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Isolation and Molecular Identification of Nitrate-Reducing Bacteria from Swiftlet Houses in Sumedang, West Java

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ABSTRACT

Edible bird's nest is an important export commodity that is currently of concern to the Indonesian government. One of the requirements for exporting edible bird's nest to the People's Republic of China is that this product must meet the requirements for nitrite content in the product below 30 ppm. This nitrite contamination can be obtained from the results of nitrogen metabolism from nitrifying bacteria. However, information on nitrite-reducing bacteria in swiftlet houses has never been reported and is urgent in controlling nitrifying bacteria in swiftlet houses. The presence of nitrite-reducing bacteria needs to be identified to prove the presence of these bacteria in swiftlet houses that have the potential to contribute to nitrite contamination in edible bird's nest. This study aims to isolate nitrate-reducing bacteria in an effort to control nitrite using bacteriophages in the future. This study targeted nitrate-reducing bacteria collected from environmental samples (waste, feces, pond water, artificial pond water, soil, swiftlet eggshells, white edible bird's nest (*Aerodramus fuciphagus*), black bird's nest (*Aerodramus maximus*)) (n=40) from two different swiftlet houses in Sumedang, West Java, Indonesia. All isolates collected were subjected to a series of microbiological tests, phenotypic characterization (Gram staining, morphology, sugar fermentation ability, enzymes, etc.) and genotyping by PCR amplification and 16S rDNA gene sequencing. Raw sequencing data were analyzed using DNASTAR® software for DNA sequence alignment and phylogenetic tree construction. In the present work, four bacteria species were identified, including *Priestia megaterium*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, and *Proteus terrae*. To our knowledge, this study is the first report of nitrate-reducing bacteria isolated from birdhouses.

Keyword: Edible Bird's Nest, Molecular Characterization, 16S rDNA, Sequencing.

ABSTRAK

Sarang burung walet merupakan komoditi ekspor penting yang menjadi perhatian pemerintah Indonesia saat ini. Salah satu syarat ekspor sarang burung walet ke negara Republik Rakyat Tiongkok adalah produk sarang burung walet harus memenuhi persyaratan kandungan nitrit dalam produk di bawah 30 ppm. Cemaran nitrit ini dapat diperoleh dari hasil metabolisme nitrogen dari bakteri nitrifikasi. Akan tetapi, informasi mengenai bakteri pereduksi nitrit di rumah burung walet belum pernah dilaporkan dan menjadi urgensi dalam pengendalian bakteri nitrifikasi di rumah burung walet. Keberadaan bakteri pereduksi nitrit perlu diidentifikasi untuk membuktikan adanya bakteri ini di rumah burung walet yang berpotensi dalam kontribusi cemaran nitrit pada sarang burung walet. Penelitian ini bertujuan untuk mengisolasi bakteri pereduksi nitrat dalam upaya pengendalian nitrit menggunakan bakteriofag di masa depan. Penelitian ini menargetkan bakteri pereduksi nitrat yang dikoleksi dari contoh lingkungan (limbah, feses, air kolam, air kolam buatan, tanah, kulit telur burung walet, sarang burung walet putih (*Aerodramus fuciphagus*), sarang burung walet hitam (*Aerodramus maximus*)) (n=40) yang berasal dari dua rumah burung walet yang berbeda di Sumedang, Jawa Barat, Indonesia. Semua isolat yang berhasil dikoleksi telah diuji dengan serangkaian uji mikrobiologi, karakterisasi fenotipe (pewarnaan Gram, morfologi, kemampuan fermentasi gula-gula, enzim, dll) dan genotipe dengan amplifikasi PCR serta sekuensing gen 16S rDNA. Data mentah hasil sekuensing dianalisis dengan menggunakan perangkat lunak DNASTAR® untuk penyejajaran sekuen DNA dan konstruksi pohon filogenetik. Berdasarkan hasil penelitian, empat bakteri berhasil terindentifikasi antara lain *Priestia megaterium* (99,5%), *Pseudomonas putida* (98,5%), *Stenotrophomonas maltophilia* (99%), dan *Proteus terrae* (99,4%). Penelitian ini memberikan informasi pertama mengenai bakteri pereduksi nitrat yang diisolasi dari rumah burung walet.

