ingest gluten. As a result of gluten digestion, a seven amino acid peptide called gliadorphin (Tyr-Pro-Gln-Pro-Gln-Pro-Phe**)** is released, and this peptide causes allergic responses in celiac patients. Gliadorphin has amino acid sequence homology in four amino acid positions with BCM-7 (Tyr-Pro-Phe-Pro-Gly-Pro-IIe). That was why the effect of BCM-7 on immune response of celiac patients was also examined. However, any significant cytokine secretion was not observed in PBMC of celiac patients as a result of BCM-7 stimulation. Therefore, further investigation was not carried out in celiac patients in the current study.

Table 4.16. Producing cells, target cells and functions of six target cytokines in second experimental set (Janeway *et al.*, 2005).

Cytokine	Producing Cell	Target Cell	Function
IL1β	monocytes macrophages B cells DC	Th cells, B cells, NK cells	Growth and differentiation of monocytes and DC, co-stimulation , maturation and proliferation, activation, inflammation, acute phase response, fever
IL-2	Th1 cells	activated T and B cells, NK cells	growth, proliferation, activation
IL-4	Th2 cells	activated B cells, macrophages, T cells	B-cell proliferation. IgE production
IL-6	monocytes macrophages Th2 cells stromal cells	activated B cells, plasma cells, stem cells	Synergistic effects with IL-1 or TNFalpha.
IL-10 (cytokine synthesis inhibitory factor)	Th2 cells	T cells, Macrophages, B cells	Inhibits cytokine production, potent suppressant of macrophage functions
TNFα	macrophages, mast cells, NK cells	Macrophages, tumor cells	CAM and cytokine expression, cell death

In the third set of experiment, sample sizes of healthy and diabetic groups were increased slightly: 4 healthy individuals and 6 diabetes patients were employed. Because of the difficulty in finding the voluntary blood donors higer number of subjects could not be found to participate in the experiments. Unfortunately because lower concentrations (40 μg/ml) for BCM-7 and scrambled peptide were employed in the third experimenl set, compared to the "optimum" concentration which was (100 μg/ml) and was used in the second set of experiment (see Table 2.8 in Materials and Methods), these preliminary IL-10 secretion results were not conclusive. In this set of experiment, IL-10 secretion upon exposure to LPS (0.5 μg/ml) in diabetics was observed to be less than that of healthy. However, it was significantly ($p<0.03$) more in another experiment where 17 diabetics and healthy individuals were examined and LPS concentration was 1 μg/ml in that experiment. If opposite result observed in the present study is mainly due to the small sizes of the treatment groups (high genetic variability was also observed in the previous study) of the present study and partly due to the lower LPS concentration, it can be assumed that IL-10 secretion is relatively high in diabetics. Hence, increased production of regulatory IL-10, in PBMC cell cultures of type 1 diabetic patients suggest a deficiency in mononuclear cell activation and consequently a deficient immune response, which may be one of the reasons that type 1 diabetic patients have increased incidence of extra cellular infections (Foss *et al.*, 2007). Possibly related with IL-10 and infections relationship Eming *et al.*'s (2007) study showed that IL-10 can impede wound repair in mice.

Remembering that enteroviruses (Graves *et al.*, 1997) have been implicated in the cause of type I diabetes, deficient immune response against them might be increasing the risk of developing type I diabetes in individuals with high IL-10 secretions upon exposure to LPS.

It must be emphasized once more that in the present study, IL-10 was not higher in diabetics compared to healthy, on the contrary was lower. In the present study significantly more increase of IL-10 in diabetics upon exposure to LPS+BCM-7

compared to that of healthy was suggesting further susceptibility to type-I diabetes in the presence of BCM-7. However, the same increase in healthy individuals upon the exposure to LPS+SP (but not in diabetics) weakens the argument of negative BCM-7 effect in diabetics. These tests must be repeated once more with higher number of observations and perhaps with higher concentration of LPS as well as higher concentrations of BCM-7 and SP.

Moreover, CFSE assay was carried out in order to reveal the presence of T and B cell proliferation after specific stimulations in PBMCs of six individuals (two healthy individuals, two diabetic and two celiac patients) from the second experiment set. As a result, cell proliferation was not observed in any of the stimulations except in positive control phytohaemagglutinin (PHA) stimulations. In the literature, Kayser and Meisel's (1996) study is a similar study which investigated human peripheral blood lymphocyte proliferation after stimulation with four bioactive peptides (Tyr-Gly, Tyr-Gly-Gly, BCM-7 and beta-casokinin-10) derived from bovine milk proteins. They concluded that lymphocyte proliferation was observed at high concentrations of BCM-7 and beta-casokinin-10. However, the main drawback of this study was that human peripheral blood lymphocytes were activated by ConA, which is a strong mitogen that may have caused overestimation of amount of lymphocyte proliferation. Additionally, subjects in this study were only healthy individuals. Therefore, CFSE assay proliferation results in the present study could be more reliable from these aspects.

In conclusion, although differential health effects of A1 and A2 milk is still controversial and even if A1 beta-casein and hence BCM-7 has no adverse health effects on human, the presence of this probability should be a reason to keep A1 beta-casein frequency at low levels in native cattle breeds (SAR, EAR and AB) and to forbear them from contamination by A1 beta-casein from European cattle breeds.

CHAPTER 5

CONCLUSION

1. For milk protein loci, 11-14 % deviation from the Hardy-Weinberg equilibrium was observed and almost all of them were slightly significant ($p<0.05$*). Therefore, it can be assumed that these deviations do not indicate close genetic relatedness or inbreeding. Absence of inbreeding was confirmed once more by the insignificant Fis values of the populations. Furthermore, because these breeds did not exhibit deviations from Hardy-Weinberg equilibrium in any particular locus, it can be concluded that there was no methodological error in the genotyping of the individuals. Hence, conclusions based on these loci can be used for future management, with confidence.

2. By using two methods (ACRS and SSCP) simultaneously during beta-casein genotyping, in most occasions the results were double checked and hence confidence to the beta-casein genotyping results was increased.

3. Genetic similarity among the native cattle breeds of Turkey, based on both milk trait loci (beta-casein, kappa-casein, BLG, as well as prolactin, DGAT1, growth hormone AluI, growth hormone MspI) and microsatellite loci (ETH10, ETH225, HEL5, ILSTS005) was as follows: Southern Anatolian Red and Eastern Anatolian Red breeds are the most similar ones, Anatolian Black is also genetically close to this pair, while Turkish Grey seems to be the most distinct from the rest. Nevertheless, the degree of differentiation among these breeds is much lower than those of other breed groups observed in Europe.

4. Turkish Holstein breed seemed to be genetically close to Turkish Grey with respect to milk trait loci (for instance, beta-casein A1 allele frequency is high in both). Nonetheless, microsatellite-based analysis indicated that this similarity is not for the whole genome and that Turkish Grey may have acquired milk trait genes from another breed, alternatively, similarity may be because of the random genetic drift experienced by Turkish Grey.

5. B alleles of beta-casein, kappa-casein and BLG genes, which are associated with high cheese yield and quality in the literature, were observed to be relatively high in Turkish breeds and seemed to be in linkage disequilibrium. These observations suggest that the milk of Turkish native breeds is advantageous for producing high-quality and high-yield cheese. Perhaps, it can be tried to increase the B allele of kappa-casein gene in order to achieve a better quality of cheese in Turkey. The major weakness of the suggested management policy is the absence of cheese quality data of individuals with known genotypes.

6. No conclusive results in relation to the tests searching for the effect of BCM-7, and hence of A1 beta-casein, on human peripheral blood mononuclear cells were obtained. However, a possible effect of BCM-7 in increasing secretion of IL-10 immunosupressor cytokine was suggested. Additionally, an experimental protocol was optimized that will guide further researchers while judging the effect of BCM-7 on human health.

7. Although differential health effects of A1 and A2 milk is still controversial and even though A1 beta-casein, and hence BCM-7 may not have adverse health effects on humans, the presence of this probability should be a reason to keep A1 beta-casein frequency at low levels in native cattle breeds (Southern Anatolian Red, Eastern Anatolian Red and Anatolian Black) and to forbear them from contamination by A1 beta-casein present in European cattle breeds.

8. When native breeds are introgressed by economically important breeds, perhaps the sperm of bulls must be screened for E allele of kappa-casein, which is known to have detrimental effects on cheese quality, as well as A1 allele of beta-casein.

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APPENDICES

APPENDIX A

SELECTED ELECTROPHORETOGRAMS OUT OF MORE THAN 40 GELS FROM OPTIMIZATION STEPS OF SINGLE STRAND CONFORMATION POLYMORPHISMS (SSCP) METHOD

Tested Different Gel Conditions:

- 29:1 ratio of acrylamide:bisacrylamide stock solution
- Gel with 5% glycerol
- 12%, 14%, 19%, 20% different gel concentrations
- 0.5X Tris Boric Acid EDTA (TBE) and 1X TBE as buffer solutions

Tested Gel Running Conditions:

- Different power combinations of 180 volts, 200 volts, 220 volts, 250 volts and 1 watt, 4 watt, 6 watt.
- At 4 ºC, 15 ºC, 18 ºC and room temperature.
- 10 hours, 18 hours, 20 hours, 22 hours, 24 hours, 26 hours, 30 hours, 36 hours and 48 hours running time.

