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RESEARCH ARTICLE

Impact of Ultrasonication on Milk Fat Globule Size and Inhibition of Salmonella enteritidis Growth

Umiatin Umiatin[1]*, Dalia Sukmawati[2], Taryudi Taryudi[3], Alluma Alluma[1], Maria Ulfa[1]

[1]*Department of Physics, Faculty of Mathematics and Natural Science, Jakarta State University, Jakarta, Indonesia*
[2]*Department of Biology, Faculty of Mathematics and Natural Science, Jakarta State University, Jakarta, Indonesia*
[3]*Department of Electronics, Faculty of Engineering, Jakarta State University, Jakarta, Indonesia*

*Corresponding Author: umiatin@unj.ac.id

Abstract: One significant effect of ultrasonic treatment is the inactivation of microorganisms. This characteristic causes ultrasound to be widely used in food processing, healthcare devices, and water treatment. Ultrasonication is an innovative technology that holds promise for accelerating processes and producing high-quality products. The ultrasonic process in milk processing includes homogenization and sterilization. Milk homogenization standardizes fat globule size, while sterilization targets pathogenic microbes harmful to humans**.** This study aims to evaluate the effect of ultrasonication on milk fat globule size and inhibition of *Salmonella* enteritidis growth, as well as the stability of pH values and temperature increase. To determine the effect of ultrasound on milk fat globule size, exposure was conducted for 30, 40, and 50 minutes on raw milk. The samples were then photographed, and the size of the formed fat globules was measured using a pixel-based approach. Furthermore, to determine the effect of ultrasonication on bacterial growth inhibition, exposure was conducted for 5 minutes, 15 minutes, and 25 minutes on UHT milk that had been cultured with *Salmonella* enteritidis bacteria. The ultrasonic wave frequency used in this study was approximately 35 kHz. The results showed a significant reduction in fat globule size after ultrasonication. The study also demonstrated that ultrasonication inhibited the growth of *Salmonella* enteritidis. Ultrasonication increased the temperature but did not affect the pH value. It can be concluded that ultrasonication has the potential to make the milk processing process more efficient because it can reduce the size of milk fat globules and inhibit pathogenic bacteria, where the current process is carried out through homogenization and pasteurization.

Keywords: Ultrasonication, Milk, Fat Globule Size, Salmonella Enteritidis UNJCC B-9

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Introduction

Milk is a functional food that serves as an essential source of macronutrients and micronutrients. Macronutrients include water, fat, protein, and lactose, while micronutrients include vitamins, enzymes, minerals, lipids, and dissolved gases [1]. Approximately 80% of annual milk production comes from cows, while the remaining portion comes from other dairy animals such as buffalo, goats, camels, and sheep [2-3]. The nutrients in milk are crucial for human health, easily digestible, and readily absorbed [4]. In 2022, total milk consumption in Indonesia reached 989,154 tons, whereas India was the world's highest milk consumer, with 85 million tons [1].

In general, the dairy processing industry consists of several stages: fresh milk reception, mixing and heating, filtration, homogenization, pasteurization, cooling, and packaging. Milk is prone to instability phenomena such as fat floating during processing and storage. To enhance sensory quality and milk stability, homogenization is a crucial step in milk processing. Homogenization is a process of standardizing particle size to maintain the stability of a mixture formed from two immiscible phases, commonly referred to as an emulsion. Homogenization aims to break down fat globules from an initial size of 5 μm to 2 μm or less. Improving milk quality and stability through homogenization involves two mechanisms: reducing fat globule size and increasing fat interaction with the protein matrix [5].

Milk is also susceptible to microbial contamination due to its high nutrient content, making it an ideal substrate for the growth of pathogenic bacteria [6]. Salmonella bacteria are among the most common foodborne pathogens worldwide. Salmonella are rod-shaped, Gram-negative, and facultatively anaerobic bacteria [7]. The optimal growth temperature for Salmonella is 37°C, but growth can occur within a temperature range of 5°C to 45°C. These bacteria can thrive in environments with a pH range of 4.0 to 9.0, with an optimal pH of around 7.0 [8]. Consuming milk contaminated with Salmonella can cause illnesses with varying severity. Symptoms may include fever, nausea, vomiting, diarrhea, and abdominal pain, and in severe cases, it can even lead to death [9]. Conventional microbial inactivation methods using heat include pasteurization through High-Temperature Short-Time (HTST) or Low-Temperature Long-Time (LTLT) techniques [10].

