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INFLUENCE OF DIFFERENT MILK-CLOTTING ENZYMES ON THE QUALITY AND SHELF LIFE OF SEMIHARD CHEESES

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KEY WORDS:
semihard cheese, milk-clotting enzymes, chymosin, pepsin, microbial coagulant, proteolysis, bitter taste, rheology, microstructure

ABSTRACT

The study examined semi-hard cheeses made with milk-clotting enzymes (MCEs) of animal origin (Naturen Extra with a mass fraction of chymosin 95%, "Bovine Pepsin" with a mass fraction of chymosin 10%), microbial origin (Fromase 750 XLG) and recombinant origin (Chy-max Extra and Chy-max Supreme), at an introduction dose of MCE of 1,500 to 6,000 IMCU per 100 kg of milk. In cheeses at the age of 7, 60, and 150 days, pH, the degree of proteolysis, the content of peptides with a mass of 1–5 kDa, and the compressive stress at fracture were determined. Cheeses produced with a dose of MCE of 5,000–6,000 IMCU/100 kg of milk had a substantially ($p < 0.05$) lower pH compared to cheeses made with doses of MCEs of 1,500–3,000 IMCU/100 kg of milk. At the same time points, there were no significant differences ($p > 0.05$) in the degree of proteolysis between cheeses made using diverse types of MCEs at the same dose. The exceptions were cheeses produced with Chy-max Supreme, which had a substantially ($p < 0.05$) lower level of proteolysis. Sensory assessment of the bitter taste intensity in cheeses is proportional to the content of peptides with a molecular weight of 1–5 kDa. With an increase in the MCE dose, the content of peptides with a molecular weight of 1 to 5 kDa increases in cheese. Chy-max Supreme forms the least number of bitter peptides in cheeses ($p < 0.05$). There are no significant differences ($p < 0.05$) in the magnitude of the compressive stress at fracture between the cheese variants produced with the same MCE doses of diverse types at the same time points.

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ВЛИЯНИЕ РАЗЛИЧНЫХ МОЛОКОСВЕРТЫВАЮЩИХ ФЕРМЕНТОВ НА КАЧЕСТВО И СРОКИ ХРАНЕНИЯ ПОЛУТВЕРДЫХ СЫРОВ

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КЛЮЧЕВЫЕ СЛОВА:
полутвердые сыры, молокосвертывающие ферменты, химозин, пепсин, микробный коагулянт, протеолиз, горький вкус, реология, микроструктура

АННОТАЦИЯ

Исследовали полутвердые сыры изготовленные с молокосвертывающими ферментами (МФ) животного происхождения (Naturen Extra с массовой долей химозина 95%, «Говяжий пепсин» с массовой долей химозина 10%), микробного происхождения (Fromase 750 XLG) и рекомбинантного происхождения (Chy-max Extra и Chy-max Supreme), при дозе внесения МФ от 1500 до 6000 IMCU на 100 кг молока. В сырах в возрасте 7, 60 и 150 сут определяли pH, степень протеолиза, содержание пептидов с массой 1–5 кДа, напряжение сжатия при разрушении. Сыры, изготовленные с дозой МФ 5000–6000 IMCU/100 кг молока, имели достоверно ($p < 0,05$) более низкий уровень pH в сравнении с сырами, произведёнными с дозами МФ 1500–3000 IMCU/100 кг молока. В одинаковые моменты времени, отсутствовали достоверные отличия ($p > 0,05$) по степени протеолиза между сырами, изготовленными с использованием МФ разного типа в одинаковой дозе. Исключением были сыры, изготовленные с МФ Chy-max Supreme, в которые имели достоверно ($p < 0,05$) более низкий уровень протеолиза. Сенсорная оценка интенсивности горького вкуса в сырах пропорциональна содержанию в них пептидов с молекулярной массой 1–5 кДа. При повышении дозы внесения МФ, в сырах повышается содержание пептидов с молекулярной массой от 1 до 5 кДа. МФ Chy-max Supreme образует в сырах наименьшее количество горьких пептидов ($p < 0,05$). Отсутствуют достоверные отличия ($p < 0,05$) по величине напряжение сжатия при разрушении между вариантами сыров выработанных с одинаковыми дозами МФ разных типов в одинаковые моменты времени.

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1. Introduction

Semi-hard cheeses are quite expensive, and keeping their quality for a long time is a critical issue. The reasons limiting the shelf life of semi-hard cheeses include proteolysis of cheese mass proteins.

Milk-clotting enzyme (MCE) is used as an essential component in the production of semi-hard cheeses. Part of the MCE added to milk passes into the composition of the cheese curd, where it retains its activity throughout the entire period of cheese maturation and storage [1,2]. MCE penetrates casein micelles and slowly hydrolyzes casein molecules into large peptides [3]. Under the action of proteolysis, the casein net, which makes up the power frame of the cheese, weakens, and the texture of the cheese softens. The cleavage of polypeptide bonds is an important part of the ripening process of semi-hard cheeses, leading to the transformation of the cheese consistency from a solid, hard, and slightly cohesive one typical of young cheeses to a cohesive, plastic, and homogeneous texture typical of mature cheeses [4]. As a result of proteolysis, peptides are released, which serve as predictors for the formation of flavoring substances because of biochemical reactions in cheeses [5]. However, an excessively high degree of proteolysis leads to the formation of an undesirable texture for cheese: viscous, sticking to the knife when cutting. Also, during deep hydrolysis of caseins, peptides with a bitter taste are formed [6–8].

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Excessively fast proteolysis leads to rapid overripening of the cheese and limits the shelf life of the cheese [9]. Finding a balance between cheese flavor development and long shelf life of cheese is of great interest. Considering the key role of MCE in the cheese maturation, the selection of MCE with certain properties can be one of the methods for creating a technology for cheese with a pronounced taste and a long shelf life. Currently, the cheesemaker has access to numerous industrially produced brands of MCEs of different origin (animal, microbial and recombinant types), which have different properties [10–12]. The purpose of this study is to evaluate the quality (taste and texture) of semi-hard cheeses made using MCEs from different origins.

2. Materials and methods

2.1. Materials

For the purposes of test manufacture, milk was used from the single supplier-manufacturer — AgriVolga, LLC (Yaroslavl region, Burmasovo village). In the cheese production, a lactic acid starter based on the BK-Uglich-No. 4 bacterial concentrate (the Federal State Budgetary Scientific Institution "Experimental Biofactory", Russia) was used; it consisted of a set of cultures of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, and was preliminary activated in sterilized milk. Milk-clotting enzyme preparations of various origins (animal, microbial and recombinant) and with various levels of milk-clotting and proteolytic activity were used for milk coagulation:

- Chy-max® Supreme 1000 (recombinant camel chymosin with a modified amino acid sequence; nominal milk-clotting activity — 1,000 IMCU/g; proteolytic activity — 0.28 units of PA /g; manufacturer — Chr Hansen A/S, Denmark);
- Chy-max® Extra 600 Liquid (recombinant calf chymosin of genetic variant "B"; nominal milk-clotting activity — 600 IMCU/g; proteolytic activity — 0.48 units of PA/g; manufacturer — Chr Hansen A/S, Denmark);
- Naturen® Extra 220 NB (enzymatic extract from the stomachs of calves with a mass fraction of chymosin of at least 95%; nominal milk-clotting activity — 220 IMCU/g; proteolytic activity — 0.95 units of PA/g; manufacturer — Chr Hansen A/S, Denmark);
- Fromase® 750 XLG (protease of the fungus *Rhizomucor miehei*; nominal milk-clotting activity — 750 IMCU/g; proteolytic activity — 56.91 units of PA/g; manufacturer — DSM Food Specialties, France);
- FS10 "Bovine pepsin" (enzymatic extract from the stomachs of adult cattle with a mass fraction of chymosin of at least 10%; nominal milk-clotting activity — 1,000 IMCU/g; proteolytic activity — 14.39 units of PA/g; manufacturer — Zavod endokrinnykh fermentov, LLC, Russia).

2.2. Methods

2.2.1. Methods for studying the properties of milk-clotting enzymes

The determination of the total milk-clotting activity was conducted according to GOST ISO 11815–2015[1].

