T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOMOLECULAR SCIENCES

DETECTION OF A2 BETA CASEIN IN COW SERUM AND MILK SAMPLES AND IN-VITRO DIGESTIBILITY TEST

MASTER OF SCIENCE THESIS

MERVE ALKAN

Thesis Supervisor

ASSOC. PROF. SERCAN KARAV

ÇANAKKALE – 2023

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ÇANAKKALE – 2023

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Merve ALKAN

31/08/2023

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Merve ALKAN

Çanakkale, August 2023

İNEK SERUM VE SÜT ÖRNEKLERİNDE A2 BETA KAZEİN TESPİTİ VE İN-VİTRO SİNDİRİLEBİLİRLİK TESTİ

ÖZET

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31/08/2023, 35

Süt proteinleri, çok çeşitli farklı moleküler yapılara ve özelliklere sahip heterojen bir polimerik bileşik grubudur. Kazeinler, peynir altı suyu proteinleri, enzimler, minör proteinler ve nitrojen bileşikleri olarak bulunurlar. Kazeinler, inek sütünün toplam proteinlerinin yaklaşık %80'ini oluşturur. β-kazein, toplam kazeinlerin yaklaşık %37'sini oluşturan kazeinlerin önemli bir parçasıdır. β-kazein içinde, genetik olarak belirlenmiş bir dizi varyant vardır. Bununla birlikte, inek sütünde bulunan β-kazeinin on üç genetik varyantı vardır. A1 β-kazein (A1-süt) ve A2 β-kazein (A2-süt) olarak adlandırılan A1 ve A2 en yaygın varyantlardır.

Bu araştırmanın amacı inek serumu ve süt örnekleri kullanılarak A2 beta-kazein içeren sütün belirlenmesi ve A2 beta-kazeinin sindirilebilirliğinin incelenmesidir. A1 ve A2 sütleri ve bunların sindirim sağlığı üzerindeki etkisi ile ilgili birçok bilimsel çalışma, A2 sütünün A1 süte göre daha kolay sindirildiğini göstermektedir. A1 sütü sindiriminden sonra bazı gastrointestinal rahatsızlık problemlerine ve laktoz intoleransına neden olur, bu problem A1 protein sindirimi ile BetaCasoMorphin-7 (BCM-7) oluşumu ile ilişkilidir.

Bu çalışmada inek serumları in vitro olarak analiz edilerek A2 genotipli inekler belirlenmiştir. Bu ineklerden Elisa yöntemi ile sağılan sütlerde A2 beta-kazein saptanmıştır. A2 süt tüketiminin sindirilebilirliği in vitro sindirim yöntemiyle incelenmiştir. Bu

çalışmanın sonuçları, A2 süt tüketiminin önemini ve sindirilebilirlik üzerindeki olumlu etkilerini göstermektedir.

Anahtar Kelimeler: Süt, A1 beta kazein, A2 beta kazein, BCM-7

DETECTION OF A2 BETA CASEIN IN COW SERUM AND MILK SAMPLES AND IN-VITRO DIGESTIBILITY TEST

ABSTRACT

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31/08/2023, 35

Milk proteins constitute a heterogeneous collection of polymeric entities characterized by a diverse array of molecular structures and properties. These proteins manifest in the forms of caseins, whey proteins, enzymes, minor proteins, and nitrogenous components. Within the compositional makeup, caseins collectively account for approximately 80% of the entirety of bovine milk proteins. Notably, a pivotal component of caseins is β-casein, which contributes approximately 37% to the cumulative casein content.

The domain of β-casein itself is marked by a genetic diversity, encompassing a range of variants. Among these, thirteen distinct genetic variants of β-casein have been identified within the repertoire of cow's milk. Paramount among these variants are A1 β-casein (commonly referred to as A1-milk) and A2 β-casein (known as A2-milk), which represent the most prevalent genetic differentiations within the β-casein category.

The aim of this study is to figure out the milk containing A2 beta-casein using cow serum and milk samples and to examine the digestibility of A2 beta-casein. Numerous studies comparing the effects of A1 and A2 milk on digestive health demonstrate that A2 milk is more easily digested than A1 milk.

In this study, cows with A2 genotypes were determined by analyzing cow sera in vitro. A2 beta-casein was detected in the milk milked from these cows by the Elisa method. The digestibility of A2 milk consumption was examined by an in-vitro digestion method. The findings derived from this study underscore the significance of A2 milk consumption and its notable positive impacts on digestibility.

Keywords: Milk, A1 beta casein, A2 beta casein, BCM-7

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SYMBOLS and ABBREVIATIONS

2-AA	2-Aminobenzoic Acid
%	Percent
ANOVA	Analysis of Variance
Asn	Asparagine
dH_2O	distilled water
et. al	Others
EndoBI-1	Endo-β-*N*-acetylglucosaminidase
g	Gram
GI	Gastrointestinal
h	hour
HexNAc	*N*-acetylglucosamine
Kg	Kilogram
LB	Lysogeny broth
M	Molar
MALDI-TOF MS	Matrix-Assisted Laser Desorption-Ionisation-Time of Flight Mass Spectrometry
mg/mL	Milligram per milliliter
min	minute
OD	Optical Density
pH	Power of Hydrogen
PNGase-F	Peptidyl *N*-Glycosidase F
RPM	Revolutions per minute
s	second
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSF	Simulated Salivary Fluid
Thr	Threonine

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CHAPTER 1

INTRODUCTION

1.1. A1 and A2 Milk

Cow's milk protein content generally consists of two main groups; caseins (80%) and whey (20%). cattle lines; It contains 4 types of casein in the chromosome 6 region as αs1, αs2, β and κ. β casein accounts for 45% of bovine milk and has 12 genetic disorders as A1, A2, A3, B, C, D, E, F, G, H1, H2 and I.

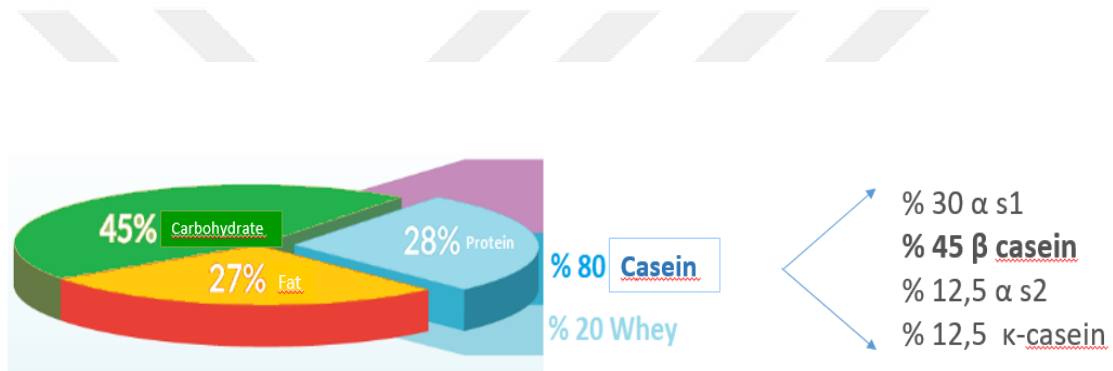

Figure 1. Cow's milk content.

β-casein variants have a significant role in cheese production and quality. The most common beta casein variants in cattle are A1 and A2. Each cow contains two copies of the beta casein gene; A1/A1, A1/A2, or A2/A2.

