Review

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Review

Health-Related Outcomes and Molecular Methods for the Characterization of A1 and A2 Cow's Milk: Review and Update

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Simple Summary: This review provides an update on the impact of A1 and A2 milk studies on milk consumption trends. Because it can cause a worldwide panic and have very negative economic repercussions, the data from the updated literature must be interpreted extremely carefully. As this paper reveal there are more *in vitro* and *in vivo* animal studies than human clinical trials. Clinical trials are more important because of the subject's significance from the perspective of human health and because of the outcomes of in vitro and animal studies.

Abstract: A new trend in cow's milk has emerged in the market called type A1 and A2 milk. These products have piqued the interest of both consumers and researchers. Recent studies suggest that A2 milk may have potential health benefits, which is why researchers are investigating this product further. It is interesting to note that the A1 and A2 milk types have area-specific characteristics compared to breed-specific characteristics. Extensive research has focused on milk derivatives obtained from cow's milk, primarily through in vitro and animal studies. However, few clinical studies have been conducted in humans, and the results have been unsatisfactory. New molecular techniques for identifying A1 and A2 milk may help researchers to develop new studies that can clarify certain controversies surrounding A1 milk. It is essential to exercise extreme caution when interpreting the updated literature. It has the potential to spread panic worldwide and have negative economic implications. Therefore, this study aims to investigate the new aspects of A1 and A2 milk in different research areas and clarify some aspects regarding these two types of milk.

Keywords: A1 milk; A2 milk; beta-casomorphin 7; bioactive peptides; dairy cows; human health

1. Introduction

The interest in the distinction between A1 and A2 milk began in the early 1990s following hypothesized that the protein in the milk of some cows contained a component likely to cause diseases such as type 1 diabetes mellitus (DM-1) [1–3] and coronary heart disease (CHD) [4]. In 1992, Elliot found that there was more than 10-fold difference in the incidence of type 1 diabetes in Samoan children living in New Zealand and those living in Samoa, which was attributed to the amount of milk they consumed. Samoan children who consumed a greater amount of milk had a higher incidence of DM type 1. He also observed that Maasai children in Kenya were less likely to develop type 1 diabetes even though they consumed a lot of milk [3]. These findings underlined the need for a more in-depth study of the biochemical differences between A1 and A2 beta-casein and their implications for dietary guidelines and public health policies.

The subject matter has generated considerable interest beyond the academic community, attracting the attention of the media, the scientific community, and the business community alike.

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This widespread interest highlights the potential of these findings to impact nutritional science, public health strategies, and the dairy industry. Therefore, a multidisciplinary approach is necessary to further elucidate the implications of milk protein composition on human health.

Proteins are large organic compounds that play a central role in the structure of all living organisms. In addition to their structural and functional role, proteins are also a fundamental component of animal and human nutrition, as they are a source of energy, nitrogen and essential amino acids [5]. Dietary proteins can also be a source of biologically active peptides that are inactive in the precursor protein sequence but can become active when released by hydrolysis during food processing. Food-derived bioactive peptides can influence physiological functions, including modulation of intestinal secretion and motility, blood pressure, thrombotic, antioxidant, antimicrobial and immunomodulatory activities in humans. Some of these effects are mediated through interaction with the opioid system and are therefore referred to as opioid peptides. Opioid peptides can be formed from milk, cereal, vegetables, and meat/poultry. The most studied so far are those derived from cow's milk [5,6] and are primarily based on in vitro and animal studies but limited clinical human studies with poor results [7]. Therefore, more robust scientific evidence is needed to reach a consensus on the role of A1 or A2 bovine milk for health [7]. This report is therefore intended to update the important aspects of A1 and A2 cow milk.

2. Milk Composition

Milk is defined as a whitish opalescent fluid containing milk proteins, fats, lactose and various vitamins and minerals, which is produced by the mammary glands of all adult female mammals after calving and serves as food for their young. Beyond its primary biological role, milk serves as the principal raw material in the production of diverse dairy products.

