Evaluating the Effectiveness of Ozonation for Microbial Reduction in Fresh Cow Milk: A Case Study from Bandung District

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Abstract: Ozonation is a microbial inactivation technology expected to reduce microbes in fresh cow milk without compromising its nutritional content. This study evaluates the effectiveness of ozonation in reducing the microbial load in fresh cow milk from Bandung District. Using 15-minute ozone treatment, microbial levels were reduced by up to 86.6%, though only 45% of the samples achieved reductions above 50%. T-test results suggest the reductions are not statistically significant with a 95% confidence level ($p > 0.05$).

Keywords: effectiveness; fresh cow milk; microbial load; ozonation; ozone; standard

1. Introduction

Milk is a vital dietary component rich in protein, calcium, and vitamins, but it is highly susceptible to microbial spoilage due to its high water content[1,2)]. The microbiological quality of milk is a key factor in determining its safety for consumption[3)]. One common method for microbial examination is the total plate count (TPC) test. The TPC level in milk can indicates the cleanliness of the milk from the milking process, the barn environment, and and post-milking handling[4)]. Standards regarding microbial contamination limits in fresh cow milk products have not been established by the Codex Alimentarius Commission (CAC), an international organization that develops international food standards, guidelines and codes of practice. In Indonesia, the amount of microbial contamination is required in SNI 3141.1:2011 entitled Fresh Milk—Part 1: Cows. The Indonesian National Standard (SNI) is a standard that applies nationally in Indonesia, which is applied voluntarily to obtain quality assurance for the products produced[5,6)]. SNI 3141.1:2011 states the maximum amount of microbial contamination in the Total Plate Count (TPC) is 1 x 10^6 CFU/ml. Compliance with such standards is particularly important given the country's substantial milk consumption.

Conventional methods to control microbial contamination in milk include heating and the use of antibiotics. Heating is most commonly used technique, but it can reduce milk's nutritional content and quality, especially when initial microbial contamination is high[7)]. Meanwhile, administering antibiotics to dairy cows may lower bacterial counts but poses risks of antimicrobial resistance and antibiotic residues in milk, raising public health concerns[8)]. It is also important to address the issue of antibiotic misuse, which can contribute to antibiotic resistance in cows[9,10)]. In addition, the interactions among vaccination, drugs, and herbal therapies given to cows, must be considered to avoid negative effects on milk production. These challenges highlight the need for safer, non-thermal alternatives that ensure microbial safety while preserving the natural characteristics of milk.

Ozonation has emerged as a promising non-thermal technology for microbial control in the dairy industry. In recent years, the use of ozonation in industrial application has increased significantly. Ozone is a highly reactive oxidizing agents that exhibits broad-spectrum antimicrobial efficacy, capable of eliminating bacteria and fungi without leaving toxic residues[11,12)]. The application of ozone is well established in various material processing

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and treatment processes, including use in cooling towers to suppress bacterial growth, reduce corrosion, and increase efficiency[13–15]. In addition, ozone technology employed for disinfection and sterilization processes can significantly improve efficiency, taking into account time criteria and economic factors[16]. In the context of milk safety, ozonation effectively kills microbes without pesticides or other chemicals that can degrade milk quality or harm consumer health, while also increasing cost efficiency. Several studies have shown that ozonation treatment has been shown to reduce microbial load in milk, with efficiency depending on treatment duration and the milk type. After 15 minutes of ozonation, the microbial load including *Enterobacteriaceae* (0.96 log), *mesophilic aerobes* (0.60 log), *psychotropic bacteria* (0.13 log), molds and yeasts (0.48 log), and *Staphylococcus sp*. (1.02 log)[17]. Another study reported decreases ranging from 1.7 to 2.16 log cycles in both whole and skim milk following treatment[18]. However, the method showed limited efficacy against *Staphylococcus aureus*, achieving only 0.42 log reductions in skim milk and 0.21 log in whole milk[19]. In contrast, ozonation was more effective against *Pseudomonas*, with reductions of 1 to 4 logs after 5–10 minutes, and greater effectiveness was observed in skim milk compared to whole milk after 15 minutes of exposure[20]. Other study show that ozonation for 20 minutes on fresh cow milk can eliminate the fishy odor of fresh milk[21]. Optimization of ozonation exposure time and concentration is critical to achieving consistent microbial reduction[22]. Ozonation proved to be more effective in air than in water on all examined organisms, except *Staphylococcus aureus*[23]. Ozonation was also most effective in decreasing microbial counts in samples with low fat content[24]. In the dairy industry, ozone is also used for various purposes, such as treating bovine mastitis, achieving a 60% recovery rate in infected cows without antibiotics, and ensuring the microbial safety of milk[25]. Furthermore, application of ozonation to pasteurized milk at concentrations of 5 and 10 ppm has been shown to significantly enhance shelf life of pasteurized milk[26].

