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Penulis : Etin Diah Permanasari, Muhammad Ibadurrohman, Susilo Susilo

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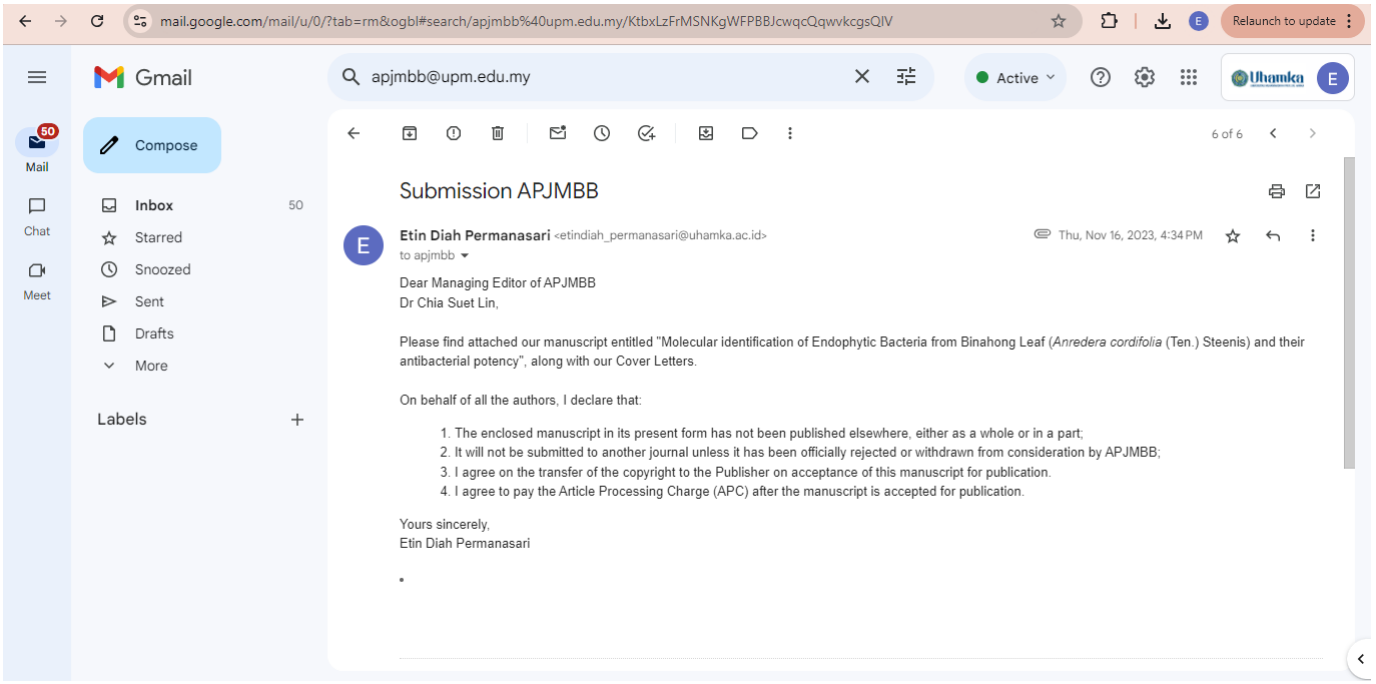

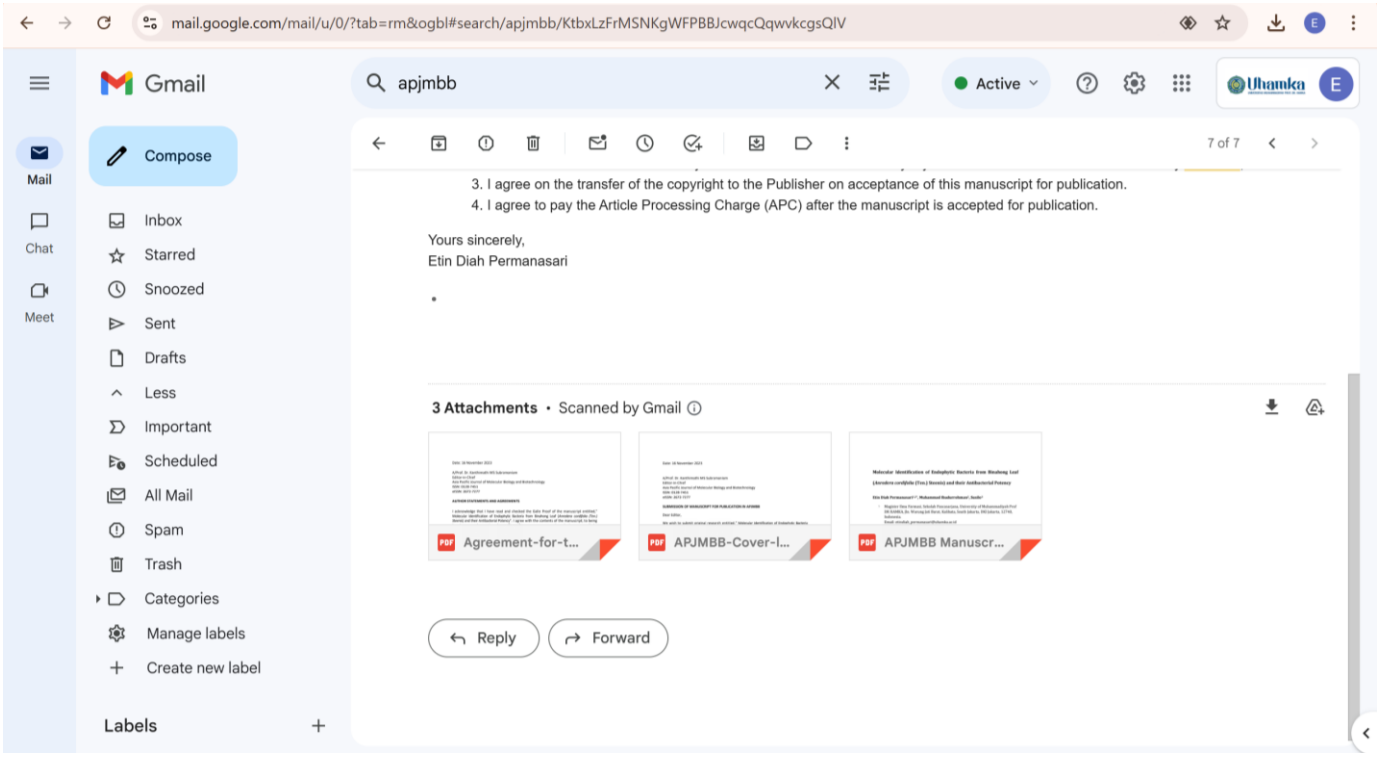

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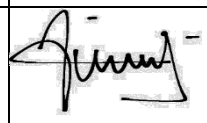
Muhammad Ibadurrohman	ibadman46@gmail.com	
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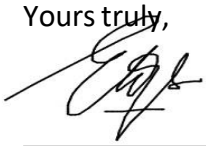

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SUBMISSION OF MANUSCRIPT FOR PUBLICATION IN APJMBB

Dear Editor,

We wish to submit original research entitled," Molecular Identification of Endophytic Bacteria from Binahong Leaf (*Anredera cordifolia (Ten.) Steenis*) and their Antibacterial Potency"and agreed for the manuscript to be published in Asia Pacific Journal of Molecular Biology and Biotechnology. All the authors declare no conflicts of interest otherwise disclose in the manuscript. There is/was no significant financial support in this project which could influence the findings. All the authors have read and approved the manuscript and hold full responsibilities of its validity.

In this paper, we discuss on the isolation and identification of endophytic bacteria from binahong leaf (*Anredera cordifolia (Ten.) Steenis*) which exhibit antibacterial properties. The metabolites from endophytic bacteria of the leaves of (*Anredera cordifolia (Ten.) Steenis*) exhibit strong antibacterial activities against *Streptococcus mutans* and *Lactobacillus acidophilus*. It was identified molecularly as *Bacillus sp strain x20* with the similarity value of 100% using 16S rRNA sequencing technique.

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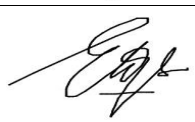
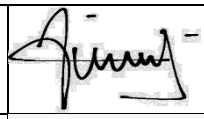
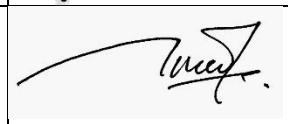
Etin Diah Permanasari	Ph.D	A,B,C,D,E,F,G,H		16-11-2023

Isna Rasdianah Aziz	B.Sc	C,D,E		16-11-2023
Susilo	M.Sc	E,F,G		16-11-2023

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A. Conceptualisation and design
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APJMBB Manuscript:

Molecular Identification of Endophytic Bacteria from Binahong Leaf (*Anredera cordifolia* (Ten.) Steenis) and their Antibacterial Potency

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from *A. cordifolia* (Ten.) *Steenis* leaves. Two isolates with the codes of DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antibacterial activity against the bacteria of dental caries, which

are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The isolate of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* and *L. acidophilus*. The isolate of DBA2 was then continue for identification. The molecular identification was performed by 16S rRNA gene sequencing method using PCR technique. The genomic DNA of DBA2 was extracted with the *Geno Plus™ Genomic DNA Extraction Miniprep System.* Amplicons were then purified and sequenced by using GenBank National Centre Biotechnology Information (NCBI) database. The endophytic bacterial isolate DBA2 from the leaves of *A. cordifolia* was identified molecularly as *Bacillus sp strain x20* with the similarity value of 100%. Future studies are required to analyse the bioactive compounds, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah, Mashuri, & Abidin, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound,

diabetic, hematoma, and hyperuricemia (Yuniarti & Lukiswanto, 2017; Mutiarawati, Muljati, & Christyaningsih, 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita, Wijayati, & Mursiti, 2017; Surbakti, Queljoe, & Boddhi, 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Mengga, Rampe, & Sangande, 2022; Veronita, Wijayati, & Mursiti, 2017; Ainurrochmah, Ratnasari, & Lisdiana, 2013; Sasebohe, Prakasita, & Aditiyarini, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Xie, Yang, Tang, Chen, & Li, 2015; Basile, Giordano, López-Sáez, & Cobian, 1999). Flavonoids are the group which effectively inhibits the growth of viruses, bacteria, and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie, Yang, Tang, Chen, & Li, 2015; Veronita, Wijayati, & Mursiti, 2017). Several flavonoid compounds from the binahong leaves have been isolated such as vitexin, isovitexin, and morin (Alba, Pelegrin, & Sobottka, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, defines as a microorganism that usually live as symbionts inside a plant (Nxumalo et al, 2020). These

endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant. They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms.

Desriani et al (2014) state that 37 of the endophytic bacteria isolates were obtained from the binahong leaves in which their fifteen isolates exhibited antibacterial properties. The endophytic bacteria from the *A. cordifolia* (Ten.) Steenis leaves has ever been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on to *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Hussein, Saleem, & Merdad, 2023; Zhang, 2013). The identification was performed using 16S ribosomal RNA (16S rRNA) sequencing method. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, *soybean meal, corn step liquor*, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and *Deionized demineralized water* (ddh2O) from Sigma Aldrich, Ltd. The kits were *Geno Plus™, Genomic DNA Extraction Miniprep System* from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, *Nutrient Agar* medium, and *Nutrient Broth* medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tanggerang, Province Banten, Indonesia. The leaves were identified at Pusat Penelitian Konservasi and Kebun Raya, BRIN, Bogor, Jawa Barat, Indonesia. The leaves were washed by running tap water and carefully dried. The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then ground in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further step.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was

carried out using gram staining to analyse the colour and shape of bacterial colony cells. Gram staining was carried out by smearing one tube of bacterial colony thinly on an object glass, then the bacteria were confirmed first by adding 0,5% of crystal violet solution for the next 5 minutes. Drop Lugol's solution was added. The preparate were shaken until no dye flows over the glass object. The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was taken and put into a 10 ml *Nutrient Broth* test tube. Incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes with a speed of 2000 rpm. The obtained supernatant was used to screen the antibacterial potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml *Nutrient Broth* test tubes for 24 hours at 37° C. The culture was then poured into a ± 20 ml petri dish until they were solid. The sterile paper disk was then soaked with the supernatant, then placed in the NB medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. The isolate with the largest zone of inhibition was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018). The sequencing results were finally analysed by a Basic Local Alignment Search Tools (BLAST) using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the high similarity value with the existing bacterial species (Nxumalo et al, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Determination was performed to confirm that the plants used in this study as a source to isolate the endophytic bacteria are the accurate plant species. According to the determination result, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was used as a prior step for the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to *Nutrient Agar*

Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 isolates were similar, suggesting that these two isolates were the same isolates. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 isolates were shown in Tabel 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The largest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 isolates against the bacteria of dental caries.

This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah, Ratnasari, & Lisdiana, 2013; Nxumalo, Ngid, Shandu, & Maliehe, 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two isolates, DBA2 isolate was chosen as potential isolate as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The extracted genome was run by PCR using the 16S rRNA gene as a target. PCR products were run under a 0,8% gel electrophoresis. The finished gel was then analysed under UV. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2.

The DNA bands were then purified by Zymo Spin Column PCR Purification kit. The 16S rRNA gene sequence was performed for the purified products. The sequences of DNA were used to find similarity based on the 16S rRNA gene and later to determine the highest similarity species of organisms. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level. In addition, it is said to be the same genus if the identity is 96% - 99%. Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the DBA2 isolate had a level of similarity to *Bacillus sp. strain x20* with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify the species of the endophytic bacteria as *Bacillus sp. strain x20*. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus sp strain x20* is also a Gram-positive bacterium producing organic acids and phosphatases (Gond, Bergen, Torres, & White, 2015; Zhao, et al., 2023). *Bacillus sp strain x20* was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids and phosphatases produced by Bacillus strain will solute the inorganic phosphorus into a plant-available form, therefore the microorganism can promote plant growth (Zhao, et al., 2023). It would be interesting to examine the bioactive compounds from the cultivation of *Bacillus sp strain x20* which can serve as antibacterial agents for dental caries treatment.

CONCLUSION

This study revealed that the endophytic bacteria *Bacillus sp strain x20* was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

Isolates	*Shapes*	*Colour*	*Texture*	*Margins of Colony*	*Surface*	*Consistency*	*Staining*
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

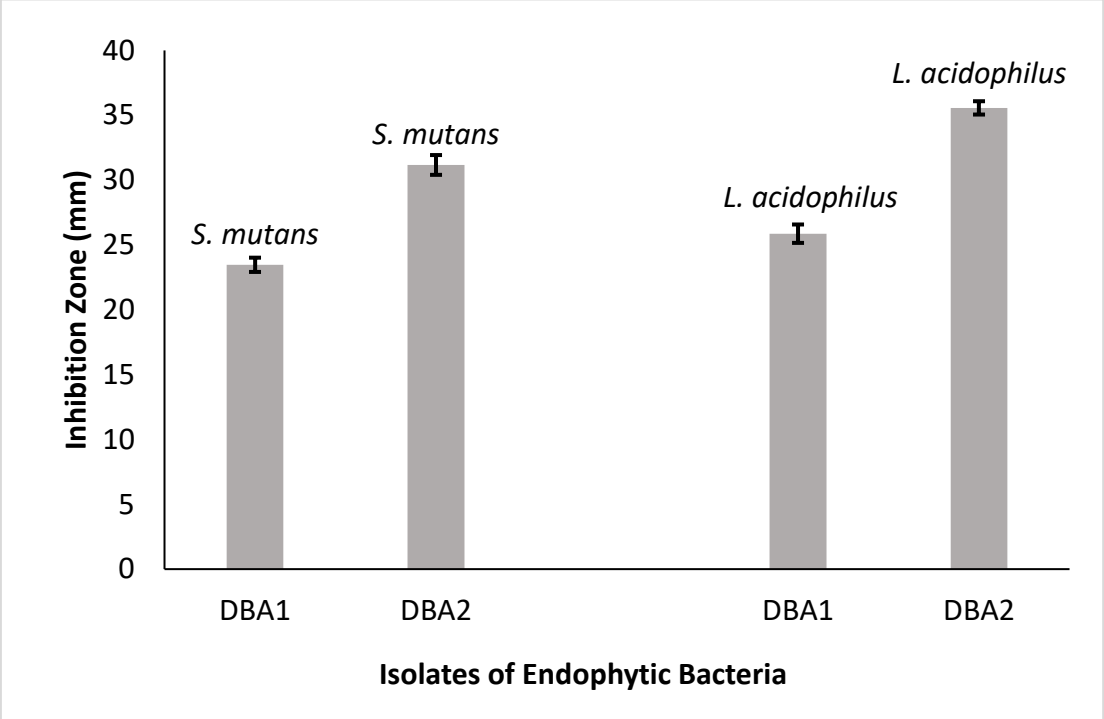


Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

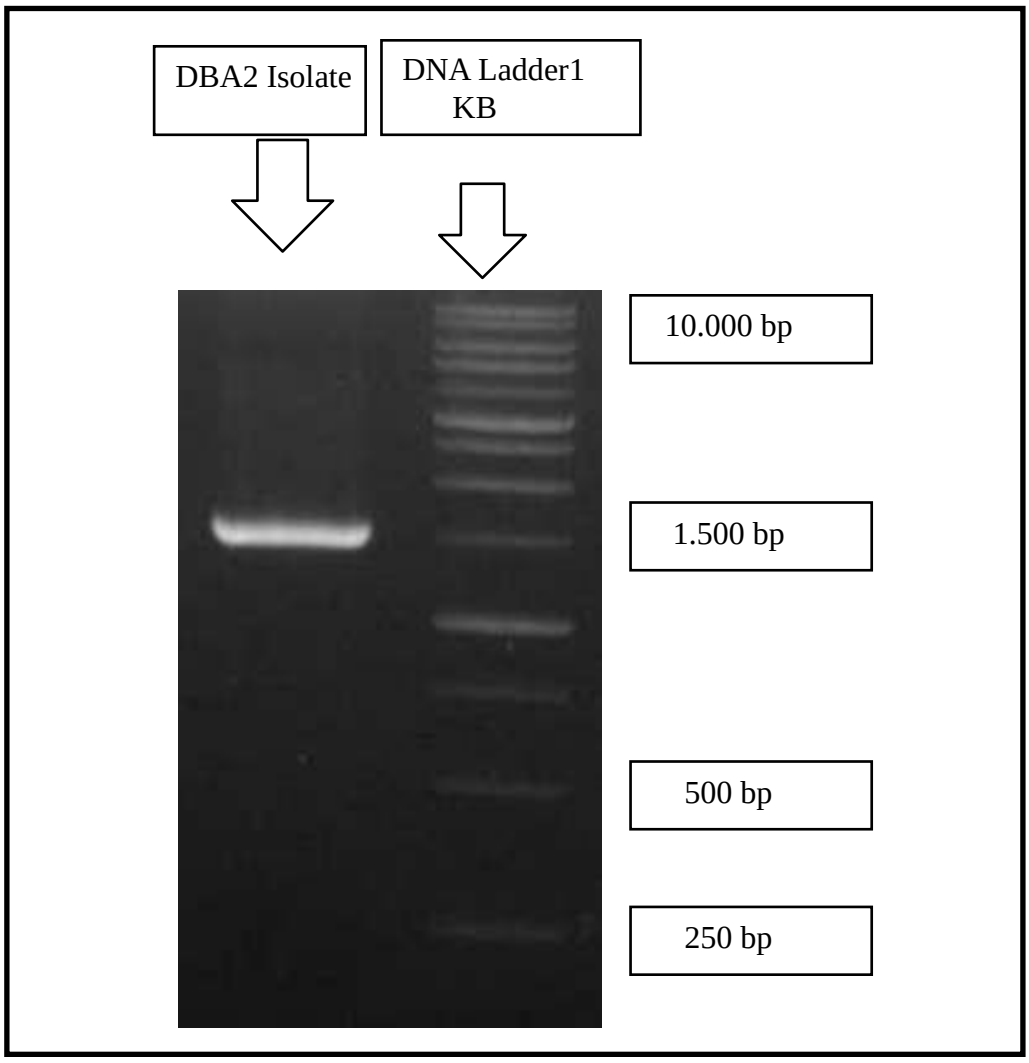


Figure 2. The gel electrophoresis of PCR product of DBA2 isolate using 16S ribosomal RNA gene

