Identification of Pathogenic Bacterial Contamination of *Salmonella* sp. in Fishery Products Using SNI ISO 6579:2015

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Abstract
Indonesia is the largest archipelagic and maritime country in the tropical region with quite large potential for exploiting marine resources, especially in the fisheries sector. Quite a large amount of fishery products are marketed through modern markets and traditional markets in the form of fresh or frozen fish. Poor sanitation and hygiene in the processing and marketing of fishery products often lead to the spread of harmful bacteria, including *Salmonella* sp. This research aimed to detect *Salmonella* sp. contamination on five samples of fishery products, namely milkfish, tuna, catfish, shrimp and frog legs using the SNI ISO 6579:2015 method. The results showed that initial enrichment and selective media isolation detected *Salmonella* sp. in some samples, but biochemical tests ultimately confirmed that all samples were negative. This outcome complies with SNI ISO 6579:2015, which requires that food, including fish, must not contain *Salmonella* sp
Keywords: *Salmonella* sp., Fishery products, Food safety, SNI ISO 6579:2015

INTRODUCTION

Indonesia is the largest archipelagic and maritime country located in the tropical region, with a total of 17,001 islands as of 2022 (BPS, 2022). This condition provides Indonesia with significant potential for utilizing marine resources, particularly in the fisheries sector (Laheng et al., 2022). According to the Decree of the Minister of Marine Affairs and Fisheries of the Republic of Indonesia No.KEP.45/MEN/2011 concerning the Estimation of Fish Resource Potential in the Fisheries Management Area of the Republic of Indonesia, the potential of fishery resource products is divided into several groups, namely small pelagic fish, large pelagic fish, demersal fish, consumable reef fish, lobster, and squid.

The substantial fishery products are marketed through both modern and traditional markets in the form of fresh or frozen fish. In addition to being marketed in fresh or frozen form, fishery products are also processed into various fish-based products by processing companies. These products not only meet domestic demand but are also exported to various countries to increase foreign exchange earnings (Effendi & Wijaya, 2019).

The fish processing must consider sanitation and hygiene, which are the primary requirements to prevent contamination in processed fishery products. Sanitation and hygiene are key factors in producing fish products that are safe for consumption. Damage to fishery products is often caused by microbiological factors such as pathogenic bacteria or fungi that can produce toxic substances, resulting in spoilage of fishery products. This damage can reduce the quality and safety of food, especially in traditional processed fish products. Severe damage can even cause foodborne disease if consumed by humans (Heruwati, 2002).

Salmonella sp. is a pathogenic bacterium that causes foodborne diseases and frequently contaminates fish. Good sanitation measures and hygienic practices are crucial to prevent contamination by pathogenic bacteria such as *Salmonella* sp. in fish and other fishery products (Apelabi et al., 2015). In humans, infection caused by *Salmonella* sp. is called salmonellosis, which can damage the epithelial tissue of the human intestine. In severe cases, salmonellosis can cause severe dehydration or even blood infection (sepsis), which can be life-threatening. Previous research conducted by Wibisono (2017), stated that 20 out of 42 samples of milkfish sold in the Sidoarjo fish market tested positive for *Salmonella* sp. contamination. Another study also mentioned that shrimp, as a raw material for fish cakes, was detected to contain *Salmonella* sp. contaminants (Sartika et al., 2016). Research conducted by Amalia (2022), also showed that 9 out of 40 fish

samples, consisting of 2 tilapia and 7 milkfish from the Yogyakarta region, tested positive for *Salmonella* sp. contamination.

Given the high risk of contamination by pathogenic bacteria such as *Salmonella* sp. in fishery products, the Indonesian government has implemented a strict monitoring system for the export and import of fishery products. Every fresh or processed fish product to be exported or imported must undergo a process of supervision and testing by the Center for Quality Control and Supervision of Marine and Fishery Products (BPPMHKP) Surabaya I. This institution plays a crucial role in ensuring that fishery products circulating in the international market meet the established food safety standards. One of the tests conducted by BPPMHKP Surabaya I is the detection of *Salmonella* sp. contamination using the SNI ISO 6579:2015 guidelines. This testing covers various types of fishery products such as demersal fish, pelagic fish, farm fish, shrimp, and frog legs.

METHOD

This study used a qualitative descriptive approach to analyze *Salmonella* sp. contamination in fish samples. A total of 5 samples representing various fishery groups were taken from traditional markets and fish processing industries, as represented in Table 1.