Pasteurization is a key step in milk processing that helps prevent product spoilage by eliminating and reducing the growth of pathogenic bacteria, ensuring milk safety for consumption. Pasteurization involves heating at 72-75°C for 15-16 seconds or 80-85°C for 10-15 seconds, as well as Ultra-High Temperature (UHT) sterilization at 135-150°C for 4-15 seconds. Although Escherichia coli can be destroyed through pasteurization, reports indicate that it could form biofilms on pasteurization equipment, leading to pasteurization failures. The stage in milk processing that causes the most significant thermal impact is pasteurization, which can reduce nutrient content [11].

The demand for dairy processing technology is increasing, requiring the production of high-quality, fresh milk that meets consumer expectations while ensuring efficiency in processing time and costs [12]. Ultrasonic wave technology has emerged as a potential solution in the dairy processing industry. Ultrasonic technology utilizes mechanical waves at frequencies above the human hearing threshold (>20 kHz). These waves can be categorized into two frequency ranges: (1) low-energy ultrasound (high frequency >100 kHz, low power, intensity <1 W/cm²) and (2) high-energy ultrasound (low frequency 20-500 kHz, high power, intensity >1 W/cm²). Low-energy ultrasound is used in non-destructive analytical techniques to measure the physicochemical properties of food, such as firmness, acidity, sugar content, ripeness, and protein interactions, while high-energy ultrasound is applied to alter the physical and chemical properties of food and inactivate microorganisms in food. In terms of cost, ultrasonic equipment is relatively inexpensive compared to other technologies, with low operational costs and an environmentally friendly alternative to thermal pasteurization [13].

In recent years, research, exploration, and application of the physical and chemical effects of ultrasonication in the dairy industry have gained popularity. Ultrasonic wave technology can be used to reduce the number of pathogenic bacteria in food products, such as Escherichia coli [14]. Pathogenic bacterial inactivation has been reported to cause changes in volatile compounds, potentially affecting the sensory quality of milk [15]. Further research is needed to confirm its effectiveness in controlling pathogenic bacterial contamination in dairy products. Therefore, this study aims to analyze the impact of ultrasound on the milk fat globule size and inhibition of bacteria growth.

Methods

Milk Collection and Treatments

To determine the impact of ultrasonication on the milk fat globule size, raw milk samples were obtained from the Farm Milk dairy farm in Cipayung District, East Jakarta. Each experiment used a 200 ml milk sample. The milk samples were first processed through heating or pasteurization at 90°C for 5 minutes. This heating process aimed to eliminate bacteria present in the milk.

Next, the samples were cooled to 50°C before undergoing ultrasonication treatment with varying durations of 0, 30, 40, and 50 minutes. The four samples were used for fat globule size analysis.

To evaluate the effects of ultrasonication on the growth inhibition of pathogenic bacteria, UHT milk, which is already sterile, was used to ensure that no other microbial contamination was present in the sample. At this stage, the UHT milk was inoculated with Salmonella enteritidis. The ultrasonication time for the UHT milk samples inoculated with these bacteria was 5, 15, and 25 minutes. The differences in ultrasonication duration for fat globule size reduction and bacterial inhibition were based on previous research that concluded effective bacterial inactivation with ultrasonication of less than 30 minutes. This also avoids high temperature increases that could potentially damage milk proteins [16].

Bacterial Cell Preparation and Ultrasonic Treatment

The next step was to rejuvenate the Salmonella enetritidis bacteria and inoculate them into the milk samples. In the rejuvenation stage, the first step was to prepare a bacterial growth medium, NA (nutrient agar). 2.8 grams of NA was placed in an Erlenmeyer flask with 100 ml of distilled water, then heated until the medium turned clear yellow. Afterward, 5 ml of the solution was poured into 10 test tubes. The mixture was then autoclaved at 121°C for 15 minutes. After cooling, the Salmonella enteritidis bacteria isolate was taken, streaked onto the NA medium, and incubated for 24 hours.

For the inoculation stage, 10 bottles were filled with 9 ml of UHT milk. The bottles were then autoclaved at 121°C for 15 minutes. After cooling, 1 ml of bacterial suspension was added to each bottle. The bottles filled with UHT milk and bacterial suspension were vortexed to mix. Bacterial counts were directly counted in the control samples, while ultrasonic exposure was applied to the treatment samples. Four group samples were made, consisting of one control sample and 3 samples that were exposed to ultrasonic waves for 5, 15 and 25 minutes, which was lebeled as C (control) and U (ultrasonication) involve U5, U15 and U25. Each group was technically replicated into two samples.

