Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 133 (2019) 85-97 EISSN 2392-2192

The utilization of lactic acid bacteria from rusip to inhibit the formation of histamine on salted-boiled mackerel tuna - *Euthynnus affinis* (Cantor, 1849)

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ABSTRACT

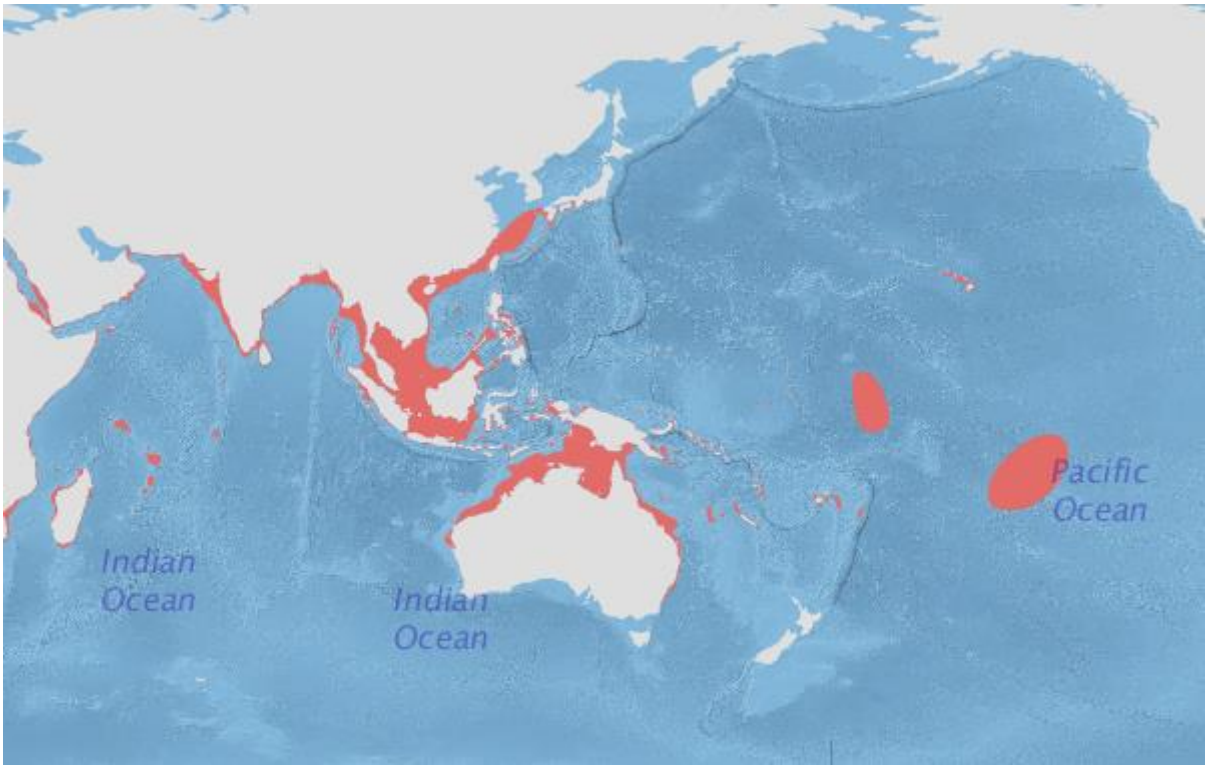
This research aims to determine the best concentration of Rusip fermentation solution for inhibiting histamine formation and its effect on the quality of salted-boiled mackerel tuna (*Euthynnus affinis*). The studies were conducted in August-September 2018 at the Fisheries Product Processing Laboratory and Microbiology and Molecular Biotechnology Laboratory of Fisheries and Marine Sciences Faculty, Padjadjaran University. This work used an experimental method with 4 variables and 3 repetitions, namely salted-boiled mackerel tuna (*Euthynnus affinis*) that soaked in rusip fermentation solution with a concentration of 0%, 10%, 20%, and 30% for 30 minutes and storage at some above room temperature (25-27 ºC). Parameters observed included TPC, Histamine levels, pH, and organoleptic values (aroma, appearance, and texture). The result showed that the use of 30% rusip fermentation solution was able to inhibit the formation of histamine and produced the lowest histamine levels of 29.80 mg/100 g. The number of histamine-forming bacteria is positively correlated with histamine content during storage. Based on the organoleptic value (aroma, appearance, and texture), the use of 30% rusip fermentation solution was still received by panelists and fulfilled the best quality requirements according to SNI 2717-1-2009 until the fifth day of storage compared to the other treatments.