Table 1. Fish samples tested

Sample type	Product type	Sample code
Milkfish	Fresh	262
Mackarel tuna	Fresh	286
Catfish	Fresh	263
Vannamei shrimp	Fresh	265
Frog legs	Fresh	257

The sample numbering in Table 1 corresponds to the sample entry data at BPPMHKP Surabaya I.

Time and Place of Research

The research was carried out from March to April 2024 at the Microbiology Laboratory of the Center for Quality Control and Supervision of Marine and Fishery Products, Surabaya I, located in Gedangan District, Sidoarjo Regency, East Java.

Tools and Materials

The tools used in this study were incubator, stomacher, bag filter, ose needles, pH meter, laminar air flow, analytical balance, hot plate, autoclave, erlenmeyer flask, measuring cup, petri dish, test tube, bunsen burner, dropper, micropipette, tip, vortex, refrigerator, tray, dissecting set, laboratory coat, gloves, and mask.

The materials used in this study were Buffered Peptone Water (BPW), Rappaport-Vassiliadis Medium With Soya Broth (RVS Broth), Muller-Kauffmann Tetrathionate novobiocin Broth (MKTTn Broth), Xylose Lysine Deoxycholate Agar (XLD Agar), Tryptone Soya Agar (TSA), Triple Sugar Iron Agar (TSIA), Lysine Decarboxylase Broth (LDB), Urea Agar, β-galactosidase, MR-VP Broth, Semi Solid Nutrient Agar, Aquadest, Kovac's Reagent, methyl red indicator, gram staining media, VP reagent, samples of milkfish, mackerel tuna, catfish, shrimp, and frog legs.

Procedure

Preparation Stage

1. **Sample Preparation**

   Samples were prepared using aseptic technique by cutting into small pieces until the sample weight reached 10 grams. The samples were then placed in sterile containers or plastic bags.

2. **Sample Homogenization**

A total of 10 grams of prepared sample was put into a bag filter, then 90 mL of BPW media was added. The ratio between sample and BPW media was 1:9. Homogenization was carried out using a stomacher for 2 minutes.

Testing Stage

1. **Pre-Enrichment**

   Samples that had been homogenized with BPW media were then incubated in an incubator at 37°C ± 1°C for 24 hours ± 3 hours.

2. **Enrichment**

   A total of 0.1 mL of culture from BPW media was inoculated into 10 mL of RVS media and incubated at 41,5°C ± 1°C for 24 hours. A total of 1 mL of the same culture was inoculated into 10 mL of MKTTn media and incubated at 37°C ± 1°C for 24 hours.

3. **Isolation on Selective Media**

   Cultures from MKTTn and RVS media suspected to contain *Salmonella* were inoculated on XLD selective media and incubated at 37°C ± 1°C for 24 hours ± 3 hours. *Salmonella* colonies on XLD are characterized by pink with or without shiny spots, or almost entirely black. If *Salmonella* is indicated with these characteristics, the colonies are inoculated on TSA media and incubated at 37°C ± 1°C for 24 hours ± 3 hours.

4. **Biochemical Confirmation Test**

   TSIA Test

   Inoculation on TSIA slant agar by streaking and stabbing the bottom of the media, incubate at 37°C ± 1°C for 24 hours ± 3 hours. Positive results if the slant part of the media is alkaline (red), the bottom of the media is acidic (yellow), with gas and H2S formation (blackening).

   Urea Test

   The inoculated colony was streaked on the surface of the urea test media. It was incubated at 37°C ± 1°C for 24 hours ± 3 hours. A positive reaction was indicated by the breakdown of urea which released ammonia, then changed the color of phenol red to pink to dark red.

   LDB Test

   The bacterial colony was inoculated just below the surface of the LDB liquid media. It was incubated at 37°C ± 1°C for 24 hours ± 3 hours. Turbidity and purple color after incubation indicated a positive reaction. Yellow color indicated a negative reaction.

   VP Test

   One loop of colony was suspended in a sterile tube containing 3 mL of VP media. It was incubated at 37°C ± 1°C for 24 hours ± 3 hours. After incubation, two drops of creatine solution, three drops of 1-naphthol ethanolic solution and two drops of potassium hydroxide solution were added. The mixture was shaken after the addition of each reagent. The formation of pink to bright red color within 15 minutes indicated a positive reaction.