Selected electrophoretograms:

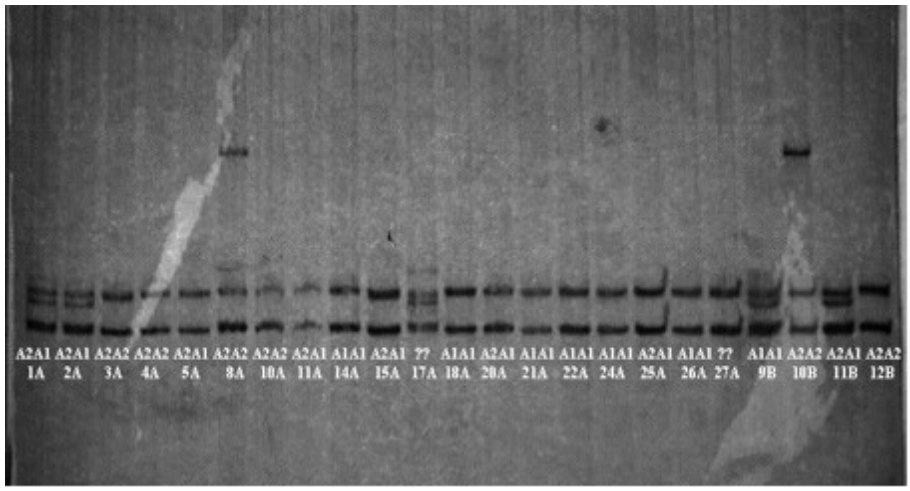

Figure A.1. 12% gel; without glycerol

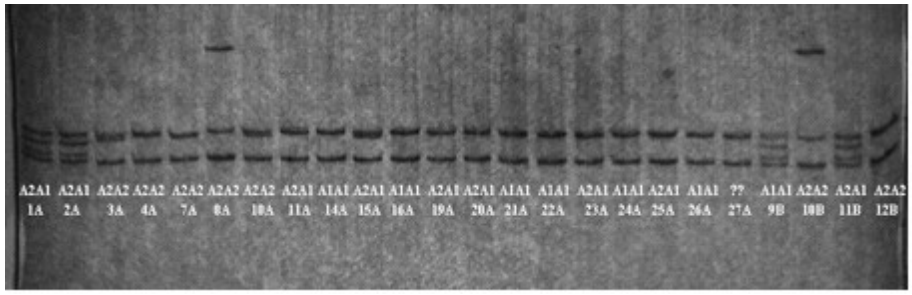

Figure A.2. 17% gel; without glycerol

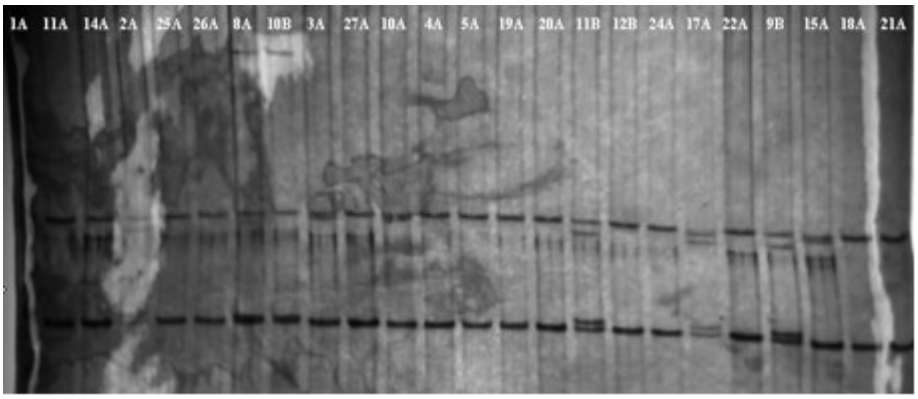

Figure A.3. 17% gel; without glycerol; 48 hours running time with 1XTBE buffer

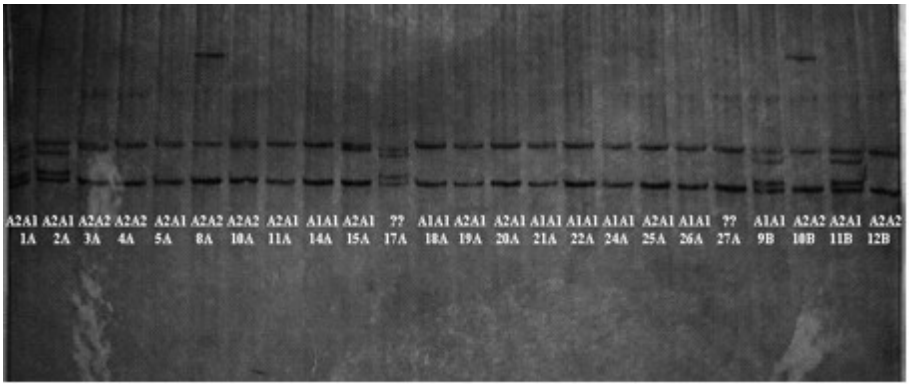

Figure A.4. 19% gel; without glycerol

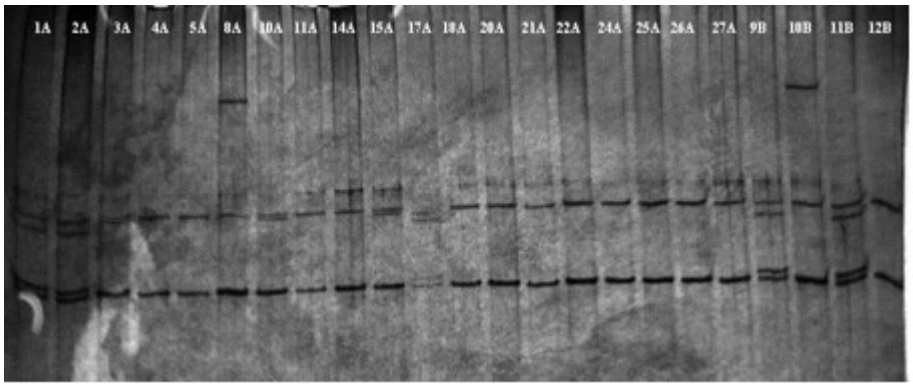

Figure A.5. 20% gel; without glycerol; 48 hours running time

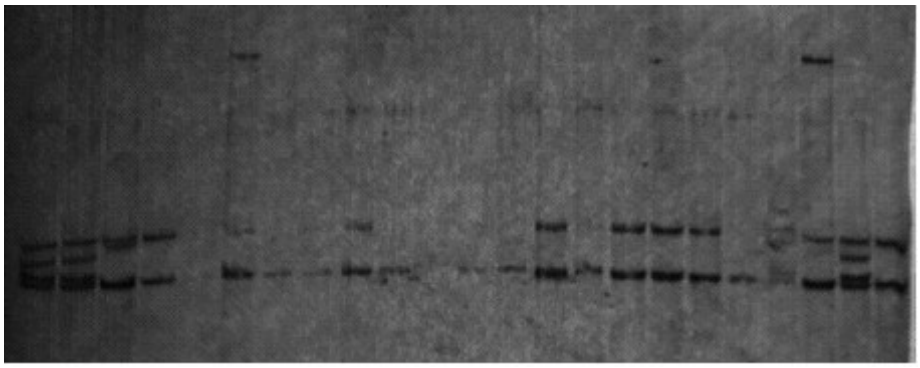

Figure A.6. 20% gel; without glycerol; from 29:1 acrylamide:bisacrylamide stock solution

Selected electrophoretograms after optimization of SSCP conditions:

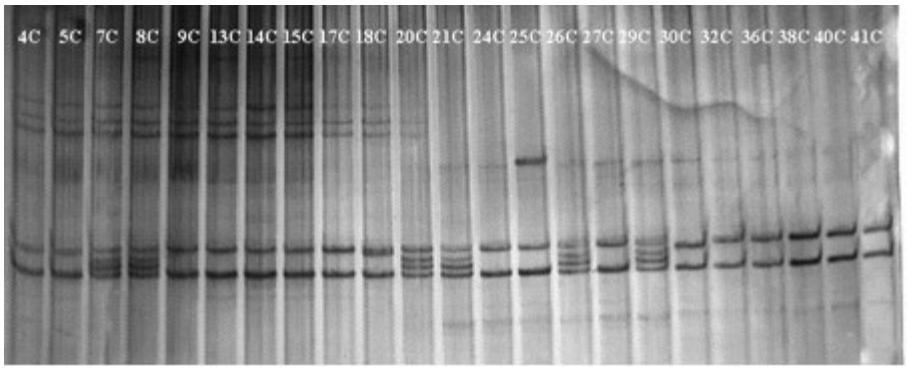

Figure A.7. 17% gel; without glycerol; 99:1 acrylamide:bisacrylamide with 24 hours running time

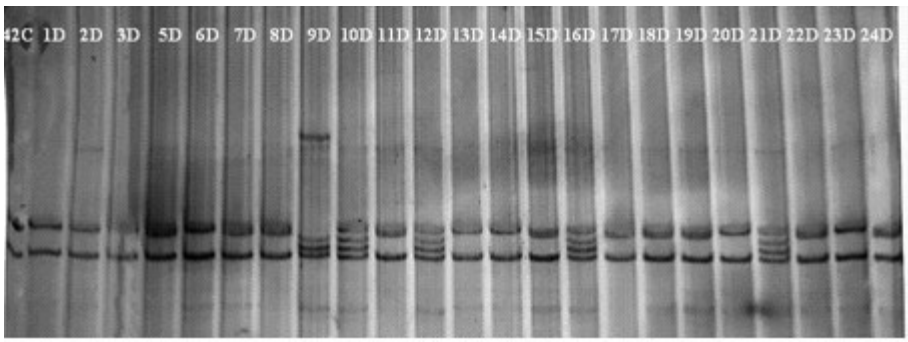

Figure A.8. 17% gel; without glycerol; 99:1 acrylamide:bisacrylamide with 24 hours running time

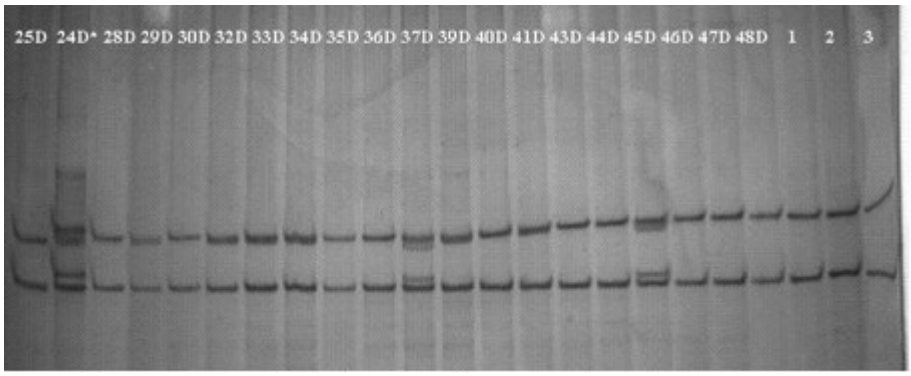

Figure A.9. 17% gel; without glycerol; 99:1 acrylamide:bisacrylamide with 24 hours running time

