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ORIGINAL RESEARCH PAPER

THE CHANGE OF CASEIN DURING STORAGE OF COOLED MILK AND ITS INFLUENCE ON THE QUALITY OF FERMENTED DAIRY PRODUCT

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Abstract: The duration of raw cooled milk storage until processing has a big influence on the properties and quality of fermented dairy products. Proteins undergo significant changes. At low temperatures, β-casein is removed from casein micelles to the milk plasma. As a result, the size of micelles decreases; under the enzymes` action, $\beta$-casein breaks down into $\gamma$-casein and components of the proteose-peptone fraction. The increase of $\gamma$-casein content and proteose-peptone fraction has a negative influence on the rate of proteins` acid coagulation, syneresis properties, milk heat resistance as well as other functional milk properties. Moreover, under the action of native and microbial proteinases in raw cooled milk during its prolonged storage, bitter peptides are formed, likewise amino acids break down into compounds with unpleasant putrid taste and odor, which can be transferred to fermented dairy products. This article shows the changes in the size of casein micelles during the refrigerated milk storage at temperature 4 ± 2 °C by 2 - 8 %; the concentration of casein γ-fraction by 23 - 25 %; milk heat resistance and total bacterial count. The results of the carried-out experiments let to sum up that the use of milk stored for 24 hours after acceptance decreases effective viscosity of a product by 27 - 31 %, water holding capacity by 30 - 32 %, together with the acquiring of off-flavor and bitterness by the final fermented dairy product.

Keywords: *milk heat resistance, milk storage, size of casein micelles, total bacterial count, γ-casein*

INTRODUCTION

The duration of raw cooled milk storage until processing has a great influence on its functional properties, therefore, on the quality of the final product.
During the milk storage on farms and dairy plants, almost all the constituent parts and properties of milk change. Proteins as well as fat undergo significant changes. This decreases physicochemical, organoleptic and functional properties of milk [1 – 4].
The decomposition of proteins in raw cooled milk can be caused by indigenous milk proteinases coupled with proteolytic enzymes of the psychotropic bacteria of the genera *Pseudomonas, Psychrobacter, Acinetobacter*, etc. At low temperatures (2 - 6 °C), *β*-casein is transferred from casein micelles to the milk plasma. As a result, the size of casein micelles decreases, then, under the action of enzymes, *β*-casein breaks down into *γ*-casein and components of the proteose-peptone fraction. Thus, through the proteolysis in raw milk during its prolonged storage, the content of *γ*-casein and proteose-peptone fraction increases which may have a negative effect on the rate of proteins` acid coagulation, syneresis properties, milk heat resistance, and other functional properties of milk [1, 2, 5 – 10].
Moreover, since the psychotropic bacteria growth in raw milk during its prolonged storage, bitter peptides are formed, and amino acids break down into compounds with unpleasant putrid taste and odor [2, 6, 11, 12]. Then, unpleasant taste is transferred to the fermented dairy product produced from this milk.
The purposes of this research were to study the degree of the structure decomposition of protein components and their functional properties, depending on the duration of raw milk storage, and the decomposition effect on the quality of fermented dairy products.

MATERIALS AND METHODS

Physicochemical and microbiological properties of cow`s milk

Raw cow`s milk was collected in three farms of the Leningrad area in all seasons of the year from 2018 to 2019. Fermented dairy product (curdled milk) was processed from this milk.
Milk samples were obtained in the farms after milking and cooling, stored at temperature 4 ± 2 °C, and were investigated after storage for 12, 24, and 48 h.
In milk samples, acidity was controlled by titrimetric and potentiometric methods; density, milk solids non-fat (MSNF), total protein content, and total fat content were determined using a milk analyzer “Klever – 1M” (“Biomer”, Krasnoobsk, Russia). The total calcium content was determined by the complexometric method.
The milk heat resistance was determined by a modified alcohol test, expressing the result in milliliters of 78 % ethanol used before the visible coagulation of proteins occurred. Milk was considered as heat resistant if more than 1.5 cm^3 of 78 % ethanol was used for proteins` coagulation (for milk with a high heat resistance more than 2 cm^3 of ethanol was needed).
The total bacterial count in milk was determined on fixed stained smears.

