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Exploration of pathogenic bacteria from lettuce (*Lactuca sativa*) in a Canteen at Universitas Padjadjaran

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Abstract

Background: Lettuce (*Lactuca sativa*) is commonly consumed raw as a salad, making it highly susceptible to contamination by pathogenic bacteria. This study aims to identify the presence of pathogenic bacteria on raw lettuce to offer an alternative method for handling these pathogens and improving public health.
Method: In this research, lettuce samples were bacteriologically tested to identify the presence of pathogenic bacteria. Isolation was performed using selective media, Salmonella-Shigella (SS) agar and Eosin Methylene Blue (EMB) agar, to obtain pathogenic bacteria. Identification was conducted through macroscopic and microscopic examinations, as well as biochemical tests, including indole, Methyl Red, Voges-Proskauer, citrate, motility, and catalase tests.
Results: The results of the study revealed the presence of four bacterial isolates: SS 1, SS 2, EMB 1, and EMB 2. Based on the study and identification using "Microbiology: A Laboratory Manual 12th Edition," isolates SS 1, EMB 1, and EMB 2 were identified as *Alcaligenes* sp., while isolate SS 2 was identified as *Proteus* sp.
Conclusion: These findings provide essential knowledge for managing pathogenic bacterial contamination on lettuce, potentially enhancing food safety and public health.

Keywords: Food safety; Lettuce; Pathogenic bacteria; Public Health

INTRODUCTION

Vegetables are a primary component of human diets due to their essential role in providing nutrition, especially as sources of vitamins (C, A, B1, B6, B9, E), minerals, fiber, and phytochemicals (1). Some phytochemicals found in vegetables are powerful antioxidants known to reduce the risk of chronic diseases by protecting the body from damage caused by free radicals. Consuming vegetables can improve digestive health, vision, and reduce the risk of cancer, heart disease, stroke, anemia, diabetes, peptic ulcers, and other chronic conditions (2).

Food safety and quality remain major issues today, affecting both the production and distribution of fresh vegetables. The inconsistent quality of vegetables and high levels of contamination can be attributed to the low application of production technologies and post-harvest handling of vegetables. Several studies have shown that the level of pathogenic microorganism contamination in post-harvest handling of vegetables by farmers and in traditional markets reaches 10^6-10^7 CFU/g of sample (3). A colony-forming unit (CFU) measures the number of viable cells that can grow into individual colonies on an agar plate (4–6). These levels do not meet the standard, which is set at 10^3 CFU/g of sample (3).

The Indonesian government regulates pathogenic microorganism contamination in fresh produce as part of its foodborne pathogen management efforts. The National Food Agency Regulation of the Republic of Indonesia Number 10 of 2024, on "Maximum Contamination Levels in Fresh Food Distribution", establishes standards for ready-to-eat fresh vegetables that are consumed without cooking, such as lettuce, tomatoes, and cucumbers (7). According to this

regulation, microbial contamination limits are set at "Negative/25 g" for *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC), and at 10^2 CFU/g for *Listeria monocytogenes*. This regulatory standard reflects Indonesia's current practices for controlling foodborne pathogens.

One type of vegetable commonly consumed raw as a salad is lettuce. Consuming raw lettuce is considered to have better nutritional value and taste. However, consuming raw lettuce can lead to contamination by pathogenic bacteria. This is due to the low microbiological quality of fresh vegetables in Indonesia (3). Raw lettuce salads are a potential source of contamination, spreading pathogenic microorganisms to humans (8).

Lettuce (*Lactuca sativa*) is a vegetable plant belonging to the Asteraceae (Compositae) family. This plant has leaves with varying shapes, colors, and sizes depending on the variety (9). Lettuce contains various nutrients such as fiber, vitamin A, phosphorus, iron, iodine, zinc, copper, calcium, cobalt, potassium, and manganese, making it beneficial for health. Generally, fresh lettuce is consumed as a salad or used in hamburgers and traditional dishes like gado-gado (10,11). Lettuce also contains various bioactive compounds beneficial to health. Some of these bioactive compounds include folate, beta-carotene, lutein, and phenolics. Previous studies have shown that lettuce exhibits anti-inflammatory, cholesterol-lowering, and anti-diabetic activities, influenced by its bioactive compounds (12).