While ozonation has been widely explored as a non-thermal microbial reduction method, limited studies have focused on its localized application in Indonesia's dairy supply chain. This study aims to evaluate the microbial reduction potential of 15-minute ozonation treatment on fresh cow milk from Bandung District, and to assess whether treated milk meets the Indonesian National Standards (SNI 3141.1:2011).

2. Material and Method

The hypothesis of this study states that ozonation will significantly reduce the microbial load in fresh cow milk. The following methodological approach was created to ensure accurate and reliable data collection and analysis.

2.1. Research Design

This study combined observational methods with laboratory analyses conducted at two locations. The data include fresh milk test results, both before (untreated) and after ozonation. Sampling observations were perform on the farm, while laboratory analyses were carried out at the Biotechnology Laboratory of the National Research and Innovation Agency (BRIN) in Serpong to evaluate changes in the microbial load of the milk.

The primary reference was SNI 3141.1:2011, a standard for fresh milk. SNI 3141.1:2011 states that the maximum of microbial load in Total Plate Count (TPC) is 1 x 10^6 CFU/ml. Laboratory testing to determine microbial load was conducted using the Total Plate Count (TPC) test parameter in accordance with SNI ISO 4833-1:2015. The test material used in this study was fresh cow milk sourced from farms around the Bandung district. Ozonation of the fresh cow milk samples was performed for 15 minutes using an ozone generator machine. This study did not include control sample (untreated and stored under the same conditions) for comparison, as the focus was on microbial reduction before and after ozonation treatment.

Ozonation time affects ozone's effectiveness, particularly its solubility. Longer ozonation allows more ozone to react with the components in the material[27]. According to the literature, ozone treatment under five minutes is ineffective at reducing the bacterial count in milk[17]. A ten-minutes ozone treatment is less effective than a fifteen-minute ozone treatment. After a 15-minute ozone treatment, populations of *Enterobacteriaceae*, *mesophilic aerobes*, *psychotropic* bacteria, molds and yeasts, and *Staphylococcus* sp. were all reduced

Before ozonation, microbial contamination testing was carried out to determine the microbial load in fresh cow milk. Ozonation was performed for 15 minutes at room temperature (25^{o}C) using an ozone generator with an output of 1000 ppm on 150 ml milk samples (20 samples). After treatment, microbial contamination testing was repeated to assess the effect of ozonation.

2.2. Sampel Data

This study utilized 20 fresh cow milk samples collected from cattle farms in Bandung District. This location in West Java was selected as the sampling site because the province has the highest population density and is the leading producer of cow milk in Indonesia. A convenience sampling technique was employed, allowing samples to be obtained based on ease of access[28]. The decision to collect 20 samples also considered the accessibility of the farms and the availability of research resources. Other reason is due to limited resources (research funding and testing laboratories) and research time (since microbial testing had to be conducted immediately to prevent contamination or excessive microbial growth). The limitation of this sampling are related to representativeness and

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generalizability. Since all 20 samples were collected from a single district (Bandung, West Java), the findings may not reflect the broader microbiological profile of milk from other regions in Indonesia, particularly areas with different farming practices, cow breeds, or environmental conditions. All cows providing milk samples were the Friesian Holstein breed. The dairy farms maintained hygiene cowshed floors, clean feed and water troughs, and clean area around the cowshed. Based on field observations and farm records, 15 dairy cow samples had been vaccinated once with the Aftopor vaccine, 4 dairy cow samples were vaccinated twice with the Aftopor vaccine type, and 1 dairy cow samples was unvaccinated. The fresh milk samples were collected during the current milking session (not from previous days). Immediately after collection, samples were placed in a cold box to preserve their quality.

