International Journal of Design & Nature and Ecodynamics
Vol. 20, No. 5, May, 2025, pp. 1093-1102
Journal homepage: http://iieta.org/journals/ijdne

Effect of Ozonation on Microbiological Quality, Nutritional Value, and Shelf Life of Fresh Cow's Milk

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https://doi.org/10.18280/ijdne.200514

Received: 31 January 2025
Revised: 18 May 2025
Accepted: 24 May 2025
Available online: 31 May 2025

Keywords:
ozonation, food quality, food safety, fresh cow's milk, national standards

ABSTRACT

The purpose of this study is to evaluate how effectively ozonation influences the quality of fresh cow's milk, particularly in terms of reducing microbiological contamination levels in accordance with national guidelines. This research was conducted experimentally using the ozonation method and included laboratory testing with duplicate repetitions. A total of 70 fresh cow's milk samples, each representing one dairy cow, were collected from three cities in West Java, Indonesia. The sampling method used was convenience sampling, which involves selecting samples from the target population based on ease of access. West Java is one of the largest milk-producing provinces in Indonesia. The research showed that with ozonation treatment, the inactivation rate of microorganisms reached 94.50%. *E. coli* and *Staphylococcus aureus* were the two pathogenic bacteria that were inactivated at rates of 97.19% and 95.97%, respectively. The decrease in standard deviation from 2.4E+08 to 1.7E+08 suggests the treatment significantly improved the uniformity of microbial content in the milk samples. The initial temperature and exposure time were 1°C for 10 minutes. The ozonation process had no effect on the milk's color, taste, or consistency, except for a slight odor alteration noted post-treatment. In addition, ozonation increased protein and fat levels proportionally. Ozonized milk products had a longer shelf life, exceeding 19 hours at room temperature. This study showed that ozonation effectively reduces microbiological contamination in fresh cow's milk and fulfills national standards. These findings are valuable for the dairy industry in improving product quality and safety.

1. INTRODUCTION

Milk is one of the most important foods for meeting human nutritional needs. Several studies show that milk is a food rich in nutrients [1]. Milk contains water, fat, protein, vitamins (A, D, E, K, and C), carbohydrates, and minerals [2]. However, this rich nutrient composition also makes milk an ideal medium for microbial growth, posing significant safety risks if not properly treated [3]. Fresh cow's milk is susceptible to environmental influences, especially microbial or bacterial contamination. Fresh cow's milk typically contains more than three million microbes per mL. The high microbial load in fresh milk causes it to spoil quickly, as lactose is broken down into lactic acid. Once microbial levels reach a critical threshold, the milk begins to decompose. According to SNI 3141.1:2011 (Fresh Milk—Part 1: Cows), the maximum allowable microbial contamination, as measured by Total Plate Count (TPC), is 1×10^6 CFU/mL [4]. By fulfilling this standard, fresh milk is hoped to be guaranteed safe for consumption and further processing.

In the field, farmers check the quality of fresh milk quickly and practically by physically checking the smell, taste, color, and consistency of milk. Further examinations include checking for the absence of dirt or foreign objects, measuring specific gravity, and conducting alcohol tests. Fresh milk typically has a specific gravity of 1.0260-1.0280. The nutrient composition of fresh milk is reflected in its specific gravity, which should correspond to at least 2.7% protein, 3% fat, and 8% non-fat dry matter. If the fresh milk alcohol test is positive, the fresh milk is rejected even though the specific gravity reaches 1.03. In the laboratory, fresh milk must be examined more thoroughly, including testing for metal content: lead (Pb) $\leq$ 0.02 µg/mL, arsenic (As) $\leq$ 0.1 µg/mL, and mercury (Hg) $\leq$ 0.03 µg/mL.

Cow breeders try to extend the shelf life of raw milk using cooling or heating techniques. Heating is one of the most popular procedures used to minimize microbial contamination in milk [5]. Although this technique is simple and effective, it may reduce the nutritional value and quality of milk, particularly in low-quality milk with high microbial

contamination [6]. This limitation has driven interest in ozonation as a non-thermal alternative that can achieve microbial safety without compromising quality. As an inexpensive, non-thermal, and environmentally friendly alternative for food processing, ozonation technology has attracted significant interest. Ozone is an allotropic form of oxygen and a potent oxidant that dissociates spontaneously by releasing a third oxygen atom [7]. It is often used to treat drinking water, vegetables, and fruits. Ozone can inactivate microbes without affecting milk's safety and quality, thereby reducing milk's microbial contamination [8]. Ozone is a gas composed of three oxygen atoms with a molecular weight of 48 g/mol. It is unstable and rapidly decomposes into molecular oxygen (O_2) and a single oxygen atom (O). Ozone is a highly oxidative substance with broad-spectrum antibacterial properties. It can inactivate vegetative and sporulating cells in yeasts, fungi, and viruses, as well as degrade mycotoxins [9].

Ozone is highly soluble in water and has a boiling point of -112°C. Ozone has a solubility 20 times higher than that of oxygen. Its specific smell makes it simple to identify even at low doses (0.01-0.05 ppm). In addition, ozone can oxidize iron and manganese, decompose sulfites and surfactants, and remove turbidity. Ozone has strong antimicrobial properties [9, 10]. In inactivating viruses, ozone is more potent than chlorine and is more profitable because it does not leave residue and does not change the taste.

Ozone eliminates microbes through progressive oxidation of vital cell components [11]. Because lipoproteins and lipopolysaccharides are key components of gram-negative bacterial membranes, ozone attacks these layers by oxidizing sulfhydryl groups in enzymes or lipoproteins. The main target of ozonation is the cell wall, which loses its permeability barrier and leads to cell lysis. Polyunsaturated fatty acid oxidation causes cell disintegration and permeability loss. Furthermore, sulfhydryl groups and amino acids in proteins, enzymes, and peptides—as well as nucleic acids—are oxidized upon exposure to ozone. Ozone breaks down the mantle layer of spores, exposing the cortex and core to the environment [9, 12]. After exposure to 0.8 mg/L of ozone for 60 seconds, only 1% of *E. coli* and *Streptococcus faecalis* remained [12, 13].

