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Research Article

Escherichia coli and *Salmonella sp.* on some lettuce in the traditional market of Banyuwangi Regency

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ABSTRACT

Escherichia coli is the main pathogenic bacteria that cause diarrhea in humans and is the fourth-highest cause of death in all age groups. *Salmonella sp* is the cause of typhoid fever and findings of 350-810 cases each year. Diarrhea and typhoid fever are closely related to consuming raw vegetables like lettuce. This study aimed to analyze the contamination of *E. coli* and *Salmonella sp* on some lettuces in traditional markets of Banyuwangi Regency. This research was an analytic descriptive study using a cross-sectional design at the Laboratory of Microbiology, STIKES Banyuwangi, from 6th to 11th June 2022, using six lettuces as research samples selected by accidental sampling. All lettuce samples in this study were contaminated with more than 1,100 CFU/ml *E. coli*. All samples in this study were positively contaminated by *Salmonella sp* with various contamination numbers, such as 0.82x104; 0.47x104, 1.90x105, 2.05x106, 1.00x105 and 1.25x106 CFU/ml, respectively. All six lettuces obtained from the Traditional Market of Banyuwangi Regency did not meet the microbiological quality specified by SNI, indicating that they were unsuitable for direct (raw) consumption.

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INTRODUCTION

Both *E. coli* and *Salmonella sp.* are opportunistic bacteria, a group of bacteria that only causes severe infection in populations with weakened immune systems compared to healthy people. Nevertheless, infections caused by opportunistic bacteria remain possible and lead to serious health problems (Stec et al., 2022). *E. coli* is the main coliform that causes diarrhea in humans. Diarrhea is a non-communicable disease that remains a problem in developing countries and the fourth-highest cause of death in all age groups (Ummah, 2022). Diarrhea was found in 49.21% of cases in East Java in 2021 across all age groups. (Dinas Kesehatan Provinsi Jawa Timur, 2022). The incidence of diarrhea is closely related to the consumption of raw vegetables (Ibrahim & Sartika, 2021).

Salmonella sp is a gram-negative enteric pathogen that typically infects the digestive tract. The main transmission route for this bacterium is via food or drink infected with *Salmonella sp* from the patient's feces (fecal-oral transmission). This bacterium is the cause of typhoid fever, with fever and diarrhea as the main clinical symptoms (Jajere, 2019).

Approximately 350-810 cases of typhoid fever per 100,000 population are reported annually in Indonesia, with a prevalence of 1.6%. It is still recorded as an infectious disease, with the fifth-highest number of cases occurring in all age groups in Indonesia. This systemic infection is also known as the 15th most common cause of death among all groups in Indonesia (Khairunnisa et al., 2020).

Indonesia is one of the countries in Asia where the majority of the population has a habit of consuming raw vegetables because they are considered fresh and able to maintain the integrity of the nutrients in the vegetables. Indonesians usually serve some raw vegetables as "*lalapan*" side by side with other dishes. Lettuce is an example of a vegetable that is usually consumed raw/fresh or only boiled for a very short time (Amrinanto et al., 2019; Rahmi et al., 2021).

Research by Laila et al. (2022) showed that 60% of subjects who had suffered from typhoid fever did not know that consuming raw vegetables was a risk factor for the disease. Some of them had a habit of eating raw vegetables directly, and other subjects used to wash vegetables using stagnant water in a container, not flowing water (Laila et al., 2022).

The finding of *Salmonella sp* in lettuce has been reported several times, one of which was in a review article written by Rahman et al. (2022), which reviewed the positive results of *Salmonella sp* in fresh lettuce sold in the US retail market (Rahman et al., 2022). This finding violated the regulation of National Standardization Agency / Badan Standardisasi Nasional (BSN) in Standard Nasional Indonesia (SNI) 7388:2009 about maximum microbial contamination in food, which mentions three indicators of hygiene for direct consumption of fresh vegetables, such as MPN index of *E. coli* (<3/gram) and *Salmonella sp.* (negative/25 gram) (Badan Standarisasi Nasional, 2009).

