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Comparative Analysis of Alkaline Phosphatase Activity and Aerobic Spore-Forming Bacteria in Pasteurized Milk

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Abstract

The remaining amounts of alkaline phosphatase (ALP) enzyme in pasteurized milk should be within certain limits. High ALP enzyme level may indicate that the milk has not been pasteurized sufficiently or may be due to excessive growth of spore-forming bacteria in the milk. For this purpose, pasteurized milk samples (n=50) taken from local markets of Manisa were screened for ALP activity and spore-forming bacteria. The ALP activity of most pasteurized milk was below the 350 mU/L limit and ranged from 0 to 500 mU/L. ALP activity was found to be high in only 4% of the 50 milk samples analyzed. The presence of spore-forming bacteria was investigated in suspicious and high ALP activity samples and representative colonies of bacteria that were selected and identified based on biochemical tests. All of the isolates were *Bacillus* spp. and *Bacillus cereus* was detected in only one sample. As a result, a positive correlation between ALP activity and the existence of *Bacillus* spp. was observed for pasteurized milk.

Keywords: Alkaline phosphatase, *Bacillus* sp., colourimetric method, quality control, pasteurized milk, spore-forming bacteria.

1. Introduction

The microorganism load of raw milk is quite high, so it may contain pathogens and spoil in a short time. Therefore, consumption of raw milk may cause health problems. Pasteurization is the most widely adopted and effective method that ensures the destruction of all spoilage and pathogenic microorganisms. One of the most important factors that shorten the shelf life of pasteurized milk is the contamination of spore-forming or non-spore-forming bacteria into the product after heat treatments [1]. Such contaminations generally occur during advanced pasteurisation processes, and, the shelf life of the milk is shortened. These pasteurized products offered for consumption directly concern public health and are very important. The shelf life of pasteurized milk is generally linked to raw milk quality and post-pasteurization contamination control [2].

One of the most important quality measures of the milk to be pasteurized is its hygienic quality. In this case, the number of bacteria, bacterial types, and toxins should be considered. During the pasteurisation process, 1% of the bacteria remain alive, meaning that if the conditions applied to milk are adequately observed, approximately 99% bacterial reduction can be achieved. [3]. Therefore, the type of bacteria is as important as the number of bacteria in raw milk. If the number of thermophile and heat-resistant (thermoduric) bacteria is high, their reduction in the pasteurization norm is quite difficult [1,2]. To increase the microbiological safety of pasteurized milk, it is necessary to remove all pathogens that may be present in it and pasteurization is recommended for this. Thermoduric bacteria isolated from processed dairy products include *Micrococcus*, *Microbacterium*, *Enterococcus*, *Arthrobacter*, *Lactobacillus*, *Streptococcus*; as well as spore formers such as *Paenibacillus*, *Bacillus*, and *Clostridium* [4,5]. The most common *Bacillus* species in fresh pasteurized milk are generally *B. cereus*, *B. licheniformis*, *B. subtilis* and *B. circulans* [6]. *B. cereus* strains and related organisms have been found to cause milk-sweet curdling and creaminess. Other milk spoilage associated with *Bacillus* and other psychrotrophic spore formers is the coagulation of milk proteins as well as the formation of a bitter taste [7,8].

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Alkaline phosphatase (ALP) is an enzyme, which is normally present in raw milk. Once milk is pasteurized, this enzyme becomes completely inactive. Therefore, the high amount of ALP remaining in pasteurized milk will indicate that the milk has not been pasteurized under appropriate conditions [9]. ALP shows a slightly higher temperature resistance than pathogenic bacteria, which die at pasteurization time and temperature. According to the European Union (EU) and the Food and Drug Administration (FDA), the recommended ALP level should be below 350 mU/L to ensure the safety of pasteurized milk and extend its shelf life [10,11]. Values higher than this ALP level may result from insufficient pasteurization or contamination with raw milk. Therefore, measuring ALP activity in pasteurized milk is recognized as a method for rapidly determining the quality of milk pasteurization.

The present study aimed to report the investigation of ALP activity and aerobic spore-forming bacteria in pasteurized milk. In addition, within the scope of the study, aerobic spore-forming bacteria analysis of pasteurized milk with cultural methods will be performed to reveal whether there is a correlation with the results obtained with the highest ALP measurement. Thus, it will be revealed whether the appropriate pasteurization conditions are followed in pasteurized milk and whether the high amounts of ALP are of microbial origin or not.

