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Assessment of Seasonal and Diurnal Variations of Alkaline Phosphatase Activity in Pasteurized Milk

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Abstract: The present study was conducted to detect the concentration levels of ALP (alkaline phosphatase) in pasteurized milk and determine whether the pasteurization was successful or not, according to WHO Directives, which clearly state that the ALP (alkaline phosphatase) substance in pasteurized milk must be totally inactivated, by implementing a newly developed method. The study, additionally, focused on repeatability, stability of results, the effect of the environmental temperature, the effect of the different origins of the milk and convenience with respect to performance characteristics of three methods for the detection of ALP. The milk samples were collected from different areas of Greece during February 2016–February 2018 and May 2019–January 2020. The novel enzymatic biochemical method, named the "AP test", showed superior characteristics for a diversity of materials such as milk, whey, cheese and butter in comparison to the other two methods that were used for screening and quantitative estimation of the concentration of ALP in samples.

Keywords: food microbiology; food industry; alkaline phosphatase; milk; milk pasteurization

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1. Introduction

The vast majority of milk products are subjected to a definitive thermal treatment (i.e., pasteurization). Taking under consideration the complexities of modern milk processing equipment, global distribution systems with their flaws and increasing regulatory and industry demands due to global population increase, the alkaline phosphatase (ALP) test has been adopted by many countries as the standard assay for rapid validation of the milk pasteurization process. Although the majority of dairy products in the markets are of bovine origin, there has been observed an increasing demand for dairy products originating from other milks such as caprine and ovine [1]. The alkaline phosphatase (ALP) test is mandatory in the dairy industry to check for proper pasteurization of the milk. The milk as raw material might be contaminated by a variety of microorganisms which in turn could be detrimental to human health such as various *Brucella* species such as *Brucella. melitensis* or *Mycobacterium* spp. and others. ALP is inactivated at temperatures somewhat higher than the critical temperature of reduction by 5 logarithms of heat resistant pathogens such as *Coxiella brunetti* [2]. It is for this reason that the EC Regulation 853/2004 imposes the ALP test to all kinds of milk with the exceptions of milk for fermented products whose fermentation period lasts at least two months or of milk originating from Brucella-free holdings. However, even in the latter cases, pasteurization is advisable to destroy not only other pathogens but also spoilage microorganisms. The pasteurization temperatures (72 °C for 15 s or 63 °C for 30 min or any other time–temperature equivalent combination) alter the stereochemical structure of the enzyme, thus rendering it inactive. The test should be performed right after milk heating to avoid reactivation. The outcome is significantly influenced—among other factors—by the season, by the lactation stage, by the fat content

of the milk and by the udder health [1]. The premise of the ALP test is based on the thermal inactivation characteristics of the ALP enzyme endogenous to milk [3–10].

ALP activity in raw ovine milk is estimated to be about three times higher and in caprine milk about five times lower than in bovine milk and is highly variable between breeds. Since the caprine ALP shows low activity, the standard tests—manufactured for the detection of the bovine milk—might show many false negative results, due to the relatively high detection limit. A more suitable test should be able to detect ALP in lower activity levels such as the ones in caprine milk.

The need for a fast, reliable and cost-effective validation method with superior technical characteristics on the ALP enzyme has led to the development of various testing methods, including the method we present in this study. An overview of the research and methods relevant to the scope of this method is given to this article.

We conducted research and developed a simple hypersensitive rapid test for the detection of alkaline phosphatase in pasteurized milk. It simplifies the testing procedure, is not influenced by exogenous factors (ambient temperature, humidity) and it is not limited by the use of accompanying equipment.

The aim of the present article is to provide essential information for a new systematic approach to the procedures of proper, valid pasteurization testing. This approach has been developed to ensure that the pasteurization process has been carried out correctly, aiming to safeguard the consumer's health and reduce person-hours in dairy processing.

