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A NATURAL OPIOID DRUG RELEASED BY A1 BETA-CASEIN MILK PROTEIN DIGESTION WAS REPORTED TO BE RELATED TO VARIOUS PATHOLOGIES

UN DROG NATURAL DE TIP OPIOD ELIBERAT PRIN DIGESTIA PROTEINEI DIN LAPTE BETA-CAZEINĂ VARIANTA A1 A FOST RAPORTAT ÎN RELAȚIE CU DIFERITE PATOLOGII

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ABSTRACT | REZUMAT

The paper reviews literature data with respect to the risk of beta-casein A1 milk variant (A1 β-CN) consumption in humans and etiopathogenic mechanisms involved. This allele of β-CN particularly came into notice by the possibility of its cleavage during the digestion process, with the release at the level of the digestive system of a strong opioid named beta-casomorphin-7. This opioid is associated with pathological conditions in humans by affecting the digestive, circulatory, and nervous systems (for instance, diabetes mellitus type I, ischemic heart disease, autism, and schizophrenia). The debated topic is important for public health, but also for animal science, completing the multitude of studies focused on associations of different allelic variants within each type of casein or whey proteins with various technological properties of milk and even with certain qualitative and quantitative characteristics. This time, a potential alarm signal was debated for a more efficient selection of dairy cows for the benefit of consumer health and not only in terms of the profitability of their breeding. Although there are evidences of negative effects on human health of liberated bioactive beta-casomorphin-7, these evidences cannot be generalized and are mostly dependent on the individual reactivity.

Keywords: milk, risk assessment, cattle selection, health management

Această lucrare revizuiește datele din literatura de specialitate cu privire la riscul consumului la om de lapte ce conține varianta A1 a beta-cazeinei (A1 β-CN) și mecanismele etiopatogenice implicate. Această alelă a β-CN a apărut în atenția publică în special prin posibilitatea scindării sale în timpul procesului de digestie, cu eliberarea la nivelul sistemului digestiv a unui opioid puternic numit beta-casomorfină-7. Acest opioid este asociat cu diverse patologii la om, prin afectarea sistemului digestiv, circulator și nervos (de exemplu, diabetul zaharat de tip I, boala ischemică a inimii, autismul și schizofrenia). Subiectul dezbătut este important pentru sănătatea publică, dar și pentru știința animalelor, fiind completat cu numeroase studii axate pe asocieri ale diferitelor variante alelice din cadrul fiecărui tip de cazeină sau proteine din zer cu diferite proprietăți tehnologice ale laptelui și chiar cu anumite caracteristici calitative și cantitative ale sale. De această dată, a fost dezbătut un potențial semnal de alarmă pentru o selecție mai eficientă a vacilor de lapte în beneficiul sănătății consumatorilor și nu numai, în ceea ce privește profitabilitatea creșterii lor. Deși există dovezi ale efectelor negative asupra sănătății umane ale beta-casomorfinei bioactive eliberate, aceste dovezi nu pot fi generalizate și sunt în mare parte dependente de reactivitatea individuală.

Cuvinte cheie: lapte, evaluarea riscului, selecția vacilor, managementul sănătății

Milk, as a mammary gland secretion of female mammals in postpartum, represents a whole-food that contains important nutritive constituents (15,40). In an overview, milk is composed of water (84.8-88%) and solids (12-15.2%). Among solids, lactose and fats share the most important contents (26.32-40% and 18.42-50%, respectively), followed by protein (18.42-33.33%) and minerals (5.26-8.33%). Caseins represent the most important fraction of milk proteins with a share of ~80%, while whey proteins, mainly represented by α-lactalbumin and β-lactoglobulin, but also included immunoglobulins, glycomacropeptides, serum albumin and minor proteins, such as lactoperoxidase, lysozyme, and lactoferrin, which are the difference in total protein content (14, 32, 36).