   Indole Test

   One loop of colony was suspended in a test tube containing 5 mL of tryptone/tryptophan media. It was incubated at 37°C ± 1°C for 24 hours ± 3 hours. After incubation, 1 mL of Kovacs reagent was added. The formation of a red ring indicated a positive reaction. A yellowish-brown ring indicated a negative reaction.

RESULT AND DISCUSSION

The identification of *Salmonella* sp. contamination in fishery products follows the guidelines of SNI ISO 6579:2015, which includes pre-enrichment, enrichment, isolation on selective media, and biochemical confirmation tests.

Pre-Enrichment and Enrichment Bacterial

In the pre-enrichment stage, the sample was introduced into BPW media and incubated at 37°C for 24 hours. BPW is a non-selective liquid medium rich in nutrients that allows for the general growth of bacteria, including *Salmonella* sp. The 24-hour incubation period is necessary to provide sufficient time for *Salmonella* to adapt to the new environment and enter the logarithmic growth phase (Pratiwi, 2018). The main objective of the pre-enrichment stage is to increase the

number of *Salmonella* that may be present in low quantities in the sample, making them easier to detect in the subsequent enrichment stage.

The results of the pre-enrichment incubation are then continued to the enrichment stage by growing bacteria on selective media RVS and MKTTn. These media function to cultivate *Salmonella* sp. while suppressing the growth of other unwanted contaminant bacteria (Torrico et al., 2022).

