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IDENTIFICATION OF BACTERIA FROM FOOD COURT WASTEWATER BASED ON 16S rRNA GENE SEQUENCES

Identifikasi Bakteri dari Air Limbah Food Court Berdasarkan Sekuen Gen 16S rRNA

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ABSTRACT

Environmental safety and public health are greatly influenced by the microbiological elements such as bacterial community, where these organisms can be used as indicators of environmental quality and sources of potential biological hazards. The identification of bacteria in wastewater is an important aspect of environmental microbiology studies. The purpose of this study was to identify the bacterial species in food court wastewater at State University of Surabaya (UNESA) Campus 1. The methods used were exploratory, including bacterial cultivation, DNA extraction, quantitative and qualitative DNA analysis, amplification of 16S rRNA gene fragments, sequencing, and phylogenetic tree reconstruction. From phylogenetic analysis, five test isolates were related to the families Enterobacteriaceae, Aeromonadaceae, Chromatiaceae, and Halomonadaceae. Isolates BU4 and BU5 were most closely related to genus Aeromonas with a similarity of 98.60% and 97.27%, respectively, while isolate BU3 was closely related to genus Klebsiella with a similarity of 96.91%. Meanwhile, isolates BU1 and BU2 cannot yet be identified into a bacterial genus. The results of this study provide important information regarding the prediction of pathogenic bacteria types, which can serve as the basis for recommending further action to prevent disease, improve water quality, and increase the sterility of the food court area.

Keywords: *Bacteria, Food court, Phylogenetics, 16S rRNA, Wastewater*

ABSTRAK

Keamanan lingkungan dan kesehatan publik sangat dipengaruhi oleh unsur mikrobiologi seperti bakteri yang dapat menjadi indikator kualitas lingkungan dan sumber potensi bahaya biologis. Identifikasi bakteri di selokan air merupakan aspek penting dalam studi mikrobiologi lingkungan. Tujuan penelitian ini untuk mengidentifikasi jenis bakteri di air limbah food court Universitas Negeri Surabaya (UNESA) Kampus 1. Metode yang digunakan yakni eksploratif meliputi peremajaan bakteri, ekstraksi DNA, analisis kuantitatif dan kualitatif DNA, amplifikasi fragmen gen 16S rRNA, sekuensing dan rekonstruksi pohon filogenetik. Dari analisis filogenetik, kelima isolat uji memiliki kekerabatan dengan famili Enterobacteriaceae, Aeromonadaceae, Chromatiaceae, dan Halomonadaceae. Isolat BU4 dan BU5 memiliki kekerabatan terdekat dengan Aeromonas caviae dengan similaritas 98,60% dan 97,27% serta isolat BU3 berkerabat dekat dengan Klebsiella pneumonia dengan similaritas 96,91%. Sementara itu, isolat BU1 dan isolat BU2 berkerabat dengan genus Enterobacter namun memiliki similaritas yang rendah yakni 89,74% dan 83,31%. Hasil penelitian ini menunjukkan bahwa isolat BU4 dan BU5 merupakan bakteri genus Aeromonas dan isolat BU3 merupakan genus Klebsiella. Sementara isolat BU1 dan BU2 belum dapat diidentifikasi pada level genus. Penelitian ini memberikan informasi penting mengenai prediksi ragam bakteri patogen yang dapat mendasari rekomendasi tindak

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lanjut untuk mencegah penyakit, perbaikan kualitas air, serta peningkatan sterilitas area food court.