Bovine milk protein, similar to milk protein of almost all livestock animals, is mainly represented by four caseins, alpha-s1-casein (αs1-CN), beta-casein

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(β-CN), alpha-s2-casein (αs2-CN), and kappa-casein (κ-CN), while the gamma-casein (γ-CN) is considered to be a product of β-CN degradation. The share of the four caseins in the total caseins' content of milk is 39-46%,25-35%,8-11%, and 8-15%, respectively. These caseins have an important role in micelles formation as a result of their association with calcium and phosphorus (32, 40). As all body's proteins, those of milk have a genetic determinism and are encoded by genes, each one with two or more autosomal and codominant alleles. The four caseins are encoded by four tightly linked genes on a 185-250 kb cluster mapped on the 6th bovine chromosome, corresponding to αs1-, β-, αs2-, and κ-CN protein, and which are named *CSN1S1, CSN2, CSN1S2,* and *CSN3*, respectively. Considering the main whey protein in cattle, alpha-lactalbumin (α-LA) is encoded by the *LAA* gene mapped on chromosome 5, whereas beta-lactoglobulin (β-LG), by *LGB* gene, is mapped on chromosome 11 (11,22).

Milk proteins are an important source of bioactive peptides as a result of the primary protein enzymatic cleavage during the process of digestion and even food processing (13, 32, 36). Most of them are beneficial for human health, excepting those resulted from β-CN digestion encoded by the alleles carrying Histidine at position 67 of the mature protein (His67). Most of the reports primarily included in this class of risk the A1 variant of β-CN, although the B variant is also a carrier of His67 (13). However, the β-casomorphine-7 (BCM-7), a heptapeptide released by the „A1 – type of milk", acts as a morphine and many reports associated its presence in milk or gastrointestinal human tract with allergies or other diseases, such as those of digestive, cardiovascular, endocrine, and brain systems (15, 19, 36, 40, 41). The presented review focuses on the characterization of the main β-CN allelic variants and the relationship of the „A1 – type of milk" with human health status, in the context of cattle selection.

THE β-CN CHARACTERIZATION IN THE CONTEXT OF BOVINE MAJOR PROTEIN

The bovine milk protein, briefly divided into caseins, and α-lactalbumin and β-lactoglobulin, as the main whey protein, express a genetic polymorphism, if we consider the possibility of finding of two or more alleles of the same encoding gene at the level of various populations. Many allelic variants have a great impact in terms of dairy cattle breeding and are constituted as genetic markers in the process of selection for yield traits, milk composition, and milk properties (25). Their nomenclature is based on a progressive alphabetical order, usually reflecting their bands of electrophoretic mobility on various gel substrates.

The vast majority of milk protein in cattle is synthesized based on the information encoded on six major genes, four for caseins (*CSN1S1, CSN2, CSN1S2,* and *CSN3*) and two for whey proteins (*LAA* and *LGB*). The genes for caseins are autosomal, codominant, and clustered on a segment of 185-250 kb located at position 6q31-6q33, in the following order of their final products: αs1-, β-, αs2-, and κ-CN (11,22). Their total lengths vary among 8.5 kb (for *CSN2*), and 18.5 kb (for *CSN1S2*), with intermediate values of 13 kb (for *CSN3*) and 17.5 kb (for *CSN1S1*). Although they are clustered, the first three genes are more closely linked than the *CSN3* gene, which is at least 70 kb away from them (21). Compared to caseins, whey protein genes are much smaller, of 2 kb total length (for *LAA* gene) and 4 kb total length (for *LGB*). Their localization is on different chromosomes, like the 5th one, for the *LAA* gene, and the 11th, for the *LGB* gene (11).

As a result of various electrophoretic techniques applied, for each gene of milk protein at least two or more alleles were identified. Moreover, various nucleotide substitutions or deletions translated into amino acids substitution and a segment deletion within the synthesized polypeptide chain were found (12,26,38).

Numerous research investigations conducted in the latest decades have established important benefits of these alleles' documentation. The benefits are relevant in terms of selection in dairy herds for yield traits, composition, and technological milk properties. In various cattle breeds, there is a well-known positive association of B alleles of αs1-, β- and κ-CN, and of β-LG with higher milk, protein, fat, and subsequent cheese yields and improved coagulation ability as a result of a better reaction with chymosin, a significantly lower clotting time and a higher rate of curd formation (1, 8, 10, 23, 29, 31, 32, 34, 35).