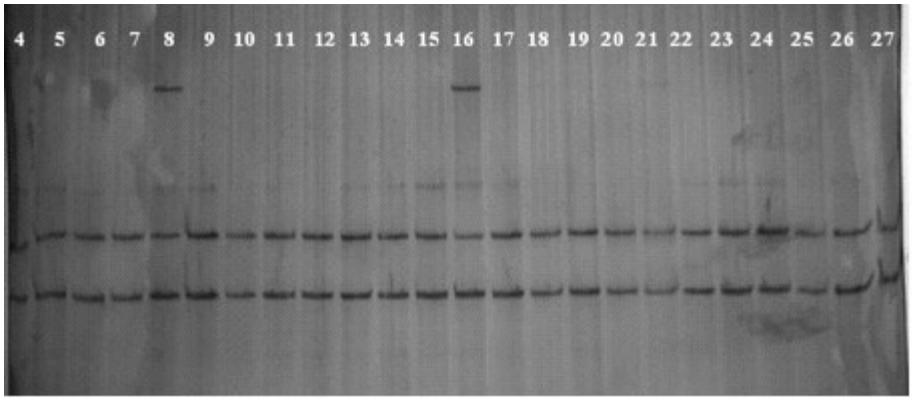

Figure A.10. 17% gel; without glycerol; 99:1 acrylamide:bisacrylamide with 24 hours running time

APPENDIX B

KATILIMCI BİLGİLENDİRİLMİŞ ONAMA FORMU

BİLGİLENDİRME

Sığır sütü beta-kazein bileşeninin A1 tipi insan sindirim sisteminde beta-kazamorfin-7 peptidini oluşturmaktadır. Peptidin Tip-I diyabeti oluşturan nedenlerden biri olabileceği düşünülmektedir. Çalışmamızda, bu küçük peptidin insanlarda bağışıklık sistemini uyarıp uyarmadığı sağlıklı ve Tip-I diyabetli insanlarda karşılaştırmalı olarak araştırılmak istenmektedir.

Böyle bir bağışıklık sistemi cevabı, katılımcılardan alınan kandan tek çekirdekli kan hücrelerinin ayrıştırılması, hücre kültürü yapılması ve hücre kültürlerine peptit uygulaması ve daha sonra ELISA metodu kullanılarak sitokin üretiminin miktarının belirlenmesi ile saptanacaktır. Bu ön araştırma ile A1 tipi inek sütü içildiği zaman bazı insanlarda ortaya çıkabilecek sağlık problemlerinin anlaşılmasına katkıda bulunabilecektir.

Katılımcılardan alınan kan örnekleri, sadece bahsedilen araştırma için kullanılacaktır.

KATILIMCI ONAMI

Aşağıda imzası bulunan ben, "İnsan ve Hayvan Sağlığı ve Peynir Verimi ile İlişkisi Olan Süt Proteinlerinden β-Kazein, β-Laktoglobulin ve K-Kazein'in Bazı Genotiplerinin DNA'ya Dayalı Yöntemlerle Tanısı" başlıklı Orta Doğu Teknik Üniversitesi, Biyolojik Bilimler Bölümü'nde Prof. Dr. İnci TOGAN danışmanlığında yürütülmekte olan doktora tezi için yapılması planlanan çalışma hakkında doktora çalışması sahibi Havva Dinç'ten tam olarak bilgi aldığımı beyan ederim.

Aşağıda imzası bulunan çalışma sahibi ben, Havva Dinç'ten bu bilgileri aldıktan sonra, yapılması planlanan çalışmanın özelliklerini ve sonuçlarını anlıyorum. Araştırma sonuçlarının eğitim ya da bilimsel amaçlarla kullanılması sırasında mahremiyetime saygı gösterileceğine inanıyorum. Bu şartlar altında sözkonusu araştırmaya kendi rızamla, hiçbir baskı ve zorlama olmaksızın katılmayı kabul ediyorum.

Tarih: 28.04.2009

Bilgilendirmeyi yapan	**Katılımcı**	**Kanı Alan Kuruluş ve Kuruluş Görevlisi**

Havva DİNÇ

---

1. Cinsiyetiniz nedir?

2. Kaç yaşındasınız?

3. Tip I diyabet hastası mısınız?

4. Tip I diyabet hastası iseniz, kaç yaşında tip-I diyabet oldunuz?

5. Ailenizde tip-I diyabet hastası var mı? Varsa akrabanın yakınlığı kaçıncı derecedir?

6. Ailenizde tip-II diyabet hastası var mı? Varsa akrabanın yakınlığı kaçıncı derecedir?

7. Ailede şizofreni ve otizm gibi hastalıklar var mı? Varsa akrabanın yakınlığı kaçıncı derecedir?

8. Süt alerjiniz var mı? Varsa tarif ediniz.

9. Sizce bağışıklık sisteminiz kuvvetli mi?

APPENDIX C

GENOTYPES

Table C.1. Genotypes of Turkish Grey individuals in the current study for three milk protein genes.

Sample No.	Beta-casein	Kappa-casein	BLG	Sample No.	Beta-casein	Kappa-casein	BLG
1A	A2B	AA	BB	**26A**	A1A1	AB	AA
2A	A2B	AA	AA	**27A**	A2A1	AB	AA
3A	A2A2	AA	AB	**28A**	A2A2	AB	BB
4A	A2A2	AA	AB	**29A**	A2A1	AA	AB
5A	A2A1	AB	AB	**30A**	A2A2	AA	BB
6A	A2A2	AA	AB	**31A**	A1A1	AB	BB
7A	A2A2	AA	AB	**32A**	-	AB	AB
8A	A2A2	AA	BB	**33A**	A2A1	AA	AB
9A	-	AB	AB	**34A**	-	AA	AB
10A	-	AA	AA	**35A**	A2A2	AA	AB
11A	A2A1	BB	AB	**36A**		BB	AB
12A	A2A1	BB	AA	**37A**	A2A2	AA	AB
13A		AA	AA	**38A**	A2A2	AB	BB
14A	A1A1	BB	AB	**39A**	A1A1	AB	AA
15A	A2A1	AA	AA	**40A**	-	BB	AA
16A	-	AA	AA	**41A**	-	AB	BB
17A	-	AA	BB	**42A**	A2A2	AA	AB
18A	A1A1	AB	AB	**43A**	-	AA	AB

Table C.1. (continued)

19A	A2A1	AB	AB	**44A**	-	AA	AA
20A	A2A1	AB	BB	**45A**	A2A1	AA	AA
21A	-	AA	BB	**46A**	A1A1	AB	AA
22A	A1A1	AA	AB	**47A**	A2A1	AB	AB
23A	A2A1	AB	BB				
24A	A1A1	AB	AB				
25A	A2A1	AB	AB				

Table C.2. Genotypes of Eastern Anatolian Red individuals in the current study for three milk protein genes.

Sample No.	Beta-casein	Kappa-casein	BLG	Sample No.	Beta-casein	Kappa-casein	BLG
1B	-	BB	BB	**22B**	A2A2	AB	AB
2B	A2A2	AA	BB	**23B**	-	AB	AB
3B	A2A1	AA	AB	**24B**	A2A2	AB	BB
4B	A2A2	AA	BB	**25B**	A2A1	AA	BB
5B	A2A2	AB	BB	**26B**	A2A2	AA	BB
6B	-	BB	BB	**27B**	A2A2	AA	AB
7B	A1B or BB	AB	BB	**28B**	A2A2	AA	AB
8B	A2A2	AB	BB	**29B**	A2A2	AA	BB
9B	A1B or BB	AB	AB	**30B**	A2A2	AA	AB
10B	A2A2	BB	BB	**31B**	A2A2	AA	AB
11B	A2B	BB	BB	**32B**	A2A2	AA	AB
12B	A2A2	AB	AB	**33B**	A2B	AA	AA
13B	-	AA	BB	**34B**	A2A2	AA	BB
14B	-	BB	AB	**35B**	A2A1	AA	AB
15B	A2A2	BB	AB	**36B**	-	AB	BB
16B	-	BB	AB	**37B**	-	AA	BB
17B	-	AA	BB	**38B**	-	AB	BB
18B	A2A2	AA	BB	**39B**	-	AA	AB
19B	-	AB	BB	**40B**	-	BB	AB
20B	A2A2	AA	BB	**41B**	-	AA	BB
21B	A2A1	AB	BB				

Table C.3. Genotypes of Anatolian Black individuals in the current study for three milk protein genes.

Sample No.	Beta-casein	Kappa-casein	BLG	Sample No.	Beta-casein	Kappa-casein	BLG
1C	A2A2	AA	BB	**22C**	-	AB	AA
2C	A1A1	AB	BB	**23C**	-	AA	AB
3C	-	AB	BB	**24C**	-	AB	AB
4C	A2A2	AA	AA	**25C**	-	BB	BB
5C	A2A2	AB	BB	**26C**	-	BB	BB
6C	-	AB	BB	**27C**	-	AB	AB
7C	A2B	AB	AB	**28C**	-	AA	AB
8C	A2B	AA	BB	**29C**	A2B	AA	BB
9C	A2A1	AA	AB	**30C**	A2A2	AA	AB
10C	-	AA	AA	**31C**	-	AB	BB
11C	-	AB	AA	**32C**	-	AA	AA
12C		AA	AB	**33C**	-	AB	BB
13C	A2A2	AB	BB	**34C**	-	AB	BB
14C	-	BB	BB	**35C**		AA	BB
15C	-	AA	AB	**36C**	A2A2	AA	AA
16C		AB	AB	**37C**	-	AB	BB
17C	-	BB	AA	**38C**	A2A2	AB	AA
18C	-	AA	BB	**39C**	A2A1	AA	AA
19C	-	AB	BB	**40C**	A2A2	AB	AB
20C	-	AA	AB	**41C**	A2A2	AA	AB
21C	-	BB	BB	**42C**	A2A2	AB	AA

Table C.4. Genotypes of Southern Anatolian Red individuals in the current study for three milk protein genes.

