Liquid smoke inhibits growth of pathogenic and histamine forming bacteria on skipjack fillets

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Abstract. Pathogenic bacteria were analyzed using the most probable (MPN) method, and histamine level was analyzed using spectrofluorometer. The best liquid smoke concentration was determined using a sensory hedonic test including smell, taste and texture. In addition, water content and pH were also analysed. Concentration of liquid smoke varied between 0, 0.4, 0.8, 1.0 and 1.2%. The best concentration of liquid smoke was 1%. Fresh fillet dipped in 1% liquid smoke showed significantly decreased total plate count (TPC), from 4.1 x 10^3 CFU/g in fresh fillet to 7.4 x 10^2 CFU/g in fillet dipped for 20 minutes. Analysis of pathogenic bacteria showed a positive result in fresh fillet, and a negative result in fillets dipped in 1% liquid smoke, after 2 days of incubation at 30^{o}C. Analysis of anti-pathogenic bacteria showed the positive results in fresh fillet, and negative result fillets dipped in 1% liquid smoke. Further analysis of histamine content also supports our finding, in which the level of histamine goes down from 19.55 to 18.56 when dipped in 1% liquid smoke solution for 20 minutes. The results indicated that dipping fillet in liquid smoke is a very effective treatment to prevent pathogenic bacteria and histamine forming bacteria.

Keywords: histamine, liquid smoke, pathogenic, skipjack fillets

1. Introduction

Smoking fish has been used as a preservation technique for centuries, especially in Manado, Indonesia. Today, the use of liquid smoke flavoring as an alternative to traditional smoking treatment has been studied [1]. Liquid smoke preparations can be easily controlled and evaluated for composition and consistency of application to optimize antimicrobial properties and histamine potential [2]. Two main compounds in liquid smoke that are known to have bactericidal and bacteriostatic effects i.e. phenols and organic acids [3-5]. Two main compounds usually work together as a synergetic preservative. The liquid smoke presented an inhibiting effect on *Escherichia coli*, *Salmonella choleraesuis*, *Staphylococcus aureus* and *Listeria monocytogenes* bacteria [6, 7].

Fish family of Scombridae, Carangidae, Clupeidae, Engraulidae, such as skipjack, bonito, scad, mackerel, sardines, anchovies, etc., contained more naturally occurring histidine in their flesh. Therefore, fish that are prone to histamine development must be handled properly, chilled rapidly, and kept cool and gentle to ensure their quality and safety. Histamine is produced if amino acid histidine is decomposed by decarboxylase enzyme of histamine-forming bacteria such as *Enterobacter* sp., *Klebsiella* sp., *Morganella*, etc [8]. Histamine can develop if the temperature of fish is allowed to remain above 40^{o}F (4^{o}C). Histamine is not destroyed by cooking, so the best way to prevent histamine poisoning is to prevent the development of histamine-forming bacteria. Fish meat contains more than 50 mg/100 g (50 mg%) of histamine and can cause symptoms of allergy, such as nausea, vomiting, oral

burning sensation, itching, red rash (flushing), and hypotension. Raw materials containing more than 20 mg% of histamine (20 mg/100 g) are rejected for processing, and some European countries such as Germany allow a maximum of 10 mg% histamine.

The number of fresh Skipjack fish that exist in abundance prompted many producers to process the fish into economical products, including canned, frozen, and curing products. In North Sulawesi, smoked skipjack is one of the most famous exotic curing products. However, besides being rich in nutrition and having a specific taste, the problem that is often experienced by the industry is the presence of pathogenic bacteria and histamine-forming bacteria in those products, and this makes fresh skipjack to decrease very fast in quality and thus unsafe for consumption. Fish meat contains very little connective tissue and high natural cathepsin enzyme, so it is very easy to be digested by that autolysis enzyme, which softens the meat and makes it a good medium for the growth of microorganisms especially pathogenic bacteria and histamine-forming bacteria. Therefore, many studies have been done to prevent autolysis before processing, and recently one among the experiments gave liquid smoke as a preservative or antibacterial.