Kata Kunci: Sarang Burung Walet, Karakterisasi Molekuler, 16S rDNA, Sekuensing

INTRODUCTION

Southeast Asia, including Indonesia, Malaysia, and Vietnam, is the world's largest producers in the edible bird's nest sector (Benjakul and Chantakun, 2022; Ningrum, 2023) Edible bird's nest, harvested from swiftlets, are highly prized and desired luxury food owing to their rich nutrient contents and medicinal values (Hui-Yan et al., 2021) However, nitrite contamination is becoming a more acute problem for the industry, with significant health risks (Ito et al., 2021; Lee et al., 2021) High nitrite concentrations are a severe health concern, as long-term consumption of high levels of nitrites is linked to an increased risk of cancer in humans (Silke, 2024)

Recent studies have indicated that specific microorganisms can influence nitrite accumulation in birdhouses, particularly bacteria involved in the nitrogen cycle (Ningrum et al., 2022; Yeo et al., 2021) The nitrogen cycle consists of nitrification, which converts ammonia to nitrate, and denitrification, which reduces nitrate to nitrite (Widiyani et al., 2021) There is special concern about nitrate-reducing bacteria in this context, as these organisms can promote nitrite accumulation by converting nitrate to nitrite in birdhouse environments (Besson et al., 2022). Identifying these bacteria is crucial for understanding the microbial ecology of birdhouses and mitigating nitrite contamination. For example, one promising approach for controlling bacterial populations is using bacteriophages, viruses that specifically infect and lyse bacteria (Rodrigues et al., 2021). Bacteriophage therapy has gained increasing attention as an environmentally friendly and targeted method to control harmful bacteria in aquaculture (Liu et al., 2022). In birdhouses, bacteriophages could potentially target nitrate-producing bacteria, reducing nitrite levels and enhancing product safety. However, the success of bacteriophage therapy depends on accurately identifying the bacterial species responsible for nitrite accumulation. This study aims to isolate and characterize nitrate-reducing bacteria from birdhouse environmental samples, potentially as a host candidate for the bacteriophage for future nitrite control in birdhouses.

MATERIALS AND METHODS

Enrichment cultures

Sewages, feces, pond water, artificial pond water inside of birdhouses, soil, egg skin, edible bird's nest (*Aerodramus fuciphagus*), and black bird's nest (*Aerodramus maximus*) (n=40) were collected aseptically in January 2024 from the artificial birdhouses at Sumedang, West Java, Indonesia. Enrichment cultures were performed for 7 days in culture media (Itoh et al., 2013) with modifications. Each 1000 mL of culture media contained 2 g ammonium sulfate, 0.5 g sodium carbonate, 0.5 g dipotassium phosphate, 50 mg magnesium sulfate heptahydrate, 5 mg calcium chloride dihydrate, 2 mg manganese sulfate tetrahydrate, 0.2 g EDTA, 0.18 Fe(II) sulfate, 0.1 mg copper sulfate pentahydrate, 0.05 mg sodium molybdate, 0.001 mg cobalt chloride hexahydrate, 0.1 mg zinc sulfate heptahydrate, and 0.5 g glucose. A 25 g sample was added to an Erlenmeyer flask containing 225 mL of liquid nitrification media and homogenized. The mixture was cultured for seven days in liquid nitrification media, pH 7.8, at 28°C at 130 rpm with shaking.

Isolation of culturable bacteria

The samples were diluted in series until a dilution factor of 10^{-6} was achieved, and then 1 mL of each dilution was added to a petri plate. An amount of 8 mL nitrification media was inoculated in plates and incubated at 30°C for 48 hours. The isolates obtained from pure culture were subjected to Gram staining, morphology, motility test, indole production, urease production, citrate utilization ability test, gelatin hydrolysis test, sugar fermentation profile testing, and catalase activity test.

DNA isolation, PCR, and sequencing

The genomic DNA was extracted using the Geneaid™ DNA Isolation Kit, following the manufacturer's instructions (Geneaid™, Taiwan). The sections of 16S rRNA in each DNA were amplified with universal bacterial primers 27-F (AGAGTTTGATCMTGGCTCAG) and 1492-R (TACGGYTACCTTGTTACGACTT). For the PCR amplification, 1 µL from each oligonucleotide primer (at a final concentration of 10 pmol), 50 ng of DNA template, 12.5 µL of master mix (GoTaq), and a final volume of 25 µL with nuclease-free water were used. The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 45 seconds, and extension at 72°C for 2 minutes, with a final extension step at 72°C for 8 minutes. The PCR results were detected by agarose gel electrophoresis. The amplicon was then sequenced for 16S rDNA with Sanger sequencing method.