The traits and genetic benefits of the Friesian Holstein breed in producing high milk production have been extensively researched [29]. Holstein cattle can produce milk more optimal than other breeds when they given optimal nutrition, good health care, and effective reproductive management[30]. Meanwhile, the Aceh cattle breed is known for its unique characteristics and premium-quality milk[31]. The high milk production in Aceh cattle breed can be attributed to several factors, including the utilization of nutrient-rich indigenous green fodder, sufficient water availability[32], genetic factors[33], and reproductive planning and management of Ongole crossbreeds [34] .

2.3. Analysis

Bacterial culture method was used in the laboratory to determine the quantity of bacteria present in milk. This methods involves growing bacteria from the sample on artificial media in the laboratory to determine both the number and type of bacteria present. The procedure is performed aseptically to prevent cross-contamination and ensure the accurate results. Sample were inoculated into the appropriate culture media and then examined to measure and identify the bacteria present. The bacterial count was determined by diluting the sample using either the serial or direct method. The diluted sample is then inoculated onto the culture media, and after incubation, the number of colonies formed was counted as an indicator of the bacterial population (see Figure 1 and Figure 2). Figure 1 shows the number of colonies formed after the sample was inoculated into the culture medium, allowing for colony counting. Figure 2 shows the method for calculating bacterial counts in a sample using serial dilution and the plate count method. The dilution process is carried out to reduce the concentration of microorganisms in the sample, so that colonies can be observed and counted accurately. This step is crucial to ensure that the observed colonies reflects an accurate and reliable estimate of microbial population[35].

Total Plate Count (TPC) is a method used to quantify bacterial cultures. It represents the number of mesophilic aerobic bacterial colonies found per gram or per milliliter of test sample. Total Plate Count (TPC) is commonly used to analyze microbiological standards in processed foods. Total Plate Count (TPC) method follows the established

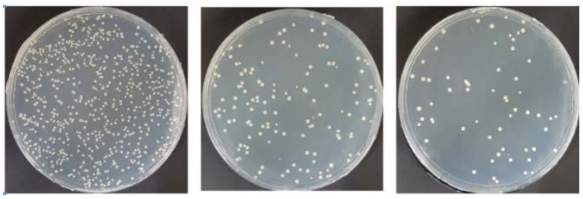

Fig. 1: Bacterial colonies in Petri dishes are present in samples[36]

