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Effect of pasteurisation on physicochemical and microbiological properties of milk

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Abstract. Study was to determine the effect of pasteurisation regimes on the quality and microbiological safety of milk. Five independent production batches of milk from the "Vereya" and "Bozhentsy" brands were examined. The samples were pasteurised at 63°C for 30 minutes, 72°C for 15 seconds and 85°C for 10 seconds, after which their parameters were determined immediately after treatment, and on the third and seventh days of refrigerated storage. Raw milk of the "Vereya" brand was characterised by higher protein and dry matter content and greater viscosity, whilst "Bozhentsy" milk had higher titratable acidity and initial microbial count. All the treatment regimes studied reduced alkaline phosphatase activity to below the established limit, and *Salmonella spp., Listeria monocytogenes, Escherichia coli* and coagulase-positive staphylococci were not detected in the pasteurised samples. The logarithmic reduction in the total number of microorganisms was 3.40, 3.91 and 4.33 $\log_{10}$, respectively, for the treatment regimes of 63°C for 30 minutes, 72°C for 15 seconds and 85°C for 10 seconds. Treatment at 85°C resulted in the lowest residual microbial count; however, this was accompanied by the retention of only 69.1% of soluble whey proteins, a colour difference of ΔE^* 2.50 and an increased aftertaste of boiled milk. The 72°C for 15 seconds treatment ensured the retention of 87.8% of soluble whey proteins, the highest sensory acceptability after seven days of storage, and a slower recovery of the microflora compared with the 63°C for 30 minutes treatment. The results obtained can be used by process engineers at Bulgarian dairy processing plants to select pasteurisation parameters, by quality control laboratories to compile testing programmes, and by producers of drinking milk to adjust production processes without compromising the microbiological safety or consumer properties of the products

Keywords: alkaline phosphatase; soluble serum proteins; sensory acceptability; mesophilic microorganisms; psychrotrophic microflora; colour difference; cold storage

Introduction

Research relevance is determined by the need to determine the optimal pasteurisation regime for milk, one that ensures its microbiological safety whilst minimising changes to its physicochemical and sensory properties. When assessing the stability of yoghurt enriched with 5% and 10% chia seeds, G. Nakov *et al.* (2024) monitored the physicochemical, microbiological and sensory changes over a 21-day storage period at a temperature of 4 ± 1°C. During this period, the pH of the control sample decreased by 0.17 units, whilst in the variants containing 5% and USD 10 chia, it decreased by 0.08 and 0.13 units, respectively; the number of lactic acid bacteria decreased from 9.005 to 8.775 log CFU/g in the control and from 8.495 to 8.150 log CFU/g in the sample with a 10% additive. The protein composition and biologically active components of donkey milk were systematised by N.N. Zheleva (2022), which focused primarily on lysozyme, lactoferrin and whey

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proteins, which are sensitive to the conditions of collection and processing. According to the summarised data, the average concentration of lysozyme was approximately 1.07 g/l, its activity ranged from 1,670 to 11,531 U/ml, and the lactoferrin content varied from approximately 0.0048 to 0.080 g/l. Changes in the composition of traditional sheep's cheese made from the milk of the Karakachan breed during ripening were studied by S. Stankov *et al.* (2023). The dry matter, total protein and fat contents increased by approximately 7, 3 and 4% respectively; the water content decreased by 7%; and on day 60%, the proportions of saturated and unsaturated fatty acids accounted for 66% and 34% of their total amount.

A comparison of traditional thermal and non-thermal methods of treating raw cow's milk, with equivalent microbiological efficacy, was conducted by J. Chen *et al.* (2024). The application of high hydrostatic pressure of 400 MPa for 5 minutes resulted in a loss of approximately 6% of IgG and 16.2% of lactoferrin, whilst at 600 MPa the corresponding losses reached 79.4% and 31.36% and following ultra-high-temperature treatment – 97.16% and 70.85%. In a study by T. Yu *et al.* (2022), the effects of high hydrostatic pressure of 600 MPa for 15 minutes, pasteurisation at 72°C for 15 seconds, and the sequential application of both treatments during 30 days' storage at 4°C were compared. The total count of aerobic bacteria after pressure treatment remained below 2.22 log CFU/ml, whereas in pasteurised and combined-treated milk it exceeded 5.00 log CFU/ml; however, the sensory evaluations of the samples after pressure treatment were lower. The effect of pasteurisation temperature and cold storage conditions on the growth of spore-forming bacteria was determined by T.T. Lott *et al.* (2023) for milk treated at 75, 85 or 90°C for 20 s. On day 14, the average microbial count was 1.82, 3.55 and 6.86 log CFU/ml at storage temperatures of 3, 6.5 and 10°C, respectively, whilst the calculated time to reach 10^6 CFU/ml was reduced from 68 to 27 and 10 days. The microbiota of 17 samples of commercial pasteurised milk with a shelf life of 1 to 15 days were analysed by J. Zhao *et al.* (2024). Proteobacteria, Firmicutes and Bacteroidetes accounted for 94.54 ± 4.22% of the bacterial community, whilst the Enterococcus faecium SFM2 strain isolated from milk retained 82.58 ± 0.01% of viable cells after two hours' incubation at 50°C, compared with 28.20 ± 0.04% for a strain of different origin. Changes in enzymatic activity and protein composition during the industrial stages of milk pasteurisation, whey production and its reheating during the manufacture of whey protein concentrate were assessed by J. Haas *et al.* (2025). Following the first heating, xanthine oxidase activity decreased by approximately 15%; in unpasteurised whey, 25-45% of the initial activity was retained, whereas after pasteurisation, only 6-12% remained; at the same time, the concentrations of lactoferrin, IgA and IgM decreased.

The effects of thermal pasteurisation and sterilisation on the microbiological safety, protein profile, vitamin content and sensory properties of milk, as summarised by A. Rabbani *et al.* (2025). According to the data cited by the authors, following high-temperature short-time pasteurisation, 59-76% of immunoglobulin activity and 70-90% of lactoperoxidase activity were retained, whilst heating at 80°C for 15 s resulted in the retention of over 75% of lysozyme activity. The practical impact of cold chain disruptions on the stability of pasteurised milk was examined by M. Al-Farsi *et al.* (2021) based on the results of a survey of 281 retail outlets and 12-day storage of products from two manufacturers at temperatures of 5 and 8°C. The temperature exceeded 5°C in 50.2% of the outlets surveyed, whilst the total microbial count ranged from 3,960 to 8,680 CFU/ml in the products of the first manufacturer and from 15 to 48 CFU/ml in those of the second; the safe storage period at 5°C was determined to be nine days. The analysed studies demonstrated that changes in milk depend on the composition of the raw material, the processing regime, residual microflora and storage conditions. However, these studies did not comprehensively compare treatment regimes of 63°C for 30 minutes, 72°C for 15 seconds and 85°C for 10 seconds on independent batches of milk from two Bulgarian manufacturers, whilst simultaneously assessing the physicochemical, microbiological and sensory parameters over a seven-day storage period.

Study aimed to determine the effect of three pasteurisation temperature-time regimes on the physicochemical, microbiological and sensory properties of milk from the "Vereya" and "Bozhentsy" brands, and to identify the most balanced treatment regime. To achieve this aim, the following objectives were set: to compare the initial characteristics of the milk from both brands; to assess changes in its quality and microbiological safety following pasteurisation at 63°C for 30 minutes, 72°C for 15 seconds and 85°C for 10 seconds, and during seven days' cold storage; to determine the processing regime that ensured adequate microbiological safety whilst minimising changes in the physicochemical and sensory properties.

Materials and Methods

A laboratory-based experimental study was conducted in June 2026 at the Department of Milk and Dairy Products Technology and the University Laboratory of the University of Food Technologies, Plovdiv, Bulgaria. Microbiological tests were conducted at the accredited food testing laboratory LB Bulgaricum n.d., registration number of the Bulgarian Accreditation Service (n.d.) No. 138 LI. Samples for microbiological analysis were collected under aseptic conditions into separate sterile, airtight containers and labelled with the brand code, batch number, pasteurisation conditions and test date. They were transported to the laboratory in thermal

containers with ice packs at a temperature of 2-6°C, which was continuously monitored by a Testo 174 T electronic data logger. The duration of transport did not exceed four hours, and microbiological analysis commenced immediately upon the samples' arrival at the laboratory. Samples found to have compromised seals, incorrect labelling or deviations from the required temperature range were excluded from analysis.

The study was based on a factorial design with repeated measurements and covered two factors, both between and within production batches: the brand of milk and the pasteurisation temperature-time profile. For parameters determined during storage, the time of analysis was an additional factor. A production batch of milk was considered to be an independent experimental unit. Each batch was divided between an untreated control and three pasteurisation regimes; therefore, the variants C, P1, P2 and P3, formed from a single batch, were treated as paired observations. Parallel laboratory measurements were not considered as separate independent observations.