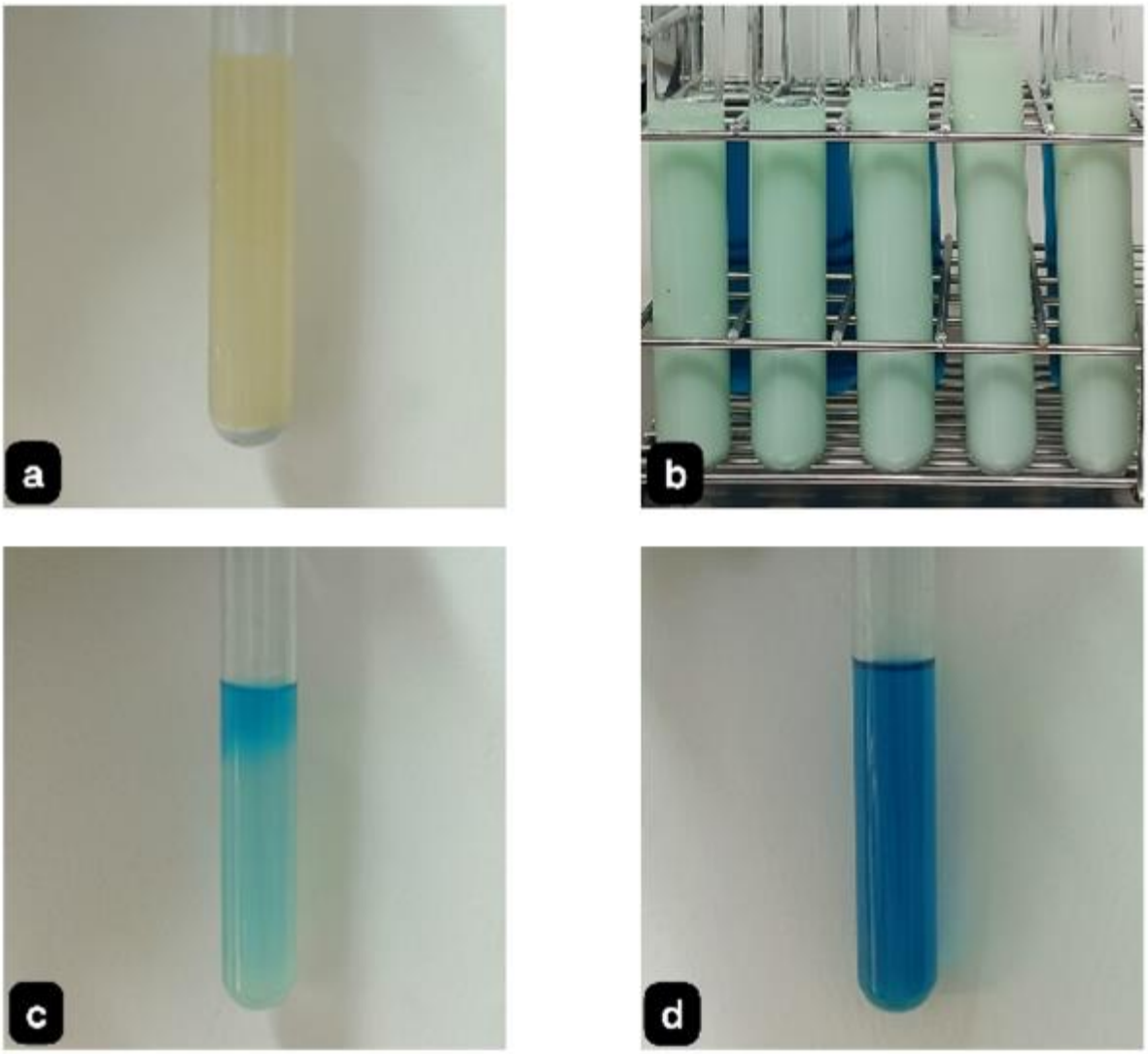

Figure 1. Positive result in RVS media (a), negative result in RVS media (b), positive result in MKTTn media (c), negative result in MKTTn media (d)

The results of the enrichment stage revealed that 4 out of 5 samples, identified by codes 262, 286, 263, and 265 (Table 2), tested positive in RVS media, as indicated by turbidity. They also tested positive in MKTTn media, characterized by turbidity and pellet formation at the bottom of the tube after incubation (Figure 1). In contrast, the sample with code 257 tested negative at the enrichment stage, with the media remaining clear. These changes in color and turbidity result from microbial growth in the media. The findings from this stage indicate the presence of microbial content in the samples; however, further testing is required to confirm the presence of *Salmonella* sp. (Sutaryana, 2018).

Bacterial Isolation on Selective Media

Samples suspected of being positive for *Salmonella* sp. during the enrichment stage were further isolated using XLD medium. XLD medium contains sodium deoxycholate, a selective inhibitor that effectively suppresses the growth of coliform bacteria without compromising the viability of *Salmonella* sp. Sodium deoxycholate, a type of bile salt, exhibits bacteriostatic properties specifically

targeting gram-negative bacteria susceptible to bile salts, such as coliforms, while allowing *Salmonella* sp. to thrive. This selective capability makes XLD medium highly effective for isolating and identifying *Salmonella* in samples potentially containing a variety of bacterial species (Hasanah et al., 2023).

Figure 2. Bacterial isolation on XLD media

During the isolation stage using XLD medium (Figure 2), suspected *Salmonella* colonies are identified by their pink appearance with black centers. The carbohydrate source in XLD medium, which can be fermented by most *Salmonella* strains, results in the formation of red colonies with black deposits (Wibisono, 2017). Samples suspected to contain *Salmonella* on XLD medium are labeled with codes 262, 263, and 265 (Table 2).

Colonies presumed to be *Salmonella* from the XLD medium were subsequently transferred to TSA medium for colony multiplication before confirmation testing. TSA is a general-purpose nutrient-rich medium that supports the growth of most bacteria, including *Salmonella* sp. It is commonly used to regrow *Salmonella* isolates from selective media prior to biochemical confirmation tests (Brashears et al., 2001).

