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Delving into the A1/A2 milk hypothesis: A comprehensive analysis of milk proteins and their impact on human health

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Abstract
The A1 vs. A2 milk controversy have captured the attention of consumers, researchers, and dairy enthusiasts alike. This review delves into the scientific literature to provide an in-depth analysis of the A1 vs. A2 milk debate, aiming to rule out facts from fiction. We have explained in depth the origins of this controversy, the biological basis of A1 and A2 milk, and the purported health implications associated with the milk type. It is essential to consider genetic variations in cow breeds and individual tolerance. A1 and A2 milk are defined by distinct beta-casein proteins, generating different digestion by-products. A1 milk, associated with beta-casomorphin-7 (BCM-7) production, faces claims of causing various health issues, from digestive discomfort to chronic diseases. Nevertheless, scientific consensus is lacking. Despite the debate, the market for A2 milk has expanded, reflecting consumer interest. In conclusion, this multifaceted issue calls for further research to provide definitive answers and guide consumer choices.

Keywords: Bovine, A1 and A2 milk, genetic variation, beta-casomorphin-7 (BCM-7)

Introduction
Milk is often hailed as a well-rounded diet, encompassing a wide range of essential nutrients, although it is deficient in iron content. It truly stands as a natural blessing, especially for nurturing newborns, as it provides the vital micronutrients crucial for both human and neonatal animal growth and overall health. India is the largest milk producer and maintaining its top position as the world's foremost milk-producing nation, with a total milk output of 221.06 million tonnes. This marked a significant 5.29% increase compared to the preceding year. Within India's diverse dairy landscape, indigenous cattle played a role in contributing 10.35% to the nation's overall milk production, while non-descript cattle and non-descript buffaloes contributed 9.82% and 13.49%, respectively, to the total milk production (BAHS, 2022) [2]. Despite being regarded as a comprehensive source of nutrition, the discourse took on a new facet in 1992 when New Zealand scientists unveiled a link between the type of milk consumed and the incidence of type-1 diabetes, prostate cancer, and ovarian cancer (Elliott, 1992) [16].
Considering the nutrient status of the milk, caseins constitute nearly 80% of total protein present in the cow milk. Casein contains total four different subtypes, among that β-casein is one of most present subtype in cow milk. On the basis of genetic characterization, total 13 variants of β-casein exist, among these, A1 and A2 are the most common variant in milch cattle globally. A1 beta-casein and A2 milk differ in the amino acid composition at the 67th position within their protein structure. In A1 milk, this position contains a histidine molecule, whereas in A2 milk, it contains proline. Beta-casomorphin-7 (BCM-7) is a bio active peptide which is released upon digestion of A1 milk in the small intestine. This particular peptide has opioid-like properties, and it is known to exert an inhibitory effect on the immune system (Elliott, 1992) [16]. There is ongoing research and some suspicion that the consumption of A1 beta-casein and the subsequent release of BCM-7 may be associated with several health issues.

These potential concerns include an increased risk of developing type 1 diabetes (DM-1), coronary heart disease (CHD), infant mortality, and autism.

In 2007, the publication of the book titled "Devil in the Milk" had a significant impact by associating the intake of A1 β-casein with the development of Type 1 diabetes (Woodford, 2009) [82]. This association caused considerable concern among milk consumers and led to a notable increase in the demand for A2 milk in both Australia and New Zealand. The book's argument was rooted in a thorough examination of the existing scientific literature, referencing more than 100 number of scientific reports that explored the potential contrary properties of A1 milk consumption. It delved into the mechanism by which beta-casomorphin-7 (BCM-7) enters the bloodstream, particularly in individuals who may suffer from a condition known as leaky gut syndrome. The book posited that BCM-7, a bioactive peptide released during the digestion of A1 milk, could find its way into the bloodstream via the compromised gut barrier, thus raising concerns about its potential health impacts. Furthermore, it extended its claims to suggest that the entry of BCM-7 into the bloodstream might also occur in infants, thereby raising additional concerns about the health implications of A1 milk consumption among young children. The book went so far as to draw connections between the presence of BCM-7 and the emergence of symptoms associated with conditions like autism and schizophrenia. It is essential to note that the assertions made in the book were considered quite controversial and sparked a great deal of debate and further research. While they prompted increased interest in A2 milk as a potential alternative, the exact relationship between A1 milk, BCM-7, and these health conditions remained a topic of ongoing scientific investigation and discussion. However, it's essential to note that this is an area of active scientific investigation, and the exact relationship between A1 beta-casein, BCM-7, and these health conditions is still a subject of debate and further research. In 2008, the New Zealand Food Safety Authority (NWFSA) sought the assistance of the European Food Safety Authority (EFSA) to address growing concerns among consumers regarding A1 milk. The EFSA conducted an inclusive scientific analysis, and their statement in 2009 concluded that there was no clear cause-and-effect association between the dietetic intake of beta-casomorphin-7 (BCM-7) and several ailments. Furthermore, the information stated that the oral consumption of BCM-7 or associated peptides was not linked to the development of non-communicable diseases, contradicting some earlier studies (EFSA. 2009). As a result, the EFSA did not recommend a formal risk assessment for food-derived peptides. Findings from this report were widely accepted by food safety authorities in Australia, New Zealand, and other nations, providing reassurance to clients of regular milk. Nevertheless, since the testimony acknowledged the possibility of certain individuals experiencing gastrointestinal changes, the attention turned toward researching digestive issues associated with A1 milk, especially among individuals with milk intolerance.

As part of a crossbreeding program, Indian cattle were bred with European breeds to create high-yield milk-producing varieties. However, a concern arose because these crossbred cows produced A1 milk. In India, the majority of milk is still sourced from buffaloes, which produce A2 milk. The controversy surrounding A1 milk is an urgent issue that requires attention, but its harmful effects have yet to be definitively established. The present review attempted to investigate various information available on A1 and A2 milk debate till date to get the comprehensive knowledge about the same.

Casein protein in bovine milk

Milk, a complex fluid, primarily comprises approximately 85% water, while the remaining 15% consists of essential components, including lactose (milk sugar), protein (Comprising approximately 3.5 to 3.9% of milk's composition), fat, and minerals. Bovine milk is a rich source of amino acids, proteins, lipids, vitamins, minerals, growth factors, hormones, immunoglobulins, and various bioactive compounds. The composition of milk is subject to variation due to factors such as the age and breed of the animal, the stage of lactation, dietary factors, and the overall health of the udder tissue. One of the key constituents of milk is beta-casein, constituting approximately 30% of the total protein content (Figure 1). In addition to beta-casein, milk comprises other proteins and lipids, with proteins making up approximately 3-3.5% of milk's composition. Casein is the predominant protein fraction in milk, accounting for 76-86% of the total milk proteins, while whey proteins make up the remaining 14-24%. In bovine milk, various casein fractions exist, including β-Lactoglobulin (making up approximately 50% of whey protein), α-Lactalbumin (comprising around 20%), Proteose/Pentones (approximately 20%), immunoglobulins (about 8%), and certain fractions of blood albumins. The composition of milk, with its intricate balance of water, proteins, fats, and minerals, plays a critical role in nutrition and is influenced by a multitude of factors that are pivotal in understanding its biological significance. The colloidal complexes of casein micelles are formed through interactions between proteins and calcium, while milk lipids are emulsified in globules within membranes, and most minerals are present in lactose solution.

