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Examination of *Escherichia coli* Bacteria in Blood Cockle Satay (*Anadara granosa*) Sold at Surabaya Traditional Market, Indonesia

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ABSTRACT

Cockle satay is one of the Surabaya local food made from the blood cockle (*Anadara granosa*). Blood cockle, commonly known as a filter feeder, is found in many Surabaya traditional markets. However, it potentially accumulates pollutant substances, both heavy metal or microbial so that improper handling and processing can cause pathogenic bacteria contamination. The present study aimed to investigate the contamination of *Escherichia coli* (*E. coli*) bacteria in blood cockle satay (*Anadara granosa*) sold at Surabaya traditional market. The current study used a descriptive observational research design with a quantitative approach. A total of 11 samples were employed using cluster sampling. The obtained data were compared with those of Bergey's manual of determinative bacteriology and Indonesian national standard. Based on the obtained results, five samples included *E. coli* with negative Methyl Red (MR) characteristics, negative Voges-Proskauer (VP) negative citric and positive indole. The Most Probable Number test for six samples indicated a value of <3.0 mpn/gr for one sample, 3.0 mpn/gr for two samples, and 3.6 mpn/gr for three samples. It can be concluded that the blood cockle satay samples sold at Surabaya traditional market (Indonesia) were contaminated with *E. coli* bacteria.

Keywords: Blood Cockle, *Escherichia coli*, Food product, Indonesia

INTRODUCTION

Surabaya is a city located in East Java which has recently turned into a blood cockle manufacturing site. Blood cockle producers in Surabaya have experienced an increase from 193.5 to 273.1 tons during 2013-2014 (Diskanlut-JatimProv, 2014, 2013). In Surabaya, the blood cockle is commonly consumed as a local food named cockle satay.

Blood cockle (*Anadara granosa*) is a kind of cockle that is popular in society and is the economic income source or society food in coastal areas (Susanti and Kristiani, 2016). The blood cockle in Surabaya is easy to find in traditional markets and supermarkets (Juniawati, 2005). The blood cockle flesh consists of a total protein 27.26% (bk), total fat of 2.54% (bk), and 48.01% carbohydrate. The blood cockle flesh mineral content consists of Ca 318.67 ppm, Cu 4.26 ppm, Fe 1720.46 ppm, and Zn 81.16 ppm (Ischak, 2015).

Blood cockle is a fishery product with a high-water content which makes it vulnerable to microbiological damage. The filter feeder life cycle of blood cockles has led to the accumulation of pollutant substances, including both heavy metal or microbial contaminants. As a result, the improper handling and processing of the blood cockle can cause pathogenic bacterial contamination (Retyoadhi et al., 2005). Pathogenic bacteria that are commonly found in seafood include *Salmonella* sp., *Staphylococcus* aureus, *Escherichia coli*, and *Vibrio* sp. (Putri et al., 2014).

Escherichia coli is a type of bacteria most likely to contaminate food (Faridz and Hafiluddin, 2007). The *E. coli* often contaminates food and is the indicator of feces contamination (Singh and Prakash, 2008). The maximum limit of *E. coli* in cockle products is <3 per gram of cockle flesh based on 3 or 5 dilution tubes of the most probable number (MPN) test (Indonesia, 2006).

The *E. coli* is a rod-shaped, facultatively anaerobic, and Gram-negative bacterium with flagella peritricate (Fardiaz, 1993). It normally exists in the human digestive tract (Brooks et al., 2007). The *E. coli* in the colon is pathogenic if it exceeds the normal amount. Certain strains can cause inflammation in the stomach and intestine membranes (gastroenteritis). In case it lives outside the intestine as in the urinary tract, it can cause inflammation of the mucous membranes (Pelczar et al., 2016). Based on the above-mentioned points, it is necessary to examine the *E. coli* contamination of cockle satay sold in the Surabaya traditional market since any *E. coli* contamination can lead to a disease outbreak.

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MATERIALS AND METHODS

Design

The employed method in the current study was observation. The samples were observed from various aspects related to the isolation and identification of *E. coli* bacteria in cockle satay from the Surabaya traditional market.