A major global health issue is the presence of pathogenic bacteria in food, often due to inadequate sanitation and improper food handling. Pathogenic bacteria can directly contaminate both raw and processed foods. The most common foodborne pathogenic bacteria include *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Staphylococcus aureus*, *Proteus* spp., *Serratia* spp., *Aeronomas* spp., *Alcaligenes* spp., *Clostridium* spp., among others (13–16). These pathogens can cause serious gastrointestinal diseases, leading to significant health problems globally (15).

The World Health Organization (WHO) estimates that about 600 million people fall ill each year after consuming food contaminated with pathogenic bacteria, resulting in 420,000 deaths worldwide (17). Sources of food contamination can include bacteria, viruses, or parasites. Pathogenic bacteria transmission can occur orally when contaminated food is ingested, entering the digestive tract (15). Generally, bacterial infections can be treated with antibiotics. However, microbial resistance to antibiotics has become one of the greatest threats to public health and food safety (17).

Based on this background, early detection is necessary to ensure food safety and protect public health from microbial contamination in food (18). This study aims to isolate and identify pathogenic bacteria from raw lettuce salads through bacteriological testing. The results of this research can serve as an alternative for addressing pathogenic bacteria in raw lettuce salads, thereby enhancing public health.

METHOD

This descriptive exploratory research was conducted from October to November 2022. The isolation and identification processes were carried out at the Microbiology Laboratory, Building D6, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Lettuce samples were obtained from a canteen at Universitas Padjadjaran. The samples were weighed to 2 g, cut into small pieces, and placed in 9 mL of physiological NaCl for serial dilution. From the dilutions of 10^{-3}, 10^{-4}, and 10^{-5}, 1 ml was taken and streaked using the spread plate method on selective media, SS and EMB. The streaked media were incubated at 37 ºC for 24 hours (19–21).

After incubation, Gram staining was performed on colonies from the three media. A portion of the colonies was taken using a sterile loop and spread on a glass slide with physiological NaCl solution. The slides were then dried and fixed over a Bunsen burner flame. The slides were stained with crystal

violet for 1 minute, washed with distilled water, followed by Lugol's iodine for 1 minute, and washed again with distilled water. They were then decolorized with 95% alcohol for 5-10 seconds, rinsed with distilled water, and counterstained with safranin for 30 seconds. The slides were observed under a microscope to examine cell morphology and color (19–21).

Bacterial isolates were aseptically inoculated into SIM media in test tubes and incubated at 37 ºC for 24 hours. After incubation, Kovac's reagent was added to the media. A positive result was indicated by a red coloration on the surface of the media, showing the bacteria's ability to break down the amino acid tryptophan (22).

Bacterial isolates were inoculated into MR-VP broth and incubated for 24 hours. For the Methyl Red (MR) test, 3-5 drops of methyl red were added to the broth and mixed. A positive result was indicated by the broth turning pink, whereas a negative result was indicated by the broth turning yellow. A positive result showed that the bacteria could convert glucose into acidic products such as lactic acid, formic acid, or acetic acid. For the Voges-Proskauer (VP) test, 5 drops of alpha-naphthol reagent and KOH were added to the broth and mixed. The reaction was allowed to proceed for 15-20 minutes. A positive result was indicated by a color change to pink or red, signifying the presence of acetoin. This indicated that the bacteria could convert glucose into acetyl methyl carbinol (22).

Isolates were inoculated on citrate agar slants and incubated for 24 hours. A positive result was indicated by a color change in the medium from green to blue, demonstrating the bacteria's ability to convert citrate into oxaloacetate. For the motility test, bacterial isolates were stabbed into semi-solid NA media in test tubes using a sterile inoculating needle. The media were incubated at 37 ºC for 24 hours. A positive result was shown by the spread of bacterial growth around the stab line, indicating motility. If the bacterial growth remained as a single line, the bacteria were non-motile (22).