Kata kunci: *Air limbah, Bakteri, Filogenetik, Food court, 16S rRNA*

INTRODUCTION

Urbanization, industry, and increasing human population in developing countries contribute to the production of wastewater (Islam, 2023). Wastewater is a complex mixture consisting of various organic and inorganic components originating from human, industrial, and environmental activities (Chan et al., 2022; Jamrah et al., 2023; Kumari et al., 2025). The content of organic and inorganic materials in wastewater creating a suitable habitat for the growth of microorganisms such as bacteria. Wastewater provides a great opportunity for the growth of various bacteria because it can provide moisture, warmth, and shelter or symbiosis with other organisms (Santoshi & Kiran, 2022). In wastewater environments, bacteria may act as the main drivers of water management systems with varying mechanisms in degrading pollutants (Aragaw, 2021). They decompose organic pollutants, nutrients, and solids, thereby improving water quality (Singh et al., 2021). However, these bacteria also have a pathogenic potential. Thus, wastewater composition can indicate the state of an environment and can be used as a parameter for environmental safety. This parameter is related to the presence of pathogenic bacteria, which requires further investigation with microbiological approach.

Bacteria found in wastewater can vary from pathogenic to commensal. Pathogenic bacteria, such as *Escherichia coli* and *Salmonella* spp. can cause gastrointestinal diseases and other infections if it gets into the human or animal food chain (Ringler et al., 2000) Research by Sebayang et al. (2025) shows that *E. coli* and *Coliform* bacteria, which are harmful to human health, were found in domestic sewage, with all samples testing positive for *Coliform* at levels exceeding 3000 colonies/100 mL. This indicates the potential for serious biological contamination, as some species of *Coliform* bacteria can cause stomach ache, vomiting, fever, or diarrhea. Selvarajan et al. (2021) also showed that there are several bacteria in domestic wastewater, such as the genera *Klebsiella, Corynebacterium, Bordetella,* and *Staphylococcus*, which are dangerous pathogenic bacteria. Meanwhile, non-pathogenic bacteria can play a role in bioremediation and the degradation of organic matter (Feng et al., 2018). Commensal bacteria found in wastewater, namely *Enterococcus* sp. and *Enterobacter* sp. which were identified by Byappanahalli et al. (2012). These commensal bacteria play a role in processing dye waste and degrading plastic (Eslami et al., 2019; Sun et al., 2024). Therefore, the identification of bacteria present in drainage channels may help in understanding the composition of the environmental microbiota but also plays a crucial role in health risk assessment and water quality management (Ammar & Farag, 2019).

Microbiological research on wastewater has actually been carried out quite a lot, but most of it still focuses on household, industrial, or hospital waste. (Akhtar et al., 2021). While studies specifically examining food court wastewater are still limited, particularly from campus environments like the Food Court at State University of Surabaya. This is despite the fact that wastewater from foodcourt at State University of Surabaya differs in its characteristics, containing a mixture of food scraps, oil, high levels of organic matter, and wastewater from kitchen equipment washing and toilet flush. The food court also lacks a fully enclosed drainage system and lacks a clear water-flow route to the main drainage system. Because food court is a public space, it is necessary to carry out an environmental hazard analysis based on microbiological approach, such as bacterial identification. Some studies rely on morphological and biochemical identification. However this method is less accurate for identification of bacteria at the species level (Golnari et al., 2024). So, it is necessary to carry out a

molecular approach identification based on 16S rRNA gene phylogenetic analysis (Weinroth et al., 2022). This analysis is important because it can provide insight into whether wastewater bacteria are closely related to disease-causing bacteria, thus impacting campus environmental health (Aminu et al., 2024).

Methods for identifying bacteria in wastewater include microbiological and molecular techniques, such as bacterial culture, Gram staining, PCR (Polymerase Chain Reaction) techniques, and sequencing. These techniques enable researchers to identify specific types of bacteria and understand their role in the wastewater ecosystem (Semenov & Overbeek, 2021). The importance of identifying bacteria in wastewater lies in their ability to provide information about the potential health risks and environmental impacts of pollution. Through a better understanding of the bacterial variation, control and prevention measures can be designed to reduce health risks and maintain ecosystem sustainability (Zhu & Chen, 2020). Based on this, it is necessary to conduct research on wastewater from the UNESA food court to identify various types of bacteria and perform phylogenetic analysis to determine their relationship with other species. It is hoped that this research may provide recommendations to the authorities regarding wastewater management policies and zero waste efforts in the campus environment.

