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EFFECT OF FREQUENCY AND DURATION OF THERMOSONICATION ON THE PHYSICAL, CHEMICAL AND MICROBIOLOGICAL QUALITY OF COW'S MILK

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ABSTRACT

Milk is a food product that contains many nutrients. Thermosonication is a method combining ultrasonic and medium-temperature heating that can be used as an alternative pasteurisation method because it can maintain the nutrients contained in milk while providing safety assurance for consumption. This study aimed to determine the effect of frequency and duration of thermosonication on the physical, chemical and microbiological quality of cow's milk. The experimental design used was a completely randomised design (CRD) with two factorials of frequency (20 and 22kHz) and duration of thermosonication (5, 10, 15 and 20 minutes). The data obtained were analysed with ANOVA and BNJ further tests at the 5% level. The best treatment was 22kHz frequency for 20 minutes with the results of viscosity 3.80 ± 0.000 cP, pH 6.8 ± 0.00, fat content 3.22 ± 0.00%, protein content 2.19 ± 0.01% and total bacteria 0.53 ± 0.67 log CFU/ml, and was able to reduce *Escherichia coli* bacteria by 2.95 log CFU/ml.

Keywords: *Escherichia coli*, Milk, Thermosonication

INTRODUCTION

Food products that are preferred by consumers are those that have good taste, colour, flavour and nutritional value. In general, fresh food contains the good nutritional value and is needed by the body, but has a short shelf life, one of the examples is milk. Food processing processes commonly applied in the dairy processing industry are thermal processes or high-temperature heating such as pasteurisation and sterilisation (Nunes & Tavares, 2019). Heating methods aim to ensure food safety and extend shelf life by inactivating spoilage and pathogenic microorganisms (Koca, 2018). However, as the heating temperature used increases, it can cause degradation of vitamin C, folate, thiamine, pyridoxine and vitamin B12, enzyme degradation, hydrolysis of proteins and lipids in food as well as changes in flavour and maillard reactions (Muehlhoff *et al.,* 2013; Sakkas *et al.,* 2014). Therefore, a food processing process is required that can not only inactivate bacteria but also overcome the problems that occur during heating such as physical and chemical changes, and nutritional and organoleptic properties of the food. This can be done by utilising ultrasonic waves (Abdullah & Chin, 2014; Bui *et al.,* 2020)

The application of room-temperature ultrasonics in food processing is not effective in inactivating microbes, so a heating process is required to obtain dairy products that are safe for consumption (Abrahamsen & Narvhus, 2022; Chouliara *et al*., 2010; Van Hekken *et al*., 2019). Thermosonication is a method combining ultrasonic and moderate heating at 37-75 °C and a frequency of 20 kHz to inactivate enzymes and pathogenic microorganisms (Lee *et al.,* 2013). Thermosonication can be used as an alternative method and has been recognised for its effectiveness in food processing as it can be widely applied with low processing costs and lower processing temperatures compared to conventional thermal processes to achieve the same microbial lethality values and produce high quality, safe and environmentally friendly food products in accordance with consumer demands (Abdullah & Chin, 2014; Chávez-Martínez *et al*., 2020), including in milk processing.

The basic principle of ultrasonics is to induce acoustic cavitation due to the formation, growth and collapse of large bubbles that cause higher energy liberation. The alternating compression and expansion creates areas of pressure changes that result in the cavitation phenomenon (Dolas *et al*., 2019). The collapse of these cavitation bubbles has a highly destructive effect on the chilling of bacterial cells (Triawan *et al*., 2019). The effects on food products are affected by several factors such as temperature, processing time, frequency, ultrasonic power (Carrillo-Lopez *et al*., 2021; Scudino *et al*., 2020). Several previous studies proved that thermosonication causes changes in several food product such as, buffalo milk fat globule size decreases at 20 kHz, 25 °C (Abesinghe *et al*., 2020), denaturation of sweet whey protein at 20 kHz, 65 °C (Barukčić *et al.,* 2015), increase in cow's milk fat content at 24 kHz,63 °C (Bermúdez-Aguirre *et al*., 2008), appearance of burnt off-flavors, 2008), the appearanceof burnt off-flavor in milk at 24 kHz, 74 °C (Marchesini *et al*., 2015), no significant change in the pH of cow's milk at 24 kHz, 55 °C (Juraga *et al*., 2021), reduce total microbes and can maintain the antioxidant activity of galangal rice at 22 kHz, 60 °C (Safitri *et al.*, 2022). Based on this, this study was conducted to determine the effect of frequency and duration of thermosonication with the addition of moderate heating on the physical, chemical and microbiological quality of cow's milk. It is hoped that through this research, thermosonication treatment will be able to produce good quality pasteurized milk that is safe for consumption.