For ultrasonication, the ultrasonic generator JYD-1500 E with a frequency of ± 35KHz and power 600W was used. The control was kept at room temperature while the other 3 samples were being treated with ultrasonic. After the ultrasonic treatment was completed, the samples were conditioned for 24 hours at room temperature, then the bacterial colonies formed were counted.

Microscopic Analysis

Bacterial counts were determined using the Total Plate Count (TPC) method. In this method, milk samples containing Salmonella enteritidis were cultured on Plate Count Agar (PCA) media. The PCA petri dishes containing Salmonella enteritidis were incubated for 24 hours. The bacteria proliferated and formed visible colonies on the PCA, allowing for direct colony counting without the need for a microscope. The bacterial count was calculated using the following equation:

$$\frac{CFU}{ml} = \frac{number\ of\ colonies\ x\ dilution\ factor}{sample\ volume} \qquad (1)$$

pH Measurement

The pH test of the milk samples was conducted to determine the effect of ultrasonic wave exposure on the pH value of the milk. The pH of both control and treated samples exposed to ultrasonic waves was measured using a pH meter.

Results and Discussion

Impact of Ultrasonication on the Milk Fat Globules Size

Fat in milk exists in the form of milk fat globules, which consist of triglycerides surrounded by a thin membrane known as the milk fat globule membrane. The milk fat globule membrane contains polar lipids, cholesterol, and specific membrane proteins, acting as an emulsifier for milk fat while protecting it from enzymatic degradation and coalescence. The size of the fat globules significantly influences the quality of dairy products, particularly their sensory and rheological properties. Therefore, the homogenization process is applied in milk processing to reduce the size of milk fat globules [17].

After ultrasonication, the milk samples were observed under a microscope as shown in Figure 1.

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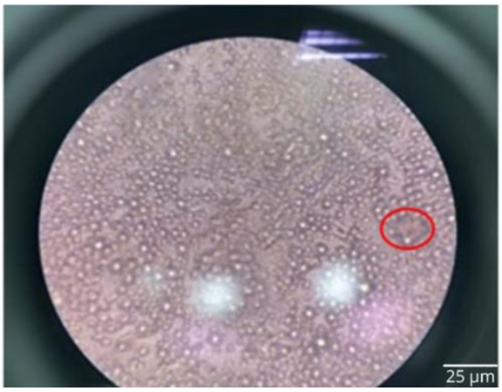

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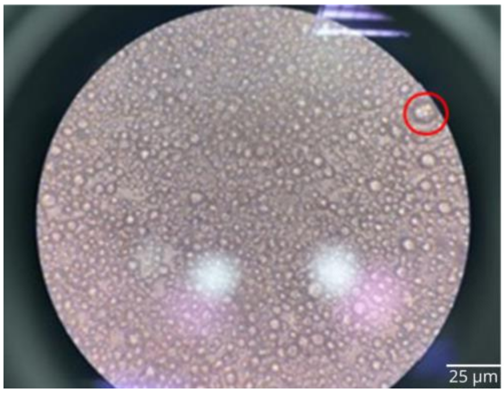

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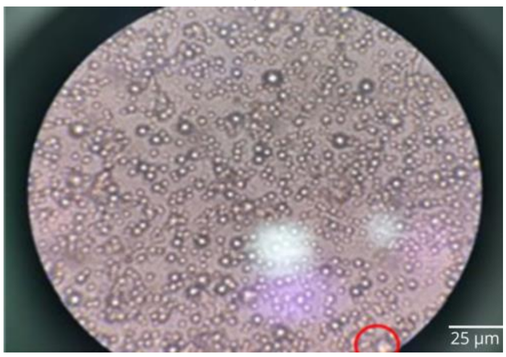

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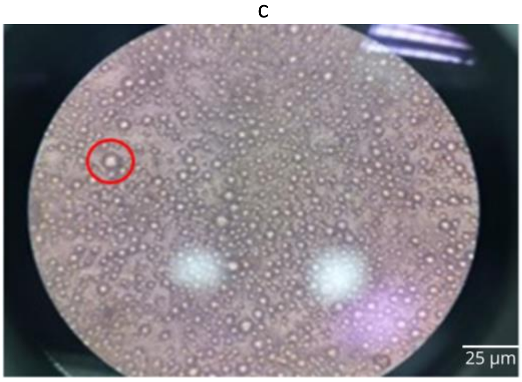

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Fig. 1: Micrograph results of milk sample subjected to ultrasonication at frequency ±35kHz. a) without homogenization, b) homogenization for 30 minutes, c) homogenization for 40 minutes, d) homogenization for 50 minutes. The red circle indicates the largest size of the milk fat globule that has been analyzed. Sample were observed using 1000x magnification

The analysis of milk fat globule size was conducted using Image J software, by calculating the diameter of the largest fat globule in each sample. Table 1 below presents the results of the fat globule size analysis.

