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Identification of Pathogenic Bacteria in Fresh and Smoked Fish of the Tuna (*Thunnus* sp.) with SNI 01-2332.2-2006

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ABSTRACT

Traditional fish preservation aims to reduce the water content in fish bodies to prevent bacterial growth. In Ternate City, the smoked fish processing at CV. X is still traditionally done and does not follow the good manufacturing practices (GMP) or standard sanitation operating procedures (SSOP). This study aimed to identify bacteria in both fresh and smoked tuna species (*Katsuwonus pelamis*, yellowfin Tuna, and *Euthynnus alletteratus*) in Ternate City. The research was conducted at CV. X, with bacterial identification testing performed at the Class I Fish Quarantine Lab at Babullah Airport from September to November 2014. Data analysis for bacteria included biochemical testing and bacterial count calculation based on SNI 01-2332.2-2006. The results showed that 14 types of bacteria were found in fresh fish, including *Vibrio* sp., *Pseudomonas* sp., *Enterobacteria* sp., *Actinobacillus* sp., *Pasteurella* sp., *Nitrobacter freundii*, *Yersinia pestis*, *Yersinia* sp., *Proteus mirabilis*, *Pasteurella* sp., *Alcaligenes* sp., *Escherichia coli*, and *Proteus* sp. Bacteria identified in smoked fish products included *Salmonella* and *Proteus* sp. However, the bacterial counts were below the total plate count (TPC) limit specified by SNI 01-2332.3-2006, which is 5x10^5 CFU/g. Therefore, it is concluded that the fish can be consumed safely and properly.

INTRODUCTION

The tuna is a source of animal protein that is widely consumed by the people of North Maluku since it is readily available and affordable. The tuna species, with significant market and economic value, include the albacore (*Thunnus alalunga*), the Atlantic bluefin tuna (*T. thynnus*), the bigeye tuna (*T. obesus*), the Pacific bluefin tuna (*T. orientalis*), the southern bluefin tuna (*T. maccoyii*), the yellowfin tuna (*T. albacares*), and

the skipjack tuna (*Katsuwonus pelamis*) **(Erauskin-Extramiana *et al.*, 2019)**. Tuna is a pelagic fish that is highly perishable, like other fish species. It is very susceptible to oxidation, so exposure to air should be minimized to prevent spoilage. Fresh tuna should be consumed within 1-2 days after purchase **(Agustini, 2002)**. To maintain the quality of fresh fish, efforts are needed to preserve it by smoking (locally called "fufu fish"). The process applied for the ataining a smoked fish is a form of fish preservation that the people of North Maluku widely carry out, because the process of smoking is very effective and efficient. The smoking process involves the penetration of volatile compounds from burning wood into the fish. This method, which uses wood as a heat conductor, imparts specific flavors and aromas to the fish and extends its shelf life due to the antibacterial properties that inhibit enzymatic activity. This process ultimately affects the quality of the smoked fish **(Nurmianto, 2018)**.

Knowledge of fish preservation using the smoking method which has been carried out by the community for generations by using mangrove wood or wood from coconut trees or other wood obtained from the surrounding environment and mixed with coconut shells or sawdust. This wood mixture produces smoke that determines the quality of the fish, enhancing its preservation as quickly as possible. If the wood used is bad, the quality of the smoked fish will not be good, the color will be dark, and the taste will be bitter.

The stages of wood pyrolysis during fish smoking are as follows (**Belichovska *et al.*, 2019**):

1) Air evaporation occurs (temperature 100- 170^{0}C)
2) Hemicellulose decomposition (temperature 200- 260^{0}C)
3) Cellulose decomposition (temperature 260- 320^{0}C)
4) Lignin decomposition (temperature 310- 500^{0}C)

The traditional fufu fish processing process by people of North Molucas causes producers not to pay attention to the quality of fufu fish products. A study conducted on food processing equipment found that mercury, chromium, iron, manganese, lead, zinc, cobalt, nickel, and other metals can be found in food every day, with varying concentrations. Such ingredients are obtained in household appliances, and they are a potential source of contamination of processed food **(Cederberg *et al.*, 2015)**.

