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Investigation of the influence of milk protein genotype on the process of fermentation of milk curds by mesophilic lactic acid streptococci

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Abstract. The relevance of this study lies in the fact that the majority of commercial milk processed into fermented dairy products is a mixture of β-casein genotypes. The increasing use of A2 milk in the dairy industry necessitates the development of scientifically grounded technologies for the production of fermented products, as casein genotype modifications can affect the course of technological processes and require adjustments to production parameters. The study aimed to determine the impact of genetic modification of the β-casein protein in cow's milk on the

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fermentation process of milk mixtures during the production of fermented dairy products. Research methods included chemical, physical, organoleptic, and microbiological methods (determination of the quality indicators of raw milk and fermented milk curds), and technological methods (determination of acid formation activity and viscosity). The initial characteristics of raw milk were investigated according to genotype, revealing no direct correlation. Changes in the physicochemical, microbiological, and organoleptic properties of fermented milk curds during biotechnological processing and storage were examined. Optimal technological process regimes were selected, and technological methods were chosen to minimise the influence of secondary factors on the experiment. A mixed culture preparation of mesophilic lactic acid lactococci, including *L. Lactis ssp.*, was chosen as a starter culture. It was established that all 13 milk samples were suitable for biotechnological processing using mixed cultures of mesophilic lactic acid bacteria. As a result, it was concluded that the production of fermented dairy products from raw milk with the presented β-casein genetic modifications, A1A1, A1A2, and A2A2 – using *L. lactis ssp.* cultures – is possible using the classical technology and does not require adjustment of the technological conditions. The obtained results can be used in the dairy industry when developing technologies for fermented dairy products from A2 raw milk

Keywords: A2 milk; technological properties; biotechnological processing; starter culture; fermented dairy products

INTRODUCTION

The advancement of biotechnology and genetics could be a pivotal approach to addressing a global human problem such as food security. This encompasses not only the quantity of food but also its impact on the human body. Thanks to breakthroughs in agricultural animal genetics, it has become possible to address one of civilization's challenges – food allergies. Fermented dairy products remain the most consumed segment within dairy products but are inaccessible to those with milk protein allergies. Milk protein, specifically casein, which constitutes about 80% of milk proteins, is heterogeneous – represented by fractions α_{s1}-, α_{s2}-, β-, and κ-casein (Daniloski *et al.*, 2021; Lewis, 2022). The ratio of these protein fractions in milk is not influenced by the animal's diet, housing, age, or lactation period but is solely determined by the animal's genetic makeup.

According to C. Sebastiani *et al.* (2022), genetic variations in casein fractions depend on the cow breed. The most common genetic modifications of β-casein in commercial milk (A1 and A2) can occur in three variations: A1A1, A1A2, and A2A2. Researchers B. Guantario *et al.* (2020) established that β-casein of the A1 genotype is the cause of discomfort when consuming bovine milk. β-casein of the A1 genotype, as a result of proteolytic cleavage by gastrointestinal enzymes, produces a higher amount of β-casomorphin compared to β-casein of the A2 genotype.

The majority of commercial milk used for the production of fermented dairy products contains β-casein of the A1A2 genotype. However, research by M. Ramakrishnan *et al.* (2020) indicates the potential for developing dairy farming with the A2A2 genotype as a raw material for a wide range of food products. To produce A2 milk, cows must possess the appropriate genetic inheritance. The probability of forming a herd with 100% A2A2 genes is only possible if two animals carrying the A2A2 genotype are mated. M. Samilyk *et al.* (2022), O. Bordunova *et al.* (2023), and A. Dantas *et al.* (2023) have determined that milk, as a raw material for dairy product production, in addition to being safe and beneficial for the end consumer, must also possess certain technological properties that determine its processing direction and the specific production process for each type of product. These properties include rennet curdling ability, souring ability, heat resistance, and others. These technological properties of the raw material ensure the correct execution of the technological process and the production of a product that meets quality standards.