Cow's milk contains 32 g of protein per liter, with 80% casein protein (CN) and 20% whey protein (WP), based on their solubility at pH 4.6 [8–10]. The natural function of milk proteins is to provide young mammals with the essential amino acids needed for the development of muscles and other protein-containing tissues [11]. In dairy products, milk proteins also play a very important role, e.g. in processing, including undesirable behaviors such as staining on heated surfaces and gelling in processing equipment [12,13]. Among milk proteins, the most important groups are caseins and whey proteins. The caseins groups are represented by αs1, αs2-casein, β-casein and κ- casein and whey proteins by the α-lactalbumin, β-lactoglobulin, immunoglobulins [14,15]. About one third of bovine milk proteins are β-caseins (β-CN), which occur in milk in various genetically determined forms, including the A1 and A2 variants. Milk that is free of A1 β-CN is commonly referred to as A2 milk, which contains predominantly A2 β-CN, with the possible caveat of minor contamination. All other cow's milk is commonly referred to as A1 milk, which contains predominantly A1 β-CN.

A free thiol group in β-lactoglobulin (β-Lg) can be easily detected by thermal deconvolution. It is responsible for the formation of the disulfide bond that causes heat-induced aggregation of WPs and between WPs and CN micelles in milk [16]. Whey proteins, especially β-Lg, can denature or aggregate and possibly associate with CN micelles via kappa-casein (κ-CN) bonds or form κ-CN/β-Lg complexes in the serum phase depending on pH [17].

According to Akkerman et al. [18], the gross composition, the number of somatic and microbial cells and the absence of various biological contaminants influence the quality and safety of raw milk. In addition, raw milk quality based on genetics has recently been linked to a single nucleotide polymorphism in the CSN2 gene, which is necessary for β-CN coding [19].

3. Casein

Casein consists of many components, and the main types are: αs1-casein, αs2-casein, β-CN and κ-CN, which have been defined and validated by analyzing DNA sequences [12]. They are mostly present in milk in the form of CN micelles, with the surface of the micelles consisting of the amphiphilic κ-CN, which stabilize them against aggregation [20–22].

To date, 12 genetic variants of β-CN have been described in the literature [23,24], including A1, A2, A3, B, C, D, F, H1, H2, I and G. The A2 variant is considered the oldest variant. The most common

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variants are A1 and A2, B is less common and A3, I and C are considered rare. The E variant is detectable only in the Italian Piedmontese breed, and the F variant has been detected in the Emilia Romagna region (Northern Italy) at a very low frequency [24].

A1 and A2 milk is found in almost all Bos taurus populations [25]. The Holstein and Ayrshire breeds produce 63 and 67% A1 milk and 35 and 33% A2 milk respectively [26].

4. Distinction between A1 and A2 β-casein

According to the literature, more than 10,000 years ago, before they were domesticated, cows only produced the A2 β-CN protein and not the A1 β-CN protein. However, around 8,000 years ago, a natural single gene mutation occurred in Holsteins, which led to the production of the A1 β-CN protein in this breed [13].

This mutation in the β-CN gene resulted in 12 genetic variants, of which A1 and A2 are the most common. The mutation was passed on to many other breeds, mainly because Holsteins are used to genetically improve the production of other breeds. Slowly, the A1 β-CN variant became dominant in milk. While dairy herds in much of Asia, Africa and parts of southern Europe still naturally have a high proportion of cows producing A2 milk, the A1 variant of the protein is widespread in cattle in the western world [13,27].

Typically, the milk produced by Guernsey, Jersey, and Asian bovine breeds, along with human milk and that of other species such as ovine, caprine, asinine, yak, camelid, bubaline, and ovine, predominantly contains A2 β-CN. Conversely, the milk from Holstein Friesian cattle, which is the predominant dairy breed in regions such as Australia, Northern Europe, and the United States, primarily comprises A1 beta-casein. Notably, the Holstein breed exhibits a roughly equivalent distribution of both A1 and A2 β-CN variants. More than half of the Jersey breed exhibits the A2 β-CN variant, albeit with significant intra-herd variability. Similarly, over 90 percent of the Guernsey breed demonstrates the presence of the A2 β-CN variant. Since the early 1990s, scientific investigations have sought to elucidate the differences between the A1 and A2 β-CN variants, spurred by the discovery of an association between the predominance of A1 β-CN proteins in milk and the occurrence of various chronic illnesses.