The catalase test was conducted by adding 2-3 drops of H_2O_2 (hydrogen peroxide) onto a sterile glass slide. Bacterial isolates were then taken using a sterile inoculating needle and placed on the glass slide, followed by mixing with the H_2O_2 solution. A positive result was indicated by the formation of oxygen bubbles, signifying the presence of catalase enzyme activity (23).

RESULTS

1. Macroscopic Morphology of Bacterial Isolates

Based on the test results, several bacterial colonies were found to grow on Salmonella-Shigella (SS) agar and Eosin Methylene Blue (EMB) agar media at the fifth dilution. SS and EMB media are selective and differential media used to isolate Gram-negative and enteric pathogenic bacteria, including *Salmonella, Shigella, Proteus, Escherichia coli,* and *Enterobacter* (24–26). Four colonies were selected for further testing. Based on the observations, the colony morphology of the four bacterial isolates is presented in Table 1 below.

Table 1. Macroscopic Morphology of Bacterial Isolates

Colony Morphology	Isolates			
	SS 1	SS 2	EMB 1	EMB 2
Shape	C	C	C	C
Margin	E	E	E	E
Elevation	F	F	F	F
Size	S	P	S	P
Appearance	D	G	D	G
Optical property	O	T	O	T
Texture	So	So	So	So
Pigmentation	Pi	B	W	B

C: Circular; E: Entire; F: Flat; S: Small; P: Punctiform; D: Dull; G: Glistening; O: Opaque; T: Translucent; S: Smooth; Pi: Pink; B: Black; W: White

2. Gram-Staining

After identifying the bacterial colonies, Gram staining was performed to distinguish between Gram-positive and Gram-negative bacteria. The four bacterial isolates subjected to Gram staining were SS 1, SS 2, EMB 1, and EMB 2. The results of the Gram staining are shown in Figure 1.

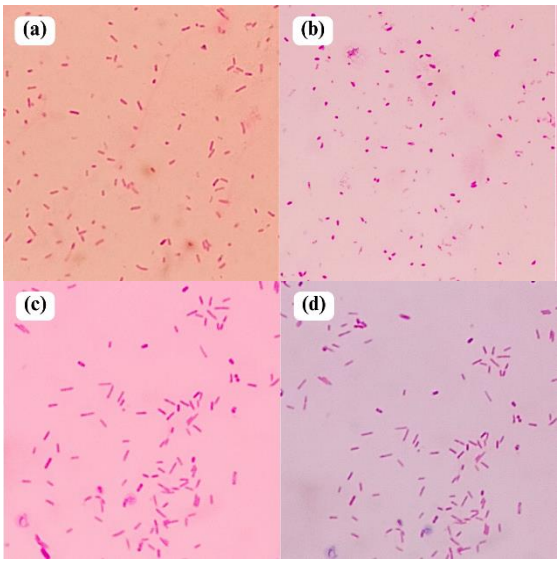

Figure 1. Gram Staining Results: (a) SS 1; (b) SS 2; (c) EMB 1; (d) EMB 2

2. Biochemical Test

After Gram staining, the IMViC tests were conducted to differentiate among the major groups of Enterobacteriaceae based on biochemical properties and enzymatic reactions with specific substrates. This series of tests includes indole, methyl red (MR), Voges-Proskauer (VP), and citrate utilization tests. Additionally, catalase and motility tests were performed on the four isolates. The results of the study are presented in Table 2 below.

Table 2. Biochemical Test Results

Isolates	Results					
	I	MR	VP	SC	M	C
SS 1	-	-	-	+	+	+
SS 2	+	+	-	+	+	+
EMB 1	-	-	-	+	+	+
EMB 2	-	-	-	+	+	+

I: Indole; MR: Methyl Red; VP: Voges Proskauer; SC: Citrate; M: Motility, C: Catalase

DISCUSSION

Based on these results, it was determined that all the isolates were Gram-negative and rod-shaped. Gram-negative bacteria have cell walls containing a thin layer of peptidoglycan and do not contain teichoic acids, resulting in a pink color after staining (27). This pink coloration is due to the loss of the primary stain when alcohol is applied, allowing the cells to retain the counterstain, carbol fuchsin (28).