One downside of using the smoking method to preserve fish is that if the water content during smoking is too low, it can lead to increased growth of fungi and bacteria. Additionally, if the smoking temperature is low and the smoking time is extended, the fish can become overly hard in texture. Balancing these factors is crucial to ensure that the fish is properly preserved and has the desired texture (**Andhikawati *et al.*, 2021)**.

To prevent a decrease in the quality of the smoked skipjack tuna microbiologically, traders must have good safety behavior by choosing the equipment

used in the form of a closed serving area, cutting tools that are easy to clean, knives that are not rusty, using gloves, and personal hygiene. To maintain the quality of smoked fish, such as the skipjack tuna, effective food safety practices are essential. Bacterial testing is a crucial measure, carried out on both fresh fish and during the smoking process, to monitor and control harmful bacteria that could compromise the fish's safety and quality. Ensuring high standards of hygiene, including clean equipment and proper handling, is vital to prevent contamination. Additionally, controlling smoking temperatures and durations is important to prevent bacterial growth and ensure the fish maintains the desired texture. Monitoring water content during smoking also plays a key role in avoiding excessive hardening and controlling bacterial proliferation. By adhering to these practices, the quality and safety of smoked fish can be significantly improved.

MATERIALS AND METHODS

This study was conducted to determine the number of bacteria and to identify the types of bacteria found in the fresh tuna and fufu tuna.

1. Materials and tools

The materials used in this research were: electric microscope, glass object, petri dish, tweezers, petri dish bunsen lamp, digital scales, tissue, aquades, autoclave, oven, erlemeyer, measuring cup, hot plate, vortex, crooked needle, ose needle, incubator, spatula, test tube, aluminum foil/cotton, camera. The tools and solutions used in this study included smoked fish and fresh fish (Madidihang fish, Skipjack fish, and Tongkol fish), as well as the following solutions: SCB (Selenite Cystine Broth), TSIA (Triple Sugar Iron Agar), HE (Hektoen Enteric Agar), XLD (Xylose Lysine Deoxycholate Agar), LIA (Lysine Iron Agar), peptone solution, NaCl solution, and lactose solution.

Data retrieval methods

This study used three types of fresh and smoked tuna: (1) the skipjack fish (*Katsuwonus pelamis*), (2) the Madidihang fish (*Thunnus albacares*), and (3) the cob fish (*Euthynnus alletteratus*).

The media used for bacterial analysis were XLD and HE agar. Fish samples, each weighing 25 grams, were diluted with 225ml of LB solution. Dilution was carried out up to 10-3. Samples from dilutions of 10-1, 10-2, and 10-3 were plated onto XLD and HE agar media to observe bacterial counts. The plating method used was the surface spread plate technique, where 0.1ml of the sample was spread onto the surface of the sterile, solidified media.

Morphological observations and Gram- staining were performed on bacterial isolates purified from XLD and HE agar media. This observation aimed to determine the shape and classification of bacteria, including Gram-positive and Gram-negative types, based on their reaction to staining and the nature of their cell walls **(AL-Fatlawy *et al*., 2020)**.

Biochemical testing is used to determine the physiological characteristics of bacteria isolated from XLD and HE media after a 24-hour incubation. This involves several tests, including the Motility Test, Catalase Test, Oxidase Test, Oxidation/Fermentation (O/F) Test, Indole Test, Ornithine Test, Citrate Test, Triple Sugar Iron Agar (TSIA) Test, and Lysine Iron Agar (LIA) Test. The results from these tests are used to identify the bacteria based on their physiological and biochemical properties. The identification process compares these results with reference data of **Cowan (1958)**, **UNESCO (1980)** and **Alsharjabi *et al.* (2019)**.

2. Data analysis

According to SNI 01-2332.3-2006, the cup count method assumes that each viable cell will develop into a single colony. Two procedures are used for cup counting: the spreading method and the pouring method on the surface of the media (Surface/Spread Plate), as specified in SNI 01-2332.3-2006.

$$N = \frac{\Sigma C}{[(1 \times n1) + (0,1 \times n2)] \times (d)}$$

The calculation for determining the number of colonies is as follows:

N : The number of colonies of the product, expressed in colonies per ml or colonies per gr.