Phylogenetic analysis

The nucleotide sequences of the bacterial isolates were compared to the genomic data of several

nitrification and denitrification bacteria present in the database maintained by GenBank (NCBI, Bethesda, USA). The analyses use the BLAST engine offered by NCBI. DNASTAR Lasergene Version 7.0 software was applied to analyze bacterial species' aligned molecular phylogenetic relationships using SeqMan and construct a phylogenetic tree using MegAlign. (Burland, 1999).

Nucleotide sequence accession number

The 16S rRNA gene sequences have been stored in the GenBank nucleic acid sequence database with accession numbers PP858222, PQ288793, PQ288795, and PQ288798.

RESULTS

In this study, 20 environmental samples were collected from two artificial birdhouses in Sumedang, West Java, Indonesia, to isolate and identify nitrate-metabolizing bacteria. The analysis used phenotypic characterization and molecular methods, PCR amplification followed by 16S rDNA sequencing. According to the phenotypic tests (Table 1) and gram staining (Fig. 1), SHW, LK2, TK, and TWK isolates investigated in this study could be reliably identified as *Priestia megaterium, Pseudomonas putida, Stenotrophomonas maltophilia*, and *Proteus terrae,* respectively.

The sequencing of 16S rDNA results identified four distinct bacterial species involving *Priestia megaterium, Pseudomonas putida, Stenotrophomonas maltophilia*, and *Proteus terrae* (Table 2). Table 2 showed that the species of 16S rRNA gene sequences in SHW, LK2, TK, and TWK isolates examined in this research were classified with sequence identities of 99.5%, 98.5%, 99.0%, and 99.4%, respectively. Figure 2 depicts typical dendrograms of the sequencing data of the 16S rRNA gene for isolates in the present study and reference strains. A phylogenetic tree was constructed (Fig. 3) to evaluate the genetic relationships of the nitrate-reducing bacterium isolates obtained in the present study to other nitrifying bacteria.

DISCUSSION

This study provides valuable insights into the microbial ecology of artificial birdhouses in Sumedang, Indonesia, specifically focusing on nitrate-reducing bacteria. Health risks and economic concerns caused by nitrite contamination have been a major issue haunting the edible bird's nest industry for decades (Yeo et al., 2021). The high nitrite levels were associated with nitrate-reducing bacteria in the birdhouse environment, posing a food safety and quality hazard in edible bird's nests. Accordingly, the identification and analysis of these bacteria are important for designing effective strategies to mitigate nitrite accumulation.

In this study, four nitrate-reducing bacteria were successfully isolated and identified as *P. megaterium*, *P. putida*, *S. maltophilia*, and *P. terrae*. This finding is important because nitrate-reducing bacteria play a role in the nitrogen cycle (Jiang et al., 2023). Although none of the species were nitrifying bacteria, which convert ammonia to nitrate, identifying nitrate-reducing organisms is still crucial to understanding how nitrite accumulates in birdhouses. The identification of *P. megaterium*, *P. putida*, *S. maltophilia*, and *P.*

Table 1. Phenotypical characteristics of SHW, LK2, TK, and TWK isolates.

Phenotypical properties	SHW	LK2	TK	TWK
Gram staining	+	-	-	-
Morphology	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped
Motility	+	+	+	+
Indole	-	-	-	+
Urease	+	(+)	+	+
Citrate	(+)	(+)	+	-
Hydrolysis of Gelatine	(+)	-	+	-
Fermentation of:				
Glucose	+	+	(-)	+
Sucrose	+	(-)	+	+
Mannitol	+	-	(-)	-
Maltose	+	+	+	+
Lactose	+	-	(-)	+
Catalase	(+)	(+)	+	(+)

+: positive reaction, -: negative reaction, (+): weak positive reaction, (-): weak negative reaction.