Table 1: Measurement of fat globule size

No	Frequency (kHz)	Time (minutes)	Diameter of fat globule (μm)
1	0	0	18.3
2	±35	30	13.6
3	±35	40	9.2
4	±35	50	8.9

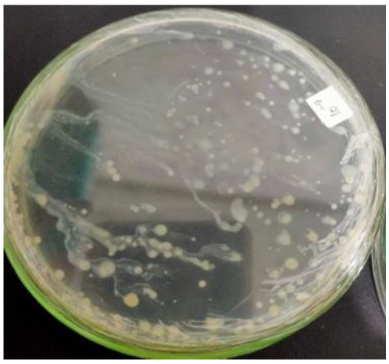
Milk samples without ultrasonication treatment had the largest globule size whereas milk samples subjected to ultrasonication for 50 minutes resulted in the smallest globule size.

The final particle size of the emulsion in ultrasonication depends on various sonication parameters (ultrasonic frequency, power, intensity, etc.), treatment duration, emulsion fat content, emulsifiers, and the amount of emulsifier used [18].

Impact of Ultrasonication on the Inhibition of Salmonella Enteritidis

Conventional heat treatment or milk pasteurization has a significant impact on milk composition, destroying protective proteins and immunoglobulins. With the increasing consumer interest in nutritious and high-quality food, there is a growing demand for the development of non-thermal treatment methods in milk processing to reduce microbial contamination [15].

In this study, milk samples containing Salmonella enteritidis were subjected to ultrasonication for 5, 15, and 25 minutes. The control sample was not exposed to ultrasonication. The bacteria from these samples were then cultured on PCA medium and incubated for 24 hours, with each condition tested in duplicate. The bacterial colony count results on PCA are shown in Figure 2 below.



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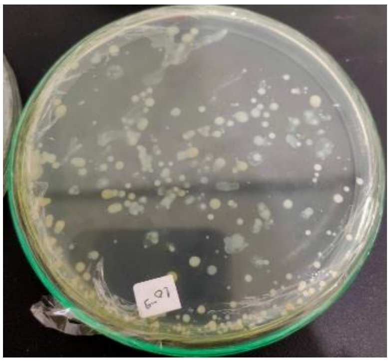

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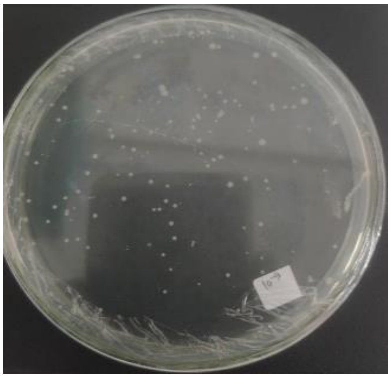

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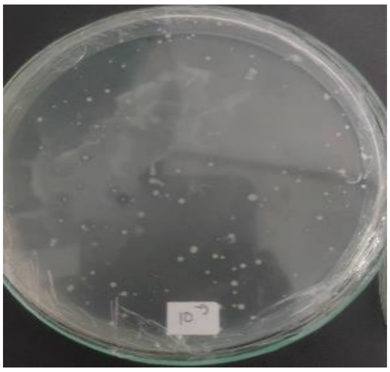

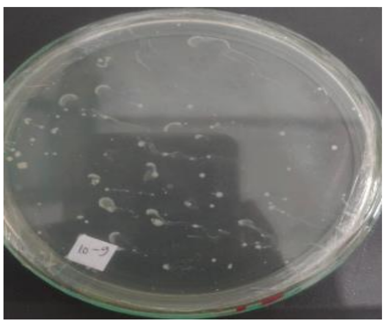

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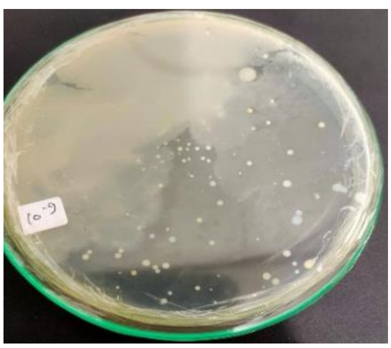