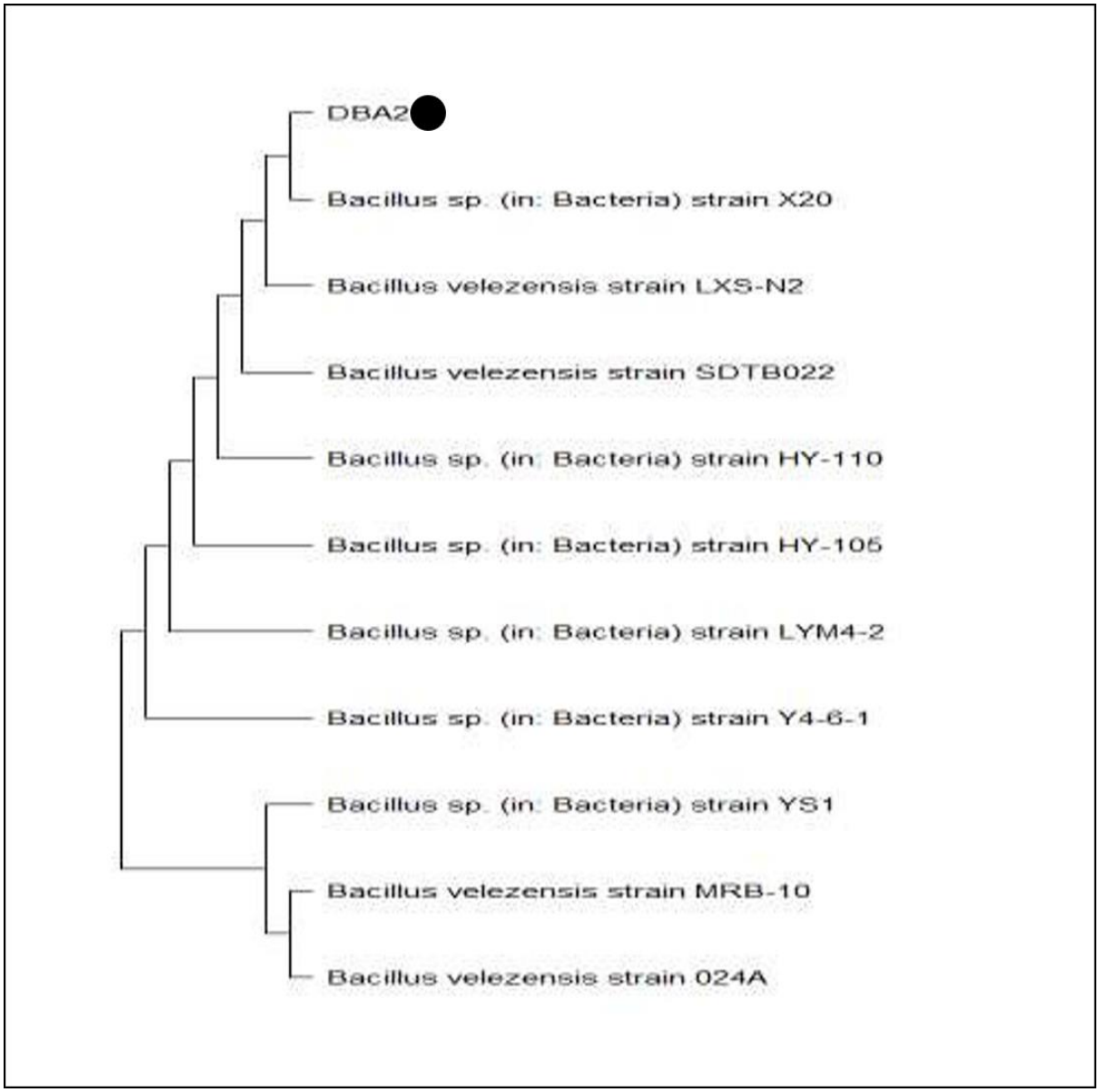


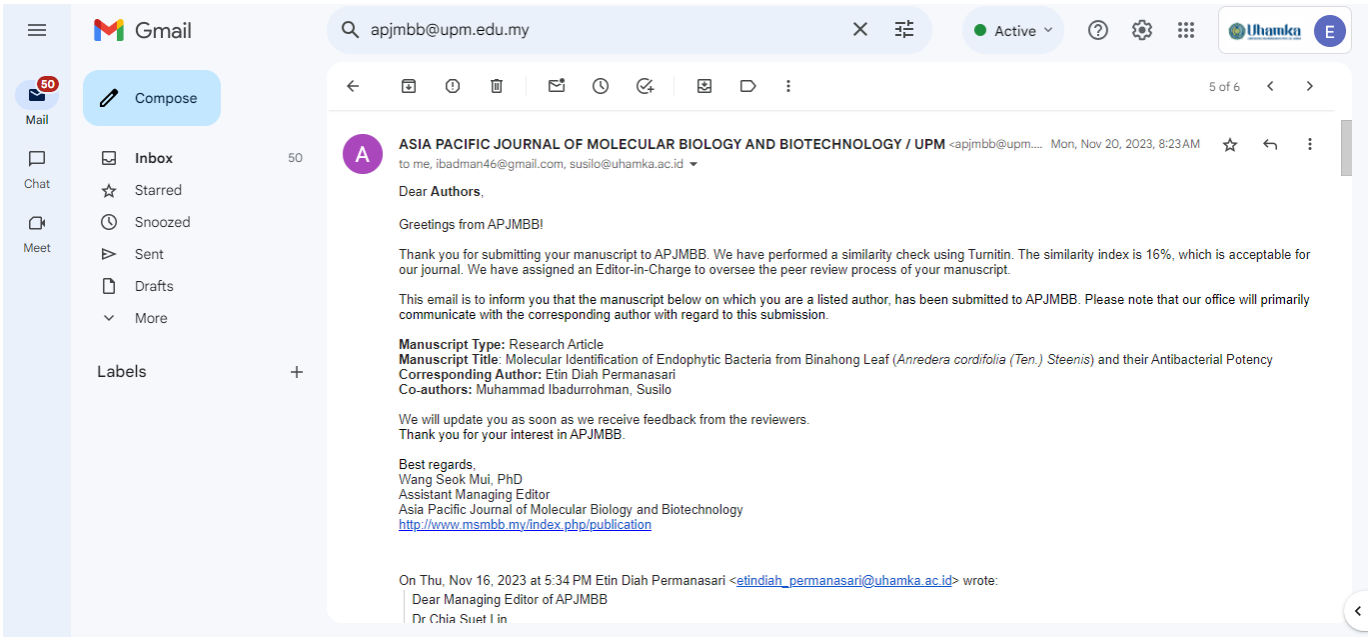
Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018).

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

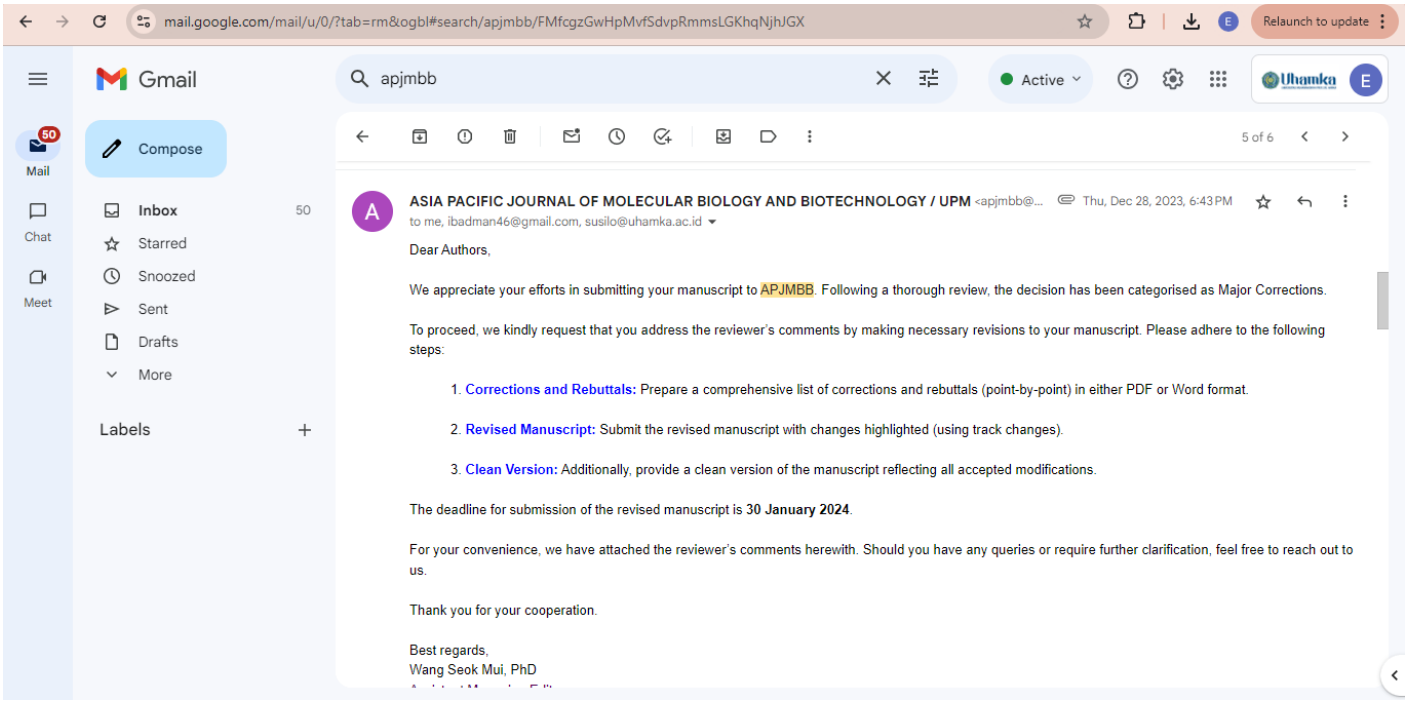
No	*Closest Relative Species Based On 16s Rrna Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus SD (in Bacteria) strain X20*	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis strain LXS-N2*	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis strain SOTB022*	OK047738	1417	2139	0.0	100.00
4	*Bacillus SD, (in Bacteria) strain HY 110*	MZ895449	1453	2139	0.0	100.00
5	*Bacillus SO (in Bacteria) strain HY-105*	MZ895445	1445	2139	0.0	100.00
6	*Bacilus SO (in Bacteria) street LYM4-2*	OP493233	1448	2139	0.0	100.00

7	*Bacillus SD (in Bacteria) strain Y4-6-1*	OP493232	1451	2139	0.0	100.00
8	*Bacillus SD. (in: Bacteria) strain YS1*	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis strain MRB-10*	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis strain 024A*	OP477121	1453	2139	0.0	100.00

2. Bukti konfirmasi penerimaan submit dari Editor (20 Nov-2024)



3. Bukti konfirmasi review dan hasil review pertama dari Editor (28 Des 2024)



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5 of 6

ASIA PACIFIC JOURNAL OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY / UPM <apjmbb@... Thu, Dec 28, 2023, 6:43 PM

to me, ibadman46@gmail.com, susilo@uhamka.ac.id

Dear Authors,

We appreciate your efforts in submitting your manuscript to APJMBB. Following a thorough review, the decision has been categorised as Major Corrections.

To proceed, we kindly request that you address the reviewer's comments by making necessary revisions to your manuscript. Please adhere to the following steps:

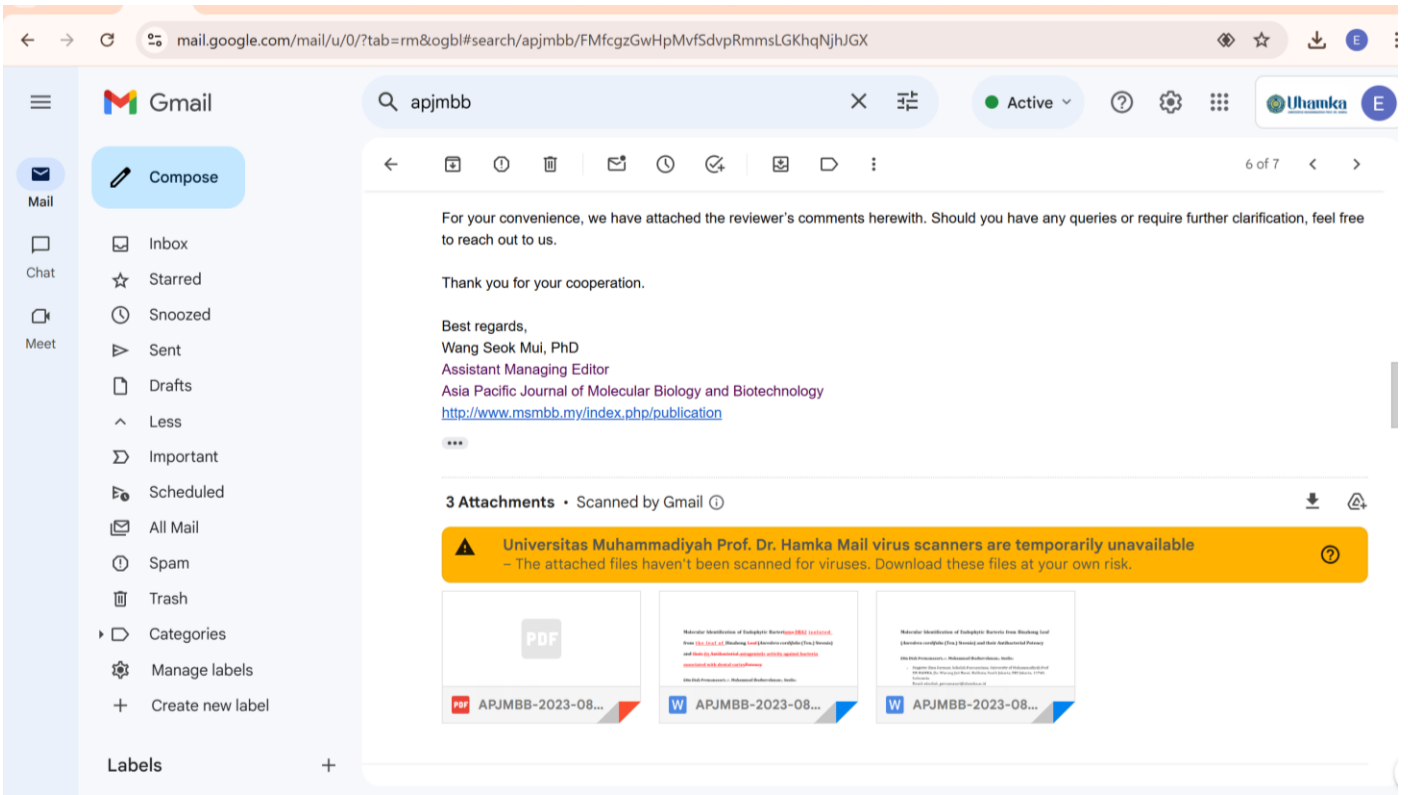
1. **Corrections and Rebuttals:** Prepare a comprehensive list of corrections and rebuttals (point-by-point) in either PDF or Word format.
2. **Revised Manuscript:** Submit the revised manuscript with changes highlighted (using track changes).
3. **Clean Version:** Additionally, provide a clean version of the manuscript reflecting all accepted modifications.

The deadline for submission of the revised manuscript is **30 January 2024**.

For your convenience, we have attached the reviewer's comments herewith. Should you have any queries or require further clarification, feel free to reach out to us.

Thank you for your cooperation.

Best regards,
Wang Seok Mui, PhD



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Categories

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Create new label

Labels

6 of 7

For your convenience, we have attached the reviewer's comments herewith. Should you have any queries or require further clarification, feel free to reach out to us.

Thank you for your cooperation.

Best regards,
Wang Seok Mui, PhD
Assistant Managing Editor
Asia Pacific Journal of Molecular Biology and Biotechnology
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APJMBB Reviewer 1,2,3 Report:

ASIA PACIFIC JOURNAL OF MOLECULAR BIOLOGY & BIOTECHNOLOGY

Reviewer's Report 1

Manuscript title:

Please complete the checklist below by ticking the appropriate boxes. In APJMBB, we aim to help scientist in improving their manuscript to a standard that is publishable. Hence, your suggestions are very much appreciated. A copy of this review form will be sent to the author(s) anonymously.

	Yes	No	remarks
Does this manuscript present new material/knowledge?		**x**	
Is the title descriptive of the content?	**x**		
Is the abstract descriptive of the content?	**x**		
Is the material/data sufficient?		**x**	
Is the statistical treatment adequate/correct?		**x**	
Are the figures/tables/photographs appropriate/necessary?		**x**	
Are the references appropriate and complete?		**x**	
Is the presentation and style adequate?		**x**	
Is English editing required?		**x**	

Comments:

The manuscript discusses the isolation of endophytic bacteria from leave of *Anredera cordifolia* (Ten.) Steenis and the authors have some expectation to obtain novel antibacterial compound from the endophytic bacteria. However there are several question need to be addressed form this manuscript. Please find all the comments on the manuscript.

ASIA PACIFIC JOURNAL OF MOLECULAR BIOLOGY & BIOTECHNOLOGY

Reviewer's Report 2

Manuscript title: APJMBB-2023-082: Molecular Identification of Endophytic Bacteria from Binahong Leaf (Anredera cordifolia (Ten.) Steenis) and their Antibacterial Potency

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	Yes	No	remarks
Does this manuscript present new material/knowledge?	✓		
Is the title descriptive of the content?	✓		
Is the abstract descriptive of the content?	✓		
Is the material/data sufficient?		✓	
Is the statistical treatment adequate/correct?		✓	
Are the figures/tables/photographs appropriate/necessary?		✓	
Are the references appropriate and complete?	✓		
Is the presentation and style adequate?		✓	
Is English editing required?	✓		

Comments:

- The authors need to define DBA1 and DBA2 better whether they are isolates or strains. Isolates refers to pure cultures obtained from isolation without knowing anything about them. Once the isolates have been characterized, isolates showing identical characteristics (Gram staining, colony & cellular morphology, or even 16S rRNA gene sequences) will be identified as the same strain. The general rule is that different isolates can be the same strain, but different strains cannot be the same isolate.
- In short, if the study has determined that DBA1 and DBA2 are the same strain, they should remove the findings about DBA1 from the abstract. However, the two strains had demonstrated different levels of antagonistic activities against the dental caries pathogens. Hence, they should be different strains.

- Keywords 16S rRNA gene instead of 16S rRNA
- Antibacterial potency in the title and antibacterial activity throughout the manuscript – suggest the term antagonistic activity
- Introduction – "Flavonoids are the group which effectively inhibits the growth of viruses, bacteria, and fungi, as it causes damage to the permeability of cell walls and inhibit motility" – this statement is inaccurate as the viruses have no cell walls
- Introduction – "These microorganisms produce similar bioactive compounds as its host plant." – this statement is disputable and has no reference to back up
- Introduction – "Desriani et al (2014) reported that out of 37 of the endophytic bacterial isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties." – more info is needed, antibacterial properties against what kind of microorganisms?
- Methods – "The leaves were washed by running tap water and carefully dried" – this is not a proper sample processing. An autoclave-sterilized distilled water should be used as the tap water contains microorganisms that can be introduced into the plant samples
- Methods – "The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%. – please clarify if NaOCl is used at 5.3%
- Methods – "The cut leaves were then ground in NA plate under aseptic condition." – please clarify, it is impossible to grind the leaves in NA plates in Petri dish. The authors should have used a sterile pestle and mortal to grind the samples, before resuspending and inoculate into NA plates
- Methods – "Gram staining was carried out by smearing one tube of bacterial colony thinly on an object glass, then the bacteria were confirmed first by adding 0.5% of crystal violet solution for the next 5 minutes. Drop Lugol's solution was added. The preparate were shaken until no dye flows over the glass object. The preparate were then analysed under microscope." – the protocol for gram staining is incomplete, lacking of safranin and so on, please provide complete procedure for the staining

- The authors do not need to italicize the DNA extraction kit, name of media used
- Methods – "The strain culture of the bacteria of dental caries (Streptococcus mutans and Lactobacillus acidophilus) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was then poured into a ± 20 ml petri dish until they were solid." – this part of the method is confusing, how did nutrient broth incubated with the pathogens solidify without agar?
- Methods – "in order to determine the high similarity value with the existing bacterial species" – suggest to correct it to in order to determine the most closely related reference bacteria in the database
- Results – "Determination was performed to confirm that the plants used in this study as a source to isolate the endophytic bacteria are the accurate plant species. According to the determination result, it was known that the leaves is Anredera cordifolia (Teen.) Steenis" – there should a specimen voucher deposited in the centre and the number of the voucher should be provided
- Results – "Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 isolates were similar, suggesting that these two isolates were the same isolates." – The two strains had later demonstrated different levels of antagonistic activities against the dental caries pathogens. Hence, they should be different strains.
- The 16S rRNA gene sequencing and analysis with reference to the NCBI database only revealed the DBA2 was a strain of *Bacillus* sp. and *Bacillus* sp. strain x20 was returned from the database search as the top match. We cannot conclude that DBA2 is *Bacillus* sp. strain x20 as it should be a different strain.

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Reviewer's Report 3

Manuscript title:

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	Yes	No	remarks
Does this manuscript present new material/knowledge?	**X**		
Is the title descriptive of the content?	**X**		
Is the abstract descriptive of the content?	**X**		
Is the material/data sufficient?		**X**	
Is the statistical treatment adequate/correct?			**NA**
Are the figures/tables/photographs appropriate/necessary?	**X**		
Are the references appropriate and complete?	**X**		
Is the presentation and style adequate?	**X**		
Is English editing required?		**X**	

Comments:

1. Minor grammatical errors in the abstract.
2. Introduction part should include more information on endophytic bacteria of earlier studies on binahong and/or other plants similar to binahong; and the need to test on dental caries-related microorganisms (was there similar studies or is binahong usually chewed traditionally to prevent caries, etc?); while reduce discussing on flavonoids since it is not being investigated or compared in this study.
3. Pg5: Endophytic bacteria isolation from the binahong leaves. The methodology here needs to be expanded more. The grounding of leaves and subsequent dilution and plating methods should be in more detail. Or cite the adaptation of a previous study method, if applicable.
4. Pg 6: Antibacterial activities screening: What are the control positive and control negatives used for analysing the antibacterial properties of the

supernatant? It would be useful to know the comparisons to standard antibiotics and interpret according to CLSI guidelines

5. The molecular determination of strains were done using 16SrRNA sequences, and Bacillus x20 strain was identified in this study. Since Bacillus is a spore forming organism, what steps were taken to rule out contamination from external sources e.g from soil. Maybe can be explained in the methodology?
6. The main objective of this study was determining antibacterial properties of potential endophytes, and subsequently identifying that endophyte. So the antibacterial properties/findings must be expanded more, as was stated in Comment 6, to include comparisons to standard antibiotics. Molecular identification is important too, but since it is not a novel organism, the focus should not be on the molecular analysis of this organism.

