Implementation of Ozonation Technology on Microbiological Quality of Milk: Study of Differences Fat and Protein Percentages Against Pathogenic Bacterial Resistance

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Abstract. The high level of pathogenic microbial contamination in milk can reduce milk quality. Milk as a source of animal protein is produced by dairy cattle with complete nutritional content. The complete nutritional content causes milk to be an ideal medium for bacterial growth. The bacteria that are found in milk are generally the result of cross-contamination. The contamination can occur due to the wrong handling of milk, both from the milking process to the processing process. Method to minimize the number of microbes due to contamination in milk have been carried out, one of which is heat treatment, but in practice, the wrong application of heat treatment can cause protein damage in milk. Another method that can be done is to use ozone technology. Ozone is one of the sterilization methods using ozone (O_3) to oxidize bacterial cell walls. Sterilization can injure or kill bacteria that found in milk. This research carried out through several stages, one of which is done by adding 2%, 4%, 6% and 8% fat and protein, each sample injected with *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* bacteria. This study aims to test the resistance of pathogenic bacteria that are commonly found in milk. The results showed an increase in the resistance of bacteria to ozone.

Keywords: Fat · Milk · Ozonation · Protein

1 Introduction

Milk as one of the livestock products produced by mammals-gland has a complete nutrition, such as protein 4.7%, fat 4.2%, carbohydrate 4.9% and mineral 0.76% [1]. The complete nutrition makes milk become the ideal media that is liked by bacteria, nonetheless the wrong handling of milk could cause cross contamination. The high bacteria population that caused by contamination can reduce the quality of fresh milk. Pathogenic bacteria such as *Escherichia coli* could ferment the lactose in milk and cause an upsurge in acid levels and produce harmful toxin [2, 3]. These pathogenic bacteria are frequently found in fresh milk, in the same way, there are many bacteria found in

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fresh milk, including *Staphylococcus aureus*, *Salmonella Sp*, *Escherichia coli*, *Listeria monocytogenes* and others bacteria [4].

The previous study has shown that pathogenic bacteria can reduce the quality of milk and produce harmful toxins. In order to anticipate the damage caused by pathogenic bacteria, there are some treatments to lessen pathogenic bacteria, as one of them is carried out to sterilize milk. There are many methods used to sterilize milk, one of the most commonly used is heat treatment, but improper heating can reduce the quality of milk. High heat treatment can destroy some vitamins, minerals and good bacteria. However, at high temperature in pasteurization, vitamin C was reduced by 20%, soluble calcium and phosphorus were reduced by 5%, thiamin and vitamin B12 were reduced by 10% [5]. The improper or excessive heating can damage the nutrition of milk. In a more detailed look, there is another method that could be used to sterilize milk, that is ozonation [6].

Ozonation is a technology that uses ozone (O_3), where ozone will turn as an oxidizing agent in sterilization products. Ozonation technology does not change the nutritional content, because ozone gas will be vanished by evaporation [7]. Ozonation is considered to be able to eliminate microbes, as a result of oxidation carried out by ozone could cause lysis in bacteria's walls. Ozone is bactericidal, that could destroy microorganisms so that spoilage due to the activity of microorganisms can be prevented. Ozonation technology is generally used to sterilize water from pathogenic microorganisms. The use of ozonation in milk can minimize the presence of pathogenic microorganisms [8].

Ozonation technology that has been applied using water can eliminate bacteria up to 94.50% [9]. The reduction in the microbial population after ozonation shows that ozonation is a technology that can sterilize food. The research that has been done shows a decrease in the microbial population, but some of the microbes still detected are thought to have been injured. Injury in bacteria is a condition where microbes are injured and can heal themselves under certain conditions, such as nutrient content and temperature. An injured microorganism is something that happens to microbes normally when the sterilization process occurs, in the injury phase, microbes can recover themselves under certain conditions [10].