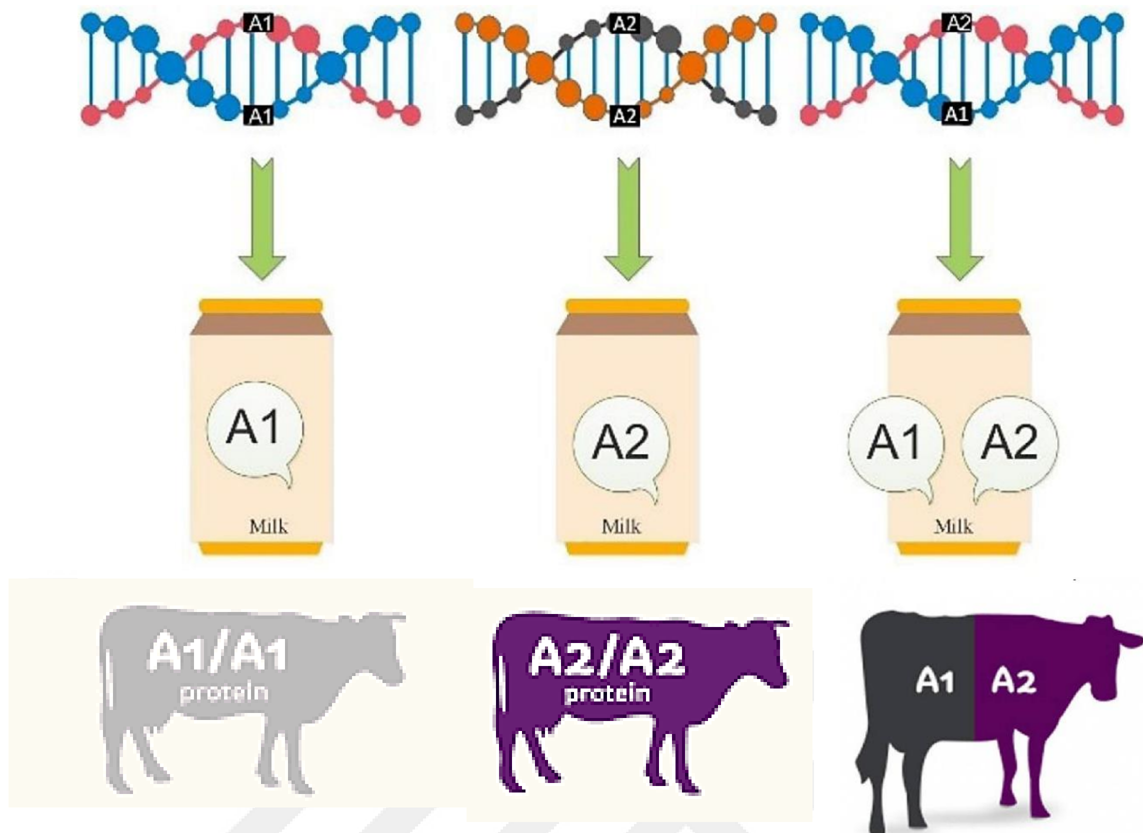

Figure 2**.** A1, A2 and A1/A2 type milk.

Bovine milk is composed of approximately 87% water and 13% milk solids, consisting of essential constituents such as lactose, fats, proteins, and minerals. Within the protein fraction of milk, whey proteins constitute 20%, encompassing key components like α- and β-lactoglobulin, transferrin, albumin, and lactoferrin. Concomitantly, the remaining 80% of the protein content is attributed to casein proteins, specifically α, β, and κ-casein (Godden, 2008).Among these, β-casein, a significant contributor to biological functionality, constitutes 45% and showcases a commendable amino acid equilibrium. Within the composition of β-casein, 209 amino acid residues are discerned, with proline comprising 16.7%, thereby inducing structural constraints on α-helix formation.

Distinct variants of β-casein emerge due to a multitude of mutations, yielding diverse forms such as A1, A2, A3, B, C, D, E, F, G, H1, H2, and I. Foremost among these are the A1 and A2 forms, recognized as the prevailing β-casein variants (Massella et al., 2017). Predominantly characterized by their allele configurations, A1 and A2 variants can manifest as A1/A1, A1/A2, or A2/A2 (Venkatachlapathy R, 2019), leading to the commonly known designations of A1 and A2 milk. Composition analysis of these milk variants shows

concordant profiles in terms of protein, fat, carbohydrates and other key components. Detailed information is given in Table 1. A single point mutation that affects the nucleotide at codon 67 of the -casein gene is responsible for the crucial distinction between the A1 and A2 forms. In particular, proline at position 67 in the amino acid sequence of A2 -casein underwent a mutation that changed it into histidine around ten thousand years ago, resulting in the A1 variant (Pal et al., 2015; Çak, 2018).

Bovines harboring the mutated form of β-casein, referred to as A1 cows, subsequently yield A1 milk as a result of this genetic transformation.

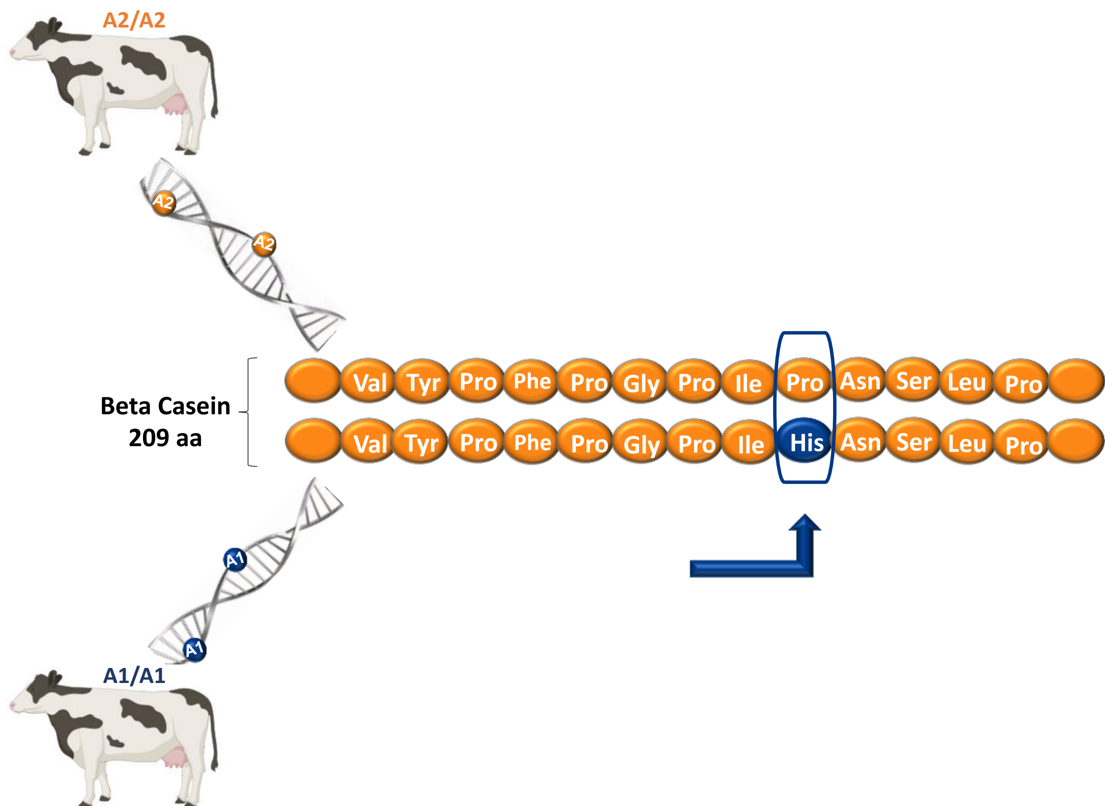

Figure 3. A1 and A2 beta-casein amino acid sequences.

1.2. A1/A2 Allele in Different Cow Breeds

All milk types originally were A2, however the genetic mutation caused A1 form which is gradually became the prevalent among cows. Some of cows still have A2 β-casein and produce A2 milk even though the percentage differ from one breed to another. Holstein breed, for instance, is a cow breed which has the lowest percentage of A2 milk in comparison to others. 35 % of Holstein Friesian produce A2 milk, whereas 48% and 16% of them produce A1/A2 milk and A1 milk, respectively. While a most of Brown Swiss breed (65%) produce A2 milk, the Guernsey is the breed which has the highest proportion of A2 β-casein at higher 90%. Though most cow breeds produce A1/A2 milk as a combination of two protein forms, A1 milk production is not observed among purebred African and Asian cattle (Ng-Kwai-Hang and Grosclaude, 2002). In similar manner, a majority of other mammalian species have not A1 β-casein and do not produce A1 milk. For instance, Indian cow and buffalo breeds have 100% A2 allele (Red Sindhi, Sahiwal, Tharparkar, Gir and Rathi), other Indian breeds, which are used in farming, have about 94% (Joshi, 2011). In addition, a study on 860 animals presenting 22 breeds of Indian cattle indicates that 90.4% have A2A2 allele and a small minority showed A1A2 genotype (0.091). In contrast, none of milch breeds including Gir, Tharparkar, Rathi, Sahiwal, and Red Sindhi showed A1A1 genotype. With the results of other studies, it is clearly seen that Indian cattle naturally carries A2 allele at a high proportion.

Table 1.
A1 and A2 frequencies among different breeds (Çak, 2018).

Breed	Region	Animal Number	A1	A2
Brown Italian	Italy	298	0.11	0.69
Brown Swiss	USA	22	0.14	0.66
Brown Swiss	USA	50	0.18	0.66
Guernsey	USA	196	0.01	0.98
Holstein	Australia	260	0.63	0.35
Holstein	Italy	1383	0.58	0.40
Holstein	USA	1152	0.43	0.55
Holstein	USA	260	0.624	0.347
Holstein	Ireland	696	0.72	0.25
Jersey	USA	37	0.22	0.49
Jersey	UK	47	0.09	0.63
Jersey	Australia	308	0.07	0.57
Jersey	Denmark	157	0.07	0.58
Jersey	USA	172	0.17	0.50
Jersey	Ireland	116	0.30	0.41
Jersey	New Zealand	1328	0.12	0.59
Ayrshire	USA	45	0.72	0.28

1.3. A1 and A2 Milk Digestion – Betacasomorphin 7 (BCM-7)

Due to the single amino acid difference in the A1 and A2 variants, specific amino acids are separated from the chain as a result of the digestion of proteins. Digestion of A1 β-casein releases β-casomorphin-7 (BCM-7) while β-casomorphin-9 (BCM-9) is released for A2 β-casein.