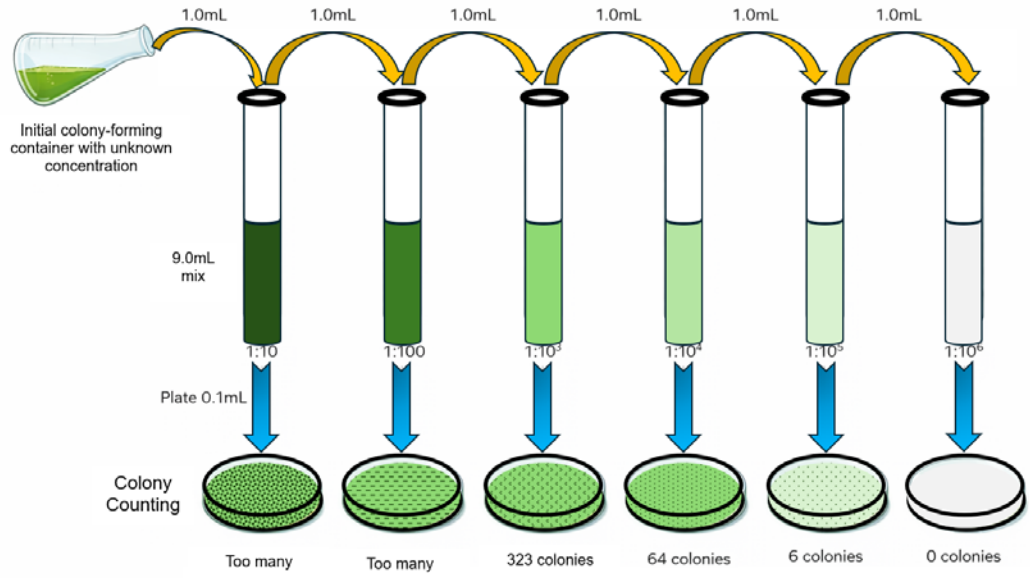

Fig. 2: Total Plate Count (TPC) testing steps: sample preparation (dilution), enrichment and confirmation

standards. Total Plate Count (TPC) to determines the number of bacteria performed in triplicate.

Microbial contamination in fresh cow milk before and after ozonation was assessed using the Total Plate Count (TPC) method in accordance with SNI ISO 4833-1:2015. This technique measures the number of viable microbes in milk samples. However, the TPC method does not detect all bacterial types capable of thriving under specific conditions. For example, lactic acid bacteria responsible for souring milk do not grow effectively on the media employed in TPC analysis. The TPC method also does not detect anaerobic or slow-growing bacteria. The TPC method also does not provide information on the specific bacterial species present in the sample. Based on SNI ISO 4833-1:2015, colony growth is carried out by calculating the Total Plate Count (TPC) in 1 mL of cow milk sample using the following formula:

$$N = \frac{\Sigma c}{(1\times n_1)+(1\times n_2)\times d} \quad (1)$$

N = Number of sample colonies (colonies/ml)
Σc = Sum of all colonies counted on the two dishes retained from two successive dilutions
n_1 = Number of plates retain in the first dilution
n_2 = Number of plates retain in the second dilution
d = Dilution corresponding to the first dilution retained

In this study, statistical tests (t-test) were conducted to compare the number of bacteria in milk samples between pre-ozonation and post-ozonation. The correlation between the number of bacteria and other pertinent variables was investigated using Spearman correlation. Data processing was followed by descriptive analysis to compare the bacterial contamination in milk pre-ozonation and post-ozonation. To enhance the robustness of the analysis, 95% confidence intervals were calculated to assess the precision of mean differences. Bivariate analysis such as Spearman correlation was performed to explore the relationship between cow age and percentage decrease in microbial load in fresh cow milk. These statistical approaches provide a comprehensive evaluation of the treatment's effectiveness.

3. Results and Discussion

The permitted microbial limits in fresh cow milk are specified in SNI 3141.1:2011 Fresh Milk—Part 1: Cows. This standard specifies quality, sampling, testing, packaging and labeling requirements for fresh cow milk. It states the maximum amount of microbial load in the Total Plate Count (TPC) is 1 x 10^6 CFU/ml. This study also used the Total Plate Count (TPC) method based on SNI ISO 4833-1:2015 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms, Part 1: Colony count at 30°C by the pour plate technique. This standard specifies a horizontal method for enumerating microorganisms that can grow and form colonies in a solid medium after aerobic incubation at 30°C. The method is applicable to products intended for human consumption and for animal feed. A limitation of the TPC method is its inability to identify the specific types of bacteria in the sample. TPC only counts fast-growing aerobic microorganisms, such as bacteria. Anaerobic microorganisms and many fungi will not grow under the conditions used with TPC.

