



Effect of cooking temperature on alkaline phosphatase in the production of raw-milk Pecorino cheese

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ABSTRACT

Alkaline phosphatase (ALP) is a native raw-milk enzyme used in many countries as the standard assay for rapidly validating the milk pasteurization process. Due to the increased restrictions on the production or import of cheeses produced from unpasteurized milk, ALP activity (<10 mU/g) in cheese was measured as a simple and reliable method to check proper milk pasteurization in cheese for both safety inspection and trading controls. In Sicily, the artisanal cheesemaking of the Protected Denomination of Origin (PDO) semi-hard cheeses made with raw sheep milk, includes the cooking of the curd, after whey separation, in a wooden vat under hot Scotta whey ($\geq 80^{\circ}\text{C}$), for 3 to 4 h, and finally is left to cool at ambient temperature. Thus, the temperatures adopted during cheesemaking may inactivate the ALP enzyme. To this purpose, the aim of this study was to demonstrate how different temperatures of Scotta whey (35°C [T35], 60°C [T60], 70°C [T70], 80°C [T80], 90°C [T90], and 100°C [T100]) used during the second cooking of Pecorino cheeses after molding for 3 h, influence the ALP activity in fresh and 3-mo aged cheese, both at core and outside. The results highlight that the rate of reduction of ALP was greater with increasing temperature of the second cooking, in particular for T 80°C curd, indicating that the use of Scotta whey $>80^{\circ}\text{C}$ could be a breakpoint able to reduce the ALP activity to values <10 mU/g. Different effects between the core and the outside portions of the experimental cheeses were found, with a decrease in ALP activity more on the outside than in the core portions, in both fresh and 3-mo aged cheeses, for T80, T90, and T100 treatments. Care must be taken in using ALP to control the use of pasteurized milk in the production of PDO cheeses without considering the cheesemaking processes, such as the second cooking, which could be equal to pasteurization, and an adequate interaction of time and temperature can reduce the ALP activity to

values comparable with cheeses produced with pasteurized milk.

Key words: alkaline phosphatase, raw-milk cheeses, Pecorino cheese, cooking temperature

INTRODUCTION

Alkaline phosphatase (ALP) is a native raw-milk enzyme, normally inactivated with high temperatures treatments (Kosikowski, 1988). An ALP test determination is used in many countries as the standard assay related to the rapid validation of the milk pasteurization process (Vamvakaki et al., 2006; Rankin et al., 2010). The rationale for its adoption is based on the fact that ALP is slightly more resistant to thermal inactivation than target bacterial pathogens. In terms of food safety, if ALP activity is greatly reduced, then the target bacterial pathogen population is at least similarly reduced, thus arguing that the pasteurization process was successful. Furthermore, due to the increased restrictions to production or import of cheeses produced from unpasteurized milk (Pellegriano and Donnelly, 2004), ALP activity in cheese was measured as a simple and reliable method to check proper milk pasteurization in cheese for both safety inspection and trading controls. Indeed, a positive result with the ALP test in cheese would indicate a raw milk product (Chávarri et al., 1998).

In the 2021 report on the activities carried out by the Inspectorate for Fraud Repression and Quality Protection of Agri-food Products and Foodstuffs (ICQRF, 2021), Control Authority of the Italian Ministry of Agriculture, in the section “Milk and Dairy Product,” it was reported that among the main infringements was “Protected Denomination of Origin (PDO) cheeses non-compliant to the product specifications (cheese produced with milk that had undergone thermic treatments while the product specifications foresee the use of raw milk).”

From personal communication from different Sicilian Protected Denomination of Origin (PDO) producers of stretched curd cheeses, we learned that they received inspection reports of fraud according to the ICQRF (2021), for having declared that the product

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was obtained from raw milk when analyses of ALP in the cheese showed very low values due to the use of pasteurized milk.

The reference ALP values in the inspection reports referred to a work by Egger et al. (2016) that proposed a limit for ALP activity (10 mU/g) in cheese from pasteurized milk, where lower values in cheese might indicate the use of pasteurized milk in the production. The same authors also declare that these indications are not applicable to stretched curd cheeses. Similar indicators have been suggested by Desbourdes et al. (2008), between 2 and 10 mU/g.

In this regard it should be noted that the REG. (EC) 1664/2006 (European Commission, 2006) establishes the ALP activity limit in pasteurized cow milk at 350 mU/L; however, for milk from other species and for cheese no limits have been established.

These circumstances have generated significant concerns in the world of producers, Italian PDO cheese producers among them. It follows that some PDO Protection Consortia have introduced the ALP test as a precaution in their control plans, as an antifraud tool to control the use of pasteurized milk in the production, to evaluate whether the traditional production process has been correctly followed (Pellegrino et al., 2021).

The target limit values of the ALP test vary according to the PDO consortium control plan, as well as for the analytical methods. For example, Grana Padano PDO poses a limit of 300 mU/L (fluorometric method), and the traditional specialty guaranteed mozzarella requires a maximum of 12 mg of phenol/g (colorimetric assays).

Several studies have reported that manufacture conditions, including the temperature at which the curd is cooked, texture, the size of the cheese wheel, and high variability can affect the residual activity of ALP with different distribution percentages depending on the part of the cheese (Cattaneo et al., 2008; Bisig et al., 2010; Egger et al., 2016). Recently Todaro et al. (2021) demonstrated that in PDO Pecorino Siciliano cheese production, the high variability between dairies, the temperature, and the time at which the curd is cooked influence residual ALP activity, with a wide variability of values and an unclear difference between cheeses produced with raw or pasteurized milk.

The agri-food sector in Sicily represents an important resource for the entire region, and dairy production has always played a central role in the livelihood of the island's economy, especially in inland areas. Several traditional historical cheeses are produced in Sicily, of which 5 have PDO, including 2 semi-hard cheeses made with raw sheep milk, Pecorino Siciliano PDO, and Piacentinu Ennese PDO (SgROI and Modica, 2022).

For these latter 2 cheeses, a reasonable trend of manufacture can be observed, with modest quantities produced. According to the data provided by the Consorzio per la Ricerca nel Settore della Filiera Lattiero-Casearia (CoRFilaC) certification agency, in 2022, 5,954 wheels of Pecorino Siciliano PDO were produced and certified, for a total of 66,827 kg, as well as 11,324 wheels of Piacentinu Ennese PDO, for a total of 47,828 kg.

Moreover, as reported by the Rapporto ISMEA-Qualivita (2022) General Report, in 2022 the PDO and Protected Geographical Indication (PGI) productions in Italy and Sicily reached a total of 4.68 billion and 87 million euros in production value, respectively, representing 59% of PDO and PGI foods, with an economic impact on cheeses in Sicily, based on 36 products, of over 3 million euros.

In Sicily artisanal cheesemaking of semi-hard cheeses (e.g., PDO Piacentinu Ennese and Pecorino Siciliano) made with raw sheep milk includes the cooking of the curd, after whey separation, in a wooden vat under hot Scotta whey ($\geq 80^{\circ}\text{C}$), derived from the production of ricotta cheese, for 3 to 4 h, and finally leaving the cheese to cool at ambient temperature (GUCE C170/2020 EUR-Lex-52020XC0518[03]; GUCE C164/2010 EUR-Lex-52010XC0624[02]; GUCE, 2010, 2020). Thus, the temperatures adopted during cheesemaking may inactivate the ALP enzyme. To this purpose, the aim of this study was to demonstrate how the temperatures of Scotta whey used during the second cooking of Pecorino cheeses after molding for 3 h influence the ALP activity in fresh and 3-mo aged cheese, both at core and outside. So far, no studies have focused on the determination of ALP activity in fresh cheeses, before aging.

For this purpose, it was decided to develop a controlled experimental protocol to study the effects of heat treatments on curd through the use of different temperatures of Scotta whey during the second cooking on ALP values in Pecorino cheeses, both fresh (immediately at the end of the cheesemaking process) and 3-mo aged.

MATERIALS AND METHODS

Because no human or animal subjects were used, this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

Experimental Design

The effects of 6 different temperatures of Scotta whey for the second cooking of Pecorino cheese were

investigated with regard to the ALP level of the cheeses. The experiment was repeated in 3 different trials, each on distinct days, by collecting and using raw sheep milk from local farms in Sicily (a different batch per trial). All 3 trials were run at CoRFi-LaC experimental cheese plant (Ragusa, Italy), and cheesemaking was performed following the traditional method of Pecorino cheese production. The curd cooking was carried out using 6 experimental thermal tanks to maintain the temperature and reproduce the condition of cooking using 6 different temperatures of Scotta whey. The scheme of the conducted experimental trials is reported in Figure 1.

Cheesemaking

For each cheesemaking trial, approximately 520 L of raw sheep milk was collected into a large vat. The mean composition of milk used in all 3 trials is reported in Table 1.

No starter culture was used, and lamb rennet paste (Calza Clemente srl, Acquanegra Cremonese, Italia; strength 1:10,000) was added at a temperature of approximately $37.4 \pm 0.3^\circ\text{C}$ in the amount of 28 g per 100 L of milk. After 60 min the curd was broken using the traditional wooden stuff called a rotula, by circular stirring. After cutting, hot water at approximately 85°C was added (60 L, corresponding to 11% vol/wt) to increase the temperature of the curd plus whey to $41.8 \pm 0.6^\circ\text{C}$.