Upon comparative analysis, A2 beta-casein milk exhibits no visual differences from its A1 counterpart when evaluated in juxtaposition. To the novice observer, both variants present equivalent aesthetic and apparent quality characteristics. Despite their congruence in containing 209 amino acids, they diverge at the molecular level at the 67th amino acid residue: A1 milk is characterized by the presence of histidine (His67), whereas A2 milk harbors proline (Pro67) at this critical juncture. This singular amino acid substitution is pivotal, influencing the health outcomes associated with the intake of A1/A1 versus A2/A2 β-CN milk. The underlying mechanism involves the differential enzymatic cleavage susceptibility at the 67th position; histidine is more readily cleaved, predisposing A1 β-CN to a higher likelihood of generating bioactive peptides, such as β-Casomorphin-7 (BCM-7), during the digestion process. This distinction is critical in understanding the divergent health implications of consuming A1/A1 compared to A2/A2 β-CN milk, emphasizing the role of this amino acid in the post-digestive bioactive peptide profile [28]. Pro67 in A2 β-CN prevents cleavage during β-CN digestion, but His67 in A1 variant permits proteolytic cleavage, releasing BCM-7 during β-CN digestion [29]. Peptides can impact our blood pressure, immune system, and even how our blood clots. It also can cause what some people like to call “intestinal discomfort”. BCM-7 is released exclusively from A1 and B variants [30,31]. A2 milk is bio-available, which means the human body can absorb lots of it without unpleasant side effects. It also does not have the penchant for creating BCM-7.

It is possible to avoid A1 in diet with dairy products by drinking goats, sheep and buffalo milk, cow's milk from indigenous African and Asian breeds or milk from genetically selected herds of European cattle that are proven to be free of His67 mutations. Many countries are in the process of establishing such herds. New Zealand, the Netherlands, Australia, the United Kingdom and the United States are just some of the countries where commercially available A1-free cow's milk is currently marketed as helpful for people with dairy intolerances. Today, commercial infant formula

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that is A1-free but contains casein is widely sold in some regions such as China and Australia, with the claim that it is easier on the baby's digestive tract [32]. However, A2 milk has been shown to be a safe substitute for human consumption, as no adverse effects of consuming A2 cow's milk have been demonstrated and its nutrient content is comparable to that of A1 milk [33]. For this reason, A2 milk was first produced in New Zealand in 2003, and other countries such as the UK, Australia, the USA, the Netherlands and China followed suit [24].

Previous studies [34,35] have demonstrated that during its simulated gastrointestinal digestion, heat treatment appears to have an impact on the release of opioid peptides from bovine milk carrying the A1/A1, A1/A2, or A2/A2 β-CN phenotype. In the study of Daniloski et al. [36] some CNs showed fluctuations between two phases, even though β-CN and κ-CN seemed to be affected by temperature effects continuously. This could be because the mineral balance shifted toward the micellar phase at higher heat treatment temperatures. Therefore, in comparison to other β-CN milks, the amino acid mutation and lower κ-CN content in A2/A2 β-CN milk may result in an increase in micelle size, a decrease in net negative charge, and a reduction in the amount of minerals. While A1/A1 and A1/A2 β-CN milks shared structural characteristics, A2/A2 β-CN milk had distinct characteristics in some areas; as a result, Principal Component Analysis was able to distinguish A2/A2 β-CN milk from the earlier milks. These results could help forecast how β-CN milk will behave throughout applicable industrial processing [36].

5. Health-Related Outcomes of A1 and A2 Milk

The differential digestibility and metabolic outcomes of A1 versus A2 milk types are underscored by the bioactive peptide BCM-7, which is exclusively produced during the digestion of A1 milk. This peptide is implicated in exacerbating health risks, notably the increased likelihood of DM-1, CHD, sudden infant death syndrome, and autism spectrum disorders [3,37]. Conversely, A2 milk is characterized by its beneficial, impacts on human health, notably through the mitigation of gastrointestinal maladies [32,38]. Despite the increasing corpus of evidence, the linkage between A1 milk consumption and the etiology of neurological conditions, such as schizophrenia and autism, remains tenuously evidenced, with a prevailing hypothesis suggesting animal studies might provide an explanatory basis for these associations [6].