Recently, an intriguing subject of debate characterized the possibility of A1/A2 milk type implication on human health, considering some reports that showed various negative implications of A1-type of milk. In fact, A1 or A2 types of milk are named so after the letters used to define two important alleles of bovine β-CN. As aforementioned, β-CN is one of the four major milk caseins, with a share of 25-30% of cow's milk protein (14,15) and 37.5% of the casein bovine milk, secondly after the share of α-caseins (~50%) (14). It is presented such a single-polypeptide chain containing 209 amino acid residues, with a molecular weight of 23,983 Da (36,39). Its gene is very polymorphic, with a total of 12 alleles translated in so many protein variants within the *Bos* genus (A1, A2, A3, B, C, D, E, F, G, H1, H2, I) (11). Among these, A1, A2, and B are usually found in cattle breeds, unlike the C and I variants. Extremely rare are the rest of them (13). The gene of β-CN includes nine exons (47), 80% of the mature protein being encoded by the nucleotide sequence of the Exon VII of the gene. The amino acid differences among the most common allelic variants are

located at this level (5). The A2 β-CN is considered to be the original form of this protein, while its structure is similar to other mammalian β-CN such as those found in milk produced by sheep, goats, buffaloes, pigs, horses, donkeys, and humans (13,15). The A1 and A2 variants are different considering the substitution of Pro67 in A2 with His67 in A1, as a result of a single nucleotide replacement in the codon sequence (a cytosine in the CCT codon of proline is substituted by adenine in CAT codon of histidine) (41). Additionally, in the B variant, Ser122 of A2 β-CN is substituted by Arg122 (32,36). Proline replacement by histidine at position 67 is responsible for a different secondary structure of the final protein and the cleavage upon digestion by enzymatic hydrolysis at this situs. As a result, there is liberated a bioactive peptide, named beta-casomorphin, which seems to be associated with some diseases affecting the gastrointestinal tract, immune and nervous systems. Although the general association is only with the A1 variant of β-CN, this cleavage occurs in all genetic variants with His67 in their structure but, indeed, is more pregnant in the case of A1 β-CN type. Of course, considering the most common alleles of β-CN in cow' milk, besides the A1 allele, the B variant may involve a similar potential risk (16).

THE A^1 TYPE OF MILK AND WHY SO MUCH CONCERN FOR IT ON HUMAN HEALTH STATUS ?

Like many other dietary proteins, those from cattle milk are a valuable source of bioactive peptides which result from the original protein fragmentation during the process of digestion and even during the processing. Recently, a possible risk was identified as a consequence of β-casomorphin-7 (BCM-7) resulting in the human gastrointestinal tract, easily absorbed by infants but also affecting adults due to its potential to activate various opioid receptors located in the nervous, endocrine, and immune systems. Therefore, without any restriction, BCM-7 may be considered an exogenous opioid peptide or exorphine, whose action may be translated into specific pathologies, such as type-1 diabetes mellitus (insulin-dependent), allergies, coronary heart disease, arteriosclerosis, autism, and schizophrenia (41,42). As valuable arguments of all these, there are some epidemiological evidences in which, for example, a low incidence of diabetes is found in countries with a low rate of milk consumption, such as Japan and, reversely, a high incidence of diabetes occurs in countries where milk consumption is frequent, such as Finland (19). More specifically, a higher intake of A2 β-CN milk was epidemiologically associated with a lower incidence of cardiovascular disease and type-1 diabetes, and a decrease in A1 β-CN milk dietary intake showed a reduction in autistic or schizophrenic symptoms (41) and even gastric or immunological disorders, too (15). As reviewed by Kamiński et al., (2007), a strong correlation between A1 β-CN milk dietary intake and ischaemic (coronary) heart disease incidence in 30-69 year-old males from 16 countries was demonstrated, and also a correlation between hypercholesterolemia or atherosclerosis and casein consumption in rabbits, pigs, monkeys, and rodents. Higher cholesterol levels and percent surface area of aorta covered by fatty streaks were reported in rabbits in the case of A1 β-CN milk dietary intake when comparing to A2 type ingestion (32,44).

Etiopathogenic mechanisms involved, although they may be different, they certainly have a common point, and this is BCM-7 (28). As Thakur and Anand (2015) reviewed, as lactoferrins, BCM-7 has opioid activity, similar to opioids or those psychoactive drugs which include morphine in their class. That means they stimulate opioid receptors which usually are found in central and peripheral nervous systems, but also in ducts area as μ opioid receptors. Clarke and Trivedi (2014) classified μ opioid receptors considering their localization and level of expression as opioid receptors with the highest level of expression (located in the hypothalamus, cerebral cortex, and spleen), moderate level (located in the cerebellum, intestine, kidney, adrenal, and reproductive organs), and with the lowest level (located in the lung and liver). They are also expressed on inflammatory cells, including lymphocytes and leukocytes, but they are not expressed in stomach, heart, or endothelium (15).