The coagulum was cut into small granules (approximately 4 mm diameter) and left to settle to the bottom of the vat. Afterward, the curd was removed and filled into perforated cylindrical plastic molds.

The drained whey derived from the cheesemaking was used to make ricotta cheese, as described by Mangione et al. (2022), and the resulting deproteinized whey, called Scotta whey, was used for the second cooking of the Pecorino cheese for 3 h. Technological parameters detected during cheesemaking are reported in Table 1.

A total of 18 cheeses per trial were obtained. Each cylinder-shaped cheese, with wheel sizes of 20 cm in diameter and 13 cm high, weighed approximately 4.4 ± 0.17 kg.

For each experimental thesis, 2 cheeses were sampled fresh, one after the second cooking (3 h) and the second after cooling (3 h) without any salting. After 24 h of production, the third cheese for each tank was dry salted using 3% of dry salt per kilogram of cheese and ripened for 3 mo in a ventilated room at 13°C with a relative humidity of about 80%. During the ripening period each cheese was hand turned and brushed weekly to remove molds.

Curd Second Cooking

To develop the experimental design (Figure 1) to carry out the second cooking, 6 plastic containers were prepared and covered with thermal glass wool on the whole surface, including the lid, to reduce heat losses during the second cooking.

The process of second cooking was divided into 2 phases: (1) the first, during which the curd is dipped in the Scotta whey, as in the traditional method, for a period of 3 h; (2) the second, cooling at room temperature (on average $16^\circ\text{C} \pm 0.1$), with measurement of the curd temperature in the following 3 h after the removal of the curd from the Scotta whey.

The experimental temperatures of the Scotta whey were 35°C (**T35**), 60°C (**T60**), 70°C (**T70**), 80°C (**T80**), 90°C (**T90**), and 100°C (**T100**). The temperature of 35°C was considered to be the control temperature. The ratio of cheese to Scotta whey was equal to 1:4 wt/wt. For each thermized tank, 3 Pecorino cheese wheels were dipped in the Scotta whey.

Curds (core and outside portions) and Scotta whey temperatures were measured every 15 min during the first phase of second cooking and every 30 min during the 3 h of cooling time.

According to Egger et al. (2016) and Pellegrino et al. (1997), who indicated that zonal differences in ALP activity were found in hard cheeses with large wheels and relatively high curd cooking temperatures, samples were taken both at the core and at the outside for each cheese wheel.

The temperature measurements were carried out using a temperature tester with a stainless-steel penetration probe (Hanna Instruments Villafranca Padovana, Padova, Italia).

Cheese Sampling

In each tank 3 Pecorino cheese samples were cooked into the Scotta whey for 3 h, as in the traditional method. For each trial, cheese sampling was distributed as follows: (1) one cheese immediately at the end of the first phase of the second cooking for 3 h (after cooking, **AC**); (2) one cheese immediately at the end of the cooling time for 3 h (after cooling time, **ACT**); (3) one cheese after 3 mo of aging (after aging, **AA**).

To sample the cheeses for analyses, the wheel of each cheese was divided into 8 slices of approximately 500 g (Figure 2). One of the 8 slices was randomly selected to take a sample of the core (**ALPc**) and outside (**ALPs**) of the wheel (Figure 2a). To prevent sample cross-contamination with microbial phosphatase, all tools and surfaces were disinfected with ethanol.

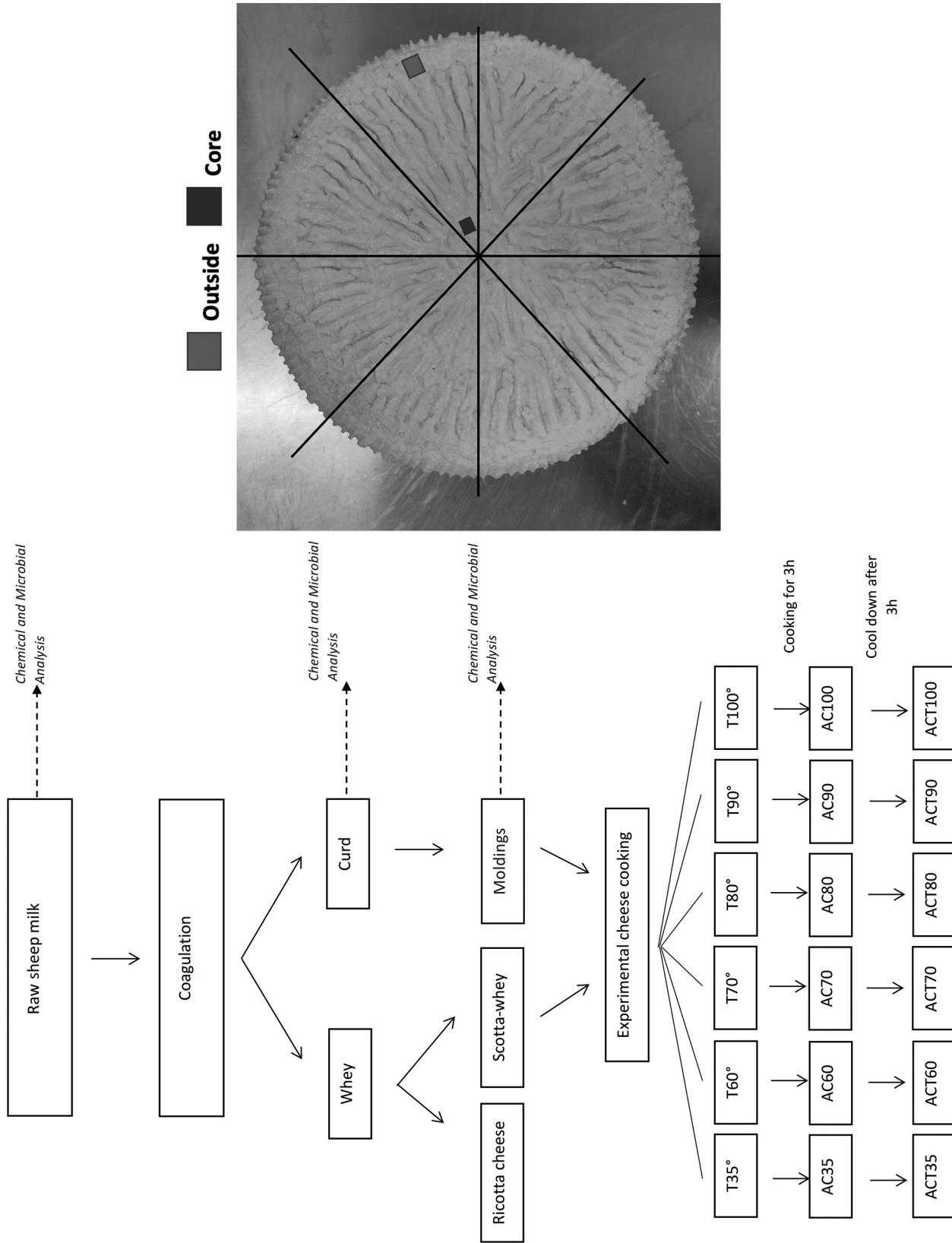


Figure 1. Scheme of experimental Pecorino cheesemaking trial to test the effect of temperatures (T; 35, 60, 70, 80, 90, and 100°C) during second cooking on alkaline phosphatase activity. AC = after cooking; ACT = cooling time.

Table 1. Chemical composition of milk, technological parameters and chemical composition of curd before first cooking, and characteristics of Scotta whey used in Pecorino cheesemaking trial¹

Item	Mean (n = 3)	SD
Milk		
Quantity (L)	510	10.00
pH	6.65	0.08
TS (g/100 g)	19.01	0.68
Protein (g/100 g)	6.09	0.17
Fat (g/100 g)	7.28	0.55
Protein in TS (g/100 g)	32.02	0.37
Fat in TS (g/100 g)	38.26	1.53
Lactose (g/100 g)	4.26	0.04
Titratable acidity (SH°)	11.90	0.30
Urea (g/100 g)	25.33	3.51
Casein (g/100 g)	4.86	0.16
ALP (mU/L)	2,838,566.7	320,015.2
Coagulation step		
T, clotting (°C)	37.43	0.32
Rennet quantity (g)	148.33	8.50
T, end of cutting (°C)	37.5	0.72
T, water for first cooking (°C)	85.5	2.18
Water quantity for first cooking (L)	60	
Final T after first cooking (C°)	41.77	0.59
Curd before first cooking		
pH	6.58	0.10
TS (g/100 g)	47.75	0.07
Fat (g/100 g)	26.25	3.75
Protein (g/100 g)	20.85	1.63
Fat in TS (g/100 g)	54.98	7.93
Protein in TS (g/100 g)	43.66	3.34
ALP activity (mU/g)	3,393.3	403.65
Scotta whey		
pH	6.05	0.03
TS (g/100 g)	6.53	0.06
Protein (g/100 g)	0.70	0.04
Fat (g/100 g)	0.14	0.02
Lactose (g/100 g)	5.00	0.03
Protein in TS (g/100 g)	10.72	0.65
Fat in TS (g/100 g)	2.19	0.22
Titratable acidity (SH°)	5.20	0.20
ALP activity (mU/L)	<10	

¹SH° = Soxhlet-Henkel degrees; ALP = alkaline phosphatase; T = temperature.

