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κ-casein polymorphism effect on technological properties of dried milk

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Abstract:
Introduction. Numerous molecular genetic studies have revealed a correlation between the polymorphism of milk protein genes and the technological properties of milk raw materials. DNA analysis, in particular, initiated research into the influence of allelic variants of κ-casein (*CSN3*) on thermal stability and cheese suitability of milk. This gives relevance to our study that compares the results of genotypic identification of lactating cows by the κ-casein gene, using raw and processed milk samples.
Study objects and methods. Our study used raw and reconstituted milk samples from first-calf cows of the black motley breed with the *AA* and *BB* genotypes of the κ-casein gene. The samples were analyzed by standardized and generally accepted chemical engineering methods, as well as by capillary electrophoresis and PCR-RFLP analysis.
Results and discussion. We compared the results of tests on thermal stability and cheese suitability of raw and reconstituted milk samples from cows with the *AA* and *BB* genotypes of the κ-casein gene. We tried out an integrated approach to monitoring milk raw materials based on the most relevant technological criteria and correlating the data with the associated *CSN3* gene identification parameters. The PCR-RFLP analysis revealed reproducible results for both raw and dried milk samples in relation to the genotypical identification by the *A*- and *B*- allelic variants of the *CSN3* gene. The tests showed higher thermal stability in the reconstituted milk from the *BB* genotype cow and better cheese suitability in the *AA* genotype sample.
Conclusion. We developed a system for evaluating milk raw materials based on the most important technological parameters in combination with their genotypic characteristics. Our research procedure can unify the accumulation of experimental data and contribute to the formation of bioinformatics algorithms. This approach can be used in mathematical modeling of criteria to evaluate the compliance of the technological properties of milk with the recommended indicators.

Keywords: Casein, genotype, PCR-RFLP, allele, milk, technological properties, whey, thermal stability

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INTRODUCTION

Each breed of lactating animals has a genetic potential that contributes to certain properties of the resulting milk. Breed characteristics determine not only the physicochemical composition of raw milk, but also its technological parameters and yield. As a result, the quality of dairy products also varies significantly [1–3].

In this regard, scientists are interested in determining genetic markers associated with the qualitative characteristics of milk. DNA technologies for developing technological properties of milk *in vivo* aim to model those parameters which are significant in certain areas of the dairy industry. Allelic variants of genes of milk proteins, hormones, and enzymes are the most probable genetic markers of milk quality. Numerous studies have analyzed the influence of cow genotypes on the rennet coagulation and thermal stability of milk. Most research has focused on α_{S1}-casein (*CSN1S1*), β-casein (*CSN2*), β-lactoglobulin (*BLG*), and κ-casein (*CSN3*) encoded by the genes of the same name [4–6].

Studies have proven that the *B* allele of the *CSN1S1* gene is associated with milk yield, while its *C* allele affects the protein milk content. Also, the *A* allele of the *CSN2* gene has a positive effect of on the thermal

stability of milk, while its *B* allele is responsible for rennet coagulation and is a synergist for the similar effect of the *CSN3 B* allele. The *BLG* gene is associated with the biological value of milk, its technological properties, and total protein, namely a high content of casein and fat fractions (*B* allele) and a high content of whey proteins (*A* allele).

The κ-casein (*CSN3*) gene determines both the protein milk content and the technological properties of milk. It is believed that its *A* allele is associated with thermal stability, while its *B* allele is responsible for a high protein content and cheese suitability [7].

Testing biomaterial by determining the κ-casein gene locus is currently one of the most widely used methods. This is due to the frequency of occurrence of its prevailing alleles and the evidence of their association with the technological parameters of milk [8–10].

Many years of research have identified more than ten allelic variants of the *CSN3* gene in cattle. The *A* and *B* alleles are most common and depend on the breed and geographic range. Scientists have also established a significant prevalence of the *A* over the *B* variant [7].

Allelic polymorphism of the κ-casein gene includes three main genotypes: *AA*, *AB*, and *BB*. Their variability affects the composition and properties of cow milk. Numerous studies of the κ-casein genotypes in domestic and foreign breeds have revealed a trend of increasing daily milk yield and decreasing protein mass fraction from the *B* allele to the *A* allele [7].

Despite some conflicting results [11], most researchers agree that the $CSN3^{AA}$ genotype has a positive effect on the thermal stability of milk, while the $CSN3^{BB}$ genotype determines its cheese suitability [12].

The polymorphism of milk proteins was initially studied on lactating cows by means of electrophoresis and isoelectric focusing. Calves and bulls were genotyped by mass testing of milk from their ancestors and offspring through the female line. Such studies took about 5–6 years. Long duration and high costs made it practically impossible to correct the desired alleles in the animal population [13].

New methods of genotyping polymorphic variants of milk proteins at the DNA level opened up wide opportunities for breeding and research. The most widely used method is the polymerase chain reaction (PCR) followed by the analysis of restriction fragment length polymorphism (RFLP) [13].

The PCR-RFLP analysis made it possible to identify the polymorphism of milk proteins using not only the animal's biomaterial, but also raw milk and processed materials. In particular, these methods enabled scientists to interpret PCR-RFLP fragments of the DNA extracted from a number of dairy products. This opened up new prospects for milk processors in predicting the technological properties of raw milk [14, 15].