The distinction in digestibility and metabolic effects between A1 and A2 milk can be attributed to the generation of the bioactive peptide BCM-7, uniquely produced during the digestion of A1 milk. This peptide is implicated in the exacerbation of health risks, including an elevated propensity for DM-1, CHD, sudden infant death syndrome, and autism spectrum disorders. The evidence, as presented by Elliot [3] and further corroborated by Aune et al. [37], highlights the critical role of BCM-7 in mediating adverse health outcomes associated with A1 milk consumption.

In contrast, A2 milk exhibits positive health effects, especially in mitigating gastrointestinal issues, a finding supported by the research of Brooke-Taylor et al. [32] and Küllenberg de Gaudry et al. [38]. However, the connection between the consumption of A1 milk and the onset of neurological disorders such as schizophrenia and autism is not robustly established. It is hypothesized that animal model studies may elucidate the underlying mechanisms for these potential associations, a notion explored by Küllenberg de Gaudry et al. [6].

A1 milk and DM-1: some studies which investigated the association between cow’s milk consumption and DM-1, found no interaction between early exposure to cow’s milk and type 1 diabetes [39–41]. This can be explained by the fact that enteral virus infection is a common cause of type 1 diabetes in children [42]. However, the type 1 diabetes risk can be associated with the differences in cow’s milk proteins [43]. So, another important factor is the magnitude/amount of milk protein exposure in cows [44]. As a result, the epidemiology data, while not showing causation, give compelling evidence that A1 β-CN is a causal factor in type 1 diabetes etiology [42]. Milk protein's diabetogenic properties were first demonstrated in BioBreeding (BB) rats, an animal model of spontaneous autoimmune diabetes. When rats were fed a typical laboratory meal, 50% of them acquired autoimmune diabetes (background rate), compared to 15% in rats fed a basic, semi-synthetic diet [2]. Later, in 2003, Laugesen and Elliot reported a strong relationship between A1 β-CN and DM-

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1 in 19 developed countries during 1990-1994 [45]. Almost all the human feeding studies have reported that there was no trace of BCM-7 in adults, but a few studies reported the presence in the infants blood [46]. These studies could not prove that BCM-7 was the cause of disease but observed aggravation in health status of the patients who were already suffering from diabetes. However, no clinical trials on human beings were carried out to study the effect of A1 β-CN on patients of DM-1.

Other studies found that BCM-7 improved intestinal mucosal immunity in mice [47] and rats [48], and that it had a positive effect on diabetes using rats [49–53] and in vitro [53].

A1 milk and CHD: McLachlan [4] reported a correlation between national A1 β-CN consumption and mortality caused by CHD in 16 countries. Tailford et al. [54] reported a rabbit experiment, where rabbits fed with 10% A1 β-CN for 6 weeks, showed larger areas of aortic fatty streaks than those which received A2 β-CN. Results of extensive studies on A1 β-CN and other dietary variables against DM-1 and CHD were reported by Laugesen and Elliott [45], where they concluded that A1 β-CN per capita supply in cow milk and cream was significantly and positively correlated with ischemic heart disease (IHD), over a 20 year period in 20 affluent countries.

However, Chin-Dusting et al. [55] did not find any difference in mice fed with A1 or A2 β-CN. Venn et al. [56] also did not find any significant difference on plasma cholesterol concentrations in human beings after consuming A1 or A2 type of dairy products.

Other effects of A1 Type β-casein: Sun et al. [57] reviewed the possibility of A1 type milk causing sudden infant death syndrome (SIDS), which is the most common cause of death in infants. They suspected that circulation of β-CN in the infant's immature central nervous system might inhibit the respiratory centre in the brainstem leading to apnea and death. Lucarelli et al. [58] indicated that drinking of cow's milk might worsen behavioral symptoms of autistic children. Reichelt and Knivsberg [59] reported the presence of opioid peptides derived from food proteins in the urine of autistic patients. The study of Sokolov et al. [60] associate the BCM-7 with autism in children based on its positive influence on behavior and learning in rats [61–63]. They confirmed this observation by measuring bovine BCM-7 content in the urine of children with autism, using a novel high-sensitivity ELISA method. However, Hunter et al. [64] and Cass et al. [65] did not find any opioid peptides in the urine of children with autism. In addition to assessing BCM-7's immunomodulatory effect in vitro [66], other studies have revealed BCM-7 as an indicator of autism [67] and atopic dermatitis in children [68].