2. Materials and Methods

In the present study, the collection of data and results was carried out in four (4) phases, so that each succeeding phase is the outcome of the previous and constitutes an "insurance policy" for the validation of the data extracted in each subsequent phase. In total, five hundred (500) samples of pasteurized milk were tested over two (2) years, coming from 1250 sheep, goat, and cow farms in mountainous and lowland areas of the Prefectures of Arta, Preveza, Aetolia–Akarnania, Achaia and Larissa and eight hundred (800) samples of raw milk were tested, coming from 17 goats of the Prefecture Aetolia–Akarnania. The aforementioned 800 samples collected from 17 goats from the area of Aetolia–Akarnania, Greece were utilized to detect ALP levels in raw goat's milk. They were divided into morning and evening sampling for: (a) each of the 17 goats and (b) for the total goats per month from May 2019 to January 2020. The aim of this comparison was to see if a high daily or seasonal ALP value has any significant interference on the derived result of any of the three methods ("AP test", Test B (Sensobiz) and Test A (Lactognost-Heyl)). This variability of mean values in ALP activity presents a pattern of large seasonal and daily fluctuations of ALP levels in raw goat milk.

2.1. Study Area and Selection Method

The animals of these farms are crossbreeds of Greek breeds such as the Mountain breed of Epirus (type Boutsiko, Laio and Belo), Agrinio, Sarakatsaniki and Karamaniki-Katsina, Frizarta, Miga, Chiotika and Ntopia Greek. Their milk production is approximately 140 kg/animal/year and the number of animals per farm ranges from 10 to 80 animals. The overall workflow for sample collection and analyses performed is presented in Figure 1.

2.1.1. Bio-Easy Rapid Tests

The SENSObiz® Biosensor System Test Kit Alkaline Phosphatase Test, a biosensor system developed by NANObiz.com.tr (accessed on 10 February 2022) is a test based on alkaline phosphatase reaction with a substrate, which induces the color change in a qualitative manner.

HEYL LACTOGNOST III kit (Rapid Phosphatase Test) is a colorimetric qualitative assay that uses three reagents, namely Lactognost I (buffer solution), II (disodium phenyl phosphate) and III (chloro imino dibromo quinine).