***Keywords*:** *Euthynnus affinis*, Lactic Acid Bacteria, Histamine, Rusip, Salted-Boiled mackerel tuna, Indo-Pacific region

(Received 22 March 2019; Accepted 08 April 2019; Date of Publication 04 August 2019)

1. INTRODUCTION

Euthynnus affinis (Cantor, 1849), the kawakawa or mackerel tuna, is a species of ray-finned bony fish in the family Scombridae. This is an Indo-Pacific species which is found from the Red Sea to French Polynesia (Map 1).



Map 1. Geographical distribution of *Euthynnus affinis* (Cantor, 1849)

Salted-boiled mackerel tuna (*Euthynnus affinis*) is one of fisheries product that has a relatively short shelf life and tends to experience spoilage rapidly. The spoilage is due to enzymatic reactions and the activity of microorganism that can produce biogenic amine compounds. One of the biogenic amine compounds formed in salted-boiled mackerel tuna is histamine. Histamine (scombrotoxin) in fish meat is produced from free histidine decarboxylation mainly from the Scombroidae family by certain bacterial species such as *Proteus* sp., *Klebsiella* sp., and *Morganella* sp. Histamine causes poisoning if consuming fish that have high histidine on fish meat. The most common symptoms are vomiting, nausea, headache, dizziness, sweating, etc. The histamine alert level in the sample is 5 mg/100 g and causes poisoning if the concentration is 50 mg/100 g (FDA 2011). Rusip is a traditional fermented fish product originating from Bangka and Lampung. Rusip is processed using anchovies (*Stolephorus* sp.) with an additional 10% palm sugar and 25% salt then stored for two weeks. Rusip is also known as a functional food product because it contains high lactic acid bacteria. The bacteria that can be found in rusip are *Lactobacillus* sp., *Streptococcus* sp., and *Leuconostoc* sp. (Yuliana *et al*. 2018). Lactic acid bacteria are microorganisms that produce lactic acid as the main metabolite and are known to have an important role as potential

biopreservative agent that can control spoilage bacteria and pathogenic bacteria. Lactic acid bacteria produce antimicrobial compounds such as organic acids, bacteriocins, hydrogen peroxide, reuterine, and CO_2. That antimicrobial compounds can destroy the cell walls of pathogenic bacteria and inhibit an enzymatic reactions. Based on that, it is necessary to conduct research that aims to determine the best concentration of Rusip fermentation solution to inhibit histamine formation and its effect on the quality of salted-boiled mackerel tuna.

2. MATERIALS AND METHODS

The main materials used are anchovies, palm sugar, salt, and mackerel tuna (*Euthynnus affinis*). Other materials were NaCl solution (saline), aquades, Eosin Methylene Blue Agar (EMBA), Man Rogosa Sharpe Agar (MRSA), Sulfanilic acid, HCl, $NaNO_2$, Na_2SO_4, $Na_3PO_4 \cdot H_2O$, n-butanol, and Na_2CO_3.

The main equipments used in this study were thermometer, colony counter, oven, incubator, magnetic stirrer, hot plate, Petri dish, spectrophotometer UV-Visible, digital scales, centrifuge, and pH meter. The method used in this study was an experimental method with 4 variables and 3 repetitions based on the rusip fermentation solution with a concentration of 0%, 10%, 20%, and 30% at some above room temperature (25 – 27 ºC). The procedure for making rusip refers to Susilowati *et al.* (2014) and salted-boiled mackerel tuna refers to the Indonesian National Standard (SNI 2717.3:2009). The parameters observed included TPC, histamine levels, pH (Bawinto 2015), and organoleptic values (aroma, appearance, and texture), based on the Indonesian National Standard (SNI 2717.1.2009). Data on TPC, pH, and organoleptic values were analyzed using the quantitative descriptive methods. Data were compared with salted-boiled fish quality requirements (SNI 2717.1:2009), and meanwhile, histamine levels were analyzed using simple regression analysis and examined using ANOVA and Duncan's Multiple Range Test with the confidence level of 95%.