Although ozone residue is toxic to aquatic life, it decomposes easily. As a result, during water flow, the ozone residue dissipates and poses no danger to organisms that use the water [14]. Mutagenic or carcinogenic compounds may be formed during the ozonation process. However, because they are unstable, they degrade within minutes and are no longer present by the time the product reaches consumers. According to Scrollavezza et al. [15], the use of ozone is more profitable than antibiotics because it can sterilize all types of pathogenic organisms (bacteria, viruses, fungi, and yeast) and works against toxins. This supports the potential of ozone treatment as a viable alternative for ensuring food safety, particularly in dairy processing, where biological contamination is a significant concern. While ozone offers effective antimicrobial action, attention must also be given to sources of contamination that occur during the handling and processing stages of milk.

Milk from a healthy cow is not necessarily safe for consumption [16]. Environmental pathogens, dairy equipment, and workers can contaminate cow's milk during the milking process [17]. Milk inside the mammary glands is considered sterile, but contamination can occur once it exits through the teat. The presence of both pathogenic and non-pathogenic bacteria greatly affects the quality of fresh milk. The number of bacteria in milk can be influenced by several factors originating from within the animal or outside the body [17, 18]. Even when milking is performed hygienically, contamination can still occur due to improper handling afterward. Milk is an excellent medium for microbial growth, so its shelf life is short [19].

According to research by Cavalcante et al. [8], ozone effectiveness varies based on the type of microbe and the time of ozone application. Ozone treatment for 5 minutes or less is ineffective in reducing the number of microbes in milk. The number of bacteria in Enterobacteriaceae (0.59 log), molds and yeasts (0.25 log), and Staphylococcus sp. (0.59 log) was greatly decreased by ozonation for ten minutes. After 15 minutes of ozone treatment, there were fewer molds and yeasts (0.48 log), mesophilic aerobes (0.60 log), psychrotrophic aerobes (0.13 log), Enterobacteriaceae (0.96 log), and *Staphylococcus* sp. (1.02 log). Ozone treatment for more than 10 minutes at appropriate concentrations can effectively reduce microbial loads to meet SNI standards while preserving milk quality [8].

Ozone treatment has proven to be a highly successful way for the dairy sector to lower microbiological contamination and maintain standards for milk quality. Ozone possesses potent sterilizing properties and has been employed in numerous applications, such as treating coliform mastitis in dairy cows, as per Shinozuka et al.'s research [20]. The study found that ozone treatment did not cause adverse effects such as iatrogenic endotoxemic shock and produced less endotoxin in milk than antibiotic treatment.

According to the investigation by Birwal et al. [21], *L. monocytogenes* could be eliminated from raw and branded milk samples by ozonating them for 15 minutes; the removal had only a little impact on the amounts of protein, carbohydrates, and calcium. Furthermore, ozonation efficiently lowers the populations of molds, yeast, psychrotrophic bacteria, mesophilic aerobic bacteria, Enterobacteriaceae, and Staphylococcus species in raw milk samples. In studies conducted by Alexopoulos et al., ozone was shown to be useful in lowering the overall quantity of mold spores in the air of cheese storage and ripening rooms as well as in decontaminating the water used to make cheese [22].

Furthermore, according to Afonso et al. [5], ozonation achieves a more substantial reduction in bacteria counts at specific concentrations and exposure times. For example, the log cycle reduction for Cronobacter in skim and whole milk powder was 2.71 and 3.28, respectively, at a concentration of 5.3 mg/L for 120 minutes. However, when the same concentration was utilized for 30 and 60 minutes, the effects were not significant. Coliform bacteria in butter samples were completely inactivated after 30 or 60 minutes of ozone treatment of raw cream at a concentration of 58.3 mg/L for butter production. Ozonation is generally a promising and successful method for lowering the number of microorganisms in dairy products, which aids in meeting required quality standards.

The purpose of this study was to evaluate the effectiveness of ozonation in improving the quality of fresh cow's milk by reducing the level of microbiological contamination according to national standards. The ozonation process was chosen because of its ability to deactivate microorganisms without damaging the nutrition and quality of milk, so that it can significantly extend the shelf life of milk. This study also aims to measure the impact of ozonation on the sensory

characteristics of milk such as color, taste, and consistency. Thus, the results of this study are expected to provide an alternative non-thermal technology that is effective and environmentally friendly for the dairy industry in maintaining product quality and safety.

2. MATERIALS AND METHODS

2.1 Ethical approval

This research has gained an ethical permit from the Ethics Committee of Universitas Brawijaya under the number No: 143-KEP-UB-2023.

2.2 Materials

Fresh cow's milk samples were tested using tools and materials available at the Biotechnology Laboratory - National Research and Innovation Agency of Indonesia (BRIN), Serpong. The sample for this research was 70 fresh cows' milk taken from cattle farms in West Java, Indonesia, namely Bogor City, Bandung Regency, and Subang Regency. The reason for sampling in West Java is that this province is the province with the largest level of cow's milk production in Indonesia. The sampling method uses the convenience sampling method, which is a way of taking samples from the target population based on ease of access [23]. 70 cow's milk samples were taken by considering ease of access to the location, including the availability of research resources. Taking samples of fresh milk from the milk can is done by placing it in sterile breast milk, storing it in a cool box, and cooling it using ice crystals to maintain the temperature of the milk sample during the shipping process so that it is no more than 4℃. Next, milk samples are taken and tested in the laboratory.

TPC tests in the laboratory use various types of equipment, namely: autoclave, hotplate, stir bar, beaker, glass bottle, Bunsen, Petri dish, color counter, Erlenmeyer, measuring cup, incubator, cotton, phemer paper, laminar air flow, vortex mixer, closed test tubes, ovens, micropipettes and tips, analytical balances, and water baths. Other ingredients are distilled water, alcohol, agar plate count, diluent buffered peptone water, and triphenyl tetrazolium chloride 0.5%.