Casein content and size of casein micelles

The casein content was determined by the method of acid precipitation. This method is based on the ability of casein to be neutralized with alkali. To do this, casein in one sample of milk is precipitated with dilute sulfuric acid and the solution is directly titrated with alkali (without filtration). In other sample, casein is precipitated and filtrated; the obtained filtrate without casein is titrated. The mass fraction of casein is calculated by the difference of alkali used by both titrations. Complete precipitation of casein occurs at an isoelectric point at milk *p*H about 4.7.

The γ-casein content was determined by a modified discontinuous polyacrylamide gel electrophoresis method using electrophoresis camera (Hoefer, Holliston, Massachusetts, United States) in the presence of sodium dodecyl sulfate [13 – 15]. After electrophoresis, the gel was soaked in Coomassie brilliant blue. All chemicals used were of reagent grade. Identification of proteins was carried out using marker proteins. The γ-casein content (γ_1-, γ_2-, and γ_3) was calculated with the Scion Image computer program and expressed in square pixels (px^2), which were conditionally converted to %. In the investigated samples, the γ-casein content fluctuated from less than 24,000 px^2 to more than 28,000 px^2.

The average size of casein micelles was measured by light scattering. The light scattering coefficient of skim milk was determined directly after diluting it with $CaCl_2$. Cuvettes with $CaCl_2$ solution and diluted milk were inserted into the sample holder of the photo colorimeter KFK-3 (AOOT "Zagorsk Optical and Mechanical Plant" (ZOMZ) Sergiev Posad, Russia). To eliminate possible errors associated with the presence of fat traces, a parallel trial was carried out examining a sample of skim milk diluted with hydrochloric acid in the same ratio as with $CaCl_2$. The mass of casein micelles without a correction for their possible coagulation in the period of measurement (B) was defined as in (Equation 1):

$$B = \frac{T}{H \cdot C} \tag{1}$$

where T is the light scattering coefficient equal to the ratio of the optical density of the milk mixture with $CaCl_2$ with the correction on presence of fat traces, cm; H is a constant value equal to $4{,}38*10^{-6}$; C is the casein content in 1 mL of milk. The mass of casein micelles with the correction on coagulation (B_1) was determined by the calculated B value, then the average size of casein micelles (D, nm) was defined as in (Equation 2):

$$D = 1.342 \cdot \sqrt[3]{B_1} \cdot 10^{-8} \tag{2}$$

Product production

To study the influence of changes in the proteins of raw milk after refrigerated storage on the quality of fermented dairy products, acid-coagulation clots and curdled milk were obtained. The experiment was carried out as following: the test milk, stored at temperature 4±2 °C during 12 and 24 h, was normalized by the fat content, pasteurized at temperature 85 - 90 °C holding for 2 - 3 min, cooled down to a temperature of

fermentation, fermented at temperature 45 °C with YO-MIX 885 (Danisco, Paris, France) cultures, containing strains of the thermophilic lactic acid bacteria *S. thermophilus* and *L. bulgaricus*, and fermented in a thermostat at temperature 42 °C until clots were formed and *p*H dropped to 4.6. Then, the product was cooled down and stored at temperature 4±2 °C for 24 h. The *p*H value of the fermented mix was controlled by the potentiometric method every hour during the fermentation process.