MATERIALS AND METHODS

Research Location and Time

The research was conducted from June 2024 to January 2025. DNA isolation and PCR were performed at the Molecular Biology Laboratory of the Faculty of Mathematics and Natural Sciences, State University of Surabaya. Sequencing was performed at First Base Malaysia.

Materials

Five bacterial isolates stock obtained from composite samples of food court wastewater from four sampling points at Campus 1 of State University of Surabaya (-7.314193 E, 112.726715 S) were used as test isolates. Material used in this study included Nutrient Agar (NA) medium, Bacterial DNA Preparation Kit (Jena Biosciences, cat.# PP-2365), TAE 1x, agarose, EtBr, TAE 10x, PCR master mix (GoTaq® Green Master Mix, Promega), Primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), primer 1492R (5'-GGTTACCTTGTTACGACTT-3') and Nuclear Free Water.

Recultivation Bacteria

Five isolates (BU1, BU2, BU3, BU4, BU5) obtained from previous study were recultured on NA media and incubated for 24 hours at 37°C. The characteristics of reculture isolates were then compared tothe stock isolates before proceeding to the next analysis.

Nucleic Acid Extraction

The stock culture was taken 1 mL for DNA extraction. DNA extraction was performed using the spin column method with the Bacterial DNA Preparation Kit (Jena Biosciences). In the first stage, cell lysis was prepared according to the type of gram bacteria, in this case, according to the procedure for gram-negative bacteria. 500 µL of culture was disrupted for 1 minute at a speed of 10,000 g. Then, the supernatant was discarded. Then, add 300 µL of lysis buffer and 2 µL of RNase A, and then vortex for 60 seconds. Add 8 µL of Proteinase K, then homogenized and incubated for 10 minutes at 60°C, then cooling down for 5 minutes. Add 300 µL of binding buffer and vortex, then place on ice for 5 minutes. After that, centrifuge as before. The steps continue with activating the column by placing the spin column in the tube, then adding 2 mL of activation buffer, then centrifuge for 30 seconds and discard the liquid that passes through. Then, the column loading stage, which involves pipetting the lysis product into the spin column, centrifuged for 1 minute, and discarding the filtrate. Next step is primary washing, which involves added 500 µL of washing buffer to the spin column and centrifuged for 1 minute, then discard the filtrate. Secondary washing is performed by adding washing buffer back into the spin column, then centrifuged for 30 seconds and discard the supernatant. Then, centrifuge again for 1 minute and discard the

supernatant to remove any remaining washing buffer. The final step is to transfer the spin column to an elution tube, then place 50 µL of elution buffer right in the middle of the column. Incubate for 1 minute at room temperature. Then centrifuge for 2 minutes and collect the eluted DNA.

Quantitative and Qualitative Analysis

The extracted DNA can then be quantitatively analyzed using a nanodrop spectrophotometer. This quantitative analysis is performed to determine the purity and concentration of the extracted DNA. The sensor on the nanodrop spectrophotometer was dripped with a blank solution, then cleaned with 70% alcohol and wiped with a tissue. One microliter of the isolated DNA was placed on the spectrophotometer sensor, and the analysis results were obtained from a connected computer device. Spectrophotometry analysis results which show a purity value of more than 1.8-2.0 indicated pure DNA and a concentration value of more than 20 mg/µL indicates success (Sophian & Syukur, 2021).