∑C : Number of colonies on all saucers counted

N_1 : The number of cups at the first diluted is calculated

n_2 : The number of cups at the second diluted is calculated

d : calculated first dilution

RESULTS

A. Bacteria found in fresh and smoked Madidihang or the yellowfin tuna (*Thunnus albacares)*

Based on the purification results for fresh Madidihang, 11 bacterial isolates were obtained. Identification of these bacteria revealed the presence of *Citrobacter freundii*, *Plesiomonas* sp., *Yersinia pestis*, *Pseudomonas* sp., *Proteus mirabilis*, *Pasteurella* sp., and *Enterobacteriaceae*. For the smoked yellowfin tuna, one bacterial isolate with the sample code TMa/Central Meat was obtained. Identification of this isolate determined it to be *Proteus* sp.

1. Bacteria found in the fresh skipjack (*Katsuwonus pelamis*)

Based on the purification results for the fresh skipjack, 10 isolates were obtained, and then identification of bacteria was carried out to determine the type of bacteria. The bacteria found in fresh skipjack were *Yersinia* sp., *Vibrio* sp., *Actinobacillus* sp., *Alcaligenesis*, *Proteus mirabillis*, and *E. coli.*

2. Bacteria found in the fresh little tunny (*Euthynnus alleteratus*)

From the results of purification for the fresh little tunny, 11 isolates were obtained, and then identification of bacteria was carried out to determine the type of bacteria. The bacteria found in fresh little tunny are *Enterobacteria, Vibrio* sp., *Proteus* sp., and *Pasteurella*. Based on the purification results for the little tunny, 1 bacterial isolate with the sample code TKa/Central Meat was obtained. From 1 bacterial isolate, identification of bacteria was carried out to determine the type of bacteria. The bacteria found in smoked tuna is the *Salmonella* sp.

B. The number of bacteria in fresh fishes

The highest number of colonies was found in the yellowfin tuna for XLD media with TMS/Head sample code, namely 3.4x104 CFU/gr. The inner surface of the fish, particularly the gills or head, is a common entry point for bacteria. The gills, being soft and moist, provide an ideal environment for bacterial growth (Table 1 & Fig. 1).

Table 1. The number of bacteria in the fresh and smoked yellowfin tuna found in different organs

CODE	XLD			Colony result/g	10^4	HE			Colony result/g	10^4
	~~10^4~~	~~10^4~~	~~10^4~~			10^4	10^4	10^4		
TMs/Head	250	210	164	34,000,00	3,4	250	205	76	25545,5	2,6
TMs/Belly	250	160	123	25,727,27	2,6	250	200	120	29090,9	2,9
TMs/Mid meat	250	200	98	27,090,91	2,7	250	120	87	18818,2	1,9
TMs/Back	250	180	112	26,545,45	2,7	250	115	60	15909,1	1,6
TMs/Tail	250	175	56	21,000,00	2,1	250	225	102	29727,3	3.0
TMa/Head	0	0	0	0	0	0	0	0	0	0
TMa/Belly	0	0	0	0	0	0	0	0	0	0
TMa/Mid meat	0	0	0	0	0	11	0	0	11	11
TMa/Back	0	0	0	0	0	0	0	0	0	0
TMa/Tail	0	0	0	0	0	0	0	0	0	0

