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The Effect of Temperature and Pasteurization Time on *Staphylococcus aureus* Isolates from Dairy Products

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Abstract. *Staphylococcus aureus* is a potential pathogenic bacterial cause of disease in humans and animals due to the ability of adhesion to epithelial tissue. Many cases of food poisoning are caused by *S. aureus* bacteria. Therefore, the purpose of this study was to determine the effect of temperature and time on the growth of *S. aureus* isolates from milk products. The samples are derived from previous research namely pasteurized milk, street vendor and café milk, milk powder, and sweetened condensed milk products. The treatment temperatures and times studied were temperature 60 ºC, 65 ºC, 70 ºC, 75 ºC, 80 ºC, and 30, 35, 40, 45, 50, 55, and 60 minutes. The results show that at temperatures of 60 ºC and 65 oC, *S. aureus* isolates did not grow at 60 minutes. All isolates of *S. aureus* died when the temperatures were increased to 70 ºC and 80 ºC, at 50 and 20 minutes, respectively.

Keywords: Staphylococcus aureus, temperature and time, pasteurization, dairy products

INTRODUCTION

Staphylococcus aureus is a Gram positive, coccus-shaped bacterium. A characteristic of *S. aureus* is its ability to produce coagulase enzymes. Coagulase enzymes are enzymes that can agglomerate plasma, producing hemolysis toxin that causes lysis in red blood cells of animals, especially cattle and sheep.[1] Contamination by *S. aureus* can be derived from the air, dust, waste, water, milk, food or on food equipment, environmental surfaces, humans or animals including cows' udders. Therefore, milking should be considered unhygienic. Milking processes that do not match standard procedures can lead to dairy products which can cause diseases in humans. This is evidenced by the presence of several typical food poisonings occurring in some countries.[2-3]

In addition to the coagulase enzyme, enterotoxin is a toxin produced by *S. aureus* strains. One of these enterotoxins is enterotoxin A which is commonly produced by S. aureus.[2] Its ability to withstand high temperatures is one of the causes of poisoning in food products, especially milk. Milk is a good nutrient for growing *S. aureus* and producing enterotoxins.[4]

Food is a good medium for the growth of bacteria, especially S. aureus, leading to toxin production when the bacteria are present in the diet. Intoxication or staphylococcal food poisoning occurs after consuming food products contaminated with *S. aureus* enterotoxins that are resistant to high temperatures.[3, 5]

Based on Fletcher et al. (2015),[6] more than 200 000 cases occur annually in the United States caused by S. aureus, with 83.5% of *S. aureus* recorded in contaminated milk. According to Gemmel (1995)[7] *S. aureus* can cause

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Scalded Skin Syndrome which is a disease in human neonates. Based on Schlievert et al. (2000)[8] *S. aureus* can cause Toxic Shock Syndrome with symptoms of fever, skin rashes, and shock. Tranter (1996)[9] said that when a person consumes 1g of toxin per 100g of food, this is enough to cause symptoms of food poisoning. Asao et al. (2003)[4] reported that more than 10 000 people had signs of food poisoning after drinking contaminated milk products and yogurt contaminated with *S. aureus* containing enterotoxin A. Scherrer et al. (2004)[10] collected samples of goat and sheep milk in Switzerland and the results show that more than 50% are positive for *S. aureus* bacteria containing enterotoxin A genes. Morandi et al. (2007)[11] in Italy also had similar results in that about 67% of *S. aureus* bacteria being found in dairy products. Shou-kui Hu et al. (2012)[5] examined some food products such as raw meat, cooked meat, whole milk, dairy products, aquatic products, and ice cream. Their results show that the highest *S. aureus* contamination was found in pure milk, which is about 30%, followed by raw meat and aquatic products at about 25% and 12%, respectively.