APJMBB Reviewer Comments-1:

Molecular Identification of Endophytic Bacteria from Binahong Leaf (*Anredera cordifolia* (Ten.) Steenis) and their Antibacterial Potency

Etin Diah Permanasari[1,2*], Muhammad Ibadurrohman[2], Susilo[3]

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from *A. cordifolia* (Ten.) *Steenis* leaves. Two isolates with the codes of DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antibacterial activity against the bacteria of dental caries, which

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are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The isolate of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* and *L. acidophilus*. The isolate of DBA2 was then continue for identification. The molecular identification was performed by 16S rRNA gene sequencing method using PCR technique. The genomic DNA of DBA2 was extracted with the *Geno Plus™ Genomic DNA Extraction Miniprep System.* Amplicons were then purified and sequenced by using GenBank National Centre Biotechnology Information (NCBI) database. The endophytic bacterial isolate DBA2 from the leaves of *A. cordifolia* was identified molecularly as *Bacillus sp strain x20* with the similarity value of 100%. Future studies are required to analyse the bioactive compounds, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA

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Commented [Rev3]: ....was then subjected for molecular identification.

Commented [Rev4]: The 16S rRNA gene was used as molecular marker for such identification. 16SrRNA gene was amplified by PCR and then sequencing.

Commented [Rev5]: GenBank is not for sequencing but for searching the data base trough Blast method

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INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah, Mashuri, & Abidin, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound,

diabetic, hematoma, and hyperuricemia (Yuniarti & Lukiswanto, 2017; Mutiarawati, Muljati, & Christyaningsih, 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita, Wijayati, & Mursiti, 2017; Surbakti, Queljoe, & Boddhi, 2018).

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It should be : Veronita, et al., 2017)

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Mengga, Rampe, & Sangande, 2022; Veronita, Wijayati, & Mursiti, 2017; Ainurrochmah, Ratnasari, & Lisdiana, 2013; Sasebohe, Prakasita, & Aditiyarini, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Xie, Yang, Tang, Chen, & Li, 2015; Basile, Giordano, López-Sáez, & Cobian, 1999). Flavonoids are the group which effectively inhibits the growth of viruses, bacteria, and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie, Yang, Tang, Chen, & Li, 2015; Veronita, Wijayati, & Mursiti, 2017). Several flavonoid compounds from the binahong leaves have been isolated such as vitexin, isovitexin, and morin (Alba, Pelegrin, & Sobottka, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, defines as a microorganism that usually live as symbionts inside a plant (Nxumalo et al, 2020). These

endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant. They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms.

Desriani et al (2014) state that 37 of the endophytic bacteria isolates were obtained from the binahong leaves in which their fifteen isolates exhibited antibacterial properties. The endophytic bacteria from the *A. cordifolia* (Ten.) Steenis leaves has ever been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on to *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Hussein, Saleem, & Merdad, 2023; Zhang, 2013). The identification was performed using 16S ribosomal RNA (16S rRNA) sequencing method. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

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Commented [Rev11]: Desrini et al (2014) already isolate 37 isolates, however this work only 2 isolates, so the question is whether this work is really able to explore novel bioactive compounds from just two isolates?

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, *soybean meal, corn step liquor*, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and *Deionized demineralized water* (ddh2O) from Sigma Aldrich, Ltd. The kits were *Geno Plus™, Genomic DNA Extraction Miniprep System* from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, *Nutrient Agar* medium, and *Nutrient Broth* medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

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Plant sample preparation

The fresh leaves were collected from Tanggerang, Province Banten, Indonesia. The leaves were identified at Pusat Penelitian Konservasi and Kebun Raya, BRIN, Bogor, Jawa Barat, Indonesia. The leaves were washed by running tap water and carefully dried. The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then ground in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further step.

Commented [Rev13]: What does the means of "ground in NA plate". It is not a common terminology in microbiology.

Commented [Rev14]: Further experiment

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was

carried out using gram staining to analyse the colour and shape of bacterial colony cells. Gram staining was carried out by smearing one tube of bacterial colony thinly on an object glass, then the bacteria were confirmed first by adding 0,5% of crystal violet solution for the next 5 minutes. Drop Lugol's solution was added. The preparate were shaken until no dye flows over the glass object. The preparate were then analysed under microscope.

Commented [Rev15]: Gram staining is used for identification of Gram type of bacteria not for color analysis.

Commented [Rev16]: Check the protocol how to do Gram staining process.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was taken and put into a 10 ml *Nutrient Broth* test tube. Incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes with a speed of 2000 rpm. The obtained supernatant was used to screen the antibacterial potential of endophytic bacteria.

Commented [Rev17]: .....inoculated into.....

Commented [Rev18]: By shaking or not?

Commented [Rev19]: At 2000 rpm

Commented [Rev20]: As a source of antibacterial subtances

Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml *Nutrient Broth* test tubes for 24 hours at 37° C. The culture was then poured into a ± 20 ml petri dish until they were solid. The sterile paper disk was then soaked with the supernatant, then placed in the NB medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. The isolate with the largest zone of inhibition was continued for the molecular identification.

Commented [Rev21]: Two bacterial strains Streptococcus mutants and Lactobacillys acidophilus, were used as strain indicator for antibacterial activity assay.

Commented [Rev22]: It should be NA not NB

Commented [Rev23]: Usually calculated by the percentage of the ratio diameter of clear zone and the diameter paper disk.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018). The sequencing results were finally analysed by a Basic Local Alignment Search Tools (BLAST) using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the high similarity value with the existing bacterial species (Nxumalo et al, 2020).

Commented [Rev24]: Implemented for BLAST

RESULTS AND DISCUSSION

Determination of the binahong leaves

Determination was performed to confirm that the plants used in this study as a source to isolate the endophytic bacteria are the accurate plant species. According to the determination result, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Commented [Rev25]: Data?

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was used as a prior step for the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to *Nutrient Agar*

Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Commented [Rev26]: Does it mean that very difficult to get the similar isolate, even it was isolated from the same sample?

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 isolates were similar, suggesting that these two isolates were the same isolates. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 isolates were shown in Tabel 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The largest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 isolates against the bacteria of dental caries.

This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah, Ratnasari, & Lisdiana, 2013; Nxumalo, Ngid, Shandu, & Maliehe, 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Commented [Rev27]: What may cause the Gram Postive is fragile against the metabolite from endophytic bacteria?

Molecular identification of endophytic bacteria isolate DBA2

Among two isolates, DBA2 isolate was chosen as potential isolate as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The extracted genome was run by PCR using the 16S rRNA gene as a target. PCR products were run under a 0,8% gel electrophoresis. The finished gel was then analysed under UV. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2.

Commented [Rev28]: This method not need to be present in this section.

The DNA bands were then purified by Zymo Spin Column PCR Purification kit. The 16S rRNA gene sequence was performed for the purified products. The sequences of DNA were used to find similarity based on the 16S rRNA gene and later to determine the highest similarity species of organisms. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018).

Commented [Rev29]: See comment above

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level. In addition, it is said to be the same genus if the identity is 96% - 99%. Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the DBA2 isolate had a level of similarity to *Bacillus sp. strain x20* with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify the species of the endophytic bacteria as *Bacillus sp. strain x20*. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus sp strain x20* is also a Gram-positive bacterium producing organic acids and phosphatases (Gond, Bergen, Torres, & White, 2015; Zhao, et al., 2023). *Bacillus sp strain x20* was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids and phosphatases produced by Bacillus strain will solute the inorganic phosphorus into a plant-available form, therefore the microorganism can promote plant growth (Zhao, et al., 2023). It would be interesting to examine the bioactive compounds from the cultivation of *Bacillus sp strain x20* which can serve as antibacterial agents for dental caries treatment.

Commented [Rev30]: It is not true. 16SrRNA gene cannot be used for species level determination. It should be done by DNA-DNA hybridization

Commented [Rev31]: Based on Table 2. Why authors specifically chose the Bacillus sp. strain x20 as closely relatives to the DBA2

Commented [Rev32]: This sentence is not totally true. It should be: "Organic acid will solubilize the inorganic phosphate, while the phosphatase will solubilize the organic phosphate."

Commented [Rev33]: Further identification of bioactive compound produced Bacillus sp. is necessary

CONCLUSION

This study revealed that the endophytic bacteria *Bacillus sp strain x20* was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

ACKNOWLEDGMENTS

We acknowledge the Department of Biology Pharmacy, Faculty of Pharmacy and Science UHAMKA for providing the laboratory facilities for our research work. We thank to the faculty member in the Department of Biology-UHAMKA and the Department of Biology Education-UHAMKA for the great discussions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

Commented [Rev35]: Table is not appropriate

Isolates	*Shapes*	*Colour*	*Texture*	*Margins of Colony*	*Surface*	*Consistency*	*Staining*
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

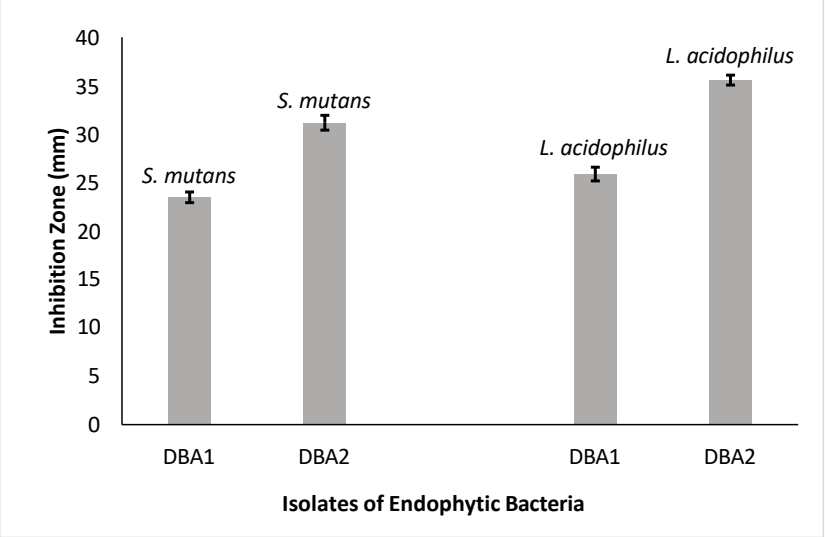

Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

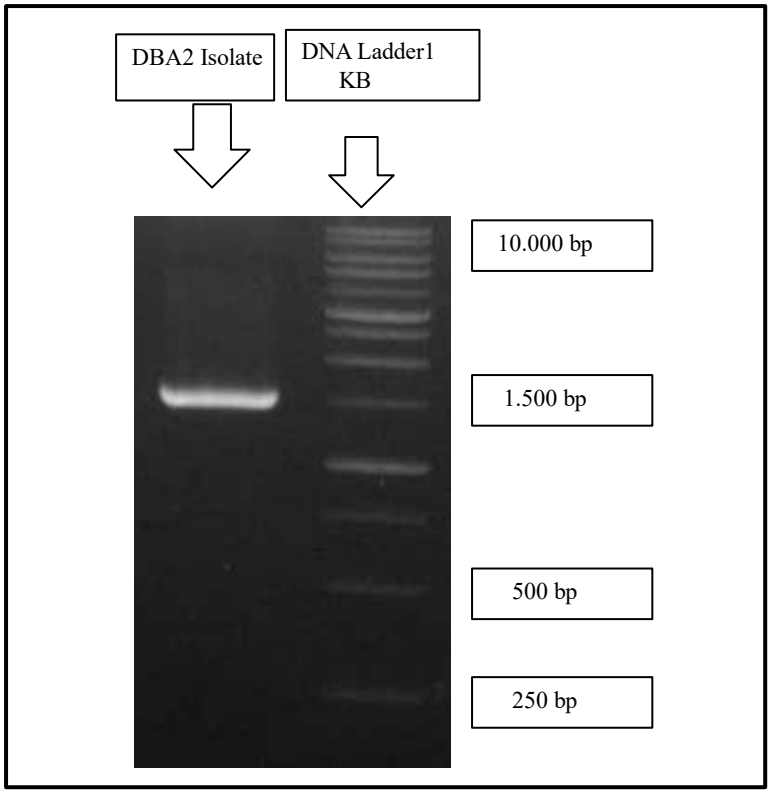

Figure 2. The gel electrophoresis of PCR product of DBA2 isolate using 16S ribosomal RNA gene

Commented [Rev36]: The gel electrophoresis of 16SrRNA gene

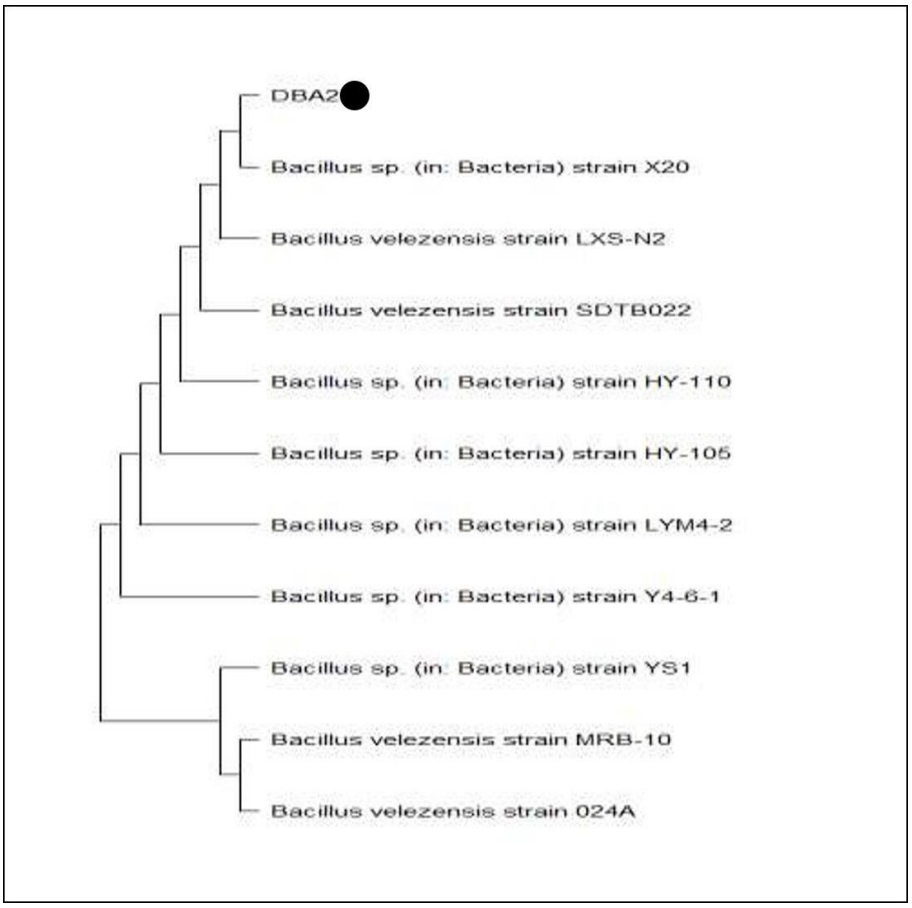

Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018).

Commented [Rev37]: This figure requires better resolution. This is also not really reflect the evolutionary relationships since the phyolgenetic tree is not rooted.

What model used in the phylogenetic tree development? Neighbor-Joining or Maximum Likelihood?

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

Commented [Rev38]: Table format is not appropriate.

No	*Closest Relative Species Based On 16s Rrna Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus SD (in Bacteria) strain X20*	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis strain LXS-N2*	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis strain SOTB022*	OK047738	1417	2139	0.0	100.00
4	*Bacillus SD, (in Bacteria) strain HY 110*	MZ895449	1453	2139	0.0	100.00
5	*Bacillus SO (in Bacteria) strain HY-105*	MZ895445	1445	2139	0.0	100.00
6	*Bacilus SO (in Bacteria) street LYM4-2*	OP493233	1448	2139	0.0	100.00

7	*Bacillus SD (in Bacteria) strain Y4-6-1*	OP493232	1451	2139	0.0	100.00
8	*Bacillus SD. (in: Bacteria) strain YS1*	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis strain MRB-10*	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis strain 024A*	OP477121	1453	2139	0.0	100.00

APJMBB Reviewer Comments-2 & 3:

Molecular Identification of Endophytic Bacteriuma DBA2 isolated from the leaf of Binahong Leaf (*Anredera cordifolia* (Ten.) Steenis) and their its Antibacterial antagonistic activity against bacteria associated with dental cariesPotency

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *Steenis* leaves. Two isolates, with the codes of DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening

for their antibacterial antagonistic activity against the bacteria of associated with dental caries, which

are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The isolate ~~of~~ DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* and *L. acidophilus*. ~~The isolate of~~ DBA2 was then ~~continue for~~further studied for identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System ~~The~~ and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene ~~sequencing method using PCR technique~~. ~~The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System~~The a~~.~~ Amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by ~~using~~ a BLAST search against the ~~GenBank~~ National Centre Biotechnology Information (NCBI) database. The endophytic bacterial ~~isolate~~ strain DBA2 from the leaves of *A. cordifolia* was identified molecularly as *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacilus* sp. strain x20 ~~with the similarity value of 100%~~. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

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INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah, Mashuri, & Abidin, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound,

diabetic, hematoma, and hyperuricemia (Yuniarti & Lukiswanto, 2017; Mutiarawati, Muljati, & Christyaningsih, 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita, Wijayati, & Mursiti, 2017; Surbakti, Queljoe, & Boddhi, 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Mengga, Rampe, & Sangande, 2022; Veronita, Wijayati, & Mursiti, 2017; Ainurrochmah, Ratnasari, & Lisdiana, 2013; Sasebohe, Prakasita, & Aditiyarini, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Xie, Yang, Tang, Chen, & Li, 2015; Basile, Giordano, López-Sáez, & Cobian, 1999). Flavonoids are the group which effectively inhibits the growth of viruses, bacteria, and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie, Yang, Tang, Chen, & Li, 2015; Veronita, Wijayati, & Mursiti, 2017). Several flavonoid compounds from the binahong leaves have been ~~isolated~~ identified, such as vitexin, isovitexin, and morin (Alba, Pelegrin, & Sobottka, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined~~s~~ as ~~a~~ microorganism~~s~~ that usually live as symbionts inside a plant (Nxumalo et al, 2020). These

endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant. They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms.

Desriani et al (2014) reported ~~state~~ that out of 37 of the endophytic bacterial isolates were obtained from the binahong leaves, ~~in which their~~ fifteen isolates exhibited antibacterial properties. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report ~~has ever~~have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against ~~to~~ *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Hussein, Saleem, & Merdad, 2023; Zhang, 2013). The identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing ~~method~~and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, *soybean meal, corn step liquor*, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and *Deionized demineralized water* (ddh2O) from Sigma Aldrich, Ltd. The kits were *Geno Plus™, Genomic DNA Extraction Miniprep System* from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, *Nutrient Agar* medium, and *Nutrient Broth* medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tanggerang, Province Banten, Indonesia. The leaves were identified at Pusat Penelitian Konservasi and Kebun Raya, BRIN, Bogor, Jawa Barat, Indonesia. The leaves were washed by running tap water and carefully dried. The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then ground in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further step.

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Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was

carried out using gram staining to analyse the colour and shape of bacterial ~~colony~~ cells. Gram staining was carried out by smearing one tube of bacterial colony thinly on an object glass, then the bacteria were confirmed first by adding 0~~,~~5% of crystal violet solution for the next 5 minutes. Drop Lugol's solution was added. The preparate were shaken until no dye flows over the glass object. The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was taken and put into a 10 ml Nutrient Broth test tube. Incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes with a speed of 2000 rpm. The obtained supernatant was used to screen the antibacterial potential of endophytic bacteria.

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Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml *Nutrient Broth* test tubes for 24 hours at 37° C. The culture was then poured into a ± 20 ml petri dish until they were solid. The sterile paper disk was then soaked with the supernatant, then placed in the NB medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. The isolate with the largest zone of inhibition was continued for the molecular identification.

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RESULTS AND DISCUSSION

Determination of the binahong leaves

~~Determination~~ taxonomic identification of the ~~was performed to confirm that the~~ plants used in this study was carried out before the specimen was ~~as a source to~~ used to isolate the endophytic bacteria ~~are~~ so that the accurate plant species was used. According to the ~~determination~~ identification result, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

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The surface sterilization was ~~used~~ done ~~as a~~ prior ~~step for~~ the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to *Nutrient Agar*

Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 isolates were similar, suggesting that these two isolates were the same isolates. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 isolates were shown in ~~Tabel~~Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The ~~largest~~ strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 isolates against the bacteria of dental caries.

This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah, Ratnasari, & Lisdiana, 2013; Nxumalo, Ngid, Shandu, & Maliehe, 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two isolates, DBA2 isolate was chosen as potential isolate as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The extracted genome was run by PCR using the 16S rRNA gene as a target. PCR products were run under a 0,8% gel electrophoresis. The finished gel was then analysed under UV. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2.