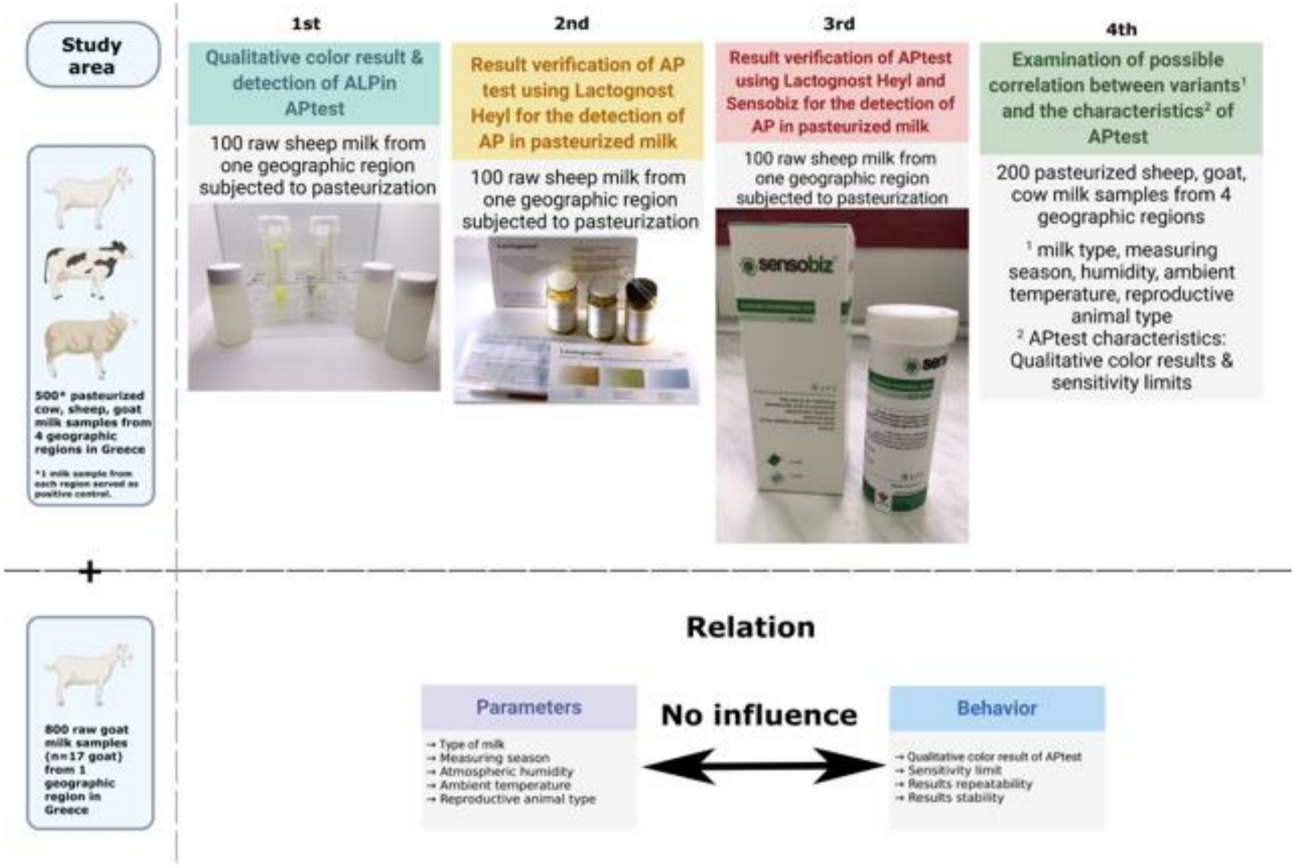

Figure 1. The workflow of the present study schematically.

The main difference between the SENSObiz® test and the HEYL LACTOGNOST III kit is that the second works with single-use test strips.

A newly developed test called the "AP test", for the detection of alkaline phosphatase activity in milk was also used.

All the above tests were conducted according to each manufacturer's instructions, and data interpretations were performed according to the manufacturer's instructions.

2.1.2. Analysis of the Alkaline Phosphatase (ALP) Standard Curve

The Electra m2 Unified Analyzer, a spectrophotometer, was used to construct the 6-point linear point measurement curve of the method with the "AP test" kit to obtain quantitative measurements of ALP presence in milk samples. Each sample was mixed with R1, R2 and R3 reagents and was incubated, using the exact quantities of reagents and samples that were used for the semi-quantitative determination. The results obtained validated the comparison method between the "AP test" kit and HEYL LACTOGNOST III. Spiked samples with 3.5 mU/L, 35 mU/L, 70 mU/L, 140 mU/L, 280 mU/L ALP and 560 mU/L ALP were used as calibrators. Reagents R1, R2 and R3 (as detailed in the Section 2.3.1.1) and deionized water were used as blank. The wavelength for the method was 340 nm.

2.2. Categorization of Milk Study Samples

The milk samples were categorized into the following groups:

A. One hundred (100) samples of raw milk (subjected to the pasteurization process) collected from 100 farms of clinically healthy animals (sheep) in mountainous areas of the Prefecture of Aetolia–Akarnania, i.e., one (1) sample from each farm taken once per farm. One (1) sample of raw sheep milk was taken randomly from one (1) of the

farms to serve as a positive control and to be used as part of the individual mixtures of pasteurized raw milk. These samples were used for: (i) the detailed recording of the qualitative color result of the newly developed test called "AP test", (ii) the detection of the sensitivity limit to alkaline phosphatase in a mixture of spiked pasteurized milk with raw milk in different proportions.

B. One hundred (100) samples of fresh sheep and goat milk collected from 100 sheep farms in mountainous areas of the Prefecture of Aetolia–Akarnania, i.e., one (1) sample from each farm was taken once per farm. The milk samples of group B were subjected to a pasteurization procedure and were then used to verify the results of the "AP test" by another certified and commercially accepted method for the detection of phosphatase in pasteurized milk. This procedure lasted 30 days divided into five (5) day calendar groups, where the repeatability of the results was certified.

C. One hundred (100) samples of fresh sheep's and goat's milk from the same animals of group B processed by the pasteurization process in the cheese dairy unit N. NAKAS & SONS, Kampos Amphilochia-30500/Greece. One (1) sample of sheep's raw milk was taken randomly from one (1) of the farms to play the role of positive control and so that researchers could use part of it in the individual mixtures of pasteurized raw milk. These samples allowed a comparative analysis of the "AP test" test with the HEYL LACTOGNOST III kit and SENSObiz. Both Lactognost III and SENSObiz are compliant with the golden standard (ISO 11816–1:2013); therefore, comparison of the "AP test" with these commercial kits renders the "AP test" compliant with the golden standard as well. The limit of detection is 350 mU/L. The samples were taken from tanks T201 (50 samples) and T202 (50 samples) and pasteurization was carried out on the NT50X pasteurizer.