ALP Activity Measurement in Milk and Cheese Samples

For determination of native and residual ALP activity levels after heat treatment, the Fluorophos methods parts 1 and 2 for milk and cheese, respectively, according to the EN ISO 11816 (ISO, 2016), were used. The residual activity of ALP was measured using a dedicated fluorimeter (FLM200, Advanced Instruments Inc., USA). The test portion of 0.075 mL of diluted ewe milk sample or cheese extract, respectively, was added to glass cuvettes with 2 mL of a specific Fluorophos substrate (FLA124, Advanced Instruments Inc., USA). The results were expressed in mU of ALP activity per liter of milk or per gram of cheese, respectively, where 1 U is the amount of ALP that catalyzes the transformation of 1 μ mol of substrate per minute. In order to be

within the linear response of the instrument (<7,000 mU/L), the test samples of both milk and cheese were diluted appropriately according to the ISO methods 11816-1 and -2, respectively. The value was corrected multiplying by the respective dilution factors.

Chemical Analysis

For each trial, bulk milk and Scotta whey were analyzed for chemical composition using a MilkoScan apparatus (Milkoscan FT 6,000 milk analyzer; Foss Electric, Hillerød, Denmark).

Statistical Analysis

Heat load for each cheese was estimated as the integral of the temperature function over time, adopted as the variable that describes the interaction between time and the temperature measured at the core and at the outside portion. The definite integral between the beginning of the second cooking and AC and ACT was estimated using the trapezoid rule, by approximating the region under the curve of the temperature function as a trapezoid and calculating its area using the following formula:

$$\sum_{k=1}^{AC \text{ or } ACT} \frac{t(x_{k-1}) + t(x_k)}{2} \Delta x_k,$$

where $t(x_k)$ is the temperature at time k and Δx_k is the time interval between measurements.

Data on ALP activity and on the integral of temperature per portion (outside and core) were statistically analyzed using a linear model with temperature of cooking (T35, T60, T70, T80, T90, and T100) and time of sampling (AC and ACT) as fixed factors. Student's t -tests ($\alpha = 0.05$) were used to determine differences between temperatures of cooking means when significant differences for that effect were found. Time of permanence above 45°C was calculated as the minutes the curd had temperature above the related temperature. Linear regression between ALP values and heat load was estimated using 3-degree polynomials.

RESULTS AND DISCUSSION

Scotta Whey Temperatures During Second Cooking

The Scotta whey used for the second cooking of the cheese, for each tank, reflected the same temperature established by the experimental scheme, 35°C (tank 35°C), 60°C (tank 60°C), 70°C (tank 70°C), 80°C (tank 80°C), and 90°C (tank 90°C), respectively, except for

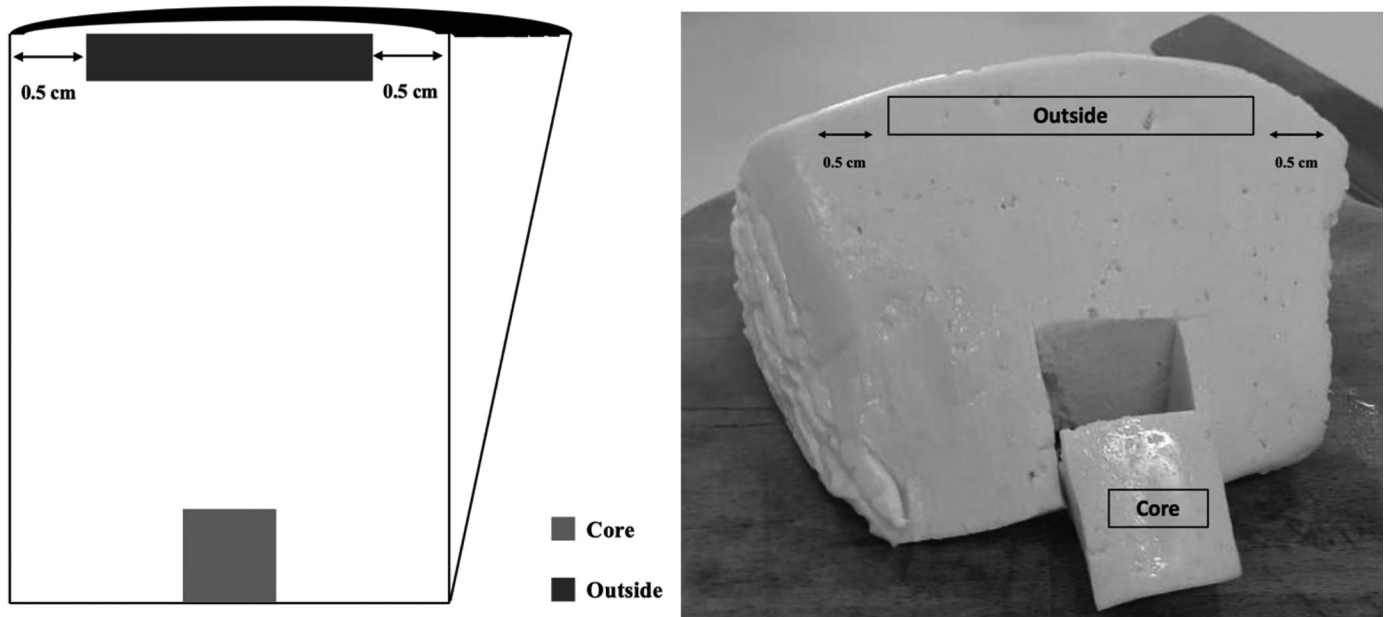


Figure 2. Sampling scheme of Pecorino cheese slice for alkaline phosphatase determination.

tank 100°C, where the maximum temperature reached by the Scotta whey during heating was $93^{\circ} \pm 5^{\circ}\text{C}$.

Mean thermal variations reached by the Scotta whey during the second cooking, for each tank, detected every 15 min, are reported in Figure 3c.

Except for the 35°C tank, whose temperature kept constant, for all other tanks, due to the use of glass wool as a thermal insulator, the starting temperatures of the Scotta whey gradually and uniformly decreased 12 degrees after 3 h.

Curd Temperatures During Second Cooking

The goal of the experiment was to verify how the use of different temperatures of the Scotta whey during the second cooking process influenced the temperature of the curd and consequently the level of ALP activity.

The starting hypothesis was to identify, if possible, the breakpoint able to thermally inactivate ALP. This could result in a good indicator as equivalent of pasteurization in traditional cheesemaking, with an easier indirect control of the presence of pathogens.

The effect of the Scotta whey at different temperatures (T35 T60, T70, T80, T90, and T100) on ALP activity was assayed both at the core and at the outside of the AC and ACT cheeses.

Averaged values of the temperatures reached at the core and at the outside, and respective ALP_c and ALP_s values of AC and ACT cheeses detected in all 3 trials, are reported in Tables 2 and 3, respectively.

Tables 2 and 3 highlight temperature values higher than 45°C (an empirical value that refers to the findings of the survey by Todaro et al. 2021), to identify a breakpoint at which the thermal increase of the curd (cheese) has effects on ALP levels, as well as in consideration of the duration for which temperatures above 45°C persist, and the relative interactions (temperature-time).

In Figure 3, mean temperatures measured for the Scotta whey during the second cooking, and those detected on the AC and ACT cheese, are reported for each experimental thesis.

As expected, the temperatures of the curds increased as the experimental temperatures of the Scotta whey increased (Tables 2 and 3; Figure 3a, b): from 47.07°C for T60, 50.12°C for T70, 54.52°C for T80, 57.88°C for T90, and 59.35°C for T100, for the core; from 49.43°C for T60, 53.35°C T70, 57.30°C for T80, 62.05°C for T90, and 62.15°C for T100, for the outside.

The temperatures of the curd rose more slowly in the core than at the outside but cooled more slowly during the cooling time, in accordance with Pellegrino et al. (1997; Figure 3).

To calculate the persistence of the target temperatures of the curds it was also necessary to consider the data of the cooling time period, at both the core and the outside, precisely because the curds placed at room temperature, after immersion in the Scotta whey, cooled slowly, with a different speed depending on the different experimental temperatures of the Scotta whey.

