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Isolation and Molecular Identification of Gram-Negative Bacteria in Tuna Sashimi (*Thunnus* spp.) Products in Manado City

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ABSTRACT

Sashimi is a typical Japanese dish consisting of thin slices of raw fish or seafood, which are served raw and therefore have the potential to be contaminated with bacteria, particularly Gram-negative bacteria. Tuna (Thunnus spp.) is one of the main ingredients of sashimi that is widely consumed in Manado City, but information on the presence and identity of Gram-negative bacteria in this product is still limited. This study aims to identify Gram-negative bacterial species in tuna sashimi circulating in Manado City molecularly based on the 16S rRNA gene through an exploratory study by taking samples from three restaurants in real operational conditions in accordance with consumer purchasing practices. Bacterial isolation was performed using MacConkey agar selective medium, followed by isolate purification, DNA extraction, 16S rRNA gene amplification using Polymerase Chain Reaction (PCR), visualization of PCR products through agarose gel electrophoresis, and analysis of sequencing results. Species identification was performed through sequence comparison using BLAST in the GenBank database. The results showed that five Gram-negative bacterial isolates were successfully identified, including *Klebsiella pneumoniae*, *Pluralibacter gergoviae*, and *Pantoea dispersa*, each exhibiting 100% similarity in the 16S rRNA gene sequence. The presence of these bacteria indicates a potential health risk to consumers, thus providing a scientific basis for quality control and improved food safety in tuna sashimi products in Manado City.

Keywords: Gram-negative bacteria; 16S rRNA gene; molecular identification; tuna sashimi; PCR.

INTRODUCTION

Sashimi is a typical Japanese dish consisting of thin slices of raw fish or seafood, so its quality and safety depend heavily on the quality of the raw ingredients and hygiene during handling and serving.[1] This product is known to have high nutritional value because it contains protein, omega-3 fatty acids, and various essential vitamins and minerals that are important for human health.[2] One of the raw materials commonly used in making sashimi in Indonesia, especially in the city of Manado, is tuna (*Thunnus* spp.) from abundant marine fishery resources.

Tuna (*Thunnus* spp.) is a high-value fishery commodity known to contain between 22.6 and 26.2 g of protein per 100 g of meat, as well as being rich in minerals and fat-soluble vitamins such as vitamin A and vitamin B complex.[3] Several species of tuna

commonly found in Indonesian waters include *Thunnus albacares*, *T. obesus*, and *T. alalunga*.[4] Despite its high nutritional value, the use of raw fish as food provides opportunities for bacterial contamination, particularly Gram-negative bacteria, during the handling, storage, and serving of sashimi products.[5]

Gram-negative bacteria are a group of bacteria with a complex cell wall structure consisting of three layers, namely an outer lipoprotein layer, a middle lipopolysaccharide (LPS) layer, and a thin inner peptidoglycan layer. This structure causes Gram-negative bacteria to be unable to retain the violet-iodine crystal complex in Gram staining and appear red.[6] Sashimi products have the potential to be contaminated with various Gram-negative bacteria, including *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., and *Serratia* spp., as well as other pathogens such as *Vibrio* spp. and *Salmonella* spp.[7] Previous studies have also reported the presence of additional Gram-negative bacteria in sashimi products, such as *Pseudomonas* spp., *Photobacterium* spp., *Acinetobacter* spp., and *Serratia* spp., indicating a potential risk of contamination for consumers.[2]

Gram-negative bacterial contamination in seafood products can cause various health problems, such as diarrhea, nausea, vomiting, abdominal pain, and fever, which generally develop into gastroenteritis. In individuals with underlying medical conditions or weakened immune systems, infections caused by Gram-negative bacteria have the potential to develop into fatal sepsis.[8,9] Additionally, the endotoxin component in the form of lipopolysaccharides (LPS) released by Gram-negative bacteria can trigger an excessive systemic inflammatory response and worsen the patient's clinical condition.[10]

Conventional bacterial identification through colony morphology observation and biochemical testing is still widely used, but these methods have limitations in distinguishing closely related species and require a relatively large amount of time and effort with limited accuracy.[11] Therefore, molecular identification methods such as Polymerase Chain Reaction (PCR) are needed to obtain more accurate identification results. One of the molecular markers commonly used in bacterial identification is the 16S rRNA gene, because this gene has conservative and hypervariable regions that allow bacterial identification down to the species level through nucleotide sequence analysis.[12,13]

Data on the isolation and molecular identification of Gram-negative bacteria in tuna sashimi in Manado City is still limited. This information is needed to identify contamination patterns and evaluate potential health risks to consumers. Conventional methods, such as morphological analysis and biochemical testing, have limitations in distinguishing closely related species and require relatively long detection times with varying degrees of accuracy.[14,11] Therefore, the application of molecular methods, such as PCR and 16S rRNA gene sequencing, is essential to obtain more accurate and reliable identification results, providing a basis for mitigating the risk of Gram-negative bacterial contamination in these products.