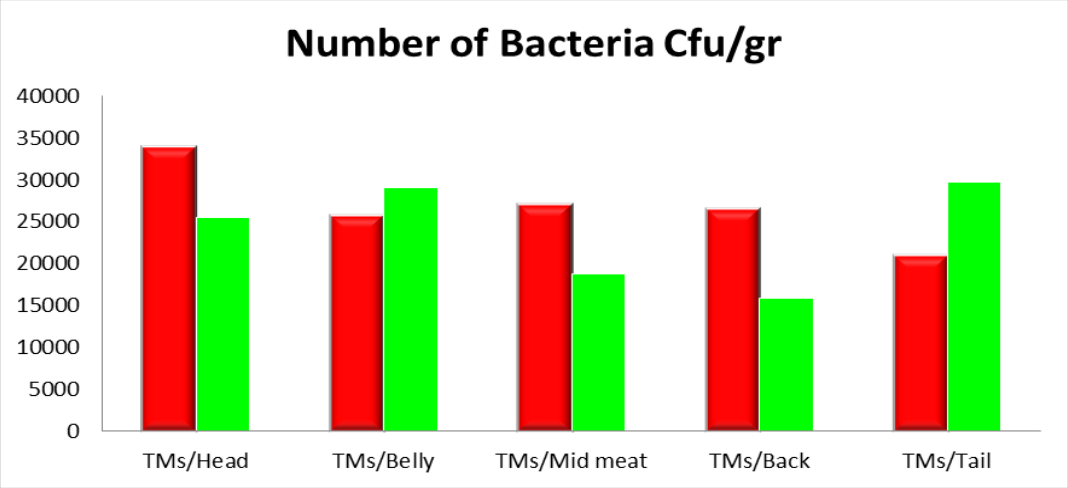


Fig. 1. The comparison number of bacteria histogram in the yellowfin tuna organs between XLD (red) and HE (green) agar media

The bacterial count is still below the total plate count (TPC) limit specified by SNI 01-2332.3-2006, which is 5x10^5 CFU/g. Therefore, the fresh yellowfin tuna produced by CV. X meets the standards for safe consumption.

In skipjack tuna, the highest number of colonies was observed on XLD media with the sample code CKS/Backsp., recording 3.1x10^4 CFU/g. According to **Jay (2000)**, fish damage can be attributed to both intrinsic factors, such as the condition of the fish when it is caught, and extrinsic factors, such as environmental conditions. Despite this, the bacterial count remains below the total plate count (TPC) limit of 5x10^5 CFU/g specified by SNI 01-2332.3-2006. Therefore, the fresh yellowfin tuna produced by CV. X meets the safety standards for consumption (Table 2 & Fig. 2).

Table 2. The number of bacteria in the fresh and smoked skipjack found in different organs

CODE	XLD			Colony results /g	10^4	HE			Colony results /g	10^4
	10^4	10^4	10^4			10^4	10^4	10^4		
CKs/Head	250	219	109	29818,2	3.0	250	244	107	31909.09	3.2
CKs/Stomach	250	170	76	22363.64	2.2	250	152	120	24727.27	2.5
CKs/Mid meat	250	218	81	27181.82	2,7	250	109	93	18363.64	1.8
CKs/Back	250	193	150	31181.82	3.1	250	130	112	22000.00	2.2
CKs/Tail	250	166	48	19454.55	1.9	250	186	148	30363.64	3.0
CKa/Head	0	0	0	0	0	0	0	0	0	0
CKa/Stomach	0	0	0	0	0	0	0	0	0	0
CKa/Mid meat	0	0	0	0	0	0	0	0	0	0
CKa/Back	0	0	0	0	0	0	0	0	0	0
CKa/Tail	0	0	0	0	0	0	0	0	0	0