MATERIALS AND METHOD

Materials

The raw material used was fresh cow's milk obtained from local farmers in Gedangan, Sidoarjo, East Java. The chemicals used for analysis are *plate count agar* (Hi-Media M091-500G), *nutrient broth* (Merck), *eosin methylene blue agar* (Merck), NaCl (Merck), purified *Escherichia coli* ATCC 25922 bacteria obtained from Laboratory *Tropical Disease Diagnostic Center* Universitas Airlangga *albumin bovine serum* (Merck 12657-5GM), follin-ciaocalteu (Merck), $CuSO_4$ (Merck), potassium sodium tartate (Merck), sodium carbonate (Merck), DPPH (Sigma), *amyl alcohol* (Merck), and other analytical materials such as aluminium foil, cotton wool, plastic wrap and alcohol.

Tools

The equipment used for milk thermosonication was a self-fabricated ultrasonic machine (Figure 1 and Figure 2) with a 5 L chamber made of *stainless steel* equipped with a thermo-controller with 365 W power and a 25 mm diameter probe. Equipment used for analysis included an autoclave (Hirayama HVE-85), *colony counter*, micropipette (Socorex acura 825), vortex mixer (Gemmy VM-300), biosafety cabinet (Thermo Scientific), plastic petri dish (OneMed), glass petri dish (Iwaki), analytical balance (Metler Toledo), viscometer (NDJ-8S), butyrometer, water bath, uv-vis spectrophotometer (Genesys 10s), beaker, erlenmeyer, test tube and volumetric flask.

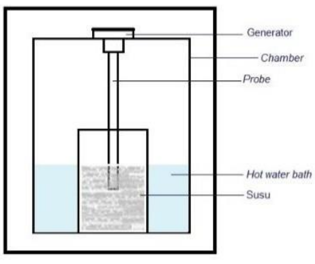

Figure 1. Design of a Thermosonic Device

Figure 2. Thermosonic Device

Research Design

The study was designed using a Completely Randomized Design (CRD) with 2 independent variables: thermosonication frequency (20 and 22 kHz) and thermosonication duration (5, 10, 15 and 20 minutes). Analysis was conducted in 2 replicates and duplicates.

Research Stages

1. Thermosonated Cow's Milk

Fresh cow's milk obtained from the farm as much as 500ml was put into a sterile thermosonic *chamber* to be heated until the temperature reached 55 °C after which 365 W ultrasonic waves were added with a frequency of 20 kHz for 5 minutes (TS20-5), 10 minutes (TS20-10), 15 minutes (TS20-15) and 20 minutes (TS20-20) and a frequency of 22 kHz for 5 minutes (TS22-5), 10 minutes (TS22-10), 15 minutes (TS22-15) and 20 minutes (TS22-20).

2. Thermal Pasteurisation of Fresh Cow's Milk (Control)

500 ml fresh cow's milk was put into the *chamber* and heated at 65 °C for 30 minutes as control behaviour.

3. Bacteria Preparation

Escherichia coli bacteria were refreshed by means of 1 ml of stock bacteria grown on 9 ml *Nutrient Broth* media then incubated for 24 hours at 37 °C.

4. Decontamination of Bacteria in Cow's Milk

Then, 1 ml of bacterial inoculum was added to 499 ml of cow's milk until a bacterial population equivalent to 104 log CFU/ml was obtained. Then the milk that had been inoculated with *Escherichia coli* bacteria was thermosonicated. Milk containing bacteria was grown on Eosin Methylen Blue (EMB) media at 37 °C for 24 hours to count the number before and after thermosonication (Kernou *et al*., 2021).