The European Food Safety Authority (EFSA), in its 2009 evaluative report, identified BCM-7 as an opioid peptide specifically liberated from the digestion of A1 beta-casein rather than A2 beta-casein. However, the investigation did not yield conclusive evidence to support the assertion that BCM-7 exerts significant physiological effects on human health [5]. In the context of research into the digestive biochemistry of milk proteins, Asledottir et al. [69] and Cattaneo et al. [70] conducted simulated gastrointestinal digestion assays that led to the observation of BCM-7 release from A2 beta-casein milk. Notably, the concentration of BCM-7 liberated from A2 milk was found to be significantly lower than that from A1 milk. Furthermore, emerging evidence suggests a potential biochemical interaction between BCM-7 and intestinal kappa-opioid receptors. Specifically, it was demonstrated that BCM-7, when administered in conjunction with enterostatin through a gastric cannula, can partially mitigate the reduction in fat absorption typically induced by a high-fat diet. This effect is attributed to the modulatory action of BCM-7 on kappa-opioid receptors, as demonstrated in the year 2000 by White et al. [71].

Further research has demonstrated that the β-CN variants A1, A2, and B produce distinct peptide profiles upon in vitro digestion. This phenomenon was explored by Lisson et al. [72] in their 2013 publication, where they hypothesized that the resultant peptide variants from each β-CN type could exhibit unique affinities for immunoglobulin E influencing binding process. Such differential binding activities suggest a nuanced immunological interaction that may influence allergenicity among the β-CN variants. Other investigations revealed an increase in the antioxidant properties of β-CN post-digestion [73–75]. These findings highlight the significant impact of β-CN digestion on enhancing its antioxidative functionality, offering insights into the protein's contribution to health-promoting dietary components.

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Weimann et al. [76] conducted a comprehensive in silico digestion analysis focusing on κ-CN, unveiling that specific genetic variants of κ-CN exhibit varied capacities in generating angiotensin-converting enzyme (ACE) inhibitor peptides. These peptides are recognized for their substantial therapeutic utility across a diverse array of pathological conditions. Notably, the ACE inhibitory peptides originating from κ-CN possess profound relevance in both the prophylaxis and therapeutic management of diseases such as cancer, neurological disorders, arthritis, and cardiovascular diseases. The pivotal role of these peptides in modulating oxidative stress and inflammation, processes fundamental to the pathogenesis of these conditions, is extensively detailed by Halliwell [77] and Valko et al. [78]. These investigations collectively underscore the therapeutic significance of ACE inhibitor peptides derived from κ-CN in disease management paradigms. Taken as a whole, the investigations show that genetic variances have a critical impact on the digestive breakdown of bioactive proteins. Petrat-Melin et al. [79] found that polymorphisms in the casein gene locus, which result in amino acid substitutions, are crucial in altering the processing mechanisms of proteins in the gastrointestinal system. This study presents evidence of the genetic basis for differential digestion and assimilation of dietary proteins, emphasising the significance of genetic determinants in nutritional biochemistry.

Additional recent research revealed that consuming A2 β-CN helped 60 volunteers with their gastrointestinal issues, and that regular milk drinking increased the amount of Bifidobacterium spp. in the distal colon. In association with this increase in the number of Bifidobacterium spp. improved symptoms of gastrointestinal discomfort were observed, such as a lower percentage of bloating and bowel movements, an increase in the frequency of bowel movements and altered stool characteristics compared to uncharacterized regular milk [80,81]. In vitro studies have demonstrated that BCM-7 stimulates the secretion of gastrointestinal mucin [82–84]. Oral administration of BCM-7 [85] and consumption of A1 β-CN [86] induced inflammatory responses in mice. The A1 milk causes gastrointestinal inflammation, discomfort in the digestive tract, and/or delayed gastrointestinal transit in humans [87–89] and rats [90]. On the other hand, mice's intestinal immunity improved after consuming A2 cow milk [91]. In some studies [6,19,38,92] the systematic reviews showed weak evidence for a positive effect of A2 bovine milk, as opposed to A1 milk [7].