This testing is a service provided at Elsa Science facilities. This laboratory examines the total number of bacterial plates in fresh milk using the SNI ISO 4833-1:2015 method, Food Chain Microbiology: Horizontal Method for Enumerating Microorganisms - Part 1: Counting colonies at a temperature of 30℃ using the pour plate technique. This method is the result of the identical adoption of ISO standards. The medium used in this study was plate count agar (Oxoid), added with 5% sterile tetrazolium chloride (Merck) as a color indicator. Peptone diluent (Oxoid) is used to dissolve formula milk into a solution. The solution of each dilution was inoculated into the solution using a sterile pipette. Medium Plate Count (PCA) is kept at 30℃ in an incubator. Each sample was tested in duplicate (duplo) as part of the technical repetitions, following the Standard Operating Procedure (SOP) of BRIN (IK-BRIN-LB-307.2.1.M-2). SOP in the testing process is determined in accordance with the rules of a competent laboratory, including having equipment calibration, competent personnel, and a supportive and appropriate location. Ozone generator model, calibration method, and dissolved ozone concentration monitoring are carried out using the SOP in the laboratory.

Liquid PCA media at a temperature of ±45℃ was poured into each cup as much as 15-20 mL, stirred until homogeneous, then allowed to stand until solid. After they were solid, all of the plates were incubated for 72 hours at 30℃ with the plates facing upside down. Colonies that grow red on the plate are observed and counted using ISO/TS 4833 procedures. If the number of colonies on the Petri plate shows between 1 and 3, the result states that microorganisms are present but less than (4×1/dilution factor) per gram or mL, or less than 40 (1×10^4 colonies/gram). The inspection results are presented as groupings based on ranking, except for grouping by the number of colonies.

2.3 Research methods

This study is a combination of experimental research and laboratory research. The data displayed is data from testing fresh milk and data from testing results from fresh milk processed by ozonation. This study examines maximum microbial contamination in fresh milk based on SNI 3141.1:2011. This study used a commercially available ozone generator device with around 600 mg/hour output. In this experimental study, ozonation treatment of fresh milk was carried out for 15 minutes.

The TPC method is used in laboratories based on SNI ISO 4833-1:2015. Not all types of bacteria can grow well in the conditions used in this method. For example, lactic acid bacteria that produce sour milk do not grow well on the media used in TPC [24]. The TPC method also does not provide information about the type of bacteria in the sample. If any harmful bacterial species exist, such as Salmonella or *E. coli*, they can harm health, even though the numbers are relatively low [11, 24].

3. RESULT

3.1 Age and dairy cows

The BRIN biotechnology laboratory tested fresh milk samples from 70 breeder cows for microbial content. Dairy cow data, pen location, vaccine data, and owner were obtained based on the cow's ear tag ID. The ear tag code was scanned using the online application "Identik PKH Kementan" to get information for this study. Based on cow age data, the largest group of cows (27.14%) is dairy cows aged 5 years. While the smallest group of cows (only 1.43%) was dairy cows aged 9 years. Dairy cows aged 2 years or more are adult cows. In this study, there were 67 or 95.71% of adult cows.

The dairy cow samples in this study came from 2 types of cows. Holstein Friesian is the most abundant type of dairy cow, namely 68%. This dairy cow type has been widely studied because it has genetic characteristics and advantages in producing abundant milk [22, 25]. If this type of cow is managed well, including good feeding and health care, it will produce optimal milk production for dairy cows [6]. The rest are Acehnese dairy cows, known for their high-quality milk, which helps meet the milk needs of the local area [26].

3.2 Evaluation of national standard requirements

The preparation and testing process occurs after the dairy cow's milk sample arrives at the laboratory. All milk samples are processed using the same procedures based on SNI ISO 4833-1:2015. Bacterial colonies were processed and counted in each petri dish for each sample using the formula shown in

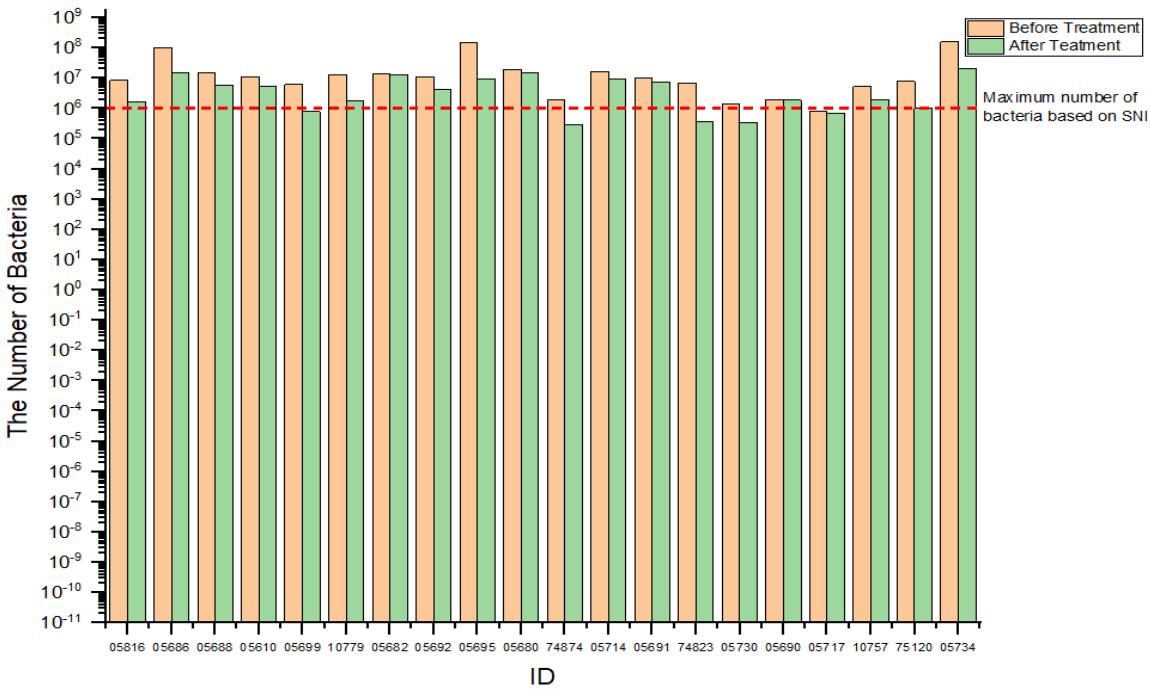
the following image. After testing 70 cow's milk samples in the laboratory using ELSA Numbers 151783/LT/LB, 153689/LT/LB, 149448/LT/LB, 153692/LT/LB, 127666/LT/LB, and 127665/LT/LB. The Total Plate Number test results were obtained in Tables 1, 2, and 3. Evaluation of dairy cow's milk samples is based on SNI as follows:

Based on the TPC test results and the maximum value of microbial contamination requirements in the Indonesian National Standard document SNI 3141.1:2011, with the title Fresh Milk - Part 1: Cows, the evaluation results showed that as many as 20% of fresh cow's milk samples met the standards. Figure 1 shows a graph of the number of microbes in cow's milk samples before and after ozonation.

Ozonation treatment has shown potential for reducing microbial counts in milk, with effectiveness varying based on treatment duration and milk composition. Studies have reported reductions in various microorganisms, including Enterobacteriaceae, mesophilic aerobic bacteria, psychrotrophs, molds, yeasts, and Staphylococcus sp., with reductions ranging from 0.13 to 1.02 log cycles after 15 minutes of treatment [8]. Another study found reductions of 1.7 to 2.16 log cycles for whole and skim milk, respectively, after ozonation [27]. However, the efficacy of ozone treatment appears to be limited for *Staphylococcus aureus*, with maximum reductions of 0.42 log CFU/mL in skim milk and 0.21 log CFU/mL in whole milk [28]. For Pseudomonas, ozonation achieved reductions of 1 to 4 logs after 5-10 minutes, with greater effectiveness in skim milk compared to whole milk after 15 minutes of treatment [29].

Table 1. Number of microbes in cow's milk from Bandung Regency before and after ozonation

No.	ID	Family	Before Ozonization		After Ozonization		Reduction (CFU/mL)	Reduction Percentage
			Result (CFU/mL)	SNI_Reg. 1.0E+06	Result (CFU/mL)	SNI_Reg. 1.0E+06		
1	02301	Holstein Friesian	3.9E+07	No	1.0E+07	No	2.900E+07	74.4%
2	02304	Holstein Friesian	1.8E+07	No	9.5E+06	No	8.500E+06	47.2%
3	02306	Holstein Friesian	2.8E+07	No	1.8E+07	No	1.000E+07	35.7%
4	02309	Holstein Friesian	1.3E+07	No	5.7E+06	No	7.300E+06	56.2%
5	02311	Holstein Friesian	8.2E+05	Yes	1.1E+05	Yes	7.100E+05	86.6%
6	02316	Holstein Friesian	7.8E+05	Yes	4.7E+05	Yes	3.100E+05	39.7%
7	02318	Holstein Friesian	1.7E+06	No	8.2E+05	Yes	8.800E+05	51.8%
8	02320	Holstein Friesian	2.2E+06	No	1.1E+06	No	1.100E+06	50.0%
9	02321	Holstein Friesian	5.6E+08	No	2.7E+08	No	2.900E+08	51.8%
10	02326	Holstein Friesian	2.4E+07	No	1.8E+07	No	6.000E+06	25.0%
11	02331	Holstein Friesian	1.7E+05	Yes	1.1E+05	Yes	6.000E+04	35.3%
12	02332	Holstein Friesian	5.9E+05	Yes	1.2E+05	Yes	4.700E+05	79.7%
13	02400	Holstein Friesian	4.3E+04	Yes	1.8E+04	Yes	2.500E+04	58.1%
14	02441	Holstein Friesian	1.2E+09	No	7.6E+08	No	4.400E+08	36.7%
15	02445	Holstein Friesian	2.6E+05	Yes	1.1E+05	Yes	1.500E+05	57.7%
16	02457	Holstein Friesian	2.9E+05	Yes	2.2E+05	Yes	7.000E+04	24.1%
17	07124	Holstein Friesian	2.5E+05	Yes	2.0E+05	Yes	5.000E+04	20.0%
18	07126	Holstein Friesian	1.5E+09	No	1.2E+09	No	3.000E+08	20.0%
19	07128	Holstein Friesian	6.2E+04	Yes	5.0E+04	Yes	1.200E+04	19.4%
20	07130	Holstein Friesian	1.5E+05	Yes	1.4E+05	Yes	1.000E+04	6.7%