Rheological properties

In the protein acid-coagulation clots obtained after fermentation, their structural-mechanical and syneresis properties were controlled. The effective viscosity of the intact destroyed, and recovered clots, and the degree of recovering of the clots were determined on a rotational viscometer "Reotest" ("RheoTest Messgerate Medingen GmbH", Germany) after holding them at 5 °C for 20 h. For measurements, a measuring cylinder S_1 was used, which was filled with 25 mL of the tested sample. The measurement was carried out at temperature 20 °C. The effective viscosity of the intact structure was determined at a shear rate of 48.6 s^{-1}; destroyed and recovered at a shear rate of 1312 s^{-1}. The structure recovering time was 15 min. The degree of the structure recovering was determined with a gradient γ = 48.6, a disruption time 24 min, a recovery time of 20 min, and temperature 20 °C. The syneresis properties were controlled by the intensity of whey separation during filtration of 100 cm^3 clot, which was mixed 3 times. The filtration was carried out through a dry folded paper filter for 30 min.

Organoleptic properties

In the curdled milk, organoleptic properties such as consistency and taste were controlled using a 5-point hedonic scale, where 1 point is a definitely unpleasant product and 5 points is a definitely pleasant product.

Statistical analysis

The quantitative results of the experimental data were evaluated by statistical analysis using a standard computer program. When calculating, a 5 % level of significance was assumed (α = 0.05).

RESULTS AND DISCUSSION

Composition and properties of milk

The average indicators of the composition and properties of milk collected in three farms of the Leningrad area are summarized in Table 1.

Table 1. *Composition and properties of milk form the Leningrad area farms (annual average value)*

Farm	Acidity [°T]	Density [kg·m^{-3}]	Content					Average size of casein micelles [nm]	Heat resistance [mL of 78% ethanol]
			MSNF [%]	Total protein [%]	Casein [%]	Fat [%]	Ca [mg%]		
1	17	1027.5	8.20	3.20	2.20	3.65	115.08	75.6	6.2
2	17	1028.0	8.32	3.03	2.00	3.62	113.50	70.7	5.0
3	17	1027.5	8.21	3.08	1.99	3.68	122.70	63.4	3.8

After studying the composition and properties of milk from three farms, it was understood that milk produced on farm 1 is characterized by a relatively high content of both protein and casein along with the largest size of casein micelles. Farms 2 and 3 have a lower level of protein. The milk from farm 3 has the smallest casein micelles, while farm 2 has an average size. The differences, probably, are caused by the level of breeding in these farms. Milk of all farms is characterized with high heat resistance. Its acidity, density, and MSNF have some differences, but in all cases, they fit the indicators for natural cow`s milk.

Effect of storage time on *γ*-casein content

The results of studying the effect of time of milk storage on the *γ*-casein content in it are shown in Figure 1.

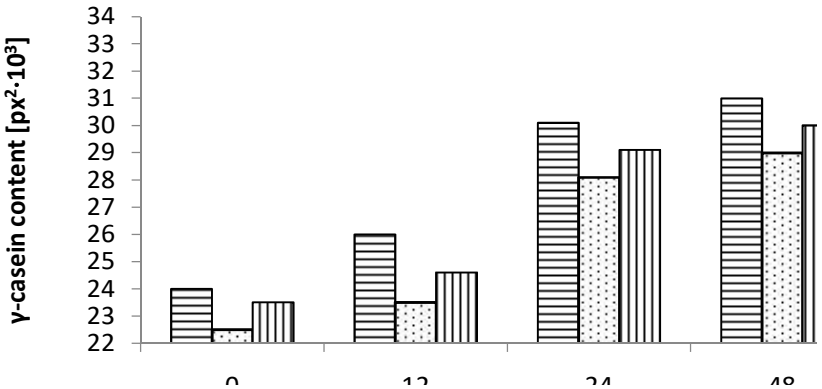

Figure 1. *Effect of milk storage time on the γ-casein content*

Figure 1 shows that the *γ*-casein content in the un-stored milk was less than 24.000 px^2; after 12 h of refrigerated storage, *γ*-casein increased to 24.600 - 26.000 px^2, i.e. by 4 - 8 %, after 24 h storage, the *γ*-casein increased dramatically to 29.000 - 30.000 px^2, which amounted to 23 - 25 % of the initial value; and after 48 h storage, the γ-casein content changed slightly and was 29.000 - 32.000 px^2. Thus, our studies shown, that the *γ*-casein content increases during storage of cooled milk. The largest increase in *γ*-casein content is observed after 24 h. Further milk storage (48 h) leads to a slight increase of *γ*-casein content.