In the Health Profile of Banyuwangi/ Profil Kesehatan Banyuwangi 2021, a total of 43,808 cases of diarrhea were reported in adults and children (Dinas Kesehatan Banyuwangi, 2022). In addition to these cases in Banyuwangi, its population's habit of consuming raw vegetables called "*lalapan*" also leads to an assumption of *Salmonella* infection as the main cause of diarrhea in this region. However, studies on diarrhea and *Salmonella sp.* in Banyuwangi remain limited.

Because the detection of *E. coli* and *Salmonella sp* from lettuce in traditional markets of Banyuwangi Regency has not been carried out,

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authors were interested in performing a study on the identification of *E. coli* and *Salmonella sp* from lettuce in traditional markets of Banyuwangi Regency.

METHODS

This research was an analytic descriptive study using a cross-sectional design performed at the Laboratory of Microbiology of STIKES Banyuwangi from June 6 to 11, 2022. The sample used in this study was six lettuces obtained from 6 different merchants at the Traditional Market of Banyuwangi Regency. Samples were selected by accidental sampling.

The equipment used in this study were: blender, test tube, Durham tube, tube rack, micropipette, blue tip, yellow tip, Erlenmeyer, petri dish, stir bar, glass funnel, beaker glass, measuring cup, wire mesh, sample bottle (with screw cap), Pasteur pipette, electric stove, inoculating loop and needle, spirits burner, LAF, incubator, and autoclave. A non-selective pre-enrichment medium such as Lactose Broth (LB), some selective media such as Brilliant Green Lactose Broth (BGLB) and Eosin Methylene Blue (EMB), and some differential selective media such as Salmonella Shigella Agar (SSA), Triple Sugar Iron Agar (TSIA), Indole, Methyl Red-Voges Proskauer (MR-VP), Simmon's Citrate (SC), and urea agar were used for bacterial identification. Fatty cotton, PZ solution, distilled water, methylated spirits, and alcohol 70% were also used in this study.

Enumeration of coliform

Total coliform is a laboratory test commonly used to indicate food, beverage, and environmental hygiene. In addition, it was used in this study to determine the hygiene of lettuce and the traditional market of Banyuwangi Regency. Contamination of total coliform was enumerated using the 3-3-3 Most Probable Number (MPN) method, which was divided into three main stages: 1) preliminary stage, 2) confirmation stage, and 3) identification stage. Preliminary stage: samples were mashed and inoculated into several tubes containing LB media sequentially and incubated at 37°C for 24 hours. Confirmation stage: each LB tube containing a sample that showed positive bacterial growth (indicated by the presence of gas in the Durham tube) was inoculated into test tubes containing BGLB media and incubated at 37°C for 24 hours. The number of positive BLGB tubes was recorded and compared with the standard MPN table to determine the total coliform. Identification stage: each BGLB

Figure 1. Lettuce used in this study

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tube containing samples that showed positive bacterial growth (indicated by the presence of gas in the Durham tube) was inoculated into EMB media in a petri dish and incubated at 37°C for 24 hours.

Identification of *E. coli*

Morphological characteristics of bacterial colonies growing on the surface of EMB media were observed after 24 hours of incubation. Specific colonies with green metallic sheen were reported as *E. coli* on EMB media (Scientific, 2022a). Identification of *E. coli* was supported by using biochemical media consisting of TSIA, Indole, MR-VP, SC, and Urea media. Characteristics of *E. coli* on those media are as follows: indole-positive, A/A and gas-positive on TSIA, MR-positive, VP-negative, citrate-negative on SC media, and urease-negative on urea media (Leboffe & Pierce, 2011).

Identification and enumeration of *Salmonella sp.*

A total of 1 ml sample was inoculated into the first test tube containing 9 ml of sterile pz solution to form a 10^{-1} dilution. The mixture of the sample and PZ solution was homogenized using a micropipette. One ml of the solution was pipetted into a sterile petri dish and liquid SSA medium was added via the pouring procedure (pour plate method). The petri dish was homogenized by moving the petri to form number 8 several times. One ml of the 10^{-1} dilution was pipetted from the first test tube into the second test tube containing 9 ml of sterile PZ solution to form a 10^{-2} dilution. The sample mixture and PZ solution in the second tube were homogenized using a micropipette. The same steps were then repeated until a 10^{-5} sample dilution was formed and the sample was inoculated into a petri dish and SSA media was added. Incubation was carried out at 37°C for 24 hours. *Salmonella sp* on SSA media has a typical transparent colony with a black center (Scientific, 2022b). Colonies with such characteristics were then counted.