MATERIALS & METHODS

This research is an exploratory study conducted from September to November 2025 at the Advanced Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University.

Samples of tuna sashimi were obtained from three different restaurants in Manado City under actual operational conditions in accordance with consumer purchasing practices. Samples were received in sealed containers and immediately transported to the laboratory within 15 minutes using temperature-controlled transportation (22–25°C) to minimize changes in sample conditions prior to analysis.

The tuna sashimi sample was processed aseptically by cutting the fish tissue into

small pieces (approximately 1–2 cm), then suspended in a sterile saline solution and homogenized using a vortex. The initial suspension was then serially diluted to a dilution level of 10^{-4} to obtain a microorganism density that would allow separate colony growth and facilitate the isolation of pure colonies.[15,16] From each dilution level, 600 µL of suspension was inoculated onto MacConkey agar medium using the spread plate method, then incubated at 37°C for 24 hours.[17]

MacConkey agar is used as a selective and differential medium for the isolation of Gram-negative bacteria, due to its ability to inhibit the growth of Gram-positive bacteria and differentiate bacteria based on their ability to ferment lactose.[18] The medium was prepared according to the manual instructions of HiMedia Laboratories and sterilized using an autoclave at 121°C for 15 minutes.[19] All glassware used in this study was first sterilized with an autoclave under the same conditions to ensure the sterility of the process.[20]

Colonies that grew well on MacConkey agar were selected based on morphological characteristics and colony density. From the three samples, two colonies were randomly selected from each, resulting in six bacterial isolates. The isolates were purified using the streak plate method until pure cultures were obtained, then incubated at 37°C for 24 hours.[21,17] The pure cultures were then used for molecular identification.

Bacterial DNA extraction was performed using the Tissue Genomic DNA Mini Kit (Geneaid) according to the manufacturer's protocol with slight modifications. The extraction process included cell lysis using GT buffer incubated at 60°C, followed by the addition of GBT buffer, centrifugation, and DNA binding to a silica column (GS Column). The purification stage was carried out through gradual washing using W1 buffer and wash buffer, followed by DNA elution using elution buffer. The purified DNA obtained was then stored at freezing temperature until it was used for 16S rRNA gene amplification.

Molecular identification was performed through amplification of the 16S rRNA gene using the Polymerase Chain Reaction (PCR) technique. The PCR reaction was performed in a total volume of 40 µL consisting of 20 µL MyTaq HS Red Mix (Bioline), 1.5 µL 16S rRNA forward primer (BKYF), 1.5 µL 16S rRNA reverse primer (BKYR), 15 µL Milli-Q water (sterile), and 2 µL DNA template. Amplification was performed using a T-Personal thermal cycler (Biometra) with initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 30 seconds, and ended with a final extension at 72°C for 30 seconds.[22]

PCR products were verified using 0.8% agarose gel electrophoresis in 0.5X TBE buffer to confirm the presence of a 16S rRNA gene fragment approximately 975 bp in size.[23] Electrophoresis was run at 100V for 30 minutes, then the gel was stained with ethidium bromide and visualized using a UV transilluminator.[22] The PCR products obtained were sent for sequencing to First Base (Malaysia) along with the primers used in the PCR.

Sequencing results in the form of chromatograms were edited using Geneious Prime version 2025.0.3 by combining DNA sequences read from the forward and reverse directions. The combination was performed using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm.[24] The forward and reverse primer regions were cut, and the consensus sequence was then converted into FASTA (Fast Alignment) format for use in molecular identification. Identification was performed using BLAST (Basic Local Alignment Search Tool), which is available online on the NCBI (National Center for Biotechnology Information) website. Species determination was based on the similarity of the 16S rRNA gene sequence, with an identity threshold of ≥97% widely used in bacterial taxonomy, and referring to the latest updates showing that sequences

from the same species generally have a minimum similarity of 97.2–100%.[13]

