

## The effectiveness of using chitosan as a natural antibacterial for maintaining the sausage quality

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### Abstract

Sausage is a food that is much favoured by the Indonesian people. Sausage belongs to a group of frozen food products that require cold storage. The weakness of sausages is to become stale when opened and exposed to the free air. It encourages sausage manufacturers to use preservatives in their products. A natural preservative that does not cause negative effects is chitosan from shrimp skin. The purpose of this study was to determine the optimum concentration of chitosan, which can be used as a natural preservative in sausages. The method used was experimental research by developing and utilizing chitosan in various concentrations, namely 1%, 1.5%, 2%, and 2.5% as experimental treatment and 0% chitosan concentration as a control treatment. The five treatments were observed in terms of organoleptic test and bacterial count test. The organoleptic test was performed at room temperature. The results showed that sausage with 1% chitosan edible film can maintain the quality of the sausage. Chitosan with a higher concentration makes the chitosan solution more concentrated, thus it becomes ineffective since it affects the appearance and performance of sausages. The use of 1% chitosan maintains the sausage quality of three days of storage and it indicates the number of bacteria is only  $1.7 \times 10^3$  CFU/g with a clear zone area of  $5.41 \pm 1.10$  mm. It concludes that the concentration of 1% chitosan is the optimal concentration that can be used to inhibit the growth of bacteria in sausages compared with other concentration treatments.

## 1. Introduction

Sausage is one of the ready-to-eat food products that can be consumed directly or further processed such as fried, baked, or used as a mixture of dishes. Sausage is included in the group of frozen food products that require cold storage to maintain its quality such that it remains good for consumption (Evitha, 2018). Sausage has a weakness that is easily stale if stored in the open air. Cooked sausages can only last one to two days at room temperature. Sausage quality can be maintained by cold storage (0-5°C) for at least 2 weeks with closed vacuum packaging and 1 week with non-vacuum packaging (Pradana *et al.*, 2019).

The weakness of sausages is easily open and exposed to free air which encourages producers to use preservatives in their products. Currently, the food preservatives that are widely used by the household to medium-scale sausage producers are borax, nitrate and nitrite. The use of chemicals without using the correct

procedures will certainly be dangerous. In the preparation of sausage in Korea, sodium nitrite is generally used as a curing agent for colour and flavour development as well as preservative effect (Park *et al.*, 1999). However, nitrite reacts with the amine in meat and may produce nitrosamine, a strong toxicant detrimental to human health. The use of hazardous materials in food is certainly not allowed because it can cause side effects on the health of consumers. Food safety and quality are fundamental concerns for consumers and the food industry (Hu and Michael, 2018).

Consumer demand for high-quality food without chemical preservatives is a challenge for the food industry. This encourages increased efforts for the discovery of new natural preservatives and antimicrobials (Nagarajan *et al.*, 2021). One way manufacturers extend the life of a product is to use natural packaging materials (bio-film) (Annu and

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Ahmed, 2021). A natural preservative that does not cause side effects that are currently being developed is chitosan (Priyadarshi and Rhim, 2020). Chitosan can be obtained from the shell extract of crustaceans such as crabs, shrimp, and lobster. Skin waste as one of the materials that can be used as the main industrial biomass source for large-scale production of chitosan can be used as a potential step to recycle waste product processing (Muxica et al., 2017).

Chitosan can be obtained by processing chitin contained in shrimp shells. Chitosan is a strong antimicrobial agent. The antimicrobial character of chitosan is due to its cationic properties. Antimicrobials in question work by killing microorganisms or inhibiting their growth. Chitin and chitosan can be easily used by utilizing the primary reactivity of primary and secondary amino and hydroxyl groups, and they can be applied in various fields ranging from the scope of health, and cosmetics to industrial activities (Dutta et al., 2004). Chitosan has unique properties that make it an ideal material for the development of edible antimicrobials (Priyadarshi and Rhim, 2020). Preliminary research conducted by the authors found that the use of chitosan on meatballs using edible film was better able to maintain the quality of meatballs organoleptically compared to chitosan mixed in meatball dough. The use of chitosan to package food is an innovative concept of biodegradable active packaging, where the developed packaging can reduce or inhibit the growth of microorganisms (Annu and Ahmed, 2021).