Sample No.	Beta-casein	Kappa-casein	BLG	Sample No.	Beta-casein	Kappa-casein	BLG
1D	A2A1	AB	AB	**26D**	-	AB	AA
2D	A2A2	AB	BB	**27D**	-	AB	AB
3D	A2A2	BB	BB	**28D**	-	BB	BB
4D	A2A2	BB	BB	**29D**	-	AA	BB
5D	-	AB	BB	**30D**	-	AA	AB
6D	A2A1	AB	AB	**32D**	A2A1	AA	BB
7D	A2A1	AA	BB	**33D**	A2A2	AB	AB
8D	A2A2	AB	AB	**34D**	-	AA	AB
9D	A2B	BB	AA	**35D**	-	AB	BB
10D	A2B	AA	AB	**36D**	-	AA	BB
11D	-	AB	BB	**37D**	A2B	AA	BB
12D	A2B	AA	BB	**38D**	A2A2	AA	BB
13D	-	AA	AB	**39D**	A2A1	AB	BB
14D	-	AA	AB	**40D**	A2A2	AB	BB
15D	A2A2	AB	BB	**41D**	A2A2	AA	BB
16D	A2B	AB	BB	**42D**	-	AA	BB
17D	A2A2	AA	AB	**43D**	A2A2	AA	BB
18D	A2A2	AB	BB	**44D**	A2A1	AB	BB
19D	A2A2	AA	BB	**45D**	A2B	BB	BB
20D	-	AA	BB	**46D**	A2A2	AA	BB
21D	A2B	AB	BB	**47D**	A2A2	BB	BB
22D	-	AB	BB	**48D**	A2A2	AA	AB
23D	-	AB	BB	**49D**	-	AB	AB
24D	-	AA	BB				
25D	A2A1	AB	AB				

Table C.5. Genotypes of Turkish Holstein individuals in the current study for three milk protein genes.

Sample No.	Beta-casein	Kappa-casein	BLG	Sample No.	Beta-casein	Kappa-casein	BLG
1G	-	AA	AB	**26G**	A2A1	AB	AA
2G	-	AB	BB	**27G**	A2A1	AA	AB
3G	A2B	AB	BB	**28G**	A2A2	AA	AB
4G	-	AA	AA	**29G**	A1A1	AA	AB
5G	A2A1	AA	BB	**30G**	-	AB	AB
6G	A1A1	AB	BB	**31G**	A2A1	AA	AB
7G	A1A1	AB	AB	**32G**	-	AA	AB
8G	-	AA	AA	**33G**	-	AA	BB
9G	A1A1	AA	AB	**34G**	-	AA	AB
10G	A2A1	AA	AB	**35G**	A2A1	AA	AB
11G	-	AA	BB	**36G**	A2A1	AB	AA
12G		BB	AA	**37G**	-	AA	AA
13G	-	BB	AA	**38G**	-	AA	AB
14G	-	AA	AB	**39G**	-	AA	BB
15G	-	AA	AB	**40G**	A2A1	AB	AB
16G	A2A1	AA	AB	**41G**	-	AA	AA
17G	-	AA	BB	**42G**	-	AB	AB
18G	-	AB	BB	**43G**	A2A1	AB	AB
19G	A2A1	AB	AB	**44G**	A2A1	AA	AB
20G	A2A1	AA	AB	**45G**	-	AA	BB
21G	A2A3 or A3A3	AA	BB	**46G**	-	AB	AA
22G	A2A1	AA	BB	**47G**	-	AA	BB
23G	-	AA	BB	**48G**	-	AB	AA
24G	A2A1	AB	BB	**49G**	-	AA	AA
25G	A2A2	AA	AA				

Table C.6. Genotypes of Holstein Candidate Bulls individuals in the current study for three milk protein genes.

Sample No.	Beta-casein	Kappa-casein	BLG
1	A2A1	AA	BB
2	A2A2	AA	AB
3	-	AB	AA
4	A2A1	AA	AB
5	A2A1	AA	BB
6	A2A2	AA	AB
7	-	AA	AA
8	A2A2	AB	AB
9	-	AA	AB
10	-	AA	AB
11	A2A1	AB	AB
12	A2A1	AA	AB
13	A2A2	AA	AA
14	A2A2	AA	AB
15	A2A2	AB	BB
16	A2A2	AB	AA
17	A2A1	AB	AA
18	A2A1	AE	-
19	A2A1	AB	AB
20	A2A2	AE	-
21	-	AA	-
22	A2A1	AE	AA
23	A2A1	AA	AB
24	-	AA	-
25	-	AA	-
26	-	AA	AB
27	-	AB	BB

APPENDIX D

P-VALUES for Fis

Table D.1. P-values (based on 18000 randomisations) for Fis values within samples presented in Table 3.11. Indicative adjusted nominal level (5%) for one table is 0.00278. (Performed by FSTAT software program)

a. Proportion of randomisations that gave a larger Fis than the observed

Loci	p-values for each breed					
	TG	EAR	AB	SAR	TH	HCB
Beta-casein	0.1867	10.000	0.3036	10.000	0.9801	10.000
Kappa-casein	0.3686	0.0232	0.5969	0.5125	0.5708	10.000
BLG	0.5287	0.9081	0.0208	0.5111	0.3173	0.7858
All	0.1318	0.1669	0.0313	0.6599	0.8521	0.9564

b. Proportion of randomisations that gave a smaller Fis than the observed

Loci	p-values					
	TG	EAR	AB	SAR	TH	HCB
Beta-casein	0.8939	0.7052	0.9273	0.1394	0.0471	0.1799
Kappa-casein	0.8466	0.9963	0.6664	0.7276	0.7652	0.2721
BLG	0.6966	0.3754	0.9969	0.8103	0.8526	0.5302
All	0.8799	0.8963	0.9772	0.3660	0.1510	0.0593

Table D.2. P-values (based on 35000 randomisations) for Fis values within samples presented in Table 4.10. Indicative adjusted nominal level (5%) for one table is: 0.00143. (Performed by FSTAT software program)

a. Proportion of randomisations that gave a larger Fis than the observed

Loci	p-values				
	TG	**EAR**	**AB**	**SAR**	**TH**
Beta-casein	0.1554	10.000	0.3074	10.000	0.9814
Kappa-casein	0.3694	0.0217	0.6051	0.5043	0.5686
BLG	0.5286	0.9089	0.0209	0.5183	0.3206
GH-MspI	0.9660	0.9829	0.5066	0.8837	0.8154
GH-AluI	0.2086	0.0434	0.9937	0.6711	0.0940
Prolactin	10.000	10.000	0.8900	0.4714	0.3103
DGAT1	0.0906	10.000	0.0009	10.000	0.0129
All	0.3294	0.9123	0.0471	0.8023	0.1306

b. Proportion of randomisations that gave a smaller Fis than the observed

Loci	p-values				
	TG	**EAR**	**AB**	**SAR**	**TH**
Beta-casein	0.9120	0.7091	0.9311	0.1411	0.0483
Kappa-casein	0.8417	0.9963	0.6706	0.7374	0.7614
BLG	0.6911	0.3834	0.9957	0.8046	0.8526
GH-MspI	0.1500	0.1349	0.7334	0.2994	0.5554
GH-AluI	0.9654	0.9971	0.0354	0.6686	0.9886
Prolactin	0.0063	0.0020	0.3140	0.8214	0.9726
DGAT1	0.9840	0.1389	0.9997	0.2991	0.9989
All	0.6709	0.0880	0.9531	0.1980	0.8697

Table D.3. P-values (based on 35000 randomisations) for Fis values within samples presented in Table 4.11. Indicative adjusted nominal level (5%) for one table is: 0.00143. (Performed by FSTAT software program)

a. Proportion of randomisations that gave a larger Fis than the observed

Loci	p-values				
	TG	**EAR**	**AB**	**SAR**	**TH**
ETH10	0.4999	0.2174	0.1462	0.1199	0.5508
ETH225	0.0565	0.6537	0.0164	0.1867	0.7374
HEL5	0.0168	0.0835	0.5991	0.0879	0.5674
ILSTS005	0.1717	0.0559	0.2730	0.4281	0.6732
All	0.0000	0.0000	0.0003	0.0000	0.7037

b. Proportion of randomisations that gave a smaller Fis than the observed

Loci	p-values				
	TG	**EAR**	**AB**	**SAR**	**TH**
ETH10	0.6509	0.8883	0.9244	0.9373	0.6088
ETH225	0.9743	0.5464	0.9941	0.9017	0.4016
HEL5	0.9935	0.9644	0.5753	0.9571	0.5716
ILSTS005	0.9023	0.9752	0.8377	0.7297	0.5571
All	10.000	10.000	0.9998	10.000	0.3474

APPENDIX E

CFSE RESULTS

Carboxyfluorescein diacetate succinimidyl ester (CFSE) assay results of each individual obtained from flow cytometry were presented in Figure 1- Figure 6. Four stimulations and the percentages of cells found in LL (lower left quadrate) were indicated on the figures.

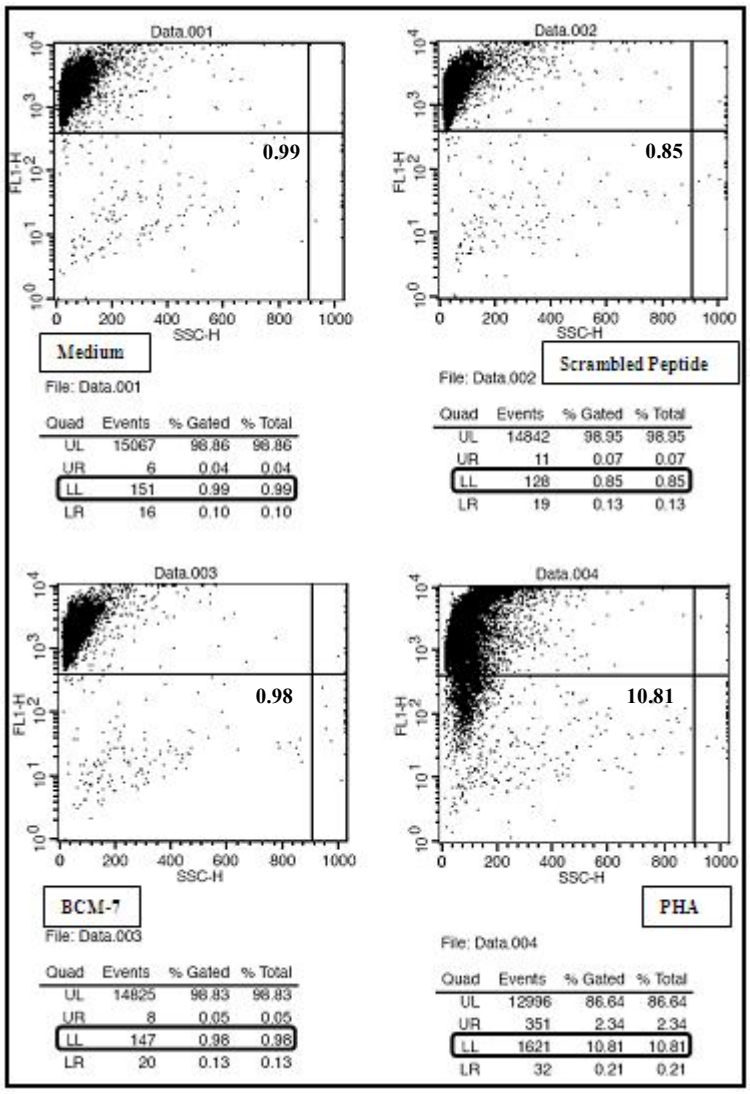

Figure E.1. CFSE staining cell proliferation results of first healthy individual (H1) after 4 stimulations (Medium, Scrambled Peptide, BCM-7 and PHA) by flow-cytometry.