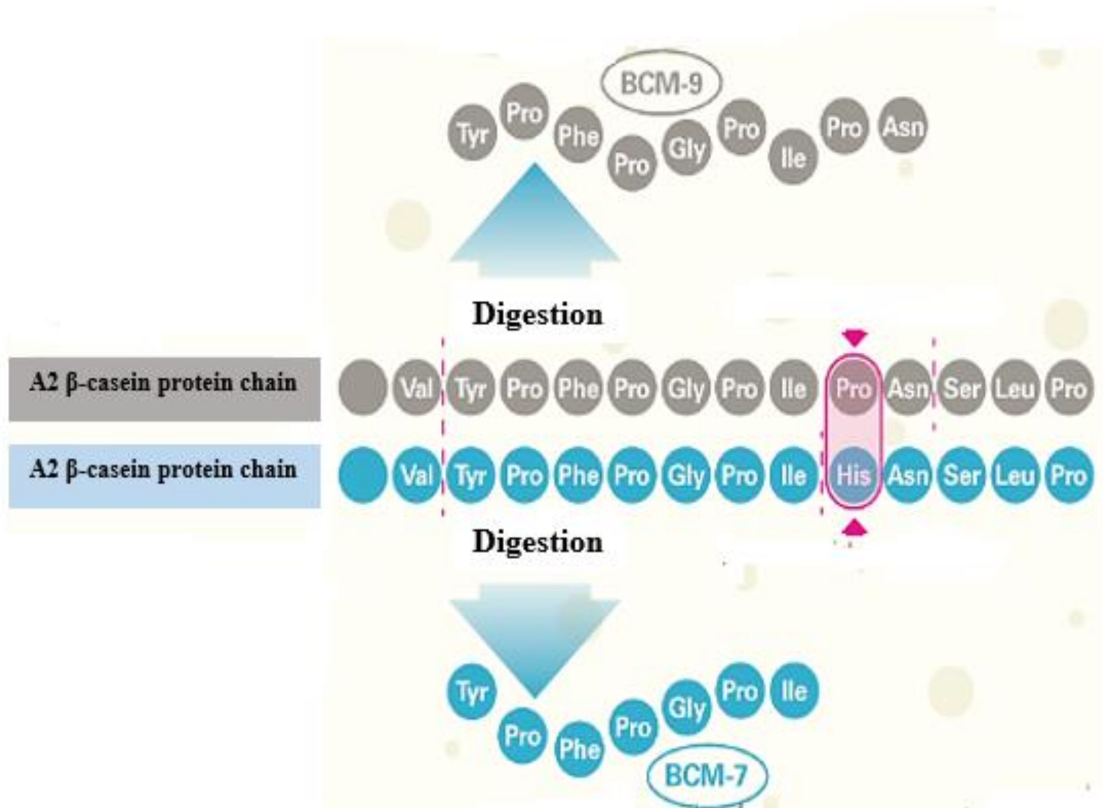

Figure 4. Digestion of A1 and A2 β-caseins.

The secondary confirmation of protein structures may be impacted by the single amino acid difference between the amino acid sequences of A1 and A2 -caseins.The physical characteristics of casein and enzymatic digestion are affected by this. Betacasomorphin-9 (BCM-9; Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn) is produced when A2 protein is digested, whereas betacasomorphin-7 (BCM-7; Tyr-Pro-Phe-Pro-Gly-Pro-Ile) is produced when A1 protein is digested (AK, 2018). Proline at position 67 of A2 -casein prohibits BCM-7 formation in the human body, but histidine at the same position in A1-casein allows cleavage by gastrointestinal enzymes to produce BCM-7. Inability of human-associated enzymes to

degradation this tiny peptide, which has morphine-like properties, results in dyspepsia. As a result, BCM-7 cannot be detected in samples of A2 cows' blood or urine, and A2 -casein is easily digested into peptides and amino acids. Contrarily, because the peptides in A1 milk cannot be converted into amino acids, consumption of this substance causes leakages in the gut that allow this molecule to enter the gastrointestinal tract and bloodstream (He et al., 2017). There are few clinical and epidemiological investigations on the relationship between consuming A1 milk and conditions like lactose intolerance, autism, diabetes, etc. Many of them often focused on lactose intolerance or gastrointestinal discomfort and A1 milk (especially formation of BCM-7). EFSA (2009), on the other hand, published a scientific report in connection with BCM-7, which explains that the relationship between the intake of BCM-7 or it related peptides and etiology of suggested diseases could not be established.

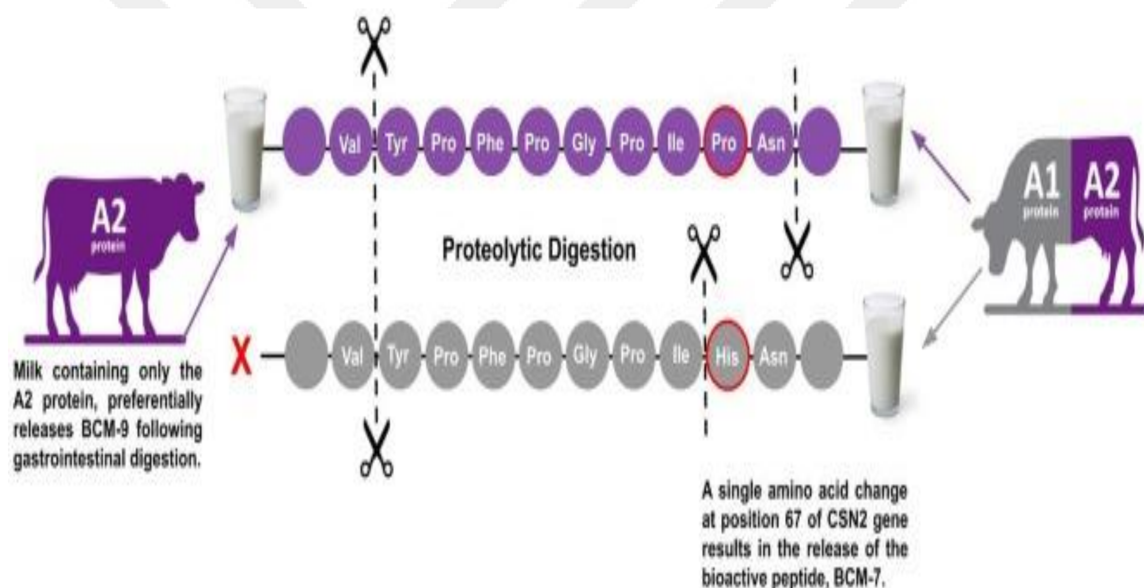

Figure 5. Formation of BCM-7 and BCM-9 from digestion of A1 and A2 milk.

1.4. Impact of A1 and A2 Milk on Human Digestion

According to the Bristol Scale, a study by Ho et al. revealed a significant difference between A1 and A2 milk consumption in terms of stool consistency. When compared to A2 milk, the results showed that drinking A1 milk was related with increased stool consistency.

Moreover, this study established a positive correlation between abdominal pain and stool consistency arising from A1 milk ingestion (r=0.52), a correlation that was not evident in the context of A2 milk digestion (r=-0.13). In a separate investigation, A2 milk consumption

among individuals exhibiting lactose intolerance exhibited a discernible reduction in intolerance symptoms, as documented by ÇAK (2018). A different study examined the gastrointestinal symptoms and hydrogen production during digestion in lactose intolerant individuals who had A2 milk, A1 milk, ordinary milk devoid of lactose, and conventional Jersey milk. According to their findings (Ramakrishnan et al., 2020), A2 milk greatly lessens gastrointestinal discomfort and symptoms. Similar studies on animals revealed that BCM-7 has a variety of effects on digestive function, including a decrease in the frequency and amplitude of intestinal contractions. According to study (Milan et al., 2020), rats fed A1 milk have a 65% increase in myeloperoxidase, an inflammatory marker. In general, it has been demonstrated that consuming A1 milk leads to gastrointestinal motility and systemic inflammation linked to BCM-7 production during its digestion. Contrarily, consumption of A2 milk did not appear to be associated with post-dairy pain, and it is thought to be a milk that may be easily drunk without experiencing any digestive discomfort (Kirk et al., 2017). Ten people who are intolerant to A1 milk did not have any stomach issues after consuming A2 milk. Another study (He et al., 2017), found that A1 milk increased symptoms and decreased lactase activity, while A2 milk reduced gastrointestinal symptoms associated with lactose intolerance.

1.5. Methods Used to Detect Beta Casein A1 and A2 Variants

Various methods used to detect beta casein A1 and A2 variants in milk are described in this section.

1.5.1. Capillary Electrophoresis

For the measurement and characterization of milk proteins, traditional methods like high-performance liquid chromatography (HPLC), isoelectric focusing, and conventional gel electrophoresis are frequently used. Although each of the methods mentioned has good quantification possibilities, it alone cannot provide a perfect separation between serum proteins and caseins (de Jong et al., 1993). Capillary electrophoresis is a high-resolution analytical technique that can simultaneously identify whey proteins and caseins, requiring simple preparation, separating ions using an applied voltage according to their

electrophoretic mobility (Cattaneo et al., 1996). J.S. In the study of Ham et al, it was determined using capillary electrophoresis how α- and β-caseins in milk and Queso Blanco cheese were affected by irradiation. The irradiated milk and cheese samples were treated with reduction buffer for the application of the capillary electrophoresis method. The resulting solution was used for the capillary electrophoresis method determined by previous studies (Revilla et al., 2005), (de Jong et al., 1993). Statistical analysis of the data obtained from the capillary electrophoresis results was performed with ANOVA. As a result of the analyses, αs1, αs0, βB, βA1, βA2 and βA3 caseins and changes due to irradiation were determined (Ham et al., 2009). With the capillary electrophoresis method used in this study, βA1 casein in A1 milk and βA2 casein in A2 milk can be determined with high yield and low sample volume.