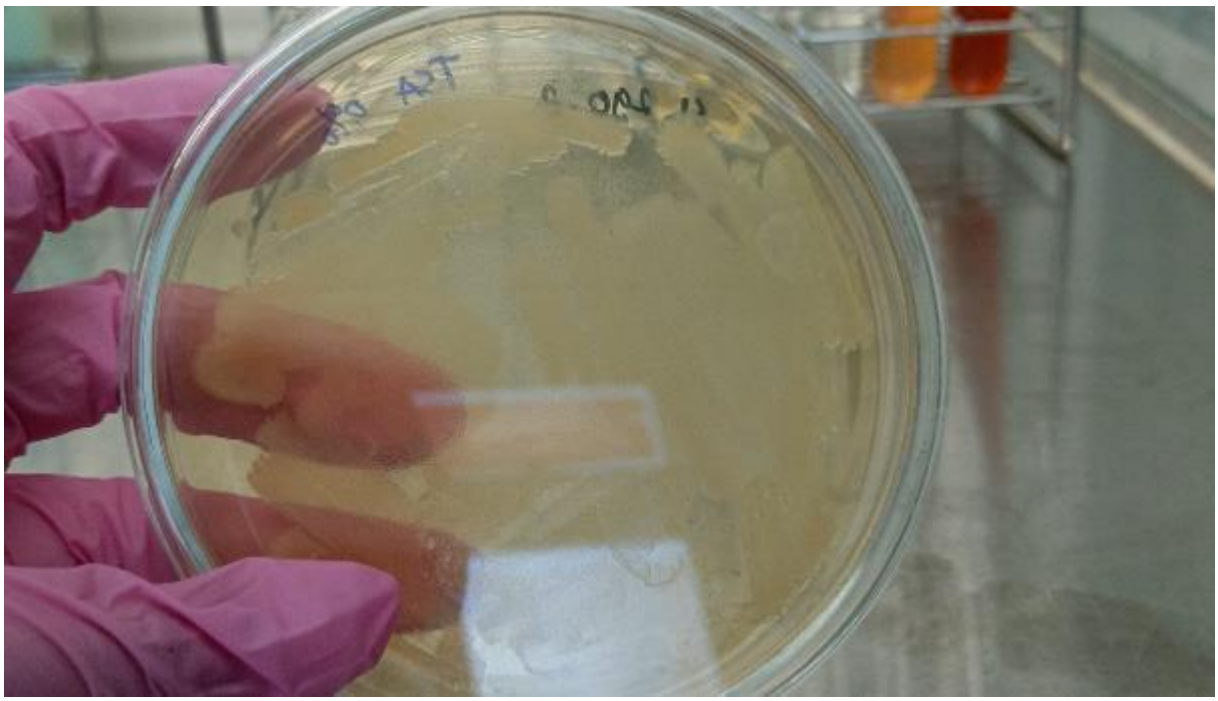

Figure 3. Bacterial Multiplication on TSA Media

Samples suspected to contain *Salmonella* bacteria were inoculated on the surface of TSA media using the streak plate technique (Figure 3). Samples were then incubated at 37°C for 24 hours. During incubation, if the sample contains *Salmonella*, bacterial colonies will grow on the surface of the TSA media with characteristics of round, smooth, convex, and white colonies. Samples suspected of *Salmonella* on TSA media with these characteristics are indicated by sample codes 262, 263, and 265 (Table 2).

Biochemical Confirmation Tests

TSIA (Triple Sugar Iron Agar) Test

The TSIA test is used to assess a bacterium's ability to produce gas through sugar fermentation. If gas bubbles are present in the medium, it indicates that the bacteria are capable of gas production during fermentation. *Salmonella* bacteria typically produce little to no gas. The TSIA test also detects the formation of hydrogen sulfide (H2S), which is indicated by a black coloration in the medium. *Salmonella* bacteria generally produce H2S, resulting in a black color in the TSIA medium (Wibisono, 2017).

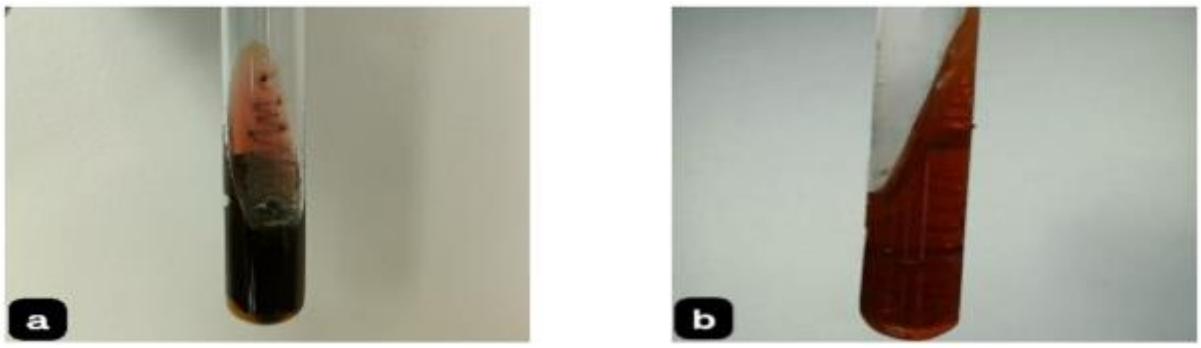

Figure 4. Positive result indicating *Salmonella* sp. in TSIA test (a), negative result not indicating *Salmonella* sp. in TSIA test (b)

The TSIA test results show that the sample with code 263 is positive for suspected *Salmonella* (K/A), as indicated by a reddish-black color change in the medium, suggesting glucose fermentation (Figure 4a). Samples with codes 262 and 265 are negative for *Salmonella*, as no color change occurred in the medium for these samples (Figure 4b). The TSIA test is designed to observe the fermentation of three sugars: lactose, sucrose, and glucose. The K/A (Alkali/Acid) reaction in

TSIA medium indicates that *Salmonella* bacteria can ferment glucose (yellow butt) but cannot ferment lactose or sucrose (red slant). This reaction is characteristic of *Salmonella* sp. An A/A (Acid/Acid) reaction, on the other hand, would indicate that the bacteria ferment both glucose and lactose (Nissa et al., 2023).