Liquid smoke is a condensed solution from a wood pyrolysis process of wood constituents such as cellulose, hemicellulose, and lignin. Pyrolysis of these compounds will produce several compounds that play a role in the preservation, color, and flavor of food ingredients [9, 10]. Phenol, carbonyl, di-phenol, formaldehyde, and acetic acid are the main compounds of more than 1000 liquid smoke compounds that have been identified successfully [11]. The most common bacteria found in fish products are *Coliform* and *Staphylococcus aureus* [12]. Liquid smoke from coconut shells had the greatest antimicrobial power against *E. coli*, *S. aureus*, *P. fluorescens* and *B. substilis* bacteria [13].

Liquid smoke is an alternative to the conventional method, because it is easy to produce, uses simple equipment, can be found also in the market, its concentration can be controlled, quality of the product including flavor can be standardized, and results in smoked fish having 100% edible portion [14]. Furthermore, he stated that liquid smoked skipjack contained *benzo (a) pyrene* below WHO standard. Some applications of liquid smoke to preserve fish product i.e. meatballs, fish sausages and tuna fish using liquid smoke with a concentration variation from 1.0% to 5% [15, 16].

Histamine is an active primary heterocyclic biological amine compound which is formed in the post-mortem phase of Scombroid and non-Scombroid fish meat which contains many free histidine, without showing decay characteristics if observed used sensory parameters [17, 18]. Histamine is stable against heating and resistant to processing including canning process [19, 20]. Tuna contains more than 2,000 mg/100 g of histidine amino acids [21]. Histidine in tuna meat is mostly a part of skeletal muscle tissue, carnosine and anserine [22]. Histidine amino acids in fish are the main component of non-carbonate buffers that protect fish from changes in pH [23].

2. Materials and Methods

2.1. Materials

First-grade fresh Skipjack (*Katsuwonus pelamis* L) was purchased at *Bersehati* fish market, Manado. The fish were put in a cool box with a fish to ice ratio of 1:2, and then transported by car for about 45 minutes to the laboratory. In the laboratory, the fish were washed and eviscerated, and fresh fillets (15x5x3cm) were prepared. Liquid smoke had been produced using smoke condensation equipment (Patent P00201405308), with coconut shell as fuel [14].

2.2. Production of liquid smoke

About 10 kg of coconut shell were used as raw materials to produced 2-3 liters of liquid smoke with a concentration of 60-70%. Coconut Liquid Smoke was produced by condensation machine in the laboratory of processing Faculty of Fisheries and Marine Science. Before used, the initial total solid concentration of liquid smoke was determined, and then the concentration of treatments were determined based on the initial concentration, used formula of $ViCi = VtCt$ [14]; where Vi is the volume of initial liquid smoke, Ci is the initial concentration of liquid smoke, Vt is the volume of liquid smoke

treatment, and Ct is the concentration of liquid smoke treatment.

2.3. Treatments

Fresh fillets were dipped in 1% liquid smoke solution for 0, 5, 10, 15, and 20 minutes, and at every one of those determined time, a sample was taken for analysis of TPC, pathogenic bacteria, histamine content, moisture content and pH. As a control, fresh fillets were also prepared at the same time without being dipped in liquid smoke, and a sample was taken at the same time as dipping treatment above. To make liquid smoked skipjack, fresh fillets were dipped in 1% liquid smoke solution for 5 minutes, then drained, and heated in an oven at 150^{o}C for 1 hour.

2.4. Laboratory analysis

Analysis has been done for total plate count using the pour plate method [24] and pathogenic bacteria: coliform and *E. coli* test using most probable method (MPN) [25]. *Salmonella* sp. was analyzed using SNI with modification [26], where the sample was grown in enrichment media of Bismuth Sultite Agar (BSA), and suspected colonies were isolated and continued with a biochemistry test, to ensure the presence of *Salmonella*. Water content was analyzed using an oven method and pH using pH meter [27]. Antibacterial activity susceptibility test was available using a solid media, based on the Kirby Bauer method. The histamine test used fluorometric [28], where the sample homogenized in methanol, and the heating, filtering and detected by using fluorometric with o-phthalaldehyde.

