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Characteristics of pasteurized and sterilized milk using lysozyme as an antibacterial agent

[1,*]Wulandari, Z., [2]Saragih, H.A. and [1,3]Budiman, C.

[1]*Department of Animal Production and Technology, Faculty of Animal Science, IPB University*
[2]*Alumnee Department of Animal Production and Technology, Faculty of Animal Science, IPB University*
Jl. Agatis, Kampus IPB Darmaga Bogor 16680, Indonesia
[3]*Biotechnology Research Institute, Universiti Malaysia Sabah, JL UMS 88400, Kota Kinabalu, Sabah, Malaysia*

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Abstract

Egg white lysozyme is a protein with a sweet taste and antibacterial activity. It is capable of hydrolyzing the bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the peptidoglycan wall of bacterial cells. However, egg white lysozyme has not been used as a biopreservative agent for milk. In this study, the impact of adding egg white lysozyme to pasteurized and sterilized milk on physical, microbiological, and organoleptic properties during refrigerated storage was investigated. A factorial randomized block design was employed and the data was analyzed using analysis of variance (ANOVA) with Tukey's post hoc test. The results showed that the milk used in this study met the minimum requirements of the Indonesian National Standard SNI 01-3141-2011 for fresh milk. The addition of lysozyme (70 µM) to pasteurized or sterilized milk did not affect the water activity (aw) or water content. However, pasteurized milk with lysozyme had significantly lower microbial populations than milk without lysozyme ($P<0.05$). On the other hand, there was no significant difference in the microbial population of sterilized milk with or without lysozyme. For both pasteurized and sterilized milk, with or without lysozyme, the microbial populations remained similar throughout 8 days of refrigerated storage. Additionally, the addition of lysozyme improved the sweetness of both pasteurized and sterilized milk. In conclusion, this study confirmed that lysozyme can be used to reduce the microbial population, particularly in pasteurized milk, and improve its sweet taste.

1. Introduction

Fresh milk, also known as raw milk, is a liquid obtained by proper milking methods from healthy cows' udders. It has not had its natural content reduced or added to and has not undergone any treatment except for cooling (BSN 2011). Cow's milk is a perishable food product due to its high water content, protein, and fat, which make it an excellent medium for microbial growth, particularly bacteria. To preserve fresh milk, pasteurization and sterilization are commonly employed.

Pasteurized milk refers to fresh milk, reconstituted milk, or recombined milk that has undergone a heating process at 63-66°C for a minimum of 30 mins or at 72°C for a minimum of 15 s, followed by immediate cooling to 10°C. The milk is then treated aseptically and stored at a maximum temperature of 4.4°C (BSN, 1995).

Another alternative to maintain milk quality is to add other ingredients exhibiting antimicrobial activity Among many antibacterial ingredients to use, bio-preservative agents are considered advantageous due to their safety and sustainability (Afiyah *et al*., 2015). Egg white lysozyme is one of the promising bio-preservative agents for improving food shelf life as this protein has antimicrobial properties through its ability to hydrolyze N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) bonds of peptidoglycan in bacterial cell walls (Alhazmi *et al*., 2014). Lysozyme exhibits antibacterial activity primarily against Gram-positive bacteria, while Gram-negative bacteria are more resistant due to the protection provided by their outer compartment membrane (Wu *et al*., 2019).

According to Carrillo *et al*. (2016), lysozyme is a natural and safe ingredient for consumption. The use of lysozyme as a food preservative is limited to 500 mg/kg in apple and pear juice, wine, and cheese, in accordance with GMP (CODEX Alimentarius Commission, 1995).

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*Corresponding author.
Email: *zakiahwu@apps.ipb.ac.id*

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Lysozyme has been used as a preservative in various food products, such as cheese and milk (Avila *et al*., 2014). However, there is currently no research on using lysozyme as a milk preservative, especially as a substitute or complement to pasteurization or sterilization treatment. Several biopreservative agents have been used in milk and milk products, including nisin, lactic acid, pediocin, lacticin, enterocin, and endolysin, as summarized by Ameer *et al*. (2020). Bacteriophages, which are viruses that infect bacteria, have also been reported to be effective in milk preservation (Singh, 2018). Importantly, lysozyme can be extracted relatively easily from egg whites compared to other biopreservatives, making it a practical option for milk preservation and a novel invention.