Urea Test

The urea test evaluates a bacterium's ability to produce the enzyme urease. In the identification of *Salmonella* spp., a negative urea test result serves as a key indicator. The absence of a color change to pink or dark red in the medium after 24 hours of incubation indicates a negative result, aligning with the characteristics of *Salmonella*. Conversely, a color change to pink or dark red signifies a positive result, which is inconsistent with *Salmonella* characteristics.

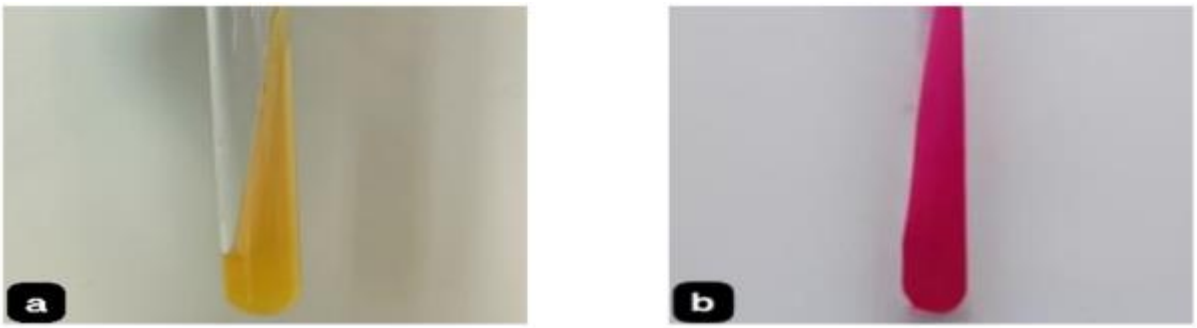

Figure 5. Negative result indicating *Salmonella* sp. in urea test (a), positive result not indicating *Salmonella* sp. in urea test (b)

Salmonella sp. lacks the ability to produce the urease enzyme, so the urea test for *Salmonella* will yield a negative result, shown by no color change in the medium (Figure 5a). The results of testing samples 262, 263, and 265, however, show a color change from yellow to pink (Figure 5b). This color change occurs when the urease enzyme catalyzes the hydrolysis of urea, producing ammonia and carbonate, which are alkaline. The resulting alkaline condition causes the medium to turn pink (Sutaryana, 2018). Based on these results, none of the samples indicate the presence of *Salmonella* bacteria.

LDB (Lysine Decarboxylase Broth) Test

The LDB (Lysine Decarboxylase Broth) test is used to detect the production of decarboxylase enzymes by bacteria. A positive result is indicated by a color change in the medium from yellow to purple after the incubation period. If the medium changes color, it suggests *Salmonella* sp. contamination in the sample. If no color change occurs, the result is negative, indicating the absence of *Salmonella* sp. bacteria. This color change happens because *Salmonella* has the ability to produce decarboxylase enzymes, which catalyze the decarboxylation of lysine into amine compounds (Torrico et al., 2022).