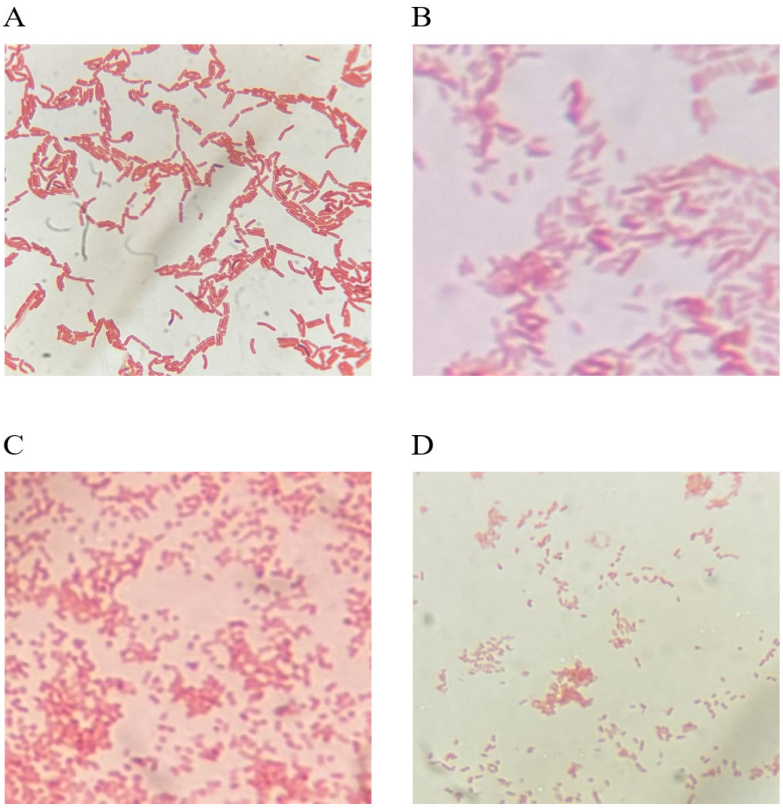

Figure 1. Morphology in gram straining. A) *Priestia megaterium*, B) *Pseudomonas putida*, C) *Stenotrophomonas maltophilia*, D) *Proteus terrae.*

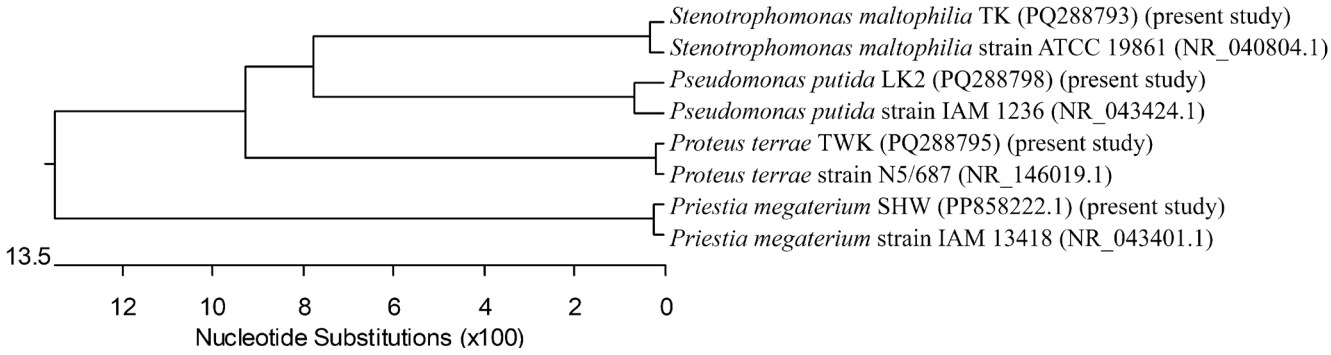

Figure 2. Phylogenetic tree of the 16S rRNA gene sequences of isolates in present study and reference strains retrieved from GenBank (NCBI).

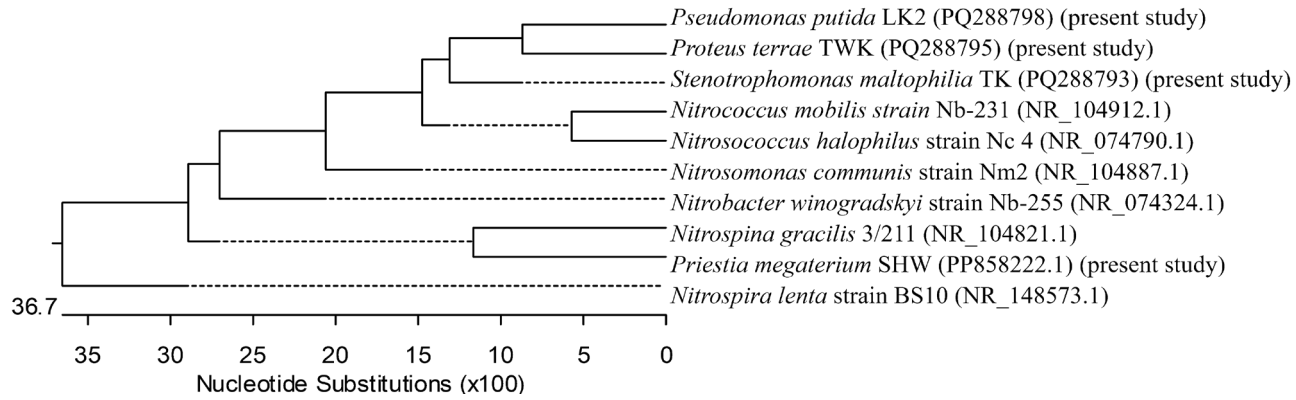

Figure 3. Phylogenetic tree of the 16S rRNA gene sequences of isolates in the present study and other key species of the nitrifying bacteria retrieved from GenBank (NCBI).