In addition to its antibacterial properties, lysozyme is also known to have a sweet taste, with a degree of sweetness of about 100 to 2000 times sweeter than sucrose sugar. This sweetness is thought to be due to the ability of this protein to interact with sweet taste receptors (TR2/TR3) on the human tongue (Maehashi *et al*., 2007). Masuda *et al*. (2005) reported that the amino acids responsible for lysozyme sweetness are Lis13, Lis96, Arg14, Arg21, and Arg73. This sweetness has encouraged many studies on this protein to be developed as a low-calorie natural sweetener to replace sugar and avoid the risk of diabetes. Wulandari *et al*. (2020) confirmed that the sweetness of chicken egg white lysozyme was also detected in lysozyme isolated from native chickens and cihateup ducks. However, the stability of the sweetness of lysozyme when it is applied as a preservative in food is still a big question.

This study aimed to determine the effect of lysozyme on the physical, organoleptic, and microbiological characteristics of pasteurized and sterilized milk stored at a low temperature in the refrigerator.

2. Materials and methods

The materials utilized in this study were fresh cow's milk, acquired from a local farm in Bogor, Indonesia, and egg white lysozyme powder (Lysolac, Bioseutica, Switzerland). For microbial analysis, materials were obtained from Oxoid (Thermo Fischer, UK), including Buffered Peptone Water (BPW) and Plate Count Agar (PCA) media. Other necessary chemicals were obtained from Sigma Aldrich (USA).

2.1 Quality testing of fresh cow milk

Testing the quality of fresh cow's milk using the MCC30 lactoscan device (Milkotronic Ltd., Nova Zagora, Bulgaria), according to the manufacturer protocol. Milk samples were placed in a container, then the quality levels were measured, consisting of solid non-fat (SNF), density, fat content, lactose, salt, and protein (Singhal *et al.,* 1997).

2.2 Milk treatments

The milk treatments were conducted by adding lysozyme at a final concentration of 70 μM, followed by exposure to either pasteurization or sterilization heating treatments. Pasteurization was carried out at a temperature of 72°C for 15 s (Badan Standardisasi Nasional 1995), while sterilization was conducted at a temperature of 115°C for 3 mins (Hendrawati and Utomo, 2017). The milk was subsequently stored at 4°C in a refrigerator for 8 days. Observations were made on days 0, 2, 4, 6, and 8 of storage, including measurements of water activity (aw) values (Association of Official Analytical Collaboration (AOAC) International, 2005), moisture content (AOAC International, 2005), microbial analysis (Badan Standardisasi Nasional, 2008), and sensory analysis.

2.3 Sensory analysis

A sample sensory analysis was conducted using an organoleptic test that evaluated the texture, shape, color, taste, and aroma of the product using the five senses. The line scalar test was employed, and twenty-five trained panelists participated in the evaluation. The variables tested included color, aroma, sweetness, and viscosity, and panelists were instructed to assess the samples using a line scale of 0-10 cm by placing a cross (x) on the line to indicate their evaluation. The results of the line scalar test were then measured using a centimeter (cm) scale ruler, with the zero point located at the left end of the line scale.

2.4 Experimental design

The experimental design utilized in this study was a randomized group design with a factorial pattern, consisting of two factors. The first factor was milk with lysozyme addition and milk without lysozyme. The second factor was the length of storage, which included 0, 2, 4, 6, and 8 days. The data obtained were analyzed for analysis of variance (ANOVA) with the Tukey test utilized as a post-hoc test. The statistical software Minitab14 (Minitab Inc., PA, USA) was used for the analysis.

3. Results and discussion

3.1 Quality of fresh cow milk

Table 1 presents the chemical composition of the milk sample used in this study, which meets the minimum Indonesian National Standard SNI 01-3141-2011 on fresh milk. The composition values were also similar to those reported by 13. Roza-Delgado *et al.* (2017), with 3.67% fat, 8.73% SNF, and 3.18% protein. Moreover, the lactoscan results in Table 1 indicated that no additional water was added to the milk, implying the absence of water-based adulteration. Therefore, the milk used in this study was of good quality and suitable for further processing.

Table 1. Properties of the fresh milk used in this study.

No.	Composition	Value
1	Fat (%)	2.39±0.21
2	Solid non-fat (%)	8.41±0.07
3	Density (kg/m^3)	30.78±0.47
4	Lactose (%)	4.62±0.04
5	Salt (%)	0.70±0.01
6	Protein (%)	3.08±0.02
7	Added water (%)	0.00±0.00
8	Temperature (°C)	28.80±1.84

Values are presented as mean±SD.