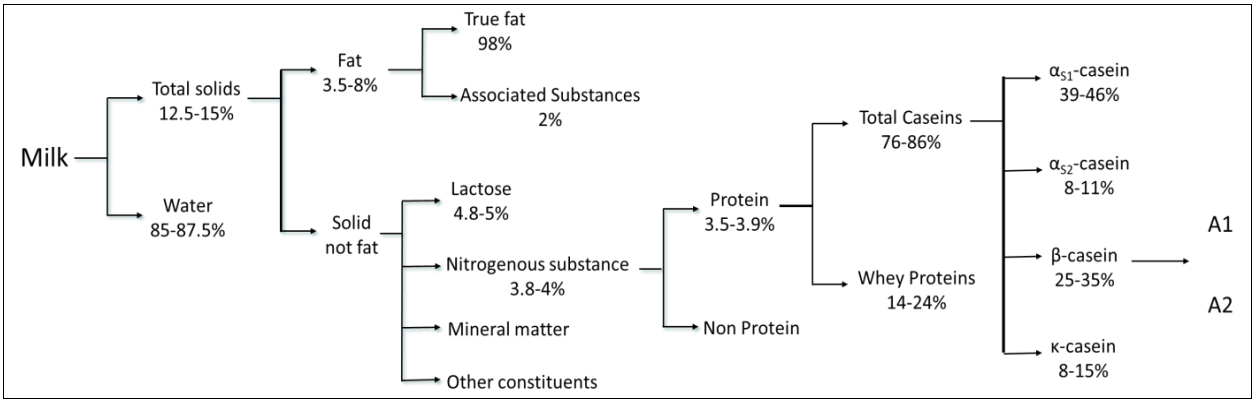

Fig 1: Milk Composition and Proportion of A1 / A2 Milk

Historical background of A1 and A2 Milk

A2 beta-casein, a protein originating from cows bred for over 10,000 years before domestication, has been associated with no known adverse effects on human health. The timeline showing the evolution of A1 and A2 milk has been given in Figure 2. However, a pivotal transformation unfolded over the past few millennia, triggered by a natural mutation within the cattle population, particularly European breeds. This genetic shift introduced a casein variant known as A1 beta-casein, gradually establishing its dominance in milk production. The genetic alteration responsible for this transition involved a change in the beta-casein gene, specifically at the 67th amino acid position among the 209 amino acids, where proline was replaced by histidine (Figure 3 and Figure 4). This genetic shift led to the emergence of A1 beta-casein, predominantly set up in the milk of crossbred cows such as Jersey, Holstein-Friesian (HF) etc.

The A1 vs. A2 milk narrative gained significant momentum starting in September 2007, when Keith Woodford's book surfaced in New Zealand. This story begins with compelling epidemiological evidence showcasing a robust correlation between nations that have a high consumption of A1 milk and elevated incidences of both type 1 diabetes and heart disease. A2 milk, in particular, contains the A2 beta-casein protein, while A1 type of milk is exclusively characterized by the presence of A1 beta-casein or the A1A2 type variant. The A1 protein variant is predominantly present in milk from crossbred and European cattle breeds, while A2 milk primarily originates from indigenous cows and buffaloes in regions like India and across Asia. This historical context sheds light on the intricate interplay between genetics, milk composition, and its potential implications for human health.

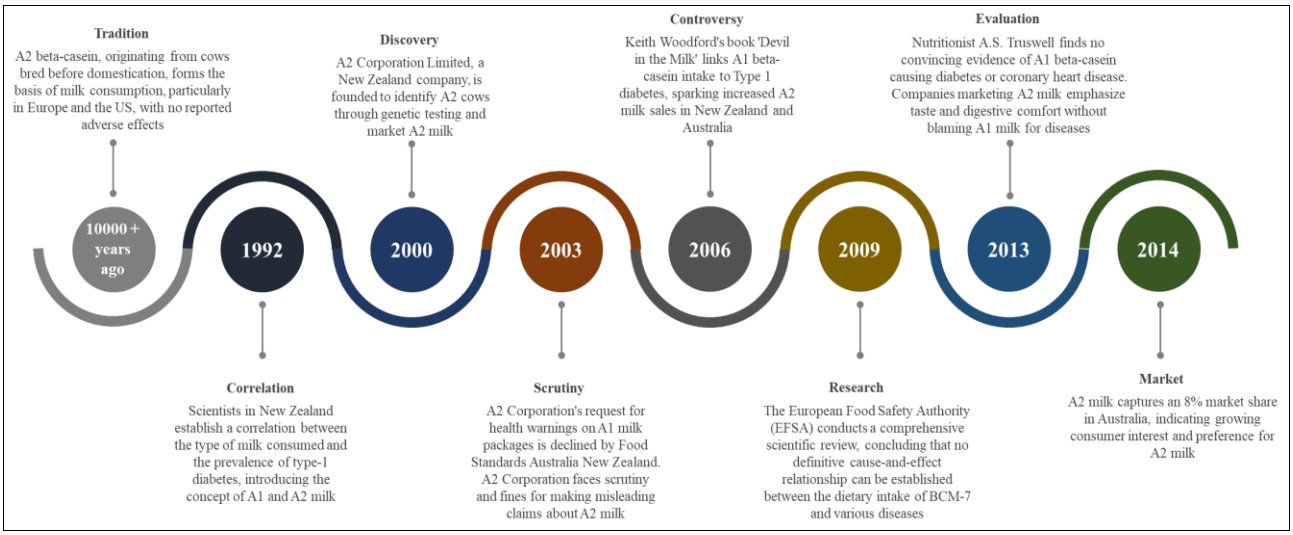

Fig 2: Evolution of A1 and A2 Milk: A Journey from Ancient Origins to Ongoing Debate