Data on the microbial load in fresh cow milk from

Table 1: The number of microbial load in fresh cow milk from the Bandung area before and after ozonation

No	ID	Before Ozonization	After Ozonization	Reduction (CFU/ ml)	Reduction Percentage (%)
		Result (CFU/ ml)	Result (CFU/ ml)		
1	ID02301	3.9E+07	1.0E+07	2.9E+07	74.4
2	ID02304	1.8E+07	9.5E+06	8.5E+06	47.2
3	ID02306	2.8E+07	1.8E+07	1.E+07	35.7
4	ID 02309	1.3E+07	5.7E+06	7.3E+06	56.2
5	ID02311	8.2E+05	1.1E+05	7.1E+05	86.6
6	ID 02316	7.8E+05	4.7E+05	3.1E+05	39.7
7	ID02318	1.7E+06	8.2E+05	8.8E+05	51.8
8	ID02320	2.2E+06	1.1E+06	1.1E+06	50.0
9	ID02321	5.6E+08	2.7E+08	2.9E+08	51.8
10	ID02326	2.4E+07	1.8E+07	6.0E+06	25.0
11	ID02331	1.7E+05	1.1E+05	6.0E+04	35.3
12	ID02332	5.9E+05	1.2E+05	4.7E+05	79.7
13	ID02400	4.3E+04	1.8E+04	2.5E+04	58.1
14	ID02441	1.2E+09	7.6E+08	4.4E+08	36.7
15	ID02445	2.6E+05	1.1E+05	1.5E+05	57.7
16	ID02457	2.9E+05	2.2E+05	7.0E+04	24.1
17	ID07124	2.5E+05	2.0E+05	5.0E+04	20.0
18	ID07126	1.5E+09	1.2E+09	3.0E+08	20.0
19	ID07128	6.2E+04	5.0E+04	1.2E+04	19.4
20	ID07130	1.5E+05	1.4E+05	1.0E+04	6.7

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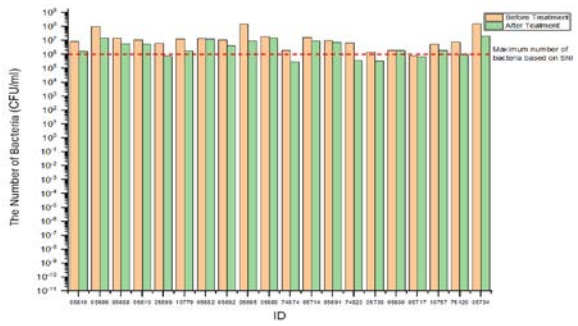

Fig. 3: The number of microbial load in fresh cow milk samples before and after ozonation

Bandung, pre-ozonation and post-ozonation, are shown in Table 1. Figure 3 shows a chart of microbial load in fresh cow milk samples before and after ozonation. The test results showed that 10 out of 20 fresh cow milk samples had microbial load higher than the standard limit outlined in SNI 3141.1:2011, which is 106 CFU/ml. Milk contamination can arise from various sources, including the environment, production equipment, and improper handling. Milk may contains dangerous microbes such as Listeria monocytogenes, Campylobacter, Mycobacterium, Salmonella, Escherichia coli, Brucella, Yersinia, Staphylococcus aureus, and Bacillus cereus that are dangerous to human health[12]. Therefore, microbial control in milk is critical for ensuring product safety and quality[37]. After ozonation process was applied to fresh cow milk samples, only 9 out of 20 samples met the SNI standard post-treatment (number of microbes above 106 CFU/ml).

From the tested samples, it was also showed varying levels of microbial load reduction in fresh cow milk. The highest microbes reduction percentage was 86.6%, and the lowest microbes reduction percentage was 6.7%. Differences in the microbial reduction percentages may occur due to variations in fat content and pH of each cow milk sample. Ozone reacts preferentially with milk fat, reducing antimicrobial efficacy in high-fat samples[38,39]. Ozone decomposes more rapidly in alkaline conditions (pH >8), shortening its active window[40,41].

