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Effects of the Autochthonous Probiotic Bacteria *Lactobacillus plantarum* B and *Lactococcus lactis* Subsp. *lactis* S1 on the Proteolysis of Croatian Cheese Ripened in a Lambskin Sack (Sir iz Mišine)

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Abstract: This study aims to determine the effects of the autochthonous probiotic bacteria *Lactobacillus plantarum* B (currently *Lactiplantibacillus plantarum*) and *Lactococcus lactis* subsp. *lactis* S1 on proteolysis during the ripening of Sir iz mišine—a Croatian cheese which ripens in a lambskin sack. Sir iz mišine was produced in four different variants: (1) from raw milk without starter cultures, and from pasteurized milk with added (2) *Lactococcus lactis* ssp. *lactis* S1, (3) *Lactobacillus plantarum* B, or (4) a starter culture consisting of a mixture of *Lactococcus lactis* ssp. *lactis* S1 and *Lactobacillus plantarum* B (1:1). The addition of *Lactobacillus plantarum* B alone or in combination with *Lactococcus lactis* subsp. *lactis* S1 noticeably increased the alpha and beta indices because of the synergistic activity between the enzymes responsible for primary proteolysis and added autochthonous bacteria. Cheese produced from raw milk had the lowest (12.16%) content of WSN%TN. The highest WSN%TN content was found in cheese produced with combined probiotic bacteria (30.40%) and *Lactococcus lactis* ssp. *lactis* S1 (29.74%). Cheese with added combined probiotic bacteria had a noticeably higher content of TCA-SN%TN, indicating a synergistic performance among autochthonous probiotic bacteria. In conclusion, autochthonous probiotic bacteria, in addition to having a functional value, can improve the ripening properties of cheese.

Keywords: Sir iz mišine; cheese in a lambskin sack; ripening; proteolysis; probiotic bacteria; *Lactobacillus plantarum* B; *Lactococcus lactis* subsp. *lactis* S1

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1. Introduction

In cheese production, ripening is the most complex technological phase, and is responsible for the formation of the characteristic sensory properties of the cheese. Biochemical processes that occur during ripening can be divided into primary (proteolysis, lipolysis, and residual lactose metabolism) and secondary processes (fatty acid and amino acid metabolism) [1–3]. Proteolysis is an important biochemical process that plays multiple roles in the formation of cheese characteristics—both desirable and undesirable. Through the combined action of rennet enzymes, endogenous milk enzymes, and enzymes originating from lactic acid bacteria (added or autochthonous), proteolysis participates in the development of cheese flavor, aroma, and texture [1,4,5]. The role of proteolytic enzymes is manifested in the degradation of casein to peptides of various lengths and free amino acids, leading to the softening of the texture and development of the characteristic cheese taste. Since casein forms the basis of cheese in which other ingredients are incorporated,

proteolysis is considered to be one of the most important processes that contribute to the development of the characteristic texture of cheese [6,7].

Sir iz mišine (cheese in a sack) is a Croatian autochthonous hard-ewe's-milk cheese. It is produced on family farms in central Dalmatia (Šibenik-Knin and Split-Dalmatia Counties). Its main specificity is ripening in a sack made from lambskin [7]. Apart from lipolysis, proteolysis is the main biochemical process that takes place during the ripening of cheese in an animal skin, and plays an important role in the development of the desired sensory properties of this type of cheese [8,9]. Cheeses that ripen in an animal-skin sack are characterized by a pronounced and piquant flavor, odor, and aroma, which are attributed to extensive proteolysis and lipolysis, due to the anaerobic conditions during ripening in the skin and the possible presence of molds on the skin [9–11]. Extensive biochemical reactions during ripening should be controlled, as this could result in a flavor, odor, and aroma of the cheese that are considered to be too intense, which could adversely affect consumer acceptability. The optimal duration of ripening of Sir iz mišine is 45 days [9]. However, due to high demand from consumers, the cheese is very often placed on the market after 30 days of ripening, without the desired traditional characteristic intensity of flavor. There are many ways in cheesemaking to speed up ripening, with the aim of producing cheese with desirable sensory properties in shorter timeframes. Some methods include raising the ripening temperature, adding lipolytic and proteolytic enzymes, and adding various starter cultures, including probiotic lactic acid bacteria. The ability of cheese to protect probiotic species from oxygen and harsh conditions in the gastrointestinal tract makes cheeses a good choice for the production of functional foods, the consumption of which may provide health benefits beyond basic nutrition [12–14]. Using lactic acid bacteria with probiotic properties has been proposed to improve cheese quality standards and sensory properties [15], but also to develop new functional foods [14]. Several studies on the production of different probiotic-based cheeses have been conducted, including Minas Frescal, Fior di Latte, Coalho, Gouda, Cheddar, Scamorza, Kalari/Kradi, Turkish white cheese, spreadable Ricotta, pasta filata-type cheeses, and Wagashi cheese, among others [12,14,16–24].