In general, the positive results obtained in animal studies regarding the health effects of A2 milk have not always been confirmed by clinical studies in humans [7]. There seems to be sufficient consensus on the positive effects of A2 milk on reducing the digestive intolerance associated with the consumption of A1 milk [81]. With regard to A1 milk, there is now a need for further clinical studies on the effects of A1 in a wide range of populations (ages, ethnicities and different genetic haplotypes) and nutritional conditions [7,32].

6. New Molecular and Biochemical Methods for Characterization A1/A2 Milk

Currently, a variety of dairy products are being distinctly labeled to indicate the presence of A2 β-CN protein, commonly referred to as A2 protein or A2 milk. This labeling practice is frequently associated with the nutritional information, aiming to inform consumers about the specific type of protein contained within these products. The differentiation between A2 and A1 β-CN proteins is of interest due to emerging research suggesting potential health benefits linked to the consumption of A2 β-CN.

By the year 2029, it is anticipated that the market for A2 milk in North America will witness exponential growth, a trend driven by consumer preferences shifting towards milk variants that provide additional health benefits in comparison to traditional milk offerings [93]. This shift is reflective of a broader consumer trend towards health-conscious dietary choices, with A2 milk being perceived as a superior alternative due to its purported digestive benefits and potential to mitigate certain health risks associated with conventional milk[93]. Also, the European market will experience significant expansion as a result of the dairy industry's efforts to conduct research and development [94]. However, Oglobline et al. [95] reported that the Dietitians Association of Australia (DAA) overall rationale was that both varieties of milk regular milk with A1 and A2 β-CN and A2 milk are safe, nourishing, and wholesome, and that consumers are free to select the one that suits them best.

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Similarly, the Australian dairy industry concurs with this viewpoint and adds that both offer the same vital nutrients, are excellent for human consumption, and are recommended in the Australian Dietary Guidelines for optimal health [95].

The four types of casein (αs1-, αs2-, β-, and κ-CN) are encoded by the genes CSN1S1, CSN1S2, CSN2, and CSN3, respectively [96]. The genetic features and breed of cows can influence the β-CN structure. According to existing literature, the oldest genetic variant of β-CN is A2, meaning that initially, all herds had only one allele of this variety [93]. When comparing crossbred dairy cattle to the original native breeds, the first group has an A1 allele frequency that is higher than the other group (as per their analysis) [97]. However, the A2 allele is still the most common in many breeds, as examined in the extensive work by Sanchez et al. [98]. It has been observed that crossbreed dairy cattle have a higher frequency of the β-CN A1 allele compared to their original native breeds. This observation suggests that genetic selection in crossbreed herds is based on protein yield, which is the total mass of protein obtained from a cow per day. It has also been noted that a higher protein yield is associated with the A1 variant [97](Nadugala et al., 2022). Due to the recent trend for A1-free milk, producers have started selecting females carrying the A2A2 genotype. According to Hoque and Mondal [99], the A2 gene frequency is more common in many imported breeds of cattle, including Guernsey, Jersey from the Channel Islands, and Asian and African cows.

Characterizing bovine herds using CSN2 genotyping tests has garnered a lot of attention in recent years. This is supported by the growing demand for only A2 dairy products, which necessitates the use of cows with A2A2 genotypes exclusively. In this regard, rhAmp® SNP genotyping and high-resolution melting (HRM) were evaluated by Giglioti et al. [100] as two methods to identify the CSN2 gene alleles in milk samples. These authors claim that genotypes from the CSN2 gene in milk can be distinguished using either approach. Nevertheless, rhAmp was ten times more sensitive than HRM at identifying the presence of A1 milk in a sample that contained only A2. The same research group developed another accurate and specific method based on real-time PCR [101] for directly detecting A1 and A2 alleles in milk. Two years latter Watanabe et al. [102] developed a highly sensitive PCR method for A1 allele detection in A2 milk samples without DNA isolation. Using the CycleavePCR technique, this method can directly amplify the β-CN gene from samples of raw milk. Genotypes obtained from the milk samples (n = 27) were completely coincident with those obtained from genomic DNA. This technique could also measure the A1 allele in the milk samples. In A2 milk, the A1 allele's detection limit was 2% [102].