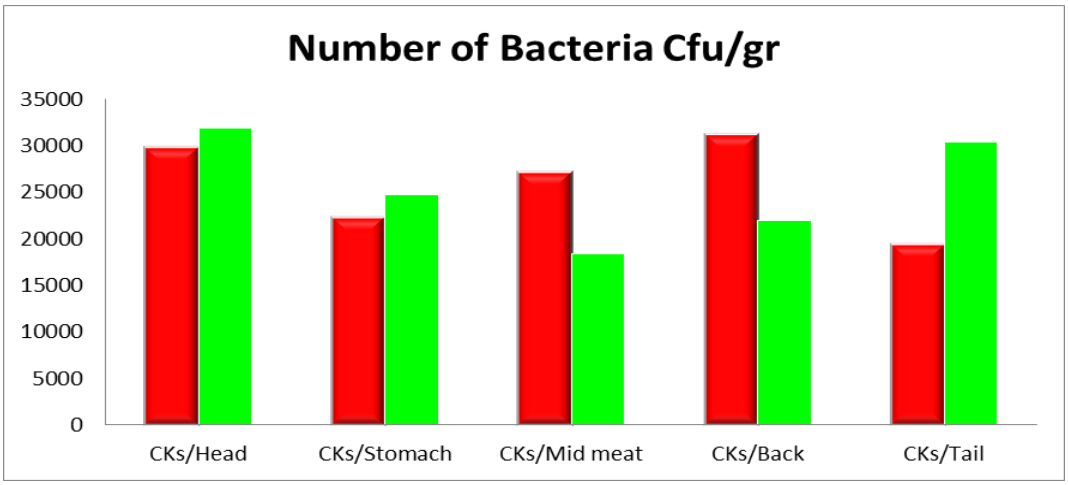


Fig. 2. The comparison number of bacteria histogram in the skipjack organs between XLD (red) and HE (green) agar media

In little tunny, the highest number of colonies was found on HE media with the sample code TKS/Central Meat, recording 4.0x10^4 CFU/g. According to the fresh fish provisions of SNI 01-2729.1-2006, the maximum allowable total plate count (TPC) is 5.0x10^5 CFU/g. Since the measured value is below this limit, the fresh fish is classified as safe and suitable for consumption (Table 3 & Fig. 3). According to **Pandit *et al.* (2008)**, the head of the tuna is a central point for blood accumulation. Additionally, the tuna's head contains toxins, and tuna is classified as a perishable fish.

Table 3. The number of bacteria in the fresh and smoked little tunny found in different organs

CODE	XLD			Colony result/ g	HE			Colony result/ g
	10^1	10^2	10^3		10^1	10^2	10^3	
TKs/Belly	250	244	252	44727,3	250	257	171	34636,4
TKs/Back	250	211	220	39181,8	250	235	118	32090,9
TKs/Tail	250	209	109	28909,9	250	118	86	18545,5
TKa/Belly	250	100	46	13272,7	250	106	43	13545,5
TKa/Back	0	0	0	0	0	0	0	0
TKa/Tail	0	0	0	0	0	0	0	0

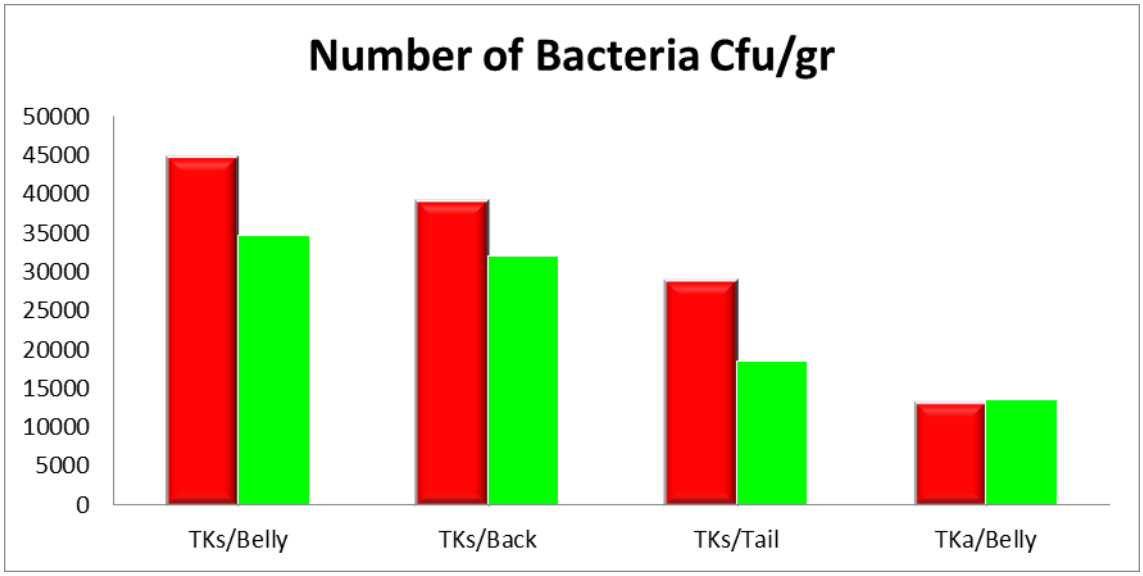


Fig. 3. The comparison number of bacteria histogram in the little tunny's organs between XLD (red) and HE (green) agar media