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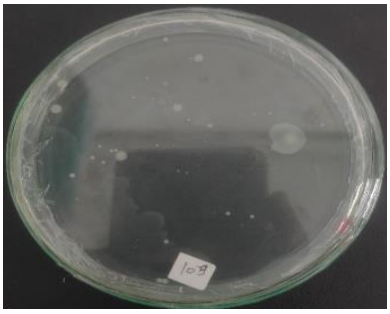

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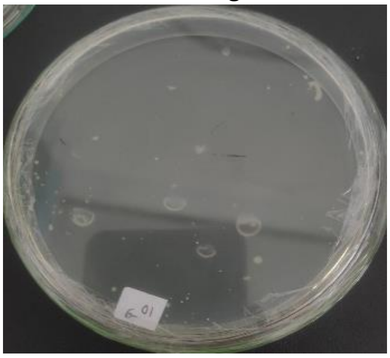

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Fig. 2: Salmonella enteritidis colony: (a dan b) control, (c dan d) Ultrasonication for 5 minutes, (e dan f). Ultrasonication for 15 minutes, (g dan h). Ultrasonication for 25 minutes

The results of the bacterial colony count for Salmonella enteritidis is presented in Table 2 below.

Table 2: Number of Salmonella enteritidis UNJCC B-9 colony

No.	Sample	Number of colony (CFU/ml) x 10^{11}		Average x10^{11} (CFU/ml)
		1	2	
1	Control	15.4	15.8	15.6
2	U-5	12.3	11.2	11.7
3	U-15	7.2	8.3	7.7
4	U-25	3.4	4.7	4.0

C: control, U-5, 15, 25: ultrasonication for 5, 15 and 25 minutes

The results in Table 2 show that the longer the ultrasonic wave exposure time on the sample, the greater the reduction in bacterial colony count in the milk sample. This reduction is caused by cavitation and thermal effects.

Ultrasonic waves possess mechanical and thermal properties that can affect the structure and activity of pathogenic microorganisms such as Salmonella. Ultrasonic waves propagate through the medium, causing molecular vibrations within it. These vibrations can stretch and compress molecular structures, altering the distance between molecules. If the ultrasonic intensity continues to increase, a condition arises where intermolecular forces can no longer maintain the original molecular structure, causing molecules to break and form cavities known as cavitation bubbles. The collapse of these cavitation bubbles generates substantial kinetic energy, which can rupture microbial cell walls and induce damaging mechanical effects. Conventional ultrasonics with frequencies ranging from 100 kHz to 2 MHz induce cavitation, which can damage DNA, enzymes, liposomes, and membranes. Research findings indicate that ultrasonics with a frequency of 22 kHz can inactivate E. coli bacteria in both standard medium (PBS pH 7.4) and milk medium with pH 6.5. This effect is comparable to the use of a cell disruptor [14, 18, 19].

Ultrasonication causes the occurrence of shear force which leads to thinning of the microbial cell walls, perforation of the cell membrane, complete rupture resulting in necrosis of the cell. In addition to mechanical effects, biological effects due to ultrasonication were reported by Liu et al. Ultrasonic cavitation also causes bacteria to undergo apoptosis or programmed cell death process, characterized by several biochemical characteristics of apoptosis including phosphatidylserine ectopion, apoptotic enzyme activation, Reactive Oxygen Species (ROS), and intracellular calcium ion [20].

The bacterial cell wall of Salmonella is primarily composed of a component known as peptidoglycan. Peptidoglycan is a complex structure consisting of sugar chains linked by peptide bonds formed from amino acids. A sudden increase in temperature can cause protein denaturation in the bacterial cell wall. This structural change in proteins can disrupt the normal function and stability of the cell wall, ultimately interfering with bacterial metabolism and survival [21].

In this study, temperature and pH measurements were also conducted on milk samples exposed to ultrasonic waves. A temperature increase occurred during the ultrasonication process, as shown in Figure 3 below.