Methods

The statistical data obtained were analysed using Analysis of Variance (ANOVA) and Honest Real Difference Test (BNJ) with a confidence level of 95% through the Minitab 18 application. The best analysis of the treatment was selected based on the lowest total microbes and continued with testing the reduction of Escherichia coli bacteria.

Analysis Procedure

The tests carried out are physical, chemical and microbiological tests. Physical tests carried out on milk are pH measurements (National Standardisation Agency, 2019) and viscosity (AMETEK, 2019). Chemical testing in the form of protein content of the Lowry method (Harjanto, 2017) and fat content of the Gerber method (Maitimu *et al.,* 2012). Microbiological testing includes total microbes carried out using *plate count* agar media (Naghili *et al*., 2013), while testing for a decrease in total *Escherichia coli* bacteria using *Eosin Methylen Blue* media (Kernou *et al*., 2021) and the calculation method used is *total plate count* (Naghili *et al*., 2013).

RESULT AND DISCUSSION

1. Viscosity

The results showed that the viscosity of fresh cow's milk was 4.5 cP and decreased after thermosonication treatment and progressively decreased as the frequency and duration of thermosonication increased. The average value of viscosity of thermosonicated cow's milk ranged from 3.80 to 4.25 cP, where the highest viscosity was found in samples thermosonicated with a frequency of 20 kHz for 5 minutes.

The decrease in viscosity is due to the physical forces generated during acoustic cavitation (Shanmugam *et al*., 2012) and decreases with increasing frequency (J. Liu *et al*., 2021) and the increasing sonication time due to the reduction of particle size in the sample (Song *et al*., 2021). Increasing the frequency causes the viscosity drop to increase. The cavitation nuclei are compressed by the acoustic waves causing the cavitation nuclei to implode which in high temperatures and pressures in an instant and affects the fluid rheology (J. Liu *et al*., 2021). Meanwhile, according to (Chávez-Martínez *et al*., 2020), the decrease in viscosity is caused by the shear force that arises during cavitation which then breaks the connection between the protein and its polymer chain.

The decrease in viscosity that occurred in the control milk was greater than the thermosonated milk. This can be caused by the temperature used where the higher the temperature, the greater the decrease in viscosity (Khalifa & Ghanimah, 2013). Control milk was LTLT pasteurised at 65 °C for 30 minutes which is higher than thermosonated milk which uses 55 °C for 5 to 20 minutes.

Table 1. Effect of Frequency and Duration of Thermosonication on Viscosity of Cow's Milk

Sample	Viscosity (cP)
Fresh Milk	4.50 ± 0.01
Control	3.60 ± 0.00
TS20-5	4.25 ± 0.07[a]
TS20-10	4.20 ± 0.00[a]
TS20-15	4.15 ± 0.07[a]
TS20-20	4.10 ± 0.00[a]
TS22-5	3.90 ± 0.00[b]
TS22-10	3.85 ± 0.07[b]
TS22-15	3.85 ± 0.07[b]
TS22-20	3.80 ± 0.00[b]

Notes: Values accompanied by different letters indicate significantly different (p≤0.05).

2. pH

The pH value can be defined as a condition of basicity or acidity. The results of the study in Table 2. show that thermosonication treatment does not have a significant effect on the pH of cow's milk. This is in accordance with the statement (Juraga *et al.,* 2021) that the thermosonication process applied to dairy products has no effect on changes in pH value. However, there was a slight decrease as the frequency and duration of thermosonication increased.

Table 2. Effect of frequency and duration of thermosonication on the pH of cow's milk

Sample	pH
Fresh Milk	6.9 ± 0.14
Control	6.9 ± 0.00
TS20-5	6.9 ± 0.00[a]
TS20-10	6.85 ± 0.07[a]
TS20-15	6.8 ± 0.00[a]
TS20-20	6.8 ± 0.00[a]
TS22-5	6.9 ± 0.00[a]
TS22-10	6.85 ± 0.07[a]
TS22-15	6.8 ± 0.00[a]
TS22-20	6.8 ± 0.00[a]

Notes: Values accompanied by the same letter do not indicate significantly different (p≥0.05).