3. RESULT

3. 1. Total Plate Count (TPC)

The total bacterial analysis aims to determine the safety limits of microbial contamination. The calculated total bacteria can be a microbiological indicator of salted-boiled mackerel tuna. Based on observations, there are three genera of Gram-negative bacteria belonging to the family *Enterobacteria*. These bacteria are also known as histamine-forming bacteria namely *Enterobacter aerogenes*, *Klebsiella pneumonia*, and *Proteus mirabilis*. The genera are presented in Table 1. During storage, the number of histamine-forming bacteria continues to increase.

This is due to an increase in pH which is an environmental factor that also affects the growth of histamine-forming bacteria and HDC enzyme activity. In addition, the cell envelope of Gram-negative bacteria is more difficult to break because it has an outer membrane, which is separated by periplasm. The outer membrane (OM) consists of phospholipids, lipopolysaccharides (LPS), integral membrane proteins, and lipoproteins (Bos 2007). The OM function as a selective barrier has additional regulators that protect bacteria from harmful compounds, such as antibiotics.

Unlike the number of histamine-forming bacteria, lactic acid bacteria actually decrease. The presence of pH and Rusip fermentation duration are factors that affect the life phase of lactic acid bacteria. Stated that the growth of lactic acid bacteria has shown a stationer phase on the fourteenth day of rusip fermentation so when fermentation is applied to salted-boiled mackerel tuna, lactic acid bacteria are considered to have entered the death phase. In this phase, there is an accumulation of lactic acid metabolites which contribute to suppressing the number of lactic acid bacteria and there is a competition between microorganisms to obtain nutrients.

Table 1. TPC on Salted-Boiled Mackerel Tuna Based on Rusip Concentration

Day	**Species Bacteria**	**Rusip Solution Concentration**			
		0%	**10%**	**20%**	**30%**
1	*Enterobacter aerogenes* (CFU/gram)	2.2×10^2	1.9×10^2	1.5×10^2	1.1×10^2
2		3.6×10^2	2.2×10^2	1.4×10^2	1.4×10^2
3		5.3×10^3	4.8×10^3	4.4×10^3	4.2×10^3
4		4.6×10^4	2.9×10^4	1.6×10^4	1.2×10^4
5		3.6×10^5	1×10^5	1×10^4	1×10^4
6		3.6×10^5	0	0	0
1	*Klebsiella pneumonia* (CFU/gram)	5×10^2	3.6×10^2	1.5×10^2	1×10^2
2		8.2×10^2	5.2×10^2	1.5×10^2	1.2×10^2
3		7.5×10^3	7×10^3	5.8×10^3	5.4×10^3
4		7.7×10^3	7.6×10^3	6.1×10^3	3.9×10^3
5		**8.2×10^5**	4.5×10^5	1.4×10^5	1.2×10^5
6		**8.1×10^5**	**5.6×10^5**	**5.1×10^5**	4.4×10^5
1	*Proteus mirabilis* (CFU/gram)	1.4×10^3	3.6×10^2	1.5×10^2	1×10^2
2		7.3×10^2	3.8×10^2	2.8×10^2	2.7×10^2
3		1.5×10^4	1.4×10^4	1.1×10^4	9.8×10^3
4		1.9×10^5	1.9×10^5	1.4×10^5	2.1×10^4
5		**7.3×10^5**	**5.7×10^5**	**5.4×10^5**	4.3×10^5
6		**1.1×10^7**	**1×10^7**	**9.1×10^6**	**8.2×10^6**

Day	Species Bacteria	Rusip Solution Concentration			
		0%	10%	20%	30%
1	Bakteri Asam Laktat (CFU/gram)	4.5×10^6	5.1×10^6	5.5×10^6	6.5×10^6
2		-	-	-	-
3		3.7×10^5	4.1×10^5	5×10^5	6.1×10^5
4		-	-	-	-
5		5.3×10^4	6.7×10^4	7.1×10^4	8.1×10^4
6		-	-	-	-

Based on Table 1, one may notice that increasing the rusip concentration is in line with the ability of lactic acid bacteria to inhibit microbial growth in salted-boiled mackerel tuna. That means the more number of lactic acid bacteria, the more antimicrobial compounds are formed. One of antimicrobial compounds produced is lactic acid. The lactic acid functions as a permeabilizer which is possible to weaken OM of Gram-negative bacteria. Treatment with lactic acid releases a large proportion of LPS from OM and creates a hydrophobic pathway for certain substances such as bacteriocins. In addition, lactic acid can act as a direct inhibitor of several species of Gram-negative bacteria and act synergistically with other antimicrobial agents to increase bacterial destruction.