The determination of the total proteolytic activity was conducted according (Anson method modification on haemoglobin) to GOST 34430–2018[2], as applied to weakly acidic proteases (at pH 5.3).

2.2.2 Cheese production process

Model semi-hard cheeses were made with a mass fraction of moisture of 42% (in fact, 41.87±0.94%), 32% of fat (in fact, 32.03±1.07%), 20% of protein (in fact, 19.96±0.53%). Our previous article [13] described the technological regulations for cheese production in detail. Briefly, the cheese production process was as follows: cheeses were made from normalized milk with a mass fraction of fat of 5% and protein of 3.2%, using a starter from mesophilic lactococci, processed at a cooking temperature of 40–41 °C without washing cheese curd with water. The resulting cheeses were salted by immersion in brine, dried, and packed in Amivak CH-B polymer film bags (Atlantis-Pak, Russia). The maturation of cheeses took place at a temperature of 11±1 °C for 60 days. Mature cheeses were stored at 4±2 °C for 90 days. Analysis of the composition and sensory properties of cheeses was performed at the beginning of the ripening period (7 days), at the end of the ripening period (60 days), and at the end of the storage period (150 days).

2.2.3. Methods for determining the composition and degree of proteolysis of cheeses

The active acidity of cheeses was determined on a pH-150MI pH meter (Izmeritelnaya Tekhnika, LLC, Russia) equipped with a FC200B combined pH electrode (Hanna Instruments Inc., USA) in a cheese suspension prepared by grinding 10 g of cheese with 10 cm^3 of deionized water.

The mass fraction of moisture in cheeses was determined by drying at a temperature of 102±2 °C according to the Russian state standard GOST 3626–73[3].

The mass fraction of fat in cheeses was determined by the acid method according to the Russian state standard GOST R55063–2012[4].

The mass fraction of total protein in cheeses was determined by the Kjeldahl method according to the Russian state standard GOST R54662–2011[5].

The degree of proteolysis in cheeses was evaluated (in percent) as the ratio of the content of water-soluble nitrogen to the content of total nitrogen in cheeses. Determination of the content of water-soluble nitrogen was conducted in aqueous extracts from cheeses obtained by the method of Kuchroo and Fox, in the modification described in [14].

The molecular weight distribution of soluble proteins in cheese was determined by high resolution gel filtration using an AKTA Pure 25 chromatographic system (Cytiva, Sweden) equipped with a Superose 12 10/300 GL column (GE Healthcare, Sweden). Eluent was aqueous solution of 0.05 M Na_2HPO_4 + 0.15 M NaCl (pH 6.50), eluent flow rate was 0.5 ml/min; detector wavelength was 280 nm. The column was calibrated according to the release time of protein substances with a known molecular weight: IgG (180 kDa), aldolase (158 kDa), BSA (69 kDa), ovoalbumin (43 kDa), β-Lg (36.0 kDa), α-La (14.4 kDa), cytochrome C (12.3 kDa), tryptophan (0.204 kDa). The calibration curve was based on a logarithmic regression model [15]. For analysis, aqueous extracts from cheeses, prepared to determine the mass fraction of soluble protein, and additionally filtered on cellulose acetate filters with a pore size of 0.45 μm, were used (Vladipor, Russia).

2.2.4 Rheology

The rheological indicators of cheeses were studied by the large strain uniaxial compression method using a structure meter «Instron 1000» (Instron Corp., USA) according to the method of Madadlou et al [16] with modifications. Cylindrical specimens 15 mm in diameter and 20 mm height were cut from the cheese block. The cheese samples were immediately placed in an air-tight container to prevent dehydration. The samples were kept for 4 hours to reach a temperature of 22±1 °C, at which the measurements were conducted. Uniaxial compression was conducted at a rate of 50 mm/min to a sample compression ratio of 60% (up to a sample height of 8 mm). As a result of measurements, a force-strain curve was obtained.

The compressive stress at fracture of the sample was calculated using the equation (1):

$$\sigma_f = \frac{F_f}{A}, \quad (1)$$

where σ_f is the compressive stress at fracture of the sample, Pa;
F_f is the force applied to the sample surface at the moment of fracture, N;
A is the cross-sectional area of the sample, m^2.

The compressive stress at fracture of the sample was calculated at the inflection point on the force-compression curve. The inflection suggests crumbliness resulting from body breakdown. The change in the cross-sectional area of the sample because of deformation was not considered.

2.2.5. Microstructure research

The microstructure of cheeses was studied by light microscopy in transmitted light, on microsections of cheeses 100±10 μm thick. Photographs were taken with a digital camera "Canon EOS600D". The photographs were corrected using the Digital Photo Professional software v.4.5 (Canon Inc.).

2.2.6. Design of the experiment and statistical data processing

The study was based on the design of a factorial experiment [17], which included two categorical factors: the "MCE type" factor and the "MCE dose" factor. The experiments were conducted in two replicates in a randomized order. Our previous article [13] provides the description of the experiment design.

To assess the joint influence of experimental factors and cheese shelf life on response variables (pH, degree of proteolysis and rheological parameters), the selected plan of the full factorial experiment was modified into a "split-plot design" [18] by introducing an additional factor "Storage duration" (Age).

Statistical processing of experimental data was conducted using the Statistica® *software package (ver. 5.5,* StatSoft, USA).

[1] GOST ISO 11815–2015 Milk. Determination of the total milk-clotting activity of bovine rennet. M.: Standartinform, 2015. — 10 p.

[2] GOST 34430–2018 Enzyme preparations for food industry. Method for the determination of proteolytic activity. M.: Standartinform, 2018. — 12 p.

[3] GOST 3626–73 Milk and milk products. Methods for determination of moisture and dry substances. M.: Standartinform, 2009. — 11 p.

[4] GOST R55063–2012 Kinds of cheese and processed cheese. The rules of tests acceptance, sampling, and control methods. M.: Standartinform, 2013. — 28 p.

[5] GOST R54662–2011 Cheeses and processed cheeses. Determination of protein mass fraction by the Kjeldahl method. M.: Standartinform, 2012. — 16 p.

Table 1. **Mean squares and probabilities (in parentheses), and R2 values for the ANOVA model for response variables (for MCEs of Naturen, Pepsin and Fromase at low and medium introduction doses)**

Таблица 1. **Средняя сумма квадратов отклонений, уровень статистической достоверности и коэффициенты детерминации модели ANOVA для переменных отклика (для МФ Naturen, Пепсин и Fromase при низких и средних дозах внесения)**

Factor	df	pH	Proteolysis degree, %	Fraction of bitter peptides (1–5 kDa), mV*s	Fracture stress, kPa
Type	2	0,0014 (–)	6,471 (***)	29362 (**)	358,4 (–)
Dose	1	0,0035 (–)	30,192 (***)	184876 (***)	381,0 (–)
Age	2	0,0698 (***)	255,206 (***)	1150686 (***)	31198,8 (***)
Type*Dose	2	0,0036 (–)	1,064 (–)	19109 (*)	182,7 (–)
Type*Age	4	0,0003 (–)	2,671 (**)	13669 (*)	85,6 (–)
Dose*Age	2	0,0031 (–)	1,406 (–)	17741 (*)	83,9 (–)
Error	28	0,0013	0,588	5081	113,8
R^2		0,85	0,97	0,95	0,95

df — number of degrees of freedom
Factor keys: Type — MCE type; Dose — MCE dose; Age — shelf life of the cheese.
Error — share of the response variable variation related to an error;
R^2 — coefficient of determination for the ANOVA model.
The level of statistical significance of the factor effect evaluation (in parentheses): "–" — not statistically significant ($p > 0.05$); "*" — $p < 0.05$; "**" — $p < 0.01$; "***" — $p < 0.001$.