The indole test was conducted to determine the ability of microorganisms to break down the amino acid tryptophan (29). Tryptophan can be deaminated and hydrolyzed by bacteria that express the enzyme tryptophanase. Indole is produced by the reductive deamination of tryptophan through the intermediate molecule indole pyruvate (30). The isolate SS 2 showed a positive indole result, while SS 1, EMB 1, and EMB 2 showed negative results. This indicates that isolate SS 2 can break down tryptophan, turning the surface of the medium red (22). The three isolates with negative results do not possess the ability to degrade tryptophan, resulting in no color change in the medium (29).

The methyl red (MR) test is used to determine the ability of microorganisms to ferment glucose with the production and stabilization of high concentrations of acidic end products (29). Various types of organic acids, such as succinic, acetic, formic, and lactic acids, are produced from glucose fermentation through the mixed acid fermentation pathway. Bacteria will produce large amounts of organic acids, resulting in a pH below 4.4. When MR reagent is added to a solution with a pH below 4.4, it turns red, indicating a positive result (31). In this study, isolate SS 2 showed a positive MR result, while isolates SS 1, EMB 1, and EMB 2 showed negative results. This indicates that isolate SS 2 can convert glucose into acidic products (22), turning the solution red when MR reagent is added, indicating a positive result (31). Isolates SS 1, EMB 1, and EMB 2 showed negative results, with no color change in the medium.

The Voges-Proskauer (VP) test was performed to determine the ability of organisms to produce neutral end products, such as acetylmethylcarbinol. The VP test uses alpha-naphthol reagent and 40% potassium hydroxide solution. To detect acetylmethylcarbinol, the end product must be oxidized to diacetyl. This reaction occurs in the presence of alpha-naphthol as a catalyst and the guanidine group present in peptone

and MR-VP media (29). The catalytic reaction of alpha-naphthol converts diacetyl to a red complex, showing a reddish-brown color as a positive result, while yellow indicates a negative result (31). In the VP test, all four isolates showed negative results. None of the isolates could produce neutral end products, such as acetylmethylcarbinol, resulting in no reddish-brown color change in the medium.

The citrate test in IMViC aims to distinguish between enteric organisms based on their ability to ferment citrate as the sole carbon source (29). The release of carbon dioxide and nitrogen forms carbonates and hydroxides (32). Citrate is produced by the condensation of active acetyl and oxaloacetate acid and is the first intermediate in the Krebs cycle. The enzyme citrate converts citrate to oxaloacetate and acetate, which are enzymatically converted to carbon dioxide and pyruvate. In this reaction, the medium becomes alkaline as carbon dioxide combines with sodium and water to form sodium carbonate, which is alkaline. The bromthymol blue indicator in the medium detects the presence of sodium carbonate, turning the medium from green to dark blue as a positive result (29). All four isolates showed positive citrate test results, indicating their ability to ferment citrate as the sole carbon source. The media for all four isolates changed from green to dark blue, indicating positive results.

The catalase test was conducted to observe the production of the enzyme catalase by microbes. This enzyme protects microbes from oxidative damage caused by reactive oxygen species. Catalase can reduce the bactericidal effects of hydrogen peroxide, with its concentration depending on the pathogenicity of the microbe. Organisms producing catalase rapidly degrade hydrogen peroxide (33). All four isolates also showed positive catalase test results, indicated by the formation of oxygen bubbles after adding hydrogen peroxide to the bacterial inoculum.

Bacterial motility was determined through the motility test. Bacterial motility is mainly due to the presence of flagella, which are the bacterial locomotor structures. This movement is known as Brownian motion (34). It is observed through the diffusion growth spreading along the inoculation line, causing the medium to become turbid (35). The motility test results showed that isolates SS 1, SS 2, EMB 1, and EMB 2 were motile, as evidenced by the turbid medium and diffusion spreading along the inoculation line.

The identification of the four isolates was based on the book "Microbiology: A Laboratory Manual 12th Edition" by Cappuccino and Welsh (29). Isolates SS 1, EMB 1, and EMB 2 showed negative indole, negative MR, negative VP, positive citrate, positive catalase, and positive motility results. According to Cappuccino and Welsh (29), these results indicate that the bacteria are *Alcaligenes* sp., with negative indole, negative MR, negative VP, and positive citrate results. *Alcaligenes* sp. also produces the enzyme catalase, showing positive catalase results indicated by the presence of oxygen bubbles. The genus *Alcaligenes* is also motile, with one or more peritrichous flagella (36).