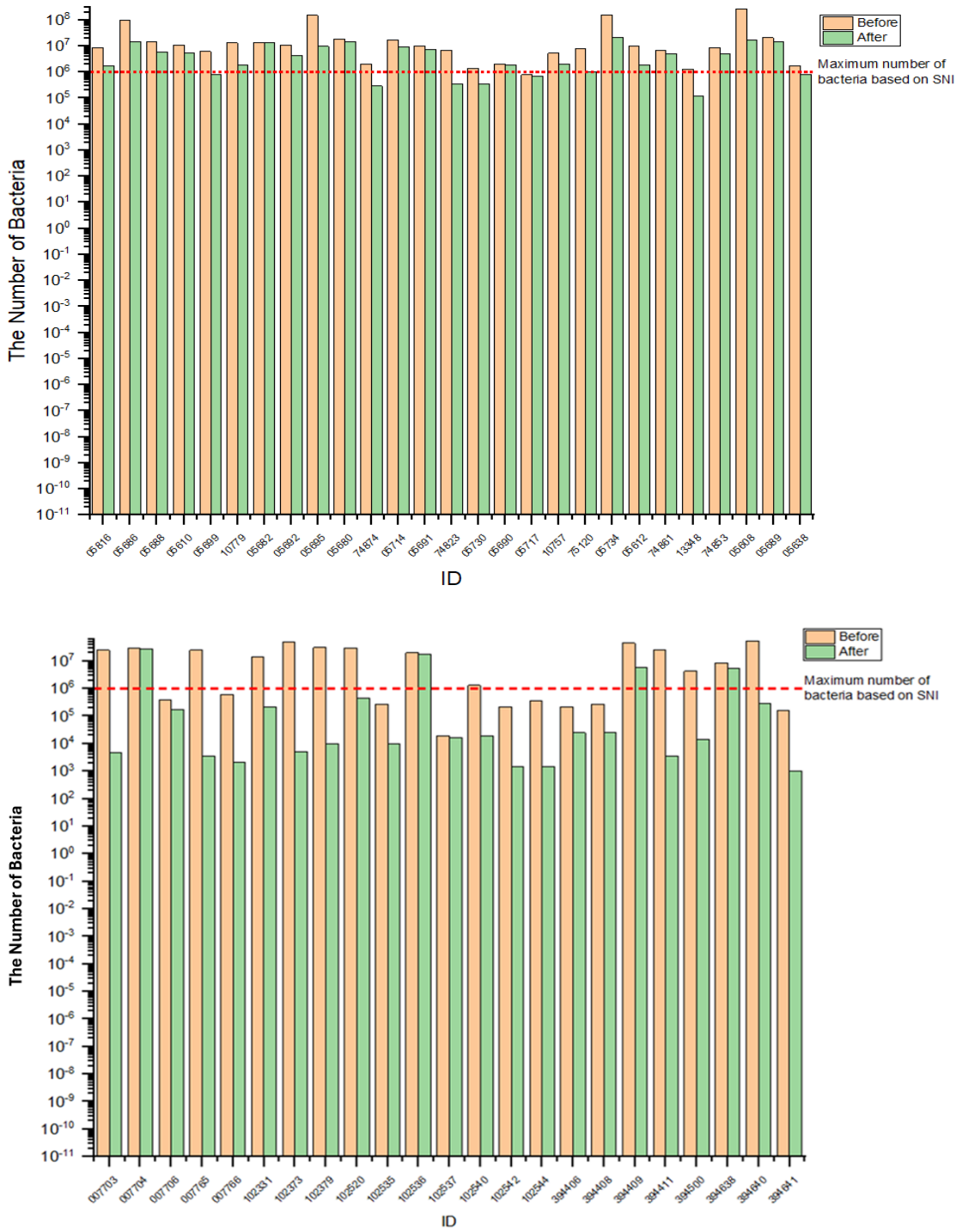


Figure 1. The quantity of microorganisms in samples of cow's milk before and after ozonation

Table 2. Number of microbes in cow's milk from Subang Regency before and after ozonation

No.	ID	Family	Before Ozonization		After Ozonization		Reduction (CFU/mL)	Reduction Percentage
			Result (CFU/mL)	SNI Reg. 1.0E+06	Result (CFU/mL)	SNI Reg. 1.0E+06		
1	05816	Holstein Friesian	8.6E+06	No	1.7E+06	No	6.900E+06	80.2%
2	05686	Holstein Friesian	1.0E+08	No	1.5E+07	No	8.500E+07	85.0%
3	05688	Holstein Friesian	1.5E+07	No	5.8E+06	No	9.200E+06	61.3%
4	05610	Holstein Friesian	1.1E+07	No	5.6E+06	No	5.400E+06	49.1%
5	05699	Holstein Friesian	6.1E+06	No	7.8E+05	Yes	5.320E+06	87.2%
6	10779	Holstein Friesian	1.3E+07	No	1.8E+06	No	1.120E+07	86.2%
7	05682	Holstein Friesian	1.4E+07	No	1.3E+07	No	1.000E+06	7.1%
8	05692	Holstein Friesian	1.1E+07	No	4.3E+06	No	6.700E+06	60.9%
9	05695	Holstein Friesian	1.5E+08	No	9.7E+06	No	1.403E+08	93.5%
10	05680	Holstein Friesian	1.9E+07	No	1.5E+07	No	4.000E+06	21.1%

No.	ID	Family	Before Ozonization		After Ozonization		Reduction (CFU/mL)	Reduction Percentage
			Result (CFU/mL)	SNI Reg. 1.0E+06	Result (CFU/mL)	SNI Reg. 1.0E+06		
11	74874	Holstein Friesian	2.0E+06	No	2.9E+05	Yes	1.710E+06	85.5%
12	05714	Holstein Friesian	1.7E+07	No	9.2E+06	No	7.800E+06	45.9%
13	05691	Holstein Friesian	9.9E+06	No	7.3E+06	No	2.600E+06	26.3%
14	74823	Holstein Friesian	6.9E+06	No	3.6E+05	Yes	6.540E+06	94.8%
15	05730	Holstein Friesian	1.4E+06	No	3.4E+05	Yes	1.060E+06	75.7%
16	05690	Holstein Friesian	2.0E+06	No	1.9E+06	No	1.000E+05	5.0%
17	05717	Holstein Friesian	8.0E+05	Yes	6.8E+05	Yes	1.200E+05	15.0%
18	10757	Holstein Friesian	5.3E+06	No	2.0E+06	No	3.300E+06	62.3%
19	75120	Holstein Friesian	7.8E+06	No	1.0E+06	No	6.800E+06	87.2%
20	05734	Holstein Friesian	1.6E+08	No	2.1E+07	No	1.390E+08	86.9%
21	05612	Holstein Friesian	1.0E+07	No	1.8E+06	No	8.200E+06	82.0%
22	74861	Holstein Friesian	6.6E+06	No	5.1E+06	No	1.500E+06	22.7%
23	13348	Holstein Friesian	1.3E+06	No	1.2E+05	Yes	1.180E+06	90.8%