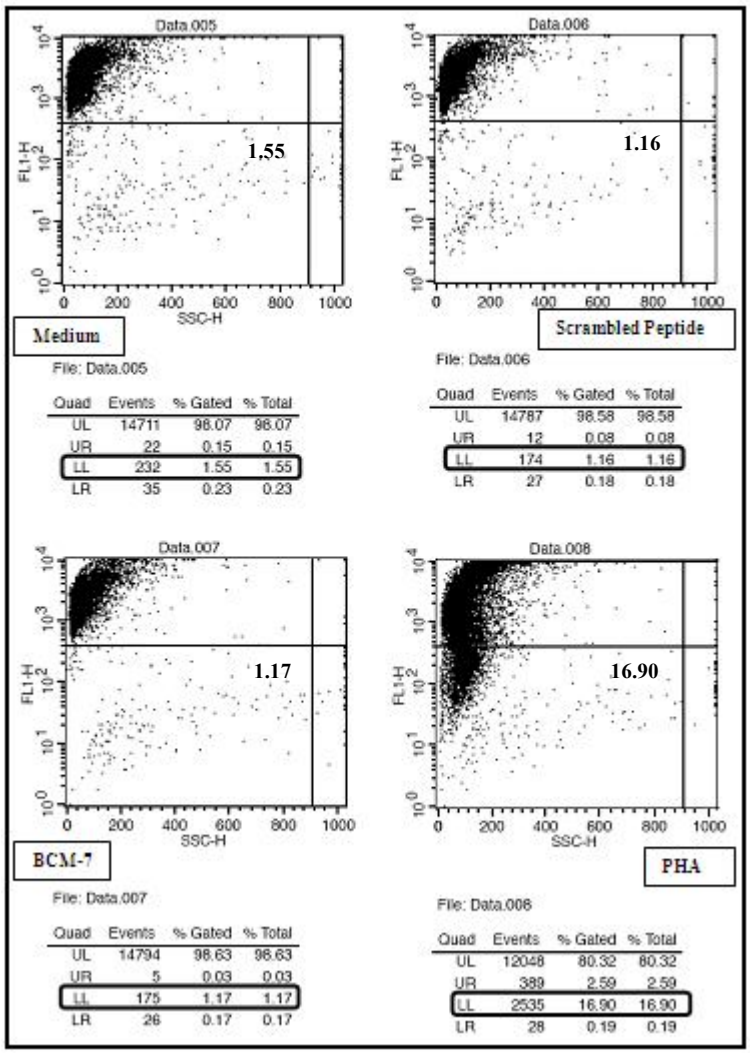

Figure E.2. CFSE staining cell proliferation results of second healthy individual (H2) after 4 stimulations (Medium, Scrambled Peptide, BCM-7 and PHA) by flow-cytometry.

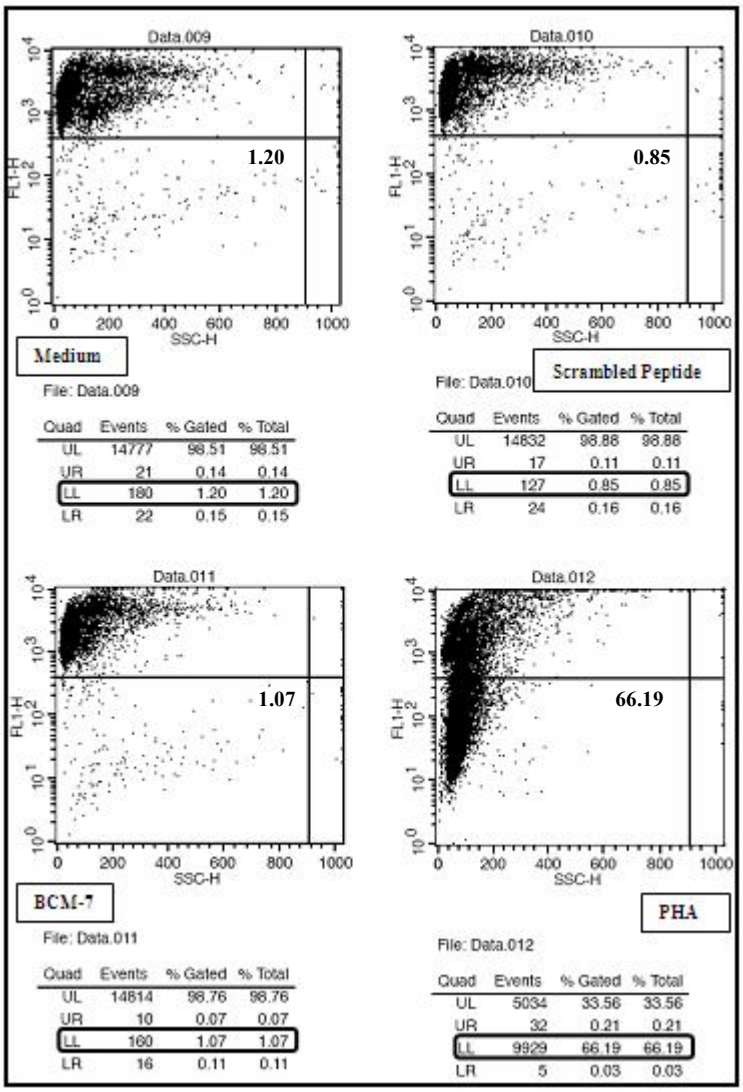

Figure E.3. CFSE staining cell proliferation results of first type-1 diabetic patient (D1) after 4 stimulations (Medium, Scrambled Peptide, BCM-7 and PHA) by flow-cytometry.

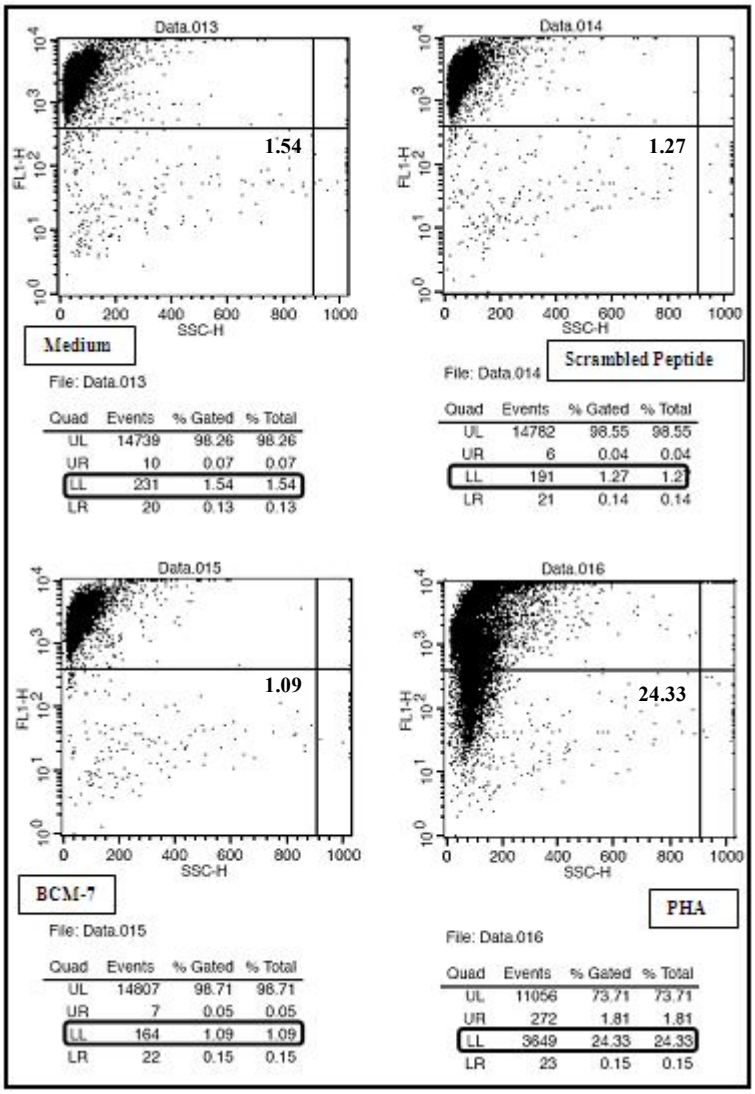

Figure E.4. CFSE staining cell proliferation results of second type-1 diabetic patient (D2) after 4 stimulations (Medium, Scrambled Peptide, BCM-7 and PHA) by flow-cytometry.