Table 2. **Mean squares and probabilities (in parentheses), and R2 values for determination of the ANOVA model for response variables (for MCEs of Naturen, Chy-max Extra and Chy-max Supreme, at medium and high introduction doses)**

Таблица 2. **Средняя сумма квадратов отклонений, уровень статистической достоверности и коэффициенты детерминации модели ANOVA для переменных отклика (для МФ Naturen, Chy- max Extra и Chy-max Supreme, при средних и высоких дозах внесения)**

Factor	df	pH	Proteolysis degree, %	Fraction of bitter peptides (1–5 kDa), mV*s	Fracture stress, kPa
Type	2	0,0035 (–)	239,382 (***)	843696 (***)	535,9 (*)
Dose	1	0,0128 (**)	47,748 (***)	272727 (***)	690,4 (*)
Age	2	0,0939 (***)	232,521 (***)	804733 (***)	32776,2 (***)
Type*Dose	2	0,0015 (–)	5,309 (***)	42513 (**)	271,4 (–)
Type*Age	4	0,0002 (–)	27,314 (***)	133447 (***)	239,9 (–)
Dose*Age	2	0,0009 (–)	0,281 (–)	33013 (**)	5,3 (–)
Error	28	0,0011	0,458	5841	108,8
R^2		0,88	0,99	0,964	0,96

df — number of degrees of freedom.
Factor keys: Type — MCE type; Dose — MCE dose; Age — shelf life of the cheese.
Error — share of the response variable variation related to an error;
R^2 — coefficient of determination for the ANOVA model.
The level of statistical significance of the factor effect evaluation (in parentheses): "-" — not statistically significant ($p > 0.05$); "*" — $p < 0.05$; "**" — $p < 0.01$; "***" — $p < 0.001$.

3. Results and discussion

The influence of the experimental factors on the response variables was evaluated: pH, the degree of proteolysis, indicators of the molecular weight distribution of proteolysis products (the content of peptides with mass of 1 to 5 kDa) and rheological indicators (compressive stress at fracture) of test cheeses.

The experiment matrix held an incomplete set of combinations of factors "MCE type" and "MCE dose". No data were available for Fromase and "Bovine Pepsin" at high introduction dose (6,000 IMCU/100 kg milk) and data for Chy-max Extra and Chy-max Supreme were also missing at low introduction dose (1,500 IMCU/100 kg milk). As a result, it was impossible to process the data by the ANOVA method simultaneously for all 5 types of the investigated MCEs with purpose of defining the influence of paired interactions of factors on the response variables. For processing, the experiment matrix was divided into two separate matrices; each matrix had data for 3 types of MCEs:

1) Calf rennet (Naturen) and rennet substitutes of animal origin ("Bovine Pepsin") and microbial origin (Fromase), at low (1,500 IMCU/100 kg milk) and medium (3,000 IMCU/100 kg milk) introduction doses;
2) Calf rennet (Naturen) and MCEs based on recombinant chymosins — Chy-max Extra and Chy-max Supreme, at medium (2,000 for Chy-max Supreme and 3,000 IMCU/100 kg for Naturen and Chy-max Extra) and high (5,000 for Chy-max Supreme and 5,000 IMCU/100 kg for Naturen and Chy-max Extra) introduction doses.

Tables 1 and 2 give the results of evaluating the effect of factors and their pair interactions on the response variables obtained by the ANOVA method.

Further, when discussing the results of the analysis of variance, we will refer to the data from Table 1 as obtained "at low and medium doses of MCEs", the data from Table 2 are obtained "at medium and high doses of MCEs", without additional interpretation of the MCE type.

The results of the analysis of variance allow drawing the following conclusions on the influence of factors on the response variables.

3.1. Active acidity (pH)

There is a statistically significant influence of the "age of cheese" factor on the pH level. The influence of cheese age on pH is associated with biochemical reactions that occur at various stages of cheese ripening and storage. If in the period from 7 to 60 days, the decrease in pH is associated with the accumulation of acid due to lactose metabolization, then in the period from 60 to 150 days, the increase in pH occurs due to the accumulation of proteolysis products with buffer properties [19,20].

There is a statistically significant effect of the MCE dose on the pH of cheeses. The use of MCEs at high introduction doses (5,000 IMCU/100 kg of milk for Chy-max Supreme and 6,000 IMCU/100 kg of milk for MCEs of Naturen and Chy-max Extra) results in cheeses having significantly ($p < 0.05$) a lower average pH level throughout the ripening and storage period than cheeses produced with medium and low doses of MCEs (1,500–3,000 IMCU/100 kg of milk).

The MCE dose influences the pH level of cheeses indirectly through the effect on the moisture content of cheeses. The data presented in a previous article [13] show that increasing the MCE dose leads to a reduction in the duration of curd processing and to the preservation of more moisture in the cheese. According to the moisture content at the beginning of ripening (7 days), cheeses made with different doses of MCEs were arranged in the following order: cheeses with a high dose of MCE (42.38 ± 1.28% moisture) > cheeses with an average dose of MCE (41.59 ± 1.22% moisture) ≈ cheeses with a low dose of MCE (41.33 ± 0.81% moisture). Cheeses with a high moisture content had a greater amount of lactose, the metabolization of which by starter microorganisms led to a decrease in pH; the greater the lactose content was, the greater the decrease in pH was.

Figure 1 shows the influence of experimental factors on the dynamics of changes in the pH of cheeses during ripening and storage.

In Figure 1, the graphs of changes in the pH show, that the pH level in the test cheeses during the ripening and storage period is within 5.05 ± 0.10 pH units and is close to the pH level at which PA of MCE was determined under "*in vitro*" conditions (pH 5.3). So, it is possible to correlate the estimate of the PA value of MCE measured "*in vitro*", which is used for a comparative assessment of MCEs of diverse types in scientific works [10,11] with the actual proteolytic action of MCE in cheeses under real conditions.

3.2. Degree of proteolysis

The results of the analysis of deviation (see Tables 1 and 2) show that there is a strong, statistically significant influence ($p < 0.001$) of all experimental factors ("MCE type", "MCE dose" and "cheese age") on the degree of proteolysis in cheeses. Figure 2 displays the graphs with the connection between the degree of proteolysis in cheeses and influencing factors.

In Figure 2, the graphs show that the degree of proteolysis in cheeses increases throughout the entire period of ripening and storage. An increase in the MCE dose leads to an increase in the rate of accumulation of proteolysis products in cheeses. However, there was no direct connection between the rate of the proteolysis process and the level of PA of MCE. Cheeses made with Chy-max Supreme, which has the lowest level of PA among all studied MCEs, have the lowest level of proteolysis. For other types of MCEs, there is no proportionality between PA of MCE and the rate of proteolysis in cheeses made with them. At the same time points, there were no statistically significant ($p < 0.05$) differences in the degree of proteolysis between cheeses produced with equal doses of MCEs of Naturen Extra, Chy-max Extra, "Bovine Pepsin" or Fromase, which had various levels of PA. Therefore, the proteolytic productivity of MCEs in cheeses cannot be assessed from the PA level of MCE measured "in vitro".

The results obtained are related to the fact that the level of proteolysis in cheeses depends not only on the total proteolytic activity of MCE, but also on the degree of MCE transition into the cheese mass. It has been set up that the amount of MCE passing from milk to the cheese mass varies