Sample of the study

The samples in the current study were obtained from a Surabaya traditional market. The investigated market was the one with the biggest income of blood cockle satay merchant. Samples taken from each market entailed 10% of the total daily sale of cockle satay. A total of 11 samples were included in the current study. Samples of blood cockle satay were then subjected to *coliform* estimator test stage, coliform strengthener test, and identification of *Escherichia coli* bacteria in the Microbiology Laboratory of the Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya in April 2017.

Parameter of the study

The parameter observed in this study was the characterization of *E. coli* and the Most Probable Number (MPN) values of *E. coli* in blood cockle satay sold in the Surabaya traditional market. The characteristic of *E. coli* was the presence of gas bubbles in the *Lactose Broth* media, the color of bacterial colonies on the *Eosin Methylene Blue* (EMB) agar media, the presence of indole and color in the MR-VP, and citrate tests. The MPN E. coli value was determined using the MPN 3 index table dilution tubes.

Data analysis

The obtained data were compared with the available data in Bergey's Manual of Determinative Bacteriology (Buddingh, 2017). The Most Probable Number (MPN) test results would be compared with SNI 01-2332.1-2006 regarding the determination of coliform and *E. coli* in fishery products.

RESULTS

Coliform estimator test result

Based on the obtained results of the coliform estimator test in Table 1, the cockle satay samples showed positive results in eight samples, namely A1, B2, B3, C1, C2, D2, E1, and E2 samples. Moreover, B2, B3, C1, D2, E1, E2 samples were positive in dilution tubes 10^{-1}. The A1 and C2 samples were positive in the 10^{-2} dilution tube. All negative results were on 10^{-3} dilution tubes. A2, B1, and D1 samples showed negative results in all dilution tubes.

Coliform strengthener test result on EMB agar media

The coliform strengthener test results on EMB agar media were conducted only on eight samples. The positive results on EMB agar media were marked by the presence of metallic green colonies. Based on Table 2, it can be observed that all samples in the strengthener test produced positive results meaning that they produced a metallic green colony on the EMB agar media.

E. coli identification result

Based on the result of the coliform strengthener test, the identification of *E. coli* was carried out in all positive samples in the coliform strengthener test, which was on eight bacterial colonies from eight samples, namely A1, B2, B3, C1, C2, D2, E1, E2 samples.

The result of *E. coli* identification would be matched with the result of the strengthener test to determine the MPN value of *E. coli* in the sample. *E. coli* identification was carried out consisting of four tests namely indole test, methyl red (MR) test, Voges Proskauer test (VP), and citrate test. The identification result was shown in Table 3. Based on table 3, it showed that the result of *E. coli* identification carried out on eight samples, as many as five samples, had the same biochemical characteristic, namely A1, B2, B3, C1, and C2 samples which were positive indole, positive MR, negative VP, and negative citrate. E1 and E2 samples had the same biochemical characteristic, namely positive indole, negative MR, negative VP, and negative citrate. The D2 sample showed biochemical characteristics of negative indole, negative MR, negative VP, and positive citrate. After the analysis, it was found that A1, B2, B3, C1, and C2 samples which included *E. coli* were characterized by negative MR, negative VP, negative citrate, and positive indole.

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Most Probable Number value of Escherichia coli in blood cockle satay sample

Based on the result of a positive *E. coli* sample identification test, MPN value analysis was then performed by matching the identification result with the result in the coliform strengthener test. The obtained results were then analyzed using the MPN table issued by Badan Standarisasi Nasional Indonesia (Indonesian National Standardization Agency, BSN, 2006). The MPN result of *E. coli* in blood cockle satay is shown in Table 4. As can be seen, only six samples, namely A2, B1, D1, D2, E1, and E2, had the MPN values of *E. coli* <3.0 MPN per gram of cockle satay flesh. A1 and C2 samples had MPN value of *E. coli* 3.0 MPN per gram of cockle flesh while B2, B3, and C1 samples had MPN value of *E. coli* 3.6 MPN per gram of cockle satay flesh.