RESULTS AND DISCUSSION

Observation of Bacterial Isolates

Isolation of bacteria from tuna sashimi samples on MacConkey agar media showed varying colony growth at each dilution factor (Figure 1). In Petri dishes with a dilution of 10-1, bacterial colonies grew quite densely but could still be observed individually. The color of the colonies varied, with most appearing pink and others appearing colorless or clear. This color difference indicates the presence of bacteria that are capable and incapable of fermenting lactose in the medium. MacConkey agar is a selective and differential medium that supports the growth of Gram-negative, rod-shaped bacteria while enabling differentiation between lactose-fermenting and non-lactose-fermenting bacteria.[25]

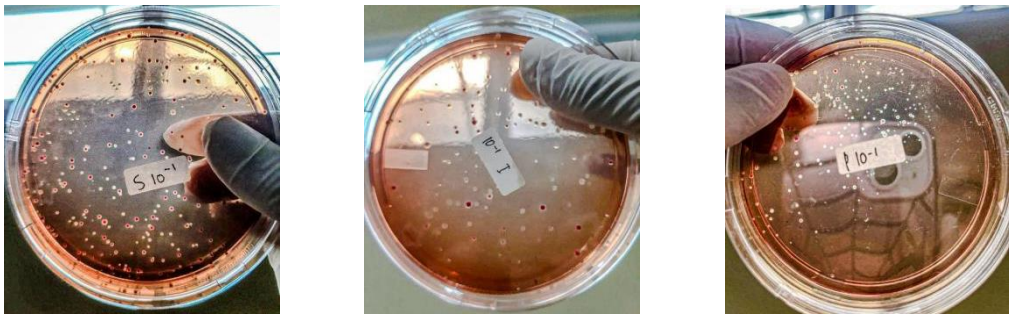

Figure 1. Bacterial culture from tuna sashimi samples with serial dilution of 10^{-1} on MacConkey agar medium

Figure 2. Pure bacterial isolates from tuna sashimi samples using the quadrant streak plate method

Figure 2 shows pure bacterial isolates obtained from tuna sashimi samples collected from three different locations in Manado City. Each isolate was labeled AP1A, AP1B, AP2A, AP2B, AP3A, and AP3B. Isolates with the code A formed pink colonies, indicating lactose-fermenting bacteria, whereas isolates with the code B formed colorless colonies, indicating non-lactose-fermenting bacteria.

Visualization of PCR products and sequencing results

The results of PCR product visualization using agarose gel electrophoresis (Figure 3) showed the presence of DNA bands in six bacterial isolates tested, namely AP1A, AP1B, AP2A, AP2B, AP3A, and AP3B. Based on comparison with the 1 kb DNA ladder, five isolates (AP1A, AP1B, AP2A, AP3A, and AP3B) showed clear single bands in the range of ±1000 bp, while isolate AP2B showed a less clear band. Isolate AP2B was not proceeded to the sequencing analysis stage because it produced a low-quality chromatogram characterized by the presence of ambiguous nucleotides (N). Low-quality PCR products are known to affect the clarity of DNA bands in electrophoresis and the quality of sequencing results.

Errors during the PCR process, including nucleotide misincorporation and byproduct formation, can result in non-homogeneous amplification products, leading to blurred DNA bands. In addition, low-quality PCR products may produce chromatograms with weak signals or overlapping peaks, resulting in base-calling failures and the presence of ambiguous nucleotides (N).[27]

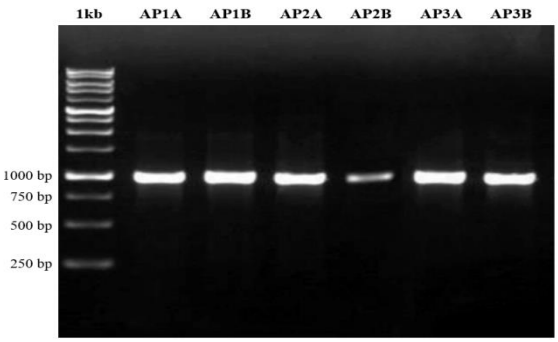

Figure 3. PCR results of bacterial isolates from tuna sashimi products

Chromatogram sequencing of the 16S rRNA gene of Gram-negative bacterial isolates

The chromatogram of the 16S rRNA gene sequencing results of Gram-negative bacterial isolates from tuna sashimi products is visualized in the form of a chromatogram showing the nucleotide base readings by the sequencer machine. Each nitrogen base is displayed as a colored peak, namely adenine (A), thymine (T), guanine (G), and cytosine (C), with the quality of the reading determined by the height of the peak and the minimal overlap between signals. Base reading quality is visualized in the form of Base Call Quality in the Geneious Prime software, which is indicated by shades of blue, where dark blue represents low base quality and light blue indicates higher quality.[28]