The technological properties of raw milk are directly dependent on the quantitative and qualitative content of its protein, fat, and carbohydrate components, acidity, and salt balance. Consequently, genetic variation in casein fractions can influence the technological process and the quality of the final product (Sokoliuk *et al.*, 2022). Therefore, it is worth investigating the impact of the milk protein genotype on biotechnological processing to understand the significance of this influence and to adjust the technological process if necessary. To study the impact of milk protein genotype on the technological process, it was decided to investigate the fermentation process of pasteurised milk blends (Giribaldi *et al.*, 2022).

T. Bintsis and P. Papademas (2022), and N. Bolgova *et al.* (2023) highlight that fermentation is the cornerstone of most fermented dairy product manufacturing processes. Milk fermentation is a process where the components of milk undergo biochemical changes under the influence of enzymes. Raw milk contains approximately 100 enzymes from various classes, which, if the primary milk processing is disrupted, can lead to spoilage. In pasteurised milk, the primary source of enzymes is starter cultures. To activate a controlled fermentation process, specially selected strains of lactic acid bacteria, propionic acid bacteria, yeasts, as well

as prebiotics and their combinations, are added to the pasteurised milk blend. The activity of these starter microorganisms leads to significant biochemical changes in all milk components, leading to the formation of various chemical substances (Liu *et al.*, 2020).

Lactose undergoes the most significant changes during directed fermentation. Under the influence of β-galactosidase, it is broken down into galactose and glucose, which, in the case of anaerobic carbohydrate oxidation via the Embden-Meyerhof-Parnas pathway, forms lactic acid as the final fermentation product (Romanchuk *et al.*, 2020; Sreenivas, 2024). Milk proteins also undergo enzymatic changes due to the action of microbial proteolytic enzymes. Proteins experience the most significant enzymatic transformation due to the action of proteases. Proteases catalyse the hydrolysis of peptide bonds in protein molecules. In the production of fermented dairy products, starter culture strains that carry out moderate proteolysis are used. Proteases produced by starter culture microflora more readily attack α_s-casein compared to β- and κ-casein, although some microorganism strains break down α- and β-fractions equally effectively (Helikh *et al.*, 2021; Kieliszek *et al.*, 2021).

In the production of fermented dairy products mixed mesophilic cultures starter cultures of *Lactococcus lactis ssp*. are commonly used. Lactococci do not possess high proteolytic activity, however, the limited breakdown of casein provides optimal consistency and structure while simultaneously increasing casein digestibility in the gastrointestinal tract and ensuring storage stability. This is because the intensity of proteolysis is somewhat inhibited by changes in hydrogen ion concentration, leading to a decrease in pH (Wang *et al.*, 2021). As a result of the biotechnological processing of milk blends, the fat component also undergoes changes, albeit to a lesser extent. The degree of lipolysis depends on the composition of the microflora, both initial and introduced and on the quantity and state of the fat globules. Uncontrolled lipolysis leads to defects and spoilage of products (Tsisaryk *et al.*, 2022; Thum *et al.*, 2022). Therefore, when selecting starter cultures, this fact is taken into account.

The aim of this study was to investigate the fermentation process of milk blends using mesophilic lactic acid bacteria *Lactococcus lactis ssp.*, to determine the impact of genetic modification of milk protein on the souring process, and to assess the physicochemical, microbiological, and organoleptic properties of milk curds both during fermentation and during storage.

MATERIALS AND METHODS

The experimental part of the study was conducted in November-December 2023 at the interdepartmental laboratories of the Faculty of Food Technology, Sumy National Agrarian University. To determine the influence of the β-casein genotype of milk protein on the fermentation process of milk curds, raw cow's milk from cows with different genotypes was used. Thirteen milk samples were collected during morning milking from cows with different β-casein genotypes (A1A1, A1A2, A2A2) at the farm of the Institute of Agriculture of the North-East of NAAS. To track the impact of the β-casein genotype on the biotechnological processing of milk, samples were grouped by genotype and initial control of all raw milk samples was conducted, as presented in Table 1. Samples 1-3 were milk from cows with an undetermined genotype, samples 4-6 from cows with the A1A2 genotype, 7-10 from cows with the A1A1 β-casein protein genotype, and samples 11-13 were A2 raw milk.