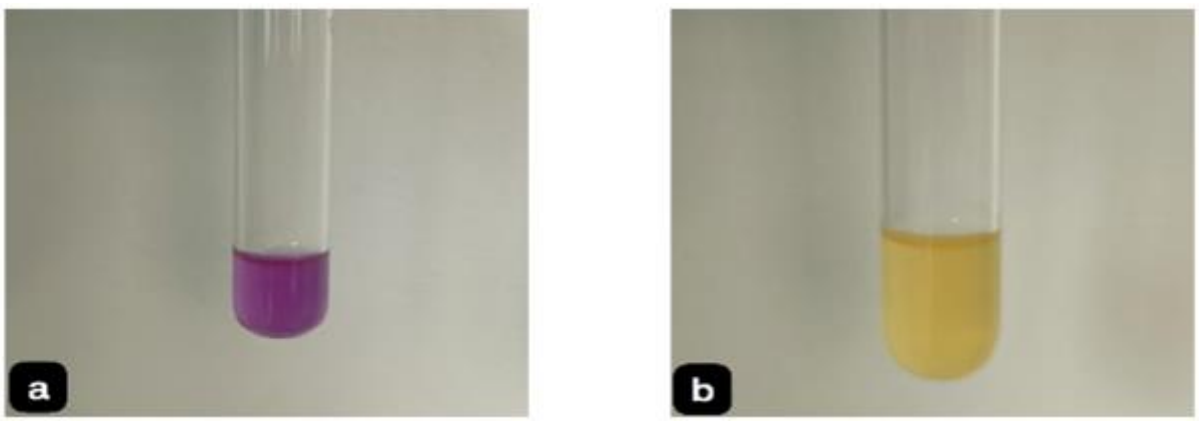

Figure 6. Positive result indicating *Salmonella* sp. in LDB test (a), negative result not indicating *Salmonella* sp. in LDB test (b)

All three test samples show positive LDB results, indicated by a change in media color from yellow to purple (Figure 6a), suggesting that the bacteria can produce decarboxylase enzymes and are indicative of *Salmonella* sp. (Hasanah et al., 2023).

VP (Voges-Proskauer) Test

The VP test is performed by adding VP reagent to the test tube and then observing the color change. If bacteria produce acetone, a dark red complex will form after the addition of VP reagent. *Salmonella* bacteria generally cannot form a dark red complex after the addition of VP reagent (negative result (-)). A negative result in the VP test indicates that the sample is indicative of *Salmonella* sp. bacteria. This is because *Salmonella* does not have a metabolic pathway that produces acetone as the end product of glucose fermentation (Susi & Muhammad, 2017).

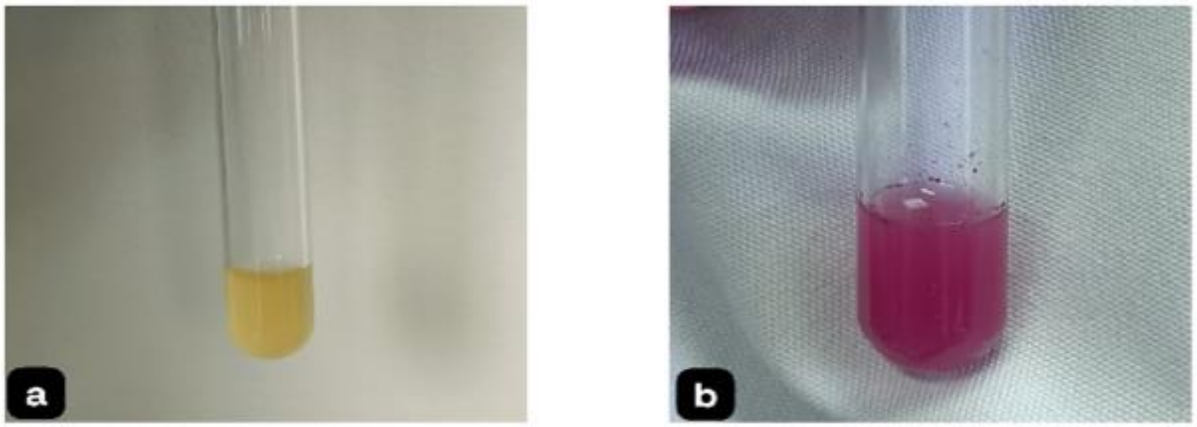

Figure 7. Negative result indicating *Salmonella* sp. in VP test (a), positive result not indicating *Salmonella* sp. in VP test (b)

All test samples—262, 263, and 265—yield negative results in the VP test, as no color change is observed following the addition of the reagent, indicating the presence of *Salmonella* (Figure 7a).

Indol Test

The indole test is designed to assess a bacterium's ability to produce metabolic byproducts such as ammonia, pyruvic acid, and indole. A positive result is characterized by the formation of a red layer on the surface of the medium (Susi & Muhammad, 2017; Hasanah et al., 2023). A key characteristic of *Salmonella* spp. is their inability to break down the amino acid tryptophan into indole, although they can ferment glucose to produce acid. Consequently, a negative indole test result indicates the presence of *Salmonella* bacteria in the sample (Hasanah et al., 2023).