Several previous studies on the effectiveness of using chitosan on some products have been carried out. Chitosan can maintain the freshness of tilapia (Mahatmanti et al., 2010). Moreover, chitosan was used to be a preservative for fried chicken (Harjanti, 2014). The chitosan edible film technique is considered to be able to coat the outer part of the food. It is protected from microbes that might enter the food. This proves that chitosan is a potential natural food preservative. The purpose of this study was to determine the optimum concentration of chitosan that can be used as a natural preservative in sausages based on the organoleptic aspects of the product, the level of inhibition of chitosan, and the number of bacteria that develop in sausages during storage.

## 2. Materials and methods

### 2.1 Materials and equipment

The tools used in this study included the incubator, laminar, analytical balance, caliper, vortex, autoclave, refrigerator, petri dish, measuring cup, micropipette, tip, Erlenmeyer, test tube, tube rack, measuring tube,

Bunsen, well, L glasses, plastic wrap, tissue, markers and label paper. While the materials used were beef sausage, Escherichia coli bacteria, sterile distilled water, 1%, 1.5%, 2%, and 2.5% chitosan solution, 1% acetic acid, 0.1% chloramphenicol, nutrient agar, Eosin Methylene. Blue Agar (EMBA) and Plate Count Agar (PCA). All tools used must be cleaned and resumed, and sterilize the tools and materials by using an autoclave for 20 mins longer at a temperature of 121°C with a pressure of 2 atm. All agar media that have been tested and homogenized by using a hot plate should also be sterilized by autoclaving.

### 2.2 Preparation of the treatment

The sample used in the form of chitosan was derived from shrimp shells. Chitosan was weighted in 1 g, 1.5 g, 2 g, and 2.5 g, respectively. The chitosan was dissolved in 100 mL of 1% acetic acid such that the extract samples were made of the concentrations of 1%, 1.5%, 2%, and 2.5%, respectively. The obtained extract was then taken at 2 mL as an antibacterial sample in the MIC test process. Furthermore, the previous 4 sausages were soaked in a chitosan extract solution that had been made 30 mins before. Later, three sausages were drained on a plate to be observed their organoleptic changes, while 1 other sausage was used as a sample to be observed the number of its bacteria.

### 2.3 Effectiveness test of chitosan as sausage preservative

Testing the effectiveness of chitosan as a sausage preservative was carried out using five test treatments. Chitosan was applied as an edible film on sausages with concentrations of 1%, 1.5%, 2%, and 2.5% as experimental treatment and 0% as control treatment. Sausage is placed in a container, which is then covered with plastic wrap to minimize contamination from outside factors. Sausages were placed at room temperature (27°C) and observed for four consecutive days. Research data was obtained from documentation and organoleptic tests. The documentation process was done by observing and photographing sausages during testing time. Furthermore, organoleptic tests were carried out to obtain the changed data in taste, color, smell/odor, and texture of sausages based on the five treatments. The organoleptic assessment was carried out by involving 30 panelists who were willing to provide a description of the test product (based on SNI No. 01-2346-2006). The panelists are staff members and researchers of the University of Jember.

### 2.4 Minimum inhibitory concentration

Nutrient Agar media was prepared from a mixture of 2.4 g of NA agar dissolved in 120 mL of distilled water in a beaker (approximately 6 Petri dishes poured with 20

mL of agar solution each). The media that will be used is cooled first before use. A total of 20 mL of bacterial agar solution was mixed with 500  $\mu$ L of *Escherichia coli* and vortexed until homogeneous. It is then proceeding with pouring the sterilized Nutrient Agar media into a petri dish. Nutrient Agar media that has been cooled and solidified is then perforated in as many as 6 holes with a diameter of 7 mm each. Those six parts then were used for 6 treatments, namely K<sup>-</sup> (aquadest), K<sup>+</sup> (Chloramphenicol 0.1%), and four chitosan treatments (1%, 1.5% 2%, and 2.5%), and each hole was filled with 50  $\mu$ L. The next step was the incubation process, it will run for 24 hrs at 37°C. After the incubation phase, observe the forming of the inhibition zone, which was indicated by the presence of a clear zone around the hole. To know in detail, the zone of inhibition was observed, measured, and photographed. The clear zone around the holes can be used as an indicator that the material used can inhibit and/or kill bacterial growth. The larger the clear zone diameter area is formed, the more effective the material can kill bacteria.