Although caseins are not digested inside mouth, they are dissolved in gut enzymes (43). In vitro experiments demonstrated successive proteolytic digestion by pepsin, pancreatic elastase, and leucine aminopeptidase, releasing, in the case of A1 β-CN, the bioactive peptide BCM-7 (32). By activating its opioid receptors, a wide range of diseases may occur. For instance, correlations were reported among BCM-7 and type-1 diabetes mellitus, and two possible mechanisms were considered: one of them is related to the stimulating of autoantibodies production that causes the auto-immune mediated killing of pancreatic β-cells and, subsequently losing of their ability to produce insulin, and the other, via opioid receptors, when its morphine-like activity affects the immune response to antigens, especially to enteroviruses or endogenous retroviruses which can damage the secretory pancreatic β-cells (15,32). BCM-7 is also recognized as a peptide with a pro-atherogenic activity and this is related to its ability to induce the oxidation of the damaging low-density lipoprotein (LDL) form of cholesterol (15, 32, 43). BCM-7, like other BCM peptides or their analogues, can cross the blood-brain barrier, exacerbating the symptomatology associated with autism and schizophrenia. The main mechanisms reported are those related to its opioid activity and the alteration of the

oxidative balance of the cells, generating oxidative stress (15). Equally important are immunological phenomena, considering that each genetic version of protein is a carrier of many epitopes (short fragments of amino acids) which act as antigens or substrates of interactions with the immune system represented by immunoglobulins, especially IgE in this case (11, 43, 44). A particular aspect needs more concern and this is related to the feeding of new-borns with infant milk formula containing milk substituents from other mammalian species than humans. Especially new-borns, but also children, develop food allergies, the most common type being that of cattle's milk proteins. This aspect the same is reported in the case of using other mammalian milk as a substitute for human milk, such as of goats and sheep, because there is a similar antigenic potential and 90% of the children who are allergic to cattle's milk are also allergic to goat or sheep milk. Thus, the ideal solution is represented by the exclusive breast milk using in babies feeding for four to six months of life, as a protective factor against further potential food allergies (40), or the use of hypoallergenic formula. An interesting fact is that, although the human milk contains BCM-7, there is no danger for the new-born, it has 10-fold less opioid activity comparing to that of bovine BCM-7 being due to their chemical differences in two amino acids (Table 1) (30).

Table 1

Amino acids differences in BCM-7 of human and bovine origin (30)

Species	Amino acids sequence
Human	H-Tyr^{51}-Pro^{52}-Phe^{53}-Val^{54}-Glu^{55}-Pro^{56}-Ile^{57}-OH
Bovine	H-Tyr^{60}-Pro^{61}-Phe^{62}-Pro^{63}-Gly^{64}-Pro^{65}-Ile^{66}-OH

The Sudden Infant Death Syndrome (SIDS) is a more important aspect related to BCMs of animal origin absorption at the intestinal level, their crossing of the blood-brain barrier, and the possibility of sudden death between the end of the first month up to the end of the first year of life due to the depression induced on the brain-stem respiratory centres (32).

Both for youngsters and adults, the A1-type of milk seems to contain an unbalanced calcium-magnesium ratio, exceeding that normal one of 2:1 at 10:1. This leads to the presence of too much calcium and a subsequent magnesium deficiency (7).