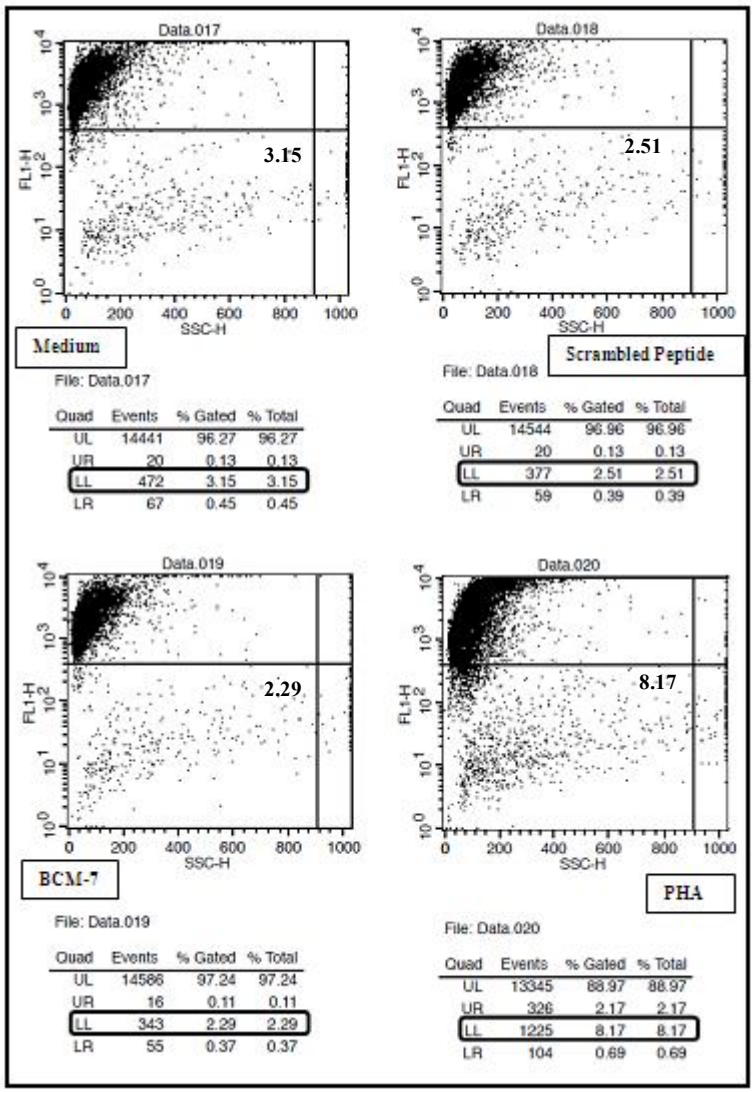

Figure E.5. CFSE staining cell proliferation results of first celiac patient (C1) after 4 stimulations (Medium, Scrambled Peptide, BCM-7 and PHA) by flow-cytometry.

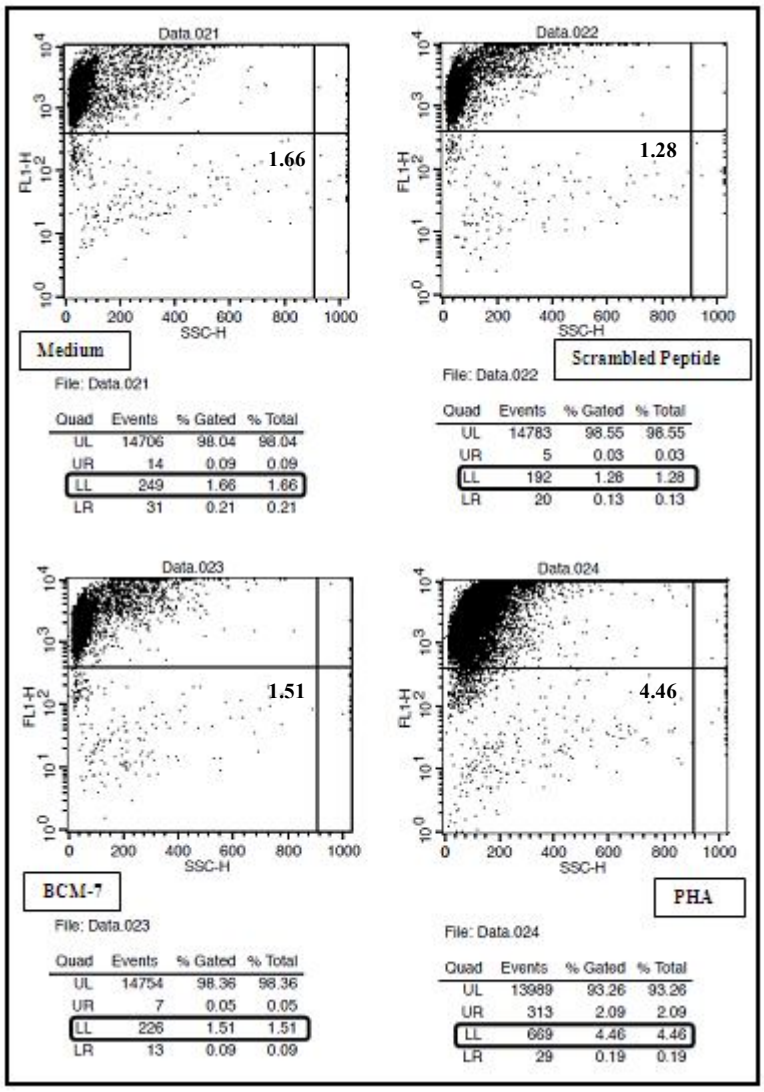

Figure E.6. CFSE staining cell proliferation results of first celiac patient (C2) after 4 stimulations (Medium, Scrambled Peptide, BCM-7 and PHA) by flow-cytometry.

APPENDIX F

FREQUENCY DISTRIBUTIONS

Table F.1. Occurrences of GH-MspI gene variants in various breeds and countries.

Allele Frequencies		Breed (N)	References
+	-		
0.55	0.45	Mazandarani (97)	Zakizadeh *et al.,* 2006
0.51	0.49	Sarabi (87)	Zakizadeh *et al.,* 2006
0.44	0.56	Golpaygani (112)	Zakizadeh *et al.,* 2006
0.83	0.17	Holstein (110)	Zakizadeh *et al.,* 2006
0.87	0.12	Beijing Holstein (543)	Zhou *et al.,* 2005
0.88	0.12	Gir (49)	Sodhi *et al.,* 2006
0.91	0.09	Kankrej (40)	Sodhi *et al.,* 2006
0.88	0.12	Rathi (46)	Sodhi *et al.,* 2006
0.94	0.06	Sahiwal (48)	Sodhi *et al.,* 2006
0.91	0.09	Tharparkar (47)	Sodhi *et al.,* 2006
0.91	0.09	Deoni (40)	Sodhi *et al.,* 2006
0.80	0.20	Gaolao (51)	Sodhi *et al.,* 2006
0.80	0.20	Hariana (45)	Sodhi *et al.,* 2006
0.91	0.09	Mewati (50)	Sodhi *et al.,* 2006
0.90	0.10	Ongole (48)	Sodhi *et al.,* 2006
0.88	0.12	Amritmahal (45)	Sodhi *et al.,* 2006
0.91	0.09	Dangi (38)	Sodhi *et al.,* 2006
0.86	0.14	Kangyam (40)	Sodhi *et al.,* 2006
0.89	0.11	Khillar (40)	Sodhi *et al.,* 2006
0.67	0.33	Nagori (40)	Sodhi *et al.,* 2006
0.79	0.21	Red Kandhari (46)	Sodhi *et al.,* 2006

Table F.1. (continued)

0.89	0.11	Umblachery (37)	Sodhi *et al.,* 2006
0.86	0.14	Angus (65)	Lagziel *et al.,* 2000
0.12	0.88	Boran (4)	Lagziel *et al.,* 2000
0.35	0.65	Brahman(23)	Lagziel *et al.,* 2000
0.74	0.26	Brown Carpathian (22)	Lagziel *et al.,* 2000
0.78	0.22	Charolais (7)	Lagziel *et al.,* 2000
0.11	0.89	Gir (18)	Lagziel *et al.,* 2000
0.94	0.06	Gelbvieh	Lagziel *et al.,* 2000
0.67	0.33	Gray Ukranian (22)	Lagziel *et al.,* 2000
1.00	0.00	Hereford (30)	Lagziel *et al.,* 2000
0.90	0.10	Holstein (911)	Lagziel *et al.,* 2000
0.85	0.15	Jersey (13)	Lagziel *et al.,* 2000
0.61	0.39	Limousin (18)	Lagziel *et al.,* 2000
1.00	0.00	N'Dama(18)	Lagziel *et al.,* 2000
0.18	0.82	Nellore (20)	Lagziel *et al.,* 2000
0.95	0.05	Norwegian Red (32)	Lagziel *et al.,* 2000
0.00	1.00	Ongole (4)	Lagziel *et al.,* 2000
0.81	0.19	Red Danish (58)	Lagziel *et al.,* 2000
0.48	0.52	Reggiana (26)	Lagziel *et al.,* 2000
0.96	0.04	Simmental (23)	Lagziel *et al.,* 2000
0.55	0.45	Siri (9)	Lagziel *et al.,* 2000
0.62	0.38	Anatolian Black (21)	Baklacı, 2005
0.78	0.22	Anatolian Grey (25)	Baklacı, 2005
0.47	0.52	Kilis (21)	Baklacı, 2005
0.74	0.26	Anatolian Yellow (21)	Baklacı, 2005
0.52	0.47	Holstein (20)	Baklacı, 2005
0.58	0.42	East Anatolian Red (26)	Baklacı, 2005
0.67	0.33	South Anatolian Red (50)	Yardibi *et al.,* 2009
0.59	0.41	East Anatolian Red (50)	Yardibi *et al.,* 2009
0.15	0.85	Nellore (111)	Unanian *et al.,* 2002

Table F.2. Occurrences of GH-AluI gene variants in various breeds and countries.