We aimed to compare the results of the κ-casein gene genotypic identification of lactating cows, using whole raw and freeze-dried milk samples. After interpreting the data, we assessed the physicochemical parameters and technological properties of the reconstituted product in order to determine the need for further processing of dried milk.

To achieve this aim, we set the following objectives:
– to genotype cows by the PCR-RFLP analysis for allelic variants of the κ-casein gene in dried and raw milk samples;
– to try out a comprehensive approach to monitoring the technological properties of reconstituted milk using multidirectional methods;
– to assess the influence of κ-casein genetic polymorphism on the technological properties of milk.

STUDY OBJECTS AND METHODS

Sampling and laboratory analysis. Milk was obtained from first-calf cows of the black-motley breed (marked 357 and 12) at the Mukhametshin farm, the Republic of Tatarstan. Their genotypes for the κ-casein gene, identified by the DNA analysis of raw milk samples, were *AA* and *BB*, respectively. Raw milk was frozen during 3–5 h after milking and delivered to the All-Russian Dairy Research Institute within two days.

After thawing, raw milk from cows with the *AA* and *BB* genotypes was sampled (*AA*[1] and *BB*[1], respectively) for extended physicochemical analysis (Table 1). The rest of the milk of each type was freeze-dried without preliminary heat treatment in order to preserve most of its native qualities.

Freeze-drying. Defrosted raw milk was freeze-dried on 1 dm^3 trays in a TG-50 apparatus (Hochvakuum, Germany) under the following conditions:
– initial temperature: –35 ± 5°C;
– final temperature: +30 ± 5°C;
– condenser temperature: –35 ± 5°C;
– residual pressure: 0.3–1.3 kPa; and
– process time: 42 ± 2 h.

Physicochemical analysis. Raw milk and then reconstituted dried milk were subjected to physicochemical analysis (Table 1) to determine the parameters of milk reconstitution [16, 17].

The moisture content was determined by thermogravimetric methods according to State Standard ISO 6731/IDF 21-2012[I].

The protein content was determined by measuring total nitrogen according to the Kjeldahl method and converting it to protein according to ISO 1871:2009[II]/

[1] State Standard ISO 6731/IDF 21-2012. Milk, cream and evaporated milk. Determination of total solids content (Reference method). Moscow: Standartinform; 2019. 6 p.
[II] ISO 1871:2009. Food and feed products — General guidelines for the determination of nitrogen by the Kjeldahl method. Geneve: International Organization for Standardization; 2009. 12 p.

Table 1 Physicochemical composition of whole milk and reconstituted dried milk samples

Indicator	± uncertainty	Whole milk samples		Reconstituted dried milk samples		
		AA[1]	*BB*[1]	*AA*[2]	*AA*[3]	*BB*[2]
Fat, %	± 0.150	3.90	3.60	3.70	3.30	3.40
Total proteins, %	± 0.060	3.14	2.81	2.96	2.65	2.67
Whey proteins, %	± 0.200	0.80	0.75	0.75	0.68	0.70
Casein proteins, %	± 0.033	2.39	2.08	2.22	2.01	1.95
Moisture, %	± 0.200	87.14	88.80	87.83	89.12	89.24
Non-fat milk solids, %	± 0.400	8.96	7.60	8.47	7.58	7.36
Lactose, %	± 0.350	5.20	4.40	4.90	4.40	4.30
Freezing temperature, °C	± 0.001	–0.526	–0.507	–	–	–
Acidity, °T	± 1.000	17.00	16.90	15.00	13.50	12.00
Ca, mg/kg	± 0.500	116.40	93.12	110.20	98.30	85.00
Active acidity, pH units	± 0.020	6.70	6.69	6.61	6.64	6.69

AA[1] and *BB*[1] are raw milk samples from cows with *AA* and *BB* genotypes

AA[2] and *BB*[2] are freeze-dried milk samples from cows with *AA* and *BB* genotypes

AA[3] is a freeze-dried milk sample with the same protein content as sample *BB*[2]

ISO 8968-1:2014 [IDF 20-1:2014][III] on a Kjeltec-2400 Auto Analyzer (Foss Electric, Denmark).

The content of non-protein nitrogen was determined by precipitating protein nitrogenous substances with trichloroacetic acid and measuring total nitrogen in the filtrate according to ISO 8968-4: 2016 [IDF 20-4: 2016][IV].

Casein and whey proteins were determined by acid precipitation of casein and measurement of total nitrogen in the filtrate according to ISO 17997-1:2004 [IDF 29-1:2004][V]. Based on the content of total and non-protein nitrogen, we calculated the ratio between whey and casein proteins.

The fat content was determined by the Gerber acid method according to State Standard R ISO 2446-2011[VI]/ ISO 19662:2018 [IDF 238:2018][VII]/ISO 11870:2009 [IDF 152:2009][VIII].

The lactose content was determined by the polarimetric method according to State Standard R 54667-2011[IX] and by enzymatic method used as a control according to ISO 26462:2010 [IDF 214:2010][X].

The freezing point for raw milk was determined by the thermistor cryoscope method according to ISO 5764:2009 [IDF 108:2009][XI].

The calcium content was measured by the titrimetric method according to ISO 12081:2010 [IDF 36:2010][XII].