The antimicrobial compounds are capable of killing *Enterobacter aerogenes* and quite capable but not optimal for inhibiting *Klebsiella pneumonia* and unable to inhibit *Proteus mirabilis*. That is because of the morphological differences between histamine-forming bacteria that affect the ability of antimicrobial resistance. Characteristics of *Proteus mirabilis* move very actively by using peritrichous flagella and can make biofilms. *Klebsiella pneumonia* is a nonmotile bacteria and has a large and regular capsule, whereas *Enterobacter aerogenes* has small capsule, even though it has peritrichous flagella so that it is easier to destroy by antimicrobial compounds.

Referring to the Head Regulation of The National Agency of Drugs and Food Control Republic Indonesia no. 16 in 2016, salted-boiled mackerel tuna with 30% rusip soaking treatment is still safe for consumption until the fifth day with the number of histamine-forming bacteria still under 5×10^5 compared to the other treatments that have histamine-forming bacteria exceeding the limit of bacterial contamination.

3. 2. Histamine Level

Histamine level in fisheries product is an indication of the quality in salted-boiled black skipjack and food safety standards. The results of histamine level analysis based on rusip concentration in salted-boiled mackerel tuna are presented in Table 2.

Based on Table 2, it can be noticed that histamine levels in all of the treatment at the beginning of storage had exceeded the limits of the histamine alert level >5 mg/ 100 g but were

still below the 50 mg/ 100 g histamine level which could cause poisoning (FDA 2011). Stated that before processed, histamine has been formed because black skipjack has high red meat compared to other fish and in red meat, containing high histidine.

Table 2. Histamine Level on Salted Boiled Mackerel Tuna Based on Rusip Concentration

Rusip Concetration	Histamine Level (mg/ 100 g)					
	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6
0%	11.77^{c}	16.16^{c}	18.46^{c}	21.78^{c}	26.31^{c}	30.90^{c}
10%	11.63^{b}	15.45^{b}	17.23^{b}	20.20^{b}	22.81^{b}	30.79^{b}
20%	11.54^{ab}	14.15^{ab}	16.39^{ab}	18.87^{ab}	21.77^{ab}	30.39^{ab}
30%	11.43^{a}	13.71^{a}	14.66^{a}	18.61^{a}	21.37^{a}	29.80^{a}

Info: the letters mean notations based on the result of algorithm calculation to compare the difference between treatment averages with the corresponding comparative value. That means the same letter in the vertical direction shows that histamine levels are not significantly different on Duncan Multiple Range Test

Histidine is classified as an essential amino acid for fish. Histidine has an important functions in the catalytic site of many proteins (enzymes), histamine formation (signaling), and purines synthesis. It has the ability to bind Zn, Fe, and Cu which affect the distribution and excretion of essential elements in the fish body. Histidine is an important buffer component in fish muscle, and is vital for continuous anaerobic energy production during burst swimming activity. But when the fish died, the enzyme still has the ability to work actively, andt because the brain as a control network no longer functions, so the enzyme system becomes uncontrolled and can destroy other organs as the antibody system cannot protect against bacterial attack. The cooking process, such as salted-boiled fish using high temperatures in boiling, can kill histamine-forming bacteria and destroy HDC enzymes but when histamine has been formed, it will be difficult to eliminate histamine that already exists in fish. Based on the statistical analysis, it can be observed that the immersion treatment using 30% rusip fermentation solution has a significant effect on histamine levels in salted-boiled mackerel tuna 0% and 10% but did not have a significant effect with 20%. That means the higher rusip concentration used, the lower histamine level in salted-boiled mackerel tuna meat is. Low histamine levels due to the role of lactic acid bacteria which produce antimicrobial compounds can kill histamine-forming bacteria and affect the performance of the HDC enzyme through inactivation of important enzymes such as ribonucleotide reductase. Metabolite compounds reduce the pH of the environment so that histamine-forming bacteria will react to maintain optimal pH through the release of a proton in the cells to prevent denaturation of cell component.

3. 3. pH

The pH indicates existence of bacteria on the product because the pH affects bacterial growth both lactic acid bacteria or histamine-forming bacteria. The pH observation of salted-boiled black skipjack during the study at room temperature storage is presented in Figure 1.