The DNA bands were then purified by Zymo Spin Column PCR Purification kit. The 16S rRNA gene sequence was performed for the purified products. The sequences of DNA were used to find similarity based on the 16S rRNA gene and later to determine the highest similarity species of organisms. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level. In addition, it is said to be the same genus if the identity is 96% - 99%. Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the DBA2 isolate had a level of similarity to *Bacillus sp. strain x20* with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify the species of the endophytic bacteria as *Bacillus sp. strain x20*. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus sp strain x20* is also a Gram-positive bacterium producing organic acids and phosphatases (Gond, Bergen, Torres, & White, 2015; Zhao, et al., 2023). *Bacillus sp strain x20* was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids and phosphatases produced by Bacillus strain will solute the inorganic phosphorus into a plant-available form, therefore the microorganism can promote plant growth (Zhao, et al., 2023). It would be interesting to examine the bioactive compounds from the cultivation of *Bacillus sp strain x20* which can serve as antibacterial agents for dental caries treatment.

CONCLUSION

This study revealed that the endophytic bacteria *Bacillus sp strain x20* was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

ACKNOWLEDGMENTS

We acknowledge the Department of Biology Pharmacy, Faculty of Pharmacy and Science UHAMKA for providing the laboratory facilities for our research work. We thank to the faculty member in the Department of Biology-UHAMKA and the Department of Biology Education-UHAMKA for the great discussions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

Isolates	*Shapes*	*Colour*	*Texture*	*Margins of Colony*	*Surface*	*Consistency*	*Staining*
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

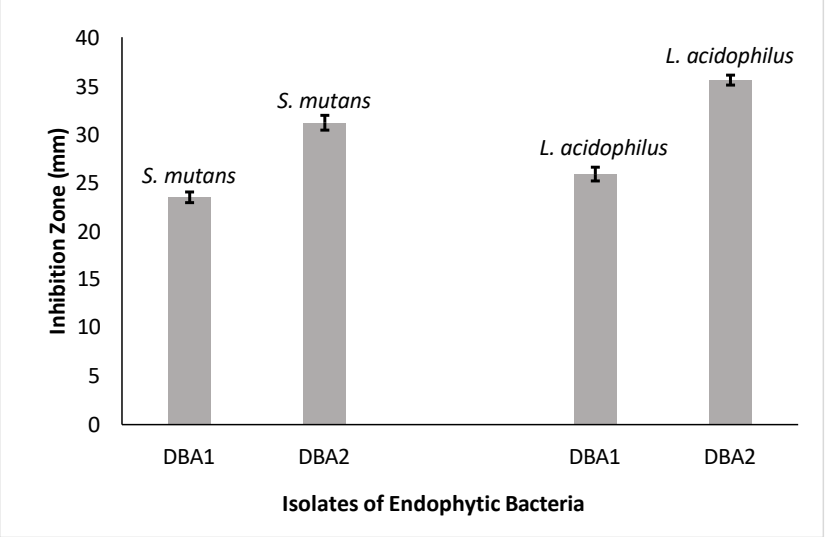


Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

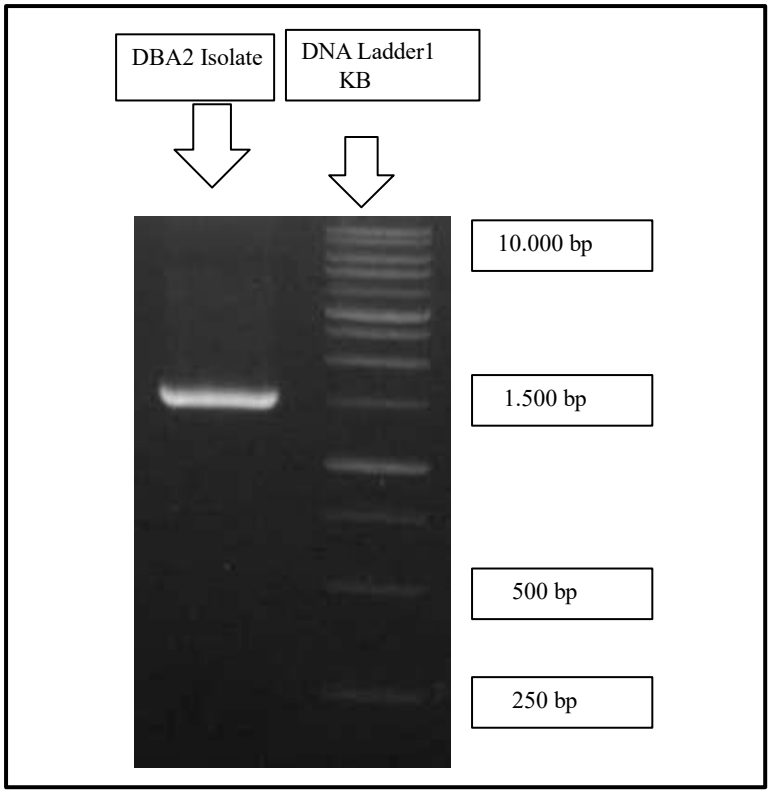


Figure 2. The gel electrophoresis of PCR product of DBA2 isolate using 16S ribosomal RNA gene

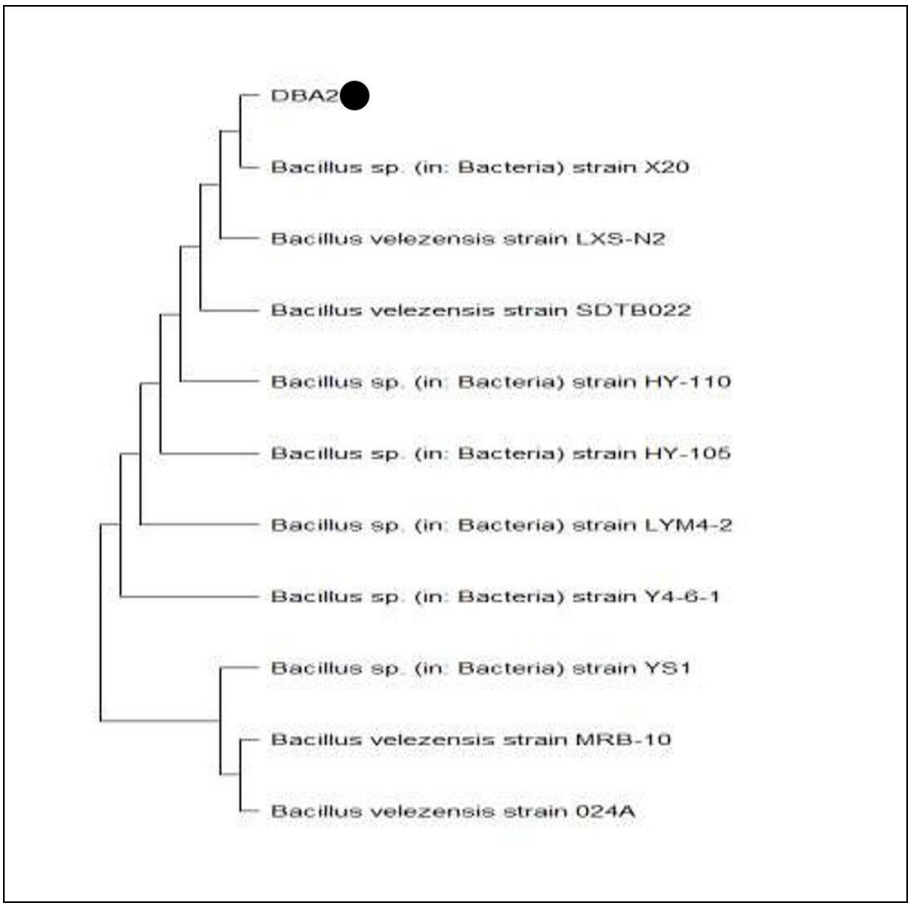


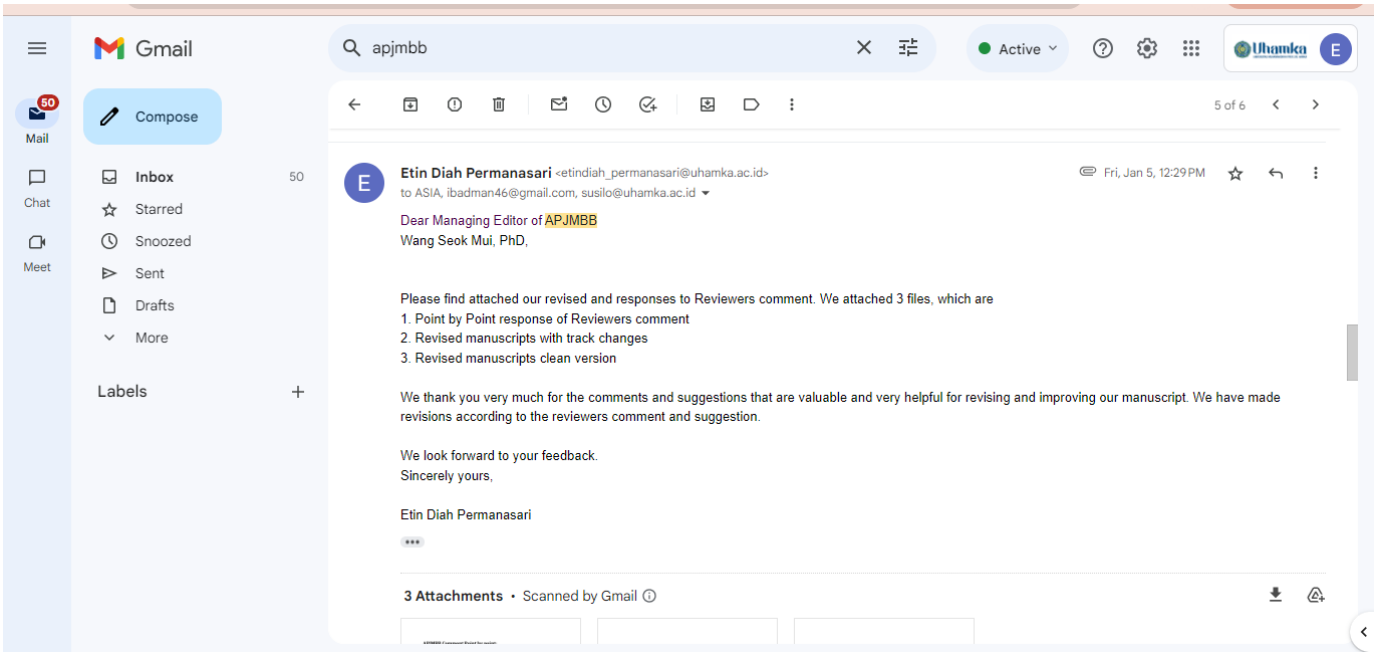
Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar, Stecher, Li, & Christina , 2018).

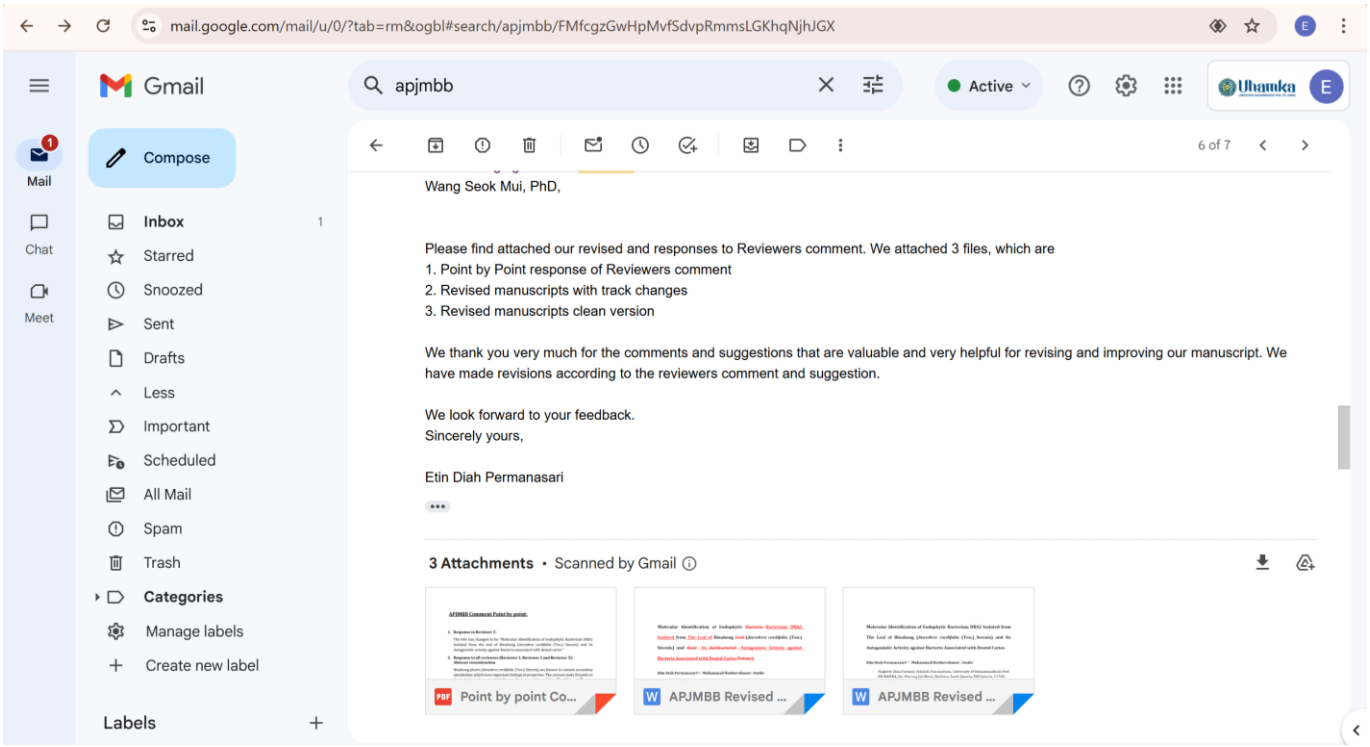
Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

No	*Closest Relative Species Based On 16s Rrna Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus SD (in Bacteria) strain X20*	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis strain LXS-N2*	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis strain SOTB022*	OK047738	1417	2139	0.0	100.00
4	*Bacillus SD, (in Bacteria) strain HY 110*	MZ895449	1453	2139	0.0	100.00
5	*Bacillus SO (in Bacteria) strain HY-105*	MZ895445	1445	2139	0.0	100.00
6	*Bacilus SO (in Bacteria) street LYM4-2*	OP493233	1448	2139	0.0	100.00

7	*Bacillus SD (in Bacteria) strain Y4-6-1*	OP493232	1451	2139	0.0	100.00
8	*Bacillus SD. (in: Bacteria) strain YS1*	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis strain MRB-10*	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis strain 024A*	OP477121	1453	2139	0.0	100.00

4. **Bukti konfirmasi submit revisi pertama, respon kepada reviewer, dan artikel yang diresubmit (5 Jan 2024):**





Point-by Point comment to the Editor:

APJMBB Comment Point by point:

1. **Response to Reviewer 2:**

   The title has changed to be "Molecular Identification of Endophytic Bacterium DBA2 Isolated from the leaf of Binahong (*Anredera cordifolia* (Ten.) Steenis) and its Antagonistic activity against Bacteria associated with dental caries "

2. **Response to all reviewers (Reviewer 1, Reviewer 2 and Reviewer 3):**
   Abstract rescontruction

   Binahong plants (*Anredera cordifolia* (Ten.) Steenis) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) Steenis. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) Steenis. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus* as respectively. The isolate of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified as *Bacillus sp*., and the top match from the database search revealed a similarity value of 100% with the reference *Bacilus sp.* strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

   Noted: The green color indicates the changes.

3. **Reviewer 1:**

- How much the different between the DBA1 and DBA2? Is there any semiquantitative data that can be provided?

  Answer: corrected to be "The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus* as respectively."

- ….was then subjected for molecular identification.

  Answer: corrected to be “The isolate of DBA2 was then subjected for molecular identification.”

- The 16S rRNA gene was used as molecular marker for such identification. 16SrRNA gene was amplified by PCR and then sequencing.

  Answer: corrected to be “~~The molecular identification was performed by 16S rRNA gene sequencing method using PCR technique~~. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene.”

- GenBank is not for sequencing but for searching the data base trough Blast method

  Answer: “Amplicons were then purified and sequenced by using GenBank National Centre Biotechnology Information (NCBI) database” corrected to be “The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database.”

- This for the plant or bacteria?

  Answer: For the bacteria, therefore we have modified by adding “strain DBA2” → corrected to be “Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.”

4. **Reviewer 1 (page 3)**

   To cite the literature, follow the journal rule or international rule how to cite the literature.

   Answer:

   The correction:

   The part of binahong plant that commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita, et al., 2017; Surbakti, et al., 2018).

5. **Reviewer 1 (page 4)**

- Is it true?

Answer: Yes, it is true. According to Arnold, et al., (2002); Potshangbam, et al., (2017), and Sharma, et al., (2021), the microorganisms produce similar bioactive compounds as its host plant.

- If the endophytic produce similar bioactive compound with the host, then it would not be possible to find the novel therapeutic agents

  Answer: Despite that most endophytic bacteria can produce the same metabolites as their host plants, but this does not rule out the possibility that these endophytic bacteria produce new compounds due to external and internal factors.

  The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

- Desriani et al (2014) already isolate 37 isolates, however this work only 2 isolates, so the question is whether this work is really able to explore novel bioactive compounds from just two isolates?

  Answer:

  Paragraph 2, Line 1: In our view, there is still the possibility of obtaining new compounds even with only two isolates.

6. **Reviewer 1 (page 5)**

- Paragraph 1, Line 3: corrected to be **ddH2O**
- Paragraph 3, Line 3: corrected to be "the cut leaves were then **put** in NA plate under aseptic condition".
- Paragraph 3, Line 5: corrected to be **further experiment**

7. **Reviewer 1 (page 6)**

- Paragraph 1, Line 1: corrected to be "Microscopic observation was carried out **using gram staining for identification of Gram type of bacteria**."
- Paragraph 1, Line 2: corrected to be "**Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023)."**
- Paragraph 2, Line 2: corrected to be "The pure isolate of endophytic bacteria **was inoculated into** a 10 ml Nutrient Broth test tube."
- Paragraph 2, Line 2: corrected to be "**Shaking incubation** was carried out for 2 days at a temperature of 25°C."
- Paragraph 2, Line 5: corrected to be "After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes **at** 2000 rpm."

- Paragraph 2, Line 2: corrected to be "The obtained supernatant was used **as a source of antibacterial substances potential** of endophytic bacteria."
- Paragraph 3, Line 2: We have added this sentence: "**Two bacterial strains *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strain indicator for antibacterial activity assay**."
- Paragraph 3, Line 5: corrected to be "The sterile paper disk was then soaked with the supernatant, then placed in the **NA** medium which has been inoculated with pathogenic bacteria."
- Paragraph 3, Line 8: We have added a sentence "**The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated**."

8. Reviewer 1 (page 7)

- Paragraph 1, Line 10: corrected to be "The sequencing results **were implemented for BLAST** using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the high similarity value with the existing bacterial species (Nxumalo et al, 2020).

- Paragraph 2, Line 3: We have attached the determination from Centre for Biosystematics and Evolution Research, National Research and Innovation Agency (BRIN) of Indonesia including with the voucher number of the specimen.

  "Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. **According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis**."

PUSAT RISET BIOSISTEMATIKA DAN EVOLUSI

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Nomor : B-804/V/DI.05.07/3/2022 Cibinong, 25 Maret 2022
Lampiran : -
Perihal : Hasil Identifikasi/Determinasi Tumbuhan

Yth.
Bpk./Ibu/Sdr(i). **Muhammad Ibadurrohman**
NPM : 1804015083
Fakultas Farmasi Dan Sains
Universitas Muhammadiyah Prof. Dr. Hamka

Bersama ini kami sampaikan hasil identifikasi/determinasi tumbuhan yang Saudara kirimkan ke "Herbarium Bogoriense", Pusat Riset Biosistematika dan Evolusi BRIN Cibinong, adalah sebagai berikut :

No.	No. Kol.	Jenis	Suku
1.	Daun Binahong	*Anredera cordifolia*	Basellaceae

Demikian, semoga berguna bagi Saudara.

Plt. Kepala Pusat Riset Biosistematika dan Evolusi
Badan Riset dan Inovasi Nasional

BADAN RISET DAN INOVASI NASIONAL * REPUBLIK INDONESIA * ORGANISASI RISET ILMU PENGETAHUAN HAYATI

Dr. Anang Setiawan Achmadi, S.KH., M.Sc

9. Reviewer 1 (page 8)

Paragraph 1, Line 6: We do not think that it is difficult to obtain the same isolate.

10. Reviewer 1 (page 9)

- What may cause the Gram Postive is fragile against the metabolite from endophytic bacteria?
  Answer:
  Paragraph 1, Line 4: We have added the sentence "**Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2**."

- Paragraph 2, Line 3: We have deleted the method.
- Paragraph 3, Line 1: We have deleted the method.