Determination of amino acid composition. The amino acid content was determined by capillary electrophoresis on a Kapel 205M system (Lumex, Russia). This method involves decomposing the samples by acid and alkaline hydrolysis (only for tryptophan), converting amino acids into free forms, obtaining phenylthiocarbonyl derivatives, and their subsequent separation and quantification. The standards of amino acids with at least 98% of the basic substance were used as analytical standards. The electrophoresis system had a quartz capillary with a total length of 60 cm, an effective length of 50 cm, and an inner diameter of 50 μm. The sample solutions were hydrodynamically introduced into the capillary at 30 mbar for 5 s. The separation voltage was 25 kV. UV detection was carried out at a wavelength of 254 nm, with a data collection rate of 2.5 Hz. The electropherograms were processed using the Elforan software. Prior to use, the capillary was washed with a 1 mol/dm^3 NaOH solution, then with ultrapure water, and finally with base electrolyte, for 5 min each.

[III] ISO 8968-1:2014 [IDF 20-1:2014]. Milk and milk products – Determination of nitrogen content – Part 1: Kjeldahl principle and crude protein calculation. Geneva: International Organization for Standardization; 2014. 18 p.

[IV] ISO 8968-4:2016 [IDF 20-4:2016]. Milk and milk products – Determination of nitrogen content – Part 4: Determination of protein and non-protein nitrogen content and true protein content calculation (Reference method). Geneva: International Organization for Standardization; 2016. 11 p.

[V] ISO 17997-1:2004 [IDF 29-1:2004]. Milk – Determination of casein-nitrogen content – Part 1: Indirect method (Reference method). Geneva: International Organization for Standardization; 2004. 8 p.

[VI] State Standard R ISO 2446-2011. Milk. Method for determination of fat content. Moscow: Standartinform; 2012. 12 p.

[VII] ISO 19662:2018 [IDF 238:2018]. Milk – Determination of fat content – Acido-butyrometric (Gerber method). Geneva: International Organization for Standardization; 2018. 15 p.

[VIII] ISO 11870:2009 [IDF 152:2009]. Milk and milk products – Determination of fat content – General guidance on the use of butyrometric methods. Geneva: International Organization for Standardization; 2009. 7 p.

[IX] State Standard R 54667-2011. Milk and milk products. Methods for determination of sugars mass fraction. Moscow: Standartinform; 2012. 27 p.

[X] ISO 26462:2010 [IDF 214:2010]. Milk – Determination of lactose content – Enzymatic method using difference in pH. Geneva: International Organization for Standardization; 2010. 11 p.

[XI] ISO 5764:2009 [IDF 108:2009]. Milk – Determination of freezing point – Thermistor cryoscope method (Reference method). Geneva: International Organization for Standardization; 2009. 17 p.

[XII] ISO 12081:2010 [IDF 36:2010]. Milk – Determination of calcium content – Titrimetric method. Geneva: International Organization for Standardization; 2010. 6 p.

Table 2 Genotyping primers for the *CSN3 A* and *B* alleles generated PCR products and RFLP fragments

Primer nucleotide sequences	PCR-product (bp)	Genotype-specific RFLP-fragments (bp)		
		AA	*BB*	*AB*
JK5: 5′-ATCATTTATGGCCATTCCACCAAAG-3′ JK3: 5′-GCCCATTTCGCCTTCTCTGTAACAGA-3′	350	*Hinf*I		
		134	265	265
		131	85	134
		85		131
				85

PCR-RFLP analysis. The next stage involved the identification of freeze-dried milk by the *A* and *B* alleles of the *CSN3* gene in order to establish correlations with the data on raw milk. The polymorphism of milk proteins in the *CSN3* gene was analyzed by PCR-RFLP.

DNA extraction from dried milk. DNA was extracted in accordance with the DNA-sorb-S-M instructions (Central Research Institute of Epidemiology, Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing). 400 μL of buffer for lysis reagent and 17 μL of lysis reagent were added to 50 mg of dried milk in Eppendorf tubes. The tubes were thermostated at 64°C for 60 min and agitated occasionally in a vortex shaker. The undissolved particles were precipitated by centrifugation at 12 000 rpm for 5 min.

200–350 μL of the supernatant was collected and transferred into tubes with 25 μL of the resuspended universal sorbent. The tubes were tightly closed and left for 10 min, occasionally shaken. After centrifugation for 1 min at 5000 rpm, the supernatant was removed. The test tubes were filled with 300 μL of washing solution No. 1, tightly closed, and resuspended. After another centrifugation for 1 min at 2000 rpm, the supernatant was removed. Then, we added 500 μL of washing solution No. 2, closed the tubes tightly, and stirred until complete resuspension. The mixture was centrifuged for 1 min at 10 000 rpm and the supernatant was removed. After a repeated washing with solution No. 2, the supernatant was removed completely. The tubes were thermostated for 5–10 min at 64°C and 50 μL of elution buffer was added. After complete resuspension, the samples were thermostated again for 5–10 min at 64°C and centrifuged for 1 min at 12 000 rpm. The supernatant with purified DNA was collected for PCR.