The aim of this study was to assess the proteolytic activity of two autochthonous probiotic bacteria—*Lactobacillus plantarum* B (currently *Lactiplantibacillus plantarum*) and *Lactococcus lactis* subsp. *lactis* S1—during ripening of Croatian Sir iz mišine, as single and mixed cultures, in comparison to the traditional Sir iz mišine produced from raw milk without the addition of any starter culture.

2. Materials and Methods

2.1. Cheese Production

Sir iz mišine was produced according to semi-hard cheese production methods in four different variants: (1) from raw milk without starter cultures (control group), (2) from pasteurized milk with added autochthonous probiotic bacteria *Lactococcus lactis* ssp. *lactis* S1, (3) from pasteurized milk with added probiotic bacteria *Lactobacillus plantarum* B, and (4) from pasteurized milk with added autochthonous starter cultures consisting of a mixture of *Lactococcus lactis* ssp. *lactis* S1 and *Lactobacillus plantarum* B at a 1:1 ratio (1.5 g of wet biomass/100 L milk). All applied strains were previously isolated and selected from traditional Sir iz mišine [25] and characterized as probiotic cultures [26]. For the production of each variant of Sir iz mišine, Dalmatian "Pramenka" sheep milk was used (approximately 80 L of a mixture of evening and morning milk). Evening milk was cooled and mixed with morning milk before cheesemaking. A commercial coagulant—100% pure chymosin (Maxiren-1800, Prodinvest, Vilnius, Lithuania)—was used, and the temperature of the milk at renneting was 33 °C. When coagulated (setting time was 40–50 min), the curd was cut into regularly shaped cubes with an average size of 2 cm using a knife. The curd grains were stirred and heated to 38-39 °C. After drying, the curd grains were the size of hazelnuts/peas. Rough curd was shaped by hand and squeezed into plastic vats using a self-molding process. After the whey was drained, the cheese curd was cut into

pieces approximately of 10 × 10 × 5 cm in size, dry-salted with large-grain sea salt (0.8 kg salt/20 kg cheese), and then put into a lambskin sack. The cheese was ripened in the lambskin sack for 45 days at 16–18 °C and relative humidity of 65–80%. For cheeses where probiotic cultures were added, pasteurized milk was used. The milk was heated to 65 °C for 30 min and then cooled to 32–33 °C. Half an hour before the addition of rennet, the cultures were added as wet biomass. The rest of the procedure was the same as in the production of the control cheese. The lambskin sacks were prepared as described in detail by Frece et al. [25].

2.2. Sampling of Cheese

Each of the four cheese variants was sampled three times during the 45-day ripening period (on days 15, 30, and 45). Before opening, the lambskin sack was placed into a specially designed sealed container from which the air was drawn out and replaced with nitrogen (so as to create a nitrogen atmosphere). Cheese samples (200 g) were taken through a larger opening within the container using sterile gloves, preventing the possibility of the development of aerobic microorganisms. Approximately 0.5 cm of the surface section of the cheese samples was discarded.