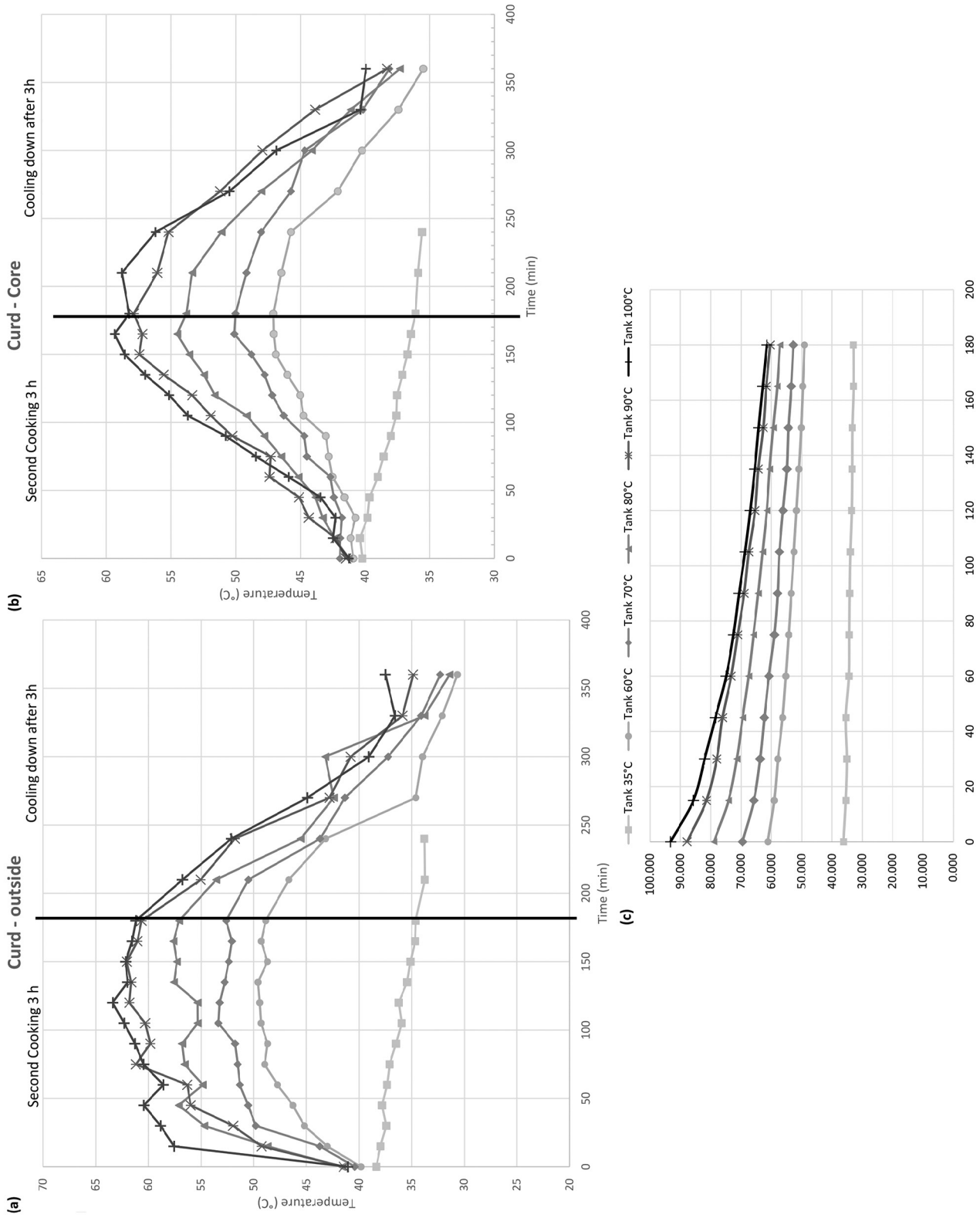


Figure 3. Temperatures of Pecorino cheese curd at outside (a) and core (b), and Scotta whey (c), measured during second cooking at different temperatures of Scotta whey (35, 60, 70, 80, 90, and 100°C).

Table 2. Temperature values (°C) reached at the core (ALPc) and outside (ALPs) of experimental Pecorino cheeses, for determination of alkaline phosphatase (ALP) activity, after cooking (AC) and after cooling time (ACT)¹

Item	Time (min)	T35	T60	T70	T80	T90	T100	
Second cooking, core	0.00	40.20 ± 1.89	40.90 ± 2.20	41.95 ± 2.13	41.25 ± 1.45	41.47 ± 1.25	41.23 ± 2.75	
	15.00	40.40 ± 1.76	41.12 ± 2.33	41.92 ± 1.93	42.35 ± 0.48	42.27 ± 1.53	42.48 ± 2.43	
	30.00	39.82 ± 1.41	40.75 ± 1.67	41.78 ± 1.88	43.28 ± 0.36	44.35 ± 2.65	44.00 ± 2.52	
	45.00	39.65 ± 1.43	41.58 ± 2.19	42.42 ± 1.13	43.83 ± 1.44	45.10 ± 3.20	45.15 ± 2.49	
	60.00	39.02 ± 1.57	42.52 ± 2.41	42.68 ± 0.84	45.15 ± 1.27	47.38 ± 3.25	46.92 ± 3.47	
	75.00	38.57 ± 1.32	42.82 ± 1.20	44.50 ± 2.38	46.48 ± 1.86	47.30 ± 2.88	48.43 ± 4.08	
	90.00	38.02 ± 1.14	43.03 ± 1.88	44.70 ± 0.26	47.78 ± 2.02	50.27 ± 4.29	50.78 ± 3.02	
	105.00	37.58 ± 0.95	44.77 ± 1.39	46.28 ± 1.73	49.15 ± 1.75	51.93 ± 3.31	53.72 ± 3.35	
	120.00	37.53 ± 0.85	45.02 ± 0.94	47.18 ± 1.44	51.62 ± 1.68	53.37 ± 1.75	55.15 ± 2.98	
	135.00	37.12 ± 0.43	46.00 ± 0.87	47.77 ± 0.33	52.45 ± 1.38	55.57 ± 3.43	57.00 ± 2.07	
	150.00	36.72 ± 0.88	46.90 ± 0.71	48.78 ± 1.28	53.58 ± 0.81	57.45 ± 1.00	58.58 ± 1.58	
	165.00	36.47 ± 0.21	47.07 ± 0.49	50.12 ± 0.50	54.52 ± 0.13	57.22 ± 1.04	59.35 ± 1.73	
	180.00	36.10 ± 0.41	47.10 ± 0.48	50.02 ± 0.93	53.82 ± 0.49	57.88 ± 0.98	58.23 ± 0.97	
	ALPc, AC cheese (mU/g)		3,625.3 ± 34.53	3,350.80 ± 426.49	3,059.53 ± 351.76	1,031.17 ± 440.98	69.23 ± 85.25	92.53 ± 82.55
	Cooling time, core	210.00	35.90 ± 0.42	46.47 ± 0.38	49.17 ± 1.79	53.37 ± 0.81	56.07 ± 2.80	58.80 ± 0.89
		240.00	35.60	45.73 ± 0.64	48.03 ± 1.16	51.10 ± 2.69	55.20 ± 2.19	56.20 ± 1.56
		270.00		42.10 ± 1.71	45.73 ± 1.50	48.00 ± 2.40	51.20 ± 2.88	50.47 ± 3.17
		300.00		40.23 ± 1.24	44.66 ± 2.33	44.10 ± 3.56	47.93 ± 3.41	46.87 ± 3.81
330.00			37.43 ± 2.08	37.43 ± 2.08	40.17 ± 2.71	41.10 ± 2.45	40.37 ± 5.43	
360.00			35.50 0.00	35.50 0.00	38.13 ± 2.10	37.30 ± 1.81	38.30 ± 4.25	
ALPc, ACT cheese (mU/g)		3,515.30 ± 353.18	2,699.80 ± 354.08	1,719.30 ± 563.91	110.99 ± 31.29	4.25 ± 1.53	1.28 ± 0.20	

¹T designations indicate temperature (°C) of Scotta whey used in cheesemaking. Values shown are mean ± SD. The bold values indicate temperature above 45°C.

Table 3. Temperature values (°C) reached at the core (ALPc) and outside (ALPs) of experimental Pecorino cheeses, for determination of alkaline phosphatase (ALP) activity, after cooking (AC) and after cooling time (ACT)¹