D. Two hundred (200) samples of pasteurized sheep, goat, and cow milk from four (4) different geographical areas of Greece with targeted calendar sampling to examine whether the qualitative color result of the "AP test", and its sensitivity limit, are affected by the type of milk, the measuring season, the atmospheric humidity, the ambient temperature or the reproductive animal's type.

2.3. Description of the Newly Developed Test "AP Test"

The "AP test" was developed with the main objective of implementing a simple hypersensitive rapid test for the detection of alkaline phosphatase in pasteurized milk. It simplifies the testing procedure and is not affected by exogenous factors (e.g., ambient temperature, humidity) and is not limited by the use of specific accompanying equipment. At the same time, an effort was made to reduce the sensitivity limit for alkaline phosphatase detection from 20 mU/L to 3.5 mU/L.

The test consists of three (3) reagents that are refrigerated at 2–8 °C and have an expiration date of two (2) years from the production date. The method used with these three (3) reagents is kinetic enzymatic with chromatographic visualization of the result.

2.3.1. Chemical Action Mechanism of the Method Using the "AP test"

Alkaline phosphatase (ALP) catalyzes the hydrolysis of p-nitrophenyl phosphate at pH 10.4, releasing p-nitrophenol and phosphate, according to the following chemical reaction:

The percentage of p-nitrophenol is compatible with the catalytic concentration of phosphatase in the sample to be tested (with a sensitivity limit of 1.5 mU/L), and the sensitivity of this percentage is enhanced by the enzyme catalyst contained in reagent R3 of the "AP test".

2.3.1.1. Contents of the Reagents for the "AP Test" Test

The AP test kit consists of three (3) individual reagents whose composition is:
Reagent R1 [Diethanolamine (DEA) 175 mL/L and Silver Iodide (AgI) 4 g/L].
Reagent R2 [p-nitrophenyl phosphate (pNPP) 18 g/L].
Reagent R3 [Trypsin 1.5 ug/L].

and used in volumes of 40, 10 and 5 mL, respectively.

2.3.2. "AP Test" Method of Use

Place as many tubes in a metal or plastic stand as there are samples to be tested by the pasteurizer and number them. Using the pipette of variable volume 100–1000 μL, take a quantity of 400 μL of reagent R1 and place it in the tubes in ascending order, change the tip, adjust the quantity to be taken from 400 μL to 100 μL and add reagent R2 to each tube. Then change the variable-volume pipette and use the 10–100 μL pipette together with the corresponding tip. Take 10 μL of reagent R3 and place it in the tubes. Finally, take the 0.5 to 10 μL variable-volume pipette with a new tip and add 8.3 μL of the milk sample to be tested to each tube. Close the tube with either hydrophobic cotton or a plastic stopper and shake the contents of the tube gently for 2–3 s.

Sampling Technique in the Research and Development Phase

For the mast excretion of reproductive animals, initially, reproductive animals (sheep, goats, cows) were milked by hand [11,12]. The milk was collected in sterile 150 mL containers and then thoroughly mixed to ensure homogeneity throughout the sample mass.

After sampling, the samples were immediately placed in a portable refrigerator and transported to the laboratory of each dairy unit, where they were processed and analyzed on the same day. When this was not possible, they were kept at a temperature of 2–4 °C to be analyzed the following day.

Samples of pasteurized milk (i.e., at time zero (0) after the end of the pasteurization process) were collected from the pasteurizer of each cheese unit directly and carefully using sterile containers. The use of sterile surgical gloves and personal surgical protection devices was mandatory in order to avoid contamination of the pasteurized milk by the user, since phosphatase is an abundant marker in the environment and contamination of the sample by exogenous phosphatase would affect our results.

Receipt of raw milk as a positive control: From the samples collected mechanically (milking), one (1) sample was randomly selected to play the role of a positive control during the color imaging and comparison of the final samples.