Table 3. Number of microbes in cow's milk from Bogor City before and after ozonation

No.	ID	Family	Before Ozonization		After Ozonization		Reduction (CFU/mL)	Reduction Percentage
			Result (CFU/mL)	SNI Reg. 1.0E+06	Result (CFU/mL)	SNI Reg. 1.0E+06		
1	007703	Holstein Friesian	2.5E+07	No	4.6E+03	Yes	2.500E+07	100.0%
2	007704	Holstein Friesian	2.9E+07	No	2.8E+07	No	1.000E+06	3.4%
3	007706	Holstein Friesian	4.0E+05	Yes	1.7E+05	Yes	2.300E+05	57.5%
4	007765	Holstein Friesian	2.5E+07	No	3.6E+03	Yes	2.500E+07	100.0%
5	007766	Holstein Friesian	6.1E+05	Yes	2.1E+03	Yes	6.079E+05	99.7%
6	102331	Holstein Friesian	1.4E+07	No	2.1E+05	Yes	1.379E+07	98.5%
7	102373	Holstein Friesian	4.9E+07	No	5.0E+03	Yes	4.900E+07	100.0%
8	102379	Holstein Friesian	3.1E+07	No	1.0E+04	Yes	3.099E+07	100.0%
9	102520	Holstein Friesian	2.9E+07	No	4.5E+05	Yes	2.855E+07	98.4%
10	102535	Holstein Friesian	2.7E+05	Yes	1.0E+04	Yes	2.600E+05	96.3%
11	102536	Holstein Friesian	2.0E+07	No	1.7E+07	No	3.000E+06	15.0%
12	102537	Holstein Friesian	1.9E+04	Yes	1.7E+04	Yes	2.000E+03	10.5%
13	102540	Holstein Friesian	1.3E+06	No	1.9E+04	Yes	1.281E+06	98.5%
14	102542	Holstein Friesian	2.2E+05	Yes	1.5E+03	Yes	2.185E+05	99.3%
15	102544	Holstein Friesian	3.5E+05	Yes	1.5E+03	Yes	3.485E+05	99.6%
16	394406	Holstein Friesian	2.2E+05	Yes	2.5E+04	Yes	1.950E+05	88.6%
17	394408	Holstein Friesian	2.7E+05	Yes	2.5E+04	Yes	2.450E+05	90.7%
18	394409	Holstein Friesian	4.4E+07	No	5.8E+06	No	3.820E+07	86.8%
19	394411	Holstein Friesian	2.5E+07	No	3.6E+03	Yes	2.500E+07	100.0%
20	394500	Holstein Friesian	4.3E+06	No	1.4E+04	Yes	4.286E+06	99.7%
21	394638	Holstein Friesian	8.7E+06	No	5.5E+06	No	3.200E+06	36.8%
22	394640	Holstein Friesian	5.3E+07	No	2.9E+05	Yes	5.271E+07	99.5%
23	394641	Holstein Friesian	1.6E+05	Yes	1.0E+03	Yes	1.590E+05	99.4%

3.3 Effectiveness of ozonation treatments in reducing microbial numbers

For the purpose of this investigation, fresh cow's milk was subjected to a 15-minute ozonation treatment in order to lower its bacterial count [8]. A normality test is performed to see the distribution of the data before evaluating the impact of ozonation treatment on cow's milk. The Kolmogorov-Smirnov test method was utilized to conduct the data normalcy test in this investigation. The table shows the SPSS output of the normalcy test findings for the microbiological data on fresh milk, both before and after treatment. The SPSS output of the normality test results for microbial data on fresh milk before and after treatment is presented in Table 4.

A sig. value is obtained using the Kolmogorov-Smirnov test results for data normalcy based on Table 4. Data on the quantity of bacteria in fresh milk before and after treatment have a (P-value) < 0.05. This indicates that the empirical distribution of the data differs from the ideal normal distribution at the 95% confidence level, indicating that the two sets of data are not normally distributed. The existence of outlier data might lead to abnormalities in the distribution of the data. As an implication of not fulfilling the normal distribution assumption, further analysis was carried out using non-parametric statistical methods. Because this research will focus on the effectiveness of ozonation treatment in lowering the number of bacteria in fresh milk, the statistical analysis chosen was to compare the average data value of the bacteria number in milk before and after ozonation treatment. In other words, this case tests the average value of two paired data samples, so the non-parametric statistical method that can be applied is the Wilcoxon test.

An overview of the Wilcoxon Signed-Rank test procedure for the quantity of bacteria in fresh milk before and after ozonation treatment is provided in Table 5. A total of 70 negative scores were obtained when the Wilcoxon Signed-Rank Test was performed with a sample size (N) = 70. This indicates that the quantity of bacteria in all cow's milk samples decreased following ozonation treatment. The total number of ratings is 2485, with an average rating = 35.5. The number of ratings is then used to calculate the Z test statistics so that a calculated Z value of = -7.271, with a sig. value is obtained = 0.000. There is a significant change in the quantity of bacteria in dairy cows' milk before and after treatment, as indicated by

the very small (<<0.05) sig. value, which suggests this at the 95% confidence level. Stated differently, this study's ozonation procedure successfully decreased the number of bacteria present in dairy cows' milk.

From the evaluation of test results and national standard requirements, only 20% of samples met the criteria before fresh dairy milk samples received treatment. However, after receiving ozone treatment for 15 minutes, it increased to 36%, meeting national standards. This means there were 17 cow's milk samples that met national standard requirements after receiving treatment, but did not previously. However, the level of reduction in bacterial numbers varied between cows with the same duration of ozone treatment. The correlation between cow age and the extent (%) of bacterial load reduction following ozonation treatment is shown in Figure 2.