At low temperatures, hydrophobic interactions are weakened; this leads to the release of *β*-casein and calcium phosphate from casein micelles, and the structure of micelles changes [10, 16 – 18]. The maximum amount of soluble *β*-casein after 48 h of milk storage at 4 °C can reach 30 – 60 % of the total *β*-casein [19, 20]. The breakdown of *β*-casein with the *γ*-casein formation occurs more active at 4 °C than at higher temperatures. The higher rate of *β*-casein proteolysis at lower temperatures compared with proteolysis at higher temperatures resulting from the different sensitivity of monomeric and micellar forms of *β*-casein to the action of enzyme. In milk at a temperature 26 °C, the enzyme substrate is in micellar form of *β*-casein, which is not sensitive to proteolysis. Moreover, the milk protease at 26 °C is immobilized, i.e. is held by the surface of casein micelles and, partly, surface of fat globules [21]. Consequently, the enzyme is bound to casein micelles like *β*-casein by hydrophobic interactions that are sensitive to temperature changes. Therefore, when milk temperature drops to 4 °C, *β*-casein and plasmin are transferred from micelles to the milk plasma due to the breaking of hydrophobic interactions. In the milk plasma, the enzyme finds its substrate and destroys it [22]. Soluble *β*-casein is exposed to the action of plasmin with the formation of *γ*-casein and phosphopeptides [21].

Protein breakdown in cooled raw milk during prolonged storage can be caused by proteolytic enzymes of psychotropic bacteria of the genera *Pseudomonas, Psychrobacter, Acinetobacter, Alcaligens*, etc. Despite the fact, that bacterial proteases mostly attack *κ*-casein than *β*- and α_s-caseins they, however, may promote the formation of bitter peptides and the breakdown of amino acids to compounds with unpleasant putrid odor and taste, which are transferred to fermented dairy products [1, 3, 11, 23, 24].

The increase of *γ*-casein, phosphopeptides, and undesired products of bacterial metabolize during milk storage may have a negative effect on the rate of acidic coagulation of proteins, syneresis properties of a protein product, milk heat resistance, and other functional properties of milk [25].

Effect of storage time on size of casein micelles

The change of the size of casein micelles during storage of raw cooled milk is shown in Figure 2.

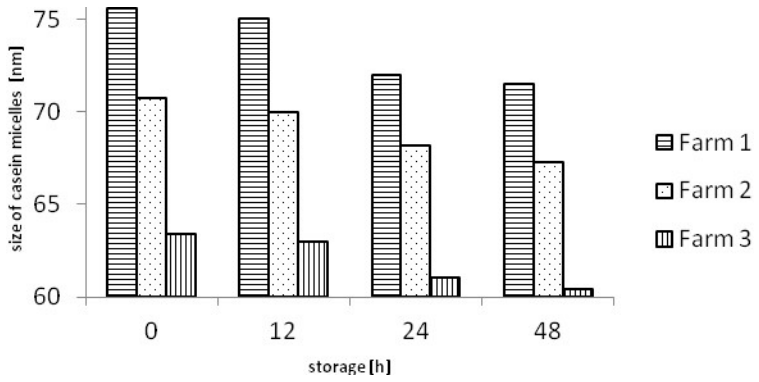

Figure 2. *Change of the size of casein micelles during storage*

Figure 2 reveals that the size of casein micelles in all the samples decreased during storage. The most drastic decrease in the size of micelles occurs during the first 24 h of milk storage from the moment of its acceptance (by 2 - 5 %). Also, another important fact is that the larger the size of casein micelles in milk, the more they decrease during storage.

This decrease can be possibly explained with the difference in the fractional composition of casein micelles, as large micelles have more *β*-casein compared to smaller micelles; *β*-casein with the temperature drop transfers to the milk plasma [5].