Alcaligenes are free-living bacteria commonly found in soil and water, but they have also been identified within the human body, likely due to the accidental ingestion of contaminated food or water (37). While *Alcaligenes* are primarily known as food spoilage agents, their presence in the human body poses a significant public health risk. These bacteria are often resistant to common antibiotics, making infections difficult to treat, and are associated with nosocomial (hospital-acquired) infections (13). *Alcaligenes* can cause opportunistic infections in humans, becoming parasitic and leading to a range of serious diseases, thus posing a major public health concern (37,38). Infections caused by *Alcaligenes* often affect the urinary tract, including the urethra, bladder, and kidneys. Other reported infections include appendicitis, abscesses, meningitis, bloodstream infections, and endocarditis (37). Given their potential to be transmitted through contaminated food, especially vegetables, and their ability to cause severe infections, the presence of *Alcaligenes* in foodborne environments and clinical settings highlights

the critical need for effective control measures to mitigate the risk to public health.

According to the book "Microbiology: A Laboratory Manual 12th Edition" by Cappuccino and Welsh (29), SS 2, which showed positive indole, positive MR, negative VP, positive citrate, positive motility, and positive catalase results, is *Proteus* sp. This identification is based on Cappuccino and Welsh (29), stating that *Proteus* sp. has IMViC results of positive indole, positive MR, negative VP, and either positive or negative citrate. *Proteus* sp. also shows positive catalase results. Yuan et al. (39) noted that *Proteus* sp. is well-known for its swarming motility.

Proteus bacteria are among the most frequent opportunistic pathogens responsible for nosocomial infections, particularly affecting individuals with weakened immune systems (40). These bacteria are significant contributors to urinary tract and wound infections, posing a substantial public health concern. In addition to their role in healthcare-associated infections, *Proteus* species are also linked to foodborne illnesses, including diarrhea and food poisoning (41). Several cases of food poisoning attributed to *Proteus* contamination have been reported, especially in relation to meat products (18). The consumption of food contaminated with *Proteus* represents a serious public health risk, as it can lead to both foodborne infections and potentially severe complications, underscoring the importance of effective food safety measures to prevent contamination and protect public health (16).

Several strategies have been proposed to control foodborne pathogens in fresh produce. Edible coatings, for example, can deliver active ingredients, such as antimicrobial agents, that inhibit pathogen growth on food surfaces (6,42). Studies also indicate that washing fresh produce with solutions like chlorine, neutral electrolyzed water, or peroxyacetic acid may increase susceptibility to decay, as yeast and bacteria can suppress fungal growth on the surface (6,43). Additionally, UV-C radiation has been shown to improve product safety by reducing bacterial contamination. A treatment combining citric acid disinfection, oregano essential oil, and UV-C radiation has been developed to enhance the quality and extend the microbiological shelf life of ready-to-eat salads (6,44,45). These approaches offer practical solutions for food service operators and public health practitioners aiming to improve the safety and quality of fresh produce in commercial settings.

CONCLUSIONS

Based on the research conducted, it was found that pathogenic bacteria are present in lettuce samples from a cafeteria at Universitas Padjadjaran. The bacterial isolates SS 1, EMB 1, and EMB 2 were identified as *Alcaligenes* sp., while the isolate SS 2 was identified as *Proteus* sp., according to "Microbiology: A Laboratory Manual 12th Edition" by Cappuccino and Welsh (2019). *Alcaligenes* sp. and *Proteus* sp. opportunistic pathogens that can cause various diseases. Therefore, the public should be cautious when consuming raw lettuce, especially from sources with questionable cleanliness, as it can serve as a medium for disease transmission. To reduce the risk of contamination, food service providers should implement effective food safety practices, such as thoroughly washing raw vegetables, using disinfecting solutions, and ensuring proper handling and storage to prevent cross-contamination. These measures can help minimize foodborne pathogen risks and improve the safety of fresh produce.

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