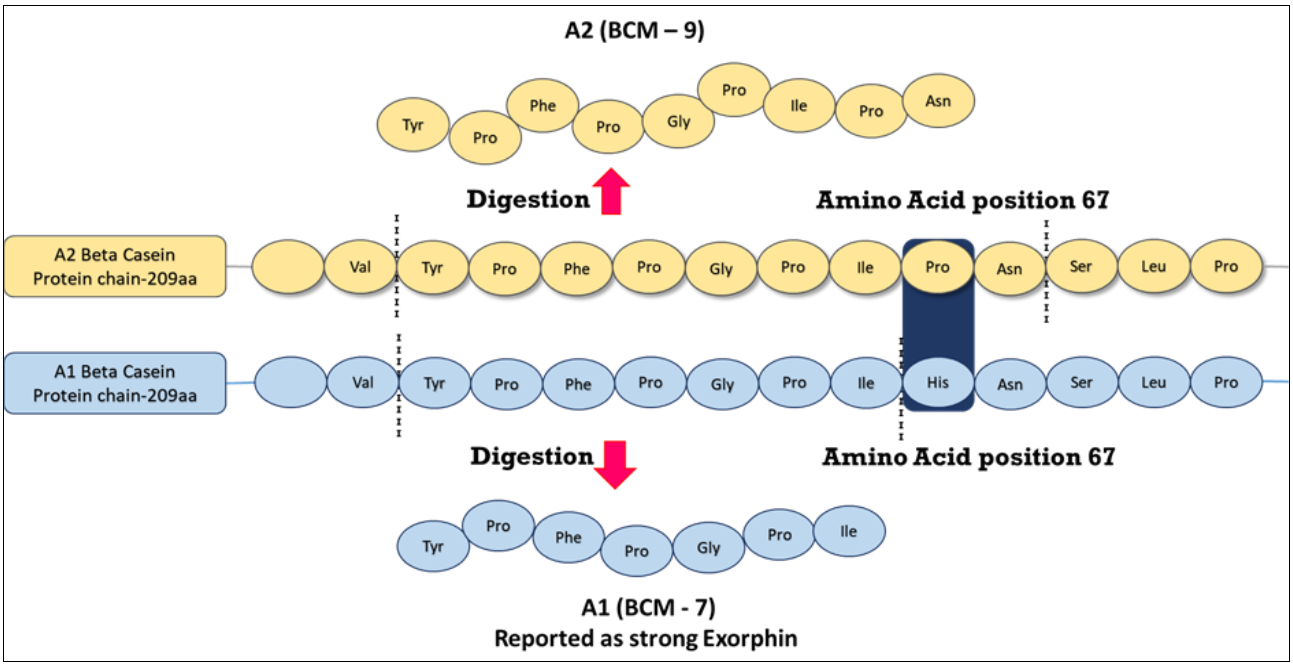

Fig 3: Amino acid structural difference between A1 and A2 milk

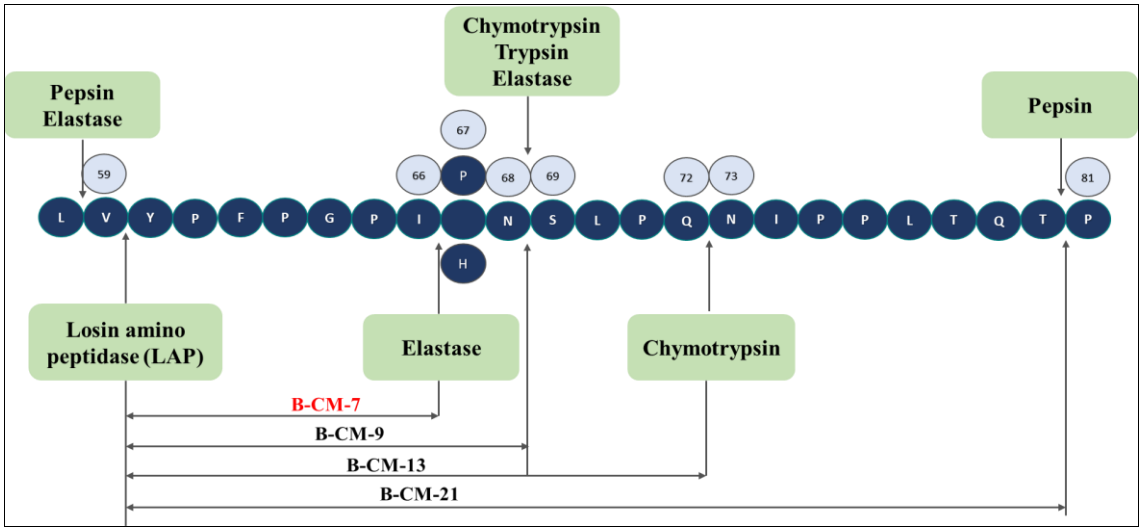

Fig 4: Splitting sites of β-casein with various digestive enzymes (Jinsmaa and Yoshikawa 1999) [29]

The A1 and A2 milk saga - market and trade perspectives

The β casein variants of A1 and A2 milk are B, C, F, G and A3, E, D, H respectively. On basis of this variants, we can trace the historical development of A1 and A2 milk, from its ancient origins to the ongoing controversy surrounding it. Over centuries, A1 milk has been a staple in the diets of people in Europe and the United States, much like crossbred cow milk has been embraced by Indian consumers for over five decades, with no reported adverse effects. In India, milk holds a revered place in mythology, culture, and daily nutrition. Despite being regarded as a complete food, some groups have discouraged milk consumption, often without substantial evidence, raising concerns about its potential links to conditions like ovarian as well as prostate cancer, type-1 diabetes, multiple sclerosis, elevated cholesterol levels, weight gain, bone health issues, and more. Naturopathy even advises asthma and psoriasis patients to avoid milk. However, the majority of people worldwide, except for a minority with lactose intolerance, have been incorporating milk into their daily diets without noticeable issues.

In 1992, the A1 and A2 milk debate took a new turn when New Zealand scientists discovered a possible connection between the incidence of type-1 diabetes and the kind of milk used up. This discovery led to the identification of A1 and A2 milk variants. Cow's milk typically consists of around 87-88% water and 12-13% solids, including lactose (4.8%), fat (3.9%), protein (3.2%), and minerals (0.7%). Of this protein content, about 80% is casein, with 30-35% being beta-casein. Significantly, this beta-casein can be categorized as either A1 or A2 beta-casein. When A1 beta-casein is digested in the small intestine, it releases a bioactive peptide known as beta-casomorphin-7 or BCM-7, which exhibits opioid-like properties. Concerns have been raised about the potential association of BCM-7 with type-1 diabetes, autism, heart disease, and infant mortality. A1 milk, primarily obtained from European cow breeds such as HF, Ayrshire, and British Shorthorn, contains this A1 beta-casein variant. In contrast, A2 milk is sourced from Jersey and Guernsey cows in the Channel Islands, the Charolais and Limousin breeds of Southern France region, and the Zebu cattle of Asia and Africa., which produce A2 milk without BCM-7. However, surveys reveal that the prevalence of A1 and A2 milk-producing cows can extend beyond specific breeds. For instance, 50-65% of HF cows in North America yield A1 type of milk, while over 90% of HF cows in Germany produce A2 milk. Furthermore, 98% of Indigenous breeds of cow and 100% of buffalo breeds yield A2 type of milk.