3.2 Water activity

The water activity (aw) of pasteurized milk, with and without lysozyme treatment and long storage time, did not show any significant difference (P>0.05). The aw values of pasteurized milk samples in this study ranged from 0.88 to 0.89 (Table 2). This indicates that the addition of lysozyme did not affect the water activity of pasteurized milk during storage. It is important to note that the range of *aw* values observed in pasteurized milk allows the growth of microorganisms such as bacteria, fungi, and molds.

Similarly, in the case of sterilized milk with and without lysozyme treatment, the results of the analysis of variance demonstrated that the length of storage did not significantly affect the aw value (P>0.05). The range of aw value in sterilized milk samples used in this study was also found to be 0.88-0.89 (Table 3). It should be noted that the aw value is closely related to the water content in the food. The water content can affect the resistance of food to microbial attack and the ability of microorganisms to grow. Bacteria generally grow and multiply at high aw values (0.91), while yeast can grow and multiply at aw values ranging from 0.87 to 0.91, and molds at even lower aw values of 0.7- 0.8. Bacteria grow optimally above aw values of 0.95 (Rawat, 2015).

3.3 Water content

The results of the analysis of variance indicate that the addition of lysozyme to pasteurized milk with prolonged storage did not have a significant effect (P>0.05) on water content. The water content of pasteurized milk with or without lysozyme addition ranged from 88.00-88.75% (Table 4). This trend was also observed in sterilized milk, which had a water content ranging from 86.75-88.75% (Table 5). The treatment of sterilized milk with or without addition of lysozyme with long storage time did not have a

Table 2. Water activity value of pasteurized milk treated with the addition of lysozyme and storage time.

Storage (day)	Treatments		Average
	A	B	
0	0.89±0.01	0.88±0.00	0.89±0.00
2	0.88±0.01	0.88±0.01	0.88±0.00
4	0.89±0.00	0.88±0.00	0.88±0.00
6	0.89±0.01	0.88±0.01	0.88±0.00
8	0.88±0.00	0.88±0.00	0.88±0.00
Average	0.89±0.00	0.88±0.00	0.88±0.00

Values are presented as mean±SD. A: Pasteurized without lysozyme, B: Pasteurized with lysozyme.

Table 3. Water activity value sterilized milk treated with the addition of lysozyme and storage time.

Storage (day)	Treatments		Average
	C	D	
0	0.88±0.00	0.89±0.00	0.88±0.00
2	0.88±0.00	0.88±0.00	0.88±0.00
4	0.89±0.01	0.89±0.01	0.89±0.01
6	0.88±0.01	0.88±0.00	0.88±0.00
8	0.88±0.00	0.88±0.00	0.88±0.00
Average	0.88±0.00	0.88±0.00	0.88±0.00

Values are presented as mean±SD. C: Sterilized without lysozyme, D: Sterilized with lysozyme.

Table 4. Water content values of pasteurized milk treated with the addition of lysozyme and storage time.

Storage (day)	Treatments		Average
	A	B	
0	88.75±0.00	88.63±0.18	88.69±0.13
2	88.00±0.71	88.25±0.00	88.13±0.50
4	88.25±0.35	88.38±0.18	88.31±0.13
6	88.50±0.00	88.50±0.35	88.50±0.25
8	88.13±0.53	88.38±0.18	88.25±0.25
Average	88.33±0.32	88.43±0.13	88.38±0.14

Values are presented as mean±SD. A: Pasteurized without lysozyme, B: Pasteurized with lysozyme.

Table 5. Water content values of sterilized milk treated with the addition of lysozyme and storage time.

Storage (day)	Treatments		Average
	C	D	
0	88.50±0.71	88.50±0.35	88.50±0.25
2	88.00±0.35	87.25±1.06	87.63±0.50
4	87.63±0.53	88.00±0.00	87.81±0.38
6	88.25±0.35	88.25±0.35	88.25±0.00
8	87.75±0.35	86.75±1.77	87.25±1.00
Average	88.03±0.16	87.75±0.71	87.89±0.39

Values are presented as mean±SD. C: Sterilized without lysozyme, D: Sterilized with lysozyme.

significant effect (P>0.05) on water content. The analysis of water content in a food system is crucial as water plays a significant role in moderating product damage. A study by Amin *et al*. (2017) suggested that a low water content often leads to a lower risk of food deterioration.