Raw milk as a percentage of phosphatase in pasteurized milk: From the samples collected mechanically (milking), one (1) sample was randomly selected to play the role of phosphatase source when added to pasteurized milk in specific proportions [13–16].

Sample Handling and Homogenization Technique in the Research and Development Phase

Mast excretion of reproductive animals: Each sample was thoroughly mixed in the sampling vial, with 20–25 inversions. The shaking was rapid and vigorous (25 inversions in 7 s). The sample, within the first 3 min of shaking, was placed in ice trays, which were thoroughly disinfected according to the prescribed disinfection standards to avoid cross-contamination by exogenous phosphatase or microbial exogenous load [17,18].

Pasteurized milk: Each sample was carefully collected from the pasteurizer and mixed thoroughly in the sampling vial, with 20–25 inversions. The shaking was rapid and vigorous (25 inversions in 7 s). Within the first 3 min after shaking, we conducted the analysis of the physio-chemical characteristics of the sample in relation to the "AP test".

Fresh milk as a positive control: One (1) sample was thoroughly mixed in the sampling vial with 20–25 inversions. The shaking was rapid and vigorous (25 inversions in 7 s). Within the first 3 min after the shaking, a volume of 8.3 μL was taken with a variable-volume pipette of 0.5–10 μL and transferred into a tube for the "AP test" test.

Raw milk as a percentage of phosphatase in pasteurized milk: One (1) sample was thoroughly mixed in the sampling vial, with 20–25 inversions. The shaking was rapid and vigorous (25 inversions in 7 s). Within the first 3 min after shaking, precise quantities of raw milk were taken with a variable-volume pipette, enough at a time to obtain a new sample volume with a ratio of pasteurized to raw milk (Table 1):

0.001% raw milk to pasteurized milk or ALP 3.5 mU/L

0.002% raw milk to pasteurized milk or ALP 7 mU/L
0.005% raw milk to pasteurized milk or ALP 17.5 mU/L
0.01% raw milk to pasteurized milk or ALP 35 mU/L
0.02% raw milk to pasteurized milk or ALP 70 mU/L
0.04% raw milk to pasteurized milk or ALP 140 mU/L
0.06% raw milk to pasteurized milk or ALP 210 mU/L
0.08% raw milk to pasteurized milk or ALP 280 mU/L
0.1% raw milk to pasteurized milk or ALP 350 mU/L

Table 1. Detection of LoD for "AP test" in pasteurized milk. Positive control = yellow-green color (presence of phosphatase in the sample). Negative control = white color (absence of phosphatase in the sample).

% Raw Milk in Pasteurized Milk	Number of Samples	"AP Test" Test Qualitative Color Result	Time Interval for Displaying the Color Effect
0.00% or undetectable phosphatase	10	White color	5 s
0.001% or phosphatase 3.5 mU/L	10	Yellow-green color	5 s
0.002% or phosphatase 7 mU/L	10	Yellow-green color	5 s
0.005% or phosphatase 17.5 mU/L	10	Yellow-green color	5 s
0.01% or phosphatase 35 mU/L	10	Yellow-green color	5 s
0.02% or phosphatase 70 mU/L	10	Yellow-green color	5 s
0.04% or phosphatase 140 mU/L	10	Yellow-green color	5 s
0.06% or phosphatase 210 mU/L	10	Yellow-green color	5 s
0.08% or phosphatase 280 mU/L	10	Yellow-green color	5 s
0.1% or phosphatase 350 mU/L	10	Yellow-green color	5 s

Preparation of Alkaline-Phosphatase-Contaminated New Samples

This was followed by vigorous manual shaking of the bottle in an arc of 30–35 cm for 1 min. The sample was homogenized in such proportions that contamination of the new sample was obtained proportionally. In this way, new samples were prepared that allowed us to collect information regarding the sensitivity limits for the phosphatase levels detection and the color representation of the samples [18].

Preparation of Alkaline-Phosphatase-Contaminated New Samples

After homogenization of each sample and preparation of the new contaminated samples (spiked), the vials were numbered in the respective percentage concentration of phosphatase in each of them, i.e., 0.001%, 0.002%, 0.005%, 0.01%, 0.02%, 0.04%, 0.06%, 0.08% and 0.1%.