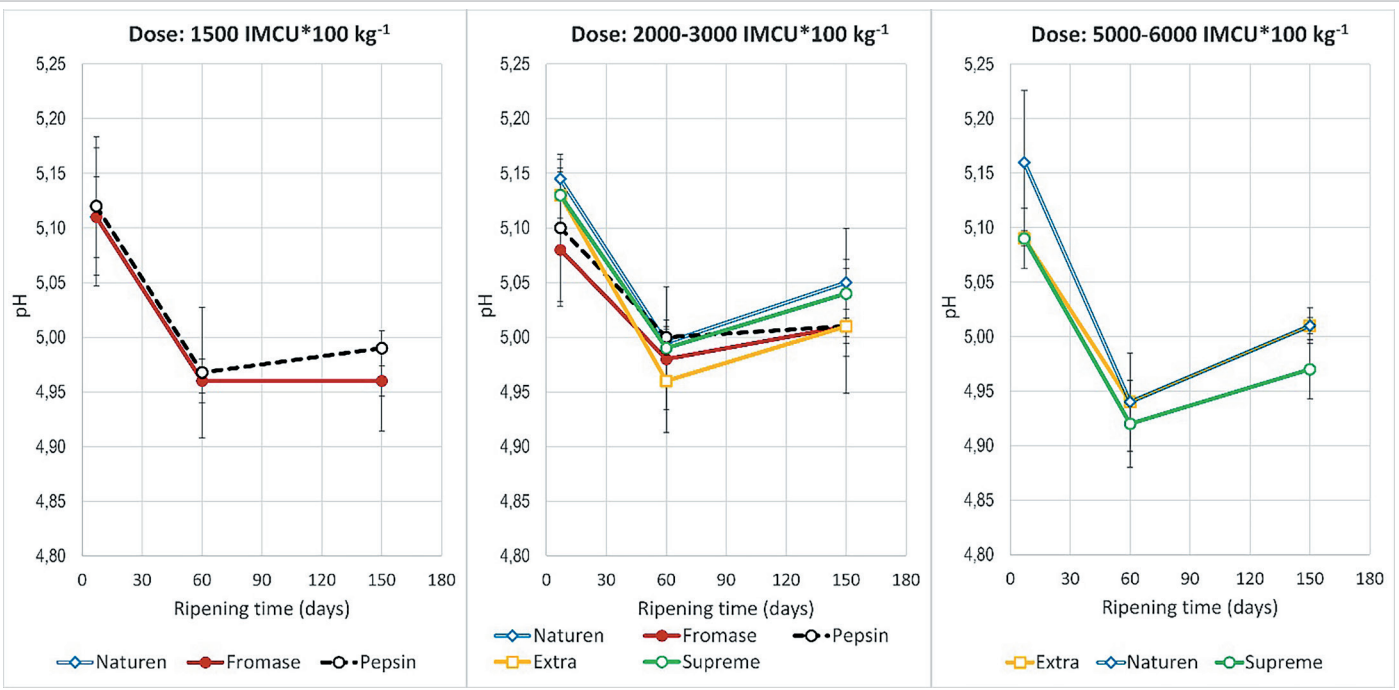

Figure 1. **Dynamics of changes in the active acidity of samples of test cheeses produced with diverse types and doses of MCEs during storage. Keys of MCE brands on the graph: Fromase — Fromase 750 XLG; Pepsin — "Bovine Pepsin" FS-10; Naturen — Naturen Extra 220; Extra — Chy-max Extra; Supreme — Chy-max Supreme. Scatter bars show "±standard deviation"**

Рисунок 1. **Динамика изменения активной кислотности образцов экспериментальных сыров, произведенных с разными типами и дозами МФП, в процессе хранения. Обозначения марок МФ на графике: Fromase — Fromase 750 XLG; Pepsin — «Пепсин говяжий» ФС-10; Naturen — Naturen Extra 220; Extra — Chy-max Extra; Supreme — Chy-max Supreme. Планки разброса показывают «±стандартное отклонение»**

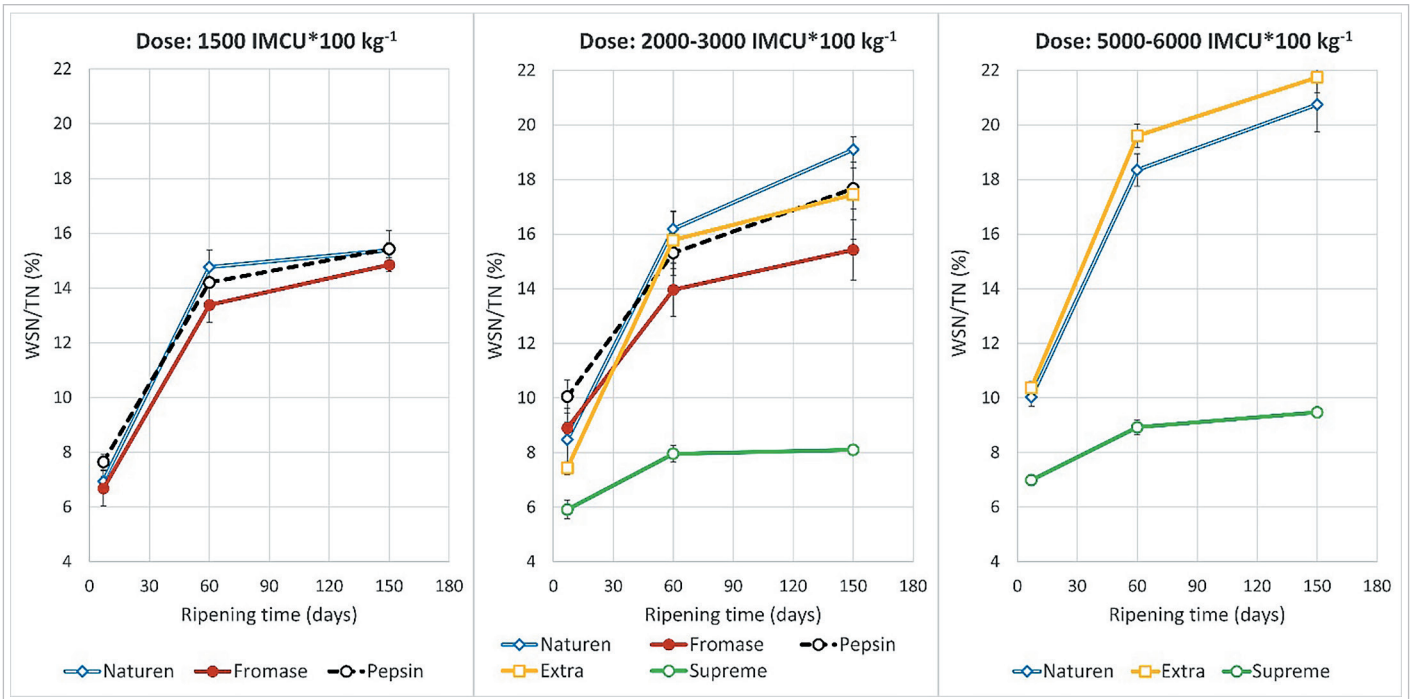

Figure 2. **Dynamics of proteolysis in samples of test cheeses produced with diverse types and doses of MCEs during storage. The keys of the MCE brands on the graph are similar to Figure 1. Scatter bars show "±standard deviation"**

Рисунок 2. **Динамика протеолиза в образцах экспериментальных сыров, произведенных с разными типами и дозами МФП, в процессе хранения. Обозначения марок МФ на графике аналогичны с Рисунком 1. Планки разброса показывают «±стандартное отклонение»**

depending on the MCE type and on the technological modes of cheese production. In the cheese curd, up to 30% or more of chymosin and no more than 2–3% of proteases of microbial origin are preserved [1,2]. Lowering the pH of the curd increases the amount of retained chymosin but does not increase the proportion of pepsin and microbial coagulants [21–24]. Small amounts of pepsin and microbial protease (Fromase) in cheese offset their high PA. As a result, cheeses made with these MCEs achieve the same or lesser degree of proteolysis than cheeses produced with calf chymosin-based MCE (MCEs of Naturen, Chy-max Extra), which have lower levels of PA (Figure 2).

3.3. Formation of small peptides

In addition to proteolytic activity, MCEs are characterized by proteolytic specificity, which is expressed in the preferential release of peptides with a certain molecular weight from the hydrolysable protein. Chymosin-based MCEs (Naturen, Chy-max Extra and Chy-max Supreme) break down caseins with the formation of a large amount of proteolysis products with a large molecular weight (over 10 kDa), including insoluble ones [6], while MCEs based on pepsin and microbial proteases (Fromase) are capable to continue the hydrolysis with the formation of a significant amount of peptides with a low molecular weight (less than 10 kDa) [25,26]. To characterize the

peptide composition of proteolysis products, the indicator "molecular weight distribution" is used.