***Table 1**. Raw milk characteristics by genotype, (n = & & &, p < 0,05)*

Sample No.	β-casein genotype	Physicochemical parameters						MAFAnM, CFU/cm³
		Fat, %	DSMS, %	Protein, %	Density, °A	Acidity,°T	pH	
No. 1	Without genotype identification	4.32	8.75	3.13	28.6	19	6.61	
No. 2		4.54	8.86	3.17	28.9	22	6.45	
No. 3		4.65	8.69	3.11	28.1	16	6.68	
No. 4	A1A2	4.24	7.91	2.82	25.3	17	6.58	
No. 5	A1A2	3.83	8.08	2.88	26.4	21	6.51	
No. 6	A1A2	2.67	8.1	2.87	27.5	15	6.74	
No. 7	A1A1	3.87	8.09	2.88	26.4	17	6.65	$4 \pm 0.5 \times 10^3$
No. 8	A1A1	4.69	8.48	3.03	27.2	23	6.4	
No. 9	A1A1	3.84	8.22	2.93	26.9	16	6.65	
No. 10	A1A1	4.09	8.36	2.98	27.2	15	6.72	
No. 11	A2A2	4.69	9.1	3.26	29.7	21	6.46	
No. 12	A2A2	4.54	8.06	2.88	25.6	21	6.5	
No. 13	A2A2	5.25	8.64	3.1	27.3	18	6.59	

***Source:** compiled by the authors*

Standard methods were used to determine the physicochemical and microbiological parameters. Fat content (Fat, %), mass fraction of dry skimmed milk solids (DSMS, %), protein content (Protein, %), and density

(°A) were determined using an EKOMILK Standard ultrasonic analyser without the use of any chemical reagents. The titratable acidity (°T) of the samples was determined by titrating the diluted milk samples (1:2 with distilled water) with a 0.1 N sodium hydroxide solution, using a 1% alcoholic solution of triphenylmethane dye as an indicator. The active acidity (pH) was determined using an Apera Instruments PH8500-DP device according to the standard (DSTU 8550:2015, 2015). Conditional viscosity was determined using a VZ-246 viscometer by measuring the time in seconds (s) for 100 cm^3 of thoroughly mixed product to flow through a 5 mm diameter nozzle of the viscometer.

Total bacterial count, characterised by the number of mesophilic aerobic and facultatively anaerobic microorganisms (MAFAnM) colony-forming units per cubic centimetre (CFU/cm^3), (MAFAnM, CFU/cm^3), was determined as a sanitary indicator of raw milk quality using the plate count method. One cm^3 of each sample was plated onto solid nutrient agar and incubated at 30 ± 1°C for 72 hours. The most probable number of viable lactococci cells (CFU/cm^3) in the fermented curds was determined using the liquid nutrient medium inoculation method. The sample of the curd was diluted in a series of ten-fold dilutions, with the last three dilutions being plated into two tubes each containing 10 cm^3 of sterilised skimmed milk. This was done to ensure that the final dilution contained no microorganisms. The inoculated tubes were incubated at 30 ± 1°C for 7 days. After incubation, the tubes were examined and those showing milk curtailment were recorded. A numerical characteristic was compiled, and the most probable number of viable cells was calculated.

Before biotechnological processing, milk samples were subjected to clarification, separation, and pasteurisation at 90 ± 2°C for 5 minutes. To minimise the impact of the milk fat fraction on the objectivity of the results, it was decided to ferment pre-skimmed samples. Skimming was carried out by separating heated (41 ± 1°C) whole milk twice using a Motor Sich-100 separator. The skimmed milk was pasteurised and cooled to the fermentation temperature of 29 ± 2°C, bypassing the cooling process. The skimmed milk was inoculated at 29 ± 2°C with a starter culture of mixed mesophilic lactic acid lactococci, trade mark "VIVO-Cottage cheese", consisting of *L. lactis ssp.* cultures. The starter culture was added in an amount sufficient to provide an initial concentration of lactobacilli cells of at least 1×10^6 CFU/cm^3. Experimental studies were conducted by current standards, using modern, generally accepted methods (Directive 2010/63/EC, 2010) and adhering to the Procedure for conducting scientific research and experiments on animals (Law of Ukraine No. 249, 2012).