Based on the DNA sequencing results (Figures 4–8), the chromatograms of the five isolates (AP1A, AP1B, AP2A, AP3A, and AP3B) generally showed sharp nucleotide peaks that were clearly separated with minimal overlap, indicating good sequence read quality. A decrease in signal clarity was observed at the beginning of the AP2A isolate sequence and at the end of the AP3A and AP3B isolate sequences, while the AP1A and AP1B isolates showed relatively stable signals. These conditions are common characteristics of Sanger sequencing results and can be corrected through the trimming process.

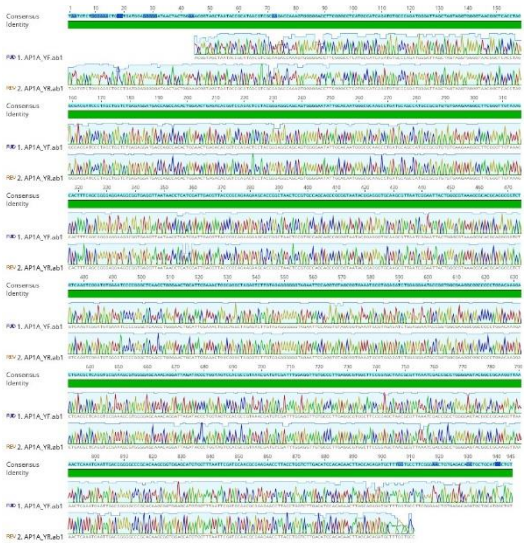

Figure 4. Chromatogram of Isolate AP1A

Figure 5. Chromatogram of Isolate AP1B

Figure 6. Chromatogram of Isolate AP2A

Figure 7. Chromatogram of Isolate AP3A

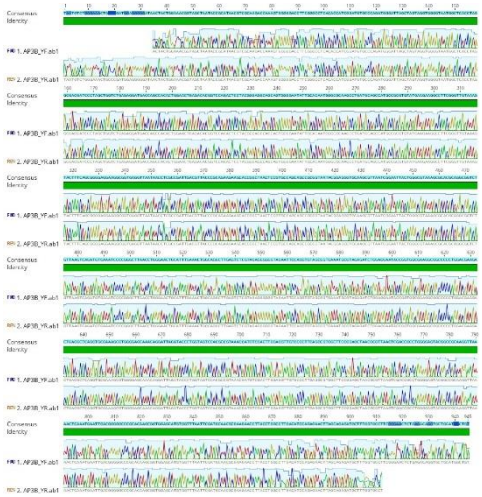

Figure 8. Chromatogram of Isolate AP3B

Identification of Gram-negative bacterial isolates based on BLAST analysis of the 16S rRNA gene

The results of BLAST analysis (Table 1) of the 16S rRNA gene sequences showed that the five Gram-negative bacterial isolates

were identified, including *Klebsiella pneumoniae*, *Pluralibacter gergoviae*, and *Pantoea dispersa*. Isolate AP1A exhibited 100% identity with *K. pneumoniae*. Isolates AP1B and AP2A were identified as *P. gergoviae*, each showing 100% identity, while isolates AP3A and AP3B exhibited 100% identity with *P. dispersa* based on the GenBank database. All isolates also showed a query cover of 100%.

Table 1. Results of BLAST analysis of 16S rRNA genes of Gram-negative bacterial isolates

Isolate	Scientific Name	Query Cover(%)	Percent Identity (%)	Accession
AP1A	*Klebsiella pneumoniae*	100	100	CP058752.1
AP1B	*Pluralibacter gergoviae*	100	100	LR699009.1
AP2A	*Pluralibacter gergoviae*	100	100	PQ336984.1
AP3A	*Pantoea dispersa*	100	100	OK255670.1
AP3B	*Pantoea dispersa*	100	100	CP157882.1

Isolates AP1B and AP2A, identified as *Pluralibacter gergoviae*, and isolates AP3A and AP3B, identified as *Pantoea dispersa*, showed identical sequencing results despite appearing as colonies of different colors on MacConkey agar medium. This condition is in line with the concept of *phenotypic variants* (PV), namely differences in colony morphology in isolates from the same species without significant genetic differences.[29] These phenotypic differences may be caused by variations in gene expression levels, including genes that affect lactose metabolism or pigment production. This suggests that although MacConkey agar is differential, the variation in colony color in isolates AP1B–AP2A and AP3A–AP3B reflects differences in gene expression rather than species differences.