The results of this study indicate that the ozonation process can reduce the microbial load in fresh cow milk. The number of bacteria in milk decreases with longer exposure to ozone[17,42,43]. Ozonation has a measurable effect on total bacterial load, where giving ozone at 3 ppm for 6 minutes can reduce the microbial load in fresh milk [42]. Other studies show a similar trend, where by extending ozonation time from 5 minutes to 10 minutes at 1.5 mg/L (equivalent to 1.5 ppm in milk) improves bacterial reduction[17]. However, longer ozonation can also reduce milk fat content [42].

Overall, the application of ozonation to fresh cow milk samples has successfully reduced the number of microbes, but the reduction percentage varied between cows. The statistics on the percentage of reduction in microbial load are presented in Table 2. Based on Table 2, the cows used as samples in this study had an average age of 5.1 years, ranging from 2.2 to 8.8 year of age. On average, the percentage of microbial load reduction after the ozonation process in fresh cow milk samples in the Bandung area was relatively low at 43.8%. As a result, ozonation did not reduce microbial loads in most samples to meet maximum number of microbes based on SNI 3141.1: 2011. Our average reduction (43.8%) with a 15-min ozonation and the statistically non-significant pre–post difference contrast with several reports that achieved larger log-reductions under different operating windows. Cavalcante et al. observed reductions across multiple microbial groups after 15 min, although the magnitudes varied by taxa (e.g., Enterobacteriaceae = 0.96 log; mesophilic aerobes = 0.60 log)[17]. Genecya et al. reported 1.7-2.16 log decreases for whole/skim milk during cold storage when ozonation was combined with pasteurization schedules[18]. Reviews also note that ozone concentration of 5-35 ppm for 5-25 minutes may deliver 99% bacterial reduction under optimized matrices[43]. Compared with these studies, our modest and variable reductions likely reflect single fixed exposure time (15 minutes) without concentration control or mass-transfer monitoring.

Figure 4 shows a scatter diagram illustrating the relationship between cow age and the reduction percentage in number of bacteria after ozonation in fresh cow milk samples from Bandung District. The diagram shows no clear linear pattern between the two variables, indicating no linear correlation between cow age and the reduction percentage in number of bacteria after ozonation. This indication is reinforced by the results of the Spearman rank correlation analysis between the two variables as shown in Table 3. The correlation coefficient was very low (-0.271), with a significance value of 0.248, which is greater than 0.05. The negative coefficient indicates that older cows tend to be associated with a smaller decrease in microbial numbers. However, this relationship is weak and not significant at the 95% confidence level ($p > 0.05$). Therefore, it can be concluded that cow age does not significantly affect the effectiveness of ozonation in reducing microbial numbers in milk samples.

Table 4 presents two groups of milk samples: before ozonation and after ozonation treatment. These two groups were compared to determine whether ozonation produced

Table 2: Statistics on the percentage of microbial load reduction in fresh cow milk

	Age (years)	Reduction Percentage (%)
N	20	20
Minimum	2.2	6.7
Maximum	8.8	86.6
Mean	5.1	43.8
Std. Deviation	1.8	21.6

Table 3: Spearman's rank correlation (spearman's rho) between cow age and percentage of microbes reduction in fresh cow milk

			Cow Age
Spearman's rho	Microbes Reduction %	Correlation Coefficient	-0.271
		Sig. (2-tailed)	0.248
		N	20

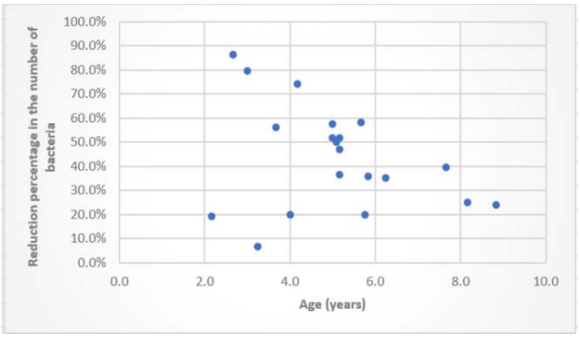

Fig. 4: Scatter diagram - The relationship between cow age and the reduction percentage in number of bacteria