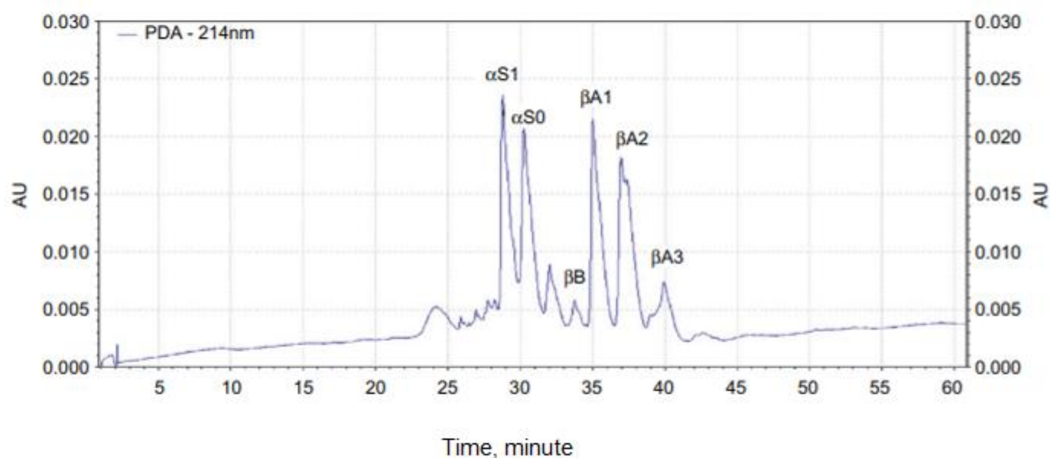

Figure 6. Electropherogram image of α- and β-caseins in Queso Blanco cheese

1.5.2. Urea-PAGE

Polyacrylamide gel electrophoresis (PAGE) is a technique that separates biological molecules (usually protein or nucleic acid) according to their electrophoretic movements and is frequently used in the fields of biochemistry, forensics-chemistry, molecular biology, genetics and biotechnology. The urea PAGE gel electrophoresis system disrupts the secondary structure of DNA or RNA molecules with urea, allowing them to be separated according to their molecular weights on a polyacrylamide gel. This system can separate

nucleic acid molecules with a length of 2-500 bases, even if there is a single nucleotide difference between them (Summer et al., 2009).

Urea PAGE is also used quite frequently in dairy technology for different samples. In particular, the Urea PAGE method has been used in many studies for the detection of different fractions of beta casein protein (Grosclaude et al., 1972; Huppertz, 2013). Since alpha casein is in a more negative form than beta casein at pH 8.6, its mobility on gel is faster than beta casein and thus can be distinguished (Swaisgood, 2003). The A1 variant has a lower net charge compared to the A2 variant due to the positively charged imidazole side chain of the amino acid His-67. Thanks to this difference in charge, two genetic variants can be detected by electrophoresis (Eigel et al., 1984). In another study (Duarte-Vázquez et al., 2018), the urea PAGE method was used to separate two variants of beta casein protein (A1, A2). In the study, milk of Holstein Friesian (HF) and Jersey cattle (JA2) was used. The PAGE system for the pretreated casein samples was carried out according to the protocol (Andrews, 1983).

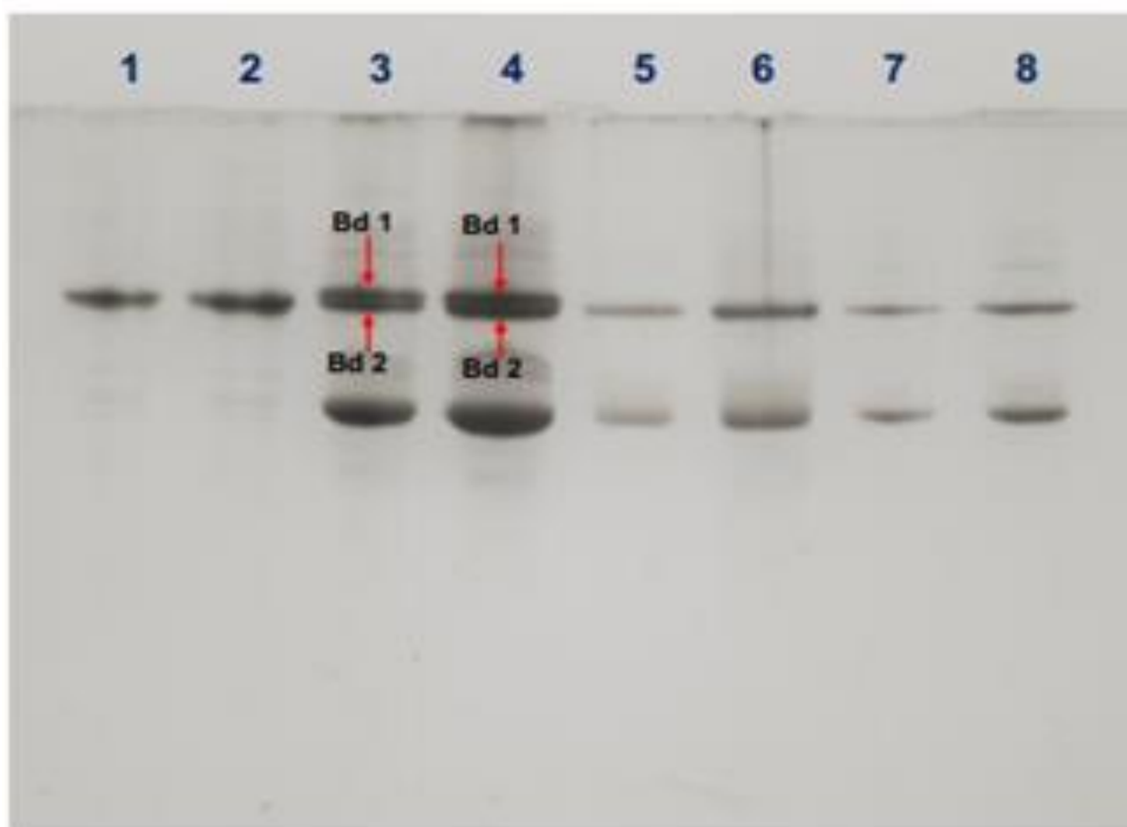

Figure 7. Urea PAGE electrophoresis result of HF, JA2 samples.

Bovine beta casein standard (Sigma Aldrich) was placed in wells 1 and 2 as a reference. HF cow's milk casein sample for wells 3 and 4; 5-8. JA2 cow's milk casein sample was loaded into the wells. For the HF casein sample, two separate bands are observed as a result of urea PAGE gel electrophoresis (Bd1, Bd2) and Bd1 beta casein A1 variant; Bd2 represents the beta casein A2 variant. On the other hand, a single band is seen for the JA2 casein sample and this band represents the beta casein A2 variant. In this study, after the urea PAGE method, the appropriate bands were cut from the gel and subjected to various pretreatments and used for HPLC-MS amino acid sequencing. Amino acid difference between A2 and A1 variants as a result of sequencing; The counterpart of amino acid His-67 in the A1 variant is shown as Pro-67 in the A2 variant.

1.5.3. HPLC-MS and RP-HPLC

High performance liquid chromatography-mass spectrometry (High Performance Liquid Chromatography-Mass Spectrometry; HPLC-MS); It is one of the widely used analytical techniques for the separation and determination of chemical and biological components by combining the mass analysis feature of liquid chromatography and mass spectrometry with the physical separation features. Theoretically, HPLC-MS method, which is based on the principle that the substances in a mixture are separated and purified in a two-phase system, one of which is stationary and the other is the mobile phase, ionized and sent to the MS unit, where they are separated at mass/charge (m/z) ratios and their mass determination is made. ; casein in milk (α-s1, α-s2, ß) etc. It has been successfully applied in the identification and quantification of protein variants. In particular, the analyzes made by HPLC-MS; The rapidity, high efficiency, accuracy and reproducibility of this technique has allowed this technique to be frequently preferred for the determination of casein variants in milk.

For example; In a study in which the casein variants in milk were determined by HPLC-MS, 55 samples were formed from milk obtained from different markets. First of all, total protein and casein concentrations of milk samples were determined by infrared (infrared) spectroscopy, and then the samples separated into phases were separated into proteins using HPLC. After this process using a reverse phase C18 column, the separated casein variants were detected using high performance mass spectrometry (HRMS). A1 and A2 casein variants in milk were determined according to the obtained chromatogram peaks (Givens et al., 2013).