Item	Time (min)	T35	T60	T70	T80	T90	T100	
Second cooking, outside	0.00	38.35 ± 0.07	39.82 ± 2.43	40.40 ± 1.85	41.6 ± 4.74	41.45 ± 2.40	41.07 ± 1.29	
	15.00	38.78 ± 1.80	42.23 ± 1.74	43.73 ± 1.48	48.85 ± 1.53	50.93 ± 2.91	59.38 ± 6.26	
	30.00	37.42 ± 2.12	45.20 ± 1.00	49.80 ± 1.49	54.70 ± 0.48	51.95 ± 4.03	58.83 ± 6.34	
	45.00	38.25 ± 0.64	45.50 ± 2.98	50.53 ± 3.22	56.23 ± 4.97	57.48 ± 3.22	62.13 ± 3.62	
	60.00	37.33 ± 1.66	47.75 ± 3.08	51.32 ± 3.88	54.83 ± 2.32	56.30 ± 2.48	58.58 ± 4.08	
	75.00	38.03 ± 1.77	48.28 ± 1.96	51.52 ± 1.94	56.80 ± 1.75	60.48 ± 1.28	61.13 ± 2.25	
	90.00	36.48 ± 0.39	48.68 ± 2.40	51.78 ± 0.90	56.80 ± 2.92	59.82 ± 2.11	61.28 ± 2.01	
	105.00	36.00 ± 0.42	49.18 ± 1.19	53.35 ± 0.82	54.88 ± 0.99	61.23 ± 2.66	60.83 ± 2.66	
	120.00	36.23 ± 0.11	49.43 ± 0.77	53.23 ± 1.37	55.33 ± 2.12	61.80 ± 1.60	63.38 ± 0.81	
	135.00	35.53 ± 0.04	48.83 ± 1.34	52.75 ± 0.85	56.95 ± 1.43	61.43 ± 1.71	61.50 ± 0.85	
	150.00	35.12 ± 0.88	48.70 ± 0.79	52.37 ± 0.60	57.30 ± 1.13	62.05 ± 1.18	62.15 ± 1.19	
	165.00	34.23 ± 0.81	48.70 ± 1.07	52.07 ± 0.34	57.15 ± 0.86	61.33 ± 0.80	60.45 ± 1.98	
	180.00	34.62 ± 0.18	48.87 ± 0.95	52.60 ± 0.26	57.08 ± 1.35	60.65 ± 1.22	61.18 ± 1.73	
	ALPc, AC cheese (mU/g)		3,684.67 ± 423.56	2,057.00 ± 445.58	653.08 ± 367.82	12.37 ± 5.74	2.99 ± 3.04	4.87 ± 7.25
	Cooling time, outside	210.00	33.75 ± 1.48	46.50 ± 1.53	50.50 ± 1.14	53.80 ± 3.42	57.30 ± 5.53	56.45 ± 3.99
		240.00	33.80	43.17 ± 2.72	43.70 ± 5.63	45.53 ± 6.85	51.80 ± 5.04	52.13 ± 2.12
		270.00		32.40 ± 3.89	41.33 ± 6.20	43.90 ± 5.55	44.20 ± 2.44	45.70 ± 3.30
		300.00		33.97 ± 0.55	37.23 ± 2.04	43.20 ± 9.71	40.80 ± 5.13	39.07 ± 5.94
330.00			32.35 ± 0.56	34.10 ± 2.87	35.40 ± 4.42	37.55 ± 3.13	36.60 ± 2.00	
360.00			30.65 ± 1.34	32.30 ± 2.40	31.40 ± 2.79	34.87 ± 4.99	37.50 ± 2.69	
ALPs, ACT cheese (mU/g)		3,720.67 ± 313.47	1,822.37 ± 622.92	255.60 ± 140.32	3.07 ± 1.36	1.20 ± 0.81	0.65 ± 0.10	

¹T designations indicate temperature (°C) of Scotta whey used in cheesemaking. Values shown are mean ± SD. The bold values indicate temperature above 45°C.

The values of temperatures reported in Table 2 and 3 show how important it is to consider the cooling time beyond the cooking time, above all for the core portion, with temperatures exceeding 45°C that might affect ALP content.

As already demonstrated by several authors, the high temperature at which the curd is heated, which remains for several hours, influences the residual ALP activity in cheese, determining a further reduction in the enzyme activity (Pellegrino et al., 1997; Bisig et al., 2010; Egger et al., 2016).

ALP Activity in Fresh Pecorino Cheeses

Table 4 reports the mean ALP values of curd before second cooking and ALP_c and ALP_s values of the AC and ACT cheeses at different temperatures. Compared with the ALP values of the curd before cooking (3,393.30 mU/g), T35 ALP values were slightly higher (on average 3,636 mU/g) in both the core and outside portions of the respective AC and CT cheeses. As with all enzymes, ALP is active in a narrow range of temperatures, with its optimum activity at 37°C and pH 9.65 and in presence of metals as calcium and magnesium. Moreover, this will be likely accompanied, in addition to native ALP, by a further increase of microbial ALP at 35°C, the optimum temperature for microbial growth (Pratt-Lowe et al., 1988; Soares et al., 2013). The differences in the means of temperatures over 45°C, during the second cooking and cooling time of the curd under the different experimental temperatures of Scotta whey, are shown in Figure 4. Curd temperatures were higher at the outside compared with the core, with a difference starting from 2°C up to 6°C with increased Scotta whey temperature.

Figure 5 shows the regression of values of ALP activity with the heat load the curds reached ACT for core ($r^2 = 0.80$) and outside ($r^2 = 0.96$) portions.

The heat load in terms of time-temperature interaction represents the discriminating factor (breakpoint) to obtain a reduction of ALP activity and demonstrates, in operational terms for cheesemakers, whether, at what temperatures, and for how long the use of Scotta whey affects ALP activity.

Mean ALP contents for T80, T90, and T100 of the cooled cheeses were significantly lower in both portions of the cheese ($P < 0.001$) compared with the other temperatures. Reduction of ALP content in both portions increased with increasing temperature of the second cooking. In particular, at the core for T80 curd a temperature of 50.58°C in 225 min was observed (cooling time included), with ALP value of 110.99 mU/g. In the outside portion a temperature of 54.73°C in 240 min

was observed (cooling time included), with ALP value of 3.07 mU/g.

The use of Scotta whey with temperatures above 90°C brought the ALP values, both at the core and the outside, lower compared with the values suggested by Egger et al. (2016), lower than 10 mU/g, and lower than those suggested by Desbourdes et al. (2008), between 2 and 10 mU/g, used to indicate dairy products produced with pasteurized milk.

The common temperature of Scotta whey used by Sicilian cheesemakers ranges between 80 and 85°C, and from the present result for T80, the ALP level of ACT cheese is low both at the core (110.99 mU/g) and at the outside (3.07 mU/g). If we compare these results with T90, it is reasonable to affirm that with Scotta whey around 85°C, even in the core, ALP content is lower than 10 mU/g. Indeed, due to heat treatment, ALP activity decreases to values more than 500-fold (Wilińska et al. 2007). Moreover, it has been proved that a proper time-temperature combination, slightly less severe than full HTST conditions (72°C, 15 s), caused inactivation of ALP (Fox and Kelly, 2006).

With a temperature of Scotta whey about 70°C (usually not used in cheesemaking), even if a mean temperature of 48.12°C in 180 min at the core and a temperature of 51.82°C in 195 min at the outside were reached, these were not enough to reduce ALP activity, with values at the core and the outside of 1,719.3 mU/g and 255.6 mU/g, respectively, in ACT cheeses. In regard to this, the control plan of the Grana Padano PDO cheese established a minimum limit of 300 mU/g to guarantee the use of raw milk in the production (CSQA Certificazioni Srl., 2019).

In summary, it is not enough to reach thermal peaks higher than 50°C lasting less than 200 min.

As further study, the persistence of each thermal level reached was taken into account rather than the simple average, adopting a synthesis parameter that identifies the heat load for each experimental thesis from T35 to T100, by calculating the temperature-time integral for both the core and the outside portion of the cheeses.

Table 5 shows the values of the heat load estimated as cumulative temperature-time integrals for the temperature >45°C of both the core and the outside, defined between the beginning and the end of cooking and between the beginning of cooking and the end of cooling with respect to the different heat treatments of the Scotta whey. The cumulative temperature values for both core and outside increased significantly with increasing temperature of second cooking (Table 5), with significantly overall higher values ACT. The values of heat loads for core (Figure 6a) and outside (Figure 6b) of curd ACT were regressed linearly

Table 4. Effects of cooking temperature (T; 35, 60, 70, 80, 90, and 100°C) on alkaline phosphatase (ALP) values in core (ALPc) and outside (ALPs) portions of fresh Pecorino cheese after cooking (AC) and after cooling time (ACT)

Cooking temperature	ALPc value (mU/g)			ALPs value (mU/g)		
	AC	ACT	SEM	AC	ACT	SEM
n	3	3		3	3	
Curd before cooking process (starting point)	3,393.3			3,393.3		
T35	3,625.33 ^a	3,515.30 ^{ab}	173.56	3,684.67 ^a	3,720.67 ^a	168.27
T60	3,350.80 ^{ab}	2,699.80 ^c		2,057.00 ^b	1,822.37 ^b	
T70	3,059.53 ^{bc}	1,719.30 ^d		653.08 ^c	255.60 ^{cd}	
T80	1,031.17 ^e	110.99 ^f		12.37 ^d	3.07 ^d	
T90	69.23 ^f	4.25 ^f		2.99 ^d	1.20 ^d	
T100	92.53 ^f	1.28 ^f		4.87 ^d	0.65 ^d	

^{a-f}Means within row for each chemical parameter with different letters are significantly different ($P < 0.05$).

against ALP values using 3-degree polynomials. Figure 6a shows that the values of ALPc of fresh cheeses are significantly reduced, to levels similar to those of cheeses obtained with pasteurized milk, when the values of cumulative temperature are over 12,000 units, reached at the experimental temperature of 80°C. This result is confirmed for the outside portion (Figure 6b), although 12,000 units are reached even with the T70 treatment.

ALP Activity on Pecorino Cheeses Aged 3 Months

Chemical analysis was performed in the experimental Pecorino cheeses after 3 mo of aging (data not reported), from which it was possible to highlight an increase in TS (1%) from T35 to T100 cheeses and relative fat losses of 3% on TS.