THE A^1 BUT NOT THE ONLY ONE

The detailed study of the literature showed that the release of beta casomorphins *(BCMs)* is also possible from the degradation of other allelic variants of *beta-casein*, apart from A1. Investigating the releasing of BCM-7 from unprocessed milk during the simulated gastrointestinal digestion by pepsin at pH 2.0, 3.0, and 4.0, and followed by hydrolysis with a mixture of pancreatic enzymes, De Noni *(2008)* found that the *B* version released a higher amount of BCM-7 comparing to A1 variant, while no quantification was obtained in the case of A2 variant. In another study, the release of BCM-7 in cattle milk with β-casein genetic variants A1, A2, F and I was assessed by *ex vivo* gastrointestinal digestion (4) and, surprisingly, all β-CN investigated variants released BCM-7 *(2).* Further investigation by Asledottir et al. (2019) aimed to study the opioid peptide BCM-7 degradation or stability during digestion using human gastrointestinal juices and swine jejunal brush border membrane peptidases. Their results showed that most of BCM-7 were degraded during gastrointestinal and brush border membrane digestion, although a small amount *(5%)* was still detected 24 hours post digestion. They concluded that it remains to be investigated whether the small amount of intact BCM-7 detected after in vitro digestion is transported via active transceptors in the human intestinal epithelial cells and penetrates to blood flow (3). Although opioid peptides in milk tend to scare consumers, there is also good news about their role in the human body. The study of Enjapoori et al. *(2019)* had in view to detect the naturally occurring BCM peptides from beta-casein in human breast milk using liquid chromatography-tandem mass spectrometry. Their study provides evidence of beta-casein-derived BCM peptides in human milk before infant digestion. They consider that proteases which are present in human milk are likely specific in their proteolysis of beta-casein. The identified bioactive BCMs, more exactly BCM -8, -9, -10, and -11, as well as the precursor peptides, meet the structural requirements to elicit opioid, but also immunomodulatory, antioxidative, and satiety functions in new-borns (20).

BETWEEN A NORMAL CONCERN AND A SCIENTIFIC HYSTERIA

Although a large amount of research proposed and tested the use of complementary and alternative therapies for children with autism, including casein exclusion diets, there was poor evidence that these diets have had any effect (37) and therefore the subject remained debatable. Undoubtedly, many reports based on epidemiological data or medical investigations demonstrated the negative potential of BCM-7 releasing from A1 cattle β-CN at the level of the human digestive system. However, humans consume cow's milk with A1 or B variants of β-CN for thousands of years, considering only the probability of A2 to A1 mutation occurring ~5,000 – 10,000 years ago, as a natural process whilst human migration and cattle domestication (14, 47). Nowadays, among various breeds of cattle the share of these alleles may be different according to va-

rious aims of selection applied. Many researchers, such as Aleandri et al. (1990), Hanusová et al. (2010), and Marchini et al. (2010), reported the A1 allele as the most prevalent in Holstein Friesian cows, while Çardak (2005) reported high frequencies of A2 allele in Holstein Friesian and Simmentaler cows from South-West Germany. In a previous study conducted by Grădinaru (2013) on three cattle populations reared in Romania, the higher rate of A2 allele was found in Romanian Spotted *(also called Romanian Simmental),* Holstein Friesian, and Montbéliarde cows, which was considered to be in agreement with the findings reported in different varieties of Holstein Friesian cows *(Italian, Estonian)* (9,10,45) and Simmentaler *(Italian) (6).* However, there is a very low probability to consume commercial cow's milk without A1 or B allelic expression of *β-CN*, unless a special selection in this regard was applied in farms for the obtaining of the labelled *„A2 milk",* as it happens in the USA, New Zealand or Australia. The casein-free diet is a viable alternative in some cases, especially in the known cases of immunological sensitivities.

In conclusion, in our opinion, there are evidences of negative effects of BCM-7 on human health, but these cannot be generalized, being mostly dependent on the individual reactivity. Therefore, dramatic actions, such as avoiding cattle's milk consumption, are not necessary unless there are obvious proofs of affecting in any way the individual health, when alternatives are better to be used.

CONCLUSIONS

The investigations on the genetic polymorphism of cattle milk proteins, although very useful in animal' selection, led us to identify some alleles whose products of hydrolysis are with potential negative effects on human health. BCM-7, an opioid-like hormone, was associated with many human pathologies, such as those of digestive and nervous systems, as well the cardiovascular and endocrine ones. The risk is open for both youngsters and adults, and possible dramatic events regarding newborns fed with infant formula including cattle's milk or other mammalian species, such as goat or sheep, may further complicate this picture. However, a scientific hysteria is not recommended as long as the subject of A1 and A2 milk remains controversial; the authorities empowered in the field have the task of investigating the real risks of *A1*-type cow's milk consumption, to set a causal relation between its hydrolysis product and various human pathologies in which it is possible to be involved.

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