2. Materials and Methods

2.1. Sampling of Pasteurized Milk

Commercially pasteurized cow's milk samples (n=50) were collected from local markets in Manisa province at different time intervals for approximately 2 years (December 2015-August 2017). Each sample consists of approximately 1 L of pasteurized, fat (approximately 3.5%), homogenized cow's milk in plastic, tetrapak or glass bottles. After sampling, each milk sample was placed in an insulated cooler box (approximately 6 ^{o}C) and transported to the laboratory no later than 90 minutes (min) after the first sample was taken. Care was taken to ensure that the samples were taken before the expiration date of the pasteurized milk.

2.2. Analysing the Alkaline Phosphatase Activity

ALP hydrolyses colourless substrate *p*-nitrophenyl phosphate (*p*-NPP) to *p*-nitrophenol (*p*-NP) and inorganic phosphate. For the determining ALP activity, a modified method by Chu *et al.* [12] was applied. Briefly; the buffer-substrate solution was prepared with carbonate buffer (10 mM, pH 10.2), containing 1 mM $MgCl_2$ with 0.2 mM of *p*-NPP in a total of 1.5 mL. Then, 200 µL of well-mixed pasteurized milk was added to 1.5 ml of buffer-substrate solution and this reaction mixture was incubated at 37 ^{o}C for 120 min. At the end of the incubation, 0.5 ml of stopper buffer (0.1 mol NaOH and 5 mmol EDTA) were added and it was rested for 3 min, centrifuged at 1400 rpm for 10 min. The supernatant was transferred to clean cuvettes and without waiting, the absorbance was measured on a 410 nm spectrophotometer. For the comparison, the negative control and positive control, boiled pasteurized milk and raw milk samples were used respectively, in the same amount and reaction conditions. ALP activity was calculated with the formula given below;

$$\textbf{ALP activity (unite/mL)} = (\mathbf{A410nm\ test} - \mathbf{A410nm\ Blank}) \times \boldsymbol{B} \div (\boldsymbol{C} \times \boldsymbol{D} \times \boldsymbol{E})$$

A= Absorbance read at 410 nm
B= Total reaction volume (mL)
C= Extinction coefficient of *p*-NP (mM), according to standard curve
D=Volume of pasteurised milk sample used (mL)
E= Reaction time (min)

The results obtained were converted to milliunits per litre (mU/L).

2.3. Determination of Aerobic Spore-Forming Bacteria

To determine whether the ALP activity was caused by aerobic spore-forming bacteria (ASFB) in the pasteurized milk samples with higher ALP levels, therefore, laboratory pasteurisation at 63 ^{o}C, 40 min (LAB pasteurisation) was performed according to the method of Rankin *et al.* [9] and the results were discussed. In this experiment, ASFB screening was performed on the samples that gave positive ALP results. 1 mL of milk sample (10^{-1} dilution) in 0.8% physiological saline was transferred in Plate Count Agar (PCA) medium by the pour plate cultivation method and incubated at 30 ^{o}C for 48 h. At the end of incubation, bacteria that could grow in the PCA medium were evaluated as ASFB and representative colonies were selected for identification by cultural and biochemical tests.

2.4. Identification of Selected Microorganisms

The liquid cultures (incubated for 48 h at 30 ^{o}C) of representative bacteria were subjected to gram staining to determine the gram reaction. API biochemical test kits (BioMérieux, France) are accepted as a reference for identifying bacterial and fungal species. API 20E biochemical tests were applied to selected bacteria. Bacterial cultures transferred to test strips were prepared fresh and applied according to the manufacturer's instructions. At the end of the incubation period, the bacterial genus/species of the isolates were identified using the numerical identification calculation method recommended by the API, through APIWEB-based software.

2.5. Statistical Analysis

All the data was represented as mean ± standard deviation of the mean (SD) using the statistical software (Minitab® 19).

3. Results and Discussion

Pasteurization is the most widely used method that ensures the destruction of all pathogenic microorganisms in milk and makes it safe for consumption. Milk is ideal for the growth and reproduction of various microorganisms that cause it to spoil prematurely. Since raw milk contains many pathogens, direct consumption is not recommended. Various factors affect the microbiological quality of pasteurized milk, such as the origin of raw milk, the heat treatment used, storage conditions, and the degree of contamination after pasteurization [3]. The purpose of milk pasteurization is to make milk safe and increase its shelf life to several weeks. However, pasteurized milk is perishable and assessing its quality is one of the most important concerns.