Table 1. Coliform Estimator Test Result on the cockle satay samples

No	Sample	Gas in Durham Tube									Number of Positive Tubes
		Dilution Tube 10^{-1}			Dilution Tube 10^{-2}			Dilution Tube 10^{-3}			
		1	2	3	1	2	3	1	2	3	
1	A1	-	-	-	+	-	-	-	-	-	0-1-0
2	A2	-	-	-	-	-	-	-	-	-	0-0-0
3	B1	-	-	-	-	-	-	-	-	-	0-0-0
4	B2	+	-	-	-	-	-	-	-	-	1-0-0
5	B3	+	-	-	-	-	-	-	-	-	1-0-0
6	C1	+	-	-	-	-	-	-	-	-	1-0-0
7	C2	-	-	-	+	-	-	-	-	-	0-1-0
8	D1	-	-	-	-	-	-	-	-	-	0-0-0
9	D2	-	+	-	-	-	-	-	-	-	1-0-0
10	E1	+	-	-	-	-	-	-	-	-	1-0-0
11	E2	+	-	-	-	-	-	-	-	-	1-0-0

Note: +: there is a gas bubble in the Durham tube, -: There is no gas bubble in the Durham tube. The number of positive tubes: Number of positive tubes from each dilution tube.

Table 2. Coliform Strengthener Test Result on EMB Agar Media

No	Sample	Colonies Color
1	A1	Metallic Green
2	A2	Not Researched
3	B1	Researched
4	B2	Metallic Green
5	B3	Metallic Green
6	C1	Metallic Green
7	C2	Metallic Green
8	D1	Not Researched
9	D2	Metallic Green
10	E1	Metallic Green
11	E2	Metallic Green

Table 3. *E. coli* Identification Result on the cockle satay samples.

Test	Sample							
	A1	B2	B3	C1	C2	D2	E1	E2
Indole	+	+	+	+	+	-	+	+
Methyl Red (MR)	+	+	+	+	+	-	-	-
Voges-Poskauer (VP)	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	+	+	+

Note: Indol is positive (+); because the surface of the media is red and negative (-); because it is yellow; MR is positive (+) because the media is red, and negative (-) because it is yellow or orange; VP is positive (+) because it is pink or red, and negative (-) because it is yellow; Citrate is positive (+) because the media is blue, and negative (-) if the color (green) is not changed.

Table 4. Most Probable Number *E. coli* in Blood Cockle Satay

No	Sample	Number of Positive Tubes			MPN[1] per gram
		Dilution 10^{-1}	Dilution 10^{-2}	Dilution 10^{-3}	
1	A1	0	1	0	3.0
2	A2	0	0	0	<3.0
3	B1	0	0	0	<3.0
4	B2	1	0	0	3.6
5	B3	1	0	0	3.6
6	C1	1	0	0	3.6
7	C2	0	1	0	3.0
8	D1	0	0	0	<3.0
9	D2	0	0	0	<3.0
10	E1	0	0	0	<3.0
11	E2	0	0	0	<3.0

[1]Most Probable Number

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DISCUSSION

Blood cockle satay is ready to eat traditional food which requires safety considerations for consumption. In case the consumed blood cockle satay contained pathogenic bacteria, it would become a source of the disease since it becomes an intermediary for the growth of pathogenic microorganisms (Novianti, 2015). One of the pathogenic bacteria that needs to be considered is *Escherichia coli* (*E. coli*). *Escherichia coli* is a group of fecal coliform bacteria (Fardiaz, 1993). Fecal coliform bacteria is an indicator of contamination because the number of colonies must be positively isolated with pathogenic bacteria. Detection of coliform is easier, faster, and simpler, compared to other pathogenic bacteria (Aminollah and Supriyanto, 2016).

The 11 blood cockle satay samples were obtained from five traditional markets in Surabaya (Indonesia). Sampling was carried out in the morning and placed in a styrofoam container filled with ice cubes during transportation to the laboratory to avoid bacterial contamination.