C. The number of bacteria in smoked fishes

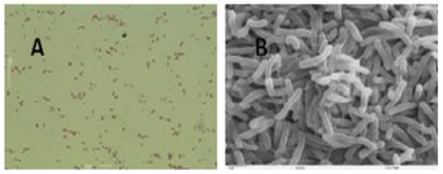
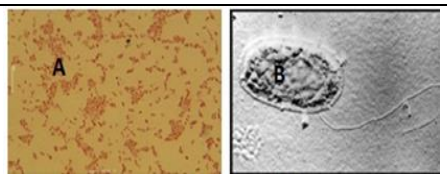
From Tables (1, 3), two bacterial isolates were found on HE agar media: One with a bacterial count of 11 CFU/g for the TMa/Central Meat samples and another with 3 CFU/g for the TKa/Central Meat samples, both for the yellowfin and smoked tuna (Fig. 4). According to the smoked fish food quality and safety standards (SNI 2725.1: 2009), the maximum allowable total plate count (TPC) for smoked fish is 5x10^5 CFU/g. Since the TPC values are below this limit, the smoked fish products are considered safe for consumption. Additionally, no bacterial isolates were found on XLD or HE media for the smoked skipjack.

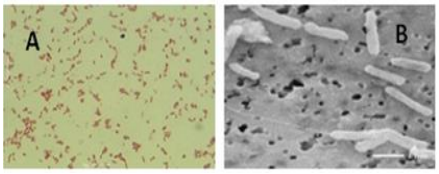
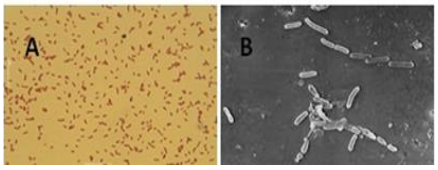
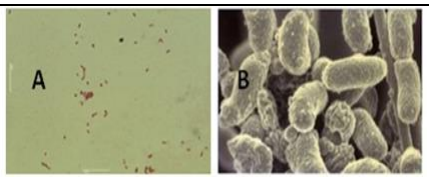
Fig. 4. The number of bacteria from middle flesh of the smoked yellowfin tuna (yellow) and the little tunny (purple) in HE agar media

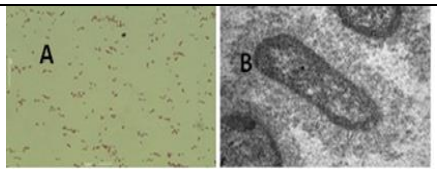
D. Types of bacteria in fresh and smoked fishes

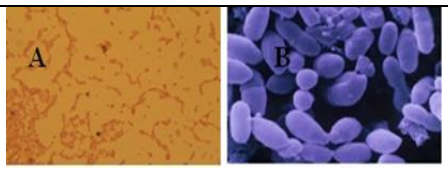
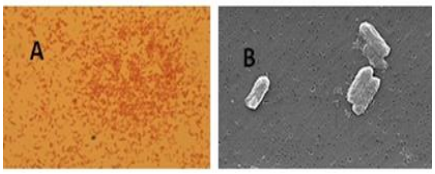
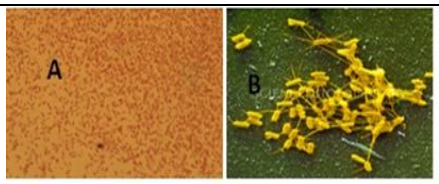
Microscopic examination revealed the identification and classification of fourteen bacterial species. Table (4) lists the different types of bacteria found in fresh and smoked fish. The classification follows the criteria set by **Buchanan and Gibbons (1974)** and **Jay (2000)**.