The presence of Gram-negative bacteria identified in this study, namely *Klebsiella pneumoniae*, *Pluralibacter gergoviae*, and *Pantoea dispersa*, indicates potential contamination risks in sashimi products. *Klebsiella pneumoniae* is a Gram-negative, rod-shaped, facultatively anaerobic, encapsulated bacterium capable of fermenting lactose. Its pathogenicity is mediated by a range of virulence factors, including a polysaccharide capsule, lipopolysaccharide (LPS), siderophores, and fimbriae, which collectively facilitate immune evasion, host cell adhesion, iron acquisition, and the induction of inflammatory responses.[30] This species is widely recognized as an opportunistic pathogen responsible for a broad spectrum of infections, such as pneumonia, meningitis, sepsis, and urinary tract infections, and represents a major contributor to both community-acquired and healthcare-associated infections.[31] In the present study, BLAST analysis of isolate AP1A 100% identity with *K. pneumoniae* strains STLIN_19 (CP058752.1). KSB1_7G (CP110723.1), and strain 189 (CP125121.1), thereby confirming its taxonomic identity and supporting its clinical relevance.

In addition to *Klebsiella pneumoniae*, other Gram-negative bacteria identified in this study exhibited notable clinical and environmental significance. *Pluralibacter gergoviae* is a Gram-negative, rod-shaped bacterium belonging to the family Enterobacteriaceae, previously classified as *Enterobacter gergoviae*. It is widely distributed in environmental sources, including humans, animals, food, wastewater, and oils. Although generally considered to have low virulence, *P. gergoviae* acts as an opportunistic pathogen and has been associated with nosocomial infections, including cases in neonatal intensive care units and immunocompromised or long-term hospitalized patients. The bacterium also exhibits potential resistance to multiple antibiotics, although some strains remain susceptible to β-lactams, fluoroquinolones, aminoglycosides, and carbapenems,

indicating its role as an environmental organism with opportunistic pathogenic potential.[32,33] The identification of *P. gergoviae* in isolates AP1B and AP2A is consistent with previous findings on *Pluralibacter* sp. strain SC (KX981861.1), which reported its broad environmental distribution and association with insect microbiota.[34] BLAST analysis of the 16S rRNA gene showed that isolate AP1B shared 98.89% identity with *Pluralibacter* sp. strain SC and 100% identity with *P. gergoviae* MGYG-HGUT-02520 (LR699009.1), while isolate AP2A showed 100% identity with strain B7114 (PQ336984.1), confirming their classification as *Pluralibacter gergoviae*.

The identification of *Pantoea dispersa* further highlights the diversity of Gram-negative bacteria detected in this study. This species is widely distributed across environmental niches, including soil, water, and plant-associated habitats, and demonstrates a strong capacity for environmental adaptation through biofilm formation and resistance mechanisms.[35,36] Although traditionally regarded as an environmental organism, *P. dispersa* has been increasingly implicated in clinical infections, including respiratory infections, neonatal sepsis, and bacteremia, even among immunocompetent individuals. BLAST analysis revealed that isolate AP3A exhibited 100% Identity with *P. dispersa* strain ZJ62 (OK255670.1), whereas isolate AP3B demonstrated 100% identity with strain XJ2 (CP157882.1), both of which have been characterized in previous genomic studies.[37,38] These results support the identification of both isolates as *Pantoea dispersa*.

CONCLUSION

Based on the results of this study, it can be concluded that Gram-negative bacteria successfully identified molecularly based on the 16S rRNA gene in tuna sashimi (*Thunnus* spp.) products obtained from three restaurants in Manado City include *Klebsiella pneumoniae*, *Pluralibacter gergoviae*, and *Pantoea dispersa*. The identification was carried out through isolation on selective media, amplification of the 16S rRNA gene using the Polymerase Chain Reaction (PCR) technique, and sequence analysis compared with the GenBank database with the highest sequence reaching 100% identity. These findings provide preliminary taxonomic information on the presence of Gram-negative bacteria in tuna sashimi products in Manado City and indicate the potential risk of microbial contamination in food products.

Declaration by Authors

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Conflict of Interest: No conflicts of interest declared.

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