Based on the previous study, the use of ozonation technology could kill and injure the existing microbes. In this study, for further research the use of sterile milk then added with different protein and fat content will be injected using pathogenic bacteria to know the effectiveness ozon to destroy the cell wall of bacteria. Bacteria need protein to grow and develop, one of which is *Staphylococcus aureus*, this bacterium requires protein to help it attach to host cells [11]. Besides protein, fat has an important role in bacterial growth and development, a certain concentration of fat is needed for bacterial growth, for example, lipolytic bacteria [12]. These studies encourage the addition of different percentages of protein and fat in fresh milk to determine the ability of pathogenic bacteria to survive after ozonation treatment.

2 Materials and Methods

The research was conducted at Brawijaya University by analyzing the microbiological quality of milk. The sample in this study was fresh milk obtained from farmer Pak Niadi, Dau, Malang.

2.1 Stage I

This stage is carried out to test the best time for ozonation technology to kill microbes in fresh milk samples using an ozone generator with the Hanaco brand.

2.2 Stage II

The study continued with the addition of skim milk and full cream milk, for 0%, 2%, 4%, 6% and 8% to increase the protein and fat content of fresh milk. The milk was then sterilized at 121 °C for 20 min using a pressure of 1.5 atm. The sterile milk then cooled down, at the time after the sample injected with *Escherichia coli, Staphylococcus aureus,* and *Listeria monocytogenes* bacteria, after the bacteria are injected, the sample then treated with ozone generator with the best time treatment obtained in the previous stage and then analyzed:

Milk Microbiological Analysis
Microbiological analysis was carried out using the media that had been determined for each bacterium.

Total Plate Count
Bacterial test using Plate Count Agar media with a dose of 22.5 g dissolved in 1 L of distilled water. Then homogenized the media and sterilized using an autoclave at 121 °C for 20 min at a pressure of 1.5 atm. Then poured media into sterile Petri dishes containing 1 mL of the sample which had been diluted using Buffered Peptone Water at a rate of 25.5 g per 1 L of distilled water, 10 mL of media was poured, then homogenized and allowed to solidify. The solidified media was incubated for 24 h at 37 °C.

Escherichia coli
Escherichia coli test using Petrifilm 3M *Escherichia coli* media. The sample will be poured using a micropipette as much as 1 mL and then flattened using a spreader and waited for 1 min. The prepared Petri film was then put into an incubator at 37 °C for 24 h.

Staphylococcus Aureus
Staphylococcus aureus test using Baird Parker Agar media with a dose of 58 g dissolved in 1 L of distilled water. Then homogenized me-dia and sterilized using an autoclave at 121 °C for 20 min at a pressure of 1.5 atm. Then poured media into sterile Petri dishes containing 1 mL of the sample which had been diluted using Buffered Peptone Water media at a rate of 25.5 g per 1 L of distilled water, 10 mL of media was poured, then homogenized and allowed to solidify. The solidified media was incubated for 24 h at 37 °C.

Listeria Monocytogenes
Listeria monocytogenes test using Brain Heart Infusion Agar media with a dose of 52 g dissolved in 1 L of distilled water. Then ho-mogenized media and sterilized using an autoclave at 121 °C for 20 min at a pressure of 1.5 atm. Then poured media into sterile

Petri dishes containing 1 mL of the sample which had been diluted using Buffered Peptone Water media at a rate of 25.5 g per 1 L of distilled water, 10 mL of media was poured, then homogenized and allowed to solidify. The solidified media was incubated for 24 h at 37 °C.

Data Analysis
This research uses quantitative research with the Completely Randomized De-sign (CRD) method which is then analyzed using ANOVA, if there is a significant difference, it will be analyzed using Duncan's Test.

3 Results and Discussion

Milk is one of the foodstuffs of livestock with complete nutritional content. Table 1 shows the difference in the number of bacteria after ozonation, there were 9.11 log CFU's/mL in the tap water sample without ozonation, this number de-creased to 8.1 log CFU's/mL after 15 min of ozonation. The treatment for 30 min of ozonation showed a drastic decrease in tap water samples, a decrease of up to 1.41 logs, where the results of tap water samples after ozonation became 7.7 logs CFU's/mL. The best ozonation treatment occurred within 30 min [13]. This preliminary study determines the use of the time that will be used as a benchmark in this study. Table 1 shows data on total bacteria before and after ozonation.