3. Results and Discussion

The best liquid smoke concentration for treatment was determined using the sensory hedonic test to the liquid smoked skipjack fillets, including smell, taste and texture; water content and pH. From the hedonic test, the best concentration of liquid smoke to smoke skipjack fillet was 1%.

3.1. Total plate count (TPC)

TPC values were used as a parameter because one cause a setback quality of fish is caused by spoilage bacteria activity. All microorganisms including pathogenic and spoilage bacteria will be encountered in TPC. The results showed that TPC values as a control (0 minute) are 4.0x10^{3} CFU/g, fresh fillets range from 4.8x10^{3} increased to 6.2x10^{4} CFU/g, while dipped in 1% liquid smoke solution range from 4.0x10^{3} decreased to 4.2x10^{2} CFU/g, was demonstrated in figure 1. Data of liquid smoked fillets with 3 times replications were 6.5x10^{2}; 1.2x10^{2} and 5.9 x10^{2} consecutively.

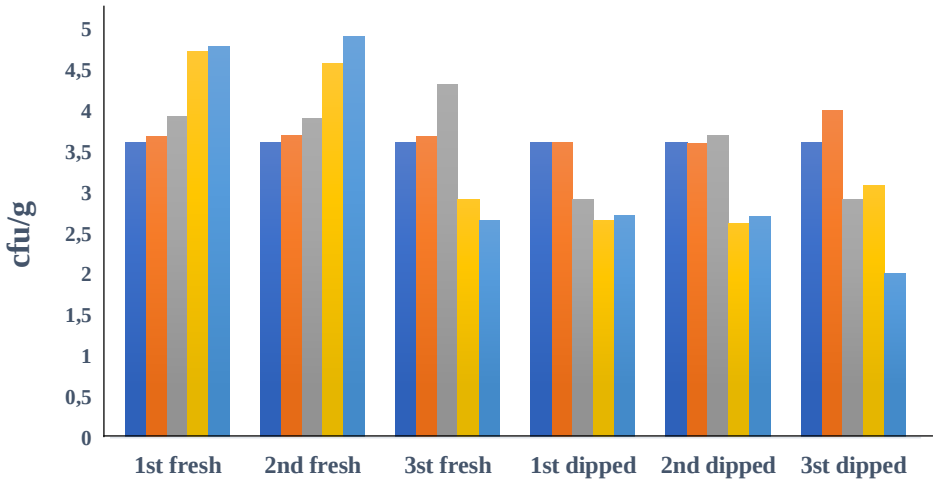

Figure 1. Total plate count of fresh and dipped fillets (: 0 minutes; 5 minutes; 10 minutes; 15 minutes and 20 minutes).

The maximum number of bacteria in fish that are suitable for consumption is 5,0x10^{5} CFU/g [25]. In accordance with [29], states that at the initial stage microbial growth has not occurred cell division.

Furthermore, after being able to adapt to their new environment, bacterial cells will grow and divide exponentially to the maximum amount. A combination of phenol functional components and high organic acid content works synergistically to prevent and control microbial growth [9], explained that the potential for smoke can extend the shelf life of products by preventing damage due to the activity of spoilage bacteria and pathogens [11]. TPC values on crushed salmon meat at the beginning of storage with the addition of various kind s of seasoning treatment [30]. TPC value is suitable for consumption in fish meatballs soaked in liquid smoke, vacuum packed, pasteurized, and stored for 10 days in refrigerator temperatures of 5-10^{o}C [31]. While other processed products such as *abon Roa* and *Cakalang pampis* packaged with MAP and stored at room temperature are still suitable for consumption up to 30 days of storage [32]. However, another research reported that an increase in the number of microbes in sardines reached more than log 7 cfu / g after 25 hours of storage at 30^{o}C [33]. These results are obtained due to storage temperature and pH optimum for microbial growth [34].