The study addressed standardised cow milk from two Bulgarian brands. Milk from the "Vereya" (n.d.) brand, which forms part of the United Milk Company's portfolio, was coded as TM-A. Milk from the "Bozhentsi" brand, produced by ELVI Ltd. (n.d.), was coded as TM-B. Milk with a target fat content of 2.5% was obtained from each manufacturer; it was sampled from the production line after clarification, separation, standardisation and homogenisation, but before industrial heat treatment. Using milk before the pasteurisation stage assessed the direct effect of the treatment regimes under investigation, rather than the consequences of reheating the finished pasteurised or ultra-pasteurised product. Five independent production batches, manufactured on different days, were sampled from each manufacturer. The total sample comprised ten batches: A1-A5 for TM-A and B1-B5 for TM-B. The interval between the sampling of the first and last batches from a single manufacturer did not exceed 30 days. From each batch, 10.5 dm^3 of milk was sampled, so the total volume of the test material was 105 dm^3. Of this volume, 1.20 dm^3 was used for the untreated control, 2.79 dm^3 each for each of the three pasteurisation regimes, and 0.93 dm^3 was retained as a reserve. This volume ensured that physico-chemical, microbiological and sensory analyses could be conducted in separate sealed containers at each stage of the analysis.

The milk was sampled directly from the process tank following standardisation and homogenisation. Before sampling, the contents of the tank were stirred following production regulations. Clean, airtight containers made of food-grade polymer were used for physico-chemical analyses, whilst pre-sterilised containers were used for microbiological tests. Each sample was labelled with the brand code, production batch number, and the date and time of sampling. After sampling, the samples were placed in thermal containers with ice packs. The temperature during transport was maintained within the range of 2-6°C and monitored using a Testo 174 T electronic data logger (Testo SE & Co. KGaA, Germany). The duration of transport did not exceed four hours. Before pasteurisation, the milk was stored at a temperature of 4 ± 1°C for no longer than 12 hours. Before a batch was included in the experiment, its appearance, colour, odour, homogeneity and the absence of flakes, sediment and mechanical impurities were assessed. The presence of antibacterial and other inhibitory substances was tested using the Delvotest T test system. Batches with a positive or inconclusive result were excluded from the experiment.

After gentle mixing, each batch was divided into four variants. The control variant K was not subjected to heat treatment and was used to determine the initial physicochemical properties and initial microbial contamination. Variant P1 was pasteurised at 63 ± 0.5°C for 30 minutes. Variant P2 was treated at 72 ± 0.5°C for 15 seconds. Variant P3 was subjected to intensive heat treatment at 85 ± 0.5°C for 10 seconds. The treatment regimes of 63°C for 30 minutes and 72°C for 15 seconds corresponded to the minimum temperature-time combinations for extended low-temperature and short-duration high-temperature pasteurisation, as established by European Union legislation (Regulation (EC) No. 853/2004 of the European Parliament and of the Council, 2004). The 85°C for 10 s treatment was used as an experimental intensified treatment to assess the additional reduction in residual microflora and heat-induced changes in the protein system, colour, viscosity and sensory profile. A total of 40 treatment variants were established: ten production batches, each of which was divided between the control and three pasteurisation regimes. These 40 variants were not considered completely independent, as four variants derived from a single batch shared a common origin.

The milk was pasteurised in a Julabo CORIO CD-B13 circulating thermostatic water bath (JULABO GmbH, Germany). For each treatment, 31 sterile, thin-walled glass containers with a capacity of 100 cm^3 were used. Each vessel was filled with 90 cm^3 of milk and sealed with a sterile lid. Thirty vessels were used for laboratory and sensory studies, whilst one identically filled vessel was used for temperature control. A type T thermocouple, connected to a Testo 176 T4 data logger, was placed at the geometric centre of the control vessel. Once the treatment was complete, the containers were transferred to an ice-water bath and cooled to a temperature not exceeding 6°C for 15 minutes. The duration of cooling and the final temperature were recorded in the laboratory report. The containers were weighed before and after heating on a Radwag AS 220. R2 analytical balance (RADWAG Wagi Elektroniczne, Poland). If the weight loss exceeded 0.5%, the relevant sample was discarded, and pasteurisation was repeated

using a reserve batch. The order of batch processing and pasteurisation regimes was determined by computerised randomisation. After pasteurisation, the containers were coded with random three-digit numbers. The specialists conducting the laboratory and sensory analyses were not informed of the brand name or pasteurisation regime until the initial data processing had been completed.

The pasteurised samples were stored in a cold room at a temperature of 4 ± 1°C. The temperature was continuously monitored using a Testo 174 T data logger. A batch was excluded from the study if the temperature decreased outside the permissible range for more than one hour. Of the 30 analytical containers for each pasteurised variant, 10 were used at the initial time point D0, four on the third day (D3) and 10 on the seventh day (D7). The remaining six containers were allocated to parallel microbiological analyses and sensory sessions. At each time point, individual containers were opened; reopening or returning the sample to storage was not permitted. Physicochemical and microbiological analyses at time point D0 were conducted immediately after cooling. Time points D3 and D7 corresponded to the third and seventh days of refrigerated storage. The untreated control milk was analysed only before pasteurisation.

The physico-chemical analysis included the determination of free and titratable acidity, density, fat content, total protein, lactose, dry matter, non-fat milk solids, freezing point, dynamic viscosity, colour characteristics, alcohol stability, concentration of soluble whey proteins and residual alkaline phosphatase activity. Active acidity was determined potentiometrically at a temperature of 20 ± 2°C using a Hanna Instruments HI 2211 pH meter (Hanna Instruments, Romania). Before each series of measurements, the instrument was calibrated using buffer solutions with pH values of 4.01, 7.01 and 10.01. The reading was recorded after the electrode had stabilised and was reported to an accuracy of 0.01 pH units. Titratable acidity was determined by the neutralisation method. To 10 cm^3 of milk, 20 cm^3 of distilled water and three drops of a 1% phenolphthalein alcohol solution were added. The mixture was titrated with a sodium hydroxide solution of 0.1 mol/dm^3 until a pale pink colour appeared, which persisted for one minute. The result was expressed in Turner degrees. Density was determined at a temperature of 20 ± 2°C using an AMT thermolactodensimeter (a milk hydrometer with a built-in thermometer) with a range of 1,015-1,040 kg/m^3. The result was corrected to a temperature of 20°C.

The mass fractions of fat, total protein, lactose, dry matter, dry non-fat milk solids and the freezing point were determined using mid-infrared spectroscopy on a MilkoScan FT1 analyser (FOSS, Denmark). Calibration and quality control of the instrument were conducted following ISO 9622:2013|IDF 141:2013 (2013). The mass fraction of dry matter was additionally verified by the gravimetric method following ISO 6731:2010|IDF 21:2010 (2010). Dynamic viscosity was determined using a Brookfield DV2T rotational viscometer fitted with an LV-1 spindle at a temperature of 20 ISO ± 0.5°C and a rotation speed of 60 rpm. The same measurement parameters were used for all samples. The result was expressed in mPa·s.

Colour was measured using a Konica Minolta CR-400 colourimeter (Konica Minolta, Japan) in the CIELAB colour space. The L^*, a^* and b^* coordinates were recorded. The overall colour difference from the raw milk of the corresponding batch was calculated using the following formula (1):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}, \quad (1)$$

where ΔE^* – total colour difference between pasteurised and unprocessed milk; ΔL^* – difference between the lightness values of the test and control samples; Δa^* – difference in colour coordinates along the green-to-red axis; Δb^* – difference in colour coordinates along the blue-to-yellow axis. Values of ΔL^*, Δa^* and Δb^* were determined as the difference between the corresponding colour coordinates of the pasteurised sample and the raw milk from the same production batch.

Alcohol stability was assessed by mixing equal volumes of milk and ethyl alcohol at concentrations of 68%, 72%, 75% and 80%. The result was defined as the highest ethanol concentration at which no flakes, clots or marked coagulation were observed. The concentration of soluble serum proteins was determined after casein precipitation at pH 4.6. Samples were centrifuged for 15 minutes at 10,000 g and 4°C. The protein concentration in the supernatant was determined using the M.M. Bradford (1976) method at a wavelength of 595 nm, with bovine serum albumin used to establish the calibration curve. The degree of preservation of soluble serum proteins was calculated as the ratio of their concentration after pasteurisation to the concentration in the raw milk from the same batch, multiplied by 100%. Alkaline phosphatase activity was determined by a fluorometric method using the Fluorophos ALP Test System (Advanced Instruments, LLC, USA) based on ISO 11816-1:2024|IDF 155-1:2024 (2024). The result was expressed in mU/l. The test was considered negative if the enzyme activity did not exceed 350 mU/l, following Commission Implementing Regulation (EU) No. 2019/627 "Laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No. 2074/2005 as regards official controls (Text with EEA relevance.)" (2019).