There are several methods for detecting microbial spoilage in dairy products after pasteurization. ALP test method in the dairy industry; can be used in pasteurized milk quality [13]. Since ALP activity is affected by different reaction conditions, it is very important to perform the process under stable reaction conditions and compare the results with the standard curve (Figure 1). In the present study, the measurement values of 50 different pasteurized milk samples analysed with the ALP activity ranged between 0-500 mU/L. ALP activity of the most pasteurised milk was below the limits of 350 mU/L (Figure 2).

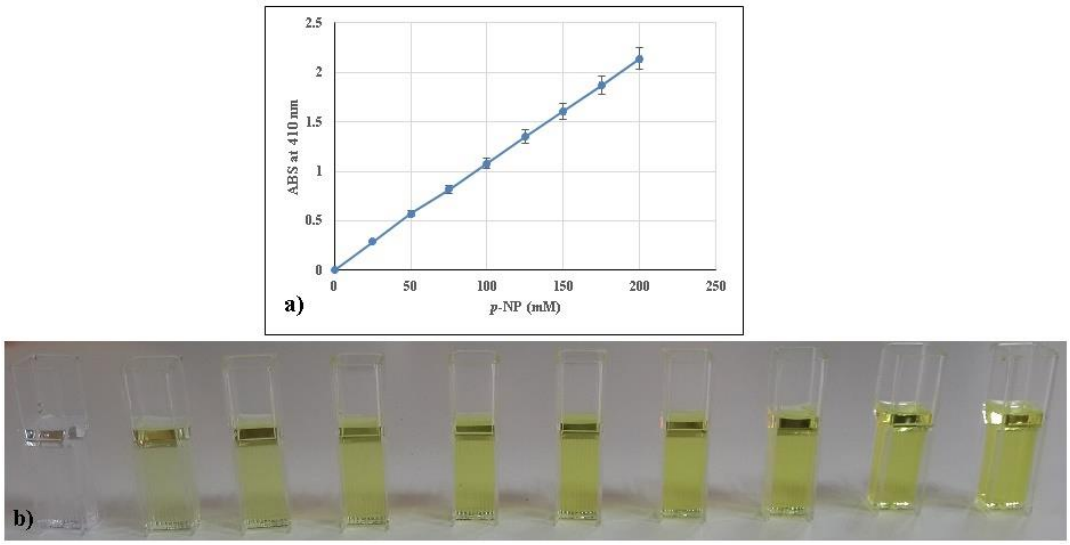

Figure 1. *p*-NP (mM) standard curve (a) and (b); the amounts of *p*-NP seen in the cuvette were 0, 25, 50, 75, 100, 125, 150, 175, 200 and 225 mM, from left to right, respectively.

When the obtained results compared the standard values, it was concluded that 2 of the 50 pasteurized milk samples were above this limit (4%, poor quality), 3 were almost at the upper limit (6%) and the rest were of good quality (90%). The results of ALP activity in pasteurised milk are presented in Figure 2. A study conducted by Rola *et al*. [10], obtained the highest ALP activity in two of 65 samples of pasteurised goat milk about 500 and 700 mU/L. Also, they found that one sample had an activity equal to 350 mU/L. Peng *et al*. [14], explained that the ALP of 451 pasteurized milk was 9.71% contained >350 mU/L. ALP activity by colourimetric method was similar to those studies and supported the present results.

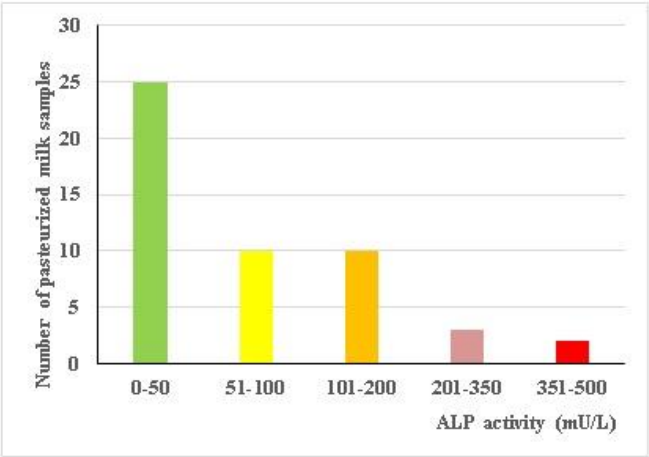

Figure 2. ALP activity of pasteurized milk samples.