RESULTS AND DISCUSSION

Analysis of the initial control of all raw milk samples from cows with different β-casein protein types did not reveal any significant differences in the analysed parameters, which is consistent with the data presented in the study of V.I. Ladyka *et al.* (2021) and the research conducted K. De Vitte *et al.* (2022). All groups of samples had average values for fat content (except for sample No. 6), protein content, and density (except for samples No. 4 and No. 12). Biotechnological processing of the experimental milk samples was carried out for 10 hours at a temperature of 29 ± 2°C, with the inoculated samples incubated in a thermostat in hermetically sealed, pre-sterilised containers. The results of the study of the fermented milk curds are presented in Table 2.

***Table 2**. Characteristics of fermented curds (n = &&&, p < 0.05)*

Sample No.	Acidity, °T	pH	Conditional viscosity, s	The most probable number of lactococci, CFU/cm^3
No. 1	77	4.66	28	$3.0 \pm 0.5 \times 10^{10}$
No. 2	73	4.63	29	$5.5 \pm 0.5 \times 10^{9}$
No. 3	76	4.5	30	$5.0 \pm 0.5 \times 10^{8}$
No. 4	73	4.74	22	$8.0 \pm 0.5 \times 10^{8}$
No. 5	70	4.6	27	$4.6 \pm 0.5 \times 10^{9}$
No. 6	72	4.6	20	$2.0 \pm 0.5 \times 10^{9}$
No. 7	76	4.6	20	$5.5 \pm 0.5 \times 10^{9}$
No. 8	70	4.71	27	$3.0 \pm 0.5 \times 10^{9}$
No. 9	71	4.58	28	$9.0 \pm 0.5 \times 10^{8}$
No. 10	75	4.59	26	$1.5 \pm 0.5 \times 10^{9}$
No. 11	75	4.59	30	$8.0 \pm 0.5 \times 10^{8}$
No. 12	76	4.6	23	$5.5 \pm 0.5 \times 10^{9}$
No. 13	78	4.53	28	$1.5 \pm 0.5 \times 10^{8}$

***Source:** compiled by the authors*

All fermented samples exhibited acidity levels consistent with those of products fermented by mesophilic lactic acid lactococci (Cheng *et al.*, 2022; Sakihara *et al.*, 2022). No deviations from the process parameters were observed. Contrary to the results obtained in the study (Kyselová *et al.,* 2019), which indicated that genetic polymorphisms of milk proteins significantly affect titratable and active acidity, the samples in this study had different combinations of protein genotypes. All fermented samples, regardless of the protein genotype in the initial raw material, had homogeneous curds with moderate acidity and viscosity after 10 hours of fermentation. Samples from A2 milk had titratable acidity in the range of 75 to 78°T and pH of 4.6 to 4.53 after 10 hours of fermentation.

Microbiological analysis of the samples to determine the influence of the β-casein genotype of cow's milk protein on the growth of lactic acid microorganisms also did not reveal any significant correlation. The sensory evaluation did not reveal any noticeable difference between the fermented samples, however, research (Gai *et al.*, 2021) indicates that curds obtained from A2 milk have l lower firmness of the formed gels, so the product may have a less firm curd but, on the other hand, it tends to be better digestible.

By analysing the research data, it is possible to compare the conditional viscosity of the samples within each group. In the group of samples from A2 milk, sample No. 12 stood out as the most fluid, with a viscosity of only 100 cm^3 in 23 seconds. The low protein content and low density of the raw material in this sample can explain this. A similar pattern can be observed in other groups of samples. For example, in samples with the A1A1 protein genotype, there was also a decrease in viscosity with low protein and density in the raw material (sample 7). In the group of samples from A1A2 milk, samples No. 4 and No. 6 had a viscosity of 20-22 seconds, and the raw material had a low protein content (2.82 and 2.87%, respectively), although only sample No. 4 had a density below the norm (25.3°A). Therefore, the data from this study confirms the direct proportionality between the viscosity of fermented curds and the protein content of the raw material (Čítek *et al.,* 2020), and does not reveal any influence of the casein genotype on the quality of fermented curds produced by mesophilic lactic acid lactococci.