3.4 Total plate count

The results of the analysis of variance have shown that the addition of lysozyme to pasteurized milk had a significant effect (P<0.05) on the total plate count (TPC), as presented in Table 6. The addition of 70 µM lysozyme to pasteurized milk reduced the number of bacterial colonies, from 2.94 CFU/mL (before the addition of lysozyme) to 2.43 CFU/mL (after the addition of lysozyme) (Table 6). According to Watts (2016), pasteurization was not intended to kill all bacteria in milk, which left the risk of post-pasteurization contamination. However, the addition of lysozyme in this study successfully killed the remaining bacteria and prevented their further growth. As reported by Shahmohammadi (2018) and Joao *et al.* (2016), lysozyme selectively kills pathogenic bacteria, which is attributed to the bacterial cell membrane properties. Shahmohammadi (2018) reported that lysozyme is more effective in killing gram-positive bacteria. The antibacterial activity of lysozyme occurs through the hydrolysis of β 1-4 glycosidic bonds of homopolymeric carbon N-acetylglucosamine (NAG) and heteropolymeric carbon N-acetylmuramic acid (NAM) of peptidoglycan in bacterial cell walls (Wua *et al.,* 2019). The active sites of lysozyme, Glutamic acid 35 (Glu35), and Aspartic acid (Asp52) act as acid catalysts and covalent bonds, respectively, to provide antibacterial activity (Held and Smaalen, 2014).

Table 6 shows no significant difference in the microbial population of pasteurized milk during storage (P>0.05), indicating that lysozyme had no significant effect on the storage of pasteurized milk. Instead, the storage temperature played a more significant role in preventing bacterial growth during storage. This is consistent with Irigoyen *et al.* (2005), who reported that a low temperature of refrigerators can retard microorganisms' growth. Additionally, Zygoura *et al.* (2004) found that storing milk at a low temperature for 7 days resulted in no changes in the microbial population of the milk.

Table 7 shows that the total microbial population of sterilized milk without lysozyme was comparable to that of milk with lysozyme (P>0.05). This is because sterilization killed all bacteria in the milk. As reported by Suwito *et al.* (2010), the sterilization heating process can kill putrefactive, pathogenic, and spore-forming bacteria, making milk safe for consumption. In addition, sterilization was treated after the treatment of milk by lysozyme which could thermally inactivate this protein. Venkataramani *et al*. (2013) reported that the optimal temperature for lysozyme activity is in the range of 69-71°C, which is considerably lower than the sterilization temperature used in this study. However, this optimal temperature is similar to the pasteurization temperature applied in this study. Therefore, it can be concluded that the antibacterial activity of lysozyme could still be observed in pasteurized milk, as shown in Table 6. Like pasteurized milk, Table 7 also shows that the bacterial population of sterilized milk, with or without lysozyme, remained comparable during storage at a low temperature.

Table 6. Total plate count values of pasteurized milk treated with the addition of lysozyme and storage time.

Storage (day)	Treatments		Average
	A	B	
Total Count (CFU/mL)			
0	2.78±0.02	2.44±0.07	2.61±0.03
2	2.65±0.26	2.40±0.00	2.52±0.18
4	3.28±0.49	2.40±0.00	2.84±0.34
6	2.82±0.20	2.40±0.00	2.61±0.14
8	3.19±0.92	2.49±0.04	2.84±0.62
Average	2.94±0.35^a	2.43±0.03^b	2.69±0.22

Values are presented as mean±SD. Values with different superscripts within the same row are statistically significantly different (P<0.05). A: Pasteurized without lysozyme; B: Pasteurized with lysozyme.

Table 7. Total plate count values of sterilized milk treated with the addition of lysozyme and storage time.

Storage (day)	Treatments		Average
	C	D	
Total Count (CFU/mL)			
0	2.40±0.00	2.40±0.00	2.40±0.00
2	2.40±0.00	2.40±0.00	2.40±0.00
4	2.40±0.00	2.40±0.00	2.40±0.00
6	2.40±0.00	2.40±0.00	2.40±0.00
8	2.77±0.53	2.40±0.00	2.59±0.38
Average	2.43±0.24	2.40±0.00	2.44±0.17

Values are presented as mean±SD. C: Sterilized without lysozyme, D: Sterilized with lysozyme.

3.5 Organoleptic test

A semi-trained panel was used for the organoleptic test to evaluate the scalar test of pasteurized and sterilized milk with 100 µM lysozyme added. The quality criteria assessed were color, aroma, sweetness, and viscosity, and the average results of the organoleptic test were presented in Table 8. The average results of the scalar test on milk color showed significant differences between treatments (P<0.05). The color assessment of pasteurized milk without lysozyme, pasteurized with

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Table 8. Average scalar test values for pasteurized and sterilized milk with the addition of lysozyme.