11. **Reviewer 1 (page 10)**

- It is not true. 16SrRNA gene cannot be used for species level determination. It should be done by DNA-DNA hybridization

  Answer:

  Paragraph 2, Line 1: This statement refers to Drancourt et al., (2000), that if similarity percentage >99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate to the species level. A 97 to 99% identity in 16S rRNA gene sequence was the criterion used to identify an organism at the genus level, and <97% identity in 16S rRNA gene sequence was the criterion used to define a potentially new bacterial species. See https://www.ncbi.nlm.nih.gov/pmc/articles/PMC87447/

- Based on Table 2. Why authors specifically chose the *Bacillus* sp. strain x20 as closely relatives to the DBA2

  Answer:

  Paragraph 2, Line 9: Because the similarity percentage is 100% and strain x20 is at the top match. Therefore, we propose that strain DBA2 is a *Bacillus* sp. strain x20.

- This sentence is not totally true. It should be: "Organic acid will solubilize the inorganic phosphate, while the phosphatase will solubilize the organic phosphate."

  Answer:

  Paragraph 3, Line 5: We made change the sentence to be "**Organic acids produced by Bacillus strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatase will solute the organic phosphorus,** therefore the microorganism can promote plant growth."

- Paragraph 3, Line 7: We made change the sentence to be "Further identification of bioactive compound produced *Bacillus* sp. is necessary."

12. **Reviewer 1 (page 11)**
    We have correction the references.

13. **Reviewer 1 (page 15)**
    We have correction the Table 1.

14. **Reviewer 1 (page 17)**
    We have correction the Figure 2 title.

15. **Reviewer 1 (page 18)**
    We have attached the high resolution for Figure 3 and We use the Neighbour-Joining

16. Reviewer 1 (page 19)

We have corrected the Table 2.

17. Reviewer 2

- The authors need to define DBA1 and DBA2 better whether they are isolates or strains. Isolates refers to pure cultures obtained from isolation without knowing anything about them. Once the isolates have been characterized, isolates showing identical characteristics (Gram staining, colony & cellular morphology, or even 16S rRNA gene sequences) will be identified as the same strain. The general rule is that different isolates can be the same strain, but different strains cannot be the same isolate.
- In short, if the study has determined that DBA1 and DBA2 are the same strain, they should remove the findings about DBA1 from the abstract. However, the two strains had demonstrated different levels of antagonistic activities against the dental caries pathogens. Hence, they should be different strains.

  Answer: We have modified in a way that the terminology of "isolate" has been changed into "strain".

18. Reviewer 2:

- Keywords 16S rRNA gene instead of 16S rRNA
  Answer: The correction was provided in the manuscript.

- Antibacterial potency in the title and antibacterial activity throughout the manuscript – suggest the term antagonistic activity
  Answer: The correction was provided in the manuscript. The term antibacterial was changed to be "antagonistic activity"

- Introduction – "Flavonoids are the group which effectively inhibits the growth of viruses, bacteria, and fungi, as it causes damage to the permeability of cell walls and inhibit motility" – this statement is inaccurate as the viruses have no cell walls
  Answer:
  We have deleted the "virus" in the sentence.
  Corrected to be "Flavonoids are the group which effectively inhibits the growth of ~~viruses,~~ bacteria, and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie et al., 2015; Veronita et al., 2017)."

- Introduction – "These microorganisms produce similar bioactive compounds as its host plant." – this statement is disputable and has no reference to back up
  Answer: We have provided the references.

Corrected to be “These microorganisms produce similar bioactive compounds as its host plant (Arnold, et al., 2020; Potshangbam et al., 2020; Sharma, et al., 2021).”

- Introduction – “Desriani et al (2014) reported that out of 37 of the endophytic bacterial isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties.” – more info is needed, antibacterial properties against what kind of microorganisms?
  Answer:
  Against *Pseudomonas aeruginosa*.
  We have modified to be: “Desriani et al (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against ***Pseudomonas aeruginosa.”***

- Methods – “The leaves were washed by running tap water and carefully dried” – this is not a proper sample processing. An autoclave-sterilized distilled water should be used as the tap water contains microorganisms that can be introduced into the plant samples
  Answer:
  Yes, it may be true. However, after cleaning with running tap water, we processed the leaves by wiping them with EtOH 75%. Therefore, we believe that our method is sufficient to sterilize the leaves surface.

- “The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%. – please clarify if NaOCl is used at 5.3%
  Answer: Yes, we used NaOCl 5.3%.

- The cut leaves were then ground in NA plate under aseptic condition.” – please clarify, it is impossible to grind the leaves in NA plates in Petri dish. The authors should have used a sterile pestle and mortal to grind the samples, before resuspending and inoculate into NA plates
  Answer:
  We have corrected the sentence to be: The cut leaves were then ~~ground~~ put in NA plate under aseptic condition.

- “Gram staining was carried out by smearing one tube of bacterial colony thinly on an object glass, then the bacteria were confirmed first by adding 0.5% of crystal violet solution for the next 5 minutes. Drop Lugol’s solution was added. The preparate were shaken until no dye flows over the glass object. The preparate were then analysed under microscope.” – the protocol for gram staining is incomplete, lacking of safranin and so on, please provide complete procedure for the staining
  Answer:

  We have corrected to be: “Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out

firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope."

- The authors do not need to italicize the DNA extraction kit, name of media used
  Answer: Yes, the correction was done.

- Methods – "The strain culture of the bacteria of dental caries (Streptococcus mutans and Lactobacillus acidophilus) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was then poured into a ± 20 ml petri dish until they were solid." – this part of the method is confusing, how did nutrient broth incubated with the pathogens solidify without agar?
  Answer:
  The sentence has been corrected to be: "The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of **NA i**n Petri dish."

- Methods – "in order to determine the high similarity value with the existing bacterial species" – suggest to correct it to in order to determine the most closely related reference bacteria in the database
  Answer:
  We have corrected to be:
  "The sequencing results were implemented for Basic Local Alignment Search Tools (BLAST) using the database from the National Centre for Biotechnology Information (NCBI) **in order to determine the most closely related** reference bacteria in the database (Nxumalo et al, 2020)."

- Results – "Determination was performed to confirm that the plants used in this study as a source to isolate the endophytic bacteria are the accurate plant species. According to the determination result, it was known that the leaves is Anredera cordifolia (Teen.) Steenis" – there should a specimen voucher deposited in the centre and the number of the voucher should be provided
  Answer: We have provided the voucher number of the specimen.
  "Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. **According to the identification result from Centre for Biosystematics**

and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis."

- Results – "Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 isolates were similar, suggesting that these two isolates were the same isolates." – The two strains had later demonstrated different levels of antagonistic activities against the dental caries pathogens. Hence, they should be different strains.
  Answer: Yes, we have corrected to be "Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 isolates were ~~similar, suggesting that these two isolates were the same isolates~~ described in Table 1."

- The 16S rRNA gene sequencing and analysis with reference to the NCBI database only revealed the DBA2 was a strain of *Bacillus* sp. and *Bacillus* sp. strain x20 was returned from the database search as the top match. We cannot conclude that DBA2 is *Bacillus* sp. strain x20 as it should be a different strain.
  Answer: "However, in our study, we believed that strain of DBA2 is *Bacillus* sp. strain x20 as it showed 100 similarity and appear in the top match."

19. Reviewer 3

Introduction part should include more information on endophytic bacteria of earlier studies on binahong and/or other plants similar to binahong; and the need to test on dental caries-related microorganisms (was there similar studies or is binahong usually chewed traditionally to prevent caries, etc?); while reduce discussing on flavonoids since it is not being investigated or compared in this study.
Answer:
We have modified the introduction, as follow:

Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* (Nursulistyarini and Ainy, 2014).

We have already added in the introduction that *S. mutans* and *L. acidophilus* are the main dental caries-related microorganism in humans, and added the information of similar studies from different medicinal plant against several dental caries-related microorganisms.

"We tested on the antagonistic activity of the isolated endophytic bacteria against ***Streptococcus mutans*** **and** ***Lactobacillus acidophilus*** **which mainly caused dental caries in human** (Hussein et al., 2023; Zhang, 2013). **Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty et al., 2017; Abdel-Aziz et al., 2020).** The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited."

See: https://www.neliti.com/publications/61154/isolation-of-endophytic-fungi-from-the-coastal-plant-terong-pungo-solanum-sp-and#cite and https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7277960/.

20. Reviewer 3

Pg5: Endophytic bacteria isolation from the binahong leaves. The methodology here needs to be expanded more. The grounding of leaves and subsequent dilution and plating methods should be in more detail. Or cite the adaptation of a previous study method, if applicable.

Answer:

We have corrected the methodology to be: "The cut leaves were then put in NA plate under aseptic condition".

21. Reviewer 3

Pg 6: Antibacterial activities screening: What is the control positive and control negatives used for analysing the antibacterial properties of the supernatant? It would be useful to know the comparisons to standard antibiotics and interpret according to CLSI guidelines

Answer:

In this study we don't use the control positive as our aim is only to screen on which the bacteria exhibited the largest inhibition zone. However, we used the media for the negative control.

22. Reviewer 3

The molecular determination of strains was done using 16SrRNA sequences, and Bacillus x20 strain was identified in this study. Since Bacillus is a spore forming organism, what steps were taken to rule out contamination from external sources e.g from soil. Maybe can be explained in the methodology?

Answer:

In the Page 9 (Paragraph 2, line 3), we have already mentioned that we added nystatin in the NA agar plate to prevent the growth of fungi or spore.

23. Reviewer 3

The main objective of this study was determining antibacterial properties of potential endophytes, and subsequently identifying that endophyte. So the antibacterial properties/findings must be expanded more, as was

stated in Comment 6, to include comparisons to standard antibiotics. Molecular identification is important too, but since it is not a novel organism, the focus should not be on the molecular analysis of this organism.

Answer:

Our objective in this study is to isolate and identify the bacteria which exhibit antibacterial activities. Therefore, our focused is on the molecular identification.

APJMBB Revised Manuscripts with track changes:

Molecular Identification of Endophytic ~~Bacteria~~ Bacterium DBA2 Isolated from The Leaf of Binahong ~~Leaf~~ (*Anredera cordifolia* (Ten.) Steenis) and ~~their~~ Its ~~Antibacterial~~ Antagonistic Activity against Bacteria Associated with Dental Caries ~~Potency~~

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *Steenis* ~~leaves~~. Two isolates, ~~with the codes of~~ DBA1 and DBA2 were isolated and

purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their ~~antibacterial~~ antagonistic activity against the bacteria ~~of~~ associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The ~~isolate~~ strain ~~of~~of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The ~~isolate~~ strain of DBA2 was then ~~continue~~ subjected for molecular identification. ~~The molecular identification was performed by 16S rRNA gene sequencing method using PCR technique.~~ The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. ~~Amplicons~~ The amplicons were then purified and sequenced, ~~by using~~ before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the ~~GenBank~~ National Centre Biotechnology Information (NCBI) database. The endophytic bacterial ~~isolate~~ strain DBA2 from the leaves of *A. cordifolia* was identified ~~molecularly~~ as *Bacillus* sp., ~~*strain x20* with~~ and the top match from the database search revealed a~~the~~ similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be

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considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases ~~(Azizah, Mashuri, & Abidin, 2022)~~ (Azizah et al., 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia ~~(Yuniarti & Lukiswanto, 2017; Mutiarawati, Muljati, & Christyaningsih, 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011)~~ (Yuniarti & Lukiswanto, 2017; Mutiarawati et al., 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids ~~(Veronita, Wijayati, & Mursiti, 2017; Surbakti, Queljoe, & Boddhi, 2018).~~ (Veronita et al., 2017; Surbakti et al., 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* ~~(Mengga, Rampe, & Sangande, 2022;~~

~~Veronita, Wijayati, & Mursiti, 2017; Ainurrochmah, Ratnasari, & Lisdiana, 2013; Sasebohe, Prakasita, & Aditiyarini, 2023)~~(Mengga et al., 2022; Veronita et al., 2017; Ainurrochmah et al., 2013; Sasebohe et al., 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties ~~(Xie, Yang, Tang, Chen, & Li, 2015; Basile, Giordano, López-Sáez, & Cobian, 1999)~~ (Xie et al., 2015; Basile et al., 1999). Flavonoids are the group which effectively inhibits the growth of ~~viruses,~~ bacteria~~,~~ and fungi, as it causes damage to the permeability of cell walls and inhibit motility ~~(Xie, Yang, Tang, Chen, & Li, 2015; Veronita, Wijayati, & Mursiti, 2017)~~(Xie et al., 2015; Veronita et al., 2017). Several flavonoid compounds from the binahong leaves have been ~~isolated~~ identified, such as vitexin, isovitexin, and morin ~~(Alba, Pelegrin, & Sobottka, 2020)~~ (Alba et al., 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are ~~defines~~ defined as ~~a~~ microorganisms that usually live as symbionts inside a plant (Nxumalo et al, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold, et al., 2020; Potshangbam et al., 2020; Sharma, et al., 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites

that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani et al (2014) ~~state~~ reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, ~~in which their~~ fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report ~~has ever~~ have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against ~~to~~ *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human ~~(Hussein, Saleem, & Merdad, 2023; Zhang, 2013)~~ (Hussein et al., 2023; Zhang, 2013). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty et al., 2017; Abdel-Aziz et al., 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, t~~T~~he identification was performed using

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16S ribosomal RNA (16S rRNA) gene sequencing ~~method~~ and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and ~~Deionized~~deionized demineralized water (dd~~H~~H_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar medium, and Nutrient Broth medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

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Plant sample preparation

The fresh leaves were collected from ~~Tanggerang~~Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), ~~Pusat Penelitian Konservasi and Kebun Raya, BRIN,~~ Bogor, ~~Jawa Barat~~West Java, Indonesia. The leaves were washed by running tap water and carefully dried. The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then ~~ground~~ put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further ~~step~~ experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining ~~to analyse the colour and shape of bacterial colony cells for identification of Gram type of bacteria~~. Gram staining was carried out firstly by ~~smearing one tube of bacterial colony thinly on an object glass, then the bacteria were confirmed first by adding 0,5% of crystal violet solution for the next 5 minutes~~an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. ~~Drop Lugol's solution was added. The preparate were shaken until no dye flows over the glass object.~~The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria ~~was taken and put into~~ was inoculated into a 10 ml Nutrient Broth test tube. Shaking i~~I~~ncubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant

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were harvested using centrifugation for 30 minutes ~~with a speed of~~ at 2000 rpm. The obtained supernatant was used ~~to screen the antibacterial~~ as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. Two bacterial strains *Streptococcus mutants* and *Lactobacillus acidophilus,* were used as strain indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was ~~then poured into a ± 20 ml petri dish until they were solid~~ then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the ~~NB~~ NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated. The isolate with the largest zone of inhibition was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT

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GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis ~~(Kumar, Stecher, Li, & Christina, 2018)~~(Kumar et al., 2018). The sequencing results were ~~finally analysed~~ implemented for ~~by a Basic Local Alignment Search Tools (~~BLAST~~)~~ using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the ~~high similarity value with the existing bacterial species~~ most closely related reference bacteria in the database (Nxumalo et al, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

~~Determination~~ Taxonomic identification of the ~~was performed to confirm that the~~ plants used in this study was carried out before the specimen was ~~as a source~~ used to isolate the endophytic bacteria ~~are~~ so that the accurate plant species was used. According to the ~~determination~~ identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was ~~used as a~~ done prior ~~step for~~ the whole isolation process. This surface sterilization method was performed to eliminate the contaminant

microorganisms that present in the surface leaves. Nystatin was added to Nutrient Agar Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

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Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 ~~isolates~~ were ~~similar, suggesting that these two isolates were the same isolates~~described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 ~~isolates~~ were shown in ~~Tabel~~ Table 1.

Antibacterial activity screening

The two ~~isolates~~ isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The ~~largest~~ strongest antibacterial activity was DBA2 with the

average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 ~~isolates~~ against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria ~~(Ainurrochmah, Ratnasari, & Lisdiana, 2013; Nxumalo, Ngid, Shandu, & Malieke, 2020)~~ (Ainurrochmah et al., 2013; Nxumalo et al., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two ~~isolates~~strains, strain DBA2 ~~isolate~~ was chosen as potential ~~isolate~~ strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. ~~The extracted genome was run by PCR using the 16S rRNA gene as a target. PCR products were run under a 0,8% gel electrophoresis. The finished gel was then analysed under UV.~~ The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2.

Molecular ~~Identification~~ identification of e~~E~~ndophytic ~~Bacteria~~ bacterium DBA2 isolated from the leaf of ~~Binahong~~ binahong ~~Leaf~~ (*Anredera cordifolia* (Ten.) Steenis) and ~~their~~ its ~~Antibacterial Potency~~antagonistic activity against bacteria associated with dental caries

~~The DNA bands were then purified by Zymo Spin Column PCR Purification kit. The 16S rRNA gene sequence was performed for the purified products. The sequences of DNA were used to find similarity based on the 16S rRNA gene and later to determine the highest similarity species of organisms.~~ It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis ~~(Kumar, Stecher, Li, & Christina , 2018)~~ (Kumar et al., 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt et al., 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt et al., 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained ~~i~~in the database that have a high level of homology. The results showed that the strain DBA2 ~~isolate~~ had a level of similarity to *Bacillus* sp.~~,~~ strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify the species of the endophytic bacteria as *Bacillus* sp.~~,~~ strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases ~~(Gond, Bergen, Torres, & White, 2015; Zhao, et al., 2023)~~ (Gond et al., 2015; Zhao et al., 2023). *Bacillus* sp. strain x20 was

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known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids ~~and phosphatases~~ produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao, et al., 2023). ~~It would be interesting to examine the bioactive compounds from the cultivation of~~Further identification of bioactive compound produced by *Bacillus* sp. strain x20 ~~which can serve as antibacterial agents for dental caries treatment~~is necessary.

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CONCLUSION

This study revealed that the endophytic bacteria *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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Molecular ~~Identification~~ identification of e~~E~~ndophytic ~~Bacteria~~ bacterium DBA2 isolated from the leaf of ~~Binahong~~ binahong ~~Leaf~~ (*Anredera cordifolia* (Ten.) Steenis) and ~~their~~ its ~~Antibacterial Potency~~antagonistic activity against bacteria associated with dental caries

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

~~*Isolates*~~	~~*Shapes*~~	~~*Colour*~~	~~*Texture*~~	~~*Margins of Colony*~~	~~*Surface*~~	~~*Consistency*~~	~~*Staining*~~
~~*DBA1*~~	~~Irregular~~	~~White~~	~~Flat~~	~~Undulate~~	~~Flat and smooth~~	~~Mucoid~~	~~+ve~~
~~*DBA2*~~	~~Irregular~~	~~White~~	~~Flat~~	~~Curled~~	~~Flat and smooth~~	~~Buttery~~	~~+ve~~

Isolates	Shapes	Colour	Texture	Margins of Colony	Surface	Consistency	Staining
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve

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Molecular ~~Identification~~ identification of e~~E~~ndophytic ~~Bacteria~~ bacterium DBA2 isolated from the leaf of ~~Binahong~~ binahong ~~Leaf~~ (*Anredera cordifolia* (Ten.) Steenis) and ~~their~~ its ~~Antibacterial Potency~~antagonistic activity against bacteria associated with dental caries

DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

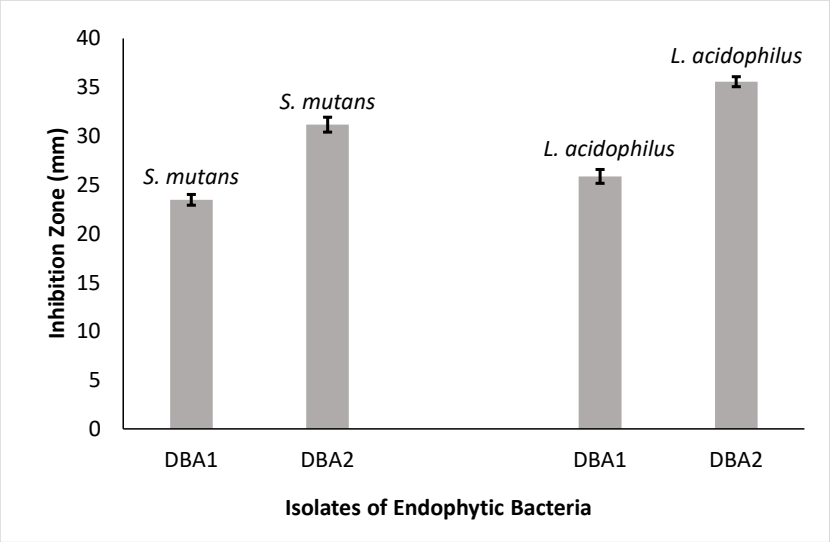


Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

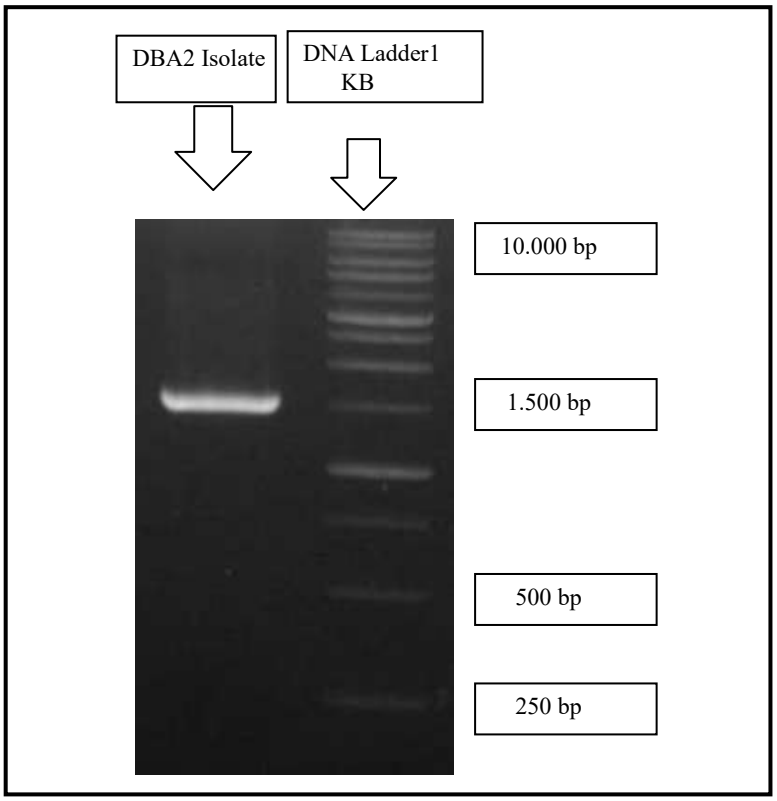
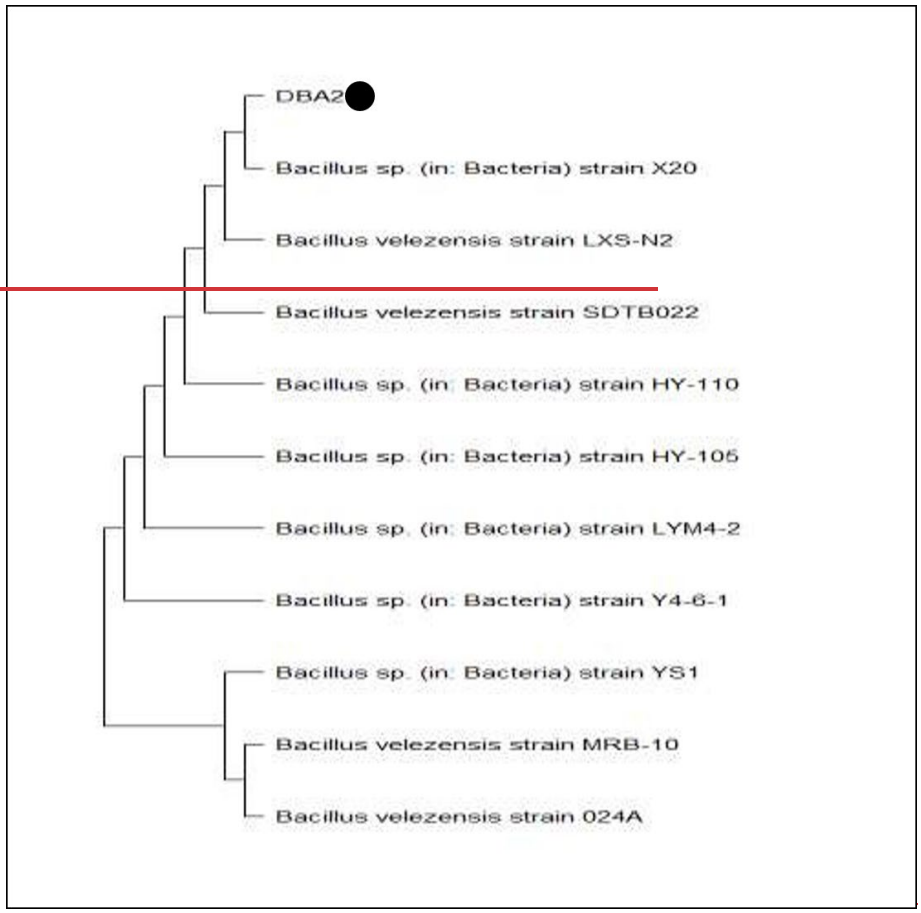


Figure 2. The gel electrophoresis of ~~PCR product of DBA2 isolate using~~ 16S ~~ribosomal~~ rRNA gene

Molecular ~~Identification~~ identification of e~~E~~ndophytic ~~Bacteria~~ bacterium DBA2 isolated from the leaf of ~~Binahong~~ binahong ~~Leaf~~ (*Anredera cordifolia* (Ten.) Steenis) and ~~their~~ its ~~Antibacterial Potency~~antagonistic activity against bacteria associated with dental caries



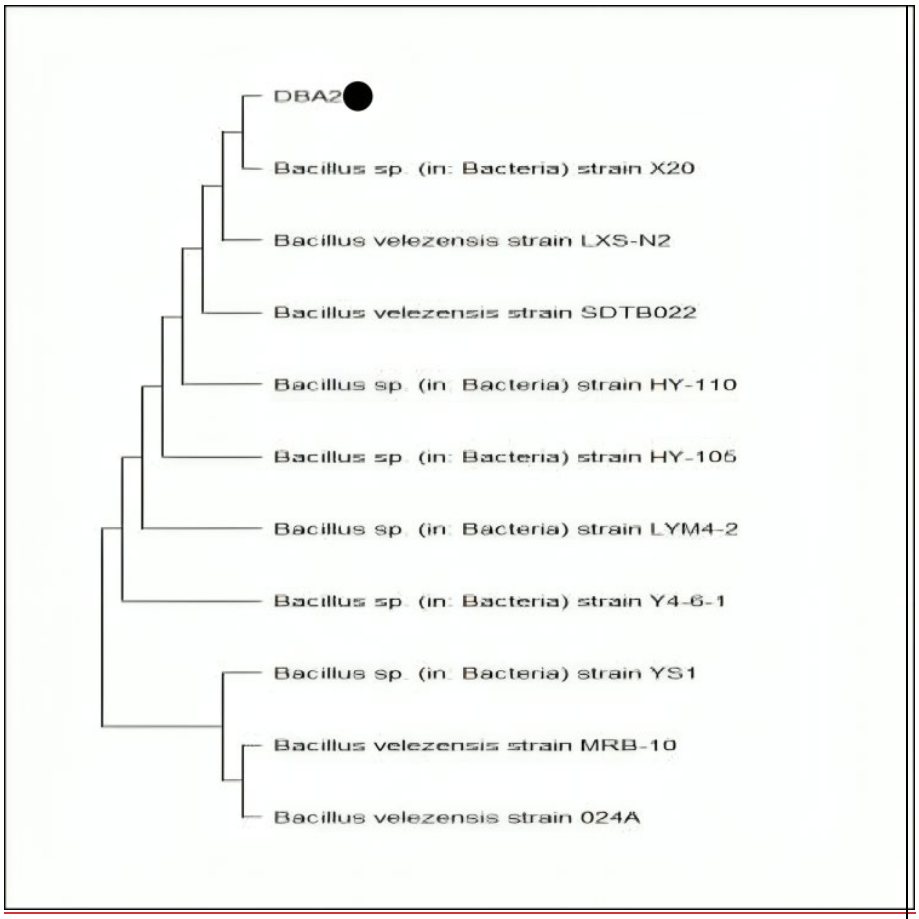


Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis ~~(Kumar, Stecher, Li, & Christina , 2018).~~(Kumar et al., 2018).

Molecular ~~Identification~~ identification of e~~E~~ndophytic ~~Bacteria~~ bacterium DBA2 isolated from the leaf of ~~Binahong~~ binahong ~~Leaf~~ (*Anredera cordifolia* (Ten.) Steenis) and ~~their~~ its ~~Antibacterial Potency~~antagonistic activity against bacteria associated with dental caries

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

~~*No*~~	~~*Closest Relative Species Based On 16s Rrna Gene Sequences*~~	~~*Genbank Accession Number*~~	~~*Base Pair Length (bp)*~~	~~*Max score*~~	~~*E Value*~~	~~*% Similarity*~~
~~*1*~~	~~*Bacillus SD (in Bacteria) strain X20*~~	~~OP537150~~	~~1530~~	~~2139~~	~~0.0~~	~~100.00~~
~~*2*~~	~~*Bacillus velezensis strain LXS-N2*~~	~~OP536155~~	~~1460~~	~~2139~~	~~0.0~~	~~100.00~~
~~*3*~~	~~*Bacillus velezensis strain SOTB022*~~	~~OK047738~~	~~1417~~	~~2139~~	~~0.0~~	~~100.00~~
~~*4*~~	~~*Bacillus SD, (in Bacteria) strain HY 110*~~	~~MZ895449~~	~~1453~~	~~2139~~	~~0.0~~	~~100.00~~
~~*5*~~	~~*Bacillus SO (in Bacteria) strain HY-105*~~	~~MZ895445~~	~~1445~~	~~2139~~	~~0.0~~	~~100.00~~
~~*6*~~	~~*Bacilus SO (in Bacteria) street LYM4-2*~~	~~OP493233~~	~~1448~~	~~2139~~	~~0.0~~	~~100.00~~

~~7~~	~~*Bacillus SD (in Bacteria) strain Y4-6-1*~~	~~OP493232~~	~~1451~~	~~2139~~	~~0.0~~	~~100.00~~
~~8~~	~~*Bacillus SD. (in: Bacteria) strain YS1*~~	~~OP493231~~	~~1449~~	~~2139~~	~~0.0~~	~~100.00~~
~~9~~	~~*Bacillus velezensis strain MRB-10*~~	~~OP493205~~	~~1441~~	~~2139~~	~~0.0~~	~~100.00~~
~~10~~	~~*Bacillus velezensis strain 024A*~~	~~OP477121~~	~~1453~~	~~2139~~	~~0.0~~	~~100.00~~

No	*Closest Relative Species Based On 16S rRNA Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00

4	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00
7	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

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Molecular Identification of Endophytic Bacterium DBA2 Isolated from The Leaf of Binahong (*Anredera cordifolia* (Ten.) Steenis) and Its Antagonistic Activity against Bacteria Associated with Dental Caries

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *Steenis*. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their

antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified as *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah et al., 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Yuniarti & Lukiswanto, 2017; Mutiarawati et al., 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita et al., 2017; Surbakti et al., 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Mengga et al., 2022; Veronita et al., 2017; Ainurrochmah et al., 2013; Sasebohe et al., 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Xie et al., 2015; Basile et al., 1999). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and

inhibit motility (Xie et al., 2015; Veronita et al., 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba et al., 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo et al, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold, et al., 2020; Potshangbam et al., 2020; Sharma, et al., 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani et al (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020). Previous study also reported

3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Hussein et al., 2023; Zhang, 2013). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty et al., 2017; Abdel-Aziz et al., 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column

PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar medium, and Nutrient Broth medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried. The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to

remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml Nutrient Broth test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. Two bacterial strains *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strain indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated. The isolate with the largest zone of inhibition was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar et al., 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo et al, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that

present in the surface leaves. Nystatin was added to Nutrient Agar Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah et al., 2013; Nxumalo et al., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar et al., 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt et al., 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt et al.,

2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify the species of the endophytic bacteria as *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond et al., 2015; Zhao et al., 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao, et al., 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

CONCLUSION

This study revealed that the endophytic bacteria *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of

dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

ACKNOWLEDGMENTS

We acknowledge the Department of Biology Pharmacy, Faculty of Pharmacy and Science UHAMKA for providing the laboratory facilities for our research work. We thank to the faculty member in the Department of Biology-UHAMKA and the Department of Biology Education-UHAMKA for the great discussions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

Isolates	**Shapes**	**Colour**	**Texture**	**Margins of Colony**	**Surface**	**Consistency**	**Staining**
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

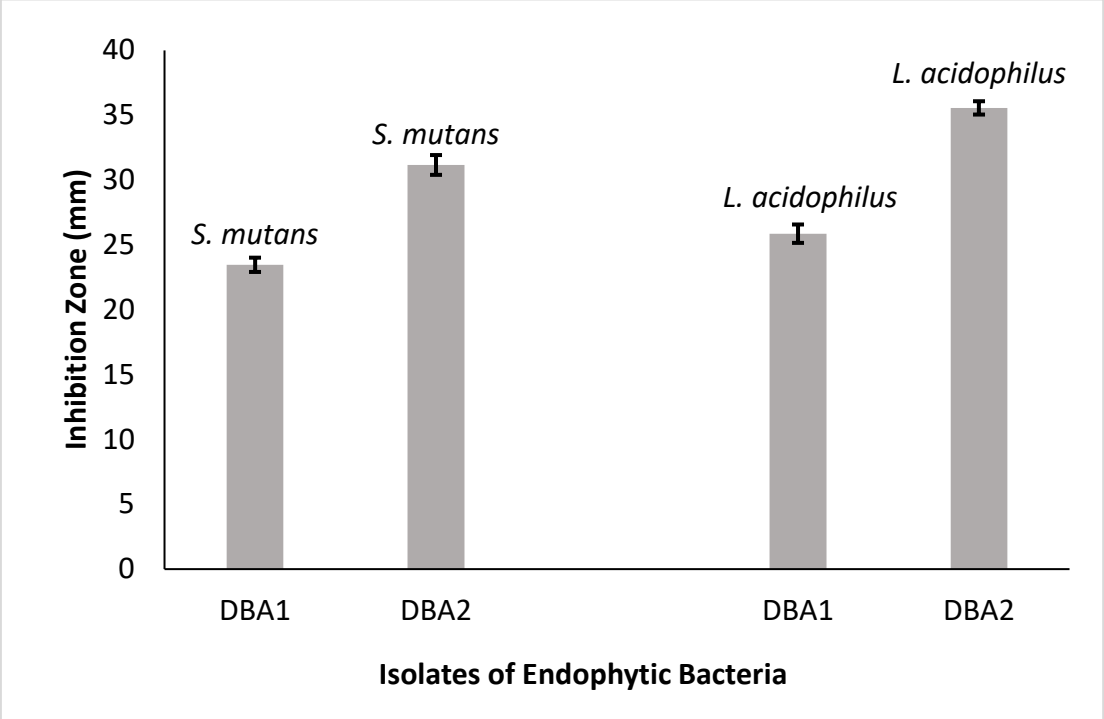


Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

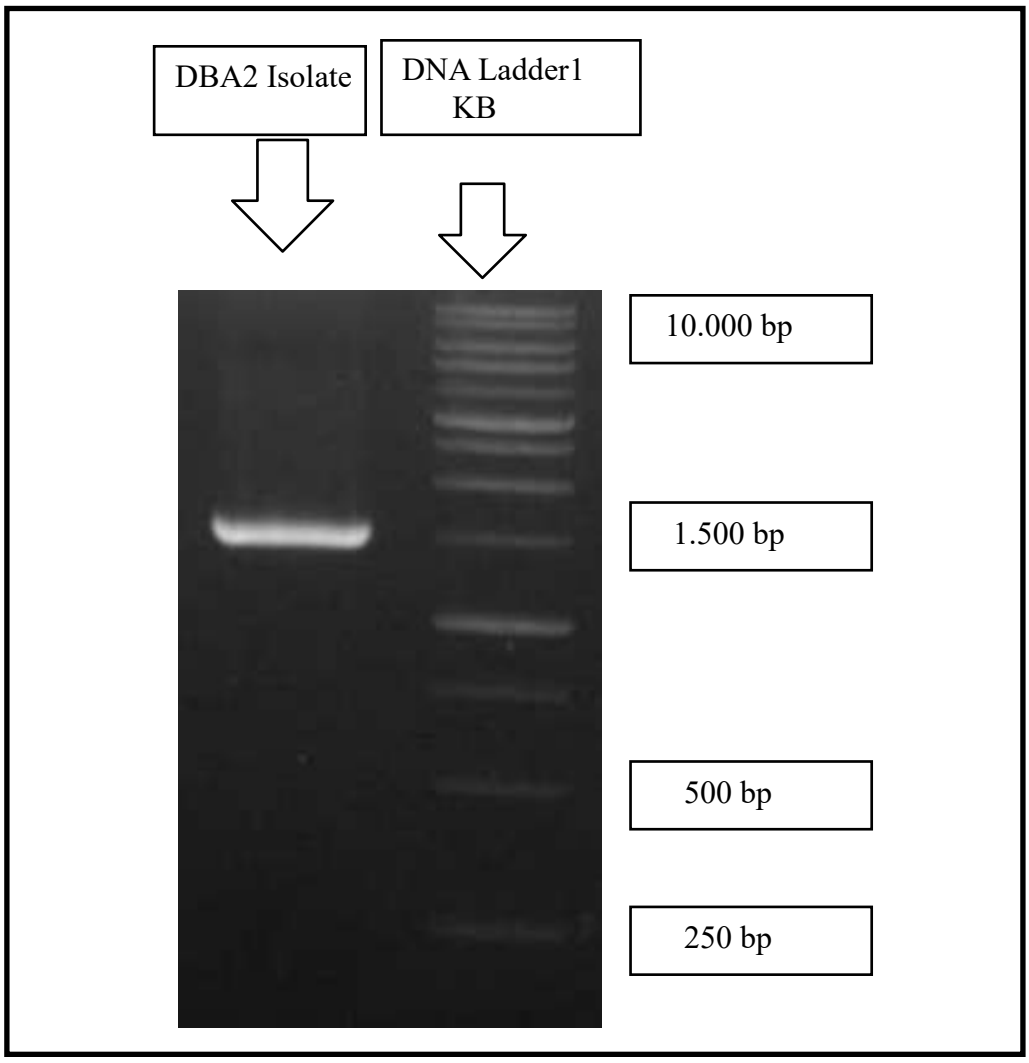


Figure 2. The gel electrophoresis of 16S rRNA gene

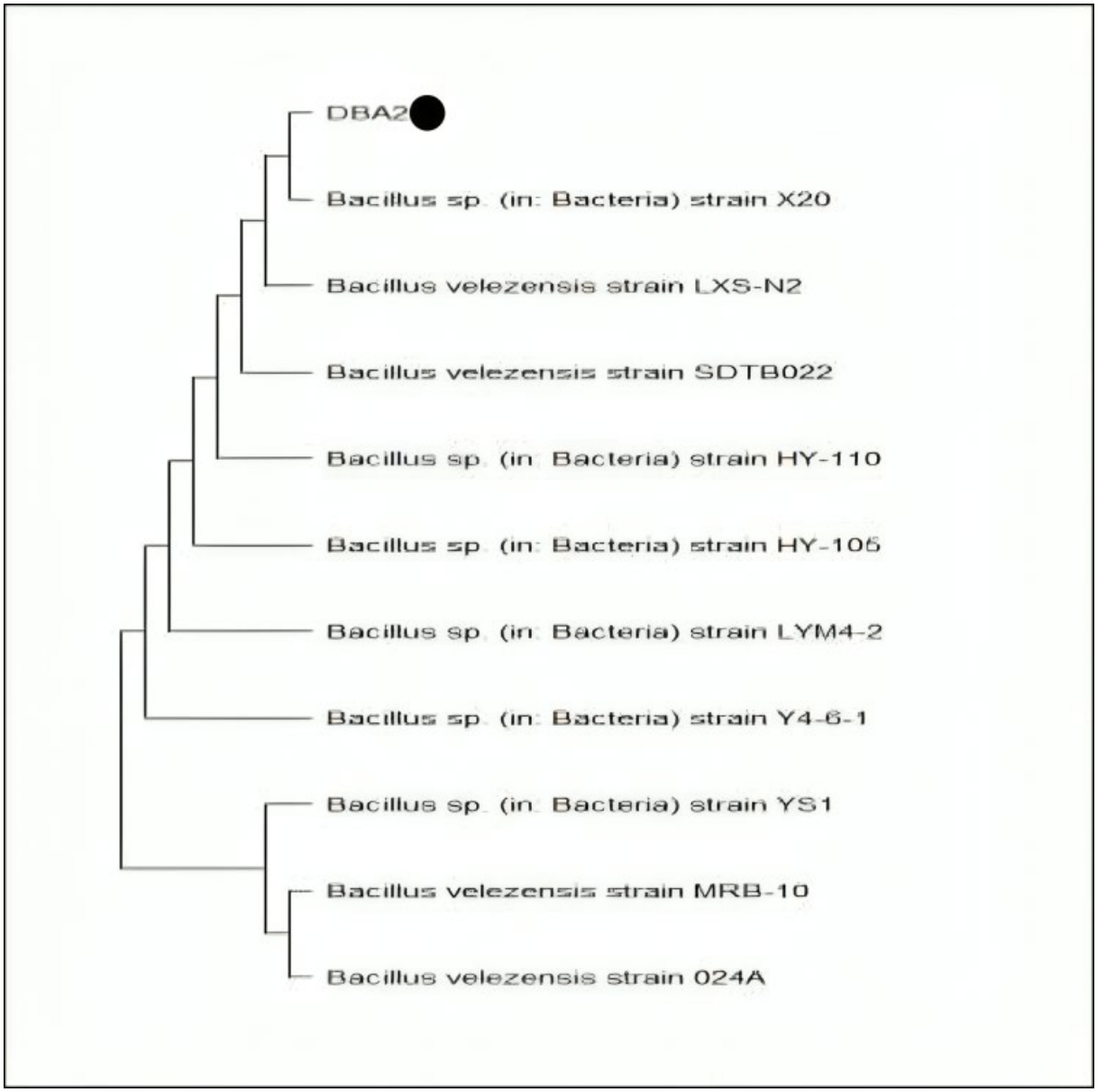


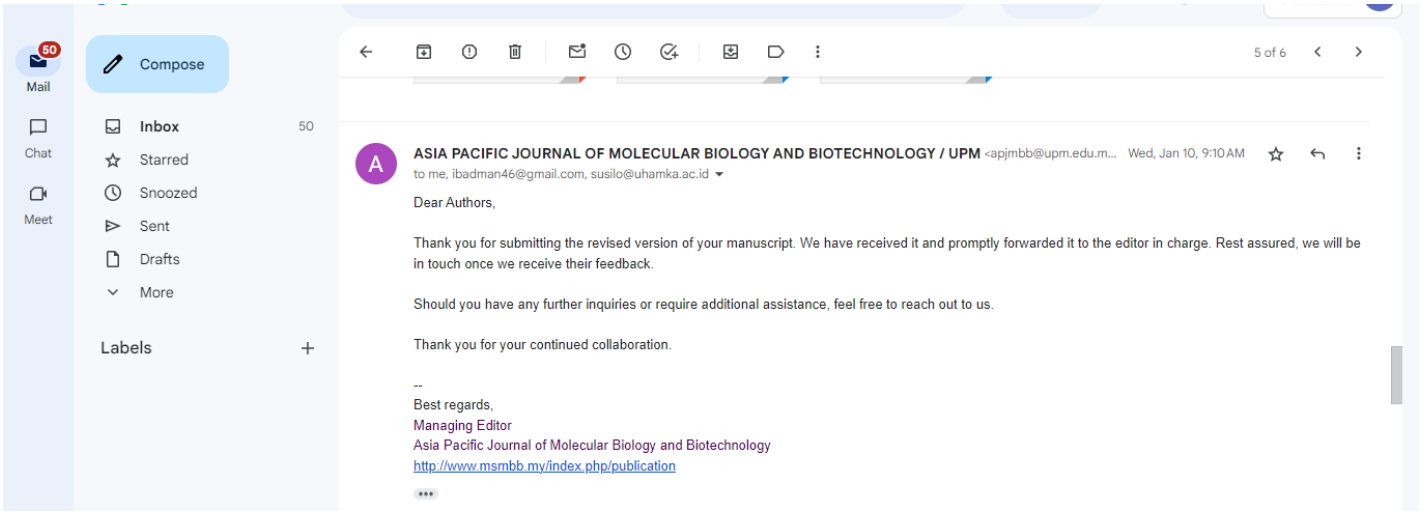
Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar et al., 2018).