S. aureus has many potential virulence factors in its pathogenesis. These virulence factors can be proteins, including enzymes and toxins. These bacteria have many surface proteins called Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM). MSCRAMM initiates infection by sticking to the tissues. MSCRAMM consists of several proteins: protein A, elastin-binding protein, collagen-binding protein, bone sialoprotein-binding, fibronectin-binding protein, and clumping factor. Protein A is a major component of the *S. aureus* cell wall. It binds to Crystallizable Fragment (Fc) in Immunoglobulin G (IgG), causing no opsonization, as well as having anti-phagocytotic effects. Fibronectin-binding protein (FnBP) is a surface protein that serves to facilitate bacteria binding to mucosal cells and tissue matrices. Clumping factor is a fibrinogen-binding protein that causes bacteria to clot when present in blood plasma.[12-13]

S. aureus can survive and grow in various ways after attachment in host cells and prosthetic surfaces. *S. aureus* has the ability to make biofilms and form a Small-Variant Colony which provides the bacteria's ability to hide in host cells without causing significant cell damage and evading both host and antibiotic systems. Due to these two factors, *S. aureus* is difficult to eradicate and can cause recurrent infections.[14]

S. aureus has microcapsules for protection from anti-phagocytosis. [14] *S. aureus* produces a variety of enzymes, such as proteases, lipases, and hyaluronidases that allow the bacteria to enter and destroy tissues and spread to nearby tissues during the infection process. β-lactam enzyme is an enzyme that inactivates penicillin while its Binding Protein (PBP) is an enzyme located in the cytoplasmic membrane and contributes to the formation of cell walls. PBP is one of the main factors in resistance.[15]

The dangerous risk of *S. aureus* infection and the presence of food poisoning cases caused by enterotoxin A encourages the authors to conduct further research using isolated samples to determine the effect of temperature and time of pasteurization that can kill *S. aureus* until it is safe for consumption.

EXPERIMENTAL DETAILS

S. aureus isolated from pasteurized milk, street vendor and café milk, powdered infant formula, and sweetened condensed milk products were collected by previous research from locations in Yogyakarta.[16-19]

Materials and tools used for research

The researchers used several ingredients such as Brain Heart Infusion as an enrichment medium, aquades, 70% alcohol, Baird Parker Agar (BPA) Oxoid as an isolation medium, peptone water which acts as a diluent, and physiological salt. The equipment used in this research includes erlenmeyer flask, incubator, autoclave, water bath, thermometer, petri dish, oven, centrifuge, test tube, measuring pipette, alcohol lamp, pipet pump, micropipettes and pipet tips, measuring cup, needle for inoculum, drigalski

Treatment temperatures and times

The temperature treatment was started by growing isolate from Brain Heart Infusion Agar (BHIA), then growing on 5 mL BHIB medium and incubated at 37 °C for 24 hours. Pellet cell cultures were harvested by centrifugation at 5,000 rpm at room temperature for 15 minutes, discarding the supernatant. The pellet is added with 9 mL of buffer

phosphate saline (BPS) and homogenized. The temperature treatment used to determine the death of *S. aureus* isolates were 60 °C, 65 °C, 70 °C, 75 °C, and 80° C for 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, and 60 min, respectively.

Microbial cell counts

Total count of *S. aureus* colonies was enumerated and duplicated by the spread plate method according to standard agar plate methods. A cell culture of 1mLwas transferred in 9 mL water peptone 1% for dilution. Cell cultures were homogenized and diluted from dilution 10-2-10-16. For enumeration of *S. aureus* from aserial dilution of BPS, they were plated on BPA with 5% egg yolk tellurite emulsion added and incubated at 37° C for 48 hours. The number of bacteria colonies counted according to standard methods were between 30 and 300 colonies.[20]

RESULT AND DISCUSSION

Milk is an important drink for humans, but we do not know the aspects of the process of making milk products or milking pure milk that lead to many people being poisoned after drinking milk. Some researchers like Thaker et al. (2013)[21] found *S. aureus* in milk and pedha samples. Possible contamination occurs due to bulk tanks, less hygienic milking processes, and udders of dirty animals. In this study, milk samples were taken from previous research including pasteurized milk,[16] vendor and café milk,[17] powdered infant formula products,[18] and sweetened condensed milk.[19]All isolates of *S. aureus* produced staphylococcal enterotoxin A.

BPA is a medium that is still often used as a selective medium for *S. aureus* bacteria.[22] Capita et al. (2001)[23] report that several organizations such as the International Organization for Standardization (ISO), the Association of Official Analytical Chemists International (AOAC), the Bacteriological Analytical Manual, the American Public Health Association and the International Dairy Federation recommend using BPA media for the isolation of S. aureus. Therefore, the researchers used BPA media to determine the growth of *S. aureus* bacteria.