CSN3 genotyping by PCR-RFLP. Amplification was performed on a Tertsik programmable amplifier (Russia). A 20 μL reaction mixture (SibEnzyme, Russia) contained 2 μL of dNTP mixture (0.25 mM each), 2 μL of buffer for Taq DNA polymerase (1×), 0.2 μL of Taq DNA polymerase (1 unit), 0.4 μL of JK5 and JK3 primers (0.5 μM each), and 2 μL of the DNA sample. The analysis was conducted in the following modes:

×1:94°C – 4 min; ×35:94°C – 10 sec, 63°C – 10 sec, 72°C – 10 sec;

×1:72°C – 7 min; storage at 4°C [18].

The genotypes for allelic variants *A* and *B* were identified by subsequent endonuclease segregation of amplicons. 10 μL of the PCR sample was treated with 5 units of HinfI restriction endonuclease in "O" SE-buffer (SibEnzyme, Russia) and incubated at 37°C overnight.

The range of genotype-specific RFLP fragments is presented in Table 2.

Detection. The incubated PCR-RFLP samples were mixed with a buffer to be loaded onto an agarose gel (4× Gel Loading Dye, Blue, Evrogen, Russia) in a 3:1 ratio. The stained amplificates were loaded into the wells of a 2% agarose gel prepared by melting 2 g of agarose (Biotechnology Grade, Amresco, USA) in 100 mL of Tris-acetate electrode buffer (500 mL of 1× TAE buffer, 15 μL of 1% ethidium bromide solution). Detection was performed by horizontal electrophoresis using a SE-2 camera (Helikon, Russia) and an Elf-4 power supply (DNA-Technology, Russia). The results were visualized on a Gel Doc XR+ gel documenting transilluminator using the Image Lab software (Bio-Rad, USA). The electrophoresis lasted 45 min and had an output voltage of 180 V and an output current of 150 mA [18].

Milk reconstitution. Dried milk samples from cows with the *AA* and *BB* genotypes of the *CSN3* gene were recombined in such a way that they reached the amount of dry solids in the original raw milk. The samples weighing 12.21 g (*AA* genotype) and 10.79 g (*BB* genotype) were introduced into 87.79 g and 89.21 g of boiled distilled water (pH 6.5 ± 0.1, 40 ± 2°C), respectively (test samples AA^2 and BB^2). Another sample was made from 10.88 g of *AA* genotype dried milk and 89.12 g of water (test sample AA^3), with a protein content unified with sample BB^2. These ratios were chosen to ensure better comparability of the experimental results. After introducing dried milk into water, the samples were kept in a magnetic laboratory stirrer at 500 rpm for 15 min and then cooled to 20 ± 2°C [19, 20].

Determination of cheese suitability. Cheese suitability was determined by a rennet-fermenting test according to State Standard 32901-2014[XIII] with some modification for reconstituted dried milk. Rennet clotting was performed with standardized milk-clotting enzymes derived from the enzymatically produced chymosin CHY-MAX M 2500 IMCU (Chr. Hansen,

[XIII] State Standard 32901-2014. Milk and milk products. Methods of microbiological analysis. Moscow: Standartinform; 2015. 27 p.

Denmark) and Microclerici 2400 IMCU (Sacco Sistem, Italy). To prepare enzyme solutions, we calculated their activity based on the activity of 1 g of rennet enzyme (100 000 IMCU). We used 0.32 g of an enzyme with a 2500 IMCU/g activity and 0.33 g of an enzyme with a 2400 IMCU/g activity. The weighed milk-clotting preparations were dissolved in 100 mL of distilled water at 30 ± 1°C and kept for 30 min in a magnetic stirrer at 300 rpm.

Washed and dried 100 mL high beakers were filled with 90 cm^3 of reconstituted milk (38 ± 1°C) measured with a cylinder. Then, 3 cm^3 of the control enzyme sample was added to each beaker. The samples were thoroughly mixed and placed in a thermostat at 38 ± 1°C until a dense clot formed. We visually assessed the quality of the clot and recorded the duration of rennet clotting. In accordance with State Standard 32901-2014[13], milk was classified as I (good), II (satisfactory), and III (unsatisfactory).

After evaluating the clot, we filtered the separated whey through a lavsan cloth used in cheese-making. The whey was thoroughly mixed and analyzed for a protein content. The percentage of total protein conversion to whey was calculated as:

$$R = (P_s \times 100)/P_m \quad (1)$$

where R is total protein conversion to whey, %; Ps is a mass fraction of total protein in whey, %; P_m is a mass fraction of total protein in reconstituted milk, %.

Based on the data, we performed a comprehensive comparative assessment of the samples' cheese suitability.

Sample preparation for assessing thermal stability. After assessing the quality of rennet clotting, we conducted a number of tests to determine thermal stability. Since samples AA^2, AA^3 and BB^2 differed in active/titratable acidity, we established their buffer capacity to minimize its effect on the protein resistance to heat exposure [21]. Then, based on each of the samples, we modelled a series of test samples with active acidity ranging from pH 6.0 to pH 7.0 and a pH interval of 0.2. Finally, all the test samples in the model series were assessed for thermal stability.

Active acidity (pH) was measured by the potentiometric method using an InoLab pH Level 1 high-precision pH meter equipped with a Sen Tix 61 pH electrode and a temperature sensor for measuring pH in the 0–14 range.

Titratable acidity was determined according to ISO 6092:1980[XIV]/ISO 6091:2010 [IDF 86:2010][XV] and correlated with pH values and subsequent data on thermal stability.