In 2000, the A2 Corporation Limited, a New Zealand company, emerged to identify A2 cows through genetic testing and promote A2 milk. In 2003, it requested health warnings on A1 milk packages from Food Standards Australia New Zealand, but this request was declined, and A2 Corporation was even asked to remove its A2 milk rights. Despite this setback, it partnered with the Australian company A2 Dairy Marketers for A2 milk procurement and marketing purpose. In 2004, both companies faced fines from the Australian government for making ambiguous rights about A2 type of milk.

In 2006, the book 'Devil in the Milk,' authored by Keith Woodford, linked A1 beta-casein consumption to Type 1 diabetes, leading to increased A2 milk sales in New Zealand and Australia. This prompted the NZFSA to commission the EFSA to deportment a inclusive systematic assessment. The EFSA's 2009 report concluded that no definitive cause-and-effect association could be conventional between dietary ingestion of BCM-7 and various diseases. Nevertheless, A2 milk continued to gain a significant market share, accounting for 8% in Australia by 2014. The A2 Corporation also expanded its A2 milk marketing efforts to the US, UK, and China. In May 2013, when asked about the current status of A1 and A2 milk, renowned nutritionist A.S. Truswell, who extensively studied investigation on the theme, specified that he found no substantial or even possible indication of A1 beta-casein being a causal reason for diabetes or coronary heart disease. He noted that companies selling A2 milk only claimed it "tastes better" or "does not cause bloating," refraining from attributing any diseases to A1 milk.

In India, the National Bureau of Animal Genetic Resources (NBAGR), the National Dairy Research Institute (NDRI), and the Indian Veterinary Research Institute (IVRI) began researching A1 and A2 milk in 2009. However, much of their work tangled reviewing investigation conducted in other nations, with a focus on reports from New Zealand emphasizing the detrimental effects of A1 milk. This research largely overlooked critical findings published by Truswell in 2005 and the EFSA in 2009. Nevertheless, the NBAGR's efforts revealed that a significant number of Indian cattle breeds possessed A2A2 genotypes, with only a limited number of cattle from Malenadu Gidda and Kherigarh breeds having A1 A2 genotypes.

Fundamental genetics of a1 and a2 milk variants

The A1 or A2 classification of cattle is determined by a couple of genes situated on the 6th chromosome, as documented by Rijnkels in 2002. These genes correspond to two major alleles, denoted as A1 and A2 beta-casein alleles. Each cow carries a pair of beta-casein genes, resulting in three possible combinations: A2A2 (Homozygous), A1A2 (Heterozygous), or A1A1 (Homozygous). It's important to note that neither allele exerts dominance over the other; instead, they exhibit codominance, meaning their effects are additive. Consequently, an A1A2 cow will produce equal amounts of A1 and A2 beta-casein. An A2A2 cow exclusively produces A2 beta-casein, while an A1A1 cow exclusively produces A1 beta-casein.

The prevalence of these alleles varies among different cattle breeds. Northern European breeds like the HF tend to carry A1 and A2 alleles in approximately equal proportions. In contrast, Southern European breeds and Jersey cows typically possess the A1 allele at around 35% and two-thirds A2. Notably, the Guernsey breed stands out with less than 10% A1 allele occurrence, while the Scottish Ayrshire breed appears to exceed 50%. Furthermore, individual herds within a breed may exhibit allele frequencies that deviate significantly from the breed's average. Understanding the genetic makeup has implications for progeny. An A2A2 cow will unfailingly pass on the A2 allele to its offspring, while an A1 cow guarantees the transmission of the A1 allele. For A1A2 cows, there exists a 50% likelihood of transmitting either allele to their progeny.

Genetic variants of β-Casein: Unraveling the fundamentals

β-Casein, a protein comprised of 209 amino acids, exhibits remarkable diversity with at least 12 known variants characterized by distinct amino acid positions (Farrell *et al.*, 2004) [20]. This diversity stems from genetic mutations, particularly within exon VII of the bovine β-casein gene on the 6th chromosome, where a crucial cytosine-to-adenine point mutation occurs. This mutation results in the substitution of proline by histidine at 67th position (Groves *et al.*, 1969) [22]. The two most prevalent forms of β-casein are A1 and A2, primarily distinguished by the amino acid residing at 67th position of the β-casein chain. A1 "like" milk features β-casein carrying A1, B, C, F, and G alleles, characterized by histidine at residue 67 (-Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66-His67-) while displaying variations at other amino acid positions. Conversely, A2 "like" milk (-Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66-Pro67-) involves β-casein containing A2, A3, D, H1, H2, and I alleles, featuring a proline residue at position 67 while also exhibiting variations at other amino acid positions (Kaminski *et al.*, 2007; Keating *et al.*, 2008) [32, 34].

To discern and analyze these β-casein variants, a range of DNA-based techniques have emerged, offering valuable insights and tools for researchers. These techniques encompass methods such as single-stranded conformational polymorphism (Barroso *et al.*, 1999) [3], PCR-RFLP (Miluchová *et al.*, 2009) [51], allele-specific PCR (Keating *et al.*, 2008) [34], real-time PCR TaqMan (Manga *et al.*, 2010) [44], and PCR-amplification created restriction site (Olenski *et al.*, 2010; Hanusová *et al.*, 2010) [53, 24], all of which facilitate the screening and characterization of these intriguing genetic alleles.

Milk protein variants in cattle: Insights into genetic diversity

Extensive research has been conducted on various cattle populations, including indigenous Zebu-type cows, robust buffaloes, and exotic Taurine-type cows, shedding light on the distribution of milk protein variants. These investigations have underscored a noteworthy pattern - the prevalence of the A1 allele, synonymous with A1 milk, is higher in exotic cattle, whereas indigenous Indian dairy cows and buffaloes exclusively carry the A2 allele, making them a reliable source of A2 milk. Among the Indian milk breeds of cows and buffaloes, the A2 allele is observed at a striking 100% frequency in breeds like Red Sindhi, Sahiwal, Tharparkar, Gir, and Rathi. In other Indian breeds utilized for agricultural purposes, this frequency hovers around 94%. In contrast, foreign breeds such as HF and Jersey cows exhibit the A2 allele at a comparatively lower frequency, approximately 60% (NBAGR, 2011). Importantly, A1 β-casein is entirely absent in the milk of pure Asian and African cattle, as reported by Ng-Kwai-Hang and Grosclaude in 2002. Therefore, our indigenous cows and buffaloes consistently yield A2 milk, establishing them as a dependable source of this sought-after variant.