The first MPN test was a coliform estimator test using Lactose Broth (LB) media with three series of tubes by diluting the sample three times, namely dilution of 10^{-1}, 10^{-2}, 10^{-3}. Each dilution was put into three test tubes which contained Durham tubes and LB media, so there were nine tubes for each sample leading to a total of 99 tubes. The tube containing the sample, the Durham tube, and LB media were then incubated at 37°C for 24 hours. The use of LB media aimed to examine the presence of lactose fermentation by bacteria. The presence of coliform bacteria in the sample was characterized by the formation of gas in the Durham tube and the change in color of the media from yellow to turbid (Fardiaz, 1993). The gas formation and discoloration of the media become turbid in the Durham tube because LB media contained lactose as a source of carbohydrates for bacteria (Jasmadi et al., 2014).

Based on the result of the study on the coliform estimator test in Table 1, it can be seen that there were only eight tubes of eight samples that showed positive results, namely A1, B1, B2, B3, C1, C2, D2, E1, and E2, producing gas in the Durham tube and the color of the media turned turbid. However, the other tubes did not show any gas in the Durham tube or changes in the color of the media. The coliform estimator test has not confirmed that a positive sample contained *Escherichia coli* bacteria because besides *E. coli* bacteria several other types of bacteria could ferment lactose, such as *Salmonella* sp. and *Acetobacter* sp. (Novianti, 2015).

Eight positive tubes were then subjected to coliform strengthener test on Eosin Methylene EMB agar media. Every sample from each tube was inoculated in EMB agar and incubated for 24 hours at 37°C. Based on the results of the study, all samples which were subjected to the coliform strengthener test were positive. The result of the coliform strengthener test was positive because in the EMB agar media there was a metallic green colony with black spots in the middle of the colony and metallic luster. The EMB Agar contained eosin and methylene blue which inhibited Gram-positive growth so that the grown bacteria were selected as Gram-negative bacteria (Leboffe and Pierce, 2011). The EMB Agar also has lactose content, so that Gram-negative bacteria that grow would be differentiated based on the characteristic that could ferment the lactose (Tille, 2015). The eight samples tested by the coliform strengthener test stage indicated high levels of *E. coli, which* could ferment lactose, sucrose, and glucose (Fardiaz, 1993).

Eight samples with high levels of *E. coli* were then subjected to a biochemical test to identify whether the bacteria characteristics were in line with the biochemical characteristics of *E. coli*. Bacteria generally obtained energy by carrying out biochemical activities from the environment through fermentation (Fardiaz, 1993). The *E. coli* was distinguished from other coliform bacteria in biochemical activity using the IMVic test (Indol, Methyl red, Voges-Proskauer, and citrate). The first test was the indole test used to determine the ability of bacteria to produce indole by breaking down tryptophan. Tryptophan was an essential amino acid that was oxidized by bacteria that involved in the formation of indole, pyruvic acid, and amino acid (Aminollah and Supriyanto, 2016).

Bacteria that had the triptonase enzyme would break down tryptophan into indole, pyruvic acid, ammonia, and energy (Fardiaz, 1993). The change in color to red on the surface of the media was a sign of the presence of indole in bacteria or culture while the absence of indole was signed by the color media was not red (Engelkirk and Duben-Engelkirk, 2008). Seven samples out of eight (i.e., A1, B2, B3, C1, C2, E1, and E2) were tested and the results showed a change in the surface color of the media to red or a positive result in the indole test after the addition of the Kovacs reagent. The red color on the surface of the media was caused by indole reacting with aldehydes (Aminollah and Supriyanto, 2016). For one of the eight samples, D2, the surface color of the media did not change to red or negative results in the indole test.

The methyl red test was conducted to find out the ability of bacteria to produce and maintain the final acid product from glucose or lactose fermentation (Fardiaz, 1993). The media would change the color into red after administration of methyl red reagent which indicated that the pH of the media decreased to 4.4 or lower, showing the existence of lactose fermentation by bacteria in the media (Engelkirk and Duben-Engelkirk, 2008). Five of the eight samples (i.e., A1, B2, B3, C1, and C2) showed a change in the color of the media to red after the addition of the methyl red reagent, meaning a positive result in the methyl red test. Three of the eight tested samples (D2, E1, and E2) did not show any changes in the

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color of the media after the addition of the methyl red reagent, which were considered as negative results in the methyl red test.