An important indicator of molecular weight distribution is the concentration of peptides with a molecular weight of 1 to 5 kDa. It is known that peptides with a molecular weight of less than 0.5 to 3 kDa relate to the formation of the bitter taste of cheese [6,27,28]. Peptides with a molecular weight of more than 6 kDa, even containing hydrophobic amino acids, do not have a bitter taste [6]. Excessive accumulation of hydrophobic peptides with a molecular weight of less than 6 kDa in cheeses is the cause of the bitter taste of the product. The most pronounced bitter taste is imparted by peptides with a molecular mass in the range of 0.5–3 kDa [27,28]. When comparing cheeses made with camel chymosins (Chy-max M) and calf chymosins (Chy-max M), a higher content of these peptides was found in cheeses made using calf chymosin, which had the consequence of more pronounced bitter taste in them [29].

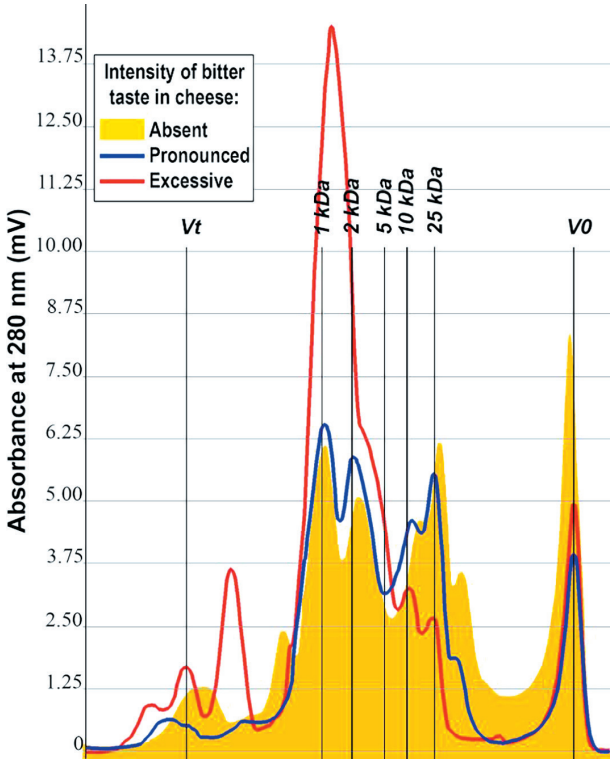

Figure 3. **Molecular weight distribution of water-soluble products of proteolysis in cheeses with different strength of bitter taste**

Рисунок 3. **Молекулярно-массовое распределение водорастворимых продуктов протеолиза в сырах с разной выраженностью горького вкуса**

Figure 3 shows the molecular weight distribution of water-soluble proteolysis products in cheeses with different severity of bitter taste (authors' own data).

The results of chromatographic studies, given in Figure 3, confirm the connection described in the literature between the strength of bitter taste and the quantitative content of peptides with a mass of 1–5 kDa in cheese.

The results of the analysis of variance (see Tables 1 and 2) show that there is a strong, statistically significant influence ($p < 0.001$) of all experimental factors ("MCE type", "MCE dose" and "cheese age") on the number of peptides with a mass of 1–5 kDa accumulated in cheeses. Figure 4 presents the graphs showing the connection between the number of peptides with a mass of 1–5 kDa accumulated in cheeses and influencing factors.

In Figure 4, the data show that with an increase in the MCE dose, the amount of 1–5 kDa peptides accumulated in cheeses increases. The MCE type affects the amount of 1–5 kDa peptides accumulated in cheese. There were no significant differences ($p > 0.05$) in the content of 1–5 kDa peptides between cheeses made with the same MCE doses of Naturen Extra, Chy-max Extra, "Bovine Pepsin" or Fromase at the same time points. In cheeses with Chy-max Supreme, the smallest quantity of bittering-forming peptides appears, which significantly ($p < 0.05$) differs from their content in cheeses with other studied types of MCEs.

Sensory assessment of the intensity of bitter taste in cheeses was proportional to the content of peptides with a molecular weight of 1–5 kDa (Figure 4).

Literature [10,30,31] hold numerous references to the ability of MCE of microbial origin, based on the *R. miehei* protease (Fromase), to form a strong bitter taste in cheeses. In our study, the degree of bitterness in cheeses made with Fromase was not greater than the degree of bitterness in other cheese varieties. At introduction dose of MCE of 1,500–3,000 IMCU/100 kg of milk, cheeses made with Fromase have the same or less pronounced bitter taste than cheeses produced with Chy-max Extra, Naturen or "Bovine Pepsin".

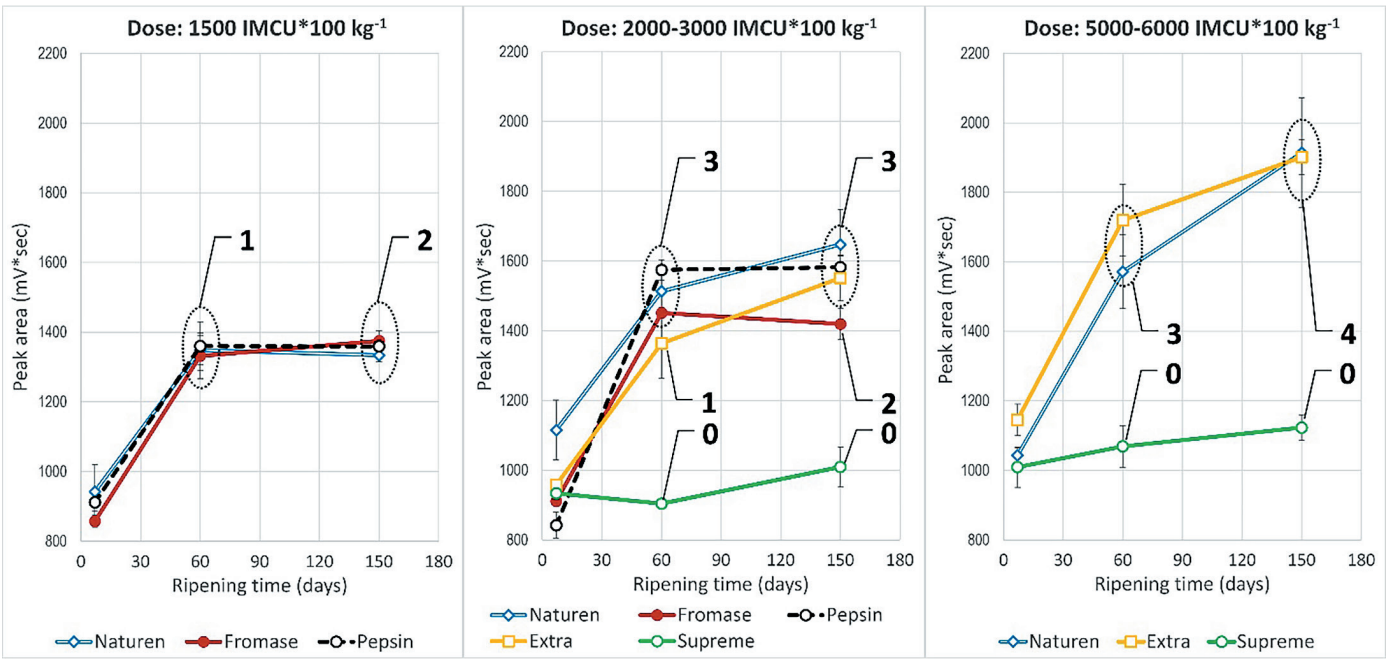

Figure 4. **Dynamics of accumulation of bitter peptides in samples of test cheeses produced with diverse types and doses of MCEs during storage. The keys of the MCE brands on the graph are similar to Figure 1. Scatter bars show " ± standard deviation". The callouts display the sensory assessment of the intensity of bitter taste in cheeses on a conditional 5-point scale: 0 — absent; 1 — very weak; 2 — weak; 3 — medium; 4 — pronounced**

Рисунок 4. **Динамика накопления горьких пептидов в образцах экспериментальных сыров, произведенных с разными типами и дозами МФП, в процессе хранения. Обозначения марок МФ на графике аналогичны с Рисунком 1. Планки разброса показывают «± стандартное отклонение». Выноски показывают сенсорную оценку интенсивности горького вкуса в сырах по условной 5-бальной шкале: 0 — отсутствует; 1 — очень слабая; 2 — слабая; 3 — средняя; 4 — выраженная**

At an equal dose of MCE, cheeses made with bovine pepsin did not differ in the strength of bitter taste from cheeses produced with calf rennet (Naturen Extra). This replicates the results of Emmons et al [32] in a comparative study of calf rennet and bovine pepsin in the production of Cheddar cheese.