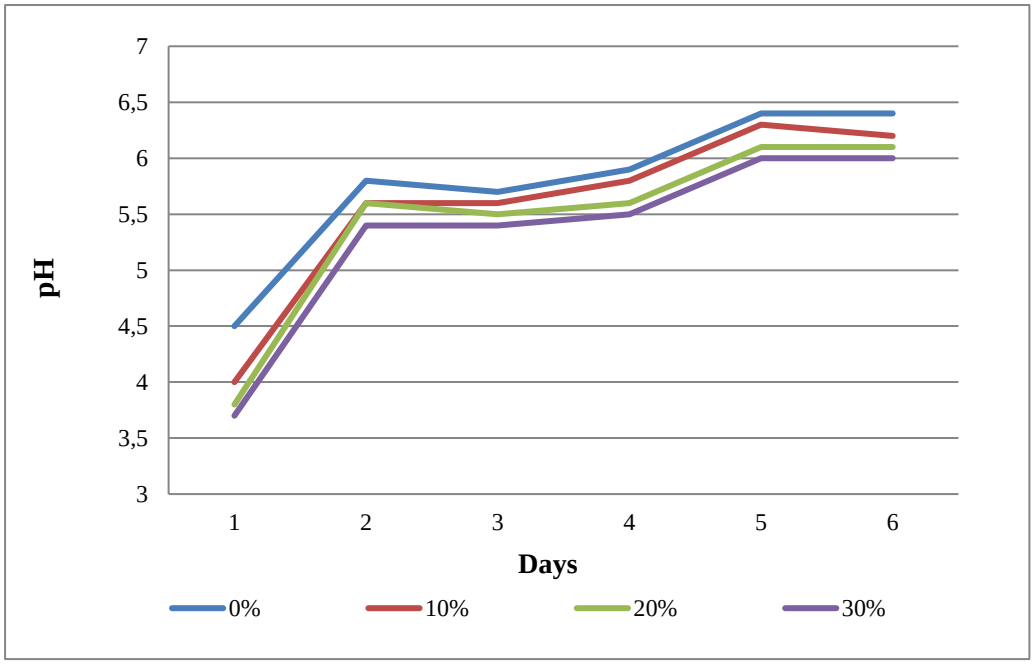


Figure 1. The pH on Salted Boiled Mackerel Tuna Based on Rusip Concentration

Based on Figure 1, rusip with a concentration of 30% has a lower pH than that after other treatments although changes in pH during storage did not show a significant effect through the same pattern between all treatments. Stated that the low pH at the beginning of storage was because antimicrobial compounds originate from an accumulation of glucose breakdown by lactic acid bacteria to produce lactic acid through the process of glycolysis. Antimicrobial compounds in salted-boiled mackerel tuna lead substrate-enzyme relationship as disturbed and will affect the catalysis reaction.

The increased pH during storage is the effect of a decrease in the number of lactic acid bacteria that affect the availability of antimicrobial compounds produced. In addition, an increased pH due to the decomposition of protein into alkaline volatile compounds, such as ammonia, trimethylamine, and other volatile compounds can reduce the organoleptic values in the product. However the antimicrobial compounds with the immersion treatment of rusip fermentation not only come from cooking but also from lactic acid bacteria found in rusip so that the number of lactic acid bacteria is higher than control and the ability to prevent histamine-forming bacteria is much better.

3. 4. Organoleptic Values

3. 4. 1. Appearance

The appearance observation of salted-boiled mackerel tuna during the study at room temperature storage is presented in Figure 2. Based on Figure 2, the appearance does not have a significant effect. The appearance of salted-boiled mackerel tuna control (0%) until the second day of storage was preferred by panelists with clean, white, intact, very bright, and no mucus characters while other treatments give a different feeling that the color of the meat is slightly dull because it is affected by the rusip color which is brownish gray.