Milk Protein and beta-casomorphins Derived Peptides

The composition of bovine milk protein is generally estimated to be around 80% casein and 20% whey, as reported by Shah (2000) [68], and Martien *et al.* (1994). However, it's worth noting that some researchers suggest whey proteins may constitute approximately 14%, as mentioned by McLachlan (2001) [47]. Casein, a major component of bovine milk protein, consists of four distinct components: αs1 (CSN1S1, 39–46%), αs2 (CSN1S2, 8–11%), β (CSN2, 25–35%), and κ (CSN3, 8–15%) of the total casein content, in accordance with findings by Roginski (2003). In contrast, human milk casein is primarily composed of β and κ caseins.

Among these casein components, β-casein emerges as the second most abundant protein and plays a pivotal role in the structural integrity of casein micelles. In fact, it constitutes approximately 30% of the total protein content in cow's milk. Bovine β-casein is characterized by its polymorphic nature, with 13 allelic variants identified to date, as documented by Kaminski *et al.* (2007) [32]. Notably, the A1 and A2 variants are the most prevalent allelic variants of β-casein in dairy cattle, as reported by Farrell *et al.* (2004) [20].

The polymorphic nature of β-casein, along with its potential influence on milk, fat, and protein yield, has spurred considerable research into evaluating this locus as a potential marker for dairy traits. The consumption of milk from certain breeds of cows, buffaloes, sheep, and goats can result in the release and potential absorption of bioactive peptides known as BCMs (beta-casomorphins). These peptides, generated during the digestion of β-casein, exhibit opioid-like effects akin to morphine and are thus termed β-casomorphins (β-CMs). Notably, β-CMs possess unique structural features that grant them a substantial and physiologically significant affinity with the binding sites of endogenous opioid receptors, as highlighted by Meisel and FitzGerald (1999) [48]. Of particular significance, the A1 beta-casein variant is known to yield BCM-7, whereas the A2 beta-casein variant does not produce BCM-7 during digestion, as corroborated by Woodford (2009) [82]. It is well-established that β-CM-7 is a potent bioactive peptide with opioid activity.

Exploring β-Casomorphins (BCMs): A Fascinating Insight

The term 'casomorphin' finds its etymological roots in 'caso,' signifying casein, and 'morphine,' a reference to Morphus, the Greek God of sleep, as elucidated by (Meisel *et al*., 1999) [48]. These peptides originate from the breakdown of β-casein in milk and exhibit opioid-like properties with pharmacological activities. Specifically, these peptides bind to μ-receptors found in various bodily locations, including the central nervous system, gastrointestinal tract, and certain immune cells, as documented by (Teschemacher *et al*., 2003) [75]. BCMs encompass a family of peptides, ranging from 4 to 11 amino acids, initially encrypted in an inactive form within the structure of β-casein. These peptides are liberated during the process of digestion, either *in-vivo* or *in-vitro*. Within this family, two of the most potent members are BCM-7 and BCM-5, corresponding to fragments f60–66 and f60–64 of β-casein, respectively, as highlighted by (Kostyra *et al*., 2004) [36].

Mechanism of BCM-7 Formation in the Small Intestine: Unraveling the Process

The distinction between A1 and A2 variants of bovine β-casein hinges on a critical difference at amino acid position 67, where A1 milk boasts histidine, and A2 milk features proline. This genetic polymorphism instigates consequential conformational alterations in the secondary structure of the expressed β-casein protein, as substantiated by research from Elliot *et al*. (1999) [16] and McLachlan (2001) [47]. The presence of histidine at amino acid position 67 in A1 β-casein triggers a specific process during the digestion of A1 milk. This process results in the release of a bioactive peptide consisting of 7 amino acids, known as beta-casomorphin 7 (BCM-7), in the small intestine. Conversely, the presence of proline at position 67 in A2 milk prevents cleavage at this particular site, leading to the generation of a different peptide, BCM-9, as described by Roginski (2003) and Kostyra *et al*., (2004) [36]. The prevailing belief is that the generation of BCM-7 serves as a pivotal factor associated with health issues linked to A1 milk consumption. Conversely, A2 β-casein has not been implicated in such health concerns, as substantiated by Kaminski *et al*. (2007) [32].

Release of β-Casomorphins from Milk

The classification of milk into two distinct types, A1 and A2 "like," hinges on a seemingly minor difference in the β-casein protein, specifically at the 67th position, where it can contain either proline or histidine. Surprisingly, this small protein variation is believed to have a substantial impact on the release of BCMs. In A1 "like" milk, where β-casein variants feature histidine at the 67th position, this site becomes susceptible to cleavage by various gastrointestinal enzymes, leading to the liberation of BCM-7. On the other hand, A2 "like" milk features β-casein with proline at this position, preventing cleavage and, consequently, the formation of BCM-7.

The release of BCM-7 has been demonstrated through simulated gastrointestinal digestion (SGID) using enzymes such as pepsin, pancreatic elastase, and leucine aminopeptidase (Jinsmaa *et al*., 1999) [29]. Demonstrated the release of β-casein metabolites by digestion with the help of pepsin and trypsin Similarly, De Noni (2008) [12] evaluated the production of BCM-5 on digestion using SGID. Overall, results indicated that the B variant released the highest amount of BCM-7, followed by the A1 variant. Notably, BCM-7 didn't get release from the A2 variant at any stage of the SGID process. De Noni and Stefano (2010) [13] quantified BCM-7 levels following SGID with digestive enzymes in various dairy products, including fermented milks, powdered milk derivatives, cheeses, and infant formulas.

Infant Formulas and BCMs

Infant formula, designed for special dietary use as a food for infants, is typically formulated to mimic human milk or serve as a suitable partial or complete substitute for it. These formulas commonly utilize purified cow's milk whey and casein as protein sources. The presence of β-caseins in infant formulas leaves room for the potential release of BCMs from these dietary products.

Hernández-Ledesma *et al.* (2004) [25] successfully recovered BCM-7 from infant formula when it underwent peptic hydrolysis at a pH of 3.5, followed by digestion with Corolase PPTM. In another study, these researchers did not detect BCM-7 in infant formula when it was digested with pepsin at pH 3.5 and porcine pancreatin. However, they did find BCM-9 in the same infant formula (Ledesma *et al*., 2007) [26]. Reported the presence of BCM-5 in peptide extracts of infant formula as well as in its pepsin-trypsin hydrolysate. The amounts of BCM-5 in the infant formula extracts and its hydrolysate were measured at 0.67 and 74.46 nmol/mL, respectively. Importantly, no BCM-7 was detected in the extracts before or after simulated gastrointestinal digestion. De Noni (2008) [12] did not observe the presence of BCM-5 or BCM-7 in the peptic digests of milk-based infant formulas at pH 2.0 or 3.5, following simulated gastrointestinal digestion. These infant formulas contained both A1 and A2 variants of β-casein. It was only upon further digestion with Corolase PPTM, a mixture of pancreatic enzymes, that BCM-7 levels ranging from 0.02 to 0.37 nmol/mL were detectable in these products.