Table 5 shows the ALPc and ALPs values of the 3-mo aged cheeses, for each temperature of cooking. Both portions revealed a partial reactivation of ALP activity after ripening, compared with the fresh cheese wheels. Although the microbial ALP contribution cannot be excluded, especially for subpasteurized curd portions, ALP reactivation may have occurred. It is known that under certain conditions of storage ALP can reactivate (Murthy et al., 1976; Prajapati and Desai, 2017). In particular, ALP reactivation always occurs at high temperatures of storage above 10°C, and in this study cheese samples were ripened at 16°C for 3 mo. Moreover, our data showed that when curds reached temperatures over the narrow range of 50 to 57°C for at least an hour during cooking, ALP increased from 6- to almost 50-fold in aged cheese compared with fresh cheese. Mcfarren et al. (1960) found that ALP reactivation occurred by increasing heat treatment over several ranges of temperatures, depending on the fat content of dairy products. Moreover, Prajapati and Desai (2017) suggested that calcium ions enhance ALP activity in milk and a temporary reduction of calcium during ther-

mal processing with a subsequent recombination during storage could be responsible for enzyme reactivation properties.

Furthermore, differently from Battistotti et al. (1997), who showed an ALP activity increase during ripening, especially on the outer layer of the cheeses, in this experiment ALP activity kept the same trend of fresh Pecorino cheese with ALP activity lower on the outer outside than in the core of cheese, the extent of which depended on cooking temperature. These results could be in part related also to the size of the cheese wheels, smaller compared with large-size wheels cheese (e.g., Emmentaler PDO, Emmentaler, and Grana Padano; Pellegrino et al., 1997; Bisig et al., 2010; Egger et al., 2016; Todaro et al., 2021), as the weight of the experimental Pecorino cheese in this study was on average 4.4 ± 0.17 kg with wheels of 20 cm in diameter and 13 cm in height. The thermal treatments were lower in duration than the Grana Padano and Parmigiano Reggiano cheeses, for which, in addition to the second cooking where the curd reaches a maximum temperature of 56°C for 70 min, a molding phase of 48 h is also expected. Due to the larger dimensions of the cheese wheels, they remain in a temperature ranging between 52 and 56°C for 8 to 10 h, thus reducing ALP values more at the core compared with the outside portion (Pellegrino et al. 1997). The ALP results of the 3-mo aged cheeses, for each temperature of cooking, confirm that the heat treatments of the Scotta whey affect ALP activity, with differences between core and outside portions. In particular, lower ALPs values were found for T80, T90, and T100 treatments, whereas higher ALPc values were found at the same experimental conditions (Table 6).

These results indicate the need to pay attention to cheese sampling, which should be representative of the whole cheeses (core and outside), for the Control Authority of the Italian Ministry of Agriculture (ICQRF) to obtain a representative ALP value.

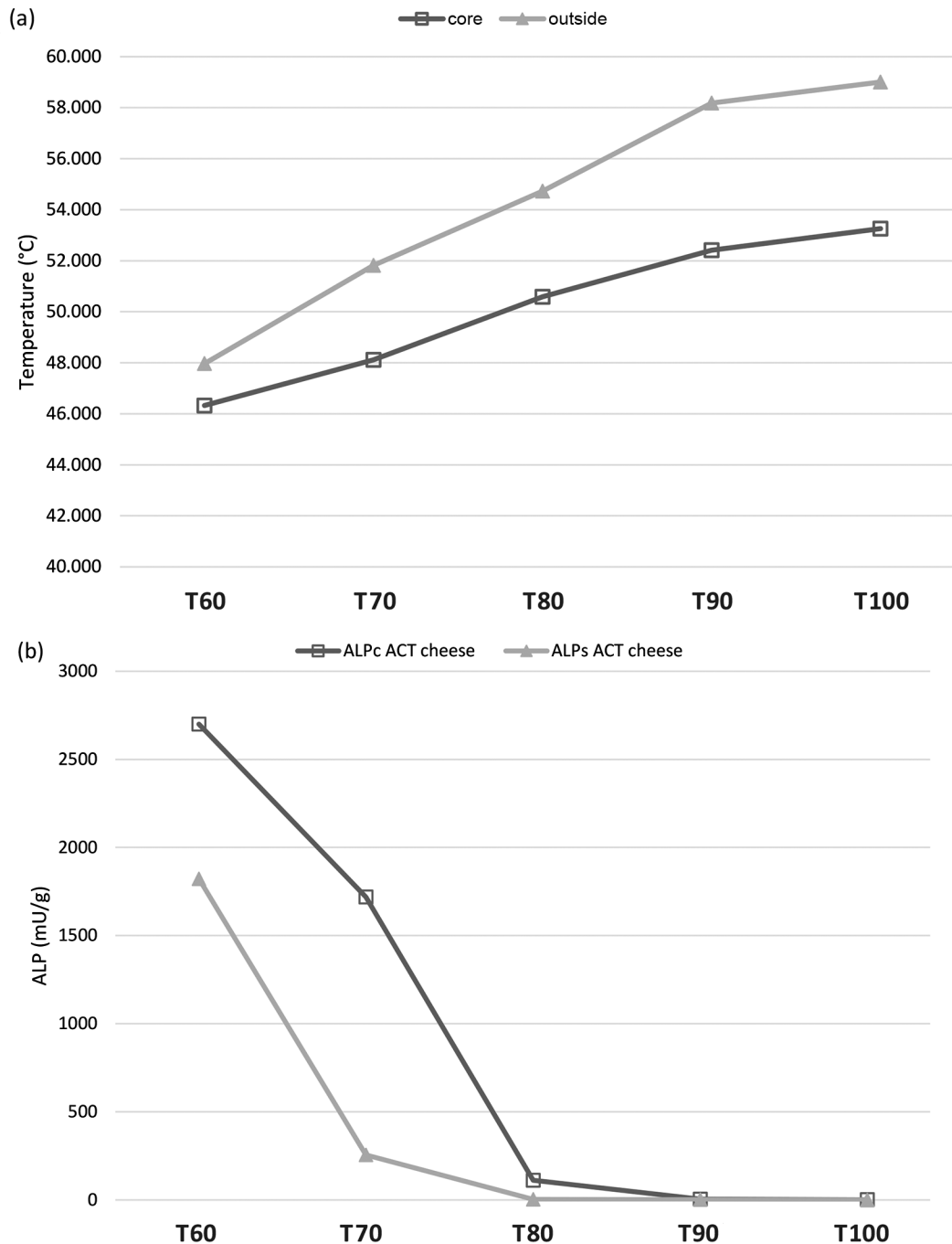


Figure 4. Mean of temperatures (T; 35, 60, 70, 80, 90, and 100°C) over 45°C reached at core (ALPc) and outside portion (ALPs) of curd (a), and alkaline phosphatase (ALP) values after cooling time (ACT) at core (black line) and outside (grey line) portion of Pecorino cheese curd under different experimental temperatures of Scotta whey (b).

Data confirmed that ALP activity is not related only to the use of raw or pasteurized milk but also to the entire cheesemaking process, in which treatments equal to pasteurization are possible, such as the second cooking of the curd. This results in lower ALP values in both fresh and aged cheeses.

Moreover, if it is assumed that the experimental thermal treatments on the curd do not negatively affect the volatile and sensory aromatic components compared with milk pasteurization treatments, in addition to the enzymatic autochthonous microflora activity, these could be helpful to maintain the specific characteris-