### 2.5 Total viable bacteria and *Escherichia coli* count

This test was conducted to determine the number of microorganisms in the tested sample (sausage) using the TPC method on two different media types, namely Plate Count Agar (PCA) and Eosin Methylene Blue Agar (EMBA). The use of PCA is intended to determine the number of colonies of microorganisms that grow on sausages, while EMBA is used to determine the presence or absence of *E. coli* contamination in the sample, by showing the presence of metallic green colonies on EMBA media.


















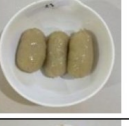

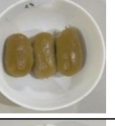





This test used five treatments, namely control (sausage without treatment), and sausage with chitosan treatment with various concentrations (1%, 1.5%, 2%, and 2.5%). For the treatment of chitosan, sausages were previously soaked in a chitosan solution for 30 mins. Next, the sausage was weighed as much as 1 g, then diluted using 9 mL sterile distilled water until a 10<sup>-3</sup> dilution was obtained. The last dilution (10<sup>-3</sup>) was then taken as much as 0.1 mL and then it was spread using L glass on the prepared medium (spread plate). The inoculation results were then incubated at 37°C for 24-48 hrs. The observation was made using a colony counter. We observe the colonies formed and count them. The number of bacteria from each treatment obtained and transformed into log 10CFU/g was then analyzed by using statistic software. If there were differences in effects between treatments after the ANOVA test, then continued with Duncan's multiple distance test using the same software.

## 3. Results

### 3.1 Organoleptic test

The research samples of sausages that undergo organoleptic changes can be identified through daily observations from day 0 to day 4. Based on observations, organoleptic changes in sausages started from day 1 in the control treatment, while the sausage treatment using chitosan coating is observed its organoleptic changes started from day 2 on the sausage texture. Based on observations, it was found that sausages with 1% chitosan coating underwent organoleptic changes longer. The changes began to occur on day 3. The results of the organoleptic test observations can be seen in Table 1.

Table 1. Sausage organoleptic test

Treatment	The observation on day				
	0	1	2	3	4
Control					
1% chitosan coating					
1.5% chitosan coating					
2% chitosan coating					
2.5% chitosan coating					

### 3.2 Minimum inhibitory concentration

The MIC Concentration Test was conducted to determine the maximum inhibition zone of the various tested treatments. The test was carried out with 3 replications for each treatment. Based on the results of the MIC test, it was found that the 1% chitosan extract solution had the most optimal results, with the diameter of the clear zone created of  $5.41 \pm 1.10$  mm. Observation of the clear zone was carried out after 24 hrs of incubation. The average value of the KHM test results can be seen in Figure 1 and Figure 2.