The next research was conducted by tested resistance of pathogenic bacteria at different levels of fat and protein with ozonation treatment for 30 min. In this step, the research carried out by injected 0.1 mL of bacterial suspension in 9.9 mL of sterile milk has mixed with the addition of full cream milk according to a predetermined percentage. Milk that has injected with bacteria then homogenized with vortex for 15 s (Table 2).

The data above shows that *Escherichia coli* increases with the addition of full cream milk, the more full cream milk is added, the more resistant *Escherichia coli* bacteria are to ozonation. Nonetheless, the data shows there are no significant effect through *Escherichia coli*. Yet, their population keep increasing by the amount of fat added. *Escherichia coli* is one of the gram-negative bacteria that has thin cell walls and lower fat content than gram-positive bacteria. The data above shows that ozonation is considered to reduce the bacterial population by up to 1 log, but the addition of fat in sample could make *Escherichia coli* survive. In this case, fat is used to protect the bacteria from the ozonation that could lysis the cell wall. Data also shows the presence of *Staphylococcus aureus*, which has a significant effect and the population keep increasing. *Staphylococcus*

Table 1. Best Time of Ozonation

Time (Minutes)	TPC (Log CFU's/mL)
0	9.11
15	8.1
30	7.7

Table 2. Total Bacteria with different fat concentration

Fat Addition using Full Cream	Total Bacteria (Log CFU's)		
	Eschericia coli	*Staphylococcus aureus*	*Listeria monocytogenes*
0%	8.2	8.83[a]	9.52[a]
2%	8.70	9.96[b]	9.02[b]
4%	8.69	9.99[b]	8.36[c]
6%	8.70	9.87[b]	8.26[c]
8%	8.82	9.98[b]	8.26[c]

Notes: Different superscripts (a-c) in the same column show highly significant difference ($P < 0.01$). Superscript order shows the total bacteria amount from lowest to highest.

aureus is one of gram-positive bacteria that has thicker cell wall than gram-negative bacteria. The data above shows that *Staphylococcus aureus* continues to increase along with the addition of fat concentration. Different conditions were experienced by *Listeria monocytogenes*, this bacteria has classified into gram-positive bacteria, exceedingly the population of *Listeria monocytogenes* tended to decrease with fat addition.

The results above show a significant effect for *Staphylococcus aureus* and *Listeria monocytogenes*, meanwhile unsignificant effect in *Escherichia coli*. The data also shows that each of *Escherichia coli* and *Staphylococcus aureus* population are keep increasing, meanwhile *Listeria monocytogenes* population is decreasing. In further observation, this could be due to the fat-addition being a protector in the process of damage caused by ozone.

The mechanism of ozonation is to destroy the permeability of bacteria, ozone destroyed the cell walls by oxidizing fats, ozone could kill microorganisms by damaging cell components in which, this mechanism will penetrate cell walls and damage cell permeability [14]. Ozonation works by oxidizing lipoproteins and lipopolysaccharides found in the cell walls of microorganisms. The addition of fat seems to increase the resistance of bacteria to the ozonation process. In bacterial resistance, fat is considered to protect bacteria from damage, especially in the heating process [15]. Fat is one of the chemical components that can increase the ability to grow and develop in bacteria [12]. Fat also can increase the resistance of bacteria to damage.

The research conducted is in line with the results of *Escherichia coli* and *Staphylococcus aureus*, but not with *Listeria monocytogenes.* The results obtained in *Listeria monocytogenes* allow for the oxidation of lipids as a result of cell wall lysis in bacteria, but another thing that could happen is the occurrence of contact between ozone and bacteria, this contact can cause lipoprotein and phospholipid complexes and cause the release of one oxygen molecule from the bacterial membrane [16].