2.3. Analysis of Proteolytic Changes of the Cheese during Ripening

To evaluate the intensity of primary proteolytic changes during the ripening of Sir iz mišine cheese, the gel electrophoresis method on polyacrylamide gel (urea–PAGE) was used. The cheese was grated, and 0.4 g of grated cheese was mixed in a tube with 5 mL of urea buffer. The contents of the tube were then heated in a water bath for 30 min at a temperature of 40 °C until the cheese had completely dissolved. Afterwards, the contents were centrifuged at 3600 rpm at 4 ° C for 10 min. The separated layer of fat was removed using a vacuum pump, and 100 µL of the supernatant was mixed with 300 µL of buffer. Then, 6 µL of the prepared sample was applied to the gel. The gels were scanned using the Gel Doc 2000 (Bio-Rad) device, which has specific densitometric values for gel bands using the Quantity One Quantitation Software (Bio-Rad), according to the method of Kalit et al. [3]. Quantification was based on the measurement of the areas of each peak of α_{s1}-casein and β-casein, along with their degradation products, as relative percentages of the total casein in the cheese samples prepared for electrophoresis that was soluble in the urea buffer. Two electrophoretic ripening indices were used: the beta index (sum γ-casein/β-casein) [27] and the alpha index (α_{s1}-I-casein/(α_{s1}-I-casein + α_{s1}-casein)) [28].

To partially evaluate primary proteolysis, and more accurately evaluate secondary proteolysis, the protein content, water-soluble nitrogen fraction in the total nitrogen (WSN %TN), and 12%-trichloroacetic-acid-soluble nitrogen fraction in the total nitrogen (TCA-SN %TN) were determined by the Kjeldahl method described in ISO 8968-1 [29]. All analyses were conducted at the University of Zagreb Faculty of Agriculture, Department of Dairy Science, Reference Laboratory for Milk and Milk Products, Croatia. The samples were analyzed in triplicate, and the results are expressed as the average values.

3. Results and Discussion

The electrophoretic profile of the ripened Sir iz mišine was typical of those for semi-hard cheeses (Figures 1 and 2). During the ripening of Sir iz mišine, α_{s1}-casein was degraded into α_{s1}-I-casein and α_{s1}-II-casein, while β-casein was degraded into γ-caseins, which is consistent with the results obtained by Magdić et al. [30], who concluded that both enzymes (chymosin and plasmin) are involved in the primary proteolysis of Istrian sheep milk cheese. The values for primary and secondary proteolysis products during cheese ripening are shown in Figures 3–6. Primary proteolysis, especially in cheese production where curd drainage is not applied at higher temperatures, involves the initial breakdown of α_{s1}-casein by chymosin [31]—causing a noticeable increase in the alpha index (Figure 3)—and to a lesser extent by plasmin [31], causing breakdown of β-casein and, consequently, increasing the beta index (Figure 4). Moreover, the addition of *Lactobacillus plantarum* B—

alone or in combination with *Lactococcus lactis* subsp. *lactis* S1—noticeably increased both indices, probably because of the synergistic activity between the abovementioned enzymes responsible for primary proteolysis and the added autochthonous probiotic bacteria. However, Bergamini et al. [32] stated that the proteolytic activity of three probiotic strains added to semi-hard cheese—either as single or mixed cultures—did not show any effect on the proteolysis of the cheeses. In the aforementioned research, only *L. paracasei* showed a limited impact at the end of the ripening. It is assumed that the higher indices in our research were attributable to a more efficient decrease in pH due to the activity of these probiotic bacteria in comparison to the acidification activity of the natural non-starter bacteria present in Sir iz mišine produced from raw milk. Lower pH of cheese causes a higher retention rate of rennet [33]. On the other hand, lower pH of the cheese body enhances the activity of aspartic acid proteinases such as chymosin [2]. Some strains have also been reported to cause changes in primary proteolysis in Cheddar cheeses manufactured without a starter culture [34]. The addition of *Lactobacillus plantarum* B—alone or in combination with *Lactococcus lactis* subsp. *lactis* S1—enhanced the degradation of β-casein (Figure 4). Before the addition of these autochthonous probiotic bacteria, the milk used for cheesemaking was pasteurized. Pasteurization inactivates thermolabile plasminogen activator inhibitors and plasmin inhibitors, increasing plasmin activity, which is mainly responsible for the degradation of β-casein [35].