Data analysis

The results of this study were presented descriptively in the table and narrative explanation.

RESULTS

All (100%) lettuce samples in this study were contaminated with >1,100 CFU/ml coliform bacteria (Table 1). This contamination was determined by the MPN method after observing that all BGLB tubes were positive (marked with gas on Durham tubes). Gas in Durham tubes indicated the contamination of Coliform bacteria, which are well known for their ability to ferment lactose, resulting in gas that accumulates in the Durham tube (Hadiansyah et al., 2021). The identification results showed that all (100%) samples were contaminated with *E. coli*. This was indicated by the growth of colonies with green metallic sheen, which are specific characteristics of *E. coli* on EMB media. This green color arises due to the fast lactose fermentation activity by *E. coli,* which produces strong acids that change the pH and indicator color in the media to green (Khakim & Rini, 2018) (Figure 2).

The identification of *E. coli* was supported by the observations on the biochemical media as listed in Tables 2 and 3. The change in the slant and base TSIA to yellow indicates the ability of *E. coli* to ferment all carbohydrates contained in the media to produce acid, which changes the pH of the media and changes the color of the phenol red indicator to yellow. The lifting of the base of the media is caused by gas production by bacteria as another result of carbohydrate fermentation activity (Kristiawan et al., 2022).

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Table 1. Enumeration of coliform bacteria

Sample	BGLB tubes with positive result			MPN index
1	3	3	3	> 1,100
2	3	3	3	>1,100
3	3	3	3	>1,100
4	3	3	3	>1,100
5	3	3	3	>1,100
6	3	3	3	>1,100

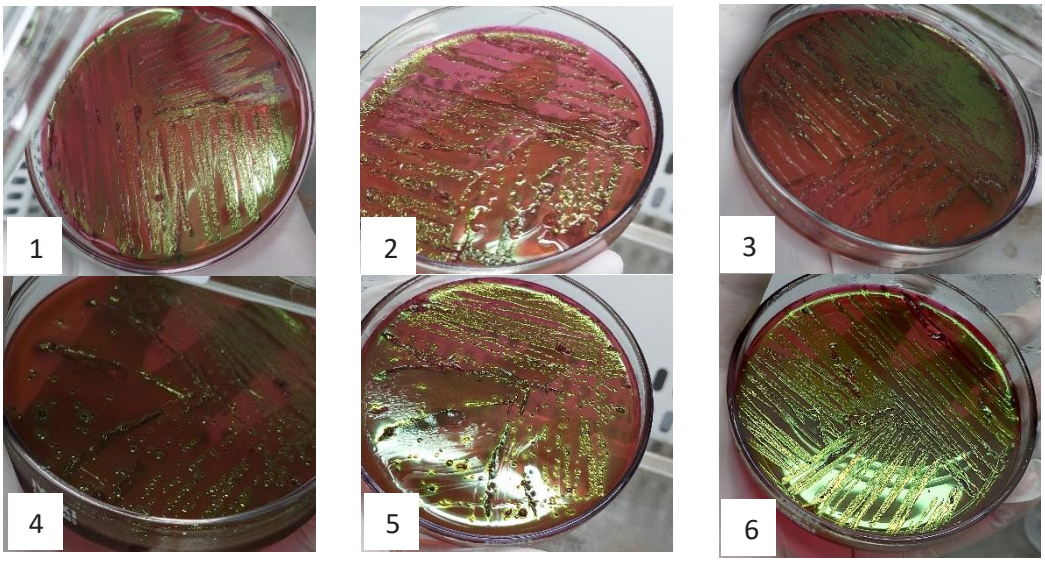

Figure 2. *E. coli* on EMB media

Table 2. Observations on EMB and TSIA media

Sample	Results on TSIA				Colony on EMB
	Slant	Base	H_2S	Gas	
1	A/+	A/+	-	+	Shape: circular, margin: entire, elevation: convex, green metallic sheen
2	A/+	A/+	-	+	Shape: circular, margin: entire, elevation: convex, green metallic sheen
3	A/+	A/+	-	+	Shape: circular, margin: entire, elevation: convex, green metallic sheen
4	A/+	A/+	-	+	Shape: circular, margin: entire, elevation: convex, green metallic sheen
5	A/+	A/+	-	+	Shape: circular, margin: entire, elevation: convex, green metallic sheen
6	A/+	A/+	-	+	Shape: circular, margin: entire, elevation: convex, green metallic sheen