Qualitative analysis was performed using electrophoresis. Electrophoresis requires a separation medium in the form of a stationary phase, such as agarose gel mixed with a buffer solution to maintain the acidity of the sample during the separation process. The speed of molecules depends on the ratio of charge to mass and also on the shape of the molecule. In qualitative testing using electrophoresis, agarose gel was made by mix 1x TAE, 50 grams of agarose, and 1 µL of EtBr. The electrophoresis tank was filled with gel and 10x TAE solution. Then, after electrophoresis for 30 minutes at a voltage of 100 volts, it is observed with a UV transluminator (Lee et al., 2012). DNA extraction results that meet the standards proceed to the PCR stage.

16S rRNA Gene Amplification

Amplification is the process of making copies of extracted DNA, which will be analyzed in the next stage. Amplification is carried out using Polymerase Chain Reaction (PCR). The DNA extraction results from each isolate are taken in 10 µL and placed in a PCR tube. Then, 12.5 µL of PCR master mix, 0.5 µL of 27F primer, 0.5 µL of 1492R primer, 10.5 µL of NFW, and 1 µL of template DNA were added. The PCR process was carried out in a Mastercycler 5330 machine (Eppendorf). The PCR cycle began with initial denaturation at 95 °C for 5 minutes, followed by 30 cycles: (denaturation at 95 °C for 1 minute, annealing at 50 °C for 1 minute, and polymerization at 70 °C for 1 minute) and final polymerization at 70 °C for 10 minutes.

Visualization of PCR Product Result

The success of the PCR was analyzed using 2% agarose gel electrophoresis by adding 1µL ONI Mark 100 RTU 1kB marker to the first . A total of 1 µL of loading dye was added to 2 µL of amplicon DNA, then homogenized using the pipetting method. Then, it was inserted into the agarose gel well. A positive result was indicated by a band with a size of ~1,500 bp.

Sequencing

Qualified PCR amplicons sent for sequencing are those with good quality and quantity, namely a concentration of approximately 20 mg/µl. The amplicons were sent to the sequencing service provider First Base Malaysia. The quality of the sequencing chromatograms was assessed manually using BioEdit software through visual inspection of peak clarity, double peaks, and ambiguous base (N) removal. Low-quality sequences were manually trimmed at the beginning and end. Then, chromatograms from 2 reaction were proceeded to make consensus sequence.

BLAST Analysis

Consensus sequence from prior analysis used as entry in BLAST analysis. The Basic Local Alignment Search Tool (BLAST) is a tool used to find regions of local similarity between protein or nucleotide sequences available at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (Wheeler & Bhagwat, 2008). Sequence identification was performed using BLASTn with default parameters on the NCBI platform with the nucleotide database (NCBI nt). Match searches were performed against the 16S ribosomal RNA sequences (Bacteria and Archaea) database as a reference base.

Results were evaluated based on percent identity, query coverage, and E-value to determine the level of taxonomic closeness. The identity threshold used to determine genus level is 95% - 98.7% and species-level matches is 98.7% (Stackebrandt & Ebers, 2006).

Reconstruction of the Phylogenetic Tree

Phylogenetic tree reconstruction was performed using BioEdit and MEGA11 software. BioEdit software was used to prepare DNA sequences, while MEGA11 was used for base sequence alignment and phylogenetic tree reconstruction. The purpose of phylogenetic reconstruction was to identify the relationships between various identified bacteria (Abdelsalam et al., 2022). Evolutionary history was explained using the neighbor-joining method. Evolutionary distances were calculated using the Maximum Composite Likelihood method. All ambiguous deletion positions from each sequence pair were removed using the pairwise deletion option.