In a similar study in which casein variants in milk were determined, reversed phase high performance liquid chromatography was used. Milk samples from 40 cows of different breeds were collected and as a result of RP-HPLC analysis, casein variants were purified and quantified. In this study, unlike the other, reverse phase C8 column was used for purification. A1 and A2 casein variants were determined by absorbance measurements at 214 nm

wavelength of the protein variants, which were separated and purified in less than 40 minutes (Bonfatti et al., 2008).

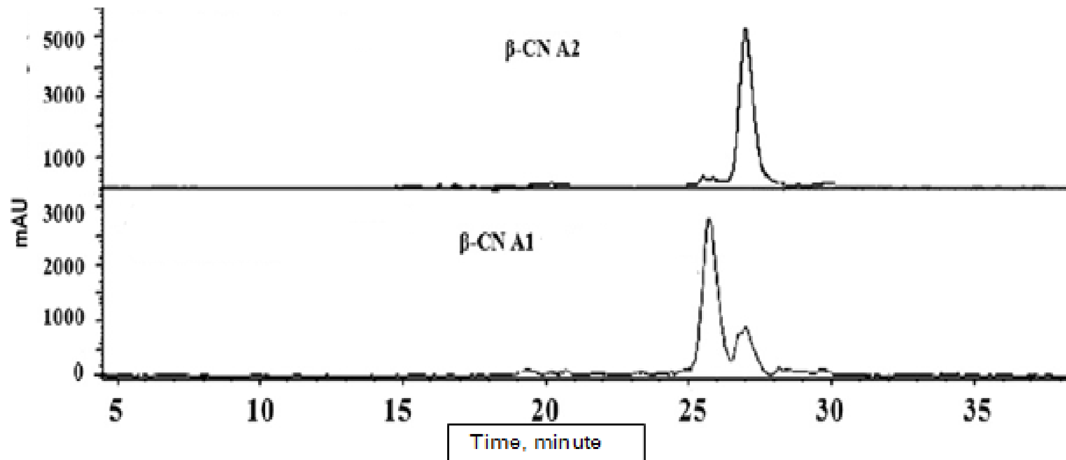

Figure 8. Chromatography Charts of A1 and A2 Casein Variants in Milk

1.5.4. Isoelectric Focusing Electrophoresis (IEF) Method

The two most common beta-casein (β-CN) variants, A1 and A2, which are characterized by casein genetic polymorphism in bovine species, have different isoelectric points (pI). Using this difference, A1 and A2 beta casein variants in milk can be detected.

Simple electrophoresis on a pH gradient constitutes isoelectric focusing. To do this, a pH gradient in the gel is required. Amphoteric compounds with low molecular weight (ampholites) are used to induce the pH gradient. The most acidic ampholites are placed in order of their isoelectric points, close to the cathode, and move when electric current is supplied. Ampholites are compounds that behave as both acids and bases and can react with both. As a result, the gel's pH gradient from the anode to the cathode decreases. Each protein gets uncharged when it reaches its isoelectric pH zone and remains stationary at that point when the protein combination is passed across a gel (Walker, 1994). The beta casein diversity in bovine milk in gel based on the difference of A1 and A2 beta casein isoelectric points visualized in the study (Caroli et al., 2015). It was reported polymorphisms that cause

beta casein diversity in animals such as sheep, goats and horses in addition to cattle (Caroli et al., 2016).

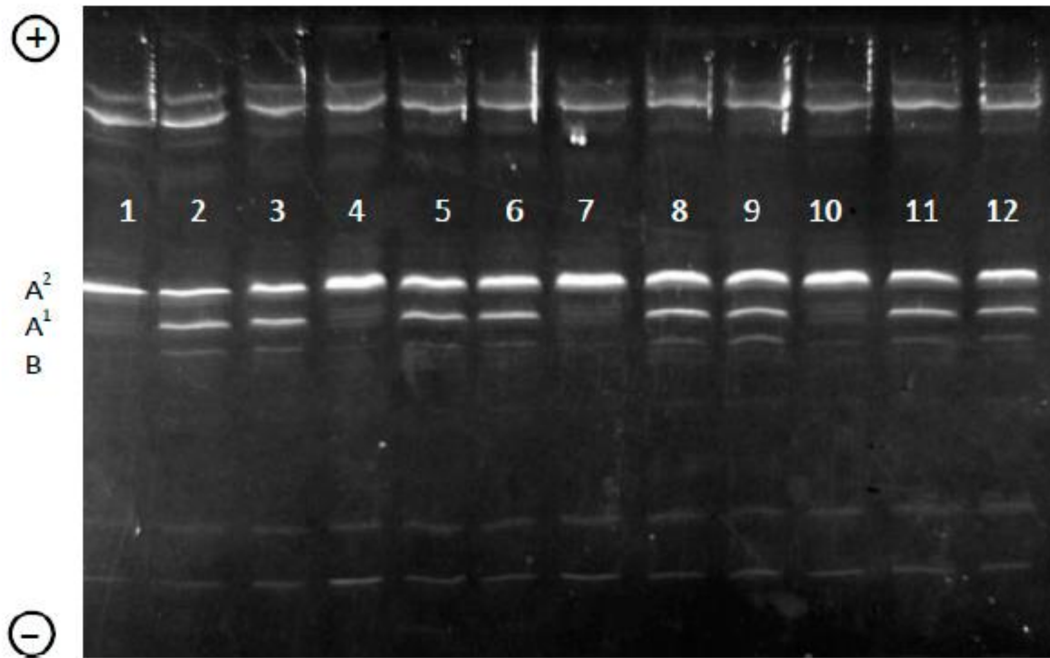

Figure 9. IEF analyzes of different milk samples known to contain A2

As a result of the analysis, while the 3 most common β-CN variants were observed, αS1-CN and κ-CN molecules were also observed in the upper (+) and lower (-) parts of the gel, respectively.

1.5.5. Enzyme-Linked Immuno Sorbent Assay (ELISA)

ELISA kits are available for the detection of bovine beta casein (β-CN) A1 and A2 variants. Briefly, the principle of the ELISA method is based on the reaction between the specific antigen-antibody. According to the sample to be studied, our sample is added to the antigen or antibody immobilized on a plastic plate and the measurement is taken at 450 nm. The concentration is calculated using the absorbance obtained as a result of the measurement

and the standards. In a study, the concentrations of beta casein variants A1 and A2 purified by HPLC were determined using the ELISA kit (Ul Haq & Ul Haq, 2020).

CHAPTER 2

PREVIOUS STUDIES

According to several research, A2 milk from cows may be healthier than A1 milk if it is dominating or unique. We are unable to definitively evaluate the impact of A1-milk and A2-milk on health on the basis of the current results. Therefore, additional research is required.

2.1. Previous Studies

A2 milk is easier to digest than A1 milk, according to numerous research contrasting the effects of milk containing A1 and A2 beta casein on digestive health. After being digested, A1 milk can cause certain gastrointestinal difficulties in lactose intolerant people. This issue is linked to the creation of BCM-7 by the digestion of A1 protein. According to a study done by Ho et al., A1 milk results in higher stool consistency on the Bristol Scale than A2 milk. The study also showed that ingestion of A1 milk, but not A2 milk digesting, is favorably associated with both abdominal discomfort and stool consistency (r=0.52). According to a research conducted by ÇAK in 2018, the consumption of A2 milk among individuals afflicted with lactose intolerance exhibited a substantial reduction in the severity of intolerance symptoms. A different study looked at the gastrointestinal symptoms and post-digestion hydrogen generation of lactose-intolerant individuals who had A2 milk, A1 milk, ordinary milk without lactose, and typical Jersey milk. According to their findings (Ramakrishnan et al., 2020), A2 milk greatly lessens gastrointestinal discomfort and symptoms. Similar studies on animals revealed that BCM-7 has a variety of effects on digestive function, including a decrease in the frequency and amplitude of intestinal contractions. According to Barnett et al., rats fed A1 milk have a 65% increase in myeloperoxidase, an inflammatory marker. In general, it has been demonstrated that consuming A1 milk leads to gastrointestinal motility and systemic inflammation linked to BCM-7 production during its digestion. Contrarily, consumption of A2 milk did not appear to be associated with post-dairy pain, and it is thought to be a milk that may be easily drunk without experiencing any digestive discomfort (Kirk et al., 2017). Ten people who are intolerant to A1 milk did not have any stomach issues after consuming A2 milk.