Consequently, during the process of raw milk storage, the size of casein micelles decreases as a result of the change of their structure and the transfer of *β*- and *κ*-casein to the milk plasma. Then, released casein fractions can be exposed to the proteases` action (containing in milk as well as produced by psychotropic bacteria) with the formation of *γ*-casein, proteose peptone fraction along with the other compounds [5, 11, 23, 26, 27].

The change of heat resistance during storage of raw cooled milk is shown in Figure 3.

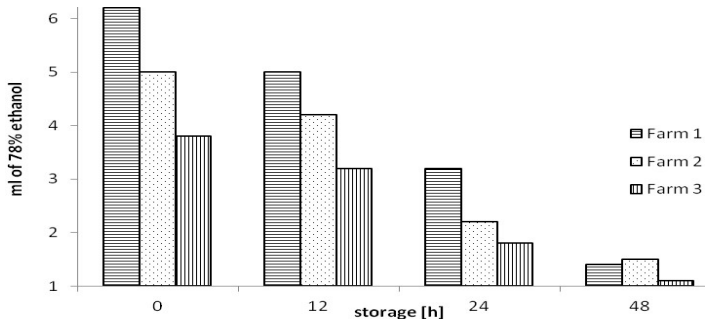

Figure 3. *Change of heat resistance during storage*

The initial heat resistance of the milk of the three farms differs. The milk of farm 1 is more heat resistant, the milk of farm 3 is less heat resistant, and the milk of farm 2 is intermediate. The difference in milk heat resistance is probably due to its different salt composition and size of casein micelles. Thus, in the milk of farm 3, the calcium content is higher, and the size of casein micelles is lower compared to the milk of two other farms (Table 1). The heat resistance of raw milk during storage at low temperatures drops drastically. The most drastic drop of heat resistance (by 2.5 - 3.0 times) occurs during 48 h of milk storage.

The decrease in heat resistance during the storage of cooled milk is explained by the change in its acidity, protein and salt composition [28, 29]. The change of acidity during storage is shown in Table 2.

Table 2. *Change of acidity during milk storage*

Milk sample	***p*H during storage**		
	12 h	**24 h**	**48 h**
Farm 1	6.62	6.60	6.59
Farm 2	6.66	6.64	6.62
Farm 3	6.65	6.63	6.61

With a change in milk *p*H, the interaction of casein with the surrounding ions changes as well as the properties of casein micelles affecting their heat resistance. The lactic acid formation causes a drop of the negative charge of casein micelles and a change in salt balance of milk; the part of colloidal calcium salts transfers into an ion-molecular state, and calcium phosphates acquire better solubility and a higher degree of dissociation [3, 20, 28 – 30]. These factors promote the increase in concentration of calcium ions in milk.

Moreover, during milk storage, enzymatic breakdown of proteins (proteolysis) occurs. The breakdown can be caused by native milk proteases, also by proteolytic enzymes of extraneous psychotropic microflora. As a result, *β*-casein, *κ*-casein, and colloidal calcium phosphate are released from the casein micelles; then, they break down into smaller fragments. These changes are followed by the decrease of charge and the size of casein micelles; resulting in the protein instability and easy coagulation if exposed to high temperatures [24, 31].

Effect of storage time on microbiological properties

The changes in total bacterial count during raw milk storage are shown in Figure 4.