Allele Frequencies		Breed(N)	References
L	V		
0.91	0.09	Mazandarani (97)	Zakizadeh *et al.,* 2006
0.84	0.16	Sarabi (87)	Zakizadeh *et al.,* 2006
0.92	0.08	Golpaygani (112)	Zakizadeh *et al.,* 2006
0.85	0.15	Holstein (110)	Zakizadeh *et al.,* 2006
0.69	0.31	Polish Friesian (214)	Grochowska *et al.,* 2001
0.94	0.06	Iranian Holstein (134)	Sadeghi *et al.,* 2008
0.86	0.14	Alenttejana (22)	Reis *et al.,* 2001
0.72	0.29	Arouquesa (24)	Reis *et al.,* 2001
0.59	0.41	Barrosá (23)	Reis *et al.,* 2001
0.87	0.12	Marinhoa (32)	Reis *et al.,* 2001
0.40	0.60	Maronesa (24)	Reis *et al.,* 2001
0.95	0.04	Mertolenga (22)	Reis *et al.,* 2001
0.93	0.07	Mirandesa (21)	Reis *et al.,* 2001
0.76	0.24	Preta (27)	Reis *et al.,* 2001
0.93	0.07	Hungarian Holstein (363)	Kovács *et al.,* 2006
0.09	0.91	Canadian Holstein Friesian (100)	Sabour & Lin, 1996
0.93	0.07	Danish Holstein (568)	Lovendahl *et al.,* 1997
0.83	0.17	Red Danish (145)	Lovendahl *et al.,* 1997
0.53	0.47	Danish Jersey (74)	Lovendahl *et al.,* 1997
0.55	0.45	Slovak Pinzgauer (80)	Chrenek *et al.,* 1998
0.72	0.28	Slovak Holstein (75)	Chrenek *et al.,* 1998
0.82	0.18	Hostein Friesian (477)	Shariflou *et al.,* 1998
0.82	0.18	Australian Holstein (384)	Shariflou *et al.,* 2000
0.59	0.41	Polish Friesian (142)	Oprzadek *et al.,* 1999
0.29	0.71	Ayrshire (100)	Sabour *et al.,*1997
0.09	0.91	Holstein (51)	Sabour *et al.,*1997
0.24	0.76	Jersey (21)	Sabour *et al.,*1997
0.81	0.18	Black & White (1086)	Dybus *et al.,* 2002
0.56	0.44	South Anatolian Red (50)	Yardibi *et al.,* 2009
0.43	0.57	East Anatolian Red (50)	Yardibi *et al.,* 2009
0.85	0.15	Podolian (132)	Dario *et al.,* 2005
0.96	0.04	German Holstein (315)	Hradecká *et al.,* 2008

Table F.3. Occurrences of DGAT1 gene variants in various breeds and countries.

Allele Frequencies		Breed(N)	References
K	A		
0.59	0.41	Polish Black&White (457)	Pareek *et al.,* 2005
0.34	0.66	German Holstein (315)	Hradecká *et al.,* 2008
0.11	0.89	Uruguayan Creole (575)	Rincón *et al.,* 2006
0.07	0.92	Fleckvieh (833)	Thaller *et al.,* 2003
0.55	0.45	German Holstein (858)	Thaller *et al.,* 2003
0.96	0.04	Gyr (53)	Lacorte *et al.,* 2006
1.00	0.00	Guzerat (53)	Lacorte *et al.,* 2006
1.00	0.00	Nellore (62)	Lacorte *et al.,* 2006
0.97	0.02	Red Sindhi (60)	Lacorte *et al.,* 2006
0.27	0.73	Holstein (50)	Lacorte *et al.,* 2006
0.61	0.39	Gyr x Holstein (53)	Lacorte *et al.,* 2006
0.83	0.17	Jersey (100)	Komisarek *et al.,* 2004
0.82	0.18	Holstein (196)	Hori-Oshima & Barreras-Serrano, 2003
0.54	0.46	Polish Holstein-Friesian (89)	Nowacka-Woszuk *et al.,* 2008
0.55	0.45	German Holstein-Friesian (1291)	Kaupe *et al.,* 2007
0.40	0.60	German Holstein-Friesian (66)	Citek et.al., 2007
0.42	0.58	German Holstein-Friesian (79)	Kaupe *et al.,* 2004
0.45	0.55	German Holstein-Friesian (28)	Thaller *et al.,* 2003
0.35	0.65	German Holstein-Friesian (40)	Winter *et al.,* 2002
0.63	0.37	Dutch Holstein-Friesian (1818 males) & New Zealand Holstein-Friesian (529 females)	Grisart *et al.,* 2002
0.40	0.60	Dutch Holstein-Friesian (1762)	Schenninka *et al.,* 2007
0.60	0.40	New Zealand Holstein-Friesian (1527)	Spelman *et al.,* 2002
0.27	0.73	Brasilian Holstein (50)	Lacorte *et al.,* 2006
0.65	0.35	Polish Holstein-Friesian (244)	Pareek *et al.,* 2005
0.40	0.60	Polish Holstein-Friesian (177)	Strzalkowska *et al.,* 2005
0.61	0.39	German Angeln (805)	Sanders *et al.,* 2006
0.13	0.87	Aberdeen Angus (43)	Kaupe *et al.,* 2004
0.38	0.62	Anatolian Black(73)	Kaupe *et al.,* 2004
0.61	0.39	Angler (48)	Kaupe *et al.,* 2004

Table F.3. (continued)

0.23	0.77	Asturian Mountain (50)	Kaupe *et al.,* 2004
0.02	0.98	Ayrshire (41)	Kaupe *et al.,* 2004
0.88	0.12	Banyo Gudali (72)	Kaupe *et al.,* 2004
0.00	1.00	Belgian Blue (beef) (30)	Kaupe *et al.,* 2004
0.03	0.97	Belgian Blue (mixed) (15)	Kaupe *et al.,* 2004
0.14	0.86	Bohemian Red (44)	Kaupe *et al.,* 2004
0.03	0.97	British Frisian (49)	Kaupe *et al.,* 2004
0.38	0.62	Casta Navarra (42)	Kaupe *et al.,* 2004
0.08	0.92	Charolais (31)	Kaupe *et al.,* 2004
0.34	0.66	Chianina (44)	Kaupe *et al.,* 2004
0.25	0.75	East Anatolian Red (50)	Kaupe *et al.,* 2004
0.00	1.00	Gelbvieh (30)	Kaupe *et al.,* 2004
0.13	0.87	German Angus (54)	Kaupe *et al.,* 2004
0.01	0.99	German Black Pied (41)	Kaupe *et al.,* 2004
0.06	0.94	German Brown (8)	Kaupe *et al.,* 2004
0.02	0.98	German Brown Swiss (48)	Kaupe *et al.,* 2004
0.42	0.58	German Holstein (79)	Kaupe *et al.,* 2004
0.06	0.94	German Simmental (126)	Kaupe *et al.,* 2004
0.00	1.00	Hereford (50)	Kaupe *et al.,* 2004
0.30	0.70	Istrian (49)	Kaupe *et al.,* 2004
0.69	0.31	Jersey (47)	Kaupe *et al.,* 2004
0.50	0.50	Maremmana (48)	Kaupe *et al.,* 2004
0.02	0.98	Menorquina (50)	Kaupe *et al.,* 2004
0.52	0.48	N'Dama (25)	Kaupe *et al.,* 2004
0.99	0.01	Nellore (46)	Kaupe *et al.,* 2004
0.01	0.99	Pezzata Rossa (47)	Kaupe *et al.,* 2004
0.01	0.99	Piemontese (40)	Kaupe *et al.,* 2004
0.00	1.00	Pinzgauer (42)	Kaupe *et al.,* 2004
0.06	0.94	Polish Red (44)	Kaupe *et al.,* 2004
0.36	0.64	Santa Gertrudis (48)	Kaupe *et al.,* 2004
0.00	1.00	Slavonian Syrmian (6)	Kaupe *et al.,* 2004
0.21	0.79	South Anatolian Red (48)	Kaupe *et al.,* 2004
0.21	0.79	Toro de Lidia (47)	Kaupe *et al.,* 2004
0.36	0.64	Turkish Grey Steppe (49)	Kaupe *et al.,* 2004
0.92	0.08	White Fulani (44)	Kaupe *et al.,* 2004

Table F.4. Occurrences of prolactin gene variants in various breeds and countries.

Allele Frequencies		Breed(N)	References
A	B		
0.73	0.27	Holstein	Chung *et al.,* 1996
0.86	0.14	Russian Ayrshire(46)	Udina *et al.,* 2001
0.91	0.09	Gorbatov Red (35)	Udina *et al.,* 2001
0.96	0.04	Argentine Creole (230)	Lirón *et al.,* 2002
0.95	0.05	Patagonian Creole (25)	Lirón *et al.,* 2002
0.82	0.18	Saavedreño (140)	Lirón *et al.,* 2002
1.00	0.00	Yacumeño (27)	Lirón *et al.,* 2002
0.91	0.09	Chusco (11)	Lirón *et al.,* 2002
0.98	0.02	Chaqueño Boliviano (30),	Lirón *et al.,* 2002
0.31	0.69	Jersey (185)	Dybus *et al.,* 2005
0.85	0.15	Black-and-White (242)	Dybus *et al.,* 2005
0.79	0.21	Lithuanian Black &White (52) and Lithuanian Red (136)	Skinkytė *et al.,*2005
0.61	0.39	German Black &White(32)	Khatami *et al.,* 2005
0.65	0.35	Yaroslavl(120)	Khatami *et al.,* 2005
0.95	0.05	Russian Black &White(32)	Khatami *et al.,* 2005
0.95	0.05	Lithuanian Light Grey(11)	Miceikienė *et al.,* (2006)
0.80	0.20	Lithuanian Black and White (36)	Miceikienė *et al.,* (2006)
0.97	0.03	Lithuanian White Backed (18)	Miceikienė *et al.,* (2006)
0.77	0.23	Lithuanian Red (26)	Miceikienė *et al.,* (2006)
0.79	0.21	Russian Red Pied (125)	Alipanah *et al.,* 2007
0.56	0.44	South Anatolian Red (40)	Öztabak *et al.,* 2008
0.74	0.26	East Anatolian Red (40)	Öztabak *et al.,* 2008
0.51	0.49	Kankrej (51)	Sacravarty *et al.,* 2008
0.58	0.42	Holstein Friesian (720)	Wojdak-Maksymiec *et al.,* 2008
0.90	0.10	Holstein Friesian (223)	Ratna Kumari *et al.,* 2008
0.55	0.45	Jersey (143)	Ratna Kumari *et al.,* 2008
0.88	0.12	Sahiwal (13)	Ratna Kumari *et al.,* 2008
1.00	0.00	Khillari (13)	Ratna Kumari *et al.,* 2008
0.90	0.10	Ongole (5)	Ratna Kumari *et al.,* 2008
0.60	0.40	Kankrej (26)	Ratna Kumari *et al.,* 2008
0.61	0.39	Gir (41)	Ratna Kumari et al., 2008

Table F.4. (continued)