Each test tube was shaken using a Vortex immediately after mixing raw milk with pasteurized milk. All mixtures were prepared within 15 min of sample homogenization.

In the following table (Table 2), the color interpretation of positive and negative controls in sheep milk is presented.

Table 2. Color interpretation of positive and negative control for "AP test".

Sample Type	Negative Indicator in Sheep Milk (Pasteurized Sample)	Positive Indicator in Sheep Milk (Raw)
"AP test" qualitative color effect	White color	Yellow-green color

2.4. Data Analysis and Statistical Analysis

All experimental procedures were repeated four times and Microsoft Excel 2016 (Microsoft Co., Redmond, WA, USA) was used for data analysis. The statistical method used for analyzing phosphatase/mL milk value was the formula calculating the mean value of daily morning and evening samples of each goat.

Statistical analysis included analysis of variance (one way ANOVA) and *t*-test with a significance level of 0.005.

3. Results

By contaminating the pasteurized milk with raw milk in various portions from 0.001% to 0.1%, a concentration gradient of ALP was achieved ranging from 3.5 mU/L to 350 mU/L. The detection limits were estimated at 3.5 mU/L, 210 mU/L and 350 mU/L for the AP test, the A test and the B test respectively (Table 2). The AP test performed well in a wide range of ambient temperatures (9–42 °C) and showed stability and repeatability of measures in samples of milk from different species (cow, sheep, goat) and from different geographical areas. In general, the behavior of the "AP test" test method in relation to result repeatability, result stability, data stability, variability of results stability, different types of milk origin, different pasteurization standards, and pasteurization periods showed that the kit produces excellent results and is not influenced by external factors [18–27].

Table 3 shows the seasonal variation of the mean values of the ALP activity in raw goat's milk, in the morning and in the evening samples. Figure 2 illustrates the same data and reveals a seasonal pattern. In the middle of the spring (May), the ALP activity is at the lowest point but gradually increases. For the morning samples, this increase is rather smooth and reaches its peak by December. ALP activity in the evening samples almost follows the same pattern except for a sudden very high peak in October. These findings—given the statistical significance—suggest large seasonal and diurnal variabilities of ALP activity in goat's raw milk. Table 4 shows comparative data (data related mostly to the technical design and implementation schedule) between the three tests used in the present study.

Table 3. Seasonal variation of the mean values of the alkaline phosphatase activity in goat's milk.

Month	Morning	Evening	Significance (*t*-Test)
May	28,350.9 ± 342.78	33,439.5 ± 664.52	t = 23.5742, $p < 0.0001$
June	10,001.7 ± 195.44	18,235.8 ± 362.39	t = 69.2802, $p < 0.0001$
July	16,448.4 ± 487.74	36,728.3 ± 7293.04	t = 9.6112, $p < 0.0001$
August	30,589.2 ± 274.84	45,489.6 ± 903.99	t = 54.6313, $p < 0.0001$
September	50,688.0 ± 792.36	97,729.2 ± 1942.11	t = 77.6879, $p < 0.0001$
October	85,458.6 ± 1033.24	226,642.5 ± 4503.91	t = 21.1938, $p < 0.0001$
November	94,777.2 ± 1145.91	136,665.0 ± 2715.85	t = 49.2262, $p < 0.0001$
December	151,138.0 ± 1744.39	190,272.3 ± 3781.97	t = 32.5495, $p < 0.0001$
January	91,131.3 ± 1101.83	108,451.8 ± 2155.19	t = 24.7883, $p < 0.0001$
Significance (ANOVA)	F = 30,448.041 $p < 0.0001$	F = 5619.99 $p < 0.0001$	

Table 4. Comparison of the methods used to detect ALP.

Test	Test A *	Test B **	"AP Test"
Incubation	1 h	-	-
Time for results	10 min after incubation	5–10 min	5 s
Temperature	37 °C	Room temperature	42 °C
Equipment	Incubator Centrifuge Waterbath	-	-

Table 4. *Cont.*

Test	Test A *	Test B **	"AP Test"
Limit of detection	350 mU/L	210 mU/L	3.5 mU/L
Sample material	Pasteurized milk, Whey, cream. butter	Pasteurized milk, HTST milk	Pasteurized milk, HTST milk, chocolate milk, whey, cream, cheese, butter

For reasons of simplification: * Test A: HEYL LACTOGNOST III kit, ** Test B: SENSObiz® test.