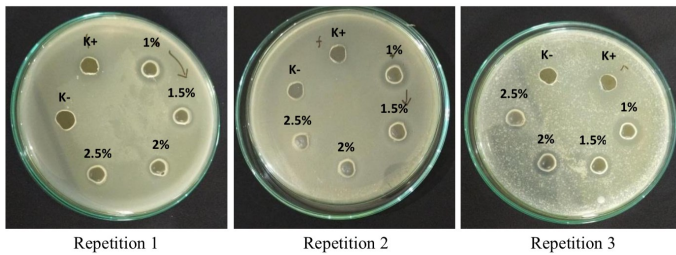


Figure 1. MIC test of chitosan to inhibit the growth of *E. coli*

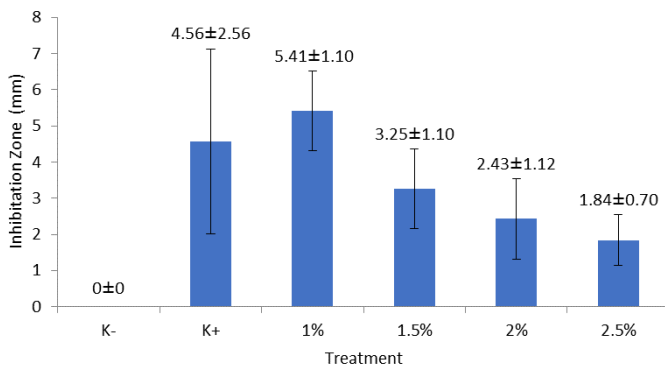


Figure 2. The average MIC test results of chitosan to inhibit the growth of *E. coli*

### 3.3 The test of bacterial count

The observation of the development of the number of bacteria was carried out to determine the number of bacteria growth. The observation of the clear zone was carried out after 48 hrs of incubation. Based on the results of testing the number of bacteria on PCA media, it was found that sausages with 1% chitosan coating were the ablest to suppress the number of bacteria that grew compared to other treatments. The results of the ANOVA test with a 95% confidence level (0.05) in Table 2 show a significance value of 0.036 ( $p \leq 0.05$ ). The results of the ANOVA analysis showed that there was a significant effect between treatments with different concentrations of chitosan on the number of bacteria that grew on sausage samples. Due to the significance value

Table 2. The results of ANOVA test on bacteria count

		Sum of Squares	df	Mean Square	F	Sig.	
Between Groups	(Combined)	366.933	4	91.733	3.920	0.036	
	Linear Term	Contrast	108.300	1	108.300	40.628	0.057
		Deviation	258.633	3	86.211	30.684	0.051
Within Groups		234.000	10	23.400			
Total		600.933	14				

of the ANOVA test 0.05, the analysis was continued with Duncan's test (Table 3) to compare all treatment pairs for the mean test. The results of total viable bacteria count on sausages with different treatment can be seen in Figure 3. Further observations with EMBA media showed that the sausage was not contaminated with *E. coli* bacteria which was characterized by the absence of metallic green bacterial colonies.

Table 3. Duncan advanced test results on the number of bacteria

Treatment	N	Subset for alpha = 0.05	
		1	2
1%	3	1.6667	
2.5%	3	5.0000	
2%	3	5.3333	
1.5%	3	7.0000	
Control	3		16.3333
Sig.		0.236	1.000

Means for groups in homogeneous subsets are displayed. Uses Harmonic Mean Sample Size = 3.000.

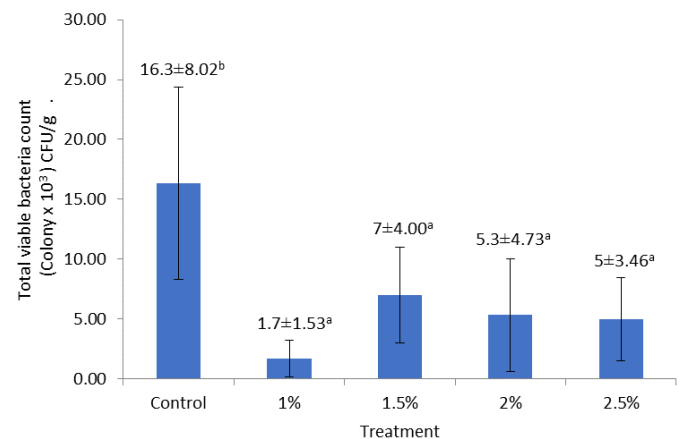


Figure 3. Total variable bacteria count of sausages with different treatment. Values are presented as mean±SD. Values with different superscripts are significantly different.