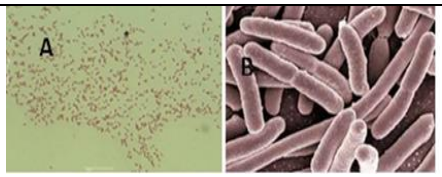
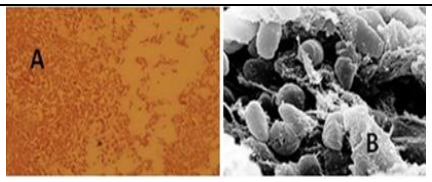
Table 4. Different types of bacteria found in fresh and smoked fish

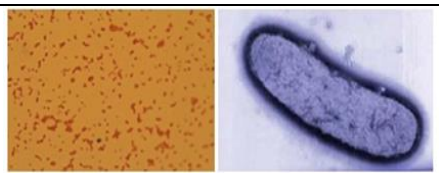
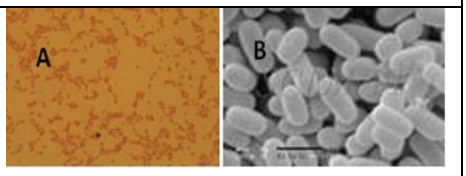
No.	Bacterium	Classification	Characterization	Performance
1.	*Vibrio* spp.	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Vibrionales Family: Vibrionaceae Genus: Vibrio	From the results of biochemical tests, Vibrio sp. bacteria can produce motile, short rods, oxidase positive, ferment glucose and Gram-negative.	
2.	*Plesiomonas* sp.	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Enterobacteriaceae Genus: Plesiomonas	Plesiomonas bacteria biochemistry test results produce oxidase and catalase (+), motile, Gram-negative, glucose (+). Bacteria belonging to the genus *Aeromonas* are short rods, Gram- negative, mobile, or motile, and positive in catalase and oxidase tests, fermenting glucose in fermentative oxidase media	

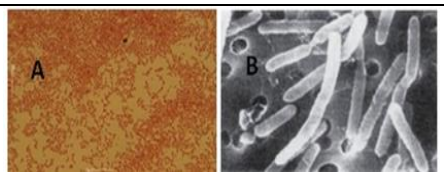
			(OF)	
3.	*Enterobacteria* sp.	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Enterobacteriaceae Genus: Enterobacter	Enterobacter bacteria biochemical test results produce glucose (+), rod-shaped, catalase (+), and Gram-negative	
4.	*Pseudomonas* sp.	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Pseudomonadales Family: Pseudomonadaceae Genus: Pseudomonas	The results of the biochemical test for Pseudomonas sp.. bacteria were Gram-negative bacteria, straight rods in pairs, and positive motile. The results of the gram staining showed that *Pseudomonas* sp. was a Gram-negative bacterium, in the form of straight rods, cells arranged singly or in pairs. The specific characteristics of the genus *Pseudomonas* sp. are straight rods, motile and do not carry out fermentation	
5.	*Actinobacillus* sp.	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria	biochemical test results for *Actinobacillus* sp. bacteria produced glucose, indole	

		Order: Pasteurellales Family: Pasteurellaceae Genus: Actinobacillus	(+), positive oxidase/catalase, motile, fermentable, short rod shape. Actinobacillus is a genus of Gram-negative, motile and non-spore forming, oval rod-shaped bacteria. Actinobacillus facultative aerobic or anaerobic bacteria, capable of fermenting carbohydrates (without gas production), and reducing nitrates	
6.	*Pasteurella* sp.	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Pasteurellales Family: Pasteurellaceae Genus: Pasteurella	Pasteurella bacteria biochemical test results produce glucose (+), oxidase/catalase (+), can ferment glucose, and gram negative. They are all oxidase positive and catalase positive but the results are biased towards other biochemical reactions	

7.	*Proteus mirabilis*	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Enterobacteriaceae Genus: *Proteus*	biochemical test results for Proteus mirabilis bacteria produced positive indole, catalase/oxidase (+), citrate (+), negative gram and round rod/stick shape. most cells are rod-shaped, 1-3 µm long and 0.4-0.6 µm wide, although short and stout, regular *cocci*	
8.	*Citrobacter freundii*	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Enterobacteriaceae Genus: Citrobacter	biochemical test results for the bacteria Citrobacter freundii Gram negative, motile, positive oxidase/catalase, glucose fermenting, and negative indole. Citrobacter freundii is an aerobic Gram-negative bacillus. These bacteria are rod-shaped but some of them are non-motile	
9.	*Proteus* sp..	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order:	biochemical test results for Proteus sp. bacteria are motile (+), produce glucose, positive urea,	