3.2. Escherichia coli

The result showed that *E. coli* count as a control was 3.6 MPN/g, and then fresh fillets range from <3.0 to 36 MPN/g, while for dipped in 1% smoke solution only <0.3 MPN/g, and *E. coli* data on liquid smoked fillets with 3 times replications were <0.3 MPN/g, 36 MPN/g, <3.0 MPN/g consecutively during 24 hours incubation. Data of *Escherichia coli* can be seen in table 1.

Table 1. *Escherichia coli* in fresh, dipped, and smoked fillets after 24 hrs incubation.

E. coli (MPN/g), 24 h						
Time (minutes)	Fresh fillet			Dipped in 1% liquid smoke solution		
	1	2	3	1	2	3
0	3.6	3.6	3.6	3.6	3.6	3.6
5	7.2	<3.0	<3.0	<3.0	<3.0	<3.0
10	7.2	<3.0	<3.0	<3.0	<3.0	<3.0
15	7.4	<3.0	<3.0	<3.0	<3.0	<3.0
20	15	36	3.6	<3.0	<3.0	<3.0

The presence of phenols and organic acids in liquid smoke prevent the *E. coli* developed. In accordance with [35] that liquid smoke can inhibit the growth of *E. coli* and is strongly bactericidal. Total *E. coli* observation results, fish meatballs soaked in liquid smoke, vacuum packed, pasteurized and stored in cold temperatures have a shelf life of about 30 days and are still received by Indonesian standard [31]. *Abon Roa* and *Cakalang pampis* smoked packaged by MAP had a negative value of coliform, *E. coli*, *Salmonella sp*, and *Vibrio* sp. [32].

3.3. Salmonella

Total *Salmonella* count as control was <3.0 MPN/g, in the fresh fillets ranges from <3.0 to 7.4 MPN/g, dipped in 1% smoke solution range from <3.0 to <3.6 MPN/g, and in liquid smoked fillets with 3 times replication only <0.3, during 24 hours incubation. Data of *Salmonella* can be seen in table 2.

Table 2. *Salmonella* sp. of fresh, dipped, and smoked fillets after 24 hours of incubation.

Salmonella (MPN/g), 24 h						
Time (minutes)	Fresh fillet			Dipped in 1% liquid smoke solution		
	1	2	3	1	2	3
0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
5	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
10	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
15	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
20	7.4	7.4	7.4	<3.0	<3.6	<3.0

Occurring antimicrobials naturally can be applied directly to food to protect food quality, extend food shelf life by inhibiting or inactivating spoilage microorganisms, and improve food safety by inhibiting or inactivating food-borne pathogens [36]. The presence of Salmonella in products can be explained mainly by hygiene failure during production [22, 37]. Total Salmonella observation results of fish meatballs soaked in liquid smoke, vacuum packed, pasteurized and stored at cold temperatures for 20 days are still accepted by Indonesian standard [31]. Another work reported that *Salmonella sp.* and *Staphylococcus aureus* bacteria were negative in all liquid smoke materials, used for Katsuobushi [38]. Because of common food-borne pathogens such as *L. monocytogenes*, *Salmonella*, *E. coli* and *Staphylococcus* have shown sensitivity to liquid smoke in vitro and in food systems, can be concluded that liquid smoke has the potential to be used as an all-natural antimicrobial in commercial applications where the smoky flavor is also desired.