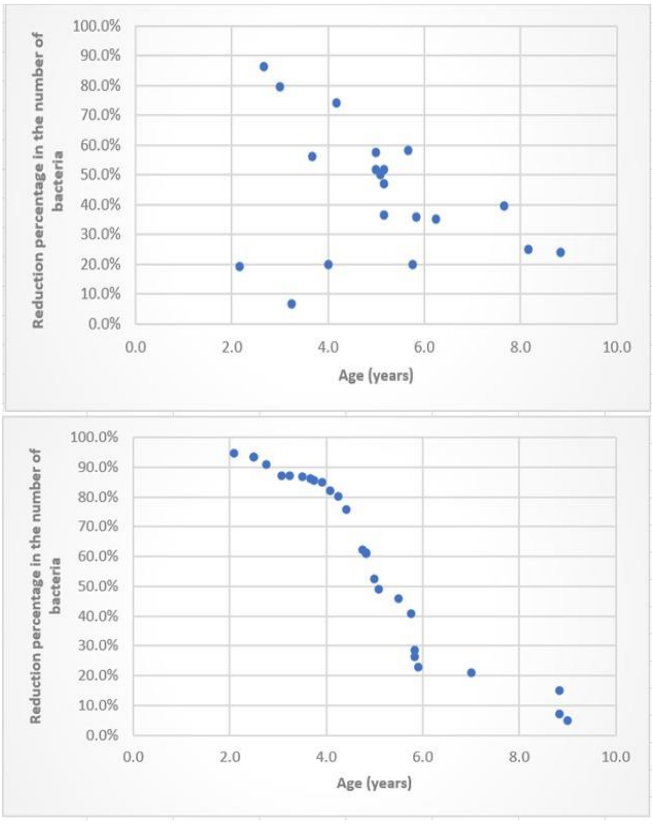
Table 4. One-sample Kolmogorov-Smirnov test

		Before	After
Sample Size		70	70
Normal Parameters[a,b]	**Mean**	66001914.29	35913012.86
	Std. Deviation	235987108.32	170429539.31
Test Statistic		.422	.478
Asymp. Sig. (2-tailed)		.000[c]	.000[c]

Note: a. Test distribution is Normal; b. Calculated from data; c. Lilliefors Significance Correction.

Table 5. Wilcoxon signed-rank test process summary

	Description	**N**	**Test Statistics[a]**	
After - Before	Negative ranks	70[a]	Z	-7.271[b]
	Positive ranks	0[b]	Asymp. Sig. (2-tailed)	.000
	Ties	0[c]		
	Total	70		
	a. After < Before b. After > Before c. After = Before		a. Wilcoxon Signed Ranks Test b. Based on positive ranks.	



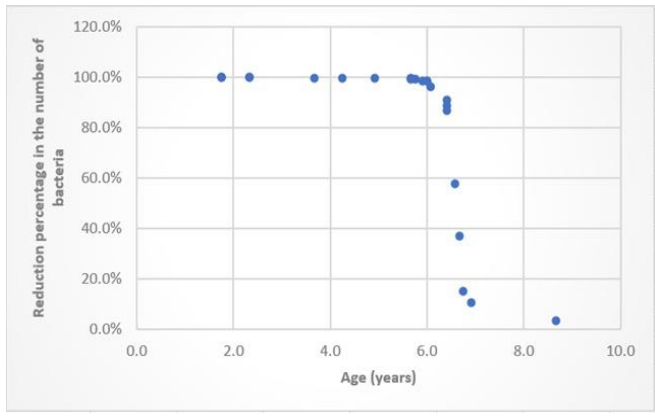


Figure 2. Scatter diagram between cow age and the percentage reduction in the number of microbes in cows

Table 6. Spearman's rank correlation (Spearman's rho) between cow age and the percentage reduction in the number of microbes in the cow's milk

		Age
Reduction Percentage	**Correlation Coefficient**	-.271
	Sig. (2-tailed)	.248
	N	20
		Age
Reduction Percentage	**Correlation Coefficient**	-.998**
	Sig. (2-tailed)	.000
	N	27
		Age
Reduction Percentage	**Correlation Coefficient**	-.998**
	Sig. (2-tailed)	.000
	N	23

A significant percentage drop (over 87%) was seen in the majority of cows, as shown in Figure 2, except for cows with sample numbers 007766 (3%), 115942 (11%), 102379 (15%), 102373 (37%), and 007764 (58%). The decrease in standard deviation from 2.4E+08 to 1.7E+08 suggests the treatment significantly improved the uniformity of microbial content in the milk samples. Figure 1 also shows that, in general, there is a tendency for the percentage reduction in bacterial numbers to increase as the cow's age increases. This finding is supported by the correlation analysis between the two factors, as shown in Table 6. Pearson correlation analysis between the percentage reduction in bacterial numbers and cow age produced a correlation value of 0.535, which was significant at the 99% confidence level. This value strengthens the assumption that there is a unidirectional relationship (positive correlation) between the percentage reduction in bacterial numbers and the cow's age.

3.4 Effect of treatment on the total number of fresh milk microorganisms

Effect of ozonation treatment on reducing the number of microbes in fresh milk. Generally, fresh cow's milk from farmers only contains *E. coli* and *Staphylococcus aureus* bacteria. The longer the ozonation treatment of the material, the more ozone concentration is dissolved and the greater the opportunity to kill the microbes in the material [30].

3.5 Shelf life of fresh cow's milk after ozonation

The fresh cow's milk used in research without ozone only

lasted 6 hours when stored at room temperature, and the alcohol test showed that it had broken. Cow's milk that has received ozone treatment can last more than 19 hours at room temperature. The longer the contact time, the longer the shelf life of fresh milk because more microorganisms are killed [15].