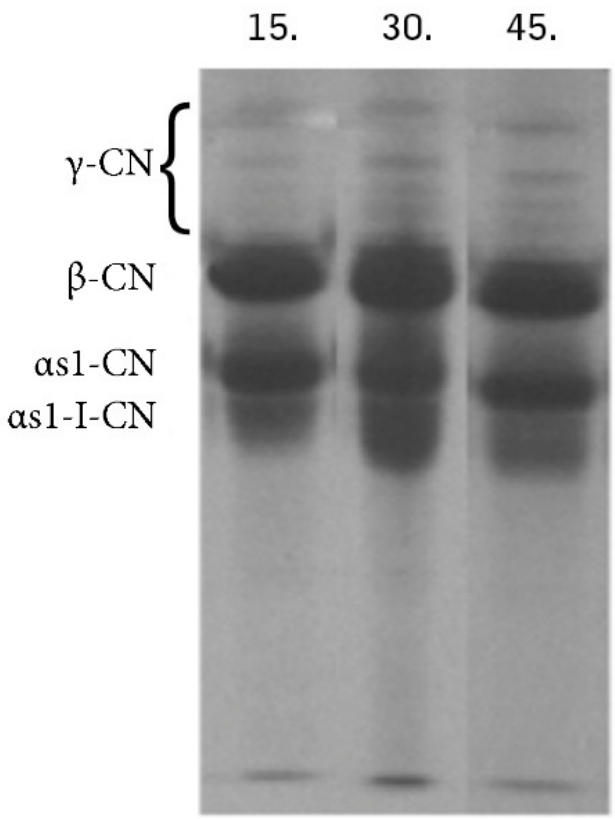

Figure 1. Urea–polyacrylamide gel electrophoresis patterns of control Sir iz mišine (without addition of starter culture) at 15, 30, and 45 days of ripening.

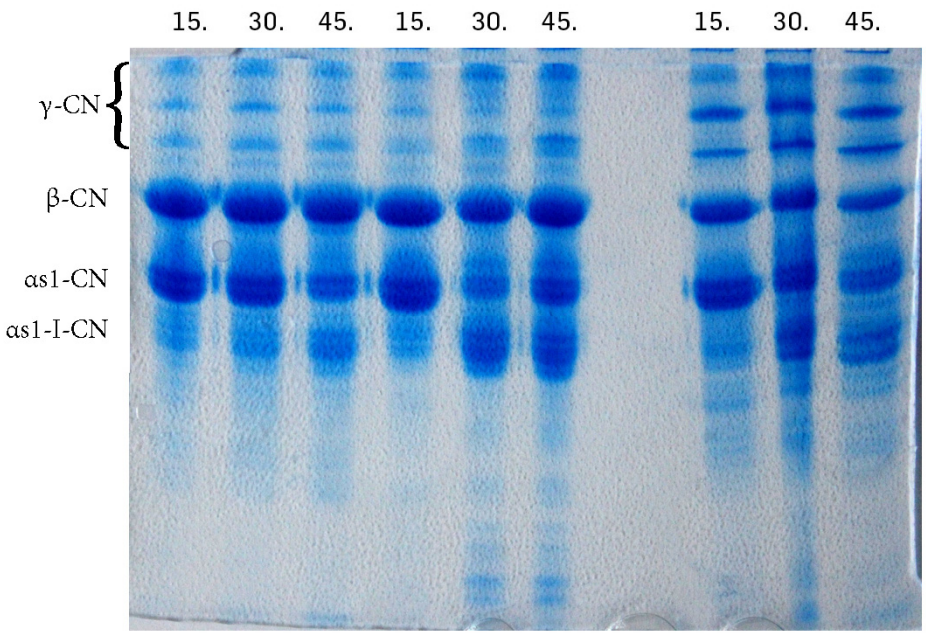

Figure 2. Urea–polyacrylamide gel electrophoresis patterns of Sir iz mišine at 15, 30 and 45 days of ripening. From right to left: *Lactococcus lactis* ssp. *lactis* S1; *Lactobacillus plantarum* B; mixed culture (*L. lactis* S1 + *L. plantarum* B).