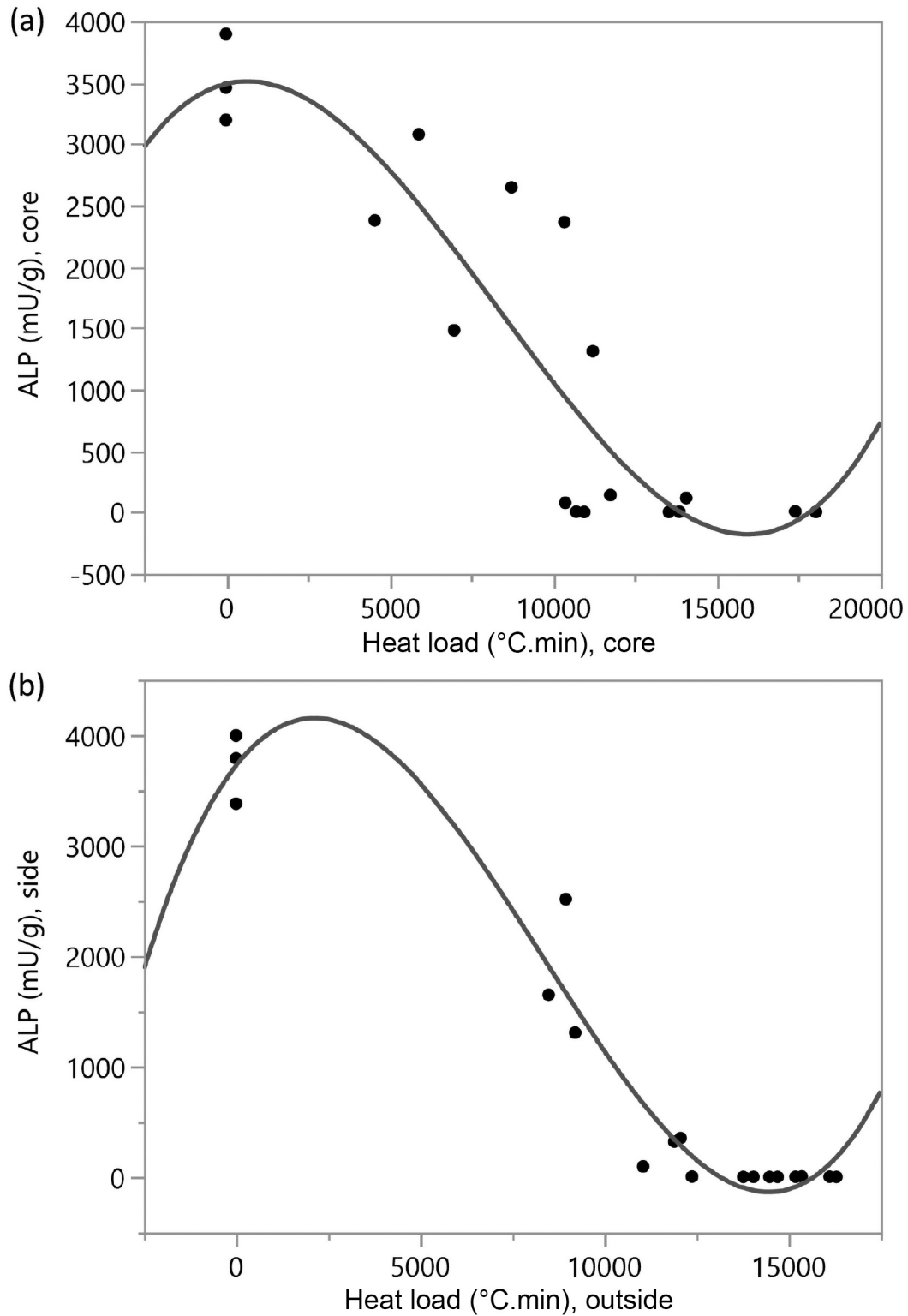


Figure 5. Regression of alkaline phosphatase (ALP) values against heat load of Pecorino cheeses after cooling time, using 3-order polynomials. Heat load ($^{\circ}\text{C}\cdot\text{min}$) = the integral of the temperature function over time, adopted as the variable that describes the interaction between time and the temperature measured at the core (a) and at the outside portion (b).

Table 5. Effects of cooking temperature (T; 35, 60, 70, 80, 90, and 100°C) on heat load for temperatures >45°C of both the core and the outside portions of fresh Pecorino cheeses after cooking and after cooling

Cooking temperature	Heat load values, core (temperature × min)			Heat load values, outside (temperature × min)		
	After cooking	Ambient temperature	SEM	After cooking	Ambient temperature	SEM
n	3	3	1,139.10	3	3	456.74
T35	0.00 ^f	0.00 ^f		0.00 ^g	0.00 ^g	
T60	3,367.25 ^e	6,389.75 ^{cde}		6,732.25 ^f	8,878.25 ^{de}	
T70	3,988.37 ^{de}	9,506.33 ^{bc}		8,401.00 ^e	11,673.00 ^b	
T80	6,199.62 ^{cd}	12,055.88 ^{ab}		9,846.50 ^{cd}	14,307.75 ^a	
T90	6,775.13 ^{cd}	13,980.38 ^a		10,074.38 ^{cd}	14,645.13 ^a	
T100	6,663.25 ^{cd}	14,164.25 ^a		10,486.63 ^{bc}	15,158.38 ^a	

^{a-g}Means within row for each parameter with different letters are significantly different ($P < 0.05$).

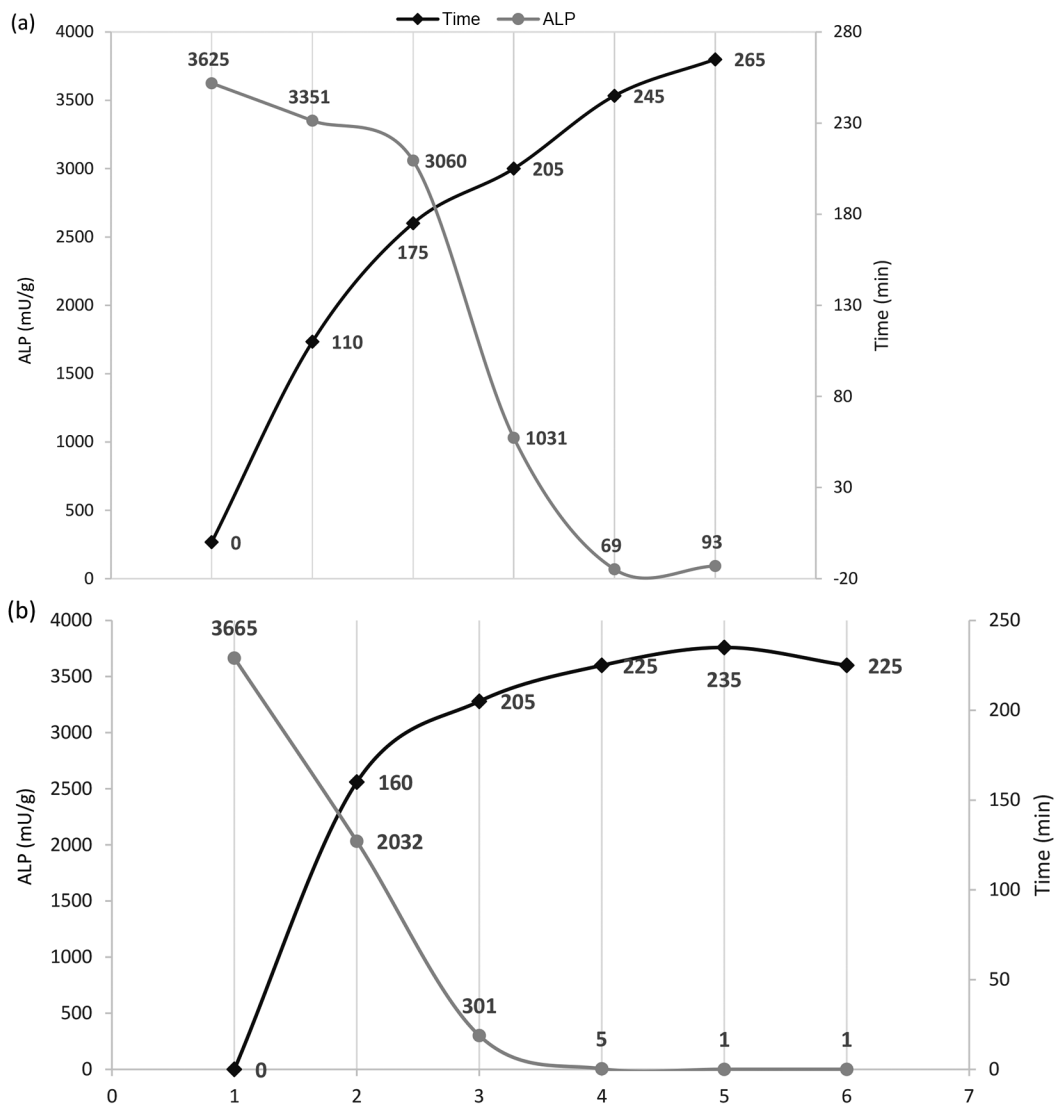


Figure 6. Heat load values for determination of alkaline phosphatase (ALP) activity in core (a) and outside (b) of Pecorino cheese curd after cooling time.

Table 6. Effects of cooking temperature (T; 35, 60, 70, 80, 90, and 100°C) on alkaline phosphatase (ALP) values in core (ALPc) and outside (ALPs) portions of Pecorino cheeses after 3 mo of aging

Cooking temperature	ALPc value (mU/g)		ALPs value (mU/g)	
	After 3-mo aging	SEM	After 3-mo aging	SEM
n	3		3	
T35	4,601.33 ^a	395.35	5,714.00 ^a	386.15
T60	3,529.73 ^{ab}		3,587.33 ^b	
T70	3,078.63 ^b		1,469.63 ^c	
T80	657.48 ^c		79.25 ^d	
T90	193.55 ^c		6.31 ^d	
T100	4.21		1.11 ^d	

^{a-d}Means within row for each chemical parameter with different letters are significantly different ($P < 0.05$).

tics of the territory of origin in the product, as well as guaranteeing consumers the food safety of the product. However, further study is needed to clarify these aspects.

Taking into the account that the production of Italian PDO cheeses plays an important role in the national economy and is often damaged by fraudulent activity, it seems to be relevant to establish possible other parameters, besides ALP, to discriminate the type of milk (raw or pasteurized) used in PDO cheese manufacture. The ultimate goal is to protect both the cheesemakers, who strictly comply with the production regulations, and the final consumers, who knowingly purchase a PDO product and pay more attention to product quality and traceability, as it is safe and guaranteed by a European brand.