Some of these samples received temperature and time treatments to determine the death of *S. aureus* isolates. Initially, the enrichment stage of the isolates was done by growing it in BHIA, inclined and incubated at 37 ºC for 24 hours. The isolates were then transferred to a 5 mL BHIB medium and incubated at 37 ºC for approximately 18 hours. The results show a change in color from yellow to clear cloudily. After turbidity, the next step is centrifugation, which aims to separate the solid (pellet) from its suspension (supernatant). Centrifugation is done at 5,000 rpm for 10 minutes. After centrifugation, the supernatant is removed and the pellet is added with physiological saline in a vortex for 30 seconds. The constant velocity and time of the vortex affect the end result. Temperatures used in this study include 60 ºC, 65 ºC, 70 ºC, 75 ºC, and 80 ºC for 0 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, and 60 minutes. Dilution used at 0 minutes = 10-2–10-16, at 30 minutes = 10-2–10-10, at 35 minutes = 10-2–10-8, at 40–45 minutes = 10-2–10-6, at 50 minutes = 10-2–10-4, at 55–60 minutes = 100–10-2. For each time, the two highest dilutions of the spread of the BPA media were then incubated at 37 ºC for 48 hours'. After that, the calculation of colonies growing in the BPA medium showed bacterial resistance at a certain temperature and time. The isolates used were five with sample code S5 (powdered infant formula), KN3 (pasteurized milk), SM2.1.1 (street and vendor milk), SKMF2.1.2 (sweetened condensed milk products), and SKMC2.2.4 (sweetened condensed milk products).