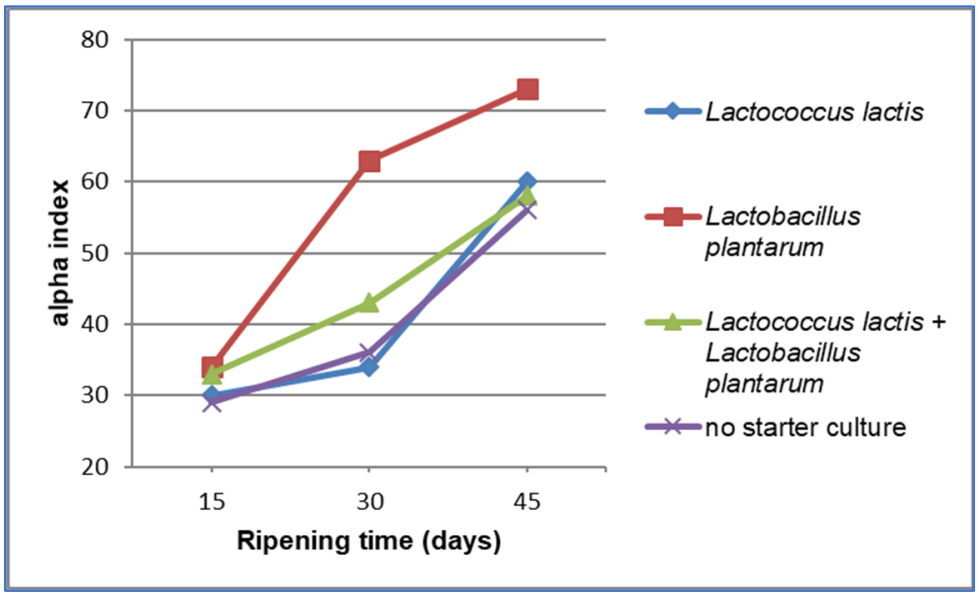

Figure 3. Alpha index of Sir iz mišine at 15, 30, and 45 days of ripening.

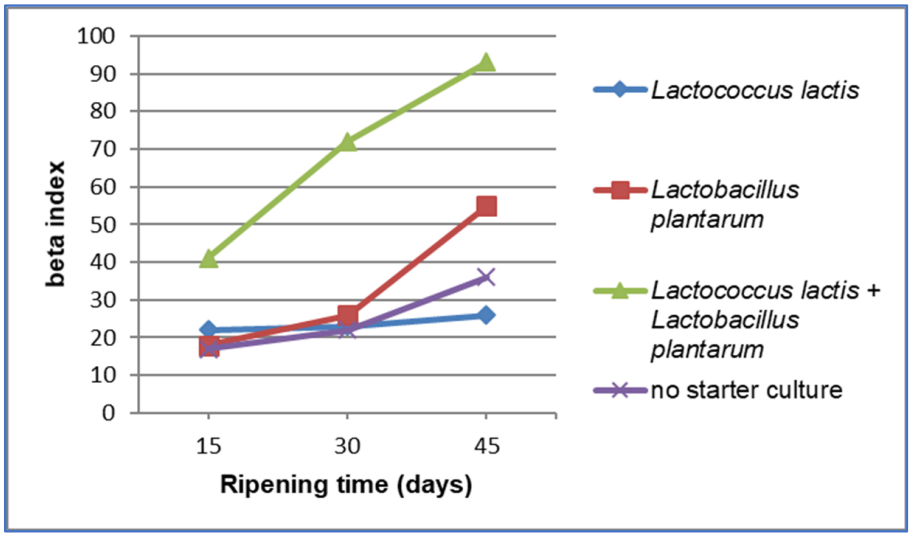

Figure 4. Beta index of Sir iz mišine at 15, 30, and 45 days of ripening.

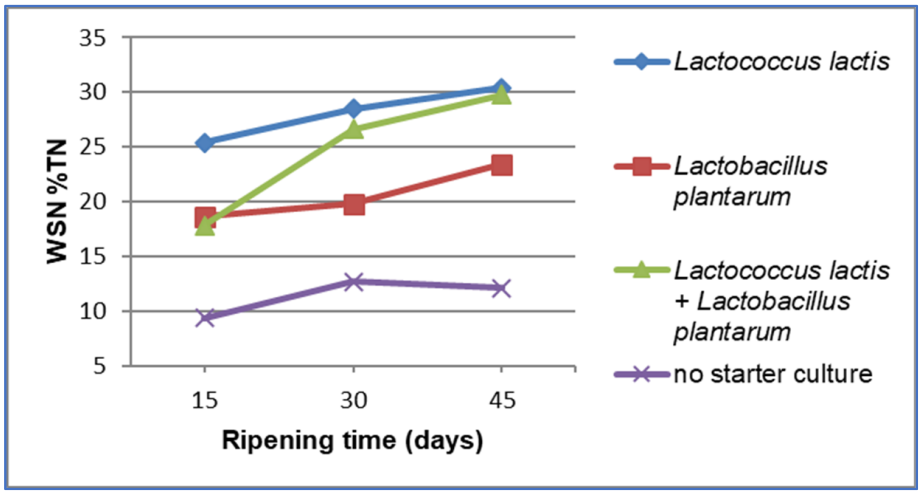

Figure 5. Water-soluble nitrogen fractions in the total nitrogen (WSN %TN) of Sir iz mišine at 15, 30, and 45 days of ripening.