The obtained data say that during the production of semi-hard cheeses it is undesirable to use MCEs based on calf chymosins of both natural origin (Naturen) and recombinant origin (Chy-max Extra) at a dose of 6,000 IMCU/100 kg of milk due to the risk of bitter cheese taste during storage.

3.4. Rheological indicators

The value of fracture stress (σf) is equal to the pressure at which the material structure fractures [33]. The higher the value of fracture stress is, the higher the strength and coherence of the cheese mass are [3]. The value of fracture stress gives information about the rheological behavior of cheeses during their chewing or industrial size-reduction operation such as shredding, grating, shearing and portion cutting.

The results of the ANOVA (see Tables 1 and 2) show that the value of fracture stress is influenced by the age of the cheese, and at medium and high doses of MCEs, by the factors of "MCE type" and "MCE dose". Figure 5 displays the graphs showing the relationship between fracture stress and experimental factors.

In Figure 5, the graphs show that, there is an increase in the average value of fracture stress over time, and then a decrease for all test variants of cheeses. There are no statistically significant differences in the magnitude of fracture stress between the variants of cheeses produced with the same doses of MCEs of diverse types at the same time points (Tukey's test, $p < 0.05$). An exception is a statistically significant higher level of fracture stress in cheeses produced with a high dose of Chy-max Supreme at the age of 150 days, in comparison with cheese variants made with Naturen and Chy-max Extra (Tukey's test, $p < 0.05$), which explains the revealed ANOVA influence of the factors "MCE type" and "MCE dose" on fracture stress.

The statistically significant dependence of fracture stress on the "Age" factor, proved by the results of the analysis of variance, means that fracture stress changes under the influence of factors, which, in turn, are influenced by the "Age" factor. According to the literature data, the structure of cheese changes over time under the influence of demineralization, proteolysis, and dehydration of the cheese mass [3].

At the first stage of maturation, the strength and plasticity of cheese consistency depends on the amount of calcium ions associated with casein molecules and combining them into aggregates [3]. As a result of the metabolization of lactose by bacteria, lactic acid accumulates in the cheese mass, accompanied by a decrease in pH. With a decrease in pH, calcium ions are separated from caseins (demineralization), resulting in a decrease in cohesion and an increase in the plasticity of the cheese mass [34], as a result of which the fracture stress index decreases.

Insignificant differences in pH between the studied cheese varieties at the beginning of the ripening period (7 days) led to the absence of differences in the level of fracture stress between them (Tukey's test, $p < 0.05$). At the age of 7 days, all cheese variants had the same consistency, which can be described as "elastic, slightly brittle".

After completion of the metabolization of lactose and stabilization of the pH in the cheese, a balance is set up between the calcium ions, which are in the casein-bound and soluble forms. A further change in the rheological properties of the cheese mass occurs under the influence of proteolysis and dehydration [19].

Figure 6 A gives the graph of the dependence of fracture stress on the degree of proteolysis. Figure 6 B gives the graph showing the dynamics of cheese dehydration.

The graph of fracture stress versus the degree of proteolysis (Figure 6A) shows that the data points are divided into 2 groups according to the minimum differences: those belonging to fresh cheeses at the age of 7 days and those belonging to mature cheeses at the age of 60 and 150 days. For the group of mature cheeses, the graph (Figure 6A) shows a trend — with an increase in the degree of proteolysis, the value of fracture stress decreases. The division of data into groups is a sign of the influence on the rheological parameters of cheeses of factors other than proteolysis. At the same time, the nature of these factors differs for fresh and mature cheeses.

In the studied cheeses, the process of conversion of lactose into lactic acid, accompanied by a decrease in pH, continues after 7 days (up to about 15 days). Therefore, the pH of the studied cheeses at the age of 60 days is lower than at the age of 7 days (Figure 1). Decrease in pH of less than 5.2 units leads to a significant removal of calcium ions from bonds with casein, as a result of which the cheese mass becomes less cohesive, and at a low moisture content in the cheese, even crumbly [34–36]. In the range of pH of 4.90–5.05, a consistency typical of Cheddar cheese is formed — dense, moderately coherent, slightly crumbly [3].

In all studied cheeses, by the end of the ripening period (60 days), the acidity was in the range of 5.0 ± 0.05 pH units (Figure 1). The consistency of cheeses at this age was characterized as "brittle, crumbly". At the same time, cheese variants made using Chy-max Supreme had a firmer and less plastic consistency compared to cheese variants produced with other types of MCEs. Figure 6A shows that data points for cheeses made with Chy-max Supreme stand out as a separate subgroup from the group of mature cheeses. Of all mature cheese samples, those made with Chy-max Supreme have the highest fraction stress with the lowest levels of proteolysis (Figure 5).

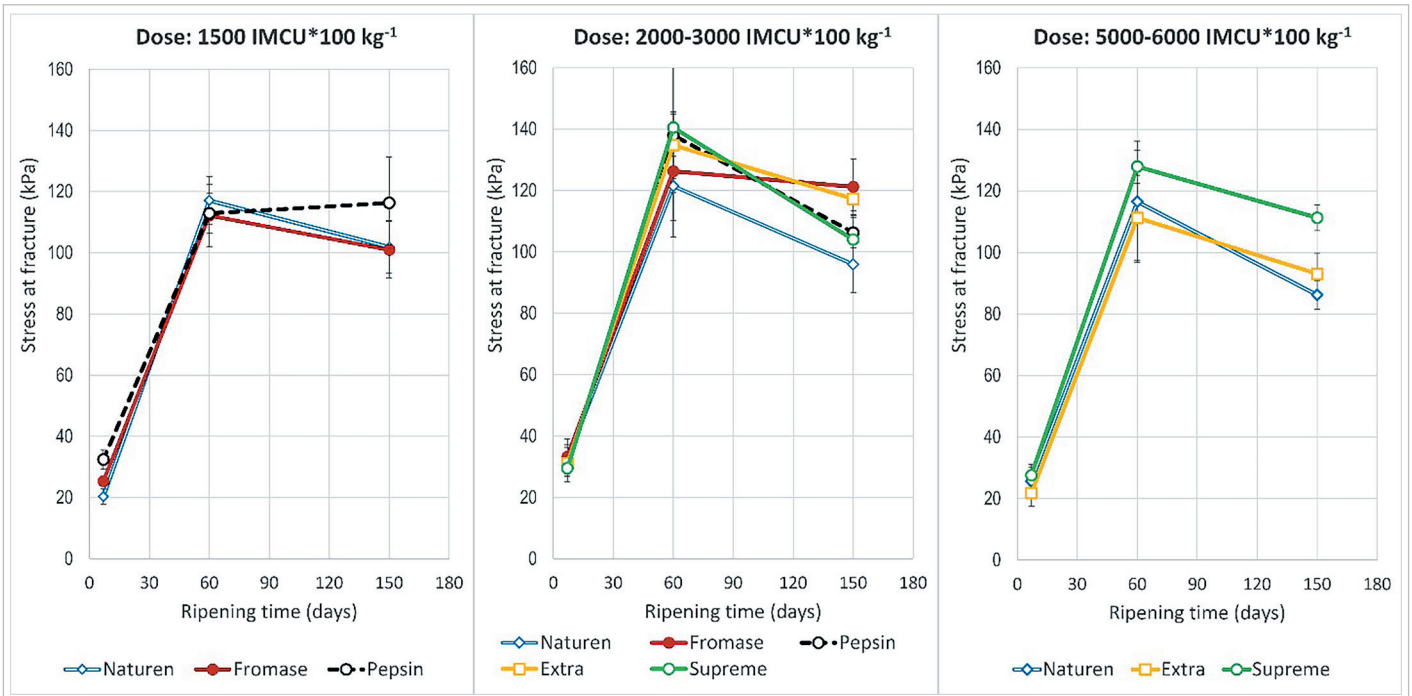

Figure 5. **Dynamics of change in the indicator " stress at fracture" in samples of test cheeses produced with diverse types and doses of MCEs during storage. The keys of the MCE brands on the graph are similar to Figure 1. Scatter bars show "± standard deviation"**