		Enterobacterales Family: Enterobacteriaceae Genus: *Proteus*	fermentation, positive catalase/oxidase, and Gram negative. *Proteus* can hydrolyze urea to CO3 and NH3 and release ammonia	
10.	*Escherichia coli*	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Enterobacteriaceae Genus: *Escherichia*	biochemical test results of bacteria E. Coli urea (-), Indole (+), produce glucose, motile, oxidase/catalase (+), and Gram negative (short rods). The morphology of this bacterium is a short rod-shaped germ (coccobasil), Gram negative, size 0.4 – 0.7 µm x 1-3 µm	
11.	*Yersinia pestis*	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Yersiniaceae Genus: Yersinia	Yersinia pestis biochemical test results showed no reaction on the O/F test, negative indole, negative motile, oxidase/catalase -/+, did not ferment glucose, and were Gram negative. Yersinia pestis is a gram-negative bacterium that	

			can grow with or without oxygen (which is called non-anaerobic quality)	
12.	*Yersinia* sp..	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Yersiniaceae Genus: Yersinia	the biochemical test results for the motile *Yersinia* sp. bacteria were positive, positive for oxidase/catalase, produced glucose, fermented glucose, indole negative, and Gram negative. Stem cell shape. Including bacteria that can move. Catalase, positive oxidase and negative H2S production	
13.	*Alcaligenes*	Domain: Bacteria Phylum: Pseudomonadota Class: Betaproteobacteria Order: Burkholderiales Family: Alcaligenaceae Genus: Alcaligenes	biochemical test results of positive motile Alcaligenesis bacteria, positive oxidase/catalase, produce glucose, not ferment, and negative diagram. Alcaligenes is a psychrophilic bacterium that can survive in a low temperature range, so it can survive in contaminating food products	

			such as fish	
14.	*Salmonella* sp..	Domain: Bacteria Phylum: Pseudomonadota Class: Gammaproteobacteria Order: Enterobacterales Family: Enterobacteriaceae Genus: Salmonella	Biochemical test results for *Salmonella* sp. bacteria produced glucose at LIA (+), negative indole, positive motile, carbonhydrate fermentation, positive catalase, negative oxidase, rod-shaped, and Gram negative. Salmonella is a facultative Gram-negative rod-shaped bacterium	

This research aims to explore fish preservation methods to maintain the quality of fish products. Smoked fish is presented as an effective alternative for preserving high-quality items. Local communities prefer smoked fish due to the enhanced flavor and texture it acquires during smoking, and they deserve to consume high-quality products. Using high-quality raw materials is crucial for ensuring a longer shelf life for the products. Smoked fish is popular in African villages **(Yusuf *et al.*, 2012; Agus *et al.*, 2013)** and is primarily produced and consumed locally. Similarly, in Indonesia, there is a challenge with the availability of suitable wood fuel for smoking. People often use dead mangrove wood, which is considered more efficient and effective. The discussion in the study emphasized the importance of effective fish preservation techniques, particularly smoking, which is both cost-effective and commonly used for seafood preservation in Ternate. It also stressed the need for good manufacturing Ppractices (GMP) and standard sanitation operating procedures (SSOP) to ensure the quality and safety of fish products. The study highlighted the presence of various pathogenic bacteria in both fresh and smoked tuna in Ternate, but noted that the bacterial counts in the fish samples were within safe limits for consumption.

CONCLUSION

The fish smoking method is a cost-effective way to preserve fish and is widely used in Ternate. Various factors influence microbial deterioration in smoked fish. To

develop effective handling, pre-treatment, and preservation strategies for smoked fish, it is crucial to thoroughly study these factors and understand the mechanisms of spoilage.

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