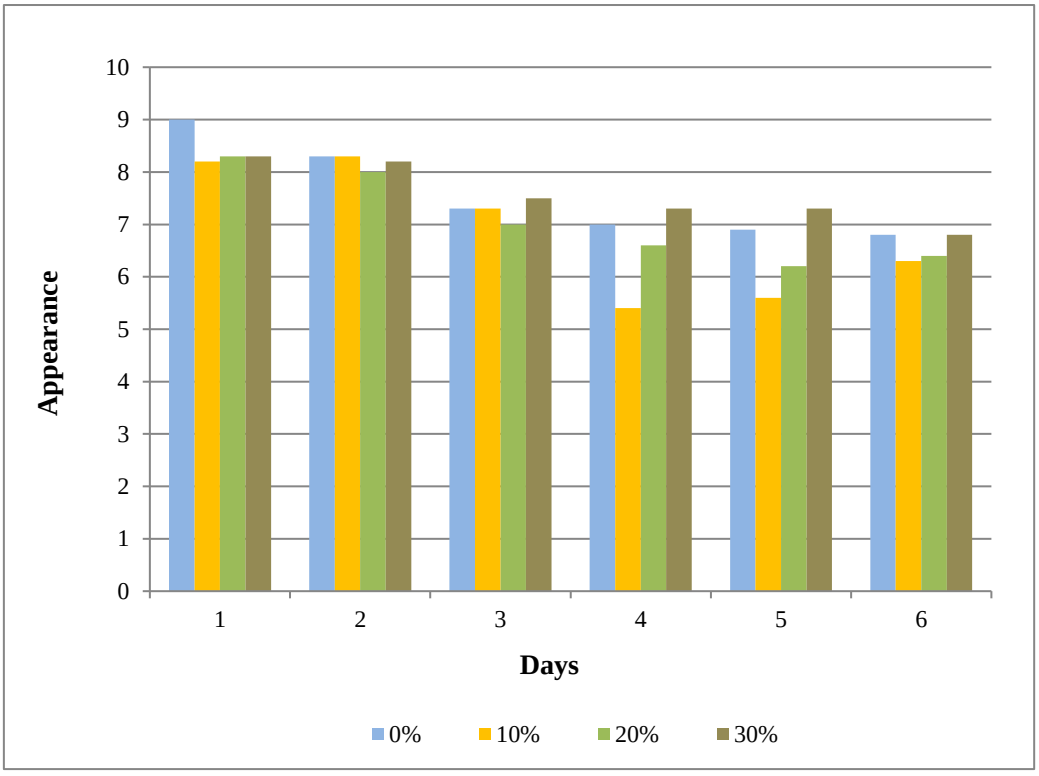


Figure 2. The Appearance's Value on Salted Boiled Mackerel Tuna Based on Rusip Concentration

During storage, the appearance value becomes decreased in all treatments. The color of salted-boiled mackerel tuna control turns dull and there were black spots on the surface of the meat. Ariyani (2010) stated that the decreased in the value of appearance was due to lipid oxidation by oxygen in the atmosphere and heat due to the influence of high storage temperatures (25 – 27 ºC).

Based on Figure 2, the panelist's acceptance of salted-boiled mackerel tuna which is soaked in 10% and 20% of the rusip is reduced on storage days 4 and 5. That is because of the uneven yellow color so that the product is less attractive. Meanwhile, the yellow color in the sample with 30% rusip concentration was light and not so visible by the panelists to be still accepted. The yellow color that appears is due to the presence of *Saccharomyces cerevisiae*.

The presence of yeast was due to the presence of compounds produced by lactic acid bacteria such as alcohol, organic acids, and other flavor compounds. Stated that the yellow color is the *Saccharomyces cerevisiae* pigment due to the sucrose fermentation process. Each yeast cell is also surrounded by a cell wall that is β-Glucan which is able to regenerate elastin to protect moisture.

3. 4. 2. Aroma

The aroma observation of salted-boiled mackerel tuna during the study at room temperature storage is presented in Figure 3.