The data shows that *Escherichia coli has* increase in 2% full cream milk addition, but being stagnant to increase slowly in 4%, 6% and 8%. *Staphylococcus aureus* has the highest population between *Escherichia coli* and *Listeria monocytogenes. Staphylococcus aureus* increase in 2% full cream addition and being stagnant to increase, but in some point insignificantly shows to decrease, meanwhile *Listeria monocytogenes* shows

Table 3. Total Bacteria with different protein concentration

Protein addition using Skim Milk	Total Bacteria (Log CFU's)		
	Eschericia coli	*Staphylococcus aureus*	*Listeria monocytogenes*
0%	8.2	8.83[a]	9.52[a]
2%	8.93	8.93[b]	8.61[b]
4%	8.31	9.25[b]	9.04[b]
6%	8.25	9.27[b]	9.13[bc]
8%	8	9.34[b]	9.23[c]

Notes: Different superscripts (a-c) in the same column show highly significant difference ($P < 0.01$). Superscript order shows the total bacteria amount from lowest to highest.

in other way, these bacteria keep decreasing in 2% and 4% full cream milk addition and stagnant in 6% and 8% full cream milk addition (Table 3).

The data above shows that there is significant effect in *Staphylococcus aureus* and *Listeria monocytogenes,* meanwhile there is unsignificant effect in *Escherichia coli* at different protein concentration. In a more detailed look, *Escherichia coli* population is increase in 2% protein addition then decrease in 4%, 6% and 8%. As stated before, *Escherichia coli* is one of the gram-negative bacteria that has thinner cell-wall than gram-positive bacteria, *Escherichia coli* bacteria as gram-negative bacteria have cell walls that contain more lipopolysaccharides than gram-positive bacteria [17]. The increased of protein content could promote the cell growth of bacteria, especially in environmental stresses. This shows that skim milk addition kept the existing of *Escherichia coli* under the pressure of ozone treatment, even there is no significant effect on it [18].

Different things with *Staphylococcus aureus*, as one of the gram-positive bacteria that has a thicker cell wall, the addition of protein has a significant effect on bacterial growth and development. The role of protein in the growth of *Staphylococcus aureus*, protein supports the growth of *Staphylococcus aureus*, this is because it has many plasmids that can code for proteins. The addition of protein has an effective way to protect *Staphylococcus aureus* from damage that caused by ozonation. Similar data were also shown by *Listeria monocytogenes* as the same gram-positive bacteria as *Staphylococcus aureus.* Pathogenic species, when it comes to environmental stress, the protein will be translocated into the cell surface to protect the bacteria [18]. *Listreia monocytogenes* has proteins in their surface. In these theories, each of *Staphylococcus aureus* and *Listeria monocytogenes* has a protein in their surface, in addition of skim milk to increase the protein content could protect these bacteria from ozone. The that bacterial resistance can be influenced by media nutrients, such as protein, carbohydrates, and fat to environmental temperature [15].

The data shows that *Escherichia coli* has increase in 2% skim milk addition, then decrease in 4%, 6% and 8% skim milk addition. *Staphylococcus aureus* in the skim milk addition shows an increase population from 0% to 8%. In the same way, *Listeria monocytogenes* has the highest population between *Escherichia coli* and *Staphylococcus*

aureus in 0% as a control treatment but then decrease in 2% but keep increasing in 4%, 6% and 8%.

4 Conclusions

The Research conducted on the implementation of ozone in dairy food products shows that the longer the time ozone is applied to the sample, the more effective ozone is in killing microbes. The interesting thing that can be underlined is the increase in bacterial resistance to the addition of protein and fat. *Escherichia coli* as one of the gram-negative bacteria increased its resistance at high-fat concentrations, but not with the addition of protein. *Staphylococcus aureus* is a gram-positive bacterium that is more resistant to ozonation, even after the addition of protein and fat, while *Listeria monocytogenes* increase with the addition of protein and decreases with the addition of fat.

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