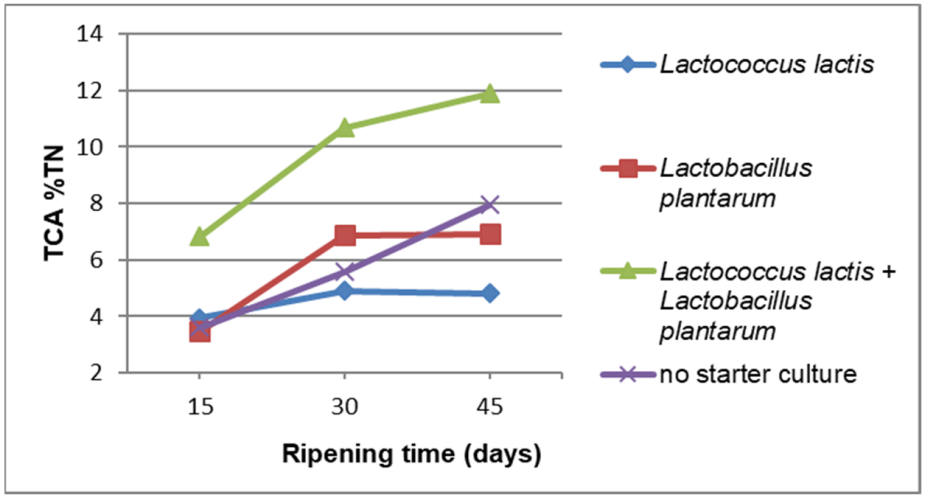

Figure 6. The 12%-trichloroacetic-acid-soluble nitrogen fraction in the total nitrogen (TCA-SN %TN) of Sir iz mišine at 15, 30, and 45 days of ripening.

WSN %TN values represent the amounts of small and medium-sized polypeptides, free amino acids, and their solids, which are formed as a result of chymosin activity, along with the milk proteases present at the beginning of the ripening and, to a lesser extent, plasmin activity [36]. Considering the results obtained in this research, the intensity of primary proteolysis of Sir iz mišine produced from raw sheep milk without autochthonous probiotic bacteria was low, while it was noticeably higher when autochthonous probiotic starter cultures were added (Figure 5). Previous research on 45-day-matured Sir iz mišine from raw sheep milk without the addition of starter cultures [37] reported intensive proteolytic reactions. The average WSN %TN content after 45 days of ripening is 21.48% [9]. As previously noted, the higher values of WSN %TN (23.41–30.4%) in this research could be attributed to the higher retention and activity of residual rennet—probably as a consequence of the more efficient acidification activity of the added autochthonous probiotic bacteria—and to the pasteurization of the sheep milk before the addition of the autochthonous probiotic bacteria.