Variable	Treatments			
	A	B	C	D
Color	8.08±1.06^{a}	8.50±0.88^{a}	2.69±1.75^{b}	3.14±1.53^{b}
Flavor	7.18±1.47	7.48±1.32	7.91±1.68	7.70±1.93
Sweet taste	4.22±1.97^{a}	6.98±1.90^{b}	4.52±2.17^{a}	7.22±1.75^{b}
Viscosity	2.07±1.72^{a}	2.41±1.38^{ab}	3.21±1.99^{bc}	3.36±2.27^{c}

Values are presented as mean±SD. Values with different superscripts within the same row are statistically significantly different ($P<0.05$). A: Pasteurized without lysozyme, B: Pasteurized with lysozyme C: Sterilized without lysozyme, D: Sterilized with lysozyme.

Color scale: 0 – yellow to 10 – white; aroma scale: 0 – sour smell to 10 – milky smell; sweetness scale: 0 – not sweet to 10 – very sweet; viscosity scale: 0 – very runny to 10 – very thick.

lysozyme, sterilized without lysozyme, and sterilized with lysozyme were 8.08 (yellow), 8.50 (yellow), 2.69 (slightly white), and 3.14 (slightly white), respectively. Milk color was influenced by the reflection of light in dispersed fat globules, calcium caseinate, and calcium phosphate, as well as the presence of water-soluble and fat-soluble pigments, such as lactoflavin, β-carotene, retinol, and xanthophylls (i.e. lutein and zeaxanthin) (Chudy *et al*., 2020). Pasteurization resulted in white-colored milk, while sterilization caused a yellowish color due to the Maillard reaction, which was the result of the reaction between reducing sugars and amino acids. The addition of lysozyme did not change the color of the milk (Reineccius, 2006). The aroma of milk was specific and rich due to the content of volatile acids and fat in milk. The results of the scalar test of variance analysis showed that there was no significant difference in the aroma of milk given different treatments ($P>0.05$). The aroma of the four milk treatments had values ranging from 7.18 to 7.91, which meant it was milky. The addition of lysozyme and different treatments did not affect the aroma of the milk. Smell and taste were easily influenced by the cow itself, feed, surrounding odors, decomposition of milk content, foreign materials, and changes in chemical reactions (Suardana and Swacita, 2009).

The sweetness of both pasteurized and sterilized milk was significantly affected by the milk scalar test ($P<0.05$). The mean sweetness ratings for milk containing lysozyme were 6.98 (sweet) and 7.22 (sweet) for pasteurized and sterilized milk, respectively. In contrast, milk without lysozyme had values of 4.22 (slightly sweet) and 4.52 (slightly sweet) for pasteurized and sterilized milk, respectively. The addition of lysozyme to both pasteurized and sterilized milk increased the degree of sweetness. Specifically, the addition of lysozyme increased the sweetness of pasteurized milk from 4.22 to 6.98 (an increase of 2.76) and the sweetness of sterilized milk by 2.70 times. This indicates that the increase in sweetness for both types of milk is the same. However, the heating treatment (pasteurization and sterilization) resulted in a decrease in sweetness due to the structural changes in amino acids responsible for sweetness, as observed in research conducted by Wulandari et al. (2020) on local poultry egg lysozyme, which has a sweetness ranging from 529 to 649 times that of sucrose.

The assessment of milk viscosity using the scalar test yielded an average result that significantly influenced viscosity ($P<0.05$). The viscosity values of all four milk treatments ranged from 2.07 to 3.36, indicating that the milk samples were still considered to be in good condition. When milk exhibits normal color, aroma, flavor, consistency, and viscosity, it is considered to be in good condition (Aritonang, 2017).

4. Conclusion

The addition of lysozyme to milk that underwent pasteurization or sterilization treatment did not affect the *aw* value and water content of the milk. However, the addition of lysozyme was able to reduce the microbial population more significantly in milk that underwent pasteurization. Meanwhile, no changes in the microbial population were observed in sterilized milk with or without the addition of lysozyme. Additionally, the addition of lysozyme was able to enhance the sweetness of both pasteurized and sterilized milk. These findings suggest that besides its antimicrobial properties, lysAozyme can also provide a sweet taste effect.

Conflict of interest

The authors declare no conflict of interest.

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