Biochemical changes to the components of milk can continue even during the storage of fermented curds. While the reduced positive temperatures and increased acidity can slow down enzymatic processes, they do not completely stop them. The fermented samples were stored at 4-6°C in hermetically sealed containers, protected from light, for 14 days. The titratable and active acidity were monitored on the 3rd, 7th, 10th, and 14th days (Fig. 1a, b).

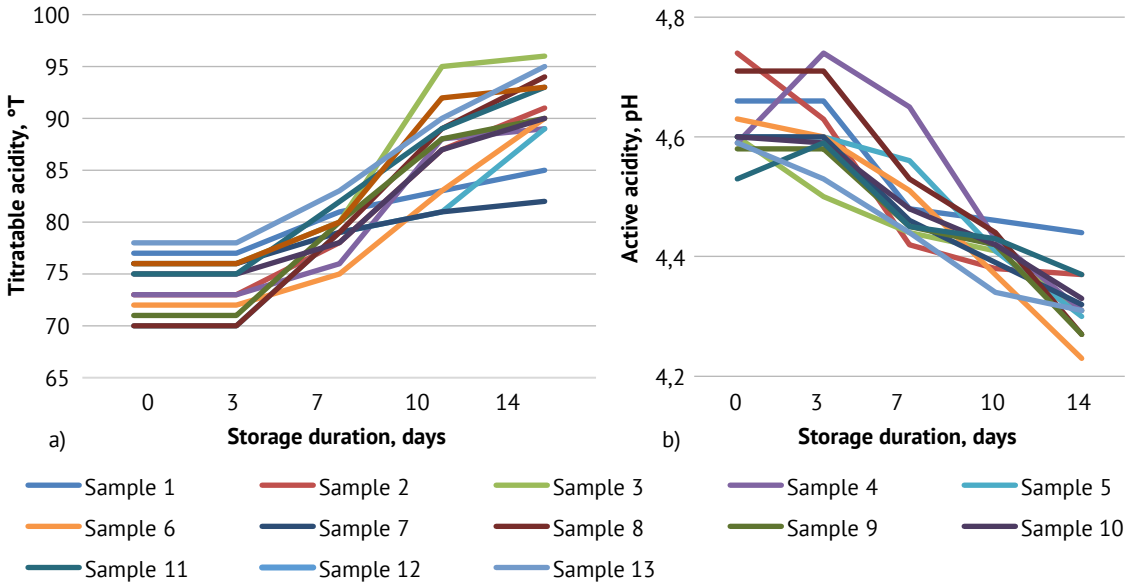

Figure 1. *Changes in titratable (a) and active (b) acidity in the fermented samples during storage*
Source: *compiled by the authors*

By analysing the data from the figures, a gradual increase in titratable acidity (Fig. 1a) and a change in active acidity (Fig. 1b) can be observed in all samples throughout storage, which is normal when using mesophilic lactococcal cultures as a starter. The conditional viscosity was determined by the duration of the flow of 100 cm^3 of curd from a viscometer with an outlet of 5 mm (Fig. 2).

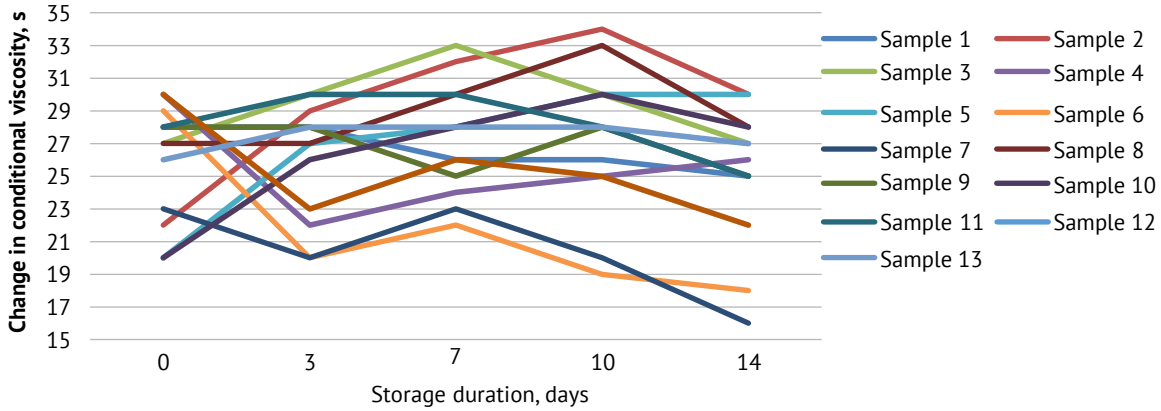

***Figure 2**. Changes in the conditional viscosity of fermented samples during storage*
***Source:** compiled by the authors*