The water-soluble proteose peptone fraction WSN %TN increased in all cheeses during ripening, with the exception of the Sir iz mišine without added autochthonous probiotic bacteria after 30 days of ripening (Figure 5). Sir iz mišine without the addition of an autochthonous probiotic starter culture had a noticeably lower content of WSN %TN in comparison to the cheeses with additional autochthonous probiotic starter cultures. The cheese with the added probiotic culture consisting of *Lactococcus lactis* subsp. *lactis* S1 had the highest content of WSN %TN (25.41%) at the beginning of ripening (15th day) compared to the cheeses with the addition of *Lactobacillus plantarum* B (18.67%), combined *Lactococcus lactis* ssp. *lactis* S1 and *Lactobacillus plantarum* B (17.84%), and without added autochthonous probiotic bacteria (9.40%). The highest increase in the WSN %TN content between the 15th and 45th days was observed for the cheese with the combined probiotic culture (11.90%), followed by the cheese with added *Lactococcus lactis* subsp. *lactis* S1 (4.99%), the cheese with added *Lactobacillus plantarum* B (4.74%), and the control cheese without an additional probiotic starter culture (2.76%). This is an indicator of the noticeable contribution of the bacterium *Lactococcus lactis* ssp. *lactis* S1—both alone, and in the form of combined culture with *Lactobacillus plantarum* B—to proteolysis during cheese ripening. The highest values of WSN %TN at the end of ripening were found in the cheeses produced with *Lactococcus lactis* subsp. *lactis* S1 (30.40%) and combined probiotic bacteria (29.74%), with values more than double in comparison to the control cheese (12.16%). This indicates that in addition to the main role of chymosin activity, added autochthonous probiotic bacteria play certain roles in these proteolytic reactions, due to cell-envelope-associated proteinases responsible mainly for the production of shorter peptides, which are then degraded by a wide range of peptidases to form free amino acids [6]. Similar results were obtained by Celik and Tarakci [38], who discovered that starter cultures contribute to proteolysis with varying breakdown capacities. Hayaloglu et al. [39] reported that Tulum cheese produced with the addition of mesophilic starters resulted in the most satisfactory scores in terms of volatile compounds composition and sensory attributes. Hayaloglu et al. [40], referring to the findings of Bedel and Kilic [41], stated that the use of starter cultures increased the levels of WSN %TN in Turkish Izmir brined Tulum cheese from 17.9% to 35.7%, which also shortened the ripening time from 90 to 45 days. These findings are consistent with results published by Kostelac et al. [15], stating that the use of probiotic bacteria improves the sensory properties of Sir iz mišine, which could decrease the ripening time from 45 days to 30 days without changing the characteristic flavor and taste of the cheese itself. Rako et al. [7] found that considerably higher values of WSN %TN (29.25%) were present in raw-sheep-milk Sir iz mišine matured for 30 days. The higher values of WSN %TN in this research could also be attributed to the higher retention of residual rennet in the curd as a consequence of adding 1.1 kg of NaCl to 100 L of milk prior to renneting, increasing the ionic strength of the milk, and leading to a decrease in the zeta potential and less negatively charged surfaces of casein micelles. As the surfaces of casein micelles become less negatively charged, increased interactions between them and chymosin molecules consequently increase the initial retention of chymosin in curd [42]. Medjoudj et al. [43] reported low WSN %TN

contents (13.04%) at the end of the ripening (64th day) of Bouhezza cheese—a raw-goat's-milk cheese matured in an animal skin. The authors of the paper attribute the reason for the low WSN %TN content to the coagulation of milk by spontaneous lactic acid fermentation, and not by using rennet.

Secondary proteolysis did not accompany primary proteolysis. During cheese ripening, the WSN %TN fraction is degraded to TCA-SN %TN, which represents the amounts of non-protein nitrogen and free amino acids that occur as a result of proteolytic activity of the starter culture and non-starter microorganisms and, to a lesser extent, chymosin activity [1,2,44,45]. The TCA-SN %TN values obtained in this research were slightly lower in comparison to the results of previous research. Tudor Kalit et al. [9] found that the average TCA-SN %TN was 10.67%, whilst in this research the values of TCA-SN %TN were lower for all cheeses, with the exception of the cheese to which both strains of autochthonous probiotic starter culture had been added (Figure 6). The obtained results confirmed good synergistic activities between these autochthonous probiotic bacteria when taking into consideration their secondary proteolytic activity—probably due to the higher microbial biomass in the cheese body [46]. Hayaloglu et al. [39] found that starter cultures had a significant impact on increasing of TCA-SN %TN in Turkish goat cheese.

4. Conclusions

The autochthonous probiotic bacteria *Lactobacillus plantarum* B and *Lactococcus lactis* subsp. *lactis* S1 isolated from Sir iz mišine—a Croatian cheese that ripens in an animal-skin sack—could be added during cheesemaking to accelerate ripening via the intensification of the proteolysis of the cheese. Each culture combination influenced the proteolysis differently. The most efficient variety in terms of proteolysis was the mixture of both autochthonous bacteria at a 1:1 ratio. These results highlight that autochthonous probiotic bacteria, in addition to having a functional value, could improve the ripening properties of the cheese. More intensive proteolysis of the cheese with added autochthonous probiotic bacteria could be a potential approach to accelerate the ripening process of this cheese, considering the fact that demand for this cheese from local consumers and tourists in Croatia is very high.

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