## 4. Discussion

### 4.1 Organoleptic test

Based on the results of the organoleptic test of sausage without chitosan, it was found that the sausage began to change in all organoleptic aspects on day 1, namely, the sausage became sour, and began to smell bad, the colour was browner, and the texture was slightly mushy. This change indicates that the sausage has oxidized and may have begun to be contaminated with spoilage microbes. Products that have fairly high

moisture content and contain high protein such as sausages require cold temperatures and vacuum storage such that the risk of potential oxidation and bacterial contamination can be minimized. Furthermore, on the 2<sup>nd</sup> and 3<sup>rd</sup> days, the sausages smell bad and start to get mouldy due to the decay process. This is due to the absence of preservatives that protect the sausage which makes the decay process takes place more quickly than other treatments. The shelf life of sausages that have been opened under normal storage conditions can only last 2 days at room temperature below 40°C (Asiah *et al.*, 2018). To get food products with a longer shelf life, efforts are needed to maintain their quality. One of them is using natural preservatives that are not harmful to human health and can maintain the nutritional aspects contained in them.

Based on the organoleptic test results of sausages with 1% chitosan coating, it was found that on day 0 to day 3 there was no significant change, both in taste, color, smell/odor, and sausage texture. This means that the sausage is still in good condition because destructive microbes have not contaminated it. This condition can be achieved with the help of chitosan, which is applied to the product (Purwanti, 2014). Bacterial contamination can be inhibited because of the interaction between chitosan and these bacteria, namely by interfering with the development of bacteria such that it reduces their toxicity (Evitha, 2018). Chitosan as a cation has the potential to interact with negative charges on the surface of bacterial cells, resulting in a weakening of the strength of the bacterial cell wall, resulting in the cell wall being unable to regulate the exchange of substances from and into the cell. It also affects the bacterial cell membrane is damaged and undergoing lysis and makes the metabolic activity will be inhibited and bacteria will die (Mahatmanti *et al.*, 2010). On the 3<sup>rd</sup> day, the sausage's colour began to change from light brown to dark brown. Discolouration as the sausage begins to oxidize. The oxidation process occurs because the sausage is exposed to free air for a long time. Previous research that used fried chicken as a test material also began to decrease the quality on the 3<sup>rd</sup> day (Harjanti, 2014). On the 4<sup>th</sup> day, there were some changes in taste, colour, aroma, and texture of the sausage. The taste of the sausage becomes sour, the colour of the sausage becomes dark brown, the aroma begins to smell bad, and the texture begins to become mushy. This was predicted to occur because the effect of chitosan as a preservative is no longer effective as a preservative on day 4, thus destructive microbes can contaminate sausages.

Based on the organoleptic test results of sausages with 1.5% chitosan coating, it was found that sausages on the 1<sup>st</sup> and 2<sup>nd</sup> days did not show significant changes

in taste, colour, smell/aroma, and sausage texture. Parameters of taste, aroma, and colour of sausage on the 3<sup>rd</sup> day began to be less enjoyable to taste and the colour of the sausage looked slightly brownish. Further, on the 4<sup>th</sup> day the taste of the sausage became bad, the taste was slightly sour, the colour of the sausage also changed to brown, the smell was bad because it started to rot and the texture started to stiffen. This fact indicates that the number of amino groups contained in the chitosan compound influences the ability of chitosan as a food preservative. The number of amine groups contained in chitosan is influenced by the value of the degree of deacetylation obtained during the process of removing the acetyl group in chitin before it becomes chitosan. Thus, the higher the degree of deacetylation, the better the ability of chitosan as a food preservative (Harjanti, 2014). The use of chitosan concentration higher than 1.5% organoleptically was not significantly different from the 1% chitosan coating. If it is done it would increase sausage production costs and did not affect the improvement of its quality. This is to the research result obtained by Pavinatto *et al.* (2019) which stated that 1% chitosan has been able to protect objects such as hydrophobic plastic capability, thus it can maintain the freshness of strawberries for up to 1 week.