Рисунок 5. **Динамика изменения показателя «напряжение сжатия при разрушении» в образцах экспериментальных сыров, произведенных с разными типами и дозами МФП, в процессе хранения. Обозначения марок МФ на графике аналогичны с Рисунком 1. Планки разброса показывают «± стандартное отклонение»**

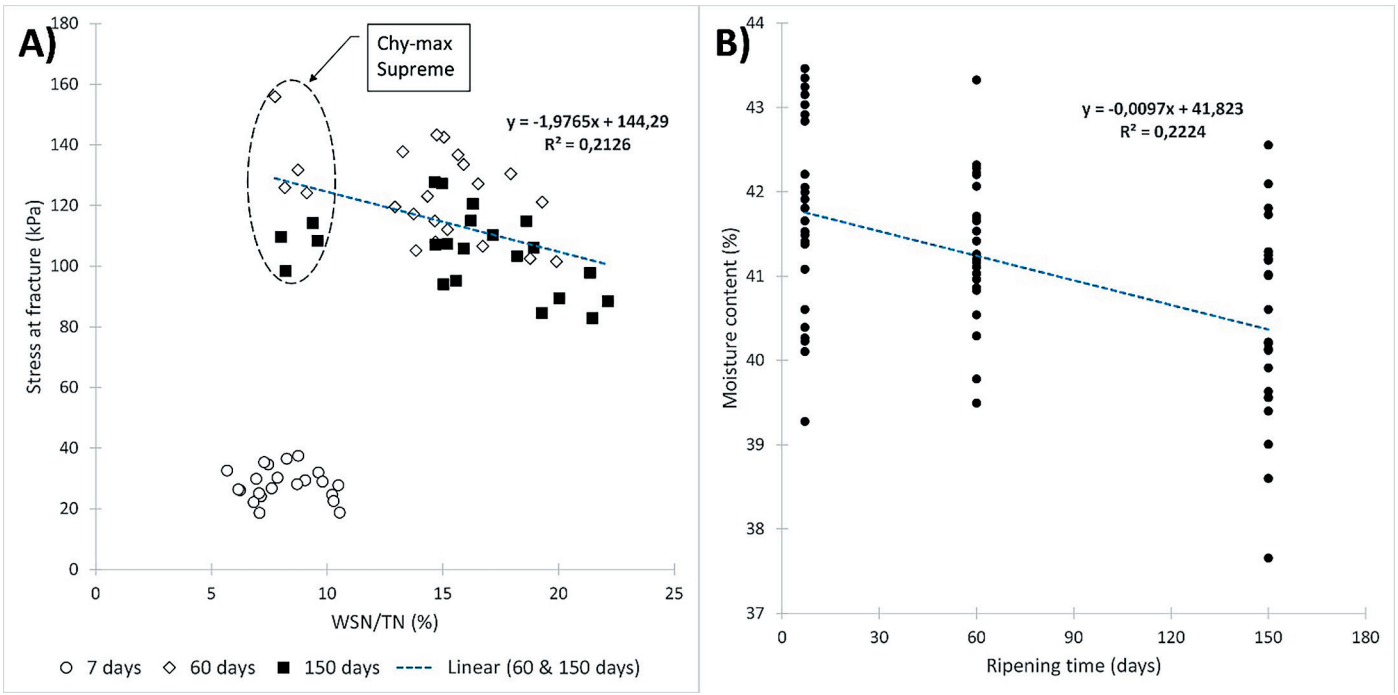

Figure 6. **Dependences: A) indicator of fracture stress of the cheese mass on the degree of proteolysis (data are presented on the indicators of cheese at the age of 7, 60 and 150 days); B) moisture content in samples of test cheeses on the age**

Рисунок 6. **Зависимости: А) показателя fracture stress сырной массы от степени протеолиза (представлены данные по показателям сыра в возрасте 7, 60 и 150 сут); В) содержания влаги в экземплярах экспериментальных сыров от возраста**

The moisture content affects the texture of cheeses. With a decrease in moisture content, the casein gel network decreases in volume and becomes denser, which leads to an increase in the density and hardness of the cheese mass [3,19]. The graph presented in Figure 6B indicates a trend towards a decrease in moisture content in cheeses during ripening and storage, as well as a significant variation in moisture content between individual cheeses at the same time points. The decrease in moisture content and the resulting compaction of the cheese mass explains the absence of a decrease in fracture stress in some types of cheeses in the period from 60 to 150 days (Figure 5), despite the process of softening of the cheese mass under the influence of proteolysis occurring in the same period. Differences in moisture content can explain the significant variation of points compared to the regression line and the low coefficient of determination ($R^2 = 0.21$) of the regression relationship between the degree of proteolysis and fracture stress in mature cheeses (Figure 6 A).

3.5. Microstructure

Microscopic studies of the structure of cheeses have been conducted. The analysis of micropreparations of test variants of semi-hard cheeses confirmed the pattern previously established in the works of other researchers [2,37,38]: the higher the degree of proteolysis is, the higher the degree of hydration of caseins is, and the higher the degree of homogeneity of the cheese structure is. Cheeses with a higher degree of proteolysis are characterized by a more uniform, finely dispersed structure. Cheeses with a lower degree of proteolysis are characterized by a more fragmented structure, with distinct boundaries between cheese grains or with traces of these boundaries. For example, Figure 7 shows photos of the microstructure of cheeses made using different doses of Naturen, at different ages, with different degrees of proteolysis and various levels of fracture stress.

The structure of cheeses at the level of microstructure forms their rheological properties [3]. The structure of a fresh cheese consists of individual pressed cheese grains with intergranular space filled with moisture (Figure 7, cheeses aged 7 days). Cheese mass, fragmented into individual grains, has a low cohesion, and is destroyed along the boundaries of grain joints under the influence of even a small load. The work of Tunik et al [39] found that Cheshire cheese, which has the same protein and fat content and pH level as Cheddar cheese, has almost 2 times lower levels of elasticity modulus (G'), viscosity modulus (G") and complex viscosity (η^*) due to the more fragmented structure. Due to the low cohesion of the structure, test cheeses at the age of 7 days had a low level of stress at fracture (Figure 5).

In maturation of cheeses, proteolysis of caseins of the cheese mass occurs. The peptides formed from caseins have a higher hydrophilicity than the original caseins. This leads to an increase in the moisture-binding capacity of the casein gel. The mechanically bound water contained in the pores of the casein net is converted into water chemically bound to the charged carboxyl and amino groups of the peptides released during proteolysis of caseins [20]. Increasing the hydration of the casein gel leads to the formation of a homogeneous, cohesive structure of the cheese mass, which has a much higher mechanical strength and requires more load to break it. As a result, in the studied cheeses at the end of the ripening period (60 days), the value of stress at fracture increases (Figure 5). Visually, the process of hydration of the casein net appears in the disappearance of the boundaries between the grains and the "gluing" of the grains (Figure 7, cheeses aged 60 and 150 days). Over time, under the action of proteolysis, an increasing number of caseins is cleaved, as a result of which the mechanical strength of the casein gel decreases. This leads to a decrease in the mechanical strength of the cheese mass and a decrease in the magnitude of the load required for its fracture [3]. As a result, cheeses aged 150 days have a lower level of stress at fracture than cheeses aged 60 days (Figure 5).

Related results were obtained by the authors when studying the effect of proteolysis on the structure of soft cheeses made using diverse types and doses of MCEs [40,41]. At the same time, the significant differences in the effect of proteolysis on the consistency of soft and semi-hard cheeses should be noted. Compared to soft cheeses, due to their lower moisture content, semi-hard cheeses have a denser casein network, which also has a higher cohesiveness, due to the higher content of calcium bound to molecules due to a higher pH level. The dense and coherent casein net of semi-hard cheeses, even after splitting some caseins because of proteolysis, retains high strength. As a result, there are no noticeable differences in rheological characteristics and sensory assessment of consistency in all variants of semi-hard cheeses made with diverse types and doses of MCEs, having differences in the degree of proteolysis. The exception is cheese made with Chy-max Supreme, which has the lowest level of proteolysis and the densest texture among all studied cheese variants.