Fermented dairy products and BCM formation

Fermented milk products, often referred to as cultured dairy items, are food products that undergo fermentation with lactic acid bacteria, such as *Lactobacillus* and *Lactococcus*. It is considered improbable for BCMs to form from fermented milk products, primarily because lactic acid bacteria contain X-prolyl-diaminopeptidyl peptidases, enzymes with specificity for proline residues. Since BCMs are proline-rich peptides, they are susceptible to degradation by these enzymes.

Nevertheless, certain studies have been conducted with X-prolyl-diaminopeptidyl peptidase-deficient bacteria, leading to the identification of BCMs released from fermented milk products. For instance, Hamel *et al.* (1985) [23] initially identified BCM immunoreactive (irBCM) material in cow's milk after incubation with lactic acid bacteria (LAB). Matar and Goulet (1996) [46], utilizing Liquid Chromatography–Mass Spectrometry (LC-MS), reported the formation of BCM-4 by *Lactobacillus helveticus* L89 from synthetic BCM-7, as well as from pasteurized milk under specific conditions. Furthermore, Schieber and Brückner (2000) [65] detected BCMs using High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) after storing yogurt, prepared from skimmed milk through fermentation with *L. delbruekii* ssp. bulgaricus Lb1466 strains, at 4 °C for three weeks. Shihata *et al.* (2000) [68] recovered BCM-11 and BCM-4 from ultra-heat treatment (UHT) milk fermented with the probiotic Lactobacillus GG strain. These peptides emerged following SGID digestion of milk completed with pepsin and trypsin.

Notably, these peptides were not present in the fermented milk before the pepsin and trypsin digestion.

Absorption Mechanisms of BCMs

In the general context of protein absorption in the intestine, proteins are typically absorbed in the form of amino acids and small peptides, usually consisting of up to three amino acid units. Despite active BCMs being comprised of 5 to 7 amino acids, research employing various *in-vivo* and *in-vitro* models has confirmed their absorption and transport across intestinal epithelial cells. Studies by Singh *et al.* (1989) [71] conducted comparative investigations on the levels of irBCM-7 in the plasma of 2-week-old, 4-week-old pups, and adult dogs after consuming bovine casein-based formula. They postulated that the immature tight junctions in the intestinal mucosa of newborns allow the passage of relatively large peptides, preventing their hydrolysis. While human and bovine immunoreactive material BCM has been detected in the blood of human infants (Kost *et al.*, 2009) [35], recent findings by Wasilewska *et al.* (2011) [79] revealed the presence of BCM-5 in the blood of human infants. However, the detection of irBCM-7 antibodies in plasma raises questions about potential cross-reactivity of antibodies with other antigen epitopes present in unexplored plasma components. Mahé *et al.* (1989) [42] suggested that brush border peptidases play a pivotal role in limiting morphiceptin transfer, with the dipeptidyl peptidase IV (DPP IV) enzyme being a major contributor. Research has demonstrated the transepithelial transport of BCM-5 and BCM-7 across human intestinal Caco-2 cells. Interestingly, the relative flux of BCM-5 increased when the cell layer was treated with a DPP IV inhibitor, as this enzyme is implicated in the hydrolysis of the peptide (Shimizu *et al.*, 1997) [69].

Physiological Implications of BCMs

BCMs have been postulated to potentially play a role in various illnesses, including heart disease, type 1- diabetes, and sudden infant death syndrome, earning them the metaphorical moniker of "the devil in the milk." Nevertheless, the available data on these peptides also include controversial reports suggesting potential physiological benefits for animals. However, it is crucial to note that the existing information is primarily drawn from epidemiological data collected in humans or derived from animal trials, often conducted *in vitro*. Consequently, further extensive research is warranted to elucidate the precise mechanisms underlying these physiological effects.

Health implications associated with BCMs

A substantial body of research has underscored the potential health complications linked to BCMs, revealing a range of adverse biological responses in various contexts:

Type-1 diabetes mellitus

Epidemiological studies have established a significant correlation between the consumption of A1 milk and the incidence of type-1 diabetes mellitus (Kaskous, S., 2020) [33]. This autoimmune disease involves the gradual destruction of insulin-producing pancreatic β-cells by autoreactive T-lymphocytes and macrophages, leading to insulin deficiency. BCMs may act as adjuvants in the autoimmune response, contributing to the destruction of β-cells in prediabetic individuals. Increased antibody production against β-casein has been observed in type-1 diabetes mellitus, particularly A1 β-casein. Moreover, A1 β-casein has been identified as diabetogenic in non-obese diabetic mice compared to A2 β-casein. Additionally, BCM-7, a key player in the potential diabetogenic pathway, suppresses immune defense mechanisms, rendering the immune system more vulnerable to certain enteroviruses implicated in pancreatic β-cell damage. This correlation continues to drive research into BCM-7's role in diabetes mellitus type-1 development.

Cardiovascular diseases

Ecological studies have associated BCM-7 consumption with cardiovascular disease mortality. Research has revealed a link between the intake of A1 β-casein and an elevated risk of cardiovascular diseases in humans. A1 β-casein consumption has been linked to conditions like hypercholesterolemia and atherosclerosis, establishing its association with heart disease incidence. Animal studies have further supported this relationship, demonstrating that rabbits fed with β-casein A1 milk exhibit higher cholesterol levels and a greater extent of aorta covered by fatty streaks compared to those fed A2 β-casein (Chia *et al.*, 2017) [9].

Psychomotor development

Studies conducted by Russian scientists have detected BCM-7 in the blood of infants fed milk formula diets. The metabolization of BCM-7 varied among babies, with some being slow metabolizers. High BCM-7 levels in the blood between feeds were associated with a higher risk of delayed psychomotor development in infants (Şahin *et al.*, 2018) [62].

Sudden infant death syndrome (SIDS)

BCM-7 has long been suspected as a potential risk factor for SIDS. Researchers have identified BCMs in the brainstems of children who died from SIDS. Polish scientists have reported that babies who experienced acute life-threatening events (ALTE) due to apnea exhibited circulating BCM-7 levels three times higher than normal children. These infants also had lower DPP IV enzyme levels, responsible for degrading BCM-7. Remarkably, bovine BCM-7 was found in the blood of breastfed infants, suggesting its transfer from the mother's stomach to the infant via human milk (Beckwith, 2003) [5].
These findings underscore the need for further investigation into the potential health implications of BCMs and their role in various health conditions, shedding light on the multifaceted nature of these bioactive peptides.