RESULT AND DISCUSSION

The total DNA obtained was then analyzed for quality and quantity using a spectrophotometer and agarose gel electrophoresis. The results of the qualitative and quantitative analysis of the DNA extraction are shown in **Table 1.**

Table 1. Analysis of the quantity of bacterial DNA extracted

Isolate code	Absorbance ratio A260/280	Concentration (ng/µL)
BU1	1.94	15.5
BU2	1.74	92.9
BU3	1.84	67.1
BU4	1.82	96.7
BU5	1.86	103.5

The results shown that the purity of the DNA samples ranged from 1.74 to 1.94 (**Table 1**). Isolates BU1, BU3, BU4, and BU5 showed good purity levels with the highest absorbance ratio, namely isolate BU1 with a value of 1.94, while sample BU2 had a slightly lower purity with an absorbance ratio of 1.74. Absorbance ratios below 1.8 indicate that the sample may be contaminated with protein or phenol. Meanwhile, ratios above 2.0 may indicate that the sample is contaminated with RNA. The concentrations of all isolates were in the range of 15.5–103.5 ng/µL. Isolate BU5 had the highest DNA concentration of 103.5 ng/µL, indicating excellent isolation results in terms of quantity. Isolate BU1 had a relatively low concentration, indicating that the amount of DNA isolated was less than that of other isolates. Overall, the quality and quantity tests demonstrated that all DNA isolates achieved a high degree of purity and adequate concentrations, making them suitable for downstream applications like PCR and sequencing.

Gene fragment amplification using PCR can exponentially duplicate the target DNA segment in three stages, denaturation, annealing, and elongation (Gerace et al., 2022). The PCR results were then further analyzed using gel electrophoresis, and a single band was formed, indicating that the 16S rRNA gene target was successfully amplified and there was no contamination (Khan et al., 2021). The results of the single band formed on the electrophoresis gel can be seen in **Figure 1**.

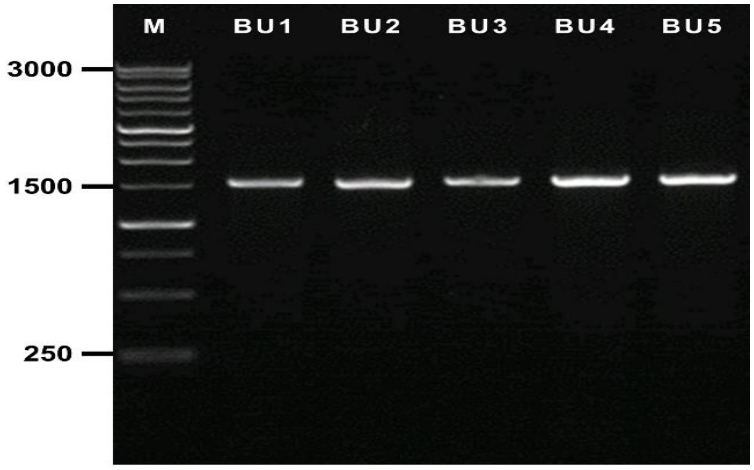

Figure 1. DNA Quality Testing using Agarose Gel Electrophoresis. M = marker/DNA ladder

Figure 1 shows the results of DNA quality testing of PCR products using agarose gel electrophoresis. The suitability of the samples for the subsequent sequencing phase was contingent upon the results of this test. Samples BU1, BU2, BU3, BU4, and BU5 have passed the examination. This was indicated by the presence of thick and clear DNA bands with a size of 1,500 bp on the agarose gel (**Figure 1**). This indicates that these five samples have successfully amplified the target DNA in sufficient quantitiesDNA sequencing produces a nucleotide base sequence, which was then aligned and analysed using BLAST. The BLAST results show bacterial species that have close similarities to the isolate, as can be seen in **Table 2** and the phylogenetic tree showing the relationships in **Figure 2** below:

Table 2. Isolate code and its closest species

Isolate code	Closest species	Sequence similarity	Accession number of the closest species
BU1	*Enterobacter sichuanensis*	89.74% (1528)	NR_179946.1
	Enterobacter quasiroggenkampii	89.74% (1538)	NR_179166.1
BU2	*Alkalimonas amylolytica*	83.31% (1510)	NR_041797.1
BU3	*Klebsiella pneumonia*	96.91% (1530)	NR_117686.1
BU4	*Aeromonas caviae*	98.60% (1494)	NR_104824.1
BU5	*Aeromonas caviae*	97.27% (1494)	NR_104824.1