Active and titrated acidity, viscosity and colour were determined at points D0, D3 and D7. Density, mass

fractions of fat, protein, lactose, dry matter and non-fat milk solids, freezing point and alkaline phosphatase activity were determined at D0. Each physicochemical determination was conducted in triplicate. For statistical analysis, the mean value of the replicates within a single production batch and under the same operating conditions was used.

For microbiological analysis, after mixing, 10 cm^3 of milk was transferred to 90 cm^3 of sterile peptone-saline solution and a series of tenfold dilutions was prepared. The number of mesophilic aerobic and facultative anaerobic microorganisms was determined by the deep-plate method on Plate Count Agar, incubated at 30 ± 1°C for 72 ± 3 hours following ISO 4833-1:2013 (2013). Psychrotrophic microorganisms were determined by surface plating on Plate Count Agar and incubated at 6.5 ± 1°C for ten days based on ISO 17410:2019 (2019). *Enterobacteriaceae* in raw milk were counted on Violet Red Bile Glucose Agar following ISO 21528-1:2017 (2017). In pasteurised samples, a detection method with pre-enrichment was used following ISO 21528-1:2017, as the expected number of microorganisms was less than 100 CFU/ml. β-glucuronidase-positive *Escherichia coli* were identified on Tryptone Bile X-glucuronide Agar at 44 ± 1°C following ISO 16649-2:2001 (2001). Typical blue-green colonies were subject to confirmation. Coagulase-positive staphylococci were identified on Baird-Parker agar with egg yolk and tellurite supplement following ISO 6888-1:2021 (2021). Typical colonies were confirmed by the coagulase test. Yeasts and moulds were identified on Dichloran Rose Bengal Chloramphenicol Agar at a temperature of 25 ± 1°C based on ISO 21527-1:2008 (2008). Yeasts were counted after three days, and moulds after five days of incubation.

Bacteria suspected of belonging to the *Bacillus cereus* group were identified on Mannitol Egg Yolk Polymyxin Agar based on ISO 7932:2004 (2004). Typical colonies were subjected to confirmatory tests. *Salmonella spp*. were identified in 25 cm^3 of milk following ISO 6579-1:2017 (2017). The method involved non-selective and selective enrichment, plating onto selective media and confirmation of typical colonies. *Listeria monocytogenes* was detected in 25 cm^3 of milk using a two-stage enrichment method and plating onto Agar Listeria according to Ottaviani and Agosti (ALOA) agar following ISO 11290-1:2017 (2017). The total count of mesophilic and psychrotrophic microorganisms was determined in the raw milk and in pasteurised samples at time points D0, D3 and D7. *Enterobacteriaceae*, E. coli, coagulase-positive staphylococci, yeasts, moulds and B. cereus were determined in raw milk and in pasteurised samples at D0 and D7. *Salmonella spp*. and *L. monocytogenes* were determined in the raw milk of each batch and in all pasteurised samples at D0. Each quantitative determination was conducted in at least two parallel dishes. The results were expressed in CFU/ml and converted to $\log_{10}$ CFU/ml before statistical analysis. The logarithmic reduction in the total number of microorganisms was calculated separately for each batch as the difference between the $\log_{10}$ of the number of microorganisms in the raw milk and the $\log_{10}$ of their number in the corresponding pasteurised variant at D0. Results below the limit of quantification were reported as "< 1.00 $\log_{10}$ CFU/ml". For the purposes of statistical analysis, such results were assigned a value corresponding to half the method's limit of quantification.

The effectiveness of pasteurisation was assessed based on a set of criteria: alkaline phosphatase activity not exceeding 350 mU/l, the absence of *Salmonella spp*. and *L. monocytogenes* in 25 ml, the absence of *E. coli* in the tested volume, and compliance with the process criterion for *Enterobacteriaceae*. Compliance with the *Enterobacteriaceae* criterion was assessed at time point D0 based on a design of n = 5, c = 2, m < 1 CFU/ml and M = 5 CFU/ml, as established for pasteurised milk at the end of the production process (Commission Regulation (EC) No. 2073/2005, 2005). The D7 results were used to characterise microbiological stability during storage, rather than to assess compliance with the process criterion at the end of pasteurisation.

Sensory evaluation was conducted for variants P1, P2 and P3. The tasters were not served any unprocessed milk. The panel comprised 12 selected and trained assessors aged between 21 and 55. Selection and training were conducted based on ISO 22935-1:2023|IDF 99-1:2023 (2023), whilst the procedure for presenting and evaluating samples was conducted following ISO 22935-2:2023|IDF 99-2:2023 (2023). The study was conducted based on the principles of voluntariness, confidentiality and personal data protection stipulated in the ICC/Esomar International Code on Market, Opinion and Social Research and Data Analytics (2025) and the European Commission (2021) Guidelines on Ethics and Data Protection. Individuals with cow's milk protein allergy, lactose intolerance, acute respiratory diseases or impairments of smell and taste were excluded from participation.

The initial sensory evaluation was conducted following the laboratory acceptance of the samples. In the table of sensory results, this baseline was designated as D0, although it referred to the initial evaluation following confirmation of safety, and not necessarily the calendar day of pasteurisation. The second session was conducted at point D7. Samples were served in white, opaque glasses, coded with random three-digit numbers. The portion volume was 30 cm^3, and the serving temperature was 17 ± 2°C. The order of presentation was randomised using a balanced scheme. During a single session, each assessor analysed no more than six samples. Between samples, participants rinsed their mouths with still water and took a break of at least one minute.

The intensity of the milky flavour, the aftertaste of boiled milk and the sulphurous note were assessed, as

well as the uniformity of texture and overall acceptability. The intensity of the sensory characteristics was rated on a scale of 0 to 10 points: 0 – characteristic absent; 1 – barely perceptible; 2 – very faint; 3 – faint; 4 – moderately faint; 5 – moderate; 6 – moderately pronounced; 7 – pronounced; 8 – strong; 9 – very strong; 10 – maximally pronounced. Consistency and overall acceptability were assessed on a nine-point scale: 1 – extremely unacceptable; 2 – very unacceptable; 3 – unacceptable; 4 – somewhat unacceptable; 5 – neutral; 6 – somewhat acceptable; 7 – acceptable; 8 – very acceptable; 9 – highly acceptable.

The temperature-time regime was determined in two stages. In the first stage, any regime that did not result in a negative reaction to alkaline phosphatase, the absence of the pathogenic microorganisms under investigation, or compliance with the *Enterobacteriaceae* criterion at D0 was excluded from further comparison. In the second stage, amongst the regimes that met the safety criteria, the following were compared: the logarithmic reduction in microflora, the total microbial count at D7, the preservation of soluble serum proteins, the colour difference ΔE^*, the change in viscosity, alcohol resistance and overall sensory acceptability. The most balanced treatment was considered to be the one that ensured a lower microbial count and a slower recovery of microflora compared with the least intensive treatment, but caused fewer changes to the protein system, colour, viscosity and sensory profile compared with the most intensive heating treatment.

For each production batch, laboratory replicates were averaged to a single value. Physicochemical parameters were reported as the arithmetic mean and standard deviation. Microbiological results were analysed after conversion to $\log_{10}$ CFU/ml. The initial characteristics of milk from TM-A and TM-B were compared using Welch's t-test. Parameters determined only at time point D0 were analysed using a two-factor mixed-effects analysis of variance model, in which the brand, pasteurisation regime and their interaction were treated as fixed effects, whilst the production batch within the brand was treated as a random effect. For parameters determined at time points D0, D3 and D7, a linear mixed-effects model was applied, which included brand, pasteurisation regime, storage duration and their interaction as fixed effects, and the production batch as a random effect. Sensory parameters were analysed using a mixed-effects model, in which the brand, pasteurisation regime and evaluation period were fixed effects, whilst the evaluator and production batch were random effects. The critical level of statistical significance was $p < 0.05$. Statistical analysis was conducted using R version 4.5.1, with the lme4 and lmerTest packages.

Before each series of tests, the equipment was checked and calibrated based on manufacturer's instructions. For physico-chemical analyses, certified buffer solutions, standard milk samples, calibration solutions and blank samples were used. In each series of microbiological tests, the sterility of culture media, solvents, pipettes and laboratory glassware was verified. The reference strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella Typhimurium* ATCC 14028, *Listeria monocytogenes* ATCC 19115 and *Bacillus cereus* ATCC 11778 were used as positive control cultures. A separate protocol was maintained for each batch, recording the brand code, batch number, date and time of sampling, transport temperature, initial milk temperature, heating time, actual exposure temperature, holding time, cooling time, final temperature, mass before and after treatment, and the date of each analysis.

Results

Physicochemical properties of milk following pasteurisation and cold storage

Before heat treatment, initial differences were observed between the two brands of milk in terms of acidity, protein content, dry matter, non-fat milk solids and viscosity. The mass fractions of fat and lactose, as well as the density and freezing point, did not differ statistically between the samples under investigation (Table 1).

Table 1. *Initial physico-chemical parameters of milk from the brands under investigation*

Indicator	Vereya (TM-A)	Bozhentsy (TM-B)	p
Active acidity, pH	6.70 ± 0.02	6.68 ± 0.02	0.041
Titratable acidity, °T	16.3 ± 0.4	16.8 ± 0.4	0.018
Density, kg/m³	1,028.8 ± 0.3	1,029.1 ± 0.4	0.184
Fat content by mass, %	2.53 ± 0.04	2.49 ± 0.05	0.176
Protein content by mass, %	3.25 ± 0.05	3.18 ± 0.04	0.013
Mass fraction of lactose, %	4.72 ± 0.06	4.67 ± 0.05	0.062
Mass fraction of dry matter, %	11.56 ± 0.10	11.41 ± 0.12	0.029
Dry skimmed milk solids, %	9.03 ± 0.07	8.92 ± 0.09	0.021
Freezing point, °C	-0.530 ± 0.003	-0.528 ± 0.004	0.354
Dynamic viscosity, mPa·s	1.83 ± 0.04	1.76 ± 0.05	0.027

Note: *M ± SD are provided for five independent production batches*

Source: *compiled by the author based on ISO 6731:2010\|IDF 21:2010 (2010), ISO 9622:2013\|IDF 141:2013 (2013)*

The higher total protein content in TM-A milk was associated with a higher mass fraction of dry matter and non-fat milk solids. This combination of differences corresponded to a higher initial viscosity of this milk. The difference in the mass fraction of fat did not reach statistical significance; therefore, the differences in rheological properties were not related to the fat phase. TM-B milk was characterised by a higher titratable acidity – 16.8 ± 0.4°T compared with 16.3 ± 0.4°T in TM-A ($p = 0.018$), as well as a lower value for active acidity – pH 6.68 ± 0.02 compared with 6.70 ± 0.02 in TM-A ($p = 0.041$). The observed difference was not accompanied by signs of instability or protein coagulation. The alcohol test confirmed the stability of all initial batches against a 72% ethyl alcohol solution. Coagulation at an ethanol concentration of 75% was observed in two batches of TM-B and one batch of TM-A. Similar values for density, freezing point and lactose mass fraction indicated a comparable degree of milk standardisation and the absence of signs of water addition. The differences identified between the brands mainly concerned the protein and dry matter components and were addressed during the subsequent comparison of the milk's response to heat treatment. The temperature-time regime had a statistically significant effect on active and titratable acidity, viscosity, colour, the concentration of soluble whey proteins and the residual activity of alkaline phosphatase. The mass fractions of fat, total protein, lactose, dry matter and non-fat milk solids did not change significantly after pasteurisation (Table 2).

Table 2. *Physico-chemical properties of milk immediately after heat treatment*

Trademark	Mode	pH	Acidity, °T	Viscosity, mPa×s	ΔE^*	Retention of soluble serum proteins, %	Alkaline phosphatase, mU/l
TM-A	K	6.70 ± 0.02	16.3 ± 0.4	1.83 ± 0.04	0	100.0	> 10,000
	P1	6.68 ± 0.02	16.6 ± 0.4	1.86 ± 0.04	0.72 ± 0.18	92.8 ± 1.9	118 ± 21
	P2	6.67 ± 0.02	16.7 ± 0.3	1.87 ± 0.05	1.08 ± 0.20	88.7 ± 2.2	47 ± 13
	P3	6.63 ± 0.03	17.2 ± 0.4	1.94 ± 0.05	2.41 ± 0.27	70.3 ± 2.8	12 ± 5
TM-B	K	6.68 ± 0.02	16.8 ± 0.4	1.76 ± 0.05	0	100.0	> 10,000
	P1	6.66 ± 0.02	17.1 ± 0.4	1.80 ± 0.04	0.79 ± 0.16	91.3 ± 2.1	132 ± 25
	P2	6.65 ± 0.02	17.2 ± 0.3	1.81 ± 0.04	1.15 ± 0.19	86.9 ± 2.4	54 ± 15
	P3	6.61 ± 0.03	17.7 ± 0.4	1.89 ± 0.05	2.58 ± 0.31	67.8 ± 3.0	15 ± 6

Note: *K – raw milk; P1 – pasteurisation at 63°C for 30 minutes; P2 – at 72°C for 15 seconds; P3 – at 85°C for 10 seconds. Modes P1 and P2 correspond to the minimum temperature-time combinations for pasteurisation laid down in Regulation (EC) No. 853/2004 of the European Parliament and of the Council "Laying down specific hygiene rules for food of animal origin" (2004). ΔE^* is calculated using formula (1) in relation to the raw milk of the relevant production batch*

Source: *compiled by the author based on Regulation (EC) No. 853/2004 of the European Parliament and of the Council (2004), Commission Implementing Regulation (EU) No. 2019/627 (2019), ISO 11816-1:2024|IDF 155-1:2024 (2024)*

Following regimens P1 and P2, changes in pH and titratable acidity remained limited. Regime P3 was accompanied by a greater decrease in pH and an increase in titratable acidity in both brands. The interaction between brand and regime for these parameters was not statistically significant, indicating a similar response in the milk from the two manufacturers. The increase in viscosity depended on the processing temperature. After P1, the viscosity of TM-A milk increased from 1.83 ± 0.04 to 1.86 ± 0.04 mPa·s, i.e., by 1.6%, whilst that of TM-B milk increased from 1.76 ± 0.05 to 1.80 ± 0.04 mPa·s, or by 2.3%. After P2, the values were 1.87 ± 0.05 and 1.81 ± 0.04 mPa·s, respectively, which exceeded the initial vaues by 2.2% and 2.8%. After P3, the viscosity increased by approximately 6% relative to the initial level. This change was consistent with a reduction in the proportion of soluble whey proteins and the formation of additional protein interactions in the milk system. The retention of the soluble whey protein fraction after P1 exceeded 90% in the milk of both brands. After P2, the proportion of undenatured proteins decreased to approximately 87–89%. The P3 regime caused a reduction in this figure by almost a third. The difference between P3 and the other two regimes was statistically significant at $p < 0.001$.

Colour changes following P1 remained below one ΔE^* unit, whilst those following P2 approached one unit. Following P3, the total colour difference exceeded 2.4 units for both brands. The change was primarily due to a decrease in lightness (L^*) and an increase in the b^* coordinate, which corresponded to an intensification of the yellow hue. The residual alkaline phosphatase activity after all treatment regimes was below the threshold level of 350 mU/l. The highest values were observed after P1, although no batch exceeded the established limit. After P2 and P3, enzyme activity decreased further. Consequently, all the treatment regimes studied ensured an adequate pasteurisation effect, and regimes P1 and P2 met the minimum temperature-time combinations stipulated by European Union legislation (Regulation (EC) No. 853/2004 of the European Parliament and of the Council, 2004). The mass fraction of fat after heat treatment varied within the range of 0.01-0.03 percentage points, that of total protein within the range of 0.01-0.04 percentage points, and that of lactose by no more than 0.03 percentage points. No statistically significant differences were found between the control and pasteurised samples for these parameters. The freezing point also remained stable. After P1 and P2, all samples

remained homogeneous in a 75% ethanol solution. After P3, coagulation at this concentration was observed in three batches of TM-A and four batches of TM-B. At an ethanol concentration of 72%, all samples remained stable. Reduction in alcohol tolerance following P3 corresponded to a more pronounced restructuring of the protein-colloid system. Over the course of seven days of cold storage, all pasteurised samples showed a decrease in pH, an increase in titratable acidity and a gradual increase in viscosity. The extent of these changes depended on the brand, the pasteurisation method and the duration of storage (Table 3).

Table 3. *Changes in acidity and viscosity of pasteurised milk during storage*

Trademark	Mode	pH, D0/D3/D7	Acidity, °T, D0/D3/D7	Viscosity, mPa×s, D0/D3/D7
TM-A	P1	6.68 ± 0.009/6.64 ± 0.010/6.58 ± 0.011	16.6 ± 0.18/17.2 ± 0.20/18.1 ± 0.22	1.86 ± 0.018/1.90 ± 0.020/1.97 ± 0.022
	P2	6.67 ± 0.009/6.64 ± 0.010/6.60 ± 0.011	16.7 ± 0.13/17.1 ± 0.14/17.8 ± 0.16	1.87 ± 0.022/1.90 ± 0.024/1.95 ± 0.026
	P3	6.63 ± 0.013/6.61 ± 0.014/6.57 ± 0.016	17.2 ± 0.18/17.6 ± 0.20/18.4 ± 0.22	1.94 ± 0.022/1.98 ± 0.024/2.05 ± 0.026
TM-B	P1	6.66 ± 0.009/6.61 ± 0.010/6.53 ± 0.011	17.1 ± 0.18/17.9 ± 0.20/19.0 ± 0.22	1.80 ± 0.018/1.86 ± 0.020/1.95 ± 0.022
	P2	6.65 ± 0.009/6.62 ± 0.010/6.56 ± 0.011	17.2 ± 0.13/17.8 ± 0.14/18.6 ± 0.16	1.81 ± 0.018/1.85 ± 0.020/1.92 ± 0.022
	P3	6.61 ± 0.013/6.59 ± 0.014/6.54 ± 0.016	17.7 ± 0.18/18.1 ± 0.20/18.9 ± 0.22	1.89 ± 0.022/1.94 ± 0.024/2.01 ± 0.026

Note: *TMan values and approximate standard errors of the mean are given. D0 – immediately after pasteurisation; D3 – the third day; D7 – the seventh day of refrigerated storage. P1 – pasteurisation at 63°C for 30 minutes; P2 – at 72°C for 15 seconds; P3 – at 85°C for 10 seconds*

Source: *compiled by the author*

The greatest change in active acidity during storage was observed following storage regime P1. In TM-B milk, the decrease in pH was more pronounced than in the corresponding TM-A variant. The interaction between brand, storage regime and storage duration were statistically significant at p = 0.038. Following P2, acidity changed more slowly than following P1. On the seventh day, the pH value remained higher, whilst the titratable acidity was lower compared with low-temperature extended pasteurisation. This trend corresponded to a less intense recovery of residual microflora. The initial acidity values after P3 were higher than after P1 and P2. The subsequent increase during storage was of a smaller relative magnitude; however, the absolute level of titratable acidity on the seventh day remained elevated due to changes that occurred directly during heating. After seven days of cold storage, changes in colour characteristics, alcohol stability and the degree of preservation of soluble whey proteins in samples of pasteurised milk were analysed (Table 4).

Table 4. *Colour characteristics, alcohol stability and preservation of soluble serum proteins after seven days of refrigerated storage*

Trademark	Mode	L*, M ± SE	a*, M ± SE	b*, M ± SE	ΔE*, M ± SE	Alcohol tolerance, % ethanol	Retention of soluble serum proteins, %
TM-A	П1	86.4 ± 0.3	-0.62 ± 0.02	9.1 ± 0.2	1.42	74 ± 1.0	96.8 ± 0.4
	П2	85.7 ± 0.4	-0.58 ± 0.03	9.6 ± 0.3	1.71	72 ± 1.2	95.9 ± 0.5
	П3	83.9 ± 0.5	-0.51 ± 0.03	10.4 ± 0.3	3.01	68 ± 1.3	93.7 ± 0.6
TM-B	П1	87.1 ± 0.3	-0.65 ± 0.02	8.8 ± 0.2	1.54	75 ± 1.0	97.2 ± 0.4
	П2	86.2 ± 0.4	-0.60 ± 0.03	9.4 ± 0.3	1.89	73 ± 1.1	96.1 ± 0.5
	П3	84.10 ± 0.5	-0.52 ± 0.03	10.6 ± 0.3	3.24	69 ± 1.2	94.0 ± 0.6

Note: *Parameters determined on the seventh day of cold storage are presented. ΔE* was calculated using formula (1) relative to the untreated milk from the corresponding production batch. Alcohol stability is expressed as the highest concentration of ethanol at which no coagulation of the milk was observed*

Source: *compiled by the author*

Viscosity increased in all experimental treatments. Following P1, the increase was mainly attributable to changes during storage, whereas following P3, a significant proportion of the increase occurred immediately after pasteurisation. The P2 treatment was characterised by the smallest overall change in viscosity between the initial state and the seventh day. Colour characteristics also changed during storage. On the seventh day, the total colour difference after P1 was 1.42 units for TM-A and 1.54 units for TM-B. After P2, the corresponding values reached 1.71 and 1.89 units. After P3, the values rose to 3.01 and 3.24 units. The increase in ΔE* during storage was mainly due to a decrease in lightness and a subsequent shift of the b* coordinate in the positive direction. The proportion of soluble whey proteins on the seventh day decreased further by 1.8-2.7 percentage points after P1 and P2, and by 3.6-4.1 percentage points after P3. The decrease following storage was less pronounced than the changes resulting directly from pasteurisation but confirmed the ongoing restructuring of the protein system.

Microbiological safety, sensory properties and a comprehensive assessment of pasteurisation methods

Before pasteurisation, TM-B milk was characterised by a higher total count of mesophilic and psychrotrophic microorganisms. *Salmonella spp.* and *Listeria monocytogenes* were not detected in any of the source batches (Table 5). The average count of mesophilic aerobic and facultative anaerobic microorganisms in raw milk was 5.42 $\log_{10}$ CFU/cm^3 for TM-A and 5.61 $\log_{10}$ CFU/cm^3 for TM-B. The average count of psychrotrophic microorganisms was 4.11 and 4.29 $\log_{10}$ CFU/cm^3, respectively. The difference between the brands was statistically significant at $p < 0.05$. *Enterobacteriaceae* in the raw milk were detected at levels of 2.72 $\log_{10}$ CFU/cm^3 in TM-A and 2.91 $\log_{10}$ CFU/cm^3 in TM-B. Low numbers of β-glucuronidase-positive E. coli were detected in two batches of TM-A and three batches of TM-B. Coagulase-positive staphylococci were detected in all batches, but their numbers did not exceed 2.1 $\log_{10}$ CFU/cm^3. Presumptive *Bacillus cereus* group bacteria were detected at levels of 1.47-1.58 $\log_{10}$ CFU/cm^3. All pasteurisation regimes resulted in a significant reduction in the total number of vegetative microflorae. The extent of the reduction increased with rising treatment temperature.

Table 5. *Changes in the mesophilic and psychrotrophic microflora of pasteurised milk*

Trademark	Mode	Logarithmic reduction following pasteurisation, M±SE	Mesophilic microorganisms, $\log_{10}$ CFU/cm^3, D0/D3/D7, M±SE	Psychrotrophic microorganisms, $\log_{10}$ CFU/cm^3, D0/D3/D7, M±SE
TM-A	P1	3.39 ± 0.08	2.03 ± 0.07/2.81 ± 0.09/4.08 ± 0.12	< 1.00/1.92 ± 0.08/3.11 ± 0.11
	P2	3.88 ± 0.07	1.54 ± 0.06/2.34 ± 0.08/3.49 ± 0.10	< 1.00/1.56 ± 0.07/2.66 ± 0.09
	P3	4.30 ± 0.06	1.12 ± 0.05/1.78 ± ± 0.07/2.86 ± 0.09	< 1.00/1.18 ± 0.06/2.05 ± 0.08
TM-B	P1	3.40 ± 0.09	2.21 ± 0.08/3.04 ± 0.10/4.37 ± 0.13	< 1.00/2.16 ± 0.09/3.39 ± 0.12
	P2	3.93 ± 0.08	1.68 ± 0.07/2.51 ± 0.09/3.71 ± 0.11	< 1.00/1.73 ± 0.08/2.85 ± 0.10
	P3	4.36 ± 0.07	1.25 ± 0.06/1.94 ± 0.08/3.02 ± 0.10	< 1.00/1.30 ± 0.07/2.17 ± 0.09

Note: *a value of < 1.00 corresponded to a quantity below the limit of quantification. Note. D0 – immediately after pasteurisation; D3 – the third day; D7 – the seventh day of refrigerated storage. Value of < 1.00 corresponds to a microorganism count below the limit of quantification*

Source: *compiled by the author based on ISO 16649-2:2001 (2001), ISO 7932:2004 (2004), ISO 4833-1:2013 (2013), ISO 21528-1:2017 (2017), ISO 21528-2:2017 (2017), ISO 6579-1:2017 (2017), ISO 17410:2019 (2019), ISO 6888-1:2021 (2021), Bulgarian Accreditation Service (n.d.), LB Bulgaricum (n.d.)*

After P1, the total number of mesophilic microorganisms decreased by an average of 3.40 logarithmic units. After P2, the reduction was close to four logarithmic units, and after P3 it exceeded 4.3 logarithmic units. The effect of the treatment regime was statistically significant at $p < 0.001$. Immediately after pasteurisation, psychrotrophic microorganisms were detected below the limit of quantification in all variants. By the third day, their growth had resumed in all groups; however, the rate of accumulation depended on the treatment regime. The fastest increase was observed after P1, and the slowest after P3. On the seventh day, the difference between P1 and P2 in terms of the total number of mesophilic microorganisms was approximately 0.6-0.7 logarithmic units. The difference between P2 and P3 was smaller. The observed trends indicated that short-term pasteurisation at 72°C resulted in a lower residual microbial count and a slower rate of its recovery than the 63°C treatment for 30 minutes.

TM-B milk was characterised by higher rates of microbial regrowth during storage, regardless of the storage conditions. On the seventh day, the difference between the brands was statistically significant for the total number of mesophilic microorganisms and psychrotrophic microflora. An interaction between brand and storage conditions was observed for the total microbial count at $p = 0.041$. Immediately after pasteurisation at time point D0, *Enterobacteriaceae*, E. coli and coagulase-positive staphylococci were not detected in any of the samples analysed. In each group, all five production batches had an *Enterobacteriaceae* count below 1 CFU/cm^3. Consequently, the number of samples with values between m and M was c = 0, and with values above M was 0. Consequently, all pasteurisation regimes met the procedural criterion for *Enterobacteriaceae*: n=5, c = 2, m < 1 CFU/cm^3, M = 5 CFU/cm^3, as stipulated in Commission Regulation (EC) No. 2073/2005 "On microbiological criteria for foodstuffs (Text with EEA relevance)" (2005). On the seventh day, *Enterobacteriaceae* remained below the limit of quantification in all variants of P2 and P3. Following P1, isolated colonies were detected in two batches of TM-B; however, the average count did not exceed 10 CFU/cm^3. Yeast and mould were not detected after pasteurisation or were below the limit of quantification. On the seventh day, low numbers were detected mainly after P1. After P2, the count remained lower, and after P3, no colonies formed in most batches.

The number of presumptive *Bacillus cereus* bacteria decreased to a lesser extent after pasteurisation than the number of non-spore-forming microflora. After P1, the count was 1.22-1.34 $\log_{10}$ CFU/cm^3; after P2, 1.18-1.31 $\log_{10}$ CFU/cm^3; and after P3, 1.15-1.27 $\log_{10}$ CFU/cm^3. The limited difference between the treatment regimes corresponded to the survival of heat-resistant spores. On the seventh day, the

number of *Bacillus cereus* increased slightly, but did not exceed 1.7 $\log_{10}$ CFU/cm^3. The increase was greatest after P1. The result confirmed that the increase in pasteurisation temperature mainly affected vegetative cells, whilst the spore fraction was preserved under all conditions. *Salmonella spp.* and *Listeria monocytogenes* were not detected in any of the pasteurised samples. The absence of pathogenic microorganisms, E. coli and coagulase-positive staphylococci, was consistent with a negative reaction to alkaline phosphatase. The sensory profile depended on the intensity of the heat treatment and the duration of storage. Modes P1 and P2 retained the distinct milky flavour and aroma, whilst after P3 there was an increase in the aftertaste of boiled milk, a sulphurous note and changes in consistency (Table 6).

Table 6. *Sensory characteristics of milk after pasteurisation and storage*

Trademark	Mode	Milk taste, D0/D7	aftertaste of boiled milk, D0/D7	Consistency, D0/D7	Overall perception, D0/D7
TM-A	П1	8.3/7.4	1.3/1.7	8.2/7.5	8.4/7.2
	П2	8.4/7.9	1.6/1.9	8.3/7.9	8.5/7.8
	П3	7.4/7.0	4.1/4.5	7.8/7.4	7.3/7.1
TM-B	П1	8.0/7.0	1.4/1.9	7.9/7.1	8.0/6.8
	П2	8.2/7.6	1.8/2.1	8.0/7.6	8.2/7.5
	П3	7.1/6.7	4.4/4.8	7.5/7.0	7.0/6.8

Note: *D0 – initial sensory session conducted following the completion of laboratory testing and confirmation of the safety of the pasteurised samples; the designation D0 in this table does not necessarily correspond to the calendar day of pasteurisation. D7 – sensory evaluation after seven days of refrigerated storage. P1 – pasteurisation at 63°C for 30 minutes; P2 – at 72°C for 15 seconds; P3 – at 85°C for 10 seconds*

Source: *compiled by the author based on ISO 22935-1:2023|IDF 99-1:2023 (2023), ISO 22935-2:2023|IDF 99-2:2023 (2023)*

Immediately after processing, the highest overall acceptability was recorded for treatment P2. The difference between P1 and P2 was not statistically significant at the initial stage but became significant after seven days of storage. The decline in acceptability of P1 was associated with a reduction in the purity of the aroma, a weakening of the milky flavour and a change in consistency. Regime P3 received lower ratings as early as the day of pasteurisation. The main difference was an increase in the intensity of the boiled milk aftertaste. In addition, a sulphurous note was detected, with an average intensity of 2.7 points for TM-A and 3.0 points for TM-B. After P1 and P2, this indicator did not exceed 1.2 points. The overall acceptability of TM-A milk was higher than that of TM-B, although the difference depended on the storage conditions and duration. After P2, the difference between the brands was smaller than after P1. The interaction between brand, storage conditions and duration were statistically significant at $p = 0.044$. After seven days, the greatest decrease in overall acceptability was observed after P1. The deterioration in the sensory profile coincided with a more rapid increase in mesophilic and psychrotrophic microflora and an increase in titratable acidity. After P3, the overall rating changed less, but remained lower due to thermal characteristics formed during heating. Mode P2 ensured the greatest stability of sensory characteristics throughout storage. On the seventh day, the milky flavour remained pronounced, and the intensity of the boiled milk aftertaste did not exceed 2.1 points. The consistency remained uniform at a level of over 7.5 points. иАn integrated comparison was conducted among treatment regimes that met the microbiological safety criteria and were characterised by a negative reaction to alkaline phosphatase. The main criteria were the logarithmic reduction in microflora, its recovery during storage, the preservation of soluble serum proteins, colour change and overall sensory acceptability (Table 7).

Table 7. *Summary performance indicators for temperature-time regimes*

Indicator	Q1 63°C, 30 min	Q2 72°C, 15 s	Q3 85°C, 10 s
Average logarithmic reduction in microflora	3.40	3.91	4.33
Total count of microorganisms on D7, $\log_{10}$ CFU/cm^3	4.23	3.60	2.94
Retention of soluble serum proteins, %	92.1	87.8	69.1
Colour difference ΔE* at D0	0.76	1.12	2.50
Overall acceptability at D0, points	8.2	8.4	7.2
Overall acceptability at D7, points	7.0	7.7	7.0
Alkaline phosphatase activity	Below 350 mU/l	Below 350 mU/l	Below 350 mU/l

Source: *compiled by the author*

Mode P1 ensured the standard pasteurisation effect and the greatest preservation of the soluble whey protein fraction. At the same time, the most intense recovery of mesophilic and psychrotrophic microflora was observed during the seven days following P1. This process was accompanied by a more rapid decrease in

pH, an increase in titratable acidity and a reduction in sensory acceptability. Regime P3 resulted in the greatest logarithmic reduction in the total number of microorganisms and the lowest microbial count on the seventh day. At the same time, following this regime, the greatest loss of soluble whey proteins, an increase in viscosity, a reduction in alcohol resistance, a marked change in colour and the development of a thermal off-flavour were observed. Regime P2 occupied an intermediate position in terms of microbiological efficacy, but outperformed P1 in terms of stability during cold storage. The difference between P2 and P3 in terms of residual microbial count was statistically significant; however, both regimes ensured the absence of indicator and pathogenic microorganisms. Following Mode P2, the retention of soluble whey proteins remained higher than after Mode P3, whilst changes in colour, viscosity and sensory profile were less pronounced. On the seventh day, Mode P2 was characterised by the highest overall acceptability among the modes studied.

Mixed-effects models confirmed a significant effect of the pasteurisation regime on alkaline phosphatase activity, soluble serum protein concentration, viscosity, colour difference, total microbial count and sensory acceptability at $p < 0.001$. The effect of brand was significant for the initial protein content, dry matter, viscosity, initial microbial contamination and changes in acidity during storage. No statistically significant interaction between brand and storage conditions was found for the mass fraction of fat, protein, lactose, dry matter and alkaline phosphatase activity. For the microbial count on the seventh day, the rate of pH change and overall sensory acceptability, such an interaction was significant. The observed relationship indicated varying intensities of post-pasteurisation changes in the milk of the two brands under identical storage conditions. Based on the combined microbiological, physico-chemical and sensory parameters, the 72°C for 15 s treatment ensured compliance with safety criteria, a lower rate of recovery of residual microflora compared with the 63°C for 30 minutes, and a lesser degree of changes in the protein system and consumer properties compared with the 85°C for 10 seconds treatment. This treatment was identified as the temperature-time combination with the smallest overall deviation in quality parameters among the variants that ensured adequate microbiological efficacy.

Discussion

Absence of statistically significant changes in the mass fraction of fat, total protein, lactose, dry matter and non-fat milk solids following pasteurisation indicated that the main compositional characteristics of milk were preserved under the tested conditions. A similar pattern was observed by S. Lalwani *et al.* (2024), who, when comparing high-temperature short-time pasteurisation, enhanced pasteurisation, extended shelf-life treatment and ultra-high-temperature heating, found limited losses of macronutrients and total mineral content. At the same time, the authors recorded a reduction in the concentrations of vitamins B_1, B_2 and B_{12} and ionised calcium following enhanced pasteurisation, suggesting the possibility of changes in components not covered by the study.

Fact that the total mass fraction of protein remained unchanged did not imply the absence of structural changes in the protein system. The retention of soluble serum proteins was 92.1% after treatment at 63°C for 30 minutes, 87.8% after treatment at 72°C for 15 seconds, and 69.1% after treatment at 85°C for 10 seconds. Thus, raising the temperature to 85°C did not result in a quantitative loss of total protein, but led to the transition of a portion of the serum proteins from a soluble state to denatured and aggregated forms. The observed simultaneous increase in viscosity and decrease in alcohol resistance following the 85°C treatment confirmed a restructuring of the protein-colloidal system, rather than a change in total protein concentration. The results were consistent with the findings of S. Lalwani *et al.* (2024), who observed an increase in the size of casein micelles following industrial heat treatment of milk, although they did not detect a significant effect of the treatment regimes studied on its overall physical stability. The discrepancy between these data and the reduction in alcohol resistance observed in the samples studied may have been due to the use of the intensive treatment regime at 85°C for 10 s, the initial composition of the milk, and the sensitivity of the alcohol test to changes in the salt and protein balance.

Observed reduction in the proportion of soluble serum proteins does not provide grounds for directly concluding that there is a corresponding decrease in the nutritional value or digestibility of milk. According to a review by M. Fatih *et al.* (2021), heat treatment altered the structure of milk proteins and the nature of their digestion in model systems; however, studies involving adults were few in number, varied in terms of treatment regimens, and were associated with a moderate or high risk of systematic error. Some studies reported changes in post-consumption amino acid kinetics and dietary nitrogen retention, but no generalised long-term physiological effect was established. Therefore, in presented study, the preservation of soluble serum proteins should be regarded as an indicator of the intensity of the technological impact and the structural nativeness of the protein fraction, rather than as a direct indicator of the product's digestibility.

The change in colour following the 85°C treatment was greater than that observed after the other two pasteurisation treatments. The overall colour difference immediately after treatment averaged 2.50 units, whereas after treatments at 63 and 72°C, the values were 0.76 and 1.12 units respectively. The intensification

of the yellow hue and the reduction in lightness may have been associated with thermal denaturation of proteins, changes in the dispersibility of the colloidal phase, and the initial stages of the Maillard reaction. H. Bi *et al.* (2023) demonstrated that the intensity of the Maillard reaction in lactose-free milk depended on both pasteurisation and the temperature of subsequent storage. In this study, furozin, 5-hydroxymethylfurfural, intrinsic fluorescence and colour were monitored, and the degree of lactose hydrolysis reached 97.08%. When comparing these results, it was necessary to address that the hydrolysis of lactose produced glucose and galactose, which had a higher reactivity than the original lactose. Therefore, the colour change in standardised milk following the 85°C treatment indicated an intensification of thermal transformations, but did not confirm the formation of specific Maillard reaction products without their chromatographic determination.

The validity of this caveat was confirmed by Y. Li *et al.* (2021), who quantified furozin, furfurols and advanced glycation end-products following low-temperature prolonged, high-temperature short-time, ultra-high-temperature and in-vessel sterilisation of milk. The authors established a relationship between the accumulation of early, intermediate and final Maillard reaction products and the intensity of the heat treatment. During seven days' storage, colour difference values continued to increase, particularly following intensive pasteurisation. A similar effect of the combination of time and temperature was observed by C. Tyl *et al.* (2024) for standard and low-lactose ultra-high-temperature milk, which was stored at 4, 20 and 30°C for 30-90 days. The most pronounced darkening was observed in low-lactose milk stored at 30°C, with the sensory panel being able to distinguish between fresh and stored samples as early as 30 days. Although the duration and temperature in this study did not correspond to the conditions of the experiment conducted, the results confirmed that thermal changes did not cease once the product had left the pasteuriser and could continue during storage.

R. Ding *et al.* (2020), in a study of milk pasteurised at temperatures ranging from 72 to 85°C with varying exposure times, identified six dominant types and 18 genera of bacteria. The proportion of Pseudomonas exceeded 42% in all samples, and six genera of microorganisms showed statistically significant correlations with amino acids, fatty acids and volatile compounds in milk. These findings explained why an increase in the total microbial count in the samples studied was accompanied by changes in odour, taste and acidity: the residual microflora could influence not only safety but also the development of sensory defects.

A limited reduction in the number of presumptive *Bacillus cereus* group bacteria following an increase in temperature confirmed the heat resistance of the spore fraction. K. Medjahdi *et al.* (2025) identified aerobic spore-forming bacteria as one of the main causes of premature spoilage of pasteurised milk. Spores could enter the milk at the farm, survive pasteurisation, persist in the production environment, germinate under refrigeration conditions and produce proteolytic and lipolytic enzymes. Therefore, increasing the pasteurisation temperature from 72 to 85°C did not eliminate the need to monitor the quality of raw materials, the sanitary condition of equipment and post-pasteurisation contamination.

Significance of initial quality of the raw material was confirmed by differences between the brands. Milk from TM-B had a higher initial level of microbial contamination and was characterised by more intense recovery of mesophilic and psychrotrophic microflora on the seventh day. F. Enayaty-Ahangar *et al.* (2021) demonstrated that the optimal strategy for extending shelf life depended on the production volume of the enterprise and the quality of raw milk obtained from different suppliers. The models proposed by the authors addressed simultaneous application of incentives for farms with low spore counts and the introduction of spore-removal technologies at the processing plant. Consequently, the same pasteurisation regime did not guarantee the same shelf life for milk with different initial levels and compositions of contamination.

The risk of spores re-entering from the production environment was also not eliminated by increasing the pasteurisation temperature. C. Pretorius & E.M. Buys (2021) found that simulated on-site decontamination had a significant effect on over 98% of *Bacillus cereus* spores, but around 0.1% remained intact. The survival of a small proportion of viable spores was of technological significance due to the possibility of their subsequent attachment, germination and the formation of sources of recontamination. In the laboratory experiment conducted, secondary contamination was minimised using sealed containers; therefore, the actual dynamics of the microflora on an industrial production line might additionally depend on the effectiveness of washing, disinfection and the aseptic conditions of the areas downstream of the pasteuriser. The identified relationship between temperature, protein changes and the sensory profile was consistent with the findings of R. Shi *et al.* (2025), according to which traditional high-temperature methods increased microbiological stability but could cause losses in natural flavour and changes in nutritional components. The authors considered new thermal and non-thermal technologies as ways of reducing the severity of such changes; however, their application required a separate technological and economic justification for each specific product.

Results of cheese studies also confirmed that the sensory effects of pasteurisation depend on the product's structure. D. Tadjine *et al.* (2020) investigated the effect of pasteurising cow's and goat's milk on the yield and physicochemical characteristics of fresh cheese. A. Rezaei *et al.* (2020) established a statistically significant effect of pasteurisation and ripening

temperature on acidity, dry matter, fat, protein, proteolysis, lipolysis and the sensory properties of traditional Motal cheese. The direct applicability of these findings to drinking milk was limited due to the concentration of solids, enzymatic processes and the prolonged ripening of cheeses; however, both studies confirmed that the initial milk matrix determined the product's response to heat treatment. The dependence of the results on product composition was also observed in the study by K. Pudtikajorn *et al.* (2021), in which pasteurised cow's milk was enriched with nanoliposomes containing fat from tuna eyes and evaluated over ten days for physical, chemical, microbiological and sensory parameters. The presence of an additional lipid phase altered the stability and perception of the product; therefore, the results obtained for standardised milk without enriching components cannot automatically be extrapolated to functional milk drinks.

S.F. Tan *et al.* (2020) found that cow's and goat's milk retained their microbiological suitability for 22 days at 8°C following high-pressure treatment. In pasteurised goat's milk at the end of storage, the number of psychrotrophic bacteria reached 9.0×10^8 CFU/ml, whilst the total microbial count was 3.5×10^8 CFU/ml. Compared with the data obtained, this confirmed that a reduction in residual psychrotrophic microflora could extend the shelf life without the need for a treatment regime that caused significant denaturation of serum proteins. G. Liu *et al.* (2020) compared high hydrostatic pressure of 600 MPa for 5 minutes, combined with microfiltration, against traditional treatment regimes of 63°C for 30 minutes and 72°C for 15 seconds. The evaluation covered microbiological inactivation, volatile compounds, protein denaturation, simulated gastric digestion and sensory properties after eight days of storage. This comprehensive approach was consistent with the rationale of the study, as the processing regime was evaluated not based on a single safety indicator, but based on a combination of microbiological and consumer characteristics. The combination of short-duration pasteurisation with high pressure was investigated by H. Gong *et al.* (2024). Treating donkey's milk at 75°C for 15 s, followed by the application of a pressure of 300 MPa, extended the shelf life by 85.71%, preserved lysozyme, α-lactalbumin and β-lactoglobulin, and reduced the total plate count, coliforms and Staphylococcus aureus. Above 400 MPa, physical stability deteriorated; this, similar to the 85°C condition in presented study, indicated the existence of a limit to the intensity of the treatment, outside of which any additional microbiological efficacy was accompanied by undesirable structural changes.

The potential of this combined approach was also confirmed by the findings of C.F. Balthazar *et al.* (2022). Pasteurisation of sheep's milk using omic and conventional heating resulted in a reduction in microflora of at least 4.2 logarithmic units, but omic heating required 72-73% less energy. Over the course of 15 days' storage, the composition of the bacterial community changed: in raw milk, Staphylococcus initially predominated, whilst Pseudomonas predominated at the end of storage. This confirmed the need to assess not only the initial reduction in microorganisms but also the structure of the residual microbiota during storage. A.W.M. Alsaedi *et al.* (2023) proposed non-thermal treatment of milk using a moderate electric field at temperatures below 42°C. Under optimal parameters of 29.8 V/cm and a flow rate of 0.018 kg/s, energy consumption was reduced by 98.58%, specific energy consumption by 98.51%, and the duration of the process by 99.09% compared with conventional pasteurisation. These results demonstrated the potential for reducing the thermal load; however, industrial application required confirmation of microbiological safety for different batches of raw materials, an assessment of electrochemical changes, and verification of stability during storage.

J.A. Ansari *et al.* (2020) considered the use of ultraviolet treatment of already pasteurised skimmed milk as an additional control measure against psychrotrophic spore-forming bacteria. This approach did not replace primary pasteurisation, but could be used as a barrier technology to reduce residual microflora. A limitation of UV treatment of milk remained its optical opacity; therefore, its effectiveness depended on the reactor design, layer thickness, agitation and the dose delivered. The approach that came closest to practical implementation of the results was the combination of pasteurisation with microfiltration. S. Gammoh *et al.* (2026), following the implementation of appropriate hygiene and production practices and a hazard analysis system, used membranes with pore sizes of 0.8 and 0.45 μm. The refrigerated storage life of whole milk was approximately two weeks, and that of milk with a fat content of 2% was approximately three weeks; denaturation of α-lactalbumin and β-lactoglobulin remained negligible. These results supported the advisability of maintaining a 72°C treatment for 15 s with a further reduction in bacterial load, rather than raising the temperature to a level at which the proportion of soluble whey proteins decreased to 69.1%.

Thus, the results of the comparison confirmed that the optimal pasteurisation regime should be determined not only by the level of microbial inactivation, but also by considering the preservation of the protein fraction, the physico-chemical properties and the sensory acceptability of the product. The most balanced regime was found to be 72°C for 15 s, which ensured sufficient microbiological safety, a negative alkaline phosphatase reaction, the preservation of 87.8% of soluble whey proteins and the highest acceptability on the seventh day of storage. This demonstrates the advisability of applying the concept of minimum sufficient heat treatment, whilst further extension of the shelf life

should be achieved primarily through quality control of raw materials, prevention of post-pasteurisation contamination, and the combination of pasteurisation with additional barrier technologies. At the same time, the findings require industrial validation, considering the seasonality of raw materials, cold chain conditions, the characteristics of processing equipment and a broader range of quality indicators.

Conclusions

Study found that the temperature-time profile of pasteurisation determined the nature of changes in the physicochemical, microbiological and sensory characteristics of milk from the "Vereya" and "Bozhentsy" brands. The initial samples differed in terms of protein content, dry matter, viscosity, titratable acidity and initial microbial contamination; however, the direction of the changes following heat treatment was similar for both brands. Pasteurisation at 63°C for 30 minutes resulted in a reduction in the total number of microorganisms by an average of 3.40 logarithmic units and the preservation of 92.1% of soluble whey proteins. At the same time, during seven days of refrigerated storage following this treatment, the most intense recovery of mesophilic and psychrotrophic microflora, an increase in titratable acidity and a decrease in sensory acceptability were recorded. The 85°C treatment for 10 s resulted in the greatest microbiological reduction, amounting to 4.33 logarithmic units, and the lowest residual microbial count on the seventh day. At the same time, it was found that only 69.1% of soluble whey proteins were retained, the colour difference increased to 2.50 units, viscosity increased, alcoholresistance decreased, and the aftertaste of boiled milk became more pronounced.

Pasteurisation at 72°C for 15 seconds resulted in a reduction in microflora of 3.91 logarithmic units, the retention of 87.8% of soluble whey proteins, a negative reaction to alkaline phosphatase, and the absence of the pathogenic and indicator microorganisms under investigation. Under this treatment regime, the highest sensory acceptability was recorded after seven days of storage, along with smaller changes in colour, viscosity and acidity compared with the more intense heating treatment. Consequently, the 72°C for 15 seconds treatment was identified as the most balanced in terms of the trade-off between safety and the preservation of consumer properties. The limitations of the study were related to the use of products from only two manufacturers, five batches of each brand and a seven-day observation period. Further studies should cover a larger number of manufacturers, seasonal milk batches, different fat contents, an extended analysis of vitamins and volatile compounds, as well as the simulation of industrial pasteurisation and packaging conditions.

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Вплив пастеризації на фізико-хімічні та мікробіологічні властивості молока

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Анотація. Дослідження мало на меті визначити вплив режимів пастеризації на якість та мікробіологічну безпеку молока. Було досліджено п'ять незалежних виробничих партій молока торгових марок «Верея» та «Боженці». Зразки пастеризували при температурі 63°C протягом 30 хвилин, 72°C протягом 15 секунд та 85°C протягом 10 секунд, після чого їх параметри визначали одразу після обробки, а також на третій та сьомий дні зберігання в холодильнику. Сире молоко марки «Верея» характеризувалося вищим вмістом білка та сухої речовини, а також більшою в'язкістю, тоді як молоко «Боженці» мало вищу титрувану кислотність та початкову мікробну кількість. Усі досліджені режими обробки знизили активність лужної фосфатази нижче встановленої межі, а *Salmonella spp., Listeria monocytogenes, Escherichia coli* та коагулазопозитивні стафілококи у пастеризованих зразках не були виявлені. Логарифмічне зменшення загальної кількості мікроорганізмів становило 3,40, 3,91 та 4,33 $\log_{10}$ відповідно для режимів обробки при температурі 63°C протягом 30 хвилин, 72°C протягом 15 секунд та 85°C протягом 10 секунд. Обробка при 85°C призвела до найнижчої залишкової кількості мікробів; однак це супроводжувалося збереженням лише 69,1 % розчинних білків сироватки, різницею в кольорі ΔE^* 2,50 та посиленням післясмаку кип'яченого молока. Обробка при 72°C протягом 15 секунд забезпечила збереження 87,8 % розчинних білків сироватки, найвищу сенсорну прийнятність після семи днів зберігання та повільніше відновлення мікрофлори порівняно з обробкою при 63°C протягом 30 хвилин. Отримані результати можуть бути використані інженерами-технологами на болгарських молочних заводах для вибору параметрів пастеризації, лабораторіями контролю якості для складання програм випробувань, а також виробниками питного молока для коригування виробничих процесів без шкоди для мікробіологічної безпеки чи споживчих властивостей продукції

Ключові слова: лужна фосфатаза; розчинні білки сироватки крові; сенсорна прийнятність; мезофільні мікроорганізми; психротрофна мікрофлора; різниця в кольорі; зберігання в холоді