Another study (He et al., 2017) found that A1 milk increased symptoms and decreased lactase activity, while A2 milk reduced gastrointestinal symptoms associated with lactose intolerance. All milk types originally were A2, however the genetic mutation caused A1 form which is gradually became the prevalent among cows. Some of cows still have A2 β-casein and produce A2 milk even though the percentage differ from one breed to another. Holstein breed, for instance, is a cow breed which has the lowest percentage of A2 milk in comparison to others. A2 milk is produced by 35% of Holstein cows, whereas A1/A2 milk and A1 milk are produced by 48% and 16% of them, respectively. While a most of Brown Swiss breed (65%) produce A2 milk, the Guernsey is the breed which has the highest proportion of A2 β-casein at higher 90%. Though most cow breeds produce A1/A2 milk as a combination of two protein forms, A1 milk production is not observed among purebred African and Asian cattle (Ng-Kwai-Hang and Grosclaude, 2002). In similar manner, a majority of other mammalian species have not A1 β-casein and do not produce A1 milk. For example, Indian cow and buffalo breeds contain 100% A2 allele (Red Sindhi, Sahiwal, Tharparkar, Gir, and Rathi), whereas other Indian breeds employed in agriculture have approximately 94% (Joshi, 2011). In addition, a study on 860 animals presenting 22 breeds of Indian cattle indicates that 90.4% have A2A2 allele and a small minority showed A1A2 genotype (0.091). In contrast, none of milch breeds including Gir, Tharparkar, Rathi, Sahiwal, and Red Sindhi showed A1A1 genotype. With the results of other studies, it is clearly seen that Indian cattle naturally carries A2 allele at a high proportion. Easily digestible A2 milk does not cause any digestive discomfort or intolerance problems. Although studies with A2 milk are quite limited, according to studies on A2 milk, A2 milk is considered a milk that even lactose intolerant individuals can digest with very few symptoms (He et al., 2017; Kirk et al., 2017; Çak, 2018; Ramakrishnan et al., 2020). Candidates' GI symptoms and hydrogen production were documented during an experiment in which 33 persons consumed four different types of milk (A2 milk, Jersey milk, A1 milk, and lactose-free milk). According to the results, lactose intolerant people experienced fewer gastrointestinal symptoms when drinking milk with A2 ß-casein as opposed to A1 milk (Ramakrishan et al., 2020). In addition, lactose intolerant individuals who consume only classic A1 milk have less abdominal pain than those who consume A1 milk. In another study, symptoms related to digestive discomfort were measured in 600 lactose-intolerant individuals who consumed A1 - A2 and only A2 milk at certain time intervals throughout the study. According to the results, those who consumed only A2 milk showed lower milk

intolerance symptoms compared to those who consumed A1 and A2 milk (Jianqin et al., 2015). In addition, A2 milk has been evaluated as a milk that can be consumed without causing any digestive discomfort (Kirk et al., 2017). When A1 consumes milk, it causes bloating, gas and discomfort. Evidence has also been presented that in some uses, the inability to digest milk sugar, which we call lactose intolerance, may be caused by A1 in general. Famous health scientist Van Miller defined it as follows: A1 is a kind of opium that affects the effects of Morphine. Mice with BCM-7 characteristics in a given environment began to exhibit behaviors with autism and schizophrenia after a while. It also has values that subvert LDL values. Dr Bob Elliot and Dr Murray Laugesen make clear the high weight of A1 with their heart disease in 2001. According to a study by Bell, Grochoski and Clarke in 2006, according to high results, the incidence of education disease and type 1 diabetes was lower in milk consumption measures including casein A2 observation, and milk consumption with A2 views did not pass with less severe autism and schizophrenia symptoms. Tailford et al. (2003) experimented on 6 rabbits that they fed A1 beta-casein and A2 beta-casein for 6 weeks, something they did. As a result of the studies, it is stated that the cholesterol body is higher in rabbits fed with A1 beta casein.

The schizophrenia and autism-related brain areas of β-casomorphin-7 (β-CM7) cells generated in A1 components were seen in the article "A Peptide Found in Schizophrenia and Autism Causes Behavioral Changes in Rats."

CHAPTER 3

MATERIAL AND METHOD

3.1 . Materials

All the materials required to complete the experiments for this thesis, including chemicals, substrates, and used laboratory equipment, will be listed under this subheading.

3.1.1. Substrates

Milk samples used for the analysis were indicated below:

- Holstein
- Jersey
- Brown-Swiss
- Guernsey

They were provided from a dairy farm of Çanakkale, Uluova Milk Trading Co.

3.1.2 Chemicals, Kits and Required Items

All laboratory equipment and chemicals used in this thesis is given below (Table 2).

Table 2.
List of materials, suppliers' catalog number and usage information

Name of Materials	Catalog Number	Information
10-kDa-cut-off centrifugal filter	UFC9010	Enzyme concentration
Ethanol	920.026.2500	Protein isolation
Sodium Chloride	31434-5Kg-R	Cell lysis
dH_2O	-	-
Master Mix (2X ExPrime Taq)	G-5000	Master mix solutions for PCR
EcoSpin Blood Genomic DNA Kit	EcoBGD-50x	DNA Isolation

3.1.3. Laboratory Equipments

In the course of conducting research for this thesis, a range of laboratory equipment was used. All equipment was accessed through the facilities of Canakkale Onsekiz Mart University (COMU) Molecular Biology and Genetics Department research laboratory. The following is a comprehensive list of all equipment used (Table 3).

Table 3.
List of Laboratory Equipment used for the thesis

Device name	Brand name
-20˚C freezer	Arçelik
Analytical balance	Shimadzu
Autoclave	NÜVE
Centrifuge	Beckman Allegra X-15R
Cooling centrifuge	Hettich Mikro 200 R
Ice generator	Izmak
Incubator	Indem Nüve EN 400
Orbital shaker	STUART
pH meter	IsoLab
Power supply	Bio Rad
Pure water system	Millipore
Thermal shaking incubator	INOVIA
Vortex	Vortex Genie 2
PCR Thermo Cycler	Thermo Fisher

3.2. Method

3.2.1. DNA Isolation

After collecting blood from individual animals into EDTA tubes, DNA isolation was performed in accordance with the EcoPURE Genomic DNA Kit instructions. NanoDrop Micro-Volume UV-Vis DNA concentration and purity of the isolates obtained using a spectrophotometer were measured. It will be used in the PCR step within the framework of the concentration values obtained.

The reaction volumes of genomic DNA and other components were determined.

Table 4.
Chemicals Used for PCR and Amounts.

Chemical	Amount
Isolated genomic DNA (~300ng)	2µL
B-casein F primer	2µL
B-casein R primer	2µL
Master Mix (2X ExPrime Taq)	50 µL
dH_2O	4 µL

3.2.2. Study of β-Casein Gene by PCR Method

Appropriate primers were designed using NCBI-Primer BLAST for the CSN2 gene region from the obtained DNA samples. PCR method was used to amplify the relevant gene fragment. To be able to determine the A1 and A2 variants of the PCR products obtained as a result of this process, cutting process was performed with NsiI-Hf restriction enzyme and the results were obtained.It was visualized and recorded by 2.5% agarose gel electrophoresis. If there are differences in the cleavage sites of the same restriction enzyme(s) in the DNA samples (may be due to mutation), the lengths of the DNA fragments formed after splicing differ. Considering this situation, genotyping evaluations were made.

3.2.3. *In-vitro* Digestibility Test of Milk Protein

Glycoproteins obtained from milk samples were tested via *In-vitro* Digestion method developed by thesis study of Kaplan M., 2022.

This digestive model incorporates the entire digestive system, including the mouth cavity, stomach, small intestine, and large intestine. For each phase, digestive solutions prepared with appropriate human-derived enzymes (amylase-oral; pepsin-stomach etc.) were used (Minekus et al., 2014). Spray dry and freeze dry powdered milk samples were prepared at appropriate concentrations and incubated in the solutions of the digestion model under appropriate conditions (temperature, pH, time). The pH values and enzyme contents of each digestion phase are different from each other. Especially in the last phase, the large intestine, recombinant microbial enzymes have a critical effect on the digestion of indigestible compounds.

Milk protein samples (1 g) was filtered using 10 kDa Amicon tube to remove contaminants including free oligosaccharides was used as a glycoprotein source that includes a high concentration of glycans. All digestive solutions were created, including simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). All digestion solutions and enzymes were preincubated at 37°C before the experiment. 1 mL of the sample was removed after each phase and kept at -20°C until the next usage. In general, in the oral phase, milk proteins which does not contain free oligosaccharides, was added to a 3.5 mL SSF stock solution. Then, human salivary a-amylase (EC 3.2.1.1, 15000 U mL-1) and 25 μL, 0.3 M $CaCI_2$ were added to the solution. Finally, 975 μL distilled water was added to the mixture and mixed well. The incubation time for the oral phase took 2 min. at 37°C shaking by hand. In the gastric phase, the final ratio of food to SGF solution was at 50:50 (v/v) after adding other components. A 10 mL liquid sample was added to a 7.5 mL SGF solution and then 1.6 mL pepsin (from porcine gastric mucosa 3200- 4500 U mg-1) was mixed with the mixture. 5 μL, 0.3 M $CaCI_2$, and 1 M HCI for keeping pH at 3.0 and 0.695 μL distilled water were added to the final mixture. The incubation time for the gastric phase was 2 hours at 37°C, 100 rpm. In the intestinal phase, the final ratio of gastric chyme to SIF stock solution was at 50:50 (v/v) after adding other chemicals and distilled water. 1 M NaOH was required to adjust pH at 7.20 mL of gastric chyme from the previous phase was mixed

with 11 mL of SIF solution. 5 mL pancreatin solution (from porcine pancreas, 800 U mL-1), 2.5 mL, 160 mM fresh bile, 40 µL, and 0.3 M $CaCI_2$ were added to the mixture. Finally, 0.15 mL of 1 M NaOH was added to adjust pH at 7.0 and 1.31 mL of distilled water was mixed with the final solution. The incubation time for intestinal digestion took 2 hours at 37°C, 100 rpm. During the experiment, 2 µL of each recombinant enzyme was integrated into appropriate phases. The last colon part where the microbial mechanism of digestion takes place includes only microbial enzymes and it was incubated overnight under the conditions of 37°C, pH 8, and 100 rpm.