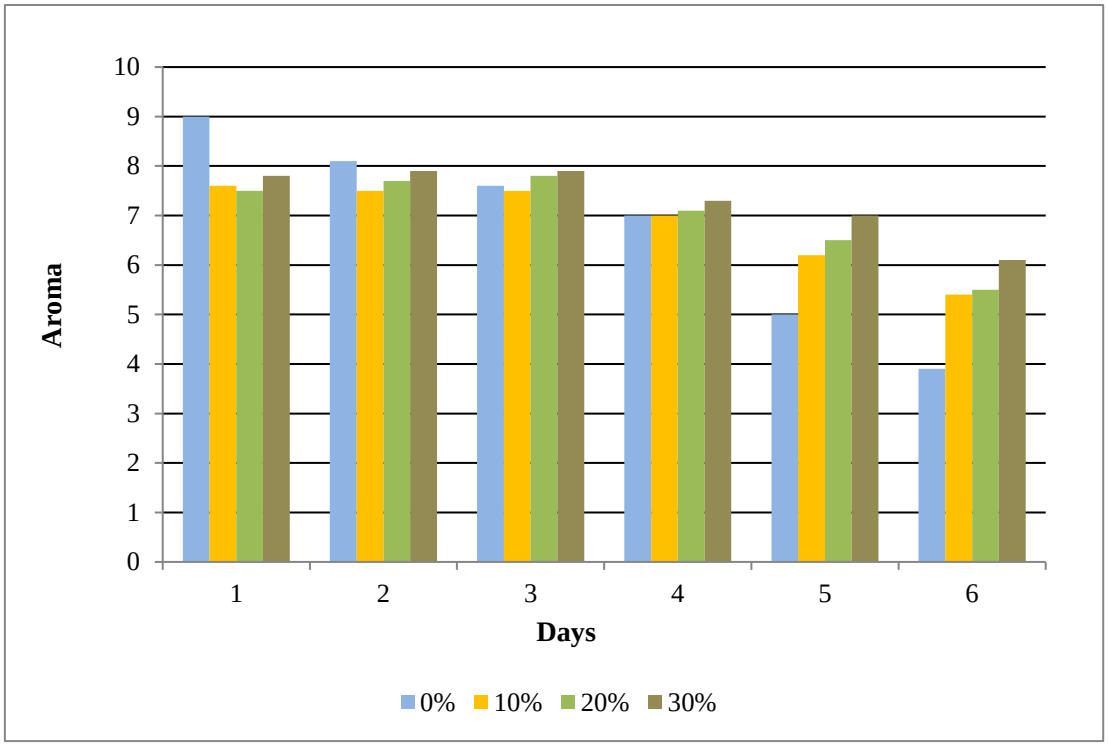


Figure 3. The Aroma's Value on Salted Boiled Mackerel Tuna Based on Rusip Concentration

Based on Figure 3, the aroma of salted-boiled mackerel tuna control at the beginning of storage was preferred by panelists. That was because the salted-boiled black skipjack control smelled of fresh and had a specific aroma compared to the other treatment. It had a specific aroma and slightly acidic from rusip. Therefore, the aroma of the acid decreases with increasing the storage time.

Based on Figure 3, it can be noted that the longer storage time results in the organoleptic value of aroma to decrease. The most significant decrease was in the control part which had smelled rancid and rather stale due to the emergence of fungi, and had passed the acceptance limit with the lowest value of 3.9 at the 6^{th} day of storage. In addition, the presence of microorganisms in salted-boiled mackerel tuna meat causes odor changes due to degradation of proteins that produce ammonia (NH_3), H_2S, indole, and histamine. Therefore, the salted-boiled mackerel tuna with treatment until the 6^{th} day of storage is still accepted by the panelists with a value above the minimum acceptance limit of 5, but only sample with 30% of rusip which still meets the best quality criteria based on SNI until the 5^{th} day of storage with organoleptic values at least 7.

The salted-boiled mackerel tuna that soaked in rusip fermented solution can reduce the rancid odor caused by the oxidation process. The activity of lactic acid bacteria that produce volatile acids and esters can inhibit bacteria and autolytic enzymes that cause decay. The combination of these compounds works synergistically in preventing the growth of microbes so as to reduce the nitrogen base in salted-boiled mackerel tuna including TMA, dimethylamine, ammonia, and other nitrogen bases.

In addition, the possibility of yeast that also grows in the rusip where the yeast produces metabolites of ethanol and CO_2 also helps to inhibit the smell of rancid on the meat. Carbon dioxide causes gram negative bacterial cells to modify the composition of membrane fatty acids so that they become more susceptible to nicin/niacin. Carbon dioxide plays a role in the efficiency of bacteriocin action.

3. 4. 3. Texture

The texture observation of salted-boiled black skipjack during the study at room temperature storage is presented in Figure 4.