Based on the studies on the determination of the conditional viscosity of fermented curds, no significant differences were found either at the end of the technological process or during storage (Table 2, Fig. 2). The degree of syneresis in all samples was low. As a result of microbiological studies of fermented samples during storage, the number of viable lactic acid bacteria cells was determined (Fig. 3). The study of the influence of the β-casein genotype of milk on the growth of mesophilic lactic acid lactococci was carried out by inoculating milk samples into tubes with sterilised skimmed milk immediately after the fermentation process and on the 3rd, 7th, 10th, and 14th days of storage, following the methodology for inoculation (DSTU 7357:2013, 2013).

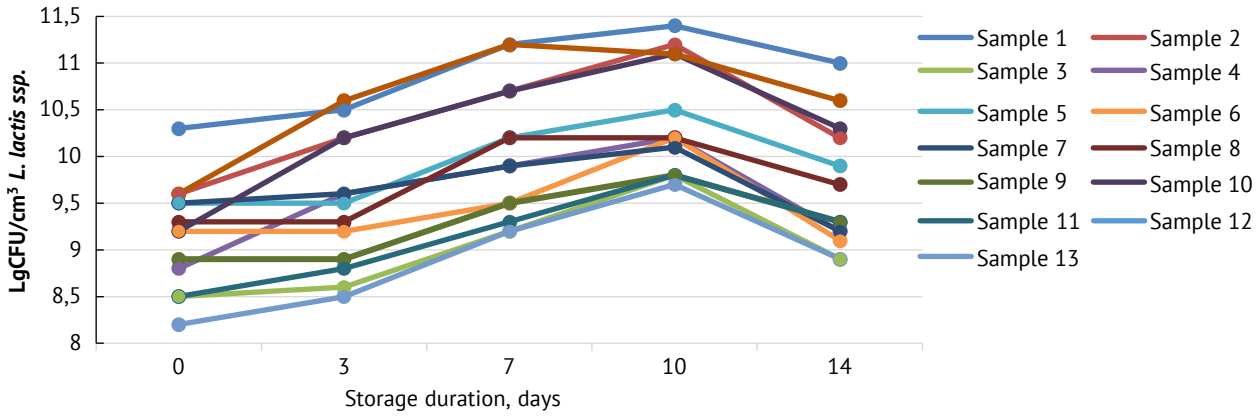

***Figure 3**. Changes in the number of viable lactococci during storage*
***Source:** compiled by the authors*

The organoleptic analysis during the storage of fermented curds involved monitoring changes in taste, smell, and texture of the samples. The duration and frequency of monitoring were similar to those of the aforementioned studies. No numerical scores were assigned. The taste of all samples changed, becoming more acidic and pronounced, corresponding relatively to the decrease in pH and increase in titratable acidity. Throughout storage, the smell of all samples remained lactic with no foreign odours. T The consistency of intact curds was firm with minor whey separation on the surface, occasionally leading to curd cracking and filling of the voids with whey. After stirring, the curds became homogeneous with satisfactory viscosity, as detailed in Figure 2.

The research results indicate a normal growth pattern of the culture in the dairy curd. Figure 3 clearly shows a stable increase in the number of viable cells up to 10 days inclusive, and the results obtained from the inoculation on the 14th day indicate a slowing down of growth and a slight decrease in viable cells. This can be explained by the biochemical conversion of lactose, the formation of lactic acid, and changes in the concentration of hydrogen ions, which negatively affect the growth of lactic acid lactococci. Similar results have not been observed in other studies. Therefore, to provide a comprehensive view of the impact of β-casein genotype on the fermentation processes of dairy mixtures in the production of fermented milk products during storage, research was conducted to monitor changes in

the physicochemical, organoleptic, and microbiological properties of fermented dairy curds during storage.