Thermal stability determination by alcohol test. The studies followed State Standard 25228-82[XVI] for reconstituted milk. The method described there is based on the ability of ethanol solutions to denature proteins in milk. The All-Russian Dairy Research Institute modified the method so that it used solutions with ethanol concentrations of 68, 70, 72, 75, 80, 85, 90 and 95% in order to expand the measurement range taking into account different pH values of the model samples. 2 cm^3 of alcohol in a certain concentration was added to Petri dishes with 2 cm^3 of the reconstituted sample and stirred in a circular motion for 2 min. Then, we assessed the consistency of the test mixtures. Coagulation of proteins in the form of visually noticeable flakes meant reduced thermal stability. We compared the data in relation to the concentration of alcohol that did not lead to protein coagulation.

Thermal stability determination by calcium chloride test. The studies involved creating an excess content of calcium ions in the samples, followed by heat exposure [21]. Heat-resistant 10 cm^3 glass vials of HC-3 type (55 mm tall, 22 mm in diameter) were filled with 10 cm^3 of reconstituted milk (20 ± 2°C) and 0.5 cm^3 of a 1% $CaCl_2$ solution. The vials were closed with rubber stoppers and placed in a compact water bath. The lid was tightened with a pressure screw so that the stoppers did not pop out when heated. The bath was quickly filled with boiling water and placed on a heated electric stove to resume boiling immediately. We controlled the time with a stopwatch and monitored the temperature. The samples were kept at 100 ± 1°C for 4 min and then rapidly cooled to 20 ± 2°C by adding cold water. Finally, the contents of the vials were transferred into Petri dishes to assess the stability of the protein fraction.

Thermal stability determination by phosphate test. The study involved adding a KH_2PO_4 solution to reconstituted milk to change the salt balance in terms of the phosphorus content, followed by heat exposure [21]. 1 cm^3 of a KH_2PO_4 solution (68.1 g per 1 L of water) was added to HC-3 vials filled with 10 mL of milk. The vials were sealed with rubber stoppers and placed in a water bath at 100 ± 1°C for 5 min. After cooling, the contents were transferred into Petri dishes to determine the presence of protein flakes.

Thermal stability determination by acid-boiling test. A combination of heat exposure and various amounts of hydrochloric acid was used as a factor of influence on milk protein [21]. Three HC-3 vials were filled with 0.5, 0.8, and 1.2 cm^3 of 0.1N HCl solution and then with 10 cm^3 of reconstituted milk. After mixing, the vials were sealed with rubber stoppers and placed in a water bath at 100 ± 1°C for 3 min. Then, the vials were cooled and checked for coagulation. The level of protein stability was characterized by the amount of the acid added.

[XIV] ISO 6092:1980. Dried milk – Determination of titratable acidity (Routine method). Geneva: International Organization for Standarization; 1980. 2 p.

[XV] ISO 6091:2010 [IDF 86:2010]. Dried milk – Determination of titratable acidity (Reference method). Geneva: International Organization for Standardization; 2010. 4 p.

[XVI] State Standard 25228-82. Milk and cream. Method of determination of termostability on alcohol test. Moscow: Standartinform; 2009. 4 p.

Thermal stability determination by heat test. For this study, we used a UKT-150 thermal stability control apparatus designed by the All-Russian Dairy Research Institute [22]. The method involved comparing the times of protein coagulation in the milk samples placed in a glycerol bath (ultrathermostat) at 130 ± 1°C. 8-mL heat-resistant molybdenum glass tubes were filled with 3 mL of reconstituted milk and tightly closed with rubber stoppers. The tubes were placed in a metal cassette holder and clamped with screws so that the stoppers did not pop out during heating.

A LOIPLT-316a ultrathermostat filled with glycerin was heated to 130 ± 1°C. The cassette holder with the test tubes was placed in the thermostat so that the tubes were immersed in the heated liquid. Immediately after that, the connecting rod was started to move the stand with the test tubes in such a way that milk slowly oscillated back and forth to avoid burning on the tube walls.

The samples were kept in the ultrathermostat at 130 ± 1°C until milk proteins started to coagulate. With the first signs of coagulation in the form of precipitation or protein flakes, the timing was stopped for the respective sample. After registering coagulation in all the samples, we turned off the connecting rod, removed the cassette holder from the UKT-150, and cooled the test tubes. Their contents were transferred into Petri dishes to determine the nature of the clots. The thermal stability of the reconstituted samples was characterized by the time of beginning of coagulation.

Assessment of amino acid balance. The balance of amino acid composition was determined by the generally accepted methods described by Donskova and Zhakslykova [23, 24]. It included the amino acid score, the ratio between essential and nonessential amino acids, the ratio between essential and total amino acids, the utilitarian coefficient, redundancy and comparable redundancy of essential amino acids, as well as the essential amino acid index.

The experiment was carried out in three repetitions. The data were processed using Microsoft Excel 2019.

RESULTS AND DISCUSSION

Qualitative and quantitative composition of raw milk. By PCR-RFLP analysis, raw milk samples were identified by the κ-casein gene as *AA* and *BB*. After freeze-drying, the samples were analyzed by PCR-RFLP again. The analyses revealed reproducible results of identification for both raw and freeze-dried milk samples by the *A*- and *B*- allelic variants of the *CSN3* gene.

The physicochemical evaluation of the milk samples from cows with the *AA* and *BB* genotypes showed significant differences in both qualitative and quantitative terms. The *AA* genotype cow milk had a higher content of fat, protein, lactose, and total dry solids (Table 1), which largely correlated with the results of other studies.

The calcium content in the BB[1] sample was below the average (120 mg/kg), unlike sample *AA*[1]. Despite

Table 3 Amino acid composition of whole milk from cows with different *CSN3* genotypes

Indicator	Actual values, g/100 g protein			Amino-acid score, %	
	FAO/WHO scale	*AA*[1]	*BB*[1]	*AA*[1]	*BB*[1]
Valin	5.00	3.21 ± 0.19	3.19 ± 0.19	64.20*	63.80*
Isoleucine	4.00	3.58 ± 0.21	3.55 ± 0.21	89.50	88.75
Leucine	7.00	4.78 ± 0.29	4.73 ± 0.28	68.29	67.57
Lysine	5.50	5.09 ± 0.31	4.90 ± 0.29	92.55	89.09
Methionine + Cysteine	3.50	2.81 ± 0.17	3.34 ± 0.20	80.29	95.43
Threonine	4.00	2.94 ± 0.18	2.92 ± 0.18	73.50	73.00
Tryptophan	1.00	1.17 ± 0.07	1.09 ± 0.07	117.00	109.00
Phenylalanine + Tyrosine	6.00	5.81 ± 0.35	5.84 ± 0.35	96.83	97.33
Essential amino acids (EAA)	36.00	29.39 ± 1.76	24.32 ± 1.46	–	–
Arginine	–	4.38 ± 0.26	3.88 ± 0.23	–	–
Histidine	–	2.02 ± 0.12	1.89 ± 0.11	–	–
Asparagine	–	5.26 ± 0.32	5.38 ± 0.32	–	–
Serine	–	3.88 ± 0.23	3.66 ± 0.22	–	–
Glutamine	–	12.89 ± 0.77	12.70 ± 0.76	–	–
Glycine	–	1.20 ± 0.07	1.21 ± 0.07	–	–
Alanine	–	2.15 ± 0.13	2.16 ± 0.13	–	–
Proline	–	6.11 ± 0.37	6.32 ± 0.38	–	–
Nonessential amino acids (NEAA)	–	37.92 ± 2.28	37.21 ± 2.23	–	–
Total amino acids (TAA)	–	67.31 ± 4.04	61.53 ± 3.69	–	–
EAA/NEAA relation	0.56–0.67	0.78	0.65	–	–
EAA/TAA relation	0.36	0.43	0.40	–	–

* limiting amino acid

AA[1] and *BB*[1] – raw milk samples from cows with *AA* and *BB* genotypes

Table 4 Balance of amino acid composition of whole milk from cows with different *CSN3* genotypes

Sample	Utilitarian coefficient	Redundancy of essential amino acids, g/100 g protein	Comparable redundancy, g/100 g protein	Essential amino acid index
AA[1]	0.79	6.28	9.78	0.87
BB[1]	0.78	6.59	10.33	0.87

the differences in the protein content between samples *AA*[1] and *BB*[1], the ratio of caseins to whey proteins was consistently equal to 75:25% and 74:26%, respectively. Thus, the polymorphism of the *CSN3* genotypes significantly affected the quantitative composition of all milk components, while the comparable ratios between whey protein and casein in raw milk remained unchanged.

Assessment of amino acid balance in raw milk. To assess the effect of the *CSN3* polymorphism on the qualitative characteristics of the protein phase of milk, we studied the amino acid composition of milk samples obtained from cows with the *AA* and *BB* genotypes (Table 3). In this study, we took into account actual differences in the mass fraction of protein in the samples.

The comparative analysis of essential amino acids in the milk samples from cows with the *AA* and *BB* genotypes (samples *AA*[1] and *BB*[1], respectively) showed similar contents of valine, isoleucine, leucine, threonine, tryptophan, phenylalanine, and tyrosine. Sample *AA*[1] had a higher content of lysine (5.09 g/100 g protein) and a lower content of methionine and cystine (2.81 g/100 g protein), compared to sample *BB*[1]. The amino acid index, i.e. a ratio between essential and nonessential amino acids, was 0.65 for sample *BB*[1] (within the FAO/WHO recommendations of 0.56–0.67 for a balanced diet) and 0.78 for sample *AA*[1], which indicated a slight imbalance in favor of essential amino acids that might slow down their metabolic availability.

The comparison of the amino acid composition of the samples with that of the "ideal" protein featured valine as a limiting amino acid in both cases. Both samples showed insufficient scores (less than 100%) of all essential amino acids, except for tryptophan. The results were used to determine the value of milk protein in the samples with the *AA* and *BB* genotypes (Table 4).

As we can see, the above differences in the amino acid composition did not have a significant effect on the biological value of milk proteins in samples *AA*[1] and *BB*[1].

The effect of the κ-casein genotype on dried milk thermal stability. Thermal stability is one of the most important indicators in the production of dairy products from reconstituted dried milk. The stability of milk during heat exposure is determined by a combination of factors, such as acidity, a concentration of free ions of calcium, magnesium, phosphorus, and citrates, the amount of total protein, the ratio between protein fractions, and the degree of their hydration. Therefore, there is no universal method to determine thermal stability that would take into account all the criteria of system variability [25].

An alcohol test, due to its simplicity, is currently the most common method to determine thermal stability in the dairy industry. However, it does not always account for all the factors affecting the stability of milk protein during heat treatment. Therefore, we used five most common methods to study the stability of casein micelles in milk with κ-casein polymorphism, namely an alcohol test, a calcium chloride test, a phosphate test, an acid-boiling test, and a heat test. The experiment was conducted in a range of 6.0–7.0 pH units with an interval of 0.2 pH units to better understand the mechanism of casein destabilization.

The analysis of thermal stability of the reconstituted milk samples obtained from cows with different *CSN3* genotypes showed significant differences between samples *AA*[2] and *BB*[2] (Table 5). In particular, at pH 6.6, sample *BB*[2] withstood the action of a 85% alcohol solution, while *AA*[2] denatured with the addition of a 68% alcohol solution. The lower thermal stability of *AA*[2] compared to *BB*[2] can be explained by a higher content of protein (by 10%), lactose (by 14%), and colloidal calcium (by 30%) in sample *AA*[2]. Sample *AA*[3], which withstood an 80% alcohol test at pH 6.6, partially confirms this hypothesis.

All milk samples acidified to pH 6.4 showed a decrease in protein thermal stability. Unlike sample *AA*[3], sample *BB*[2] failed the test even at 68% alcohol, which made it unsuitable for processing at this acidity level. We can assume that these results are associated with the *CSN3* genotypic characteristics of the cows. Sample *AA*[3] had a more stable hydration membrane during alcohol exposure at a lower acidity level.

Noteworthily, all the samples withstood the alcohol test at pH 7.0 (95% alcohol) and pH 6.8 (90–95% alcohol). On the one hand, it indicated the absence of

Table 5 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by alcohol test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA[2]	< 68%	< 68%	< 68%	< 68%	90%	95%
AA[3]	< 68%	< 68%	68%	80%	90%	95%
BB[2]	< 68%	< 68%	< 68%	85%	95%	95%

AA[2] и *BB*[2] – reconstituted dried milk samples from cows with *AA* and *BB* genotypes

AA[3] – a reconstituted dried milk sample with the same protein content as sample *BB*[2]

Table 6 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by calcium chloride test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	–	–	–	–	–	±
AA^3	–	–	–	–	–	±
BB^2	–	–	–	–	–	±

– negative result; ± conditionally positive result; + positive result

Table 7 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by phosphate test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	–	–	–	–	+	+
AA^3	–	–	–	+	+	+
BB^2	–	–	–	+	+	+

– negative result; + positive result

Table 8 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by acid-boiling test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	–	–	–	–	0.5 mL*	0.8 mL*
AA^3	–	–	–	–	0.5 mL*	0.5 mL*
BB^2	–	–	–	0.5 mL*	0.5 mL*	0.5 mL*

– negative result; *amount of 0.1N HCl withstood by milk, mL

Table 9 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by heat test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	15 min	15 min	35 min	35 min	35 min	60 min
AA^3	5 min	12 min	27 min	33 min	35 min	60 min
BB^2	15 min	25 min	33 min	43 min	90 min	> 180 min

destabilizing factors for casein micelles in this pH range. On the other hand, there was a genetic dependence: the milk from the *BB* genotype cows revealed higher thermal stability than the *AA* genotype milk (pH 6.8–7.0).

All the samples failed a test with calcium chloride used as a destabilizing agent followed by heat exposure at pH 6.0–6.8 (Table 6). At pH 7.0, the samples contained sporadic protein agglomerates, which made them conditionally thermostable. Increased amounts of calcium ions in milk, especially at higher acidity, reduced the negative charge of casein micelles and the aggregation of casein particles. An increase in the size of aggregated particles decreased their thermal stability, ultimately leading to complete destabilization during heat exposure. Thus, we found that the *CSN3* genotypes had an insignificant effect on the thermal stability of milk whose salt balance was shifted by calcium ions.

The milk thermal stability test, which involves exposure to a solution of monosubstituted potassium phosphate in combination with heat treatment, showed the stability of samples AA^2, AA^2, and BB^2 in a pH range of 6.8–7.0 (Table 7). With a decrease in active acidity to pH 6.6, samples AA^3 and BB^2 remained stable, unlike AA^2, and only AA^3 had a denatured protein, which generally agreed with the data from the alcohol test. At pH 6.4, none of the samples was heat-resistant.

The acid-boiling test revealed insignificant differences between the milk samples with different *CSN3* genotypes, which can be clearly seen at pH 6.6 (Table 8). At this acidity level, sample BB^2 withstood the addition of 0.5 mL of 0.1N HCl, remaining thermostable. The stability of all the samples at pH 6.8 can be due to a partial neutralization of the acid by the alkali solution introduced during the preparation of milk samples. According to this method, milk is considered heat-resistant if it withstands an acid-boiling test with the addition of 0.8–1.0 mL of 0.1N HCl. Thus, only sample AA^2 at pH 7.0 can be considered heat-resistant.

The heat test, which takes into account all the factors affecting protein stability in the assessment of milk thermal stability, showed a partial correlation with the results of the other tests. The reconstituted dried milk from cows with the *BB* genotype (sample BB^2) showed better protein stability at pH 6.0–7.0 during high-heat treatment, compared to the other samples (Table 9). Sample BB^2 withstood high-heat treatment at pH 7.0 for more than 180 min (after 180 min, the experiment was terminated due to the absence of such regimes in world practice), while the samples from the *AA* genotype cows were stable for only 60 min.

Decreasing active acidity to pH 6.8 reduced the thermal stability by half for all the samples, while a decrease to pH 6.6 had a significant effect only on sample BB^2, reducing its thermal stability to 43 min. It appeared that an increase in pH from 6.6 to 7.0 decreased the positively charged groups on the surface of the casein micelle, which ultimately increased the total negative charge of the casein molecule and, therefore, its thermal stability. The genotypic differences of the κ-casein fractions in samples AA^3 and BB^2 most likely affected the rate of the reaction.

Lowering active acidity to pH 6.6 and more had the opposite effect, i.e. decreased the total negative charge of the casein molecule while maintaining the reaction rate for samples AA^3 and BB^2. These genotypically different samples with a similar physicochemical composition revealed a dependence between the time of heat treatment before the onset of protein destabilization and milk acidity. A decrease in milk acidity from pH 6.6 by 0.2 units lead to a decrease in the time of high-heat treatment by an average of 10 ± 3 min.

The effect of the κ-casein genotype on dried milk cheese suitability. The ability of milk, including

Table 10 Assessment of cheese suitability of reconstituted dried milk from cows with different *CSN3* genotypes by rennet-fermenting method

Parametr	MICROCLERICI (chymosin 2400 IMCU/g)		CHY-MAX (chymosin 2500 IMCU/g)	
	AA^3	BB^2	AA^3	BB^2
Rennet clotting time, min	100	360	60	150
Clot quality group	II	III	I	II
Protein content in whey, %	0.73	1.3	0.73	0.955
Total protein conversion to whey, %	28.6	48.0	28.6	35.7

reconstituted milk, to form a dense clot under the action of milk-clotting enzymes is the most important factor in the production of cheese and curd. Literature describes quite contradictory studies into the influence of the *CSN3* genotype of cows on the cheese suitability of raw milk. Some researchers found the presence of this effect, while others reported its absence. This can be due to concomitant factors that have a significantly greater effect on the cheese suitability of milk than the *CSN3* polymorphism.

For example, Fox *et al.* reported that the level of glycosylation directly affected the susceptibility of κ-casein to hydrolysis by chymosin: it decreased with an increase in carbohydrates [26]. Other authors found that the quality of the clot and the time of its formation were affected by milk acidity, protein and fat contents, as well as the quality of enzyme preparations (mainly chymosin) obtained from different manufacturers [27, 28].

We took into account the results of previous works and tested samples BB^2 and AA^3, which was rehydrated to a similar amount of protein as BB^2 had (2.67% and 2.65%, respectively). This method of rehydrating dried milk from a cow with the $CSN3^{AA}$ genotype minimized the differences in the main components between samples AA^3 and BB^2. In addition, in order to exclude the influence of enzyme preparation quality, we used chymosin produced by two large European companies. The test results are presented in Table 10.

When using 2400 IMCU/g chymosin (MICROCLERICI, Sacco Sistem), a dense clot formed in samples AA^3 and BB^2 after 100 min and 360 min, respectively. The tests using 2500 IMCU/g chymosin (CHY-MAX, Chr. Hansen) showed a higher rate of clotting in both AA^3 and BB^2 (60 min and 150 min, respectively). We found that the rate of clotting was higher in the milk from the *CSN3 A* allele cows, compared to the *B* allele cows. In addition, we observed a similar correlation when assessing the clot quality.

When using CHY-MAX, the quality of the AA^3 clot belonged to group I, while that of BB^2, to group II. When coagulating milk with MICROCLERICI, the quality of the AA^3 clot belonged to group II, while that of BB^2, to group III. Assessing the degree of protein loss associated with its conversion into whey, we found that sample AA^3 had a protein mass fraction of 0.73%, which corresponded to 28.6% of the total protein content in milk. In this sample, the degree of protein conversion into whey did not depend on the enzyme used. However, sample BB^2 showed a protein loss of 48% (MICROCLERICI) and 35.7% (CHY-MAX), which corresponded to a conversion to whey of 1.3% and 0.96% of total protein, respectively.

CONCLUSION

Our study revealed the effect of the *CSN3* gene polymorphism on the technological properties of dried milk. We found that the reconstituted milk sample with the *BB CSN3* genotype had higher thermal stability, while the sample with the *AA* genotype had better cheese suitability. In this regard, we cannot rule out an additional effect of milk drying and reconstitution. In addition, in order to minimize the influence of other factors, we used various methods to determine thermal stability and cheese suitability. Although we found no strict correlation between them, their combination allowed us to conduct a thorough analysis of milk stability during heat treatment and rennet coagulation. This approach to a comprehensive monitoring of technological properties opens up opportunities for accumulating a significant array of experimental data. Systemic research in this direction will produce a sufficient amount of material to create bioinformatics algorithms for mathematical modeling. In the future, this might give rise to a new system of criteria to evaluate the compliance of the technological properties of milk with the recommended indicators.

CONTRIBUTION

The authors were equally involved in working on the manuscript.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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