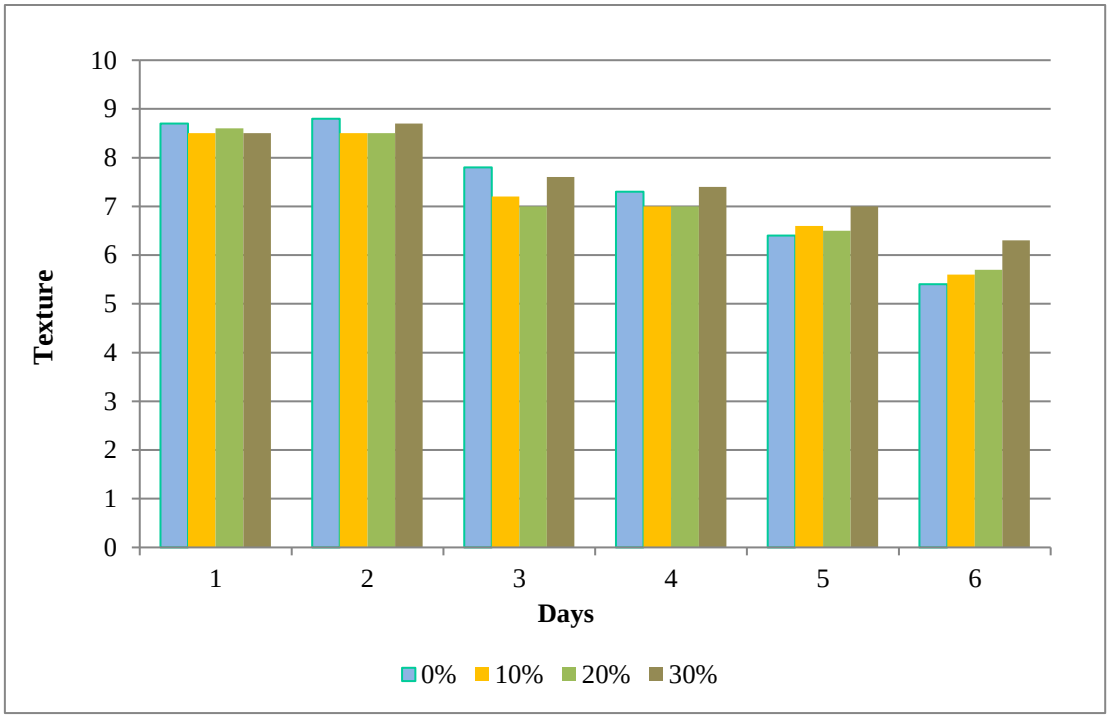


Figure 4. The Texture's Value of Salted Boiled Mackerel Tuna Based on Rusip Concentration

Based on Figure 4, the texture of salted-boiled mackerel tuna control (0%) until the second day of storage was preferred by panelists with the texture more compact and dense. Meanwhile the other treatment was compact and slightly dense. That was because the salted-boiled mackerel tuna treatment is wetter than the control, as there was a soaking treatment on rusip solution.

The salted-boiled black skipjack in all treatments was still received by the panelist until the 6^{th} day of storage, but the sample with treatment of 30% had the highest acceptance compared to the other treatments. The lowest acceptance was the control one with the texture become drier with increasing the storage time. When the processing was used, a high salt content caused free water to be pulled out. Stated that the decreasing water content caused by processing and handling was given to the mackerel tuna. Handling is done in order to reduce the water content in the body considering that most of the fish's body contains a lot of water

that is suitable for the growth of microorganisms. The handling given is osmotic pressure through the salting process. A higher concentration of salt and long boiling in high temperature (around 3 - 4 hours) can reduce the water content in the fish's body. In addition, storage at room temperature causes evaporation of water due to higher ambient temperatures than the temperature of the salted-boiled mackerel tuna so that the fish cannot resist air dehydration.

In contrast, salted-boiled mackerel tuna which is treated with immersion in rusip fermentation solution causes the water content to be lost during the process replaced with the water that comes from rusip fermentation solution. In addition, the presence of lactic acid bacteria as antimicrobial agents and *Saccharomyces cerevisiae* play a role in maintaining moisture due to the presence of β-Glucan which provides protection from environmental influences and is allegedly capable of regenerating elastin content to maintain moisture (Kusmiati 2011).

4. CONCLUSIONS

Based on the results of the study of inhibition of histamine levels using lactic acid bacteria from rusip and observation of salted-boiled mackerel tuna quality during storage at room temperature, immersion treatment in rusip cementation solution with a concentration of 30% was able to suppress the growth of histamine-forming bacteria, inhibit the development of histamine formation, and prolong the shelf life.

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