The samples from each phase (1 mL) were mixed with cold ethanol (1:4; v/v) and incubated at -20°C for 1 h to precipitate proteins. After the incubation, samples were centrifugated for 30 min under the conditions of 4°C and 4000 rpm. The supernatant parts were removed and dried using a vacuum evaporator machine. The dry samples were dissolved with 600 µL dH_2O and used in a phenol sulphuric acid assay to be quantified. As for the phenol-sulphuric acid assay, each 25 µL sample was mixed firstly with 25 µL phenol (1:1; v/v) and then 125 µL sulphuric acid in a plate. After the 20 min incubation at room conditions, concentrations were measured at OD_{490} nm.

The final product peptides passed through the in-vitro digestion model are collected in 10kDa membranes and their concentrations are determined by Qubit etc. analyzed and the results obtained were compared. Thus, the digestion rates of A2 and A1 milks were calculated.

Table 5.
List of chemicals to be used for in-vitro digestive system

No	Chemical	mol/L (M)	g/L
(1)	KCl	0.5	37.5
(2)	KH_2PO_4	0.5	68
(3)	$NaHCO_3$	1	84

Continuation of table 5

(4)	NaCl	2	117
(5)	$MgCl_2(H_2O)_6$	0.15	30.5
(6)	$(NH_4)_2CO_3$	0.5	48
(7)	HCl	6	
(8)	$CaCl_2(H_2O)_2$	0.3	44.1

Table 6.
Preparation amounts of simulated digestive fluids

No	**SSF (pH:7)**	**SGF (pH:3)**	**SIF (pH:7)**
(1)	15.1 mL	6.9 mL	6.8 mL
(2)	3.7 mL	0.9 mL	0.8 mL
(3)	6.8 mL	12.5 mL	42.5 mL
(4)	-	11.8 mL	9.6 mL
(5)	0.5 mL	0.4 mL	1.1 mL
(6)	0.06 mL	0.5 mL	-
(7)	0.09 mL	1.3 mL	0.7 mL

Table 7.
Enzymes used for the in-vitro digestive system and their preparation rates

Enzyme	Brand	Activity	Preparation Rate
α-amilase	Sigma (A1031)	300-1500 U/mL	15 mg α-amilase + 10 mL SSF
Pepsin	Sigma (P7012)	≥2500 U/mL	100 mg pepsin + 10 mL SGF
Pancreatin	Sigma (P7545)	≥100 U/mL	80 mg pankreatin + 10 mL SIF
Bile	Sigma (B8631)	-	250 mg bile + 10 mL SIF

Table 8.
Recombinant Microbial Enzymes Used in an In-Vitro Digestive System Model

	GenBank ID / Accesion Number **Locus tag**	**Microorganism**
1	ATP38112.1 CR531_08240	*Lactobacillus salivarus subsp. salivarius (Ligilactobacillus salivarius)* ATCC 11741
2	ATP36889.1 CR531_01355	*Lactobacillus salivarus subsp. salivarius (Ligilactobacillus salivarius)* ATCC 11741
3	ATP37244.1 CR531_03290	*Lactobacillus salivarus subsp. salivarius (Ligilactobacillus salivarius)* ATCC 11741

Continuation of table 8

4	SQH52440.1 NCTC11324_01490	*Streptococcus intermedius* ATCC 27335
5	ATP38122.1 CR531_08290	*Lactobacillus salivarus subsp. salivarius (Ligilactobacillus salivarius)* ATCC 11741
6	ATP37586.1 CR531_05275	*Lactobacillus salivarus subsp. salivarius (Ligilactobacillus salivarius)* ATCC 11741
7	SQH51076.1 NCTC11324_00070	*Streptococcus intermedius* ATCC 27335
8	CAR86329.1 LGG_00434	*Lactobacillus rhamnosus GG*
9	AAO77566.1 BT_2459	*Bacteroides thetaiotaomicron* ATCC 29148
10	ABR41745.1 BVU_4143	*Bacteroides vulgatus* ATCC 8482
11	AAO75562.1 BT_0455	*Bacteroides thetaiotaomicron* ATCC 29148
12	ACD04858.1 Amuc_1032	*Akkermansia muciniphila* ATCC BAA-835
13	BAQ98211.1 BBBF_1004	*Bifidobacterium bifidum* ATCC 29521
14	BAQ97897.1 BBBF_0690	*Bifidobacterium bifidum* ATCC 29521

Continuation of table 8

15	ERK41518.1 HMPREF0495_02198	*Levilactobacillus brevis (Lactobacillus brevis)* ATCC14869
16	ACJ51836.1 Blon_0732	*Bifidobacterium longum subsp. infantis* ATCC 15697
17	ACJ53413.1 Blon_2355	*Bifidobacterium longum subsp. infantis* ATCC 15697
18	QQA29671.1 I6G58_17045	*Bacteroides uniformis FDAARGOS_901* ATCC 8492
19	BAQ30021.1 BBKW_1886	*Bifidobacterium catenulatum subsp. kashiwanohense* JCM 15439
20	ABR38247.1 BVU_0537	*Bacteroides vulgatus* ATCC 8482
21	SQF24907.1 NCTC12958_01101	*Streptococcus thermophilus* ATCC 19258
22	SQF25661.1 NCTC12958_01892	*Streptococcus thermophilus* ATCC 19258
23	SQF24918.1 NCTC12958_01112	*Streptococcus thermophilus* ATCC 19258
24	ACD04701.1 Amuc_0868	*Akkermansia muciniphila* ATCC BAA-835
25	ACD04208.1 Amuc_0369	*Akkermansia muciniphila* ATCC BAA-835

Continuation of table 8

26	BAQ97280.1 BBBF 0073	*Bifidobacterium bifidum* ATCC 29521
27	CAH09389.1 BF9343_3608	*Bacteroides fragilis* ATCC 25285
28	ABR38963.1 BVU 1273	*Bacteroides vulgatus* ATCC 8482
29	ACJ53522.1 Blon 2468	*Bifidobacterium longum subsp. infantis* ATCC 15697
30	ACJ51376.1 Blon_0248	*Bifidobacterium longum subsp. infantis* ATCC 15697

3.2.4. Statistical Analysis

The data gathered during this investigation were subjected to a rigorous statistical analysis using the one-way ANOVA variance analysis and the Tukey's multiple comparisons statistical test. With a significance level of p0.05, these approaches were used to determine the dataset's statistical significance. Because it can handle intricate statistical analyses and interpretations, the NCSS 21 statistical program was used to conduct the analysis.

CHAPTER 4

RESEARCH FINDINGS

In this section, qualitative and quantitative research findings are presented in accordance with the purpose of this study.