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Table 3. Observations on IMViC and Urea media

Sample	Indole	MR	VP	SC	Urea
1	+	+	-	-	-
2	+	+	-	-	-
3	+	+	-	-	-
4	+	+	-	-	-
5	+	+	-	-	-
6	+	+	-	-	-

Table 4. Observations on SSA media

Sample	Colony on SSA	Identification
1	Shape: circular, color: transparent with black center, elevation: convex, margin: entire	*Salmonella sp*
2	Shape: circular, color: transparent with black center, elevation: convex, margin: entire	*Salmonella sp*
3	Shape: circular, color: transparent with black center, elevation: convex, margin: entire	*Salmonella sp*
4	Shape: circular, color: transparent with black center, elevation: convex, margin: entire	*Salmonella sp*
5	Shape: circular, color: transparent with black center, elevation: convex, margin: entire	*Salmonella sp*
6	Shape: circular, color: transparent with black center, elevation: convex, margin: entire	*Salmonella sp*

A positive indole test indicated by the formation of a red ring on the surface of the medium indicates the ability of *E. coli* to break down the essential amino acid tryptophan to produce indole. Indole will react with the aldehyde group in Kovac's reagent, forming a stable red ring (Khasanah et al., 2021). A positive MR test was reported in all (100%) samples in this study, which were indicated by a red ring on the surface of the media. This is based on the ability of *E. coli* to further metabolize pyruvic acid via a mixed acid pathway to produce acids that change the color of the methyl red indicator from yellow to red (Puspita et al., 2020).

The negative VP test in this study was reported based on the absence of a red ring on the surface of the media. This shows that *E. coli* cannot produce a neutral product in the form of acetoin from carbohydrate fermentation. The negative results of SC media in this study indicate that *E. coli* is unable to use citrate contained in the media in the form of sodium citrate to become ammonia, so there is no change in the pH and color of the media (Kartikasari et al., 2019). The negative urea test in this study was concluded from the absence of a change in the color of the media. This is because *E. coli* does not have the enzyme urease to hydrolyze urea to ammonia (Puspita et al., 2020).

All (100%) samples in this study were positively contaminated by *Salmonella sp*. This was indicated by the growth of specific colonies of *Salmonella sp* on SSA media, such as transparent colonies with black precipitate/ black center due to the reduction of thiosulfate

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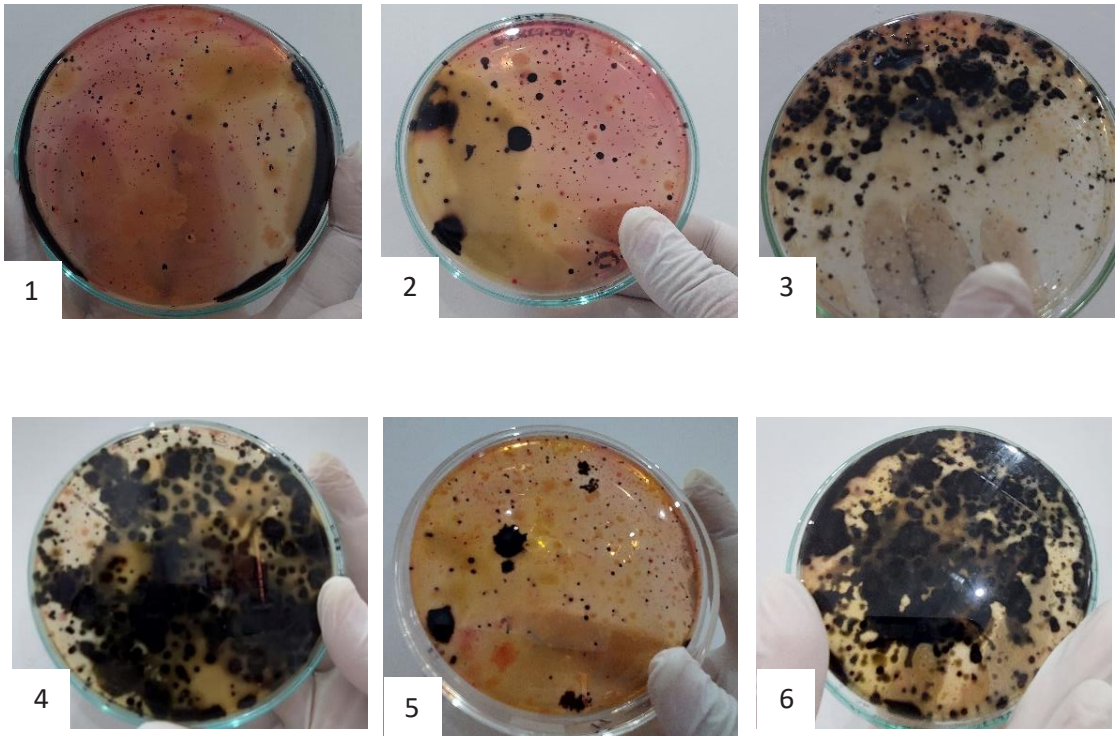