a statistically significant difference. The t-test was performed assuming two sample groups have different variances. The results show that the average between group 1 and group 2 is very different, but group 2 does show a lower average than group 1, meaning that ozonation efforts have an impact on reducing the number of microorganisms. The results of the one-tail p-value (Significance) show a value of 0.286> 0.05, meaning that the difference was not statistically significant at the 95% confidence level. Similarly, the results of the two-tail p-value (Sig.) show a value of 0.572> 0.05, meaning that there is a difference after ozonation but it is not significant. The reduction in microbial load post-ozonation was not statistically significant (p=0.572), suggesting variability and suboptimal ozone performance. This may have occurred due to high variability in microbial load between cows, milking time, and handling conditions. Inconsistent ozone exposure, where ozonation efficacy is highly dependent on exposure time, ozone concentration, temperature, and milk composition, may also affect the reduction in microbial load post-ozonation. Furthermore, some microorganisms in raw milk (e.g., psychrotrophs) are also relatively resistant to ozone.

Although statistical test results indicated no significant reduction in microbial numbers, ozonation still had a positive impact in practice. Of the 20 milk samples analyzed, 45% showed a microbial reduction more than 50%. However, this effectiveness was inconsistent due to significant variation between samples. Compared with the Indonesian National Standard (SNI) for milk quality, the ozonation effectiveness in this study reached only 55% of the standard requirement. These findings highlight limitations in the ozonation machine's performance, both in terms of capacity and process stability. Therefore,

Table 4: T-test: two-sample assuming unequal variances

	Variable 1	Variable 2
Mean	169465750	102583400
Variance	1,80664E+17	9,51966E+16
Observations	20	20
Hypothesized Mean Difference	0	
dF	35	
t Stat	0,569484513	
P(T<=t) one-tail	0,28633227	
t Critical one-tail	1,68957244	
P(T<=t) two-tail	0,57266454	
t Critical two-tail	2,030107915	

although ozonation has potential as a milk processing method, optimizing the ozonation machine's performance is necessary to achieve consistent results and meet quality standards.

This study's results showed that ozonation effectively reduces microbial load. This finding aligns with recent global studies reporting the efficacy of ozonation in reducing microbial loads in various dairy products. The application of ozone, in either liquid or gas phase, has been shown to improve microbiological safety and extend the shelf life of various food products such as vegetables, meat, fish, and beverages, without leaving harmful chemical residues[43,44]. Ozone treatment has also been proven to reduce microbial load in milk concentrate and whey concentrate, as demonstrated in a study conducted in Turkey[45].

The decision accuracy of any ozonation-based protocol matters for stakeholders who must release or withhold raw milk against SNI 3141.1:2011 (TPC $\leq 1\times10^6$ CFU/mL). In our data, only only 9 out of 20 samples met the standard post-treatment, indicating that a simple "15-minutes ozonate" rule yields variable compliance. In the milk processing industry, ozonation has also been applied for sterilization and microbial control. The advantages of the ozonation include low cost, easy of operation, and environmental friendly. Although ozonation technology offers many benefits and has significant potential for further development, it is necessary to evaluate its effectiveness in milk treatment.

4. Conclusion

While ozonation was effective in reducing microbial load in samples, only 55% met the national microbial standards post-treatment (maximum microbial according to SNI 3141.1:2011). The highest reduction in the number of microbes was 86.6%, while the lowest reduction in the number of microbes was 6.7%. The study indicates that 15-minute ozonation alone is insufficient for universal compliance. From an industrial and policy perspective, these findings highlight the importance of considering ozonation as supplementary tool rather than a stand-alone solution in dairy hygiene practice. Its integration into

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regulatory frameworks and dairy processing practices could contribute to improved milk safety. Nevertheless, this study has several limitations, including the relatively small and localized sample size, the lack of microbial species differentiation, absence of post-treatment nutrient analysis, and insufficient control of ozonation concentration. Future research should optimize ozonation parameters (exposure time, ozone concentration, etc) and evaluate impacts on milk quality (protein degradation, flavor, etc.).

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