This could help in greater transparency and improved trust both in the cheesemaking process and in the authenticity of the final products.

CONCLUSIONS

The results of the effects of thermal treatments using Scotta whey for the second cooking of raw milk Pecorino cheese wheels revealed that the rate of reduction of ALP was greater with increasing temperature of the second cooking in both portions (outside and core), for fresh and 3-mo aged cheese. Lower ALP values were found for Scotta whey T80, T90, and T100 treatments. These technological processes could be considered equal to pasteurization and contribute to controlling bacterial pathogen growth, maintaining a relevant thermophilic dairy microflora. Thus, the use of Scotta whey higher than 80°C and the relative cooling time could be a breakpoint able to reduce ALP activity to values below 10 mU/g. Due to the complexity of the matter, it is necessary that the European Union define guidelines, both for limit values of ALP from pasteurized and raw-milk cheeses and for analytical methods, which must take into account the entire cheesemaking process.

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REFERENCES

- Battistotti, B., E. Brambille, F. Cappa, and G. Ferreandi. 1997. Alkaline phosphatase in milk and ripened cheeses. *Sci. Tecn. Latt. Casearia* 48:157–162.
- Bisig, W., M. T. Fröhlich-Wyder, E. Jakob, and D. Wechsler. 2010. Comparison between Emmentaler PDO and generic Emmentaler cheese production in Europe. *Aust. J. Dairy Technol.* 65:206.
- Cattaneo, S., J. A. Hogenboom, F. Masotti, V. Rosi, L. Pellegrino, and P. Resmini. 2008. Grated Grana Padano cheese: New hints on how to control quality and recognize imitations. *Dairy Sci. Technol.* 88:595–605. <https://doi.org/10.1051/dst:2008024>.
- Chávarri, F., A. Santisteban, M. Virto, and M. De Renobales. 1998. Alkaline phosphatase, acid phosphatase, lactoperoxidase, and lipoprotein lipase activities in industrial ewe's milk and cheese. *J. Agric. Food Chem.* 46:2926–2932. <https://doi.org/10.1021/jf970968n>.
- CSQA Certificazioni Srl. 2019. Piano dei Controlli del Formaggio Grana Padano DOP. DCP001, Rev 5.0.
- Desbourdes, C., M. Nicolas, and A. C. Boitelle. 2008. Phosphatase activity in cheese. AFSSA, CRL Milk and Milk Products. 11th Workshop of the EU CRL for Milk and Milk Products, Vienna, Austria.
- Egger, L., M. Nicolas, and L. Pellegrino. 2016. Alkaline phosphatase activity in cheese as a tracer for cheese milk pasteurization. *Lebensm. Wiss. Technol.* 65:963–968. <https://doi.org/10.1016/j.lwt.2015.09.033>.
- European Commission. 2006. Commission Regulation (EC) no. 1664/2006 of 6 November 2006 amending Regulation (EC) no. 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. *Off. J. Eur. L* 338:27.
- Fox, P. F., and A. L. Kelly. 2006. Indigenous enzymes in milk: Overview and historical aspects—Part 2. *Int. Dairy J.* 16:517–532. <https://doi.org/10.1016/j.idairyj.2005.09.017>.
- GUCE (Gazzetta Ufficiale dell'Unione Europea). 2020. Application for approval of an amendment pursuant to the first subparagraph

- of Article 53(2) of the Regulation (UE) n. 1151/2012. Pecorino Siciliano n. UE: PDO-IT-0019-AM01-4.6.2018. C170/2020 EUR-Lex-52020XC0518.
- GUCE (Gazzetta Ufficiale dell'Unione Europea). 2010. Publication of an application pursuant to Article 6(2) of Council Regulation (EC) No 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. Piacentinu Ennese EC No: IT-PDO-0005-0646-25.09.2007-C164/2010 EUR-Lex-52010XC0624.
- ICQRF. 2021. ICQRF—Department of Central Inspectorate for Fraud Repression and Quality Protection of Agri-food Products and Foodstuffs, part of the Italian Ministry of Agriculture. Report of activities, 2021. Accessed Nov. 17, 2022. https://www.politicheagricole.it/ICQRF_Report_attivita_2021.
- ISO. 2016. ISO 11816-2: Milk and Milk Products—Determination of Alkaline Phosphatase Activity—Part 2: Fluorometric Method for Cheese. IOS Press.
- Kosikowski, F. V. 1988. Enzyme behavior and utilization in dairy technology. *J. Dairy Sci.* 71:557–573. [https://doi.org/10.3168/jds.S0022-0302\(88\)79592-2](https://doi.org/10.3168/jds.S0022-0302(88)79592-2).
- Mangione, G., M. Caccamo, G. Farina, and G. Licitra. 2023. Comparison of methods to homogenize ricotta cheese samples for total solids determination. *JDS Commun.* 4:5–8. <https://doi.org/10.3168/jdsc.2022-0260>.
- Mcfarren, E. F., R. C. Thomas, L. A. Black, and J. E. Campbell. 1960. Differentiation of cream reactivated from residual phosphatase in high-temperature-short time pasteurized milk and cream. *J. Assoc. Off. Agric. Chem.* 43:414–426. <https://doi.org/10.1093/jaoac/43.2.414>.
- Murthy, G. K., S. Cox, and L. Kaylor. 1976. Reactivation of alkaline phosphatase in ultra high-temperature, short-time processed liquid milk products. *J. Dairy Sci.* 59:1699–1710. [https://doi.org/10.3168/jds.S0022-0302\(76\)84427-X](https://doi.org/10.3168/jds.S0022-0302(76)84427-X).
- Pellegrino, L., G. Battelli, P. Resmini, P. Ferranti, F. Barone, and F. Addeo. 1997. Effects of heat load gradient occurring in moulding on characterization and ripening of Grana Padano. *Lait* 77:217–228. <https://doi.org/10.1051/lait:1997215>.
- Pellegrino, L., and C. Donnelly. 2004. Public health issues in dairy production and the implications on the safety of aged cheeses made with raw milk. Pages 33–55 in *Safety Assurance During Food Processing*. F. J. M. Smulders and J. D. Collins, ed. Wageningen Academic Publ., Wageningen, the Netherlands.
- Pellegrino, L., J. A. Hogenboom, V. Rosi, and P. D'Incecco. 2021. Evaluating the authenticity of the raw-milk cheese Fontina (PDO) with respect to similar cheeses. *Foods* 10:350. <https://doi.org/10.3390/foods10020350>.
- Prajapati, D., and K. Desai. 2017. Alkaline phosphatase: Significance in dairy industry. *Int. J. Res. Sci. and Management* 4:18–22.
- Pratt-Lowe, E. L., R. M. Geiger, T. R. M. Richardson, and E. L. Barrett. 1988. Heat resistance of alkaline phosphatase produced by microorganisms isolated from California Mexican-style cheese. *J. Dairy Sci.* 71:17–23. [https://doi.org/10.3168/jds.S0022-0302\(88\)79519-3](https://doi.org/10.3168/jds.S0022-0302(88)79519-3).
- Rankin, S. A., A. Christiansen, W. Lee, D. S. Banavara, and A. Lopez-Hernandez. 2010. Invited review: The application of alkaline phosphatase assays for the validation of milk product pasteurization. *J. Dairy Sci.* 93:5538–5551. <https://doi.org/10.3168/jds.2010-3400>.
- Rapporto ISMEA-Qualivita. 2022. Sulle Produzioni Agroalimentari e Vitivinicole Italiane DOP, IGPeSTG. Accessed Mar. 14, 2023. <https://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/12017>.
- Sgroi, F., and F. Modica. 2022. Localized agri-food systems: The case of Pecorino Siciliano PDO a food product of the tradition of Mediterranean gastronomy. *Int. J. Gastron. Food Sci.* 27:100471. <https://doi.org/10.1016/j.ijgfs.2022.100471>.
- Soares, C. F., L. M. Fonseca, M. O. Leite, and M. C. P. P. Oliveira. 2013. Application of Schärer's quantitative method for the determination of residual alkaline phosphatase activity in standard Minas. *Arq. Bras. Med. Vet. Zootec.* 65:1223–1230. <https://doi.org/10.1590/S0102-09352013000400039>.
- Todaro, M., V. Lo Presti, A. Macaluso, M. Alleri, G. Licitra, and V. Chiofalo. 2021. Alkaline phosphatase survey in Pecorino Siciliano PDO cheese. *Foods* 10:1648. <https://doi.org/10.3390/foods10071648>.
- Vamvakaki, A. N., E. Zoidou, G. Moatsou, M. Bokari, and E. Anifantakis. 2006. Residual alkaline phosphatase activity after heat treatment of ovine and caprine milk. *Small Rumin. Res.* 65:237–241. <https://doi.org/10.1016/j.smallrumres.2005.06.025>.
- Wilińska, A., J. Bryjak, V. Illeova, and M. Polakovič. 2007. Kinetics of thermal inactivation of alkaline phosphatase in bovine and caprine milk and buffer. *Int. Dairy J.* 17:579–586. <https://doi.org/10.1016/j.idairyj.2006.08.008>.

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