3.4. Antibacterial

The results showed that the longer the incubation time of the tested media, the higher antibacterial activity of smoke liquid which can be seen from the increased in the diameter of the inhibitory zone by all treatment concentrations, in the 48 hours. Inhibition zone of liquid smoke on *E. coli* and *Salmonella* can be seen in table 3. From data of inhibition zone in table 3, can be stated that *E. coli* and *Salmonella* categorised as sensitive because the inhibition zone was> 14. The diameter of the inhibitory zone is an indication of the sensitivity of the test bacteria, the greater the inhibitory zone, the greater the antibacterial activity [35]. Furthermore, inhibition zone > 14 is sensitive, and <11 is resistant. A large number of different genes may be responsible for anti- microbial resistance [35].

Tabel 3. Inhibition zone on *E. coli* and *Salmonella* by liquid smoke.

	Inhibition Zone Diameter (mm)							
	0.4%		0.8%		1.0%		1.2%	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
E. coli	13	20	17	19	25	28	27	35
	16	25	18	21	23	31	25	40
	14	30	15	35	17	39	21	42
	14	36	18	43	19	28	25	45
Salmonella	12	23	14	27	19	25	20	27
	14	26	15	28	25	33	24	35
	16	29	18	35	24	27	26	35
	18	30	19	35	27	39	28	40

As an antibacterial, phenol compound has a working mechanism by damaging the structure of bacterial cells and inhibits the process of cell wall formation so that it can cause lysis of the bacterial cell wall [39]. The mechanism of action of antibacterial compounds is varied and complex, in addition to damage to the bacterial cell wall, the ability of phenol compounds is also to denaturize proteins and can also cause cell death [40]. Some of the bacterial cytoplasmic membrane structures contain protein and fat. Unstable in the cell wall and cytoplasmic membrane causing the function of selective permeability, active transport function, control of bacterial cell structure to be disrupted. Disruption of cytoplasmic integrity in bacteria results in the escape of macromolecules and ions from cells. Bacterial cells become deformed and lysis occurs. Basically, the damage caused is a loss of integration and damage to the structure of cell wrapping [41].

The bacterial cell structure is the main target for the antibacterial mechanism action. The mechanism of antibacterial action is attacking the cytoplasmic membrane, loss of stability in protons and electrons and coagulation in the constituent components of the cell [42]. Antibacterial activity was influenced by several factors, namely concentration, the content of antibacterial compounds, diffusion power of extracts and inhibited types of bacteria [43].

This study shows the higher the concentration of liquid smoke, the greater the inhibition zone diameter of *E. coli* and *Salmonella* so that it can be assumed that the higher the concentration, the greater the number of antibacterial compounds released thus facilitating the penetration of these compounds into

bacterial cells with the mechanism each. Basically, there was a reaction between anti-bacterial and bacterial cells that affected the structure and shape of bacterial cells [44]. The inhibitory activity of *E. coli* and *Salmonella* from liquid smoke is caused by the influence of bioactive compounds or secondary metabolites.

3.5. Histamine

The total histamine in control was 19.55 mg/kg and in fresh fillets it increased by time, but in fillets dipped in liquid smoke the number decreased by time, and the higher the concentration the lower the histamine value, as can be seen in figure 2. The average of histamine content in liquid smoked fillets was 26.51 mg/kg. The results showed that samples have a low content of histamine. The European Legislation allowed for histamine amount to be up to a maximum of 200 mg/kg in fresh fish and 400vmg/kg in fishery products.