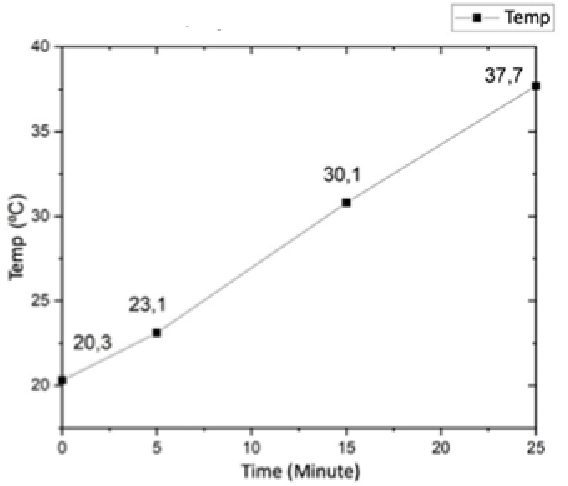

Fig. 3: Temperature increase over ultrasonication. The sample was ultrasonicated for a maximum of 25 minutes so that the temperature did not exceed 40oC. High temperature increases can inhibit pathogenic bacteria and can also damage milk nutrients

The highest temperature increase occurred after 25 minutes of exposure, reaching 37.7°C. The longer the exposure time, the greater the increase in temperature. This is due to the mechanical effect of the ultrasonic transducer, which is converted into heat energy. In addition to temperature measurement, pH measurement was also conducted on milk samples that had undergone ultrasonication. The pH level of milk can influence microbial activity, which is directly related to its safety for consumption. The pH of evaporated milk is around 6.14, which is suitable for the growth of molds, yeasts, and spore-forming bacteria, as these microorganisms can survive and perform metabolic activities within a pH range of 4-7. Through heat processing, microbial cells can experience denaturation and enzymatic damage, affecting their metabolic processes [22]. Evaporated milk has a lower pH than fresh milk [1]. The decrease in pH in evaporated milk is due to the alteration of ion balance in milk caused by heating. Heating leads to a reduction in calcium ions and phosphate ions due to molecular aggregation processes [23].

In this study, pH measurements of milk were conducted using a pH meter. The results are presented in Table 3.

Table 3: pH Test Measurement

No.	Sample	pH		Average pH
		1	2	
1	C	6.2	5.7	5.95
2	U-5	6.9	6.7	6.80
3	U-15	6.4	6.7	6.55
4	U-25	6.2	6.4	6.30

C: control, U-5, 15, 25: ultrasonication for 5, 15 and 25 minutes

From the data in Table 2, there is a noticeable difference in pH values across the samples. According to the Indonesian National Standard (SNI-3141.1:2011), the standard pH value for milk ranges from 6.3 to 6.8. The results of this study indicate that the average pH values of milk samples subjected to ultrasonication fall within this range. Further research is necessary to determine the exact impact of ultrasonication on milk pH. Nisa et al reported that variations in amplitude and duration of ultrasonication have no significant effect on the pH of goat milk yogurt, but improve gel rigidity and its microstructural homogeneity [24].

Ultrasonication may serve as a suitable method for milk processing, particularly in homogenization and sterilization of pathogenic bacteria. However, additional studies are needed to develop large-scale milk processing techniques that are cost-effective. Moreover, the effectiveness of ultrasonication is also influenced by factors such as microbial strain, suspension medium, cell size, and power input [25].

Conclusion

This study demonstrates that ultrasonic wave exposure has the potential for application in the dairy industry. In the homogenization process, ultrasonication significantly reduces the size of milk fat globules. Additionally, ultrasonication effectively inhibits the growth of Salmonella enteritidis. A longer exposure duration results in a significant reduction in bacterial colony count. Future research should focus on examining the impact of ultrasonic wave exposure on pH levels and assessment of the composition of milk to assess its quality after ultrasonication treatment.

This study confirms the promising potential of ultrasonic wave exposure for applications in the dairy industry. Ultrasonication not only enhances milk homogenization by significantly reducing the size of fat globules but also exhibits effective antimicrobial activity against Salmonella enteritidis. Extended exposure durations lead to a notable decrease in bacterial colony counts, highlighting its potential as a non-thermal preservation method. To fully optimize its industrial application, future research should investigate the effects of ultrasonication on pH stability, nutrient profile, and overall milk quality to ensure both safety and sensory acceptability. Further research is needed to conduct microstructural analysis using Scanning Electron Microscopy (SEM) to ensure the homogenization of milk fat globules.

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Author’s Contributions

Umiatin Umiatin (UU): Conceptualization, Methodology, Supervision, Writing - original draft, Writing - review & editing
Dalia Sukmawati (DS): Formal analysis, Data curation, Writing - review & editing
Taryudi Taryudi (TT): Formal analysis, Validation, Project administration
Alluma Alluma (AA): Data curation, Visualization
Maria Ulfa (MU): Data curation, Visualization

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved of the manuscript, and no ethical issues involved.

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