Table 2. Bacterial species isolated from artificial birdhouses (*Aerodramus* sp.).

Sample ID	Isolation source	16S rDNA identification	Sequence similarity (%)	Nitrogen metabolism
SHW	Black bird's nest (*A. maximus*)	*Priestia megaterium*	99.5	Nitrate-reducing bacteria
LK2	Sewages	*Pseudomonas putida*	98.5	Nitrate-reducing bacteria
TK	Soil	*Stenotrophomonas maltophilia*	99.0	Nitrate-reducing bacteria
TWK	Egg skin from *A. fuciphagus*	*Proteus terrae*	99.4	Nitrate-reducing bacteria

terrae is particularly interesting since these species perform nitrate reduction in various environments. *P. Megaterium,* previously known as *Bacillus megaterium* (Gupta et al., 2020) has capability to reduce nitrate to ammonia (Sharma et al., 2022). *P. putida* is a well-known has capability to convert nitrite to ammonia (Schmidt et al., 2022), and has been studied in agriculture (Molina et al., 2020) and industrial biotechnology (Weimer et al., 2020) for its role in nitrogen cycling. Similarly, *S. maltophilia* is recognized for its nitrate-reducing capabilities and adaptability to different environmental conditions (Xu et al., 2022), which may explain its presence in birdhouses. Though less studied, *Proteus terrae* has also been reported as a nitrate-reducer, nitrate to ammonia (Behrendt et al., 2015), suggesting its potential contribution to nitrite accumulation in birdhouses.

This discovery opens new avenues for exploring how nitrate-reducing bacteria contribute to nitrite contamination in edible bird's nest from birdhouses. The previous study (Chan, 2013) examined the sources of nitrite contamination. Swiftlet droppings and water samples were gathered from edible bird's nest production locations in Malaysia and Indonesia. Their research uncovered a significant concentration of nitrates rather than nitrites. In addition, they performed a proteomic analysis of protein extracts from edible bird's nests using mass spectrometry. This research allowed them to assume that nitrite in edible bird's nest may be the result of bacteria that reduce nitrate. The nitrate-reducing bacteria can transform nitrate in an edible bird's nest into nitrite, thereby increase the accumulation of nitrite in edible bird's nest (Widiyani et al., 2022)

This study could lay a baseline study for the development of future biotechnological interventions to regulate nitrite levels in birdhouses by isolating NITRATE-REDUCING BACTERIA. A potential method to address this challenge could be biocontrol treatment with bacteriophages. Instead of harmful chemicals, bacteriophages are harmless viruses that target and lyse only bacteria, providing pinpoint control against bacterial populations (Jones et al., 2021). Bacteriophage application could selectively reduce nitrate-forming bacteria, thereby efficiently limiting nitrite formation in birdhouses. However, the success of this approach relies on a detailed understanding of the bacterial species inhabiting birdhouses, making the results of this study an essential first step toward effective biocontrol strategies. *S. maltophilia* is the only bacterium in this study that might be serve as a potential host for such bacteriophages, given its ability to reduce nitrate into nitrite (Al-Dhabi et al., 2021). Future investigations should focus on identifying bacteriophages that have broad range for nitrifying and NITRATE-REDUCING BACTERIA and evaluating their effectiveness in controlling nitrite levels under practical conditions.

In conclusion, this study successfully identified NITRATE-REDUCING BACTERIA, involving *P. megaterium*, *P. putida*, *S. maltophilia*, and *P. terrae,* from birdhouses in Sumedang, West Jawa, Indonesia. By identifying key bacterial species responsible for nitrate metabolism, this study opens up new possibilities for addressing nitrite contamination in the edible bird's nest industry. Understanding the presence of this nitrate-reducing bacteria can inform better practices and treatments to reduce nitrite contamination effectively in the future.

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"The author declares that there is no conflict of interest with the parties involved in this research."

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