Ultrasonic processes can affect the pH value due to the formation of a certain amount of nitric acid produced by oxygen and nitrogen reactions under the hot zone due to cavitational effects (Supeno & Kruus, 2000) and disrupt the natural mineral balance present in milk (Z. Liu *et al*., 2014). In addition, the decrease of pH value by ultrasonication is due to the hydrolysis of phosphoric esters and cavitation can accelerate some reactions, as well as to lipolysis effects by enzymatic reactions of triglycerides and the release of free fatty acids into the medium (Bermúdez-Aguirre *et al*., 2009; Walstra *et al*., 2006). Heating can also cause changes where soluble calcium and phosphate become saturated and partially associated with casein micelles, indicating the milk becomes acidic due to the balancing of Ca and phosphate insolubilisation (Walstra *et al*., 2006).

3. Protein Content

It can be seen in Table 3 that thermosonication did not significantly affect the protein content of thermosonated cow's milk. This is probably because the thermosonication frequency range used is too narrow, so it is not enough to damage the protein structure in cow's milk.

Table 3. Effect of Frequency and Duration of Thermosonication on the Protein Content of Cow's Milk

Sample	Protein Content (%)
Fresh Milk	2.8 ± 0.00
Control	1.7 ± 0.04
TS20-5	2.23 ± 0.01[a]
TS20-10	2.23 ± 0.00[a]
TS20-15	2.21 ± 0.01[ab]
TS20-20	2.20 ± 0.00[ab]
TS22-5	2.23 ± 0.00[a]
TS22-10	2.21 ± 0.01[ab]
TS22-15	2.20 ± 0.01[ab]
TS22-20	2.19 ± 0.01[b]

Notes: Values accompanied by the same letter do not indicate significantly different ($p \geq 0.05$).

Atalar *et al.* (2019) in their research proved that thermosonication caused a small decrease in the soluble protein content of hazelnut milk, while research conducted by Juraga *et al.* (2021) proved that thermosonication at a frequency of 24 kHz at temperatures of 20 and 55 OC for 2.5, 5, 7.5 and 10 minutes had no significant effect on cow's milk protein. However, there was a slight decrease in protein levels as the frequency and duration of thermosonication increased. This is caused by protein denaturation which modifies the tertiary and quaternary structure of casein due to the simultaneous use of heat and ultrasonics. Modifications occur due to free radicals that induce oxidation of amino acid side groups in the protein chain (Rahman *et al.,* 2020). Changes in proteins can also occur due to the breaking of hydrophobic bonds. Ultrasonic shear forces can open whey proteins, especially α-la and β-lg, and further result in protein aggregation via hydrophobic interactions and disulfide bonds (Bui *et al.,* 2020) leading to a decrease in protein solubility (Atalar *et al.,* 2019). Short-time ultrasonics can break the protein structure into irregular fragments, whereas higher powers and longer times result in smaller particles and more free sulfihryl groups that react with themselves or oxidize to create larger aggregates (Rahman & Lamsal, 2021).

The sonication process causes the concentration of hydrogen radicals to quarter that of hydroxyl radicals and at higher concentrations, the hydroxyl radicals recombine to form hydrogen peroxide. These reactive oxygen species (ROS), namely free radicals (OH) and non-radical species (H_2O_2) can damage the quality and nutrition of proteins by initiating oxidation reactions. This process of protein oxidation is capable of altering the secondary and tertiary structures leading to subsequent changes in the physical and chemical properties of the protein, including functional characteristics. Oxidation of disulfide linkages in proteins (R-S-S-R') can form thiol bonds (R-SH and R'-SH, sulfihydirl) that can result in aggregation and re-polymerisation, leading to changes in surface hydrophobicity, solubility, water binding capacity, and gelation properties (Rahman *et al*., 2020).

Protein content in control milk was lower than in thermosonated milk. This can be caused by the temperature used in the control milk treatment being higher than the temperature of thermosonated milk. According to (Čurlej *et al*., 2022), in general, milk protein is sensitive to heat at temperatures above 60 °C which causes whey protein to denature.

The thermosonated milk and control milk contained in this study did not meet the requirements of pasteurised milk based on SNI 01-3951-2018 where the protein content of plain pasteurised milk is at least 2.7% (Badan Standarisasi Nasional, 2018). This is due to the protein content of the milk raw materials used in the study is not good or is at the minimum limit of fresh cow's milk requirements, which is at least 2.8% (Badan Standarisasi Nasional, 2011)..