Based on the organoleptic test results of sausage with 2% chitosan coating, it was found that on day 1, there was no significant change in the taste, colour, smell, and texture of the sausage. On the 2<sup>nd</sup> day, sausages decreased in value on the parameters of taste, smell and colour. Sausage coated with 2% chitosan solution on day 2 had a chitosan taste that was quite pungent, it affected the sausage's distinctive smell. The higher the amount of chitosan used as a coating material for a product, it can affect the distinctive aroma of a product thereby reducing its taste and appeal (Harjanti, 2014). Furthermore, on the 3<sup>rd</sup> and 4<sup>th</sup> day, the organoleptic parameters decreased in value where the sausage taste got worse, the taste was slightly sour, the sausage colour also turned darker brown than before, the smell was bad and it started to rot and the texture was quite stiff. This is presumably due to the effect of the nature of chitosan, namely as a purifier, the higher the concentration of chitosan given, the colour of the sausage looks lighter brown than that of the sausage which was coated with 1% and 1.5% chitosan. The lighter colour of the sausage on day 2 made the sausage less attractive and it is not like the colour of sausages in general (Nurazizah *et al.*, 2014). Apart from this, the chitosan concentration also affected the coated food texture (Chattopadhyay *et al.*, 2019).

Based on the organoleptic test of sausages with 2.5% chitosan coating, the results showed that on day 1, there

was no significant change in taste, colour, smell/aroma, and texture. On day 2, sausages decreased in value on all parameters except texture. The sausage on the 2<sup>nd</sup> day had a strong chitosan aroma that affected the sausage's distinctive smell. The higher the amount of chitosan used as a coating material for a product, it can affect the distinctive aroma of a product thereby reducing its taste and appeal (Nurazizah *et al.*, 2014). Furthermore, on days 3 and 4 the organoleptic parameters decreased in value where the sausage taste got worse, the taste was slightly sour, the sausage colour also turned brown, and the smell was bad because it started to rot. However, in terms of texture parameters, sausage still has a fairly stiff elasticity compared to other treatments. This is presumably due to the high concentration of chitosan which can produce a thicker edible film. The higher the concentration of chitosan used, the higher the residual weight (Wisuda *et al.*, 2014).

Furthermore, Hu and Michael (2018) stated that the use of certain concentrations such as 1%, 1.5% to 2% in meat protein products can affect organoleptic parameters. At a concentration of 1% chitosan, it began to affect the colour, smell and taste. At a concentration of 1.5% chitosan, it began to affect sensory attributes, while the use of chitosan at a concentration of 2%, began to affect lipid oxidation in meat. The antimicrobial action is influenced by intrinsic factors such as the type of chitosan, and the degree of chitosan polymerization and extrinsic factors such as the microbial organism, the environmental conditions and the presence of the other components. The use of chitosan in food systems should be based on sufficient knowledge of the complex mechanisms of its antimicrobial mode of action (Hafdani and Sadeghinia, 2011).

The ambient conditions, including pH, temperature and divalent metal ions also affect the antimicrobial activity of chitosan. A low pH favours the protonation of chitosan and increases its antimicrobial activity (Hu and Michael, 2018). Chitosan is a compound that is insoluble in water. A good solvent for chitosan is formic acid with a concentration of 0.2% up to concentration. However, chitosan is often dissolved in acetic acid (Ibrahim *et al.*, 2012). Chitosan is easily biodegradable and polyelectrolytic. Chitosan can easily interact with other organic substances such as protein. Chitosan has higher reactivity than chitin since it has a strong nucleophilic free amine group. This amine group is easily protonated at a pH of less than 6.5 which makes chitosan cationic so that it can bind to negative materials such as enzymes, cells, and nucleic acids so that it can inhibit and kill bacteria that interact by lysing the system (Sedjati, 2006).

#### 4.2 Minimum inhibitory concentration test

The characteristics of chitosan used as test material in this study have been by the established chitosan quality standards, namely, SNI 7949:2013 where the chitosan in this study was white, water content is 9.953% ( $\leq 12\%$ ) and ash content is 4.326% ( $\leq 5\%$ ). Moisture content and ash content are important parameters to determine the quality of chitosan. Water content affects the durability of a material, it is due to the high water content that increases the possibility of microbial attack. The ash content is a parameter to determine the minerals contained in a material that characterizes the success of the demineralization process. The lower the ash content produced, the higher the quality and level of purity of chitosan (Zahirudin *et al.*, 2008).