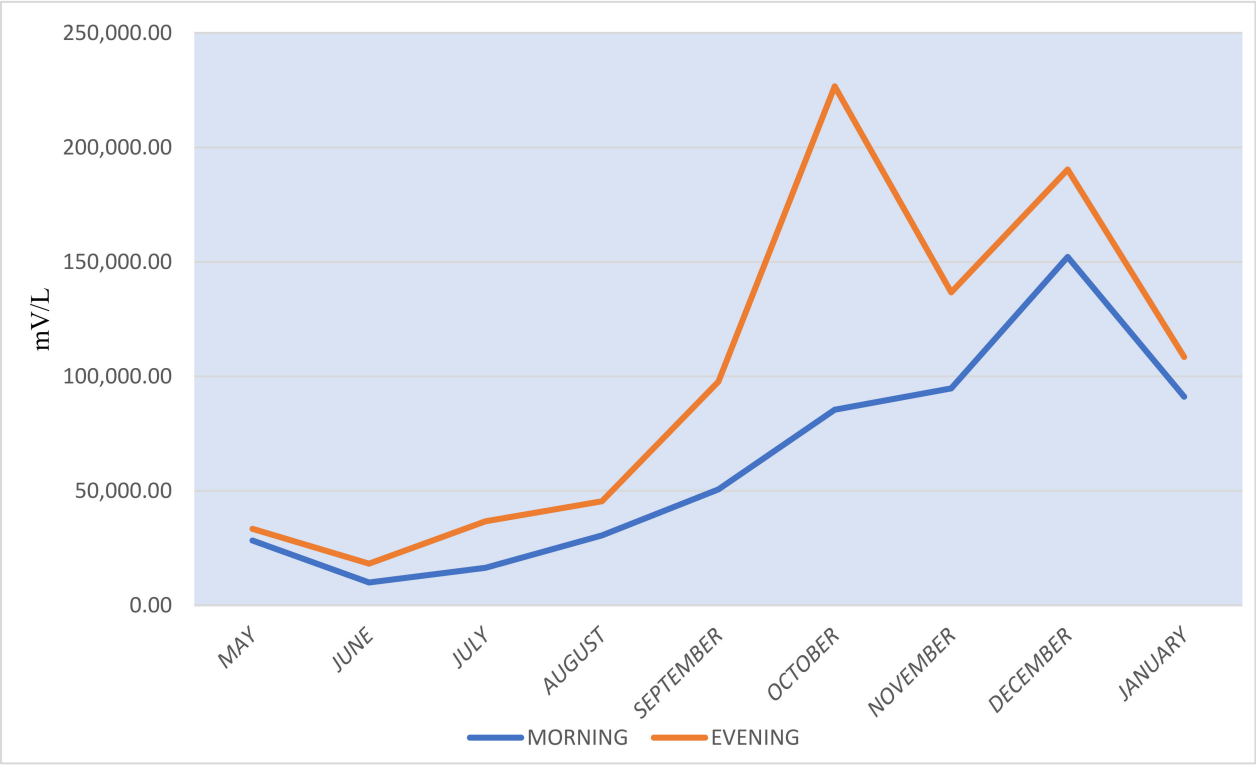

Figure 2. Schematic representation of the seasonal variation of the mean values of the ALP activity in raw goat's milk.

4. Discussion

Pasteurization is the method of choice to significantly reduce the microbial load of raw milk. Raw, unprocessed milk is an excellent substrate for various foodborne pathogens to grow, causing serious illnesses upon consumption. Through this perspective, pasteurization is more than necessary. So far, the most popular and legally mandatory method is the inactivation of ALP. This enzyme is a hydrolase that removes one phosphoric group from various compounds, a crucial step in phosphorus metabolism, and hence is abundant in nature in procaryotic and eukaryotic cells.