Immunological insights into BCMs

BCM-7 peptides have demonstrated intriguing immunological activities, implicating their involvement in various chronic inflammatory responses, including allergies, mucin production, lymphocyte proliferation, and skin reactions. This multifaceted role warrants investigation to unveil the underlying mechanisms.

Milk allergy

Milk allergy arises when the immune system erroneously recognizes a food protein as harmful, leading to an adverse immune response. Certain proteins, including peptides resistant to digestion, can stimulate B-cells to produce IgE antibodies. These antibodies bind to receptors on mast cells or basophils, prompting their degranulation and the release of inflammatory compounds like histamine. The connection between BCMs and allergies stemmed from the observation that opiate alkaloids, including morphine, induce histamine release from mast cells *in vitro* and *in vivo*, potentially triggering anaphylactoid reactions. *In vitro* experiments

incubating peripheral leukocytes with BCM-7 led to histamine release in a concentration-dependent manner. Intradermal injection of BCM-7 induced wheal and flare reactions in the skin, akin to histamine or codeine. These reactions were inhibited by H1 antagonists such as terfenadine or cetirizine, further implicating BCM-7 in allergic responses. Naloxone, an opiate receptor antagonist, inhibited histamine release and skin reactions, but only at a significantly higher concentration than BCM-7, as reported by (Kurek *et al.* 1992) [38].

Kurek and Malaczynska (1999) [37] extended this research to guinea pigs and rat peritoneal cells, observing histamine release and wheal formation and bronchial obstruction in sensitized guinea pigs upon BCM-7 exposure. Additionally, BCM-7 elicited wheal and flare reactions in healthy children, the size of which depended on concentration. These findings suggest BCM-7's role as a noncytotoxic, direct histamine releaser in humans, though its clinical relevance necessitates further exploration. Furthermore, a study examined the correlation between serum DPP IV activity and BCM content in mother's milk among healthy and allergic infants. In the allergic group, high BCM levels in mother's milk corresponded to low DPP IV activity in infant sera, indicating a potential connection between BCMs and infant allergies, as demonstrated by Wasilewska *et al.* in 2011 [79]. Recent research by Reddi *et al.* in 2012 [60] observed significant histamine release and mast cell-specific tryptase upon incubation of bone marrow-derived cells with BCM-5, shedding further light on the immunological aspects of BCMs.

Milk Intolerances

A2 milk, often touted as easier to digest, still contains lactose, a primary concern for milk intolerance. Paradoxically, BCM-7 released from A1β-casein appears to slow down food passage through the digestive system, providing more time for lactose fermentation. Additionally, some individuals may specifically exhibit intolerance to BCM-7, contributing to this phenomenon. However, further research is needed to definitively establish this hypothesis. Recognizing the potential benefits of A2 milk, A2 Corporation Ltd. was founded in New Zealand in 2000 to identify cows with A1 and A2 genetic variants of β-casein and market A2 milk. This selective distribution of bull semen enables the development of herds exclusively producing A2 variant milk. Since 2003, A2 milk has been available in New Zealand and Australia as a premium brand, offering a natural choice for superior protein quality. The company has expanded its A2 milk marketing to Asia and the United States, capitalizing on the growing consumer interest in this unique dairy product.

Functional Significance of BCM-7

Mammalian opioidergic systems encompass a network of opioid receptors and their associated opioid peptides. Depending on their location within the body, these systems play a vital role in numerous neuroendocrine regulatory functions. However the beneficial effect of BCMs such as modulation of intestinal mucus discharge, defence against noxious agents, reduces separation-induced distress vocalizations (DVs), protective and preventive effect against diabetes and oxidative stress, improves glycometabolism, increasing growth-related hormones and growth hormone receptor mRNA expression (Trompette *et al.*, 2003; Yin *et al.*, 2012) [77, 83]. Opioid receptors are distributed throughout the nervous, endocrine, and immune systems, as well as the gastrointestinal tract in mammals, where they interact with both endogenous and exogenous opioids, as discussed by (Teschemacher, 2003) [75]. BCM-7, a μ-receptor agonist, has been found to hold substantial functional significance across various domains. Research by (Trompette *et al.*, 2003) [77] suggests that BCM-7 may play a role in gut immunity, as it has been shown to induce significant jejunal mucus secretion. Moreover, oral administration of BCM-7 to diabetic rats has been associated with increased plasma insulin levels, decreased glucagon levels, and elevated superoxide dismutase and catalase activity, as reported by (Yin *et al.*, 2010) [84]. This points to BCM-7's potential protective role against hyperglycemia and oxidative stress, including inhibition of the NF-KB-iNOS-nitric oxide signaling pathway in the pancreas of diabetic rats, as demonstrated by (Yin *et al.* 2012) [85].

BCM-7 has also been linked to hormonal regulation. (Nedvidková *et al.* 1985) [52] observed that BCM-7 increased plasma prolactin levels after intraperitoneal injection. Prolactin, known for its role in lactation and immune system regulation, thus underscores the hormone's significance. Additionally, BCM-7 has demonstrated analgesic properties, as described in (Dubynin *et al.*, 2008) [14] through intraperitoneal injection in rats. This peptide has further shown effects on learning, as elucidated by (Marklakova *et al.*, 1995) [43], with BCM-7 administration prior to training enhancing the acquisition of food-reinforced tasks and increasing the number of correct trials in T-maze experiments, suggesting a role in reducing manifestations of defensive motivation. Furthermore, BCM-7 appears to modulate gene expression of regulatory peptides in G and D cells, affecting gastrin gene expression indirectly via the paracrine action of somatostatin, as demonstrated by (Zong *et al.* 2007) [87]. (Maslennikova *et al.*, 2008) [45] indicated that BCM-7 activated proliferative processes in the myocardium and various epithelial tissues of newborn rats ex-vivo.