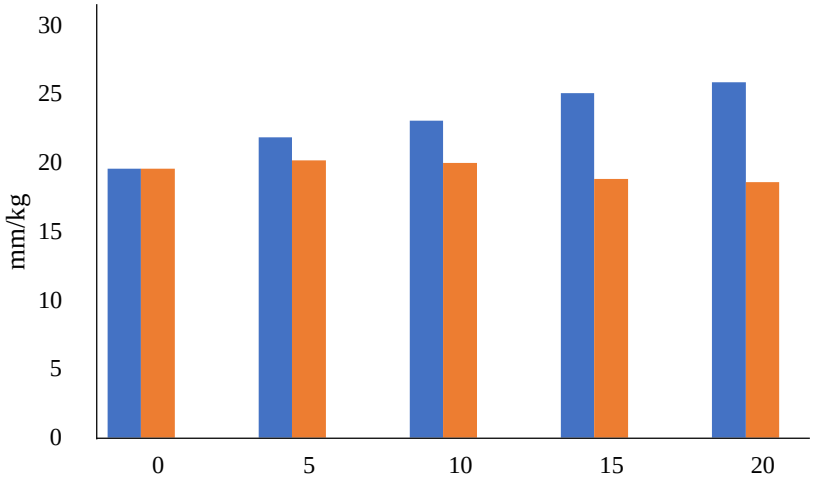

Figure 2. Histamine content of fresh and dipped fillets (0, 5, 10, 15, 20 min), fresh fillet (), dipped in 1% liquid smoke solution ().

The decrease in histidine amino acids during storage is caused by enzymatic damage due to the activity of histidine decarboxylase enzymes [45] and L-histidine ammonia-lyase which produce histamine and glutamate [46]. Damage is caused by microbiological activities, namely *Pseudomonas* sp., and *E. coli* [47], *Micrococcus luteus* [48], *Staphylococcus aureus* [49] and is one of the factors that cause the slowing down of histamine content during storage.

3.6. pH

The results showed that the pH value of fresh fillets at room temperature during 20 minutes were increased, while fillets dipped in 1% liquid smoke solution for 20 minutes were decreased. Liquid smoked fillets showed that pH values range from 5.56 to 5.58. Low pH of liquid smoke is caused by organic acids present as a result of the condensation process of smoke. Organic acids also give an effect to preserve fish and meat products. The resulting pH value is presented in table 4.

Table 4. The pH of fresh and dipped fillets.

Time (minutes)	pH					
	Fresh fillet			Dipped in 1% liquid smoke solution		
	1	2	3	1	2	3
0	5.46	5.52	5.57	5.46	5.52	5.57
5	5.48	5.57	5.53	5.39	5.38	5.43
10	5.44	5.40	5.51	5.31	5.20	5.33
15	5.38	5.56	5.28	5.20	5.23	5.23
20	5.15	5.43	5.18	4.98	5.33	5.17

Liquid smoke can generally be used as a preservative because it has a degree of acidity (pH) with a value of 2.8-3.1 [50], so it can prevent the growth of spoilage bacteria and pathogens, such as *Escherichia coli, Vibrio sp,* and *Salmonella* [18, 51].

3.7. Water content

The water content of fresh and dipped fillets can be seen in table 5. The results showed that water content in fresh fillets was higher than fillets dipped in 1% liquid smoke solution. While the water content of liquid smoked fillets ranged from 48.3 to 49.3%. Another important mechanism that is responsible for slowing down the growth rate of microorganisms, is related to the microenvironment surrounding growing colonies. Water content is important, apart from temperature, pH, and other factors including food structure. Based on the requirements of the quality and safety of fish meatballs in Indonesian standards, the maximum water content is 65% [31]. Traditional smoked fish products varied between 46-59 % [14].

Table 5. Water content of fresh and dipped fillets.

Water Content (%)						
Time (minutes)	Fresh fillet			Dipped in 1% liquid smoke solution		
	1	2	3	1	2	3
0	62.8	62.8	63.0	62.8	62.8	63.0
5	66.5	64.3	64.0	69.0	65.5	66.0
10	70.5	67.7	68.0	73.3	69.5	70.0
15	76.3	70.3	70.0	76.8	72.5	73.0
20	81.8	76.2	76.0	79.3	73.3	77.0

4. Conclusion

Liquid smoke with a concentration of 1% effectively prevented the growth of bacteria in general by lowering TPC, and also effectively prevented pathogenic bacteria i.e. *E. coli* and *Salmonella*. Liquid smoke with a concentration of 1% also lowered the production of histamine. The higher the concentration of liquid smoke the greater the inhibition zone diameter of *E. coli* and *Salmonella*.

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