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

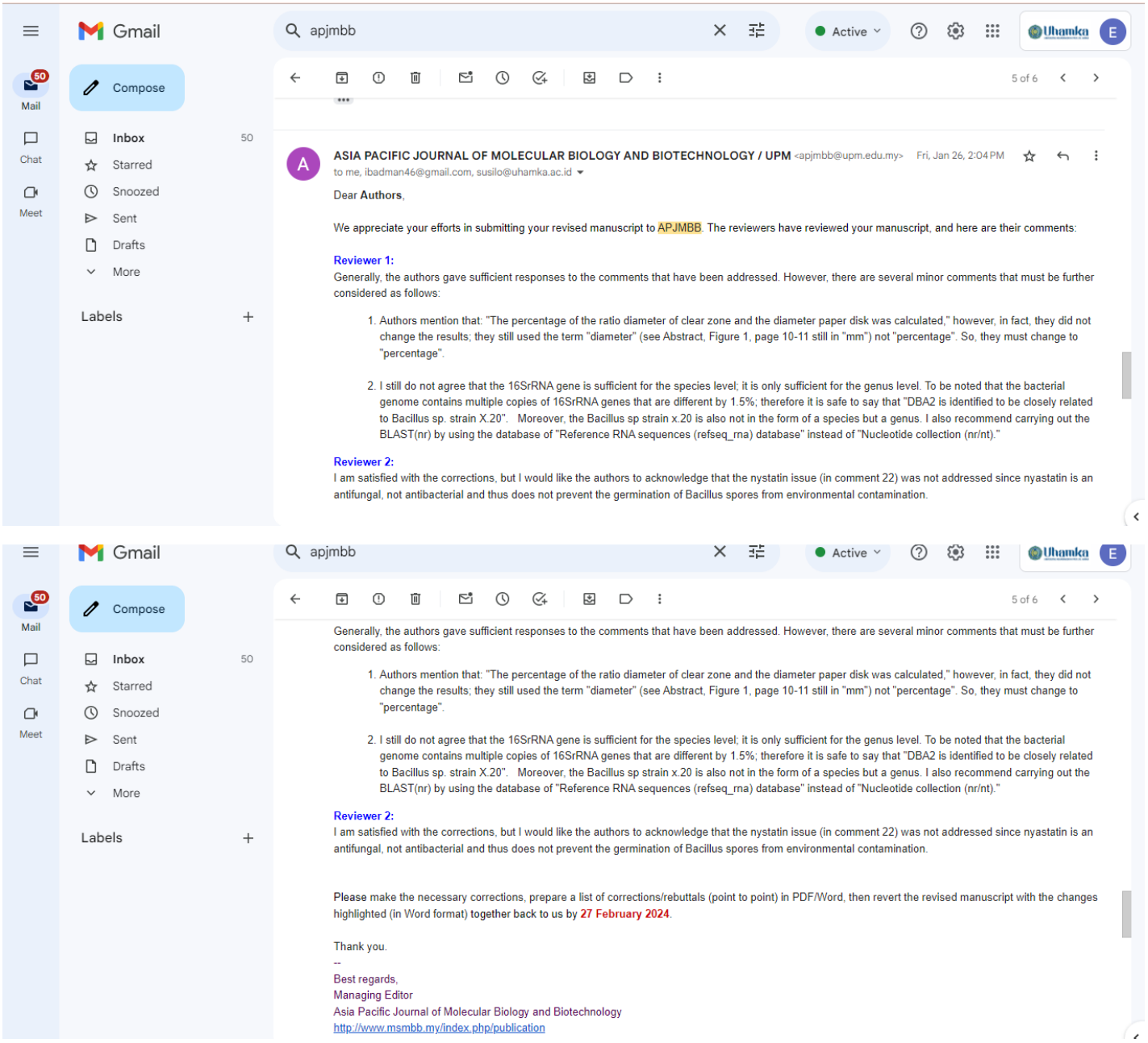
No	*Closest Relative Species Based On 16S rRNA Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00

7	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

5. Bukti penerimaan revisi pertama dari Editor (10 Jan-2024)



6. Bukti permintaan revisi kedua dari editor (26 Jan-2024)



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ASIA PACIFIC JOURNAL OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY / UPM <apjmbb@upm.edu.my> Fri, Jan 26, 2:04 PM

to me, ibadman46@gmail.com, susilo@uhamka.ac.id

Dear **Authors**,

We appreciate your efforts in submitting your revised manuscript to APJMBB. The reviewers have reviewed your manuscript, and here are their comments:

Reviewer 1:

Generally, the authors gave sufficient responses to the comments that have been addressed. However, there are several minor comments that must be further considered as follows:

1. Authors mention that: "The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated," however, in fact, they did not change the results; they still used the term "diameter" (see Abstract, Figure 1, page 10-11 still in "mm") not "percentage". So, they must change to "percentage".

2. I still do not agree that the 16SrRNA gene is sufficient for the species level; it is only sufficient for the genus level. To be noted that the bacterial genome contains multiple copies of 16SrRNA genes that are different by 1.5%; therefore it is safe to say that "DBA2 is identified to be closely related to Bacillus sp. strain X.20". Moreover, the Bacillus sp strain x.20 is also not in the form of a species but a genus. I also recommend carrying out the BLAST(nr) by using the database of "Reference RNA sequences (refseq_rna) database" instead of "Nucleotide collection (nr/nt)."

Reviewer 2:

I am satisfied with the corrections, but I would like the authors to acknowledge that the nystatin issue (in comment 22) was not addressed since nyastatin is an antifungal, not antibacterial and thus does not prevent the germination of Bacillus spores from environmental contamination.

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Generally, the authors gave sufficient responses to the comments that have been addressed. However, there are several minor comments that must be further considered as follows:

1. Authors mention that: "The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated," however, in fact, they did not change the results; they still used the term "diameter" (see Abstract, Figure 1, page 10-11 still in "mm") not "percentage". So, they must change to "percentage".

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Reviewer 2:

I am satisfied with the corrections, but I would like the authors to acknowledge that the nystatin issue (in comment 22) was not addressed since nyastatin is an antifungal, not antibacterial and thus does not prevent the germination of Bacillus spores from environmental contamination.

Please make the necessary corrections, prepare a list of corrections/rebuttals (point to point) in PDF/Word, then revert the revised manuscript with the changes highlighted (in Word format) together back to us by **27 February 2024**.

Thank you.

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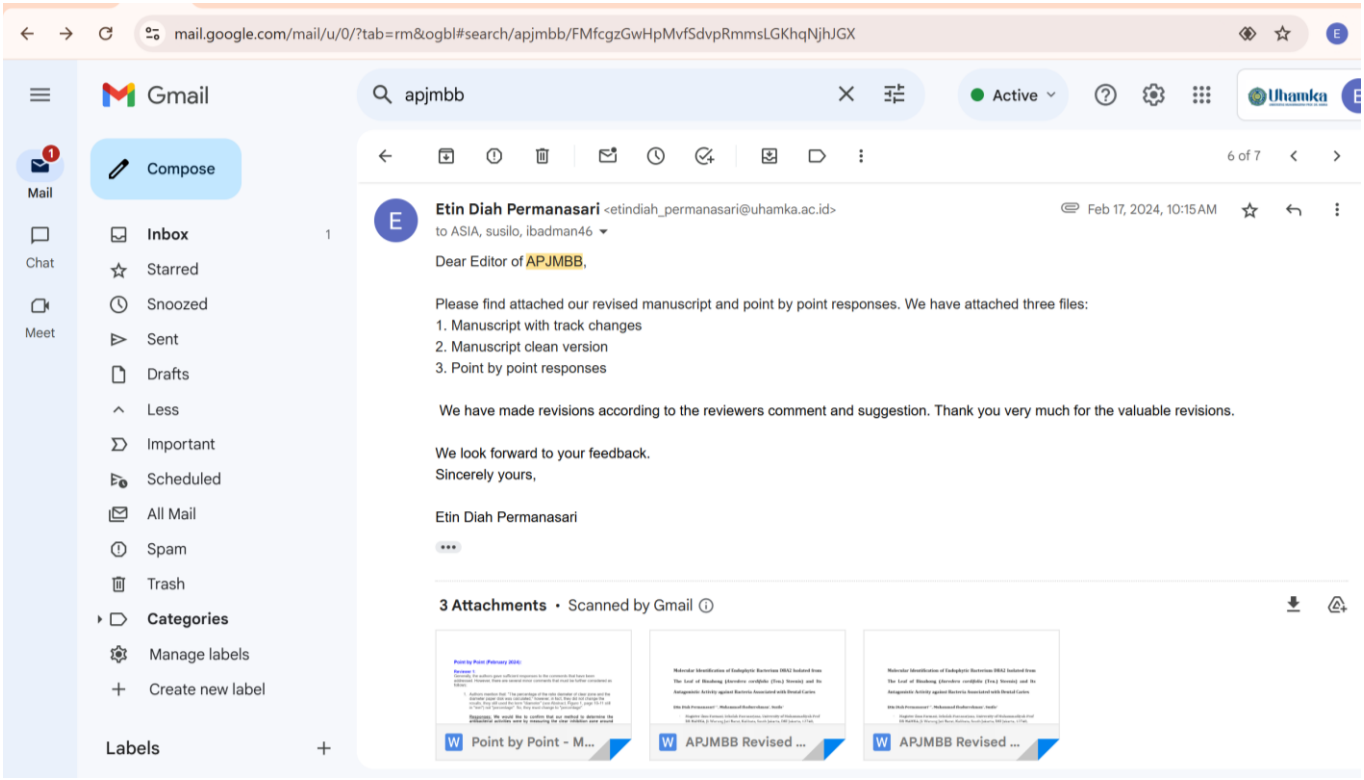
Best regards,

Managing Editor

Asia Pacific Journal of Molecular Biology and Biotechnology

http://www.msmbb.my/index.php/publication

7. **Bukti konfirmasi submit revisi kedua, respon kepada reviewer, dan artikel yang diresubmit (17 Feb 2024)**



2nd Revision: Point-by Point comment to the Editor

Point by Point (February 2024):

Reviewer 1:

Generally, the authors gave sufficient responses to the comments that have been addressed. However, there are several minor comments that must be further considered as follows:

1. Authors mention that: "The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated," however, in fact, they did not change the results; they still used the term "diameter" (see Abstract, Figure 1, page 10-11 still in "mm") not "percentage". So, they must change to "percentage".

   Responses: **We would like to confirm that our method to determine the antibacterial activities were by measuring the clear inhibition zone around disc paper in millimetre (mm). Therefore, we have revised our method in the "antibacterial activities screening" section from "The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated" into "The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against pathogenic microorganism", as described in the manuscript with the track changes. This method refers to the previous studies of (Arullappan et al., 2009; Bhandari et al., 2023; Hudzicki, 2009; Prastiyanto et al., 2021) that antibacterial activities were determined by measuring the diameters of the inhibition zones in mm against the tested organism. Therefore, we will keep using the term "mm" in the manuscript.**

   References as below:

Arullappan, S., Zakaria, Z., Fredalina Basri, D., Baru, P., Padang Tembak, J., Intan, T., Kebangsaan Malaysia, U., Raja Muda Abdul Aziz, J., & Lumpur, K. (2009). Preliminary Screening of Antibacterial Activity Using Crude Extracts of Hibiscus rosa sinensis. In *Tropical Life Sciences Research* (Vol. 20, Issue 2).

Bhandari, R., Pant, D., Kathayat, K. S., Bhattarai, R., Barakoti, H., Pandey, J., & Jamarkatel-Pandit, N. (2023). Preliminary Study on the Antibacterial Activities and Antibacterial Guided Fractionation of Some Common Medicinal Plants Practices in Itum Bahal, Kathmandu Valley of Nepal. *The Scientific World Journal*, *2023*, 1–10. https://doi.org/10.1155/2023/7398866

Blackwood, K. S., He, C., Gunton, J., Turenne, C. Y., Wolfe, J., & Kabani, A. M. (2000). Evaluation of recA Sequences for Identification of Mycobacterium species. *Journal of Clinical Microbiology*, *38*(8), 2846–2852. https://doi.org/10.1128/JCM.38.8.2846-2852.2000

Hudzicki, J. (2009). Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. *American Society for Microbiology*. www.atcc.org

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X.20". Moreover, the *Bacillus sp* strain x.20 is also not in the form of a species but a genus. I also recommend carrying out the BLAST(nr) by using the database of "Reference RNA sequences (refseq_rna) database" instead of "Nucleotide collection (nr/nt)."

Responses: As we have explained in the previous revision, We refer to the previous research by Drancourt et al., (2000) (Blackwood et al., 2000; Johnson et al., 2019; Schloss & Handelsman, 2005), that if similarity percentage >99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate to the species level.

However, we have done corrections in the manuscript to safely mention that: DBA2 is identified to be closely related to *Bacillus sp*. strain X.20 (as shown in the track changes), according to reviewer recommendation.

References as below:

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-13036-1

Prastiyanto, M. E., Dewi, N. M. B. A., Pratiningtias, T. D., Pratiwi, N. M. R., Windayani, A., Wahyunengsih, E., Astuti, Amir, E., & Wardoyo, F. A. (2021). In vitro antibacterial activities of crude extracts of nine plants on multidrug resistance bacterial isolates of wound infections. *Biodiversitas*, *22*(7), 2641–2647. https://doi.org/10.13057/biodiv/d220712

Schloss, P. D., & Handelsman, J. (2005). Introducing DOTUR, a Computer Program for Defining Operational Taxonomic Units and Estimating Species Richness. *Applied and Environmental Microbiology*, *71*(3), 1501–1506. https://doi.org/10.1128/AEM.71.3.1501-1506.2005

Reviewer 2:

I am satisfied with the corrections, but I would like the authors to acknowledge that the nystatin issue (in comment 22) was not addressed since nystatin is an antifungal, not antibacterial and thus does not prevent the germination of Bacillus spores from environmental contamination.

Responses: Thank you for the correction. We can explain here that the work was carried out by minimizing all contamination, both fungal contamination (with the addition of nystatin as an antifungal) and bacterial contamination (the media used has been sterilized and all the steps were properly maintained in the sterile conditions).

2nd Revision: APJMBB Revised Manuscripts with track changes

Molecular Identification of Endophytic Bacterium DBA2 Isolated from The Leaf of Binahong (*Anredera cordifolia* (Ten.) Steenis) and Its Antagonistic Activity against Bacteria Associated with Dental Caries

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *Steenis*. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their

antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to ~~as~~ *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah et al., 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Yuniarti & Lukiswanto, 2017; Mutiarawati et al., 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita et al., 2017; Surbakti et al., 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Mengga et al., 2022; Veronita et al., 2017; Ainurrochmah et al., 2013; Sasebohe et al., 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Xie et al., 2015; Basile et al., 1999). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie et al., 2015; Veronita et al., 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba et

al., 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo et al, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold, et al., 2020; Potshangbam et al., 2020; Sharma, et al., 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani et al (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Hussein et al., 2023; Zhang, 2013). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty et al., 2017; Abdel-Aziz et al., 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The

mediums were yeast extract, Nutrient Agar medium, and Nutrient Broth medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo et al., 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to

remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml Nutrient Broth test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. Two bacterial strains *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strain indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. ~~The percentage of the ratio diameter of clear zone and the diameter paper disk was calculated~~The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against pathogenic microorganism.

The isolate with the largest diameter of inhibition zone ~~of inhibition~~ was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar et al., 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo et al, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to Nutrient Agar Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1.

The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah et al., 2013; Nxumalo et al., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar et al., 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt et al., 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt et al., 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to ~~as~~ *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond et al., 2015; Zhao et al., 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao, et al., 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

Isolates	Shapes	Colour	Texture	Margins of Colony	Surface	Consistency	Staining
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