Determination of A1 and A2 β-CN in Milk Using Characteristic Thermolytic Peptides via Liquid Chromatography-Mass Spectrometry has been developed by the Liu et al. [103] to determine A1 and A2 β-CN variants in milk using characteristic thermolytic peptides. They show that under the optimized liquid chromatography gradient, A1 peptide and A2 peptide could be separated completely. Six commercially available milk samples including four normal milk samples and two A2 milk samples were analyzed for the application demonstration of the advantages of high sensitivity, high specificity, and high efficiency of this novel method. The A1 β-CN variant makes up the majority in all normal milk samples analyzed. In one A2-labeled milk sample, no A1 β-CN variant was detected, and in another A2-labeled milk sample, a small amount of an A1 β-CN variant was clearly detected [103].

The potential for high-quality DNA yields in large-scale screening led to the evaluation of both manual and automated DNA extraction techniques were evaluated in a recent work of Vigolo et al., [104]. The objective was to evaluate the effectiveness of such techniques in differentiating the genetic variants A1 and A2 of β-CN starting from milk somatic cells, as well as the operational cost and time required for analysis, expertise, and labor requirements. First, the most effective way to obtain large amounts of total genomic material has been shown to be automated DNA extraction from a complex matrix, such as milk. Second, the most effective and high-quality method for indirectly genotyping the cow was demonstrated by the high-performance liquid chromatography (HPLC) approach. It does, however, necessitate more analysis time. As a result, allele-specific PCR, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system-polymerase chain (ARMS-PCR), has shown to be an extremely dependable

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technique for characterizing the most prevalent β-CN variants (A1/A2). The analysis took less time with the molecular methods. Specifically, the ARMS-PCR proved to be the most affordable, rapid, and intuitive approach. Finally, a more involved and expensive process was needed to ascertain the cow's genotype using the PCR-RFLP method, which depends on the use of a restriction enzyme [104]. Although more expensive than HPLC analysis, both allele-specific PCR techniques proved to be quick and accurate in differentiating between A1 and A2 variants. To be more precise, out of all the techniques that were evaluated, PCR-RFLP was the most costly and labor-intensive, while ARMS-PCR was the fastest and required less technical expertise. All things considered, ARMS-PCR in conjunction with automated milk matrix DNA extraction is the most appropriate method for large-scale genetic CSN2 gene characterization [104].

In manufactured products such as skim milk powder, processing conditions may induce reactions between proteins and reducing sugars (Maillard reaction) that yield multiple proteoforms containing one or more sugar adducts [105]. The ultraperformance liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) intact protein method presented in the study of Fuerer et al. [106] is an effective method for the identification and quantification of proteins in dairy samples such as raw milk, skim milk powder, whey powder, final products, and samples from other species such as buffalo or human. If a protein's overall signal is adjusted for a factor associated with its glycation index, it is feasible to quantify individual proteins in processed food matrices [106]. The authors therefore noted that the major milk proteins (below 30 kDa) and their corresponding proteoforms can be easily and unambiguously detected using UPLC-HRMS method.

7. Conclusions

The emergence of comprehensive genotyping and genomic selection technologies has revolutionized our ability to analyze the frequencies of milk protein variants on an unprecedented scale. While academic literature reviews serve as an indispensable resource in this field, their inherent subjectivity—stemming from the selection bias of the researcher towards certain studies—may overlook significant contributions. Promoting A2 milk consumption, while driven by potential health benefits, necessitates a balanced approach considering the implications for genetic diversity among dairy cattle. The sustainability of dairy farming, conservation of genetic resources, economic viability, ecosystem health, and future adaptability all hinge on maintaining a broad genetic base. Therefore, strategies that promote A2 milk should also incorporate measures to preserve the genetic diversity of A1 milk breeds, ensuring a holistic approach to dairy production that safeguards both public health interests and biodiversity. This analysis shows up a discrepancy in the body of research, with a greater focus on in vitro and in vivo studies involving animals rather than human clinical trials. Considering the paramount importance of human health and the limitations inherent to in vitro and animal models, the need for extensive human clinical trials becomes evident, highlighting the critical nature of translating these preliminary findings into concrete health outcomes for the human population.

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