Figure 3. *Salmonella sp* on SSA media

Table 5. Enumeration of *Salmonella sp*

Sample	Total bacteria (CFU/ml)
1	0.82×10^4
2	0.47×10^4
3	1.90×10^5
4	2.05×10^6
5	1.00×10^5
6	1.25×10^6

to H_2S (Rahmiati, 2016), which can be seen in Table 4 and Figure 3. The use of SSA media in the Total Plate Count (TPC) method can facilitate the enumeration of *Salmonella sp* in the sample, considering that this specific media is used to distinguish *Salmonella sp* and *Shigella sp* based on the characteristics of the bacterial colonies in the media (Fatiqin et al., 2019). Six samples in this study were contaminated by *Salmonella sp* with different numbers of bacteria as follows: 0.82×10^4; 0.47×10^4, 1.90×10^5, 2.05×10^6, 1.00×10^5 and 1.25×10^6 CFU/ml, respectively (Table 5)

DISCUSSION

E. coli contamination in the six samples in this study indicated that the lettuce was unsuitable for consumption because it exceeded the limit for *E. coli* contamination in vegetables, namely <3/gram based on SNI 7388 of 2009 (Badan Standarisasi Nasional, 2009). The findings of *E. coli* in lettuces were also reported in a study by Olianovi and Pasaribu (2017), which identified *E. coli* in all (100%) samples of lettuce sold at the Grogol District market, West Jakarta, in quantities that exceeded the SNI provisions. *E.*

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coli was also reported contaminating lettuce served as fresh vegetables at food vendors around Airlangga University. It is known that *E. coli* was found in 57% of the samples (Wibowo et al., 2017).

Salmonella sp contamination in six samples in this study indicated that the lettuce was unfit for consumption because it exceeded the limit for *Salmonella sp* contamination in vegetables, namely negative per 25 grams based on SNI 7388 2009 (Badan Standarisasi Nasional, 2009). The low bacteriological quality of lettuce was also found by Ramadhani et al., who identified *Salmonella sp* in 4 (25%) samples of lettuce sold in traditional wholesale markets and supermarkets in the city of Semarang (Ramadhani et al., 2017). This finding was also supported by research by Niguma et al. on four lettuce plantations in Londrina, Brazil, which showed that 1 (1.4%) of lettuce grown using conventional techniques contained *Salmonella sp*. In contrast, *Salmonella sp* was not detected on all organically grown lettuces (Niguma et al., 2017).

Public Health Agency of Canada 2011 stated that infectious dose of non-typhoidal salmonellosis and enteric fever-causing *Salmonella sp* was 10^3 and 10^5 bacilli, respectively (Canada, 2011). However, a study by Akil and Ahmad (2019) which analyzed an estimate for the probability (dose-response model) of salmonellosis, suggested that consumption of at least 1.46 x 10^4 CFU/gr of *Salmonella enterica serovar enteritidis* or 6.4 x 10^3 of *Salmonella enterica serovar typhimurium* from food would likely cause *Salmonella sp* infection in 50% population (Akil & Ahmad, 2019). Based on those studies, the contamination of *Salmonella sp*. of 0.82 x 10^4, 0.47 x 10^4, 1.90 x 10^5, 2.05 x 10^6, 1.0 x 10^5, and 1.25 x 10^6 CFU/ml in each sample this study would likely cause salmonellosis if the lettuce is consumed raw. However, no further enumeration of *E. coli* remained one of the limitations of this study. It is known that the lowest infectious dose of *E. coli* in humans is <50 – 100 CFU, which causes diarrhea accompanied by stomach cramps, nausea, vomiting, or fever (Rahayu et al., 2018).