In milk, ALP originates from two sources [1,2]. The first is the cellular membranes of various cells of the udder. In general, ALP shows a strong affinity for membranes and thus is also located on the membrane of the lipids of the milk [28]. The other source of ALP in milk is of bacterial origin. Bacterial cells harbor ALP, and the bacterial ALP is usually more heat resistant than the other one, causing false positive results in ALP testing after pasteurization. To tackle this problem, it is advisable—after checking the pasteurization equipment for various mechanical and technical flaws—to repeat pasteurization and then check again for ALP. If the ALP test is still positive, then the enzyme is probably from bacterial origin.

ALP activity in milk is dependent on various factors such as the animal species, the health of the udder, the nutrition of the animals, the lactation period, the animal husbandry, the season, and the time of the day. The effect of the last two factors on goat's milk can be clearly seen in Table 3. Our results suggest that there are significant differences not only from month to month but also in morning and in evening milk. In Figure 2, a pattern can be observed in which ALP activity increases smoothly till August and then more rapidly until December for the morning milk, while there are two peaks for the evening milk, the higher in October and the lower in December. A sound interpretation of these findings needs of course more research but given the fact that these animals live in herds that graze all day and receive a small amount of concentrated food at night, it is the nutrition that affects to some extent the ALP concentration, since in different seasons, the biodiversity and the availability of the grazing plants varies. The steeper increases in both curves from August correspond with the beginning of the breeding season and the December peaks correspond with the beginning of the labor period, indicating a possible hormonal underlying mechanism.

Since the pasteurization temperatures reduces the ALP activity of the treated milk in a logarithmic manner, it follows that if the initial content of ALP in milk is high, then the heat treatment will not eliminate the ALP, and, hence, the control will show unsuccessful pasteurization. It seems that this could be the case in caprine milk especially after August, according to our results in which the ALP activity in caprine milk is exceedingly high. The situation can become more complicated in circumstances where there is high prevalence of subclinical mastitis—very common in Greece—since unhealthy udders increase ALP activity. On the other hand, it is important to detect any residual ALP in order to monitor the pasteurization circuit. A reliable method should be implemented, accurate and sensitive enough to detect even traces of ALP activity in caprine milk. Legislation should deal with interpretation of the results after taking into consideration the all the parameters.

Under standard conditions of assay, the ALP method has been shown to be a remarkably valuable tool for the routine assessment of milk pasteurization validation. Between the three (3) different methods mentioned in this article "AP test", showed crucial benefits, as presented in Table 4.

The "AP test" is a sensitive, state-of-the-art phosphatase detection test. It is modern, affordable (without the use of accompanying equipment), fast, ultra-sensitive, easy to use and can be performed anywhere, even at extreme temperatures [26]. With the introduction of a test with the performance characteristics of the "AP test" into the milk handling process, bacterial contamination can be dealt with, and possible outbreaks can be avoided. Another benefit of this method is that it saves valuable time, since the dairy process of the pasteurized milk can start immediately after the pasteurization without the waiting time needed to other methods, which can reach up to 60 min. The direct consequence of this is the optimal management, redistribution and/or reallocation of person-power in other tasks, an increase in the production line and a reduction in electricity cost in each dairy unit [29–32].

5. Conclusions

Caprine ALP shows significant diurnal and seasonal variation, which could affect the evaluation of the pasteurization process if the method used is not accurate enough.

The "AP test" method has been successfully tested in both room and outdoor ambient temperatures, ranging from 2 °C to 42 °C. Therefore, there is practically no geographical limitation associated with local environmental temperatures related to using this test.

No special equipment (such as centrifuges, luminometers, spectrophotometers, water baths and Petri plates) is required to perform the "AP test". This leads to a dramatic reduction in capital investment costs for each dairy plant. The test can be carried out effortlessly using cutting-edge technology, and it is affordable for all dairy plant sizes.

The "AP test" was tested with 100% result accuracy for two (2) years with 100% result repeatability in four (4) different dairy farms around Greece from different geographical

regions. This way, we were able to certify the test's accuracy, considering all the different variables (milk type, product type, temperature variations per region, food type of breeding animal, different breeds per species, different fat concentrations in raw milk, time period of milk intake).

In conclusion, the use of "AP test" is proposed as a valid method for verifying correct milk pasteurization due to its analytical, operational and financial advantages.

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