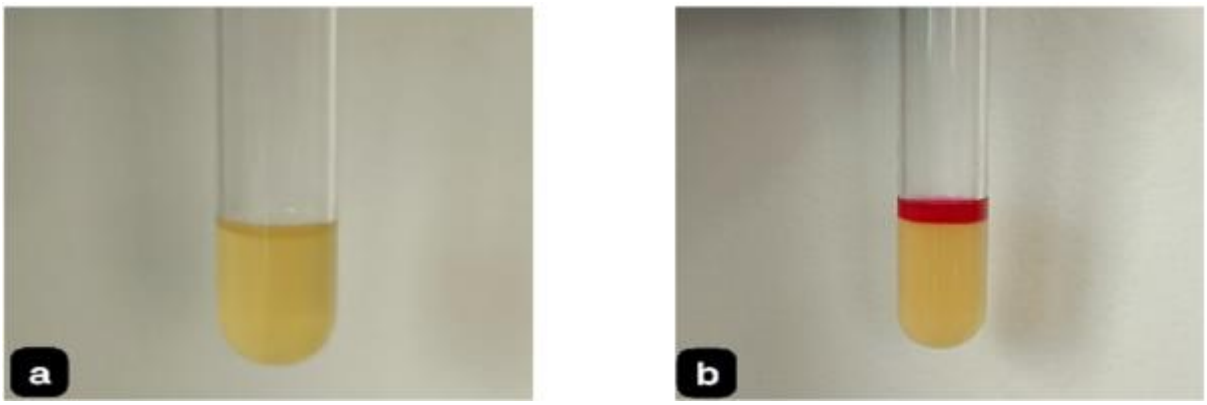

Figure 8. Negative result indicating *Salmonella* sp. in indole test (a), positive result not indicating *Salmonella* sp. in indole test (b)

The indole test result for the control is negative, as no red layer forms on the surface of the medium after the addition of Kovacs reagent. The test results for all samples are also negative, with no red layer appearing on the surface (Figure 8a).

Table 2. *Salmonella* sp. test results for fishery samples

Sample type	Test parameters									Final result
	Enrichment		Selective media isolation		Biochemical confirmation test					
	RVS	MKTTn	XLD	TSA	TSIA	Urea	LDB	VP	Indol	
Milkfish (262)	Turbid	Turbid	Red colony with black dots	White colony	A/A, gas, H_2S	+	+	-	-	(-) Negative
Mackarel tuna (286)	Turbid	Turbid	Red colony with black dots	#	#	#	#	#	#	(-) Negative
Catfish (263)	Turbid	Turbid	Red colony with black dots	White colony	K/A, gas, H_2S	+	+	-	-	(-) Negative
Vannamei shrimp (265)	Turbid	Turbid	Red colony with black dots	White colony	A/A, gas, H_2S	+	+	-	-	(-) Negative
Frog legs (257)	jernih	jernih	#	#	#	#	#	#	#	(-) Negative

Note:
Positive results for *Salmonella* sp. If: RVS (turbid), MKTTn (turbid), XLD (red colony with black dots), TSA (white colony), TSIA (K/A, gas, H2S), urea (-), LDB (+), VP (-), and indol (-). The # symbol means negative results and not continued to the next stage.

As shown in Table 2, all test samples were negative for *Salmonella* sp., as confirmed through biochemical testing. However, during the initial testing stages—pre-enrichment, enrichment, and isolation on selective media—false positive results were observed. This indicates that certain characteristics suggested the presence of *Salmonella* during these tests, but biochemical confirmation revealed no actual *Salmonella* contamination in any of the samples.

The false positives may have been caused by cross-contamination during the testing process, where non-*Salmonella* bacteria also grew on the selective media. This aligns with findings from Rahayuningtiyas et al. (2022), who reported challenges in identifying *Salmonella* due to competition from other microorganisms, particularly *Proteus* sp. On selective media such as XLD and BSA, the colony morphology of *Proteus* sp. closely resembles that of *Salmonella* sp., potentially leading to misidentification and false positives. Additionally, bacteria from the genera *Citrobacter* and *Enterobacter* share similar characteristics with *Salmonella*, enabling them to grow on selective media for *Salmonella* and contribute to false positive results during the initial testing stages (Putri, 2015).

CONCLUSION

All tested samples, including milkfish, mackerel tuna, catfish, vannamei shrimp, and frog legs, were free of *Salmonella* sp. contamination. This complies with the standard limits outlined in SNI ISO 6579:2015, which require that food, particularly fish, must be free of *Salmonella*.

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