Moreover, oral administration of BCM-7 has been shown to impact postprandial metabolism by stimulating insulin secretion, as noted in (Schusdziarra *et al.* 1983) [67]. It also positively influenced gastric antrum mucosa thickness, accelerated intestinal villi growth, and improved the development of ileum Peyer's patches during early piglet weaning, as reported by (Pan *et al.* 2006) [55]. Additionally, BCM-7 promoted growth in rats by affecting growth-related hormone and growth factor levels in rat serum, including an up-regulation of growth hormone receptor (GHR) mRNA expression in the rat liver, as detailed by (Qin *et al.*, 2004) [58]. BCM-5, originating from the enzymatic transformation of BCM-7, exhibits a range of positive impacts on physiological functions. Research has illuminated the pathway through which BCMs are released within the mammary gland, subsequently transported in the bloodstream, and ultimately engage with endogenous opioid receptors. This suggests a potential role for BCMs in the endocrinic regulation of pregnancy, as highlighted by (Teschemacher *et al.*, 1994) [76]. Furthermore, BCMs have been found to exert significant effects on the cardiovascular system, particularly in pregnant or lactating mammals. BCM-5, in particular, has been observed to have a positive inotropic effect and antiarrhythmic properties, ultimately contributing to its cardioprotective function, as described by (Mentz *et al.* 1990) [49]. (Panksepp *et al.* 1984) [56] reported the reduction of separation-induced distress vocalizations (DVs) in young domestic chicks upon administration of BCMs. Notably, BCM-5 exhibited greater potency compared to BCM-4 or

BCM-7, albeit with a duration of action lasting approximately half an hour. In terms of cognitive functions, systemic administration of BCM-5 has been linked to improvements in learning and memory disturbances resulting from cholinergic dysfunction. This effect is mediated through central mechanisms involving µ1-opioid receptors, as established by (Sakaguchi *et al.* 2006) [64]. Additionally, intracerebroventricular injection of bovine BCM-5 in mice has shown to induce amnesia at high doses, while ameliorating scopolamine-induced amnesia at low doses, as reported by (Sakaguchi *et al.* 2003) [63].

Genotype project findings

Recent investigations conducted by the National Bureau of Animal Genetic Resources (NBAGR) in Karnal, encompassing a comprehensive study of 22 desi cattle breeds, have yielded crucial insights into India's native cattle genotypes. These studies affirm that the predominant genotype among India's indigenous cows and buffaloes is A2, effectively confirming the production of A2 milk in these animals. Particularly noteworthy is the revelation that the A2 allele is present at a 100% frequency in five high-yielding milk breeds: Red Sindhi, Gir, Rathi, Shahiwal, and Tharparkar. This implies that these breeds exclusively carry the A2 allele, without any presence of the A1 allele or A1A1/A1A2 genotypes. Among the remaining breeds, the A2 allele is prevalent at an impressive 94% rate. Moreover, NBAGR's findings also indicate a moderate to high frequency of the A2 allele among breeding bulls, providing further reassurance regarding the safety of milk for human consumption in India. In stark contrast, exotic breeds such as Jersey and HF exhibit a very low availability of the A2 allele. Given the extensive use of exotic breeds in Indian cross-breeding programs and the fact that these breeds harbor the A1 allele, there arises a need for caution in future breeding activities to prevent the fixation of the A1 allele within Indian cattle breeds.

In response to these findings, NBAGR has initiated a vital project titled "Delineating Beta Casein Variants in Indian Cows and Potential Health Implications of A1A2 Milk." Additionally, the bureau offers a cost-effective service for genotyping the A1/A2 allele, utilizing milk or blood samples from cattle species. Furthermore, private companies in India have also ventured into this area by providing allele detection kits, contributing to the ongoing efforts to better understand and manage the composition of milk produced by Indian cattle.

Industrial applications of BCMs

Genetic engineering techniques have been harnessed to generate BCMs and their analogues through the enzymatic or chemical cleavage of microbial fusion proteins, a process elucidated by (Carnie *et al.*, 1989). The primary aim of producing these recombinant BCMs is to enhance animal performance, such as promoting weight gain or increasing milk yield. However, it is important to note that no notable success has been achieved in utilizing these recombinant BCMs for human nutrition thus far.

Nonetheless, pharmaceutical companies have ventured into the development of modified BCMs with the objective of enhancing their analgesic potency, reducing side effects, and prolonging their duration of action. One notable approach involved substituting L-amino acids with D-amino acids, as demonstrated by (Erll *et al.*, 1994) [19]. This alteration resulted in a significant augmentation of analgesic and antidiarrheal activity, particularly in dogs. Examples of chemically modified potent opioid peptides stemming from this effort include morphiceptin and casokefamide. Modifications applied to natural BCMs have resulted in analogues with heightened affinity for opioid receptors and altered pharmacokinetics, especially in terms of resistance to enzymatic degradation by proteolytic and peptideolytic enzymes. These substituted BCMs have exhibited greater resistance to enzymatic breakdown and have demonstrated increased opioid potency compared to their natural peptide counterparts, as outlined in the work of (Daniel *et al.*, 1990) [11].

Definitive link between A1 milk and these conditions remains elusive, with inconclusive evidence stemming from both animal and human studies. Consequently, A1 milk remains a widely consumed commodity without any bans or restrictions, as it appears that its potential influence on these disorders may be intertwined with various other factors, including environmental influences and dietary habits. Presently, India stands as the world's largest milk producer, a feat achieved through strategic measures such as the selection of high-yield breeds, improvements in fodder quality, and cattle crossbreeding programs. Notably, crossbreeding has led to the creation of high-yield cattle varieties that predominantly produce A1 milk, raising concerns within the Indian context. Nevertheless, a significant portion of India's milk production is sourced from buffalo, which predominantly yields A2 milk. In response to these concerns, the Indian Council of Agricultural Research (ICAR) has taken proactive steps to address A1 milk-related issues. ICAR is investing resources in ongoing research to monitor the prevalence of the A1 allele in crossbred cattle. Furthermore, there is a growing recognition of the need for comprehensive statistical data and human studies to better understand the potential role of A1 milk in exacerbating non-communicable disorders within the Indian population.

In conclusion, the A1 milk controversy is a topic that continues to warrant attention and scrutiny, even though concrete evidence substantiating its harmful effects remains elusive. The complex interplay of variables makes it a subject that demands ongoing research and consideration to better inform consumers and policymakers alike.

Conclusion

In the past few decades, extensive research has delved into the potential health implications of A1 milk consumption, particularly in relation to non-communicable disorders like diabetes and autism. Despite these efforts, a definitive link between A1 milk and these conditions remains elusive, with inconclusive evidence stemming from both animal and human studies. Consequently, A1 milk remains a widely consumed commodity without any bans or restrictions, as it appears that its potential influence on these disorders may be intertwined with various other factors, including environmental influences and dietary habits. Presently, India stands as the world's largest milk producer, a feat achieved through strategic measures such as the selection of high-yield breeds, improvements in fodder quality, and cattle crossbreeding programs. Notably, crossbreeding has led to the creation of high-yield cattle varieties that predominantly produce A1 milk, raising concerns within the Indian context. Nevertheless, a significant portion of India's milk production is sourced from buffalo, which predominantly yields A2 milk. In response to these concerns, the Indian Council of Agricultural Research (ICAR) has taken proactive steps to

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