Several factors might allow contamination of lettuce by *Salmonella sp*, such as consuming fresh lettuce (direct consumption without processing procedures) as fresh vegetables to accompany staple foods, contamination of springs used in irrigating lettuce gardens, contamination of organic compost animal waste used to support lettuce production, and contamination during the delivery of lettuce from plantations, markets, to individual traders (Ardiana et al., 2021).

Society, in general, has anticipated the existence of germs in vegetables by washing vegetables with water. Not only cleaning disease-causing bacteria but washing vegetables has also been agreed upon to free vegetables from dirt and pesticide residues. However, washing fresh vegetables repeatedly by immersing the vegetables in a container filled with water and alternating with other vegetables can reduce the water quality and increase the possibility of cross-contamination among vegetables (Pablos et al., 2022).

Consuming raw vegetables without further processing has been associated with many cases of *Salmonella sp* infection. The high nutrient content, humidity, and exposed surface to free air make vegetables an ideal habitat for pathogenic bacteria in food/foodborne pathogens, one of which is *Salmonella sp* (Yeni et al., 2016). This was proven in a study by Afifah and Pawenang (2019), which showed that 5 (19.2%) research subjects with a history of typhoid fever at the age of 15-44 years in the Working Area of the Tlogosari Kulon Health Center had a habit of eating raw vegetables.

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Serovar *Salmonella sp,* which was most commonly found in fresh vegetables sold at Kajang Market, Puchong Market, and Sungai Besi Malaysia Market, respectively, included: *S. weltevreden, S. agona, S. seftenberg*, and *S. albany*. The research results in these three markets also showed that the two most common serovars found in humans and animals, *S. typhimurium* and *S. paratyphi B*, were also found in 6 (5.36%) samples. Although the prevalence and contamination level of *Salmonella sp* in fresh vegetables and fruits is not as high as the prevalence in meat, the findings of *Salmonella sp* in vegetables, especially in people with a habit of consuming fresh vegetables, should not be underestimated (Salleh et al., 2003). *Salmonella sp* contamination in lettuce has been reported to cause outbreaks globally in 2012 – 2015, resulting in 136 cases of hospitalization and 3 cases of death. *S. typhimurium* was identified as the main pathogen of the *Salmonella genus,* which was the cause of the outbreak through contamination of raw vegetables (Kilonzo-Nthenge & Mukuna, 2018).

Salmonella sp and *E. coli* contamination in vegetables can be overcome by choosing the right method of washing vegetables. One recommended washing method for industrial vegetable production is a mixture of chlorine and acid. This is because chlorine at the right dose can cause inactivation of cell membranes and the function of bacterial DNA components. One attempt that society can implement as a target for lettuce consumption is to wash lettuce regularly before direct consumption, especially by using a mixture of vinegar containing acetic acid and lemon juice. This is because acetic acid can decompose enzymes that are important for the survival of bacterial cells and change the structure of bacterial cell membranes to become easily penetrated by various substances / permeable (Wiastari & Sujaya, 2021).

Several attempts to prevent *Salmonella sp* and *E. coli* infections which the consumer can implement in addition to washing vegetables are to provide good handling of fresh vegetables, such as cooking vegetables at the right temperature (not to damage the nutrients), separating vegetables that have been washed or cooked from fresh vegetables, as well as storing vegetables before and after cooking in the refrigerator. Using clean cooking utensils or cutlery and monitoring hand and environmental hygiene before contact with vegetables must also be implemented to ensure food health (Ehuwa et al., 2021).

CONCLUSION

The six samples of lettuce sold at the Traditional Market in Banyuwangi Regency did not meet the microbiological quality specified by SNI and, thus, were not suitable for direct (raw) consumption.

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