4. Fat Content

Based on Table 4, it can be seen that there is a significant interaction of frequency and duration of thermosonication on the fat content of thermosonated cow's milk. The lowest fat content of cow's milk was found in milk given 20 kHz thermosonication for 5 minutes, while the highest fat content of cow's milk was found in milk given 22 kHz thermosonication for 20 minutes.

The fat content of cow's milk increases with increasing frequency and duration of thermosonication. This is due to the simultaneous use of ultrasound and heating (temperature 55-75 °C), resulting in a decrease in the size and disruption of the fat globules, then finally breaking and producing new structures and releasing the triacylglycerol content from within the fat globules, not to mention other lipids such as cholesterol and phospholipids that usually encase the core of the fat globules are also counted after the thermosonication process, therefore the fat content of milk increases (Bermúdez-Aguirre *et al*., 2008, 2009). Research conducted by Juraga *et al*. (2021) also proved that there was a slight increase in milk fat content after thermosonication with a frequency treatment of 24 kHz at 55 °C. Meanwhile, an increase of 0.20% occurred in milk thermosonicated with a frequency of 24 kHz for 30 minutes at 63 °C (Bermúdez-Aguirre *et al*., 2008). Fat content in thermosonicated milk showed results that were not significantly different and even higher when compared to control milk.

Table 4: Effect of Frequency and Duration on Fat Content of Thermosonated Cow's Milk

Sample	Fat Content (%)
Fresh Milk	3.3 ± 0.37
Control	2.97 ± 0.05
TS20-5	3.04 ± 0.01^{e}
TS20-10	3.07 ± 0.01^{d}
TS20-15	3.11 ± 0.01^{c}
TS20-20	3.15 ± 0.01^{b}
TS22-5	3.07 ± 0.01^{de}
TS22-10	3.12 ± 0.01^{c}
TS22-15	3.17 ± 0.00^{b}
TS22-20	3.22 ± 0.00^{a}

Notes: Values accompanied by unequal letters indicate significantly different ($p \leq 0.05$).

5. Total Microbes

Based on the results of Table 6. shows the initial total microbes in cow's milk were 5.21 log CFU/ml and after thermosonication showed a decrease to 3.35 log CFU/ml to 0.53 log CFU/ml. The highest total microbes were shown at the lowest frequency and duration, while the lowest microbes were shown at the highest frequency and duration. This indicates that with the increase in frequency and the longer duration of thermosonication given, the lower the total microbes. The decrease in microbes is caused by the rupture of the bacterial cell wall. Bacterial inactivation occurs due to physical forces generated by acoustic cavitation (Chandrapala *et al*., 2012). Shock waves generated from cavitation are capable of generating shear forces that trigger stress on microbial cells and enzymes, destroying the microbial spore layer, cell wall and inner membrane, damaging structural integrity and resulting in the release of cell nuclei (Onyeaka *et al*., 2021).

Table 5. Effect of Frequency and Duration of Thermosonication on Total Microbes of Cow's Milk

Sample	Total Microbes (log CFU/ml)
Fresh Milk	5.21 ± 0.03
Control	2.90 ± 0.09
TS20-5	3.35 ± 0.07[a]
TS20-10	3.27 ± 0.13[a]
TS20-15	3.17 ± 0.24[a]
TS20-20	2.91 ± 0.29[ab]
TS22-5	2.95 ± 0.07[ab]
TS22-10	2.26 ± 0.21[ab]
TS22-15	1.95 ± 0.24[b]
TS22-20	0.53 ± 0.67[c]

Notes: Values accompanied by unequal letters indicate significantly different (p≤0.05).

Research conducted by (D'Amico *et al*., 2006) proved that a 5 log reduction in total microbes can be achieved after milk ultrasonication (20 kHz, 57 °C for 18 min). A 1.7 log CFU/ml reduction in total bacteria was achieved after a combined heating and ultrasonic process for 15 min, whereas samples that were ultrasonicised for 16 min only experienced a 1.0 log CFU/ml reduction in total bacteria (Chouliara *et al*., 2010).