To determine whether the ALP activity was caused by ASFB in the pasteurized milk samples with higher ALP

levels, therefore, laboratory pasteurisation at 63 ºC, 40 min was applied. Five samples with high ALP activity were selected and subjected to LAB pasteurization, and then ASFB bacteria were isolated using a PCA medium. After laboratory pasteurization, firstly, ALP activities were measured again for the five samples and the value was found negative for 3 samples. According to the ALP activity results of these 3 samples, it was estimated that high ALP activity may be due to inadequate pasteurization or storage conditions. Spore counts from the remaining 2 ALP positive samples were determined to be approximately between 1.0 x 10^1 and 4.0 x 10^2 cfu/mL. Therefore, it was concluded that the high ALP activity in these 2 samples may be caused by spore-forming bacteria. As a result of all isolations, 30 different isolates were obtained from the two samples and those that were similar to each other were separated and 5 strains that were assumed to represent all were selected and identified by biochemical tests. All bacteria selected for identification were gram-positive and according to the API test, the selected 5 isolates showed similarity rates ranging from 90 to 99.9%, 4 were *Bacillus* sp. the other was evaluated as *Bacillus cereus* (Table 1.).

Table 1. API 20E biochemical test results of the selected isolates.

Isolate	ONPG*	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	Possible genus/species
PALP1	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	+	-	*Bacillus cereus*
PALP2	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	*Bacillus* sp.
PALP3	-	+	-	-	V	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	*Bacillus* sp.
PALP4	+	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	*Bacillus* sp.
PALP5	+	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	*Bacillus* sp.

* (+): positive, (-): negative, (V): variable.

Peng *et al.* [14] determined a positive correlation between ALP activity and aerobic plate count, *Bacillus* and thermophilic *Bacillus* abundance, for pasteurized milk. These results confirm the current study. In another study, *Bacillus* spp. *Pseudomonas* spp. and *Acinetobacter* spp. have been identified from pasteurized milk samples [15]. In a microbiological study conducted to determine the quality of cooled raw milk, processed milk and dairy products; *Lactococcus lactis* (27.3%), *Enterobacter kobei* (14.8%), *Serratia ureilytica* (8%), *Aerococcus urinaeequi* (6.8%) and *Bacillus licheniformis* (6.8%) were detected [7]. A comprehensive microbiological analysis of 39 pasteurized milk samples, it was noted that the number of TASB was found below the spoilage limit of 10^7 cfu/mL, which is higher than the present study [16].

In this study, isolated aerobic spore-forming bacteria had higher ALP activity of pasteurized milk at a rate of 4% (2 samples). It was concluded that the other 96% (48 samples) complied with the standards. It is thought that the spore contamination in samples may result from inadequate adherence to pasteurization temperature and time or post-pasteurization processes. According to a previously reported study [17]; by contaminating pasteurized milk with various portions of raw milk from 0.001% to 0.1%, an ALP concentration gradient ranging from 3.5-350 mU/L was obtained. Therefore, to ensure that pasteurized milk is safe, it is important to perform pasteurization correctly and prevent post-pasteurization contamination. Additionally, more importance should be given to ensuring personnel hygiene in pasteurized milk facilities.

While foodborne pathogens always pose a threat to public health, determining microbial viability with traditional detection approaches has many drawbacks. Application of the ALP assay is a reliable, rapid detection technique that monitors the quality of pasteurized milk. Moreover, this method does not require as much sampling compared to traditional approaches and allows us to obtain results in a short time. Therefore, the data obtained within the scope of this study is very valuable, as it will make significant contributions to the literature in this field.

4. Conclusion

Overall, the ALP activity of pasteurized cow's milk was determined and from the positive samples, aerobic spore-forming bacteria were identified. A positive correlation between ALP activity and the existence of *Bacillus* sp. was observed for pasteurized milk. As a result, compared to the colony counting technique, the ALP method significantly reduces the analysis time and provides very useful information in researching the quality control of pasteurized milk. However, a combination of techniques is required to validate the desired sensitivity and specificity in the determination of ALP activity. Additionally, identification of *Bacillus* sp. at the species level should be supported by molecular studies.

Author's Contributions

Mustafa Oskay: Designed and performed the experiments, analysed the results, and wrote the article.

Ethics

There are no ethical issues after the publication of this paper.

Declaration of Competing Interest

The author declares that he has no conflict of interest regarding the content of this manuscript.

Data Availability

The supporting data will be made available on reasonable request.

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