4. DISCUSSION

Cow's milk samples that had received ozonation treatment showed a decrease in the number of microbial colonies and total plate number in cow's milk. Based on the TPC figures, graphs can be analyzed by sorting the magnitude of the most significant reduction in microbes in 23 cow's milk samples. This study showed the potential of ozonation as an effective intervention to meet national safety standards. By significantly reducing the pathogen load, ozonation improves milk safety, in line with regulatory limits ($\leq 1 \times 10^6$ CFU/ml). These results also support its application in the dairy industry targeting global safety standards.

The study also showed increased protein and fat levels post-ozonation, suggesting potential benefits beyond microbial reduction. Ozone can change the structure of milk proteins, which may affect their functional properties, which may indirectly increase protein levels, although not significantly [31]. Ozone can also affect the solubility of fat in milk, so that fat previously bound to milk proteins may become more easily detected by certain methods [32]. Although there is little research on the effect of ozone treatment on protein and fat stability [33], this is a positive result that could impact the nutritional value and marketability of dairy products. The study noted that ozonated dairy products had a longer shelf life, exceeding 19 hours at room temperature. This result is consistent with a study by Perna et al. [33], where ozone treatment was able to extend the shelf life of liquid milk without causing significant oxidation in the product. This is a significant improvement for areas with limited cold chain infrastructure. Longer shelf life reduces spoilage and supports wider distribution, thereby increasing economic value for dairy producers [34].

This study shows that the relationship between the trend in the number of bacterial colonies and the cow's age is inversely proportional. If the number of bacterial colonies detected decreases (according to an exponential line), the younger the cow will experience this. This finding was still being studied by a research team from Cornell University [35]. Based on the research findings, younger cows generally experienced a higher rate of reduction in milk bacteria following ozonation treatment than older cows. The cow's age factor can influence the effectiveness of reducing the number of microbes in milk. Is it because cattle with a shorter lifespan may have an immune system against various types of microbes that is not yet fully formed, so the microbial response to ozone treatment still tends to be stronger and results in a more significant reduction in bacterial numbers [35]. Several factors may contribute to this observation. The dietary regimen for younger cows typically contains higher protein levels to support growth, which may alter milk composition and microbial profiles. High-protein diets in heifers led to milk containing more ozone-sensitive bacterial communities. In contrast, older cows tend to harbor more diverse and potentially ozone-resistant microbial populations, likely due to prolonged exposure to environments and repeated milking cycles [36].

This study was limited to ozone treatment with a concentration of 600 mg per hour for 15 minutes. Further studies can be carried out through the combination of ozone concentrations and optimal treatment times for each type of product, and to ensure that the use of this technique, with different combinations of concentrations and times, in the dairy industry is safe. Future research should assess the effectiveness of ozonation against a larger range of pathogenic and spoilage microorganisms, such as spore-forming bacteria and heat-resistant species. This would give a more complete picture of ozonation's potential as a milk processing technology. additionally, Large-scale studies on the economic and logistical implications of introducing ozonation in industrial dairy environments are required. Cost-benefit analysis, energy consumption, and equipment maintenance requirements should all be considered in assessments.

5. CONCLUSIONS

Ozonation, a fresh milk processing technology, shows great potential in improving milk quality to meet national and global standards. Specifically, ozonation at 600 mg/h for 15 minutes reduced microbial contamination by 94.5%, fulfilling the SNI microbial safety standards, while also extending the shelf life of fresh milk to more than 19 hours. This technology allows for a significant reduction in microbial contamination levels without compromising the sensory quality of the product. This provides an excellent opportunity for the dairy industry to produce safe, high-quality products while expanding access to global markets. Ozonation can help local producers compete with stricter international standards by maximizing food safety. Another advantage is the ability of this technology to extend the shelf life of milk, which is very important in reducing waste and increasing distribution efficiency. In the context of industrial sustainability, ozonation also provides an environmentally friendly solution by producing products free of additional chemicals. This technology has the potential to be widely adopted by the modern dairy industry, especially in areas with limited cold chain infrastructure. The increase in protein and fat levels as a positive impact of ozonation also provides added value to fresh milk products. With this, ozonation supports the industry in creating products with higher nutritional value to meet the community's needs. Through continuous innovation, ozonation can be essential in transforming the dairy industry, oriented towards quality and sustainability.

From a health perspective, ozonation reduces the risk of foodborne diseases by eliminating dangerous pathogens such as *E. coli* and *Staphylococcus aureus*. The technology also offers a safer, chemical-free alternative for consumers, especially vulnerable groups such as children and pregnant women. In the long term, ozonation could help improve global food safety, reducing the burden of disease associated with contaminated milk. The environmental implications of technology are equally important, as ozonation produces a harmless oxygen residue. By extending the shelf life of milk, the technology contributes to reducing food waste, supporting the sustainability of the dairy supply chain. The more efficient use of energy compared to conventional methods, such as pasteurization, makes ozonation more environmentally friendly. To realize its full potential, further research is needed into optimizing ozonation parameters, such as concentration and duration of exposure. ozonation has great potential to revolutionize dairy processing in a more modern and

sustainable way. This technology addresses current industry challenges and contributes to building a safer, healthier, and more environmentally friendly world.

ACKNOWLEDGMENT

The authors express their gratitude to cattle farms in West Java, Indonesia, namely Bogor City, Bandung Regency, and Subang Regency. Thank you to management and researchers at the Research Center for Testing Technology and Standards – BRIN for their assistance and feedback on this study.

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NOMENCLATURE

CFU/mL	Colony Forming Unit per milliliter
TPC	Total Plate Number
Pb	Lead
As	Arsenic
Hg	Mercury
On	free oxygen atom or O-nasen
ppm	Parts per million
SNI	Indonesian National Standard
BSN	National Standardization Agency of Indonesia
PCA	Plate Count Agar
ISO	International Organization for Standardization

Greek symbols

%	Percent
<	Less than
>	Greater than
μ	Dynamic viscosity