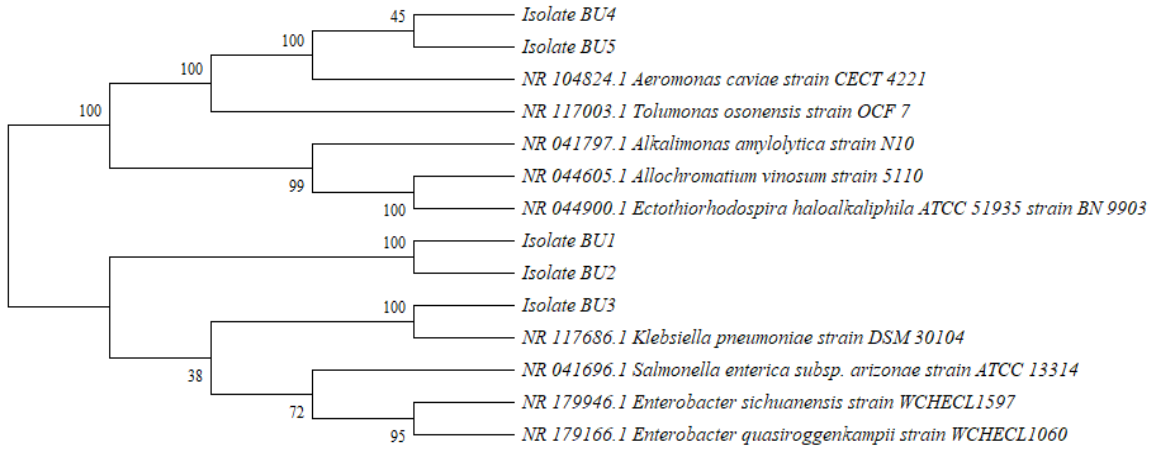

Figure 2. Phylogenetic tree result

The phylogenetic tree (Figure 2) shows the phylogenetic relationships between several bacterial references and test bacterial isolates based on the 16s rRNA gene sequence. Isolates BU4 and BU5 were most closely related to *Aeromonas caviae* bacteria with similarity values of 97.27% and 98.60%. Isolate BU3 also has a close relationship with the species *Klebsiella pneumonia* (96.91%). Isolates BU1 and BU2 have a close relationship with the genus *Enterobacter* and *Alkalimonas,* but the species cannot yet be determined because they have low similarity, that is 89.74% and 83.31%.

The phylogenetic tree uses four outgroup bacteria, namely *Tolumonas osonensis, Allochromatium vinosum, Ectotiorhodospira haloalkaliphia,* and *Salmonella enterica* (**Figure 2**). The common outgroup of the bacteria *Ectotiorhodospira haloalkaliphilia, Allochromatium vinosum, Alkalimonas amylolytica*, and *Tolumonas osonensis* was shown in the clade of isolates BU4 and BU5. Isolates BU1, BU2, and BU3 also appear to have the same ancestor as the genera *Klebsiella, Salmonella,* and *Enterobacter* as proved by the clade formation.

Based on the sequencing results and phylogenetic tree reconstruction, it is known that the types of bacteria in the food court drain water are closely-related to both pathogenic and saprophytic bacteria. *Enterobacter sichuanensis, Enterobacter quasirrogenkampii, Klebsiella pneumonia*, and *Aeromonas caviae* are pathogenic bacteria. Is a group of Gram-negative rod-shaped (bacillus) bacteria that are facultative anaerobes. They are generally motile due to having a single polar flagellum and show positive reactions in the biochemical tests for oxidase and catalase (Lau et al., 2023). Their primary habitat is aquatic environments, widely distributed in freshwater, brackish water, and even saltwater such as lakes, rivers, and estuaries (Fernández-Bravo & Figueras, 2020). Although they are normal inhabitants of these environments, *Aeromonas* are also known as opportunistic pathogens that can cause various infections in humans and animals. In humans, the most common infections are gastroenteritis due to consuming contaminated water or food, severe wound infections, and systemic infections such as septicemia in individuals with weakened immune systems (Sadeghi et al., 2024).