The decrease in total microbes in the thermosonicated milk was greater than the control milk. This is consistent with research conducted by (Jeličić *et al*., 2012) where the thermosonication process (24 kHz, 320 W, 55 °C for 6.5 and 8 min) resulted in a better microbial reduction compared to the thermal pasteurisation process (65 °C for 30 min). The combination of heating and ultrasonic treatment resulted in increased microbial mortality due to the synergistic attack on vital processes and microbial structures. Scudino *et al*. (2020) mention that another important factor in the application of ultrasonication is the temperature used, where the use of temperatures above 55 °C can increase microbial inactivation in food products. The total microbes produced in this process indicate that the thermosonication process is able to reduce the total microbes in milk to the safe consumption limit set in SNI 01-3951-2018 which is a maximum of 4.0 log CFU/ml (Badan Standarisasi Nasional, 2018).

6. Decrease in *Escherichia coli bacteria*

Escherichia coli is one of the coliform bacteria included in the *Enterobacteriaceae* family, which are bacteria that can live and survive in digestion (Rahayu & Nurjanah, 2018). The best treatment was selected based on the lowest microbial value achieved in the thermosonicated cow's milk, namely the treatment of 22 kHz frequency for 20 minutes. Sterile cow's milk was decontaminated with *Escherichia coli* bacteria and then subjected to thermosonication process with the same frequency and duration to determine how much bacteria reduction occurred in cow's milk.

The magnitude of the decrease in *Escherichia coli* bacteria can be seen in Table 6. The results show that the initial *E. coli* bacteria count was 5.82 log CFU/ml. After the thermosonication process, the number of *E. coli* bacteria decreased by 2.95 log CFU/ml to 2.87 log CFU/ml. Previous research proved that ultrasonic treatment (30kHz) was able to reduce E. coli bacteria by 0.75 log in samples of 1.5x104 CFU/ml (Sesal & Kekeç, 2014). Meanwhile, ultrasonic treatment at a frequency of 20kHz with 60 °C heating reduced *E.coli* bacteria by 3.07 log CFU/ml (Herceg *et al*., 2012).

Tabel 6. Decrease in *Escherichia Coli bacteria*

Type of Bacteria	**Number of Bacteria (log CFU/ml)**		**Decrease (log CFU/ml)**
	Initial	**End**	
E. coli	5.82±0.02	2.87±0.04	2.95

The decrease is caused by the simultaneous use of ultrasonic and heat. The temperature used in the thermosonication process of 55 °C can kill *Escherichia coli* bacteria that can live at temperatures between 10 - 45 °C (Rahayu & Nurjanah, 2018). The use of high enough temperatures can increase vapour pressure, and decrease tensile strength and viscosity, which then makes cavitation bubbles form more easily. This increased cavitation effect makes combining the ultrasonic method with heating more effective in killing microorganisms in milk at pasteurisation temperatures (Li *et al*., 2016).

Ultrasonic causes the cells of Escherichia coli to break into pieces. The cell wall turns rough and disconnects. The mechanism of microbial disintegration and cell destruction by cell death is generally attributed to pore formation or mechanical disruption of the cell or membrane. Ultrasonics at 24 kHz causes bacterial inactivation either through pore formation or mechanical disruption of cell boundaries by the shock waves generated during cavitation. This pore formation disrupts the cell mechanisms. The small pores prevent the passage of molecules that can maintain osmotic balance but cannot prevent the entry of water into the bacterial cell, resulting in an increase in cell volume that creates an increase in turgor pressure and eventually cell rupture resulting in cell death (Gera & Doores, 2011). It was also demonstrated that *Escherichia coli* cells undergo complete and irreversible cell disruption into debris after ultrasonic treatment of 20 kHz for 12 min (Liao *et al*., 2018).

CONCLUSION

The results showed that cow's milk pasteurised by thermosonication method produced good quality such as LTLT pasteurised milk. Milk that was thermosonicated with a frequency of 22 kHz for 20 minutes was the best treatment because it was able to produce the greatest reduction in total microbes in cow's milk with a viscosity value of 3.80 cP, pH 6.8, fat content of 3.22%, protein content of 2.19%, total bacteria of 0.53 log CFU/ml and was able to reduce *Escherichia coli* bacteria by 2.95 log CFU/ml. Based on these results, it can be concluded that the thermosonication method can be used as an alternative method of LTLT pasteurisation in milk processing because the application of duration, frequency and lower temperature can produce good milk quality and minimise the occurrence of damage.

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