The Voges-Proskauer (VP) test used the same media as the media used for methyl red, namely MR-VP Broth, but the reagent used was 40% KOH solution and alpha naphthol solution. The VP test was used to find out the ability of bacteria to produce methyl carbinol (acetoin, Fardiaz, 1993). Among all tested cases, eight samples did not show any changes in the color of the media after the addition of the reagent that was still yellow, so that they were considered negative in the Voges-Proskauer test. The changes in the color of the media to red indicated a positive result while the yellow color in the media or there was no change in color showed a negative result (Fardiaz, 1993). The KOH and alpha naphthol were the chemicals that detected acetoin (Aminollah and Supriyanto, 2016). The change in color to red was an indication of the formation of acetoin (Engelkirk and Duben-Engelkirk, 2008). Acetoin was an intermediate in the production of butylene glycol in carbohydrate fermentation (Hemraj et al., 2013). Two reagents used in the VP test were 40% KOH and alpha naphthol solution added to the media after incubation and exposure to oxygen. In case there was acetoin, it would be oxidized by the air and KOH become acetyl. In the next step, diacetyl reacted with the guanidine component of peptone which was a complex composition of VP media, the alpha naphthol produced a red color. Alpha naphthol played the role of catalyst and color enhancer. The VP test for *Escherichia coli* was negative because *E. coli* fermented carbohydrates into acidic products and did not produce neutral products, such as acetoin (Fardiaz, 1993).

Citrate test was a test to determine the ability of bacteria to use the citrate as the only source of carbon and ammonia salt as the only source of nitrogen (Engelkirk and Duben-Engelkirk, 2008; Fardiaz, 1993). Five of the eight tested samples (A1, B2, B3, C1, and C2) did not show any change in the color of the media indicated a negative result in the citrate test whereas the color of the media change to blue for D2, E1 and E2 samples showing the positive results on the citrate test. The utilization of citrate involved the permease citrate enzyme which broke down citrate into oxaloacetate and acetate (Hemraj et al., 2013). Oxaloacetate was further broken down into pyruvate and CO_2. The production of Na_2CO_3 and NH_3 from the utilization of sodium citrate and ammonium salt resulted in an alkaline pH leading to color changes of the media from green to blue. The *E. coli* did not use citrate as a carbon source (Fardiaz, 1993).

Based on the result of the biochemical test, the presence of bacteria in A1, B2, B3, C1, and C2 samples had the triptonase enzyme so that it could break down tryptophan into indole, ammonia, and energy. That bacteria did not produce acetoin so that the media remained yellow, but it could ferment lactose until the pH decreased which was marked by changing color into red. The bacteria were unable to neither use citrate as the carbon source and nor producing acetoin. The biochemical result of the samples was the same as the biochemical characteristic of *E. coli*, namely positive indole, positive methyl red, negative Voges-Proskauer, and negative citrate (Breed et al., 1948).

As can be seen in Table 4, the value of MPN *E. coli* in blood cockle satay showed that A1 and C2 samples had an MPN value of *E. coli* 3.0 sMPN per gram. Furthermore, A2, B1, D1, D2, E1, and E2 samples had an MPN value of *E. coli* <3.0 MPN, and B2, B3, and C1 samples had MPN value of *E. coli* f 3.6 MPN per gram. This showed that there were differences in the number of *E. coli* in blood cockle satay which was sold in Surabaya traditional markets. The difference in the number of MPN *E. coli* in the blood cockle satay was probably due to the differences in cockle satay containers sold where there were plastic-covered containers and there were non-plastic-covered containers that could cause bacterial contamination carried by flies or air and cross-contamination from other places and merchandise.

CONCLUSION

Based on the result of the study, it can be concluded that there is *E. coli* contamination in blood cockle satay sold in traditional markets in Surabaya (Indonesia) exceeding the maximum limit set in SNI 7388: 2009. This means that the maximum limit of *E. coli* contamination in blood cockle product was <3.0 MPN per gram of blood cockle satay flesh. The MPN of *E. coli* in the highest blood cockle satay was 3.6 MPN per gram of blood cockle satay flesh sold in the Surabaya traditional market (Indonesia).

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