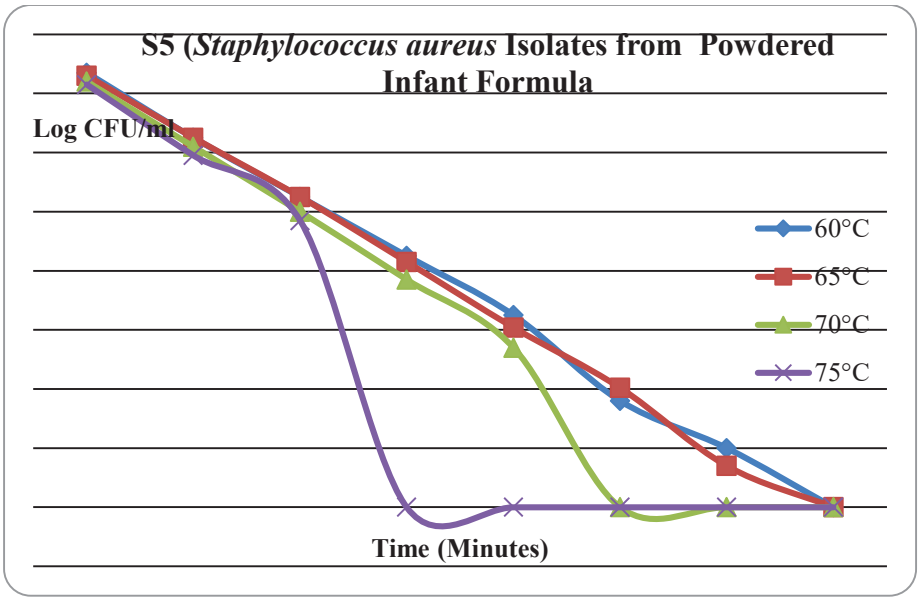

FIGURE 1. Thermal death time from 60ºC until 75ºC , sample of S5

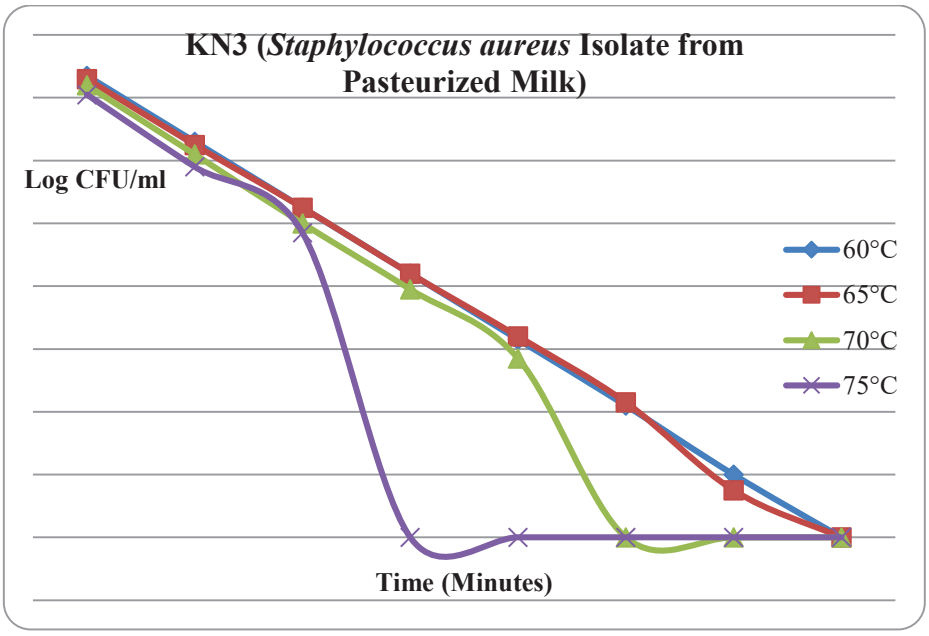

FIGURE 2. Thermal death time from 60ºC until 75ºC , sample of KN3

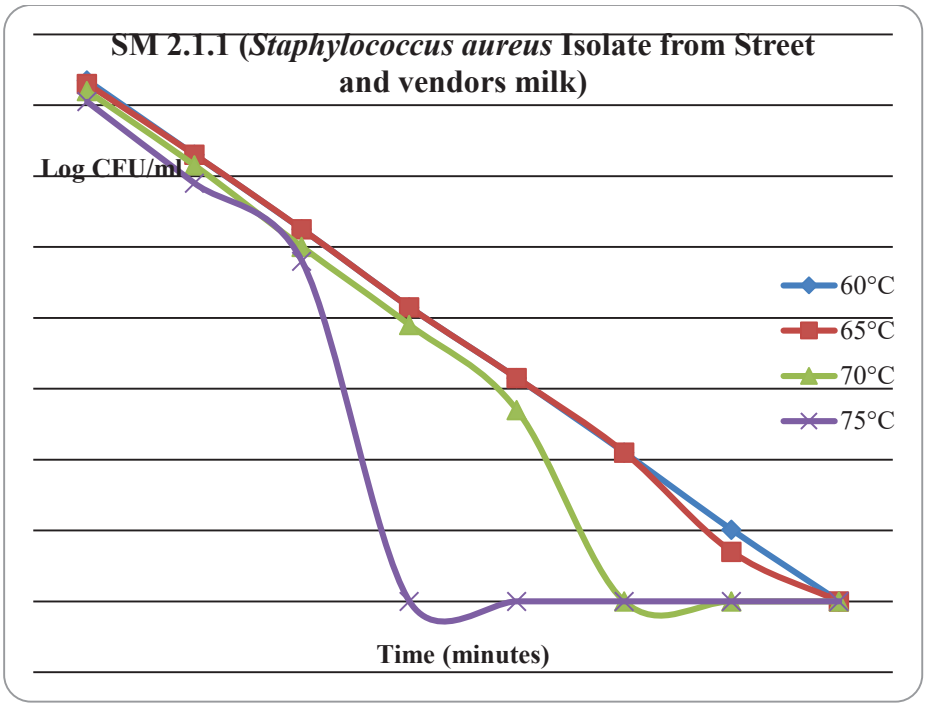

FIGURE 3. Thermal death time from 60ºC until 75ºC , sample of SM 2.1.1

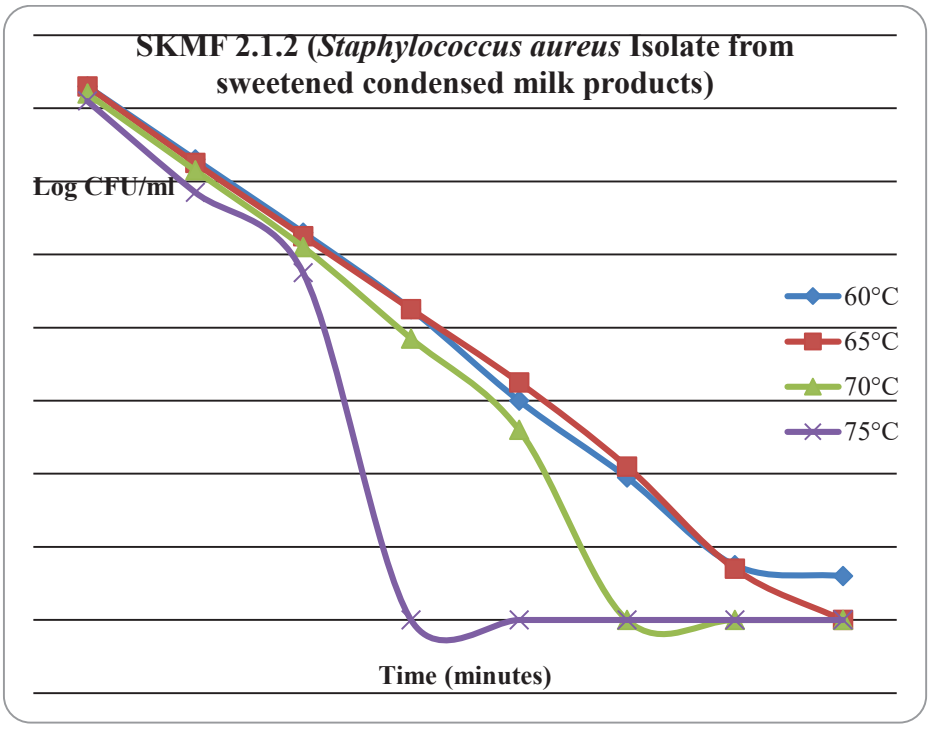

FIGURE 4. Thermal death time from 60ºC until 75ºC , sample of SKMF 2.1.2

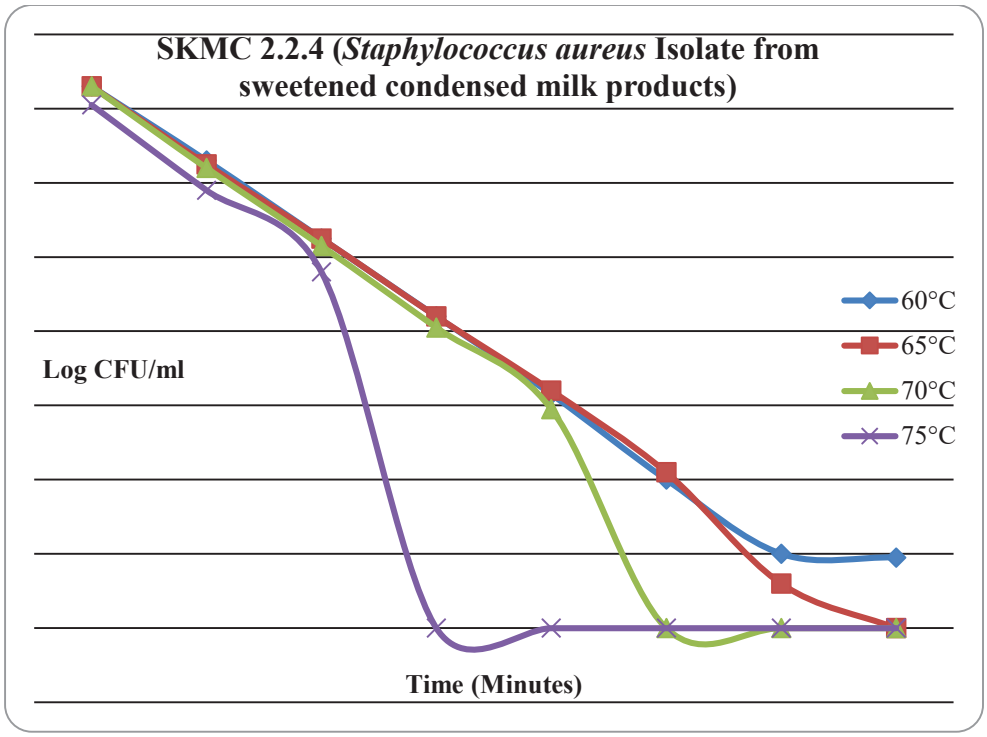

FIGURE 5. Thermal death time from 60ºC until 75ºC , sample of SKMC 2.2.4

All samples (Figure 1 until 5) show that at 60 and 65ºC have the same decline graph that is estimated to be dead *S. aureus* at 60 minutes. At 70 ºC the death of *S. aureus* occurred at 50 minutes. The temperature of 75 ºC graph of *S. aureus* mortality showed a decrease at minute 45. Therefore the researchers raised the temperature to 80 ºC and seen all the samples have the same decrease graph in minute 20 minutes.