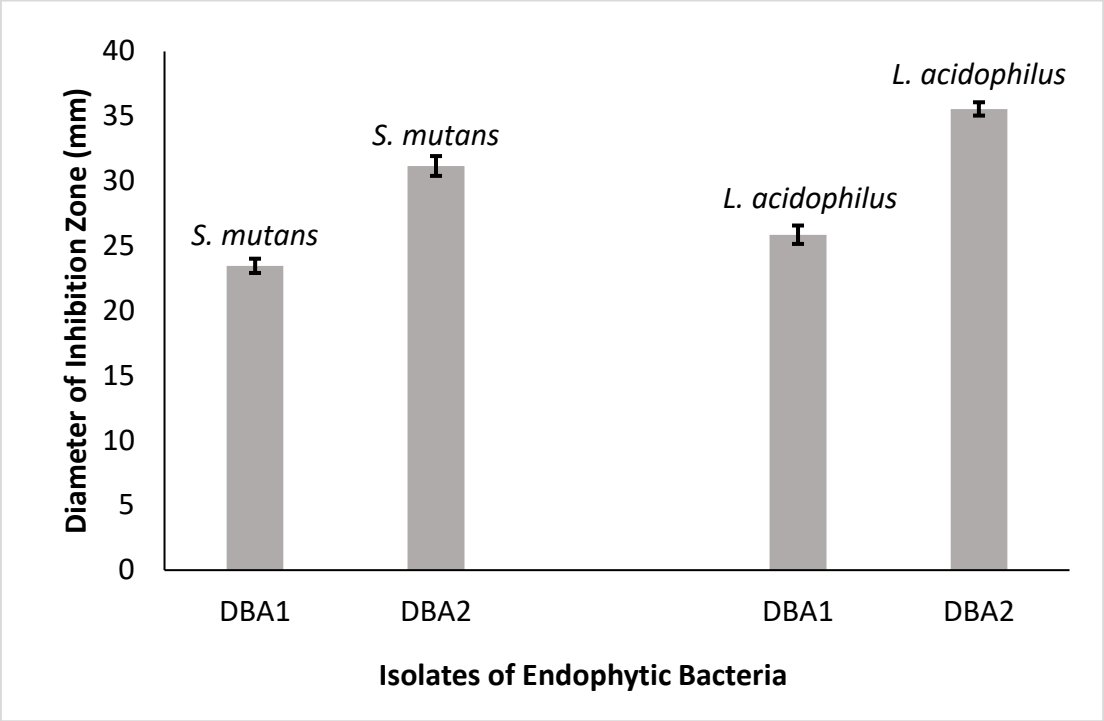

Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

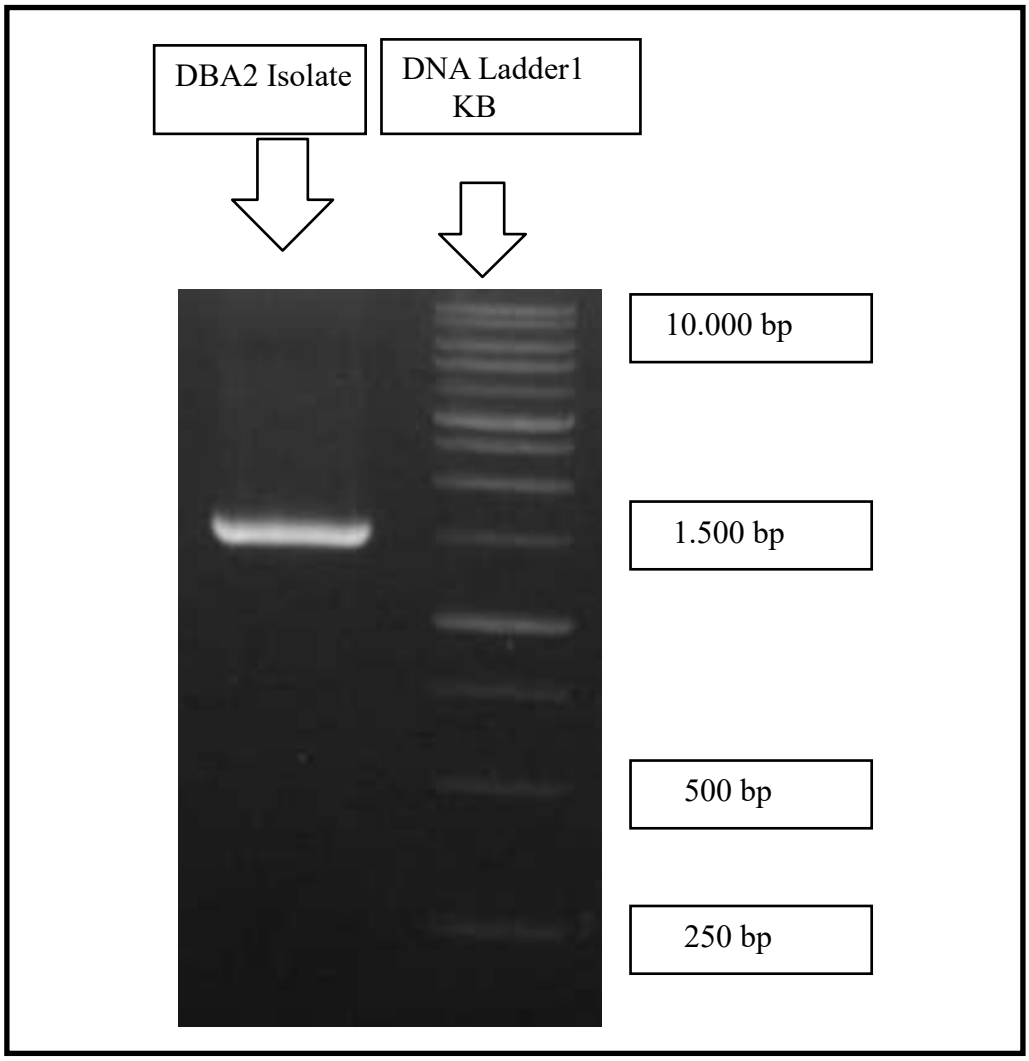

Figure 2. The gel electrophoresis of 16S rRNA gene

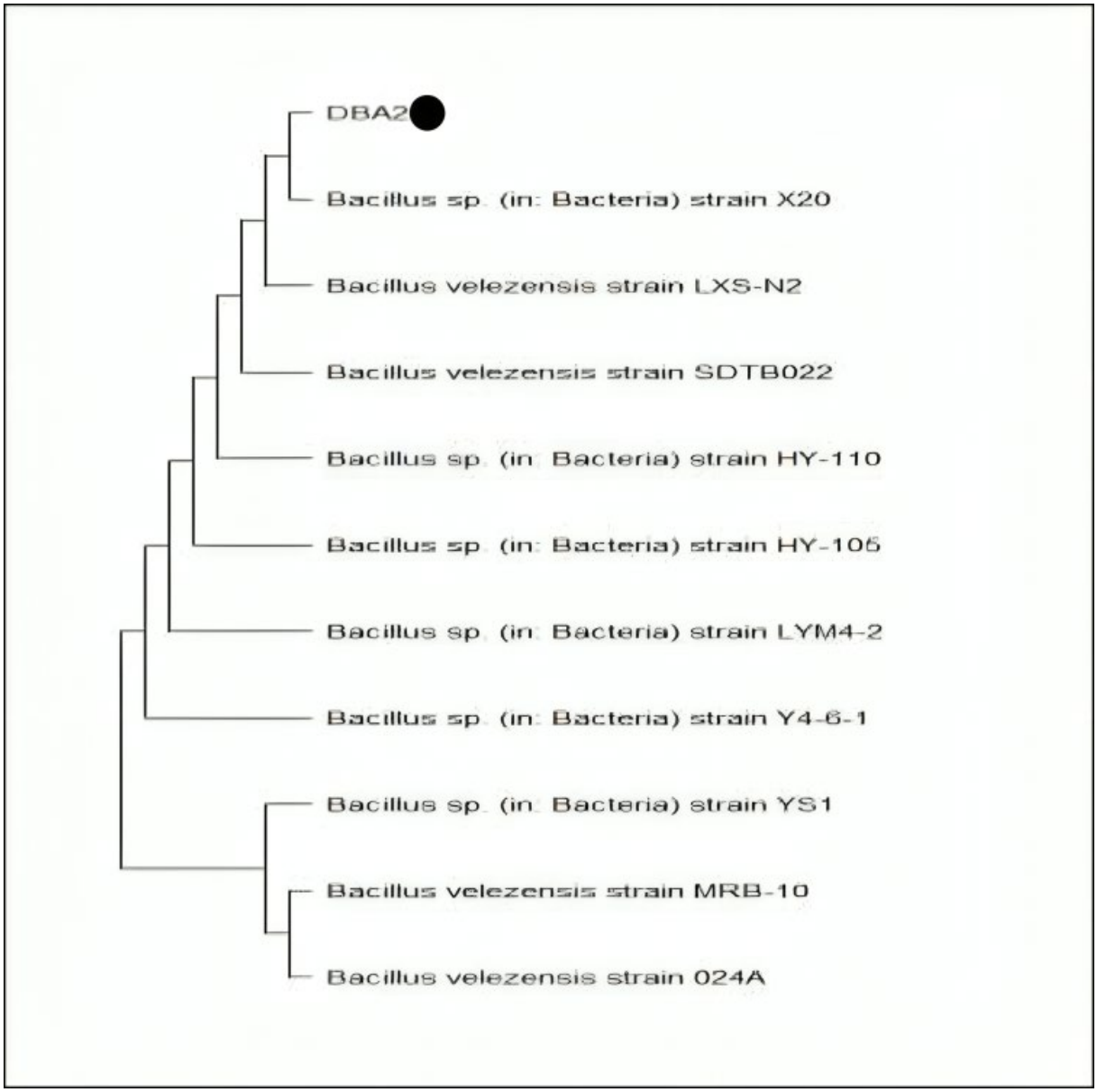

Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar et al., 2018).

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

No	*Closest Relative Species Based On 16S rRNA Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00

7	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

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ABSTRACT

Binahong plants (*Anredera cordifolia* (Ten.) *Steenis*) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *Steenis*. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *Steenis*. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their

antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to Bacillus sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah et al., 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Yuniarti & Lukiswanto, 2017; Mutiarawati et al., 2017; Laksmitawati & Simbolon, 2017; Sumartiningsih, 2011). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita et al., 2017; Surbakti et al., 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Mengga et al., 2022; Veronita et al., 2017; Ainurrochmah et al., 2013; Sasebohe et al., 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Xie et al., 2015; Basile et al., 1999). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie et al., 2015; Veronita et al., 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba et

al., 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo et al, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold, et al., 2020; Potshangbam et al., 2020; Sharma, et al., 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani et al (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo et al, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Hussein et al., 2023; Zhang, 2013). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty et al., 2017; Abdel-Aziz et al., 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The

mediums were yeast extract, Nutrient Agar medium, and Nutrient Broth medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo et al., 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

Nutrient Agar was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plate. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to

remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml Nutrient Broth test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial potential in this study was performed by the disk diffusion method. Two bacterial strains *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strain indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*Streptococcus mutans* and *Lactobacillus acidophilus*) were incubated in 10 ml Nutrient Broth test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potential by the presence or absence of an inhibition zone around the disk paper. The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against pathogenic microorganism. The isolate with the largest diameter of inhibition zone was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar et al., 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo et al, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that

present in the surface leaves. Nystatin was added to Nutrient Agar Addition as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel, et al., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah et al., 2013; Nxumalo et al., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar et al., 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt et al., 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt et al.,

2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to Bacillus sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond et al., 2015; Zhao et al., 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao, et al., 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao, et al., 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial

properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis

Isolates	Shapes	Colour	Texture	Margins of Colony	Surface	Consistency	Staining
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

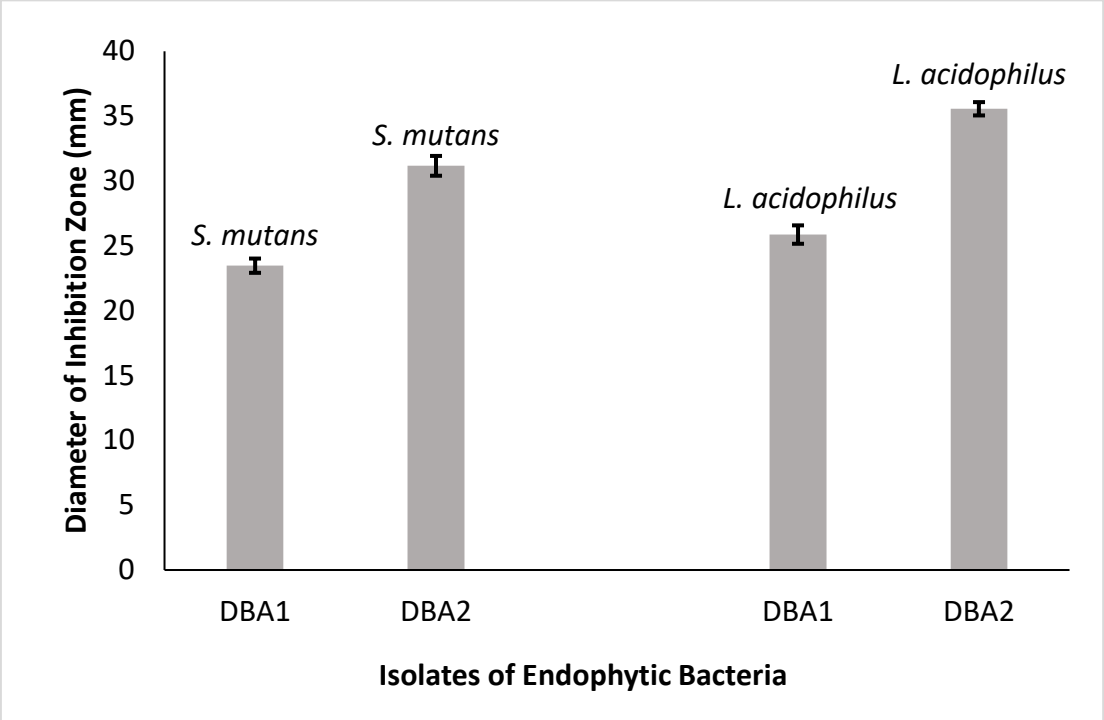


Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*

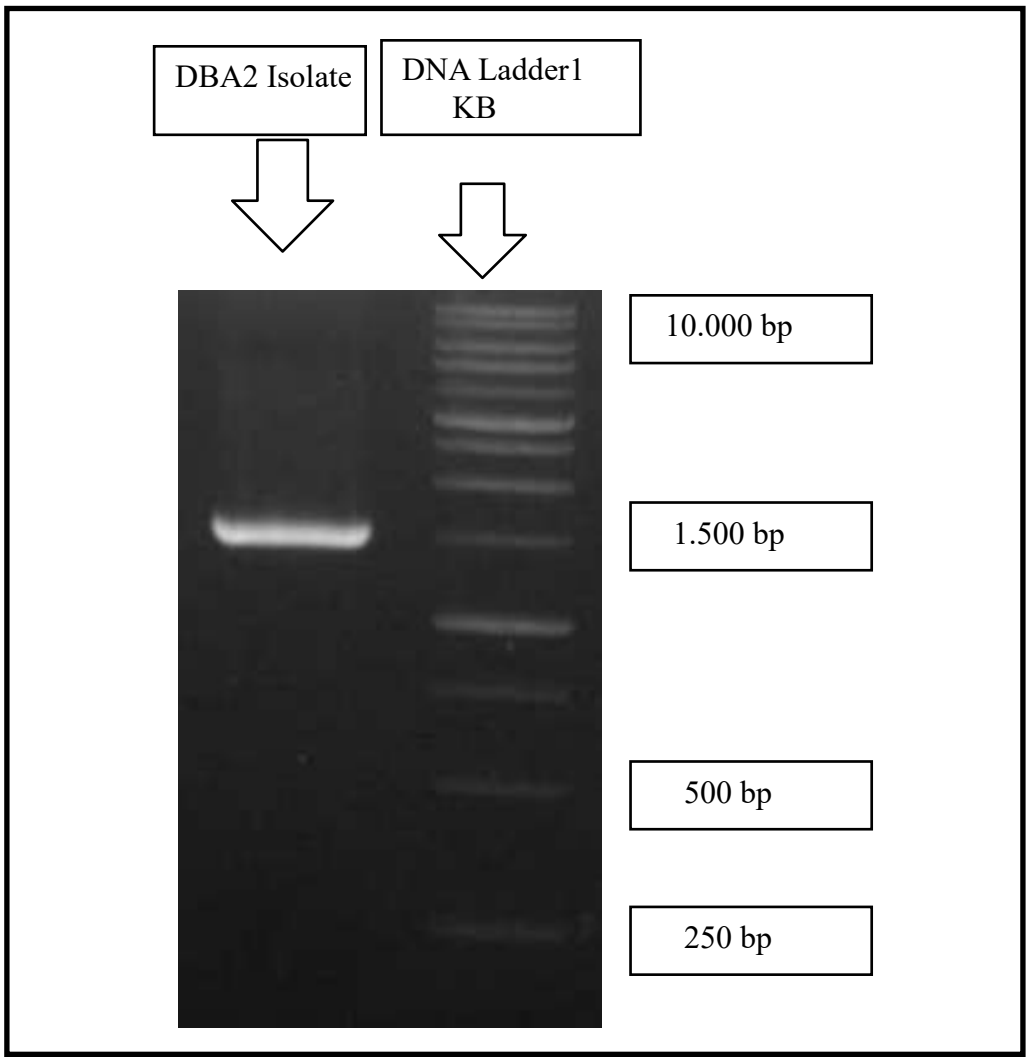


Figure 2. The gel electrophoresis of 16S rRNA gene

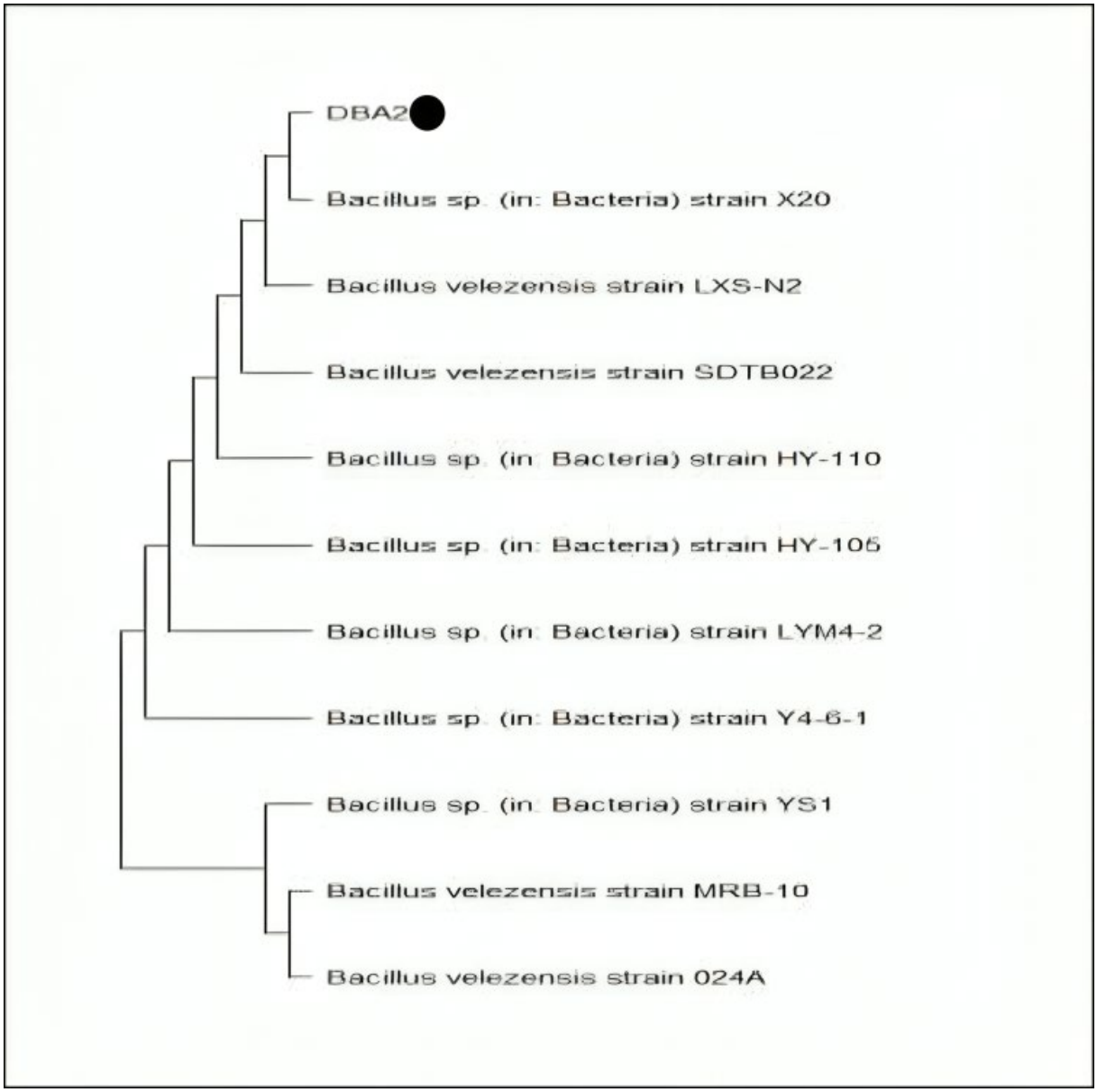


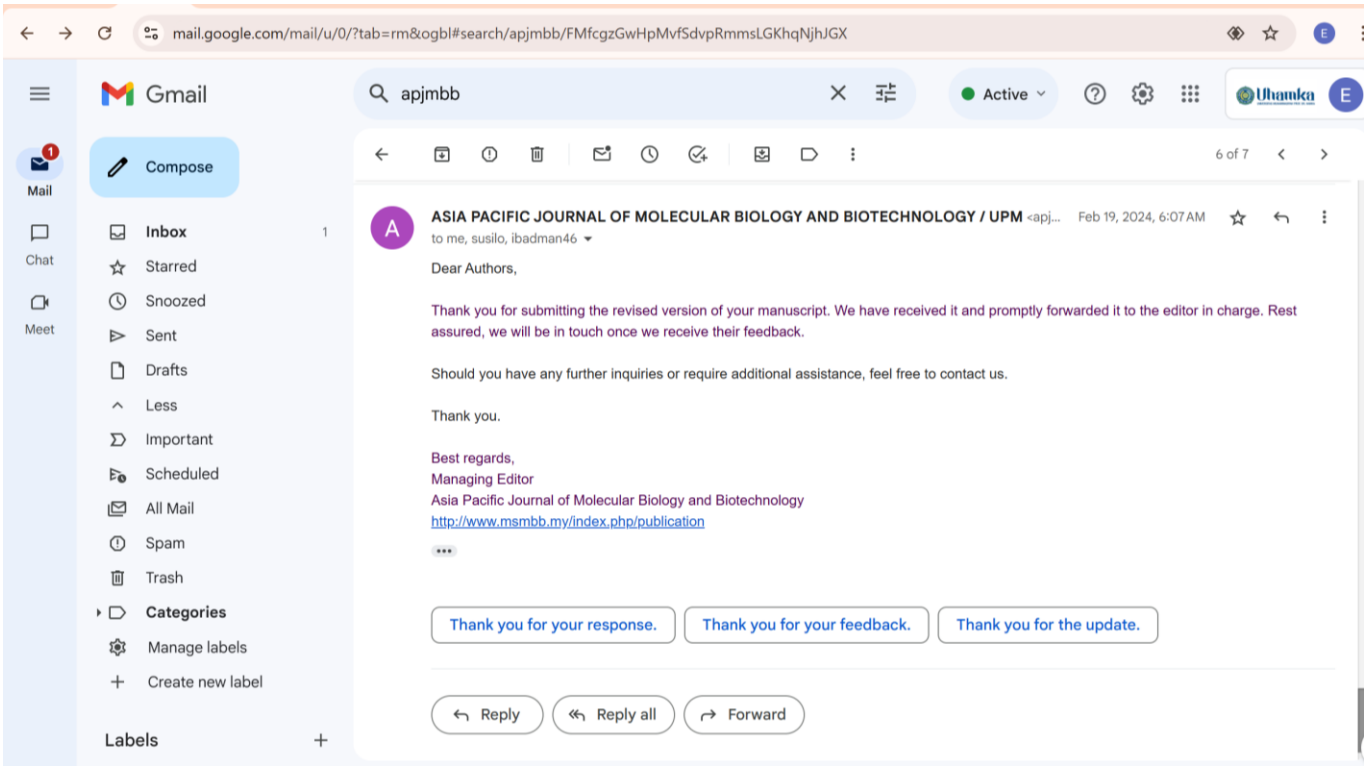
Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar et al., 2018).

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers