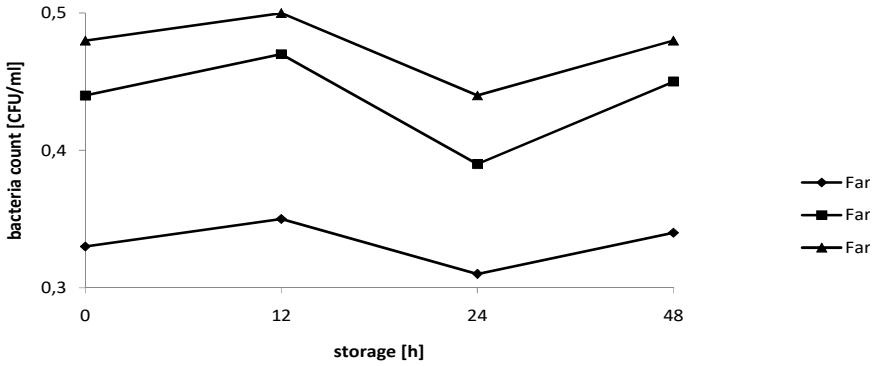

Figure 4. *Changes in total bacterial count during raw milk storage*

Figure 4 shows that during the refrigerated storage of raw milk, bacterial count changes. It slightly increases after the first 12 h, decreases drastically after 12 - 24 h, then, gradually increases.

The drop of total bacterial count during the first 24 h storage is explained by the death of mesophilic and thermoduric bacteria. Then, in conditions of prolonged storage (48 h), total bacterial count increases markedly resulting in the growth of psychotropic bacteria [24, 32].

Effect of storage time on rate of lactic acid fermentation

Based on the data obtained, we assumed that the production of fermented dairy products from raw milk stored more than 24 h after acceptance is impractical. To prove these assumptions, a fermented dairy product (curdled milk) was produced from the samples of milk collected from the dairy farms. This milk was stored for 12 h (sample 1), and

24 h (sample 2). Then, the properties of the product were studied. The rate of acid formation by the microflora of the starter in milk samples is shown in Figure 5.

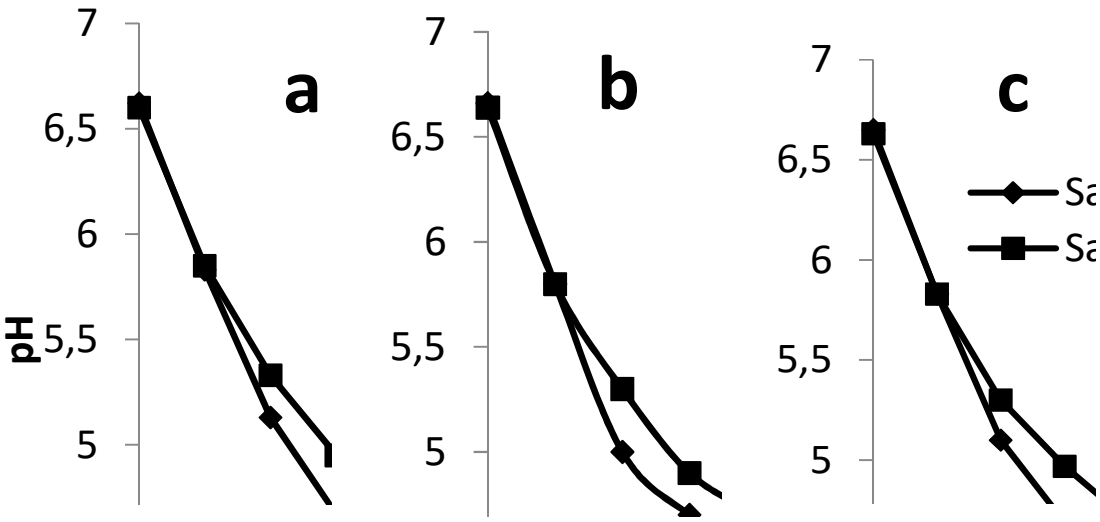

Figure 5. *Rate of acid formation*
Fermentation time, h: a – farm 1; b – farm 2; c – farm 3

Figure 5 shows that lactic acid fermentation was less intensive in the milk samples stored for 24 h. The delay in the process of fermentation ranged from 45 min to 1 h. Thus, if the *p*H of the sample 1 from milk of the farm 2 reached the value 4.6 approximately after 3.5 h; then, the sample from milk stored for 24 h after 4.5 h. A less intensive acid formation in prolonged stored milk is due to a drop of its nutritional value, probably, because of the growth of psychotropic bacteria during storage.

Effect of storage time on rheological properties

The results of rheological studies of protein clots and organoleptic properties of the curdled milk obtained from milk with different storage time are shown in Tables 3 and 4.

Table 3. *Rheological characteristics of protein clots*

Sample		Effective viscosity [Pa·s·10^{-3}]			Degree of structure recovering [%]	Whey separated [mL]
		η_{intact}	$\eta_{destroyed}$	$\eta_{recovered}$		
Farm 1	Sample 1*	560.0	15.1	16.9	8.8	25.0
	Sample 2	422.0	10.6	11.8	6.2	36.0
Farm 2	Sample 1	570.0	15.2	17.3	9.2	23.0
	Sample 2	395.0	10.0	11.3	6.1	35.0
Farm 3	Sample 1	452.0	10.9	12.5	8.9	27.0
	Sample 2	325.0	8.5	9.9	6.0	39.0

*Sample 1 is a clot produced from milk stored for 12 h at 4±2 °C; sample 2 is a clot produced from milk stored for 24 h at 4±2 °C

Table 4. *Organoleptic properties of the product*

Sample		Organoleptic properties** of curdled milk, points				
		Homogeneity and firmness of consistency	Whey separation	Off-flavors	Bitterness	Total acceptance
Farm 1	Sample 1*	4.75	0.50	0.25	0.00	4.75
	Sample 2	3.75	1.75	1.25	1.00	3.25
Farm 2	Sample 1	5.00	0.00	0.00	0.00	5.00
	Sample 2	4.00	1.25	1.25	1.00	3.25
Farm 3	Sample 1	4.00	1.50	0.25	0.50	4.25
	Sample 2	2.50	3.00	1.50	1.25	3.00

*sample 1 is a product produced from milk stored for 12 h at 4±2 °C; sample 2 is a clot produced from milk stored for 24 h at 4±2 °C

**organoleptic properties were evaluated using the hedonic sale, where 1 point is a definitely unpleasant product and 5 points is a definitely pleasant product

The data from Table 3 show the changes in effective viscosity of protein clots depending on the farm and the storage time of milk. Changes in the viscosity of the intact, destroyed, and recovered clots reveals that interactions of the condensation type (irreversibly collapsing) with non-thixotropic properties play the main role in the formation of the product's consistency. The recovery of the structure of clots was 6.0 - 9.2 %.

After 24 h storage of all milk samples, the ability of proteins to form a spatial structure during the acid coagulation decreased; which is proven by the decrease of effective viscosity of the protein clots compared to the samples produced from the milk stored for 12 h. Moreover, the ability to syneresis drastically increased. Thus, our studies have shown the reduction of structural-mechanical and syneresis properties of the protein clots because of the breakdown of casein during milk storage.

Effect of storage time on organoleptic properties

The data presented in Table 4 show that the milk storage before processing leads to the alteration of organoleptic properties of a product produced from this milk. In the curdled milk, the whey separation increased; it acquired off-flavors and tastes. The grade of the total acceptance of the product produced from milk stored for 24 h at 4 ± 2 °C decreased by 1.75 - 1.25. The appearance of off-flavors and bitterness is explained, probably, by the formation of bitter and other peptides and products of the breakdown of amino acids under the enzymes` action of psychotropic bacteria [26, 33]. According to [34], proteolytic enzymes produced by aerobic or microaerophilic psychotropic gram-negative rods *Pseudomonas, Flavobacterium* and other remain active even in pasteurized milk.

CONCLUSION

Thus, our studies have shown that, during the storage of raw milk before its processing, the change in the structure of casein micelles and the breakdown of casein under the action of native and bacterial proteolytic enzymes occur, which leads to the reduction of structural-mechanical and syneresis properties of the protein clots and the quality of the

fermented dairy products. The effective viscosity decreases by 27 - 31 %; water holding capacity by 30 - 32 %; the curdled milk acquires off-flavors and bitterness. It is important to produce fermented dairy products no later than 12 h after milk acceptance.

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