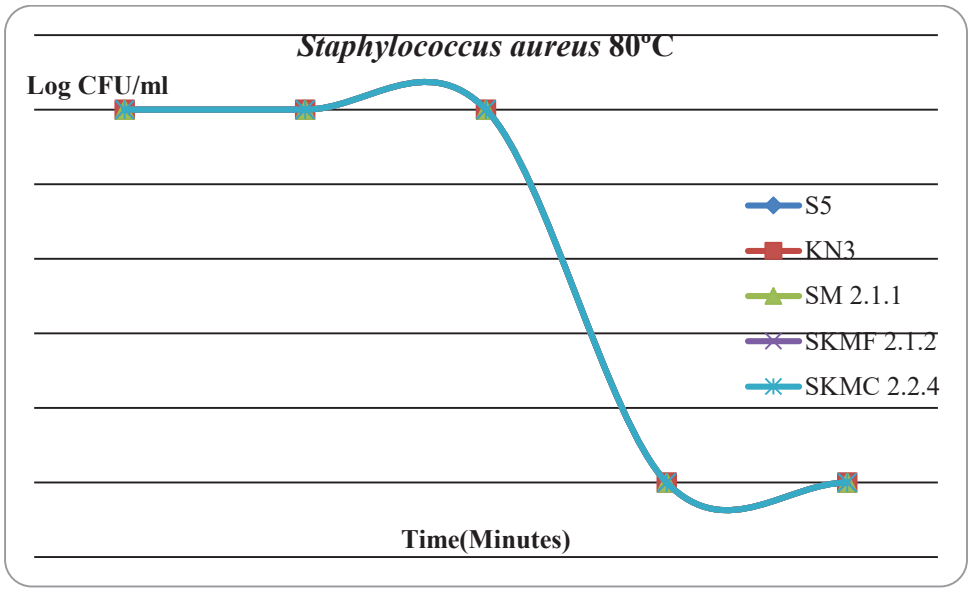

FIGURE 6. Thermal death time from all sampels

All samples (Figures 1 to 5) at 60 and 65 ºC have the same decline graph that is estimated to be the death of *S. aureus* at 60 minutes. At 70 ºC, the death of *S. aureus* occurred at 50 minutes. At a temperature of 75 ºC the graph of *S. aureus* mortality showed a decrease at minute 45. Therefore, the researchers raised the temperature to 80 ºC and saw that all samples have the same decrease graph at 20 minutes.

S. aureus has several virulence factors. The ability to form biofilms is one that can make *S. aureus* resistant to high temperatures. According to Chen et al. (2013),[24] biofilms are collections of bacteria that form layers and adhere to a surface. The embedded biofilm has an arrangement comprising an extracellular polymer matrix produced by the bacterium. Tarver (2009)[25] said that in the biofilm layer, microbes tend to grow and develop rapidly, thus forming colonies especially on the surface of nutrient-rich ingredients such as dairy foods.

The heat treatment of *S. aureus* bacteria at 80 ºC for 20 minutes (Figure 6) allowed the protein to envelop the denatured *S. aureus* so that the cell experiences death. Protein will lose shape so that its function to protect the bacterial cell does not run properly and when the temperature is raised to 80 ºC, the cell will experience plasmolysis which means peeling of the cytoplasm membrane of the cell wall due to cytoplasm penetration, allowing the liquid from outside into the cell and damaging the cell. Heating can lead to the death of bacterial cells, but the number of dead bacteria depends on the intensity of heating and heating time.[6] Research shows that the higher the temperature used, the shorter the time to kill bacteria, especially S. aureus.

The same study was conducted by Montanari (2015)[26] with a different sample of fermented sausage taken from the Department of Agri-Food Science and Technologies (University of Bologna). The results show that *S. aureus* died within 20 minutes at 80 ºC. Montanari et al. (2015)[26] said that the possible factor causing high-temperature resistance is the presence of a biofilm formation mechanism in *S. aureus* at the time of high-temperature treatment or before the temperature treatment.

SUMMARY

This study shows that the temperature and time of pasteurization that is usually used, 60–65 ºC for 30 minutes, cannot kill *S. aureus* producing enterotoxins, but the optimal temperature and time to reduce the level of contamination in food products, especially milk products, is a temperature of 80 ºC for 20 minutes.

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