4.1. PCR Fragments

Table 9.
PCR Fragments After Restriction Enzyme

PCR Product	A1A1	A2A2	A1A2
B-casein 321 bc	284/37	321	321/284/37

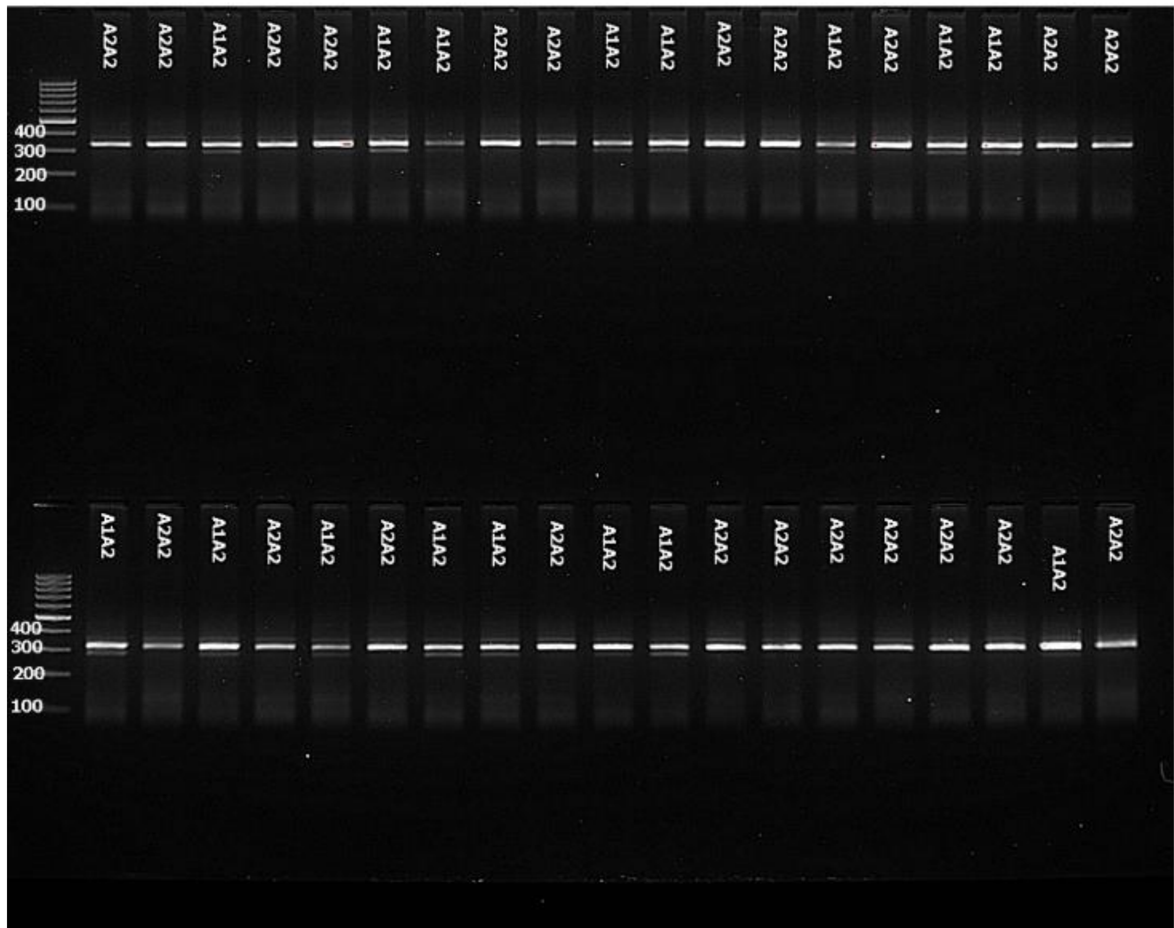

Figure 10. Visual of Samples Visualized by Gel Electrophoresis

When Figure 3 is examined, the samples with a single thick band were identified as A2A2, and when there was a thick band and a secondary band below it, it was understood to be A1A2. After this stage, Elisa experiments of milk obtained from cows that were shown to have A1 or A2 variants by genotyping were started. At this stage, in order to fully reveal the known error rate risk of Elisa tests, samples prepared at different rates were tested with LCMS and compared with Elisa (Biosensis) results. When the results were examined, it was observed that the Elisa test was successful in pure samples, but was insufficient to measure different mixtures. The reason for testing different mixtures is to reveal whether Elisa tests can be used, especially when it is necessary to determine the content of tank milk.

4.2. Elisa and LC-MS

Table 10.
A1 and A2 contents of different samples prepared after genotyping

Number	Sample	LC-MS A1 Beta Casein mg/g	LC-MS A2 Beta Casein mg/g	A1 Elisa mg/g	A2 Elisa mg/g
1	A1 – A1 (Individual)	26,3	1,43	25,3	TE
2	A2 – A2 (Individual)	0,008	33,2	TE	31,5
3	A2 – A2 (Individual)	0,332	33,8	TE	30,6
4	A1 – A2 (Individual)	26,4	22	10,7	32,5
5	A1 – A1 (Mixture)	20,7	0,78	25,9	TE
6	A2 – A2 (Mixture)	0,99	34,7	TE	30,2
7	A1 – A2 (Mixture) %50-%50	18,6	14,6	7,6	22,5
8	A1 – A2 (Mixture) %25-%75	11	20,2	3,1	25,6
9	A1 – A2 (Mixture) %5-%95	2,49	32,8	3,2	26,6
10	A1 – A2 (Mixture) %1-%99	0,83	36,0	2,4	26,7

When Table 3 is examined, it is seen that the samples obtained from individual A1A1 and A2A2 cows can be distinguished by the Elisa method, but the sample results obtained from individual A1A2 cows cannot be at the desired level. In this example, approximately 50% A1 and 50% A2 protein measurements are expected. Although the LC-MS method gave an acceptable result, the Elisa method could not give a sufficiently sensitive result. When the A1A1 and A2A2 mixture samples were examined, Elisa gave successful results like the individual samples, but the same sensitivity was not observed when A1 and A2 mixtures were prepared at different ratios. When all these results were evaluated, it was shown that

the Elisa tests were insufficient to test the ratios of A1 and A2 proteins in a sample, but were successful in validating individual samples.

4.3. In-Vitro Digestion

Milk proteins are digested to form peptides. The amount of peptide formed as a result of digestion determines the amount of digested protein.

Table 11.
Peptide concentrations

	Qubit Results After Digestion (mg/mL)
Freeze Dry A1 Milk Sample	1.15
Freeze Dry A2 Milk Sample	1.21

1 g sample was used in the study. Peptide concentration formed after in vitro digestion of A1 and A2 milk samples dried by freeze drying method was measured with Qubit device. For the in vitro digestion test, the peptide concentration formed after digestion was measured in the samples tested at equal times. Higher peptide concentration means more protein is broken down. The cleavage of the protein to form peptides explains its digestibility.

A QUBIT 3.0 fluorometer was used to measure the peptide concentrations and protein content of A1 and A2 milk samples. (Thermo Fischer Inc, CA USA). The peptide concentration released after in vitro digestion of A2 milk sample is higher than the peptide concentration released after in vitro digestion of A1 milk sample, according to the study. The findings indicate A2 milk's faster in vitro digestion.

CHAPTER 5

RESULTS AND RECOMMENDATIONS

Numerous investigations have indicated that bovine milk predominantly containing or exclusively composed of A2 beta-casein protein may potentially offer certain health advantages in comparison to A1 milk. The examination of the digestive processes connected to A1 milk that produce -casomorphin-7 (BCM-7) is the basis for these studies. The subsequent elevation of BCM-7 has been implicated in fostering inflammation, type-1 diabetes, cardiovascular ailments, autism, gastrointestinal discomfort, and other health conditions within the consumer demographic. Consequently, there exists a mounting global interest in the attributes of A2 milk, as it is perceived to present a potential solution to circumvent the adverse health effects associated with A1 milk consumption. Notably, the comparative impact of A1 and A2 milk variants on human health exhibits a nuanced spectrum of outcomes across various investigations. Given the present body of evidence, a definitive evaluation of the health ramifications attributed to A1 and A2 milk remains elusive.

Thus, it is imperative to acknowledge the need for continued and in-depth inquiries into this matter. Further comprehensive investigations, encompassing diverse methodologies and populations, are warranted to establish a more conclusive understanding of the distinctive health implications associated with A1 and A2 milk consumption.

A prominent attribute associated with A2 milk technology is its purported ease of digestibility. Nonetheless, a comprehensive review of the existing literature reveals a conspicuous absence of empirical scientific data substantiating this assertion. Addressing this discernible knowledge gap necessitates the implementation of suitable methodological approaches aimed at quantifying and elucidating the extent of digestibility within the domain of A2 dairy technology.

Through diligent measurement and investigation, the intention is to rectify this deficiency, thereby contributing substantively to the scientific discourse on A2 dairy technology. This effort also serves the purpose of augmenting the existing body of literature dedicated to this specific facet. By rigorously pursuing these endeavors, a more comprehensive understanding of the digestibility aspect inherent to A2 milk technology is envisaged, ultimately enriching both the scientific domain and the extant scholarly works.

Within this framework, the validation of A2 milk technology constitutes the initial phase. To achieve this, A1 and A2 predictive analyses were conducted employing genotyping analysis on blood samples sourced from cattle. Concomitantly, the ELISA method was employed to corroborate the A2 beta-casein content within milk samples originating from cattle that had been duly validated via A2 prediction.

An intrinsic facet of the A2 milk technology is its assertion of an easily digestible constitution. However, a dearth of scientific data addressing this claim within the corpus of literature is evident. Quantifying the enhanced digestion rate of A2 constructs posed a pivotal challenge in the study. This research endeavor is purposed to rectify these conspicuous gaps, contributing comprehensively to both the scientific domain and the literary sphere concerning A2 milk technology through judicious utilization of pertinent methodologies.

In this pursuit, a novel in vitro digestive model was employed, facilitating the quantification of liberated peptides. This approach engendered numerical insights into the disintegration kinetics of A1 and A2 milk compositions.

Subsequently, the gastrointestinal repercussions of A1 milk and its correlation with lactose intolerance were explored. A1 milk consumption has been associated with the release of β-casomorphin-7 (BCM-7), thus causing gastrointestinal upset. In contrast, A2 milk is distinguished by its comparative ease of digestion when juxtaposed with A1 milk. A cohort characterized by lactose intolerance was engaged in the study, with consumption of A2 milk, A1 milk, lactose-free milk, and conventional Jersey milk. The ensuing gastrointestinal

responses were methodically evaluated, yielding insights into digestive efficacy and ensuing discomfort. The outcomes underscored the diminished gastrointestinal symptoms linked with A2 milk consumption, offering relief from abdominal discomfort.

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APPENDICES

APPENDIX 1

ORAL PRESENTATION IN 10TH INTERNATIONAL CONGRESS ON LIFE, ENGINEERING AND APPLIED SCIENCE IN A CHANGING WORLD

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