CONCLUSIONS

Given the potential for increased production of A2 milk, there is a need to develop scientifically sound technologies for processing this milk into various dairy products. Fermentation is the basis for the production of most dairy products, therefore, research into the impact of β-casein genetic modifications on this process is relevant. Based on the results obtained through theoretical and experimental research, it can be concluded that A2 milk is suitable for the production of fermented dairy products using mesophilic lactic acid lactococci starter cultures, without significant changes to existing technological instructions and production processes. Milk with the A2A2 β-casein genotype can be fermented without any additional processing, and the resulting curds have high sensory qualities, while the physicochemical and microbiological parameters are within the norms of current regulatory documents for fermented dairy products.

Based on the theoretical and experimental research conducted, it was established that all sample groups of the milk studied were suitable for biotechnological processing using mixed cultures of mesophilic lactic acid bacteria. By the 10th hour of fermentation, the fermented samples from A2 milk had a titratable acidity within the range of 75-78°C and a pH of 4.6-4.53, which is within the normal range for fermentation using such a starter culture. Studies of changes in physicochemical, organoleptic, and microbiological parameters during storage confirmed the hypothesis that the β-casein genotype has a negligible impact on acid formation processes and the growth of microorganisms in fermented curds. The production of fermented dairy products from the studied milk using *L. Lactis ssp*. cultures can be carried out using the general technological scheme and do not require adjustment of the technological parameters.

Future research plans to investigate the impact of the genetic configuration of cow's milk proteins on the fermentation processes of milk mixtures using mixed cultures of lactic acid microorganisms and a range of probiotic cultures. The aim is to develop scientifically-based technologies for producing a wide range of hypoallergenic dairy products based on milk from cows with the A2A2 β-casein protein genotype.

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CONFLICT OF INTEREST

None.

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Дослідження впливу генотипу білка молока на процес ферментації молочних згустків мезофільними молочнокислими стрептококами

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Анотація. Актуальність роботи полягає в тому, що переважна більшість товарного молока, що переробляється на кисломолочні продукти є змішаним за генотипом β-казеїну, а поширення використання молока А2 в молокопереробній галузі потребує розробки науково-обґрунтованих технологій виробництва ферментованих продуктів, тому що модифікації генотипу казеїну можуть впливати на хід технологічних процесів та потребують коректування технологічних параметрів виробництва. Метою проведення дослідження було встановлення впливу генетичної модифікації β-казеїну білка молока корів на процес ферментації молочних сумішей під час виробництва кисломолочних ферментованих молочних продуктів. Методи дослідження – хімічні, фізичні, органолептичні, мікробіологічні (визначення якісних показників молока-сировини та ферментованих молочних згустків), технологічні (визначення активності кислотоутворення, в'язкості). Було досліджено вихідні показники молока-сировини відповідно до генотипу – прямої залежності не встановлено. Було досліджено зміни фізико-хімічних, мікробіологічних та органолептичних показників ферментованих молочних згустків в процесі біотехнологічного оброблення та протягом зберігання. Було обрано оптимальні режими технологічного процесу та обрано технологічні прийоми для мінімізації впливу другорядних чинників на хід експерименту. В якості закваски було обрано препарат змішаних культур молочнокислих мезофільних лактококів, до якого входили культури *L. Lactis ssp*. Встановлено, що всі 13 зразків молока придатні для біотехнологічної обробки змішаними культурами мезофільних молочнокислих бактерій. В результаті було узагальнено, виробництво кисломолочних продуктів з молока-сировини з представленими генними модифікаціями β-казеїну, A1A1, A1A2 та A2A2 з використанням культур *L. lactis ssp*, можливе за класичною технологією і не потребує коригування технологічних умов. Отримані результати роботи можна використовувати в молокопереробній промисловості при розробці технологій кисломолочних продуктів з молока-сировини А2

Ключові слова: молоко А2; технологічні властивості; біотехнологічна обробка; закваска; кисломолочні продукти