Bacteria in the genus *Klebsiella* are opportunistic pathogens with thick polysaccharide capsules. They form mucoid (slime-producing) colonies and are non-motile Gram-negative bacteria (Dong et al., 2022). *Klebsiella* is ubiquitous in nature, inhabiting environments such as soil, water, and plants (Cortés-Sánchez et al., 2025). In addition, these bacteria are also part of the normal flora of mammalian mucous membranes, including the human intestines and respiratory tract. *Klebsiella* can infect individuals with compromised immune systems or enter sterile parts of the body, often in hospital settings (nosocomial infections). Diseases that can be caused include urinary tract infections (UTIs), pneumonia, meningitis, wound infections, and bacteremia (bloodstream infections) that can lead to sepsis (Sun et al., 2021; Guerra et al., 2022).

The genus *Enterobacter* is a Gram-negative bacillus that is generally motile with peritrichous flagella. They part of Enterobacteriaceae family. *Enterobacter* is a facultative anaerobe capable of fermenting lactose to produce acid and gas (Rahi et al., 2024). *Enterobacter* can be found in various environments such as soil, water, waste, and plants (Moxley, 2022). Additionally, these bacteria are commonly found in the digestive tracts of humans and animals. Although often harmless to healthy hosts, *Enterobacter* can act as a significant opportunistic pathogen, particularly in hospital settings. Species such as *Enterobacter cloacae* and *Enterobacter aerogenes* are often associated with nosocomial infections (infections acquired in hospitals), causing various diseases such as urinary tract infections, bloodstream infections (bacteremia), pneumonia, and infections in surgical wounds and soft tissues (Yang et al., 2018; Janda & Abbott, 2021).

Poorly maintained wastewater systems have the potential to become incubators and media for the spread of dangerous pathogenic bacteria. Domestic waste, which is rich in organic matter and feces, is an ideal source of nutrients for pathogenic bacteria to multiply (Kumar et al., 2025).

Various wastewater treatment methods can be applied to prevent the spread of

harmful bacteria, such as filtration, flotation, adsorption, photocatalysis, electrooxidation, coagulation, and flocculation (Gogate & Pandit, 2024). Without proper management system, this wastewater can seep and contaminate drinking water sources (wells or rivers), overflow during rainfall, and may become a breeding ground for disease vectors such as flies and mosquitoes (Tariq & Mushtaq, 2023). This is where the important role of a structured wastewater management system comes in, namely through a closed piping system connected to a communal or centralized Wastewater Treatment Plant (WWTP). In the WWTP, wastewater will go through a series of biological and disinfection processes that effectively kill or drastically reduce the number of pathogenic bacteria to a safe level before being discharged back into the environment, thereby breaking the chain of disease transmission (Rawis et al., 2022). Recommendations regarding wastewater treatment include managing wastewater infrastructure, educating the public about waste management and disposal, and promoting clean and healthy living practices.

CONCLUSION

Based on research on wastewater from the food court at State University of Surabaya (UNESA) Campus 1, various types of bacteria were identified through molecular analysis of the 16S rRNA gene, including several that are potentially pathogenic to humans. Phylogenetic analysis results showed the presence of bacterial isolates closely related to the genus *Klebsiella* and *Aeromonas*. The presence of these pathogenic bacteria indicates a serious potential health risk for the community around the area, so this study emphasizes the importance of further action to improve water quality and increase food court sterility to prevent the spread of disease. However, in this study, bacterial identification using 16S rRNA gene is still not able to reveal the species of the isolates. Therefore, further analysis such as whole-genome sequencing is recommended.

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