Based on the results of the MIC test, it is known that the 1% chitosan solution has the largest clear zone value of  $5.41 \pm 1.10$  mm. The clear zone was made as evidence that 1% chitosan was more able to inhibit the growth of *E. coli* bacteria that had previously been cultured on NA media. Its ability to suppress bacterial growth is since chitosan has a positively charged polycation that can inhibit bacterial growth. The mechanism that occurs is that chitosan molecules can interact with compounds that make up bacterial cells such as proteins, amino acids, and glucose which are then absorbed to form a kind of layer, that affects the cell's experience of a lack of substances to develop. It will also inhibit bacterial metabolism and eventually cause cell death (Wulandari *et al.*, 2015). The increase in the concentration of chitosan used in other treatments did not produce a clear zone that was more maximal than a concentration of 1%, presumably because the high the concentration of chitosan resulted in a thicker extract solution, thus it is not optimal when it is tested on agar plates. This is supported by research conducted by Nurazizah *et al.* (2014), which states that the higher the concentration of chitosan the more increased the viscosity. Chitosan is a polysaccharide that has biological properties that can form a gel, it affects the water that will be bound by chitosan through hydrogen bonds.

#### 4.3 Total viable bacteria and *Escherichia coli* count

The results of testing the total viable bacteria in the sausage samples in this study showed various values. Based on observations, the number of bacterial colonies on sausages with coatings of various concentrations of chitosan solution showed that the sausages in the treatment had significant bacterial growth. This is because the water content in sausages increases and is in line with the rise of storage time. The amount of free water contained in meat is an excellent medium for the growth and activity of food-destroying microbes,

including increasing the activity of enzymes found in sausages. The lowest number of colonies at the end of the observation (48 hrs storage) was obtained in the treatment of 1% chitosan with the highest average number of bacteria, and at the same time, the number of colonies on the control sausage reached  $16.3 \times 10^3$  CFU/g. These results showed that the chitosan solution at a concentration of 1% had the highest activity which was able to maintain the quality of sausages by inhibiting the number of bacteria that grew.

Based on statistical tests with ANOVA analysis, there were significant differences between the treatments ( $p \leq 0.05$ ). This proves that the application of chitosan coating can inhibit bacterial growth compared to sausages without chitosan coating. Chitosan has antibacterial properties. As a cation, chitosan has the potential to bind to proteins contained in microbial cell structures and can cause microbes to undergo lysis and death (Puspitasari and Ekawandani, 2019) The ability to suppress bacterial growth is due to chitosan having a positively charged polymer that can inhibit bacterial growth. The mechanism that occurs is that chitosan molecules can interact with compounds that make up bacterial cells such as proteins, amino acids, and glucose and then are absorbed to form a kind of layer which causes the cells to experience a lack of substances to develop and will inhibit bacterial metabolism and eventually lead to cell death.

Based on the bacteria identification with EMBA media, it was found that there were no contaminating *E. coli* bacteria. It is suspected that the bacteria died during the heating process or the sausage-making process and the use of clean water and raw materials that were not contaminated with bacteria with good sanitation. It is known that coliform bacteria such as *E. coli* can survive for months in soil and water, but they can die by heating them at a temperature of 60°C or more within 15 mins. This is supported by the research result of Dayanara et al. (2019), which states that *E. coli* pathogenic bacteria that contaminate food can occur due to a lack of attention to sanitation, both from snacks, snack handlers, sales points, and equipment used as well as utensils that are reused without being cleaned, especially utensils used for cooked or fast foods.

#### 4. Conclusion

The chitosan coating of 1% is able to maintain the quality of sausage until the 3<sup>rd</sup> day of storage with the number of bacteria  $1.7 \times 10^3$  CFU/g with a clear zone created of  $5.41 \pm 1.10$  mm. The concentration of 1% chitosan is the optimal concentration that can be used to inhibit the growth of bacteria in sausages compared to

other treatments.

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