0.46	0.54	Red Sindhi (26)	Ratna Kumari *et al.,* 2008
0.93	0.07	Hariana (7)	Ratna Kumari *et al.,* 2008
0.00	1.00	Red Kandhari (1)	Ratna Kumari *et al.,* 2008
0.50	0.50	Dangi (1)	Ratna Kumari *et al.,* 2008
1.00	0.00	Deoni (1)	Ratna Kumari *et al.,* 2008
1.00	0.00	Tharparkar (1)	Ratna Kumari *et al.,* 2008
0.89	0.11	Montebeliard (120)	Ghasemi *et al.,* 2009

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name:	Dinç, Havva
Nationality:	Turkish Cypriot
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Phone / Fax:	+90 532 777 85 92 / +90 312 210 79 76
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Foreign Languages:	English (Advanced), German (Elementary)

EDUCATION

Degree	Institution	Graduation
Ph.D.	METU Biological Sciences Dept.	2009
M.Sc.	Hacettepe University, Dept. Science and Mathematics for Secondary Education	2005
M.Sc.	METU Biological Sciences Dept.	2003
B.Sc.	METU Biological Sciences Dept.	2001
High School	19 Mayıs Türk Maarif College Kyrenia, Cyprus	1997

PROFESSIONAL EXPERIENCE

Year	Role
2007-2009	Project Assistantship in the project entitled "In-vitro conservation and pre-molecular characterization of native domesticated animal genetic resources of Turkey-1; TURKHAYGEN-1". http://www.turkhaygen.gov.tr/ (TUBITAK–KAMAG–Project no: 106G005)

2005-2007	Project Assistantship in the project entitled "DNA based methods to identify β-casein, β-lactoglobulin and κ-casein genotypes which are milk proteins related with health and cheese yield." (TUBITAK–TOVAG –Project no: 104V137)
Fall, 2003	Participated in Assistantship of Bio 400 Biostatistics Applications Laboratory
Fall, 2002	Participated in Assistantship of Gene 205 Ecology and Evolutionary Biology Laboratory
Summer, 2000	Thirty-days' Training in the Microbiology and Biochemistry Unit of a Diagnosis and Check-up Center (Med-lab) Ankara, Turkey

ACADEMIC ACTIVITIES

Academic Publications

Dinc, H., Ozkan, E., Kepenek, E.S. and Togan, I. 2009. Polymorphisms of six milk traits related genes in native Turkish cattle breeds. Manuscript in preparation.

Berkman, C.C., **Dinc, H.**, Sekeryapan, C. and Togan, I. 2008. *Alu* Insertion Polymorphisms and an Assessment of the Genetic Contribution of Central Asia to Anatolia with Respect to the Balkans. *American Journal of Physical Anthropology*, 136(1): 11–18.

Dinç, H. and Togan, I. 2005. Allele frequencies for ten *Alu* insertion polymorphisms in Anatolian population. *Journal of Forensic Sciences*, 50(5): 1221-1222.

Academic Research Projects

In-vitro conservation and pre-molecular characterization of native domesticated animal genetic resources of Turkey-1; TURKHAYGEN-1. TUBITAK–KAMAG–Project number: 106G005; a national project. http://www.turkhaygen.gov.tr/ (2007-present).

Method development for the detection of beta-casomorphin peptides via molecular interactions in A1 type milk and milk products. An Interdisciplinary Project; Project number: ODTU-BAP-2006-07-02-03 (2006-2009).)

DNA based methods to identify β-casein, β-lactoglobulin and κ-casein genotypes which are milk proteins related with health and cheese yield. TUBITAK–TOVAG–Project number: 104V137 (2005-2007))

Mitochondrial DNA 9-bp deletion in Anatolian and Central Asian populations. Project number: ODTU-BAP-2004-07-02-00-06 (2004-2005)

Alu insertion polymorphisms in Anatolian Turks. Project number: ODTU-BAP-2002-07-02-00-55 (2002-2004)

Technical Reports

Togan, I., Koban, E. and **Dinç, H.** July, 2007. DNA based methods to identify β-casein, β-lactoglobulin and κ-casein genotypes which are milk proteins related with health and cheese yield. TUBITAK (TOVAG-104V137).

Presentations

Doğan, Ş.A., Yüncü, E., Koban, E., **Dinç, H.**, Berkman, C.C., Erol, H. and Togan, İ. 2009. Genetic Diversity in Turkish Native Sheep Breeds Based on Two Types of Genetic Markers. 4th International Symposium on Health Informatics and Bioinformatics, Ankara, Turkey, 16-17 April 2009. (Poster Presentation)

Togan, I., M.I., Soysal, E. Koban, C.C. Berkman, E. Özkan, **H. Dinç**. 2008. An Assesment of the Use of Molecular Markers in Livestock in Turkey. "Marker Assisted Selection Workshop"; Present situation of livestock animals and an application on statistical methods of animal genomic data, Tekirdağ, Turkey, 17–19 November 2008.

Dinc, H., Koban, E., Kepenek, Ş. E. and Togan, I. 2007. Screening of Beta-casein Gene Variants for the Conservation of Native Turkish Cattle Breeds. ESF workshop on "Population genetics modelling and habitat fragmentation: separating recent and ancient events for efficient conservation", Instituto Gulbenkian de Ciência, Oieras, Portugal, 19-21 September 2007. (Oral presentation)

Togan, I., Berkman, C., Koban, E., Saraç G.Ç. and **Dinc, H.** 2007. An Anatolian Trilogy With Some Implications on Conservation Issues: Arrivals of Nomadic Turks Together with their Sheep and Shepherd Dogs. ESF workshop on "Population genetics modelling and habitat fragmentation: separating recent and ancient events for efficient conservation", Instituto Gulbenkian de Ciência, Oieras, Portugal, 19-21 September 2007.

Berkman, C.C., **Dinc, H.**, Sekeryapan, C. and Togan, I. 2007. Genetic Contribution of Central Asia to Turkey and Azerbaijan and Search for Its Component to the Language Spoken in the Region. International Symposium on Health Informatics and Bioinformatics, Antalya, Turkey, 30 April - 2 May 2007. (Oral Presentation)

Dinc, H., Koban, E., Saatçi, E., Ozkan, E., Iscan, M. and Togan, I. 2007. Analysis of Polymorphism at Three Milk Protein Genes in Native Cattle Breeds of Turkey and Their Use for Management. International on Conference Environment: Survival and Sustainability, Near East University, Nicosia, Northern Cyprus, 19-24 February 2007. (Oral Presentation)

Dinc, H., Kepenek, E.S., Koban, E., Özkan, E. and Togan, I. 2007. Determination of Genotypes for Four Milk Protein Related Genes in Native Cattle Breeds of Turkey. Third Joint Meeting of Universities and Research Institutions for Animal Science of the Balkan Countries, Thessaloniki, Greece, 10-12 February 2007. (Oral Presentation)

Dinç, H., Koban, E., Çatçat, D. and Togan, I. 2006. Süt Proteinlerinden beta-kazein, beta-laktoglobulin ve κ-kazein Genotiplerinin DNA'ya Dayalı Yöntemlerle Tesbiti. 18. Ulusal Biyoloji Kongresi, Kuşadası, Turkey, 26-30 June 2006. (Poster Presentation)

Kepenek, E.S., **Dinc, H.**, Catcat, D. and Togan I. 2006. Polymorphism of Four Milk Protein and Two Disease (BLAD and CVM) Related Genes in Native Cattle Breeds of Turkey. 20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, Kyoto, Japan, 18-23 June 2006. (Poster Presentation)

Dinç, H., Şekeryapan, C. and Togan, I. 2005. Genetic Differentiation of Anatolian Turks Based on *Alu* insertions. 11th Meeting of PhD Students in Evolutionary Biology, Bordeaux, France, 5-9 September 2005. (Oral Presentation)

Dinç, H., Togan, I. 2005. Genetics of A2 Type Cow Milk and its Importance on Human Health (A Compilation); is it A1 milk or A2 milk? A Conference on Proteomic and Genomic Techniques in Conservation of Native Genetic Resources, Tekirdağ, Turkey, 15-16 June 2005. (Oral Presentation)

Organized Workshops and Symposiums

Population Genomics-II Workshop in TURKHAYGEN-1 (In-vitro conservation and pre-molecular characterization of native domesticated animal genetic resources of Turkey-1) Project, Ankara, Turkey, November 08-09, 2008.

Population Genomics-I Workshop in TURKHAYGEN-1 (In-vitro conservation and pre-molecular characterization of native domesticated animal genetic resources of Turkey-1) Project, Ankara, Turkey, July 08-10, 2008.

"Symposium on Evolution" by Evolution Working Group in Hacettepe University, May 04, 2006 and in Cultural Convention Center in Middle East Technical University, May 08, 2006, Ankara, Turkey.

Workshops and Conferences Attended

November 13-14, 2008	"7th Ankara Biotechnology Days" in Veterinary Sciences Ankara University Ankara, Turkey
June, 2005	"1st Molecular Genetic Applications Workshop" Ondokuz Mayıs University Samsun, Turkey
June 10-13, 2004	"Human migrations in continental East Asia and Taiwan; Genetic, linguistic and archaeological evidence" International Conference Swiss National Foundation for Scientific Research University of Geneva Geneva, Switzerland
June 25-27, 2003	"1st International Silk Road Symposium" International Symposium Georgian Academy of Sciences International Black Sea University Tbilisi, Georgia
March, 2003	"Red Lists, Priority Species and National Conservation Action Plans" National Workshop Turkish Bird Research Society Middle East Technical University Ankara, Turkey
October 10-13, 2001	8th National Biology Student Conference Hacettepe University Ankara, Turkey

Academic Fellowships

February-April, 2008	Fellowship from a European Project entitled "Calpastatin Biosensor for Meat Tenderness Prediction" Project acronym: TENDERCHECK Project no: COOP-CT-2006-032696 Department of Analytical Chemistry Lund University, Lund, Sweden

Experimental Skills

Wet Lab	ELISA
	Cell Culture
	DNA Sequencing
	Microsatellite Based Genotyping
	Restriction Fragment Length Polymorphisms (RFLP)
	Single Strand Conformation Polymorphisms (SSCP)
	Amplification Created Restriction Sites (ACRS)
Computer Based	Arlequin
	GENETIX
	Molecular Evolutionary Genetics Analysis (MEGA)
	Numerical Taxonomy System (NTSYSpc)
	Phylogeny Inference Package (PHYLIP)