Conclusion

Based on the data obtained, the following conclusions can be drawn:

Cheeses produced using high doses of MCEs added to milk (5,000–6,000 IMCU/100 kg of milk) have a significantly ($p < 0.05$) lower pH level than cheeses made with medium and low doses of MCEs (1500–3000 IMCU/100 kg of milk). The use of high doses of MCEs for milk coagulation results in a reduction in the processing time of the curd and the production of cheese with a high moisture content. The higher the moisture content of the cheese is, the greater the lactose content is, and the greater the decrease in pH due to the conversion of lactose to acid by starter bacteria is.

An increase in the dose of MCE added to milk leads to an increase in the rate of accumulation of proteolysis products in cheeses. At the same

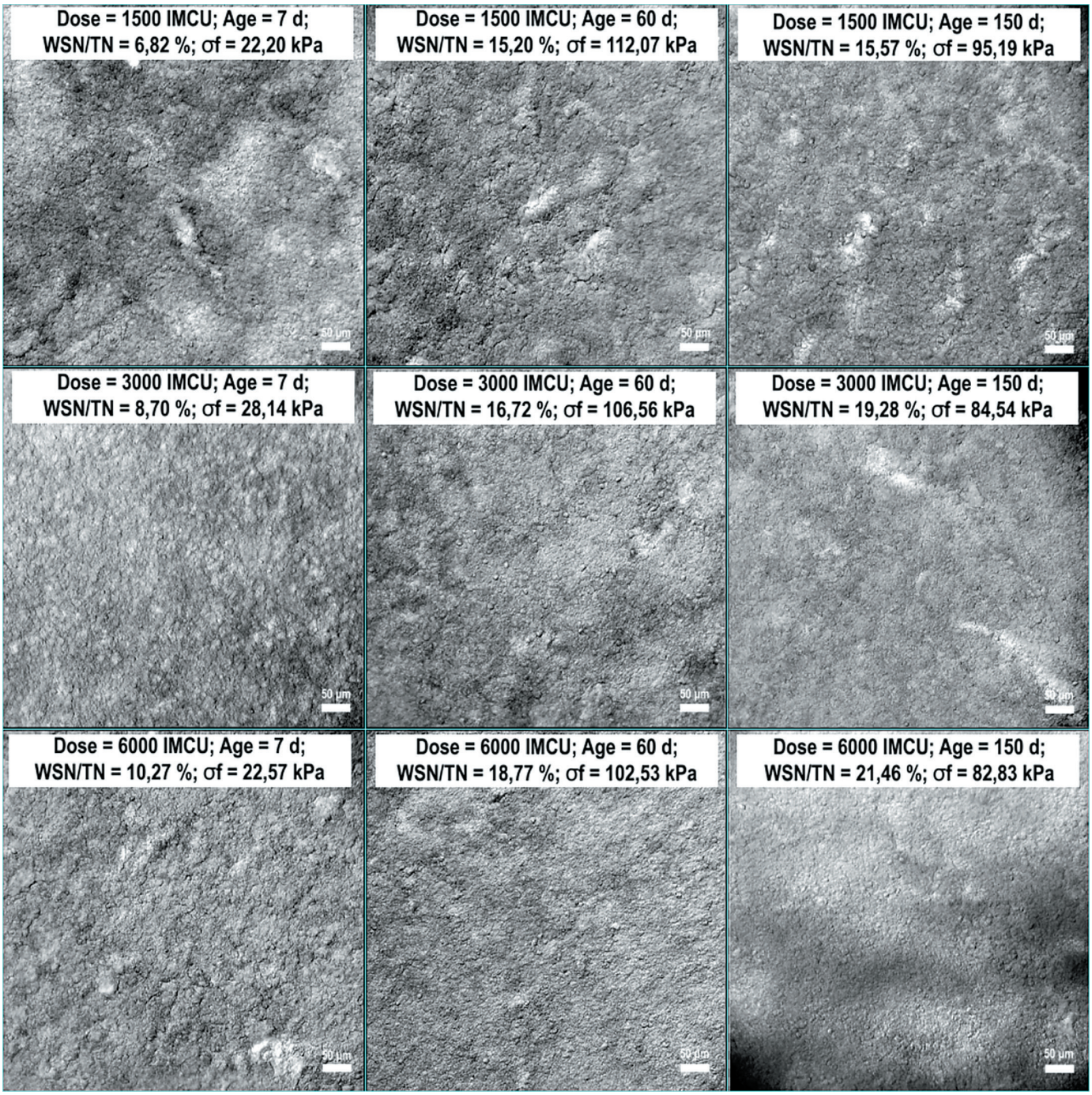

Figure 7. **Typical microstructure of cheeses produced using different doses of Naturen during ripening and storage. Dose — MCE introduction dose (IMCU/100 kg of milk); Age — cheese age (days); WSN/TN — share of soluble nitrogen from the total (%); σf — fracture stress (kPa). The scale bars are 50 µm in length**

Рисунок 7. **Типичная микроструктура сыров, произведенных с использованием разных доз МФ Naturen, в течение срока созревания и хранения. Dose — доза внесения МФ (IMCU/100 кг молока); Age — возраст сыра (сут); WSN/TN — доля растворимого азота от общего (%); σf — fracture stress (кПа). Мерный отрезок равен 50 мкм**

time points, there were no significant differences ($p > 0.05$) in the degree of proteolysis between cheeses made using the same dose of distinct types of MCEs. The only difference was cheeses produced with Chy-max Supreme, which had the lowest level of proteolysis ($p < 0.05$). The reason for the lack of differences in the degree of proteolysis between cheeses made with MCEs of Naturen or Chy-max Extra and cheeses made with MCEs of "Bovine Pepsin" or Fromase, which have a significantly higher level of PA, is a small degree of transfer of pepsin and microbial proteases from milk to cheese, which levels their high PA.

With an increase in the MCE dose, the content of peptides with a molecular weight of 1 to 5 kDa increases in cheese. Sensory assessment of the intensity of bitter taste in cheeses was proportional to the content of bitter peptides with a molecular weight of 1–5 kDa. Chy-max Supreme produces the least number of bitter peptides among all MCE variants. At MCE introduction dose of 3,000 IMCU/100 kg of milk, Fromase produces fewer bitter peptides than Chy-max Extra, Naturen or "Bovine Pepsin", which have a lower level of proteolytic activity. This shows the high quality of MCEs of microbial origin achieved to date and allows us to recommend MCEs of microbial origin to use in the production of semi-hard cheeses at a dose of up to 3,000 IMCU/100 kg of milk. At MCE introduction dose of 6,000 IMCU/100 kg of milk, cheeses made with Chy-max Extra show an accumulation of bitter peptides at the level of Naturen Extra ($p < 0.05$), which has a higher PA level than that of Chy-max Extra.

For all test variants of cheeses, over time, at first there is a tendency to an increase in the level of fracture stress, and then its decrease. There are no statistically significant differences in the magnitude of fracture stress between the variants of cheeses produced with the same doses of MCEs of diverse types at the same time points ($p > 0.05$). The structure and consistency of the test cheeses depended more on their composition (moisture, protein, fat content) and pH than on the level of proteolysis.

The consistency of the cheeses depended on the structure of the cheeses at the microstructure level. In turn, the microstructure of cheeses depended on the calcium content associated with casein molecules and the degree of casein proteolysis. The crumbling texture of fresh cheeses and low values of fracture stress were associated with a substantial leaching of calcium ions from bonds with caseins and a low water-binding capacity of native caseins. Proteolysis of caseins, which occurs during the ripening of cheeses, leads to an increase in the water-binding capacity of the cheese mass. In mature cheeses, there is a greater cohesion and a higher level of fracture stress in comparison with fresh cheeses.

The rheology of the cheese mass can be controlled through the degree of casein hydrolysis (by selecting the type and dose of MCE) or by changing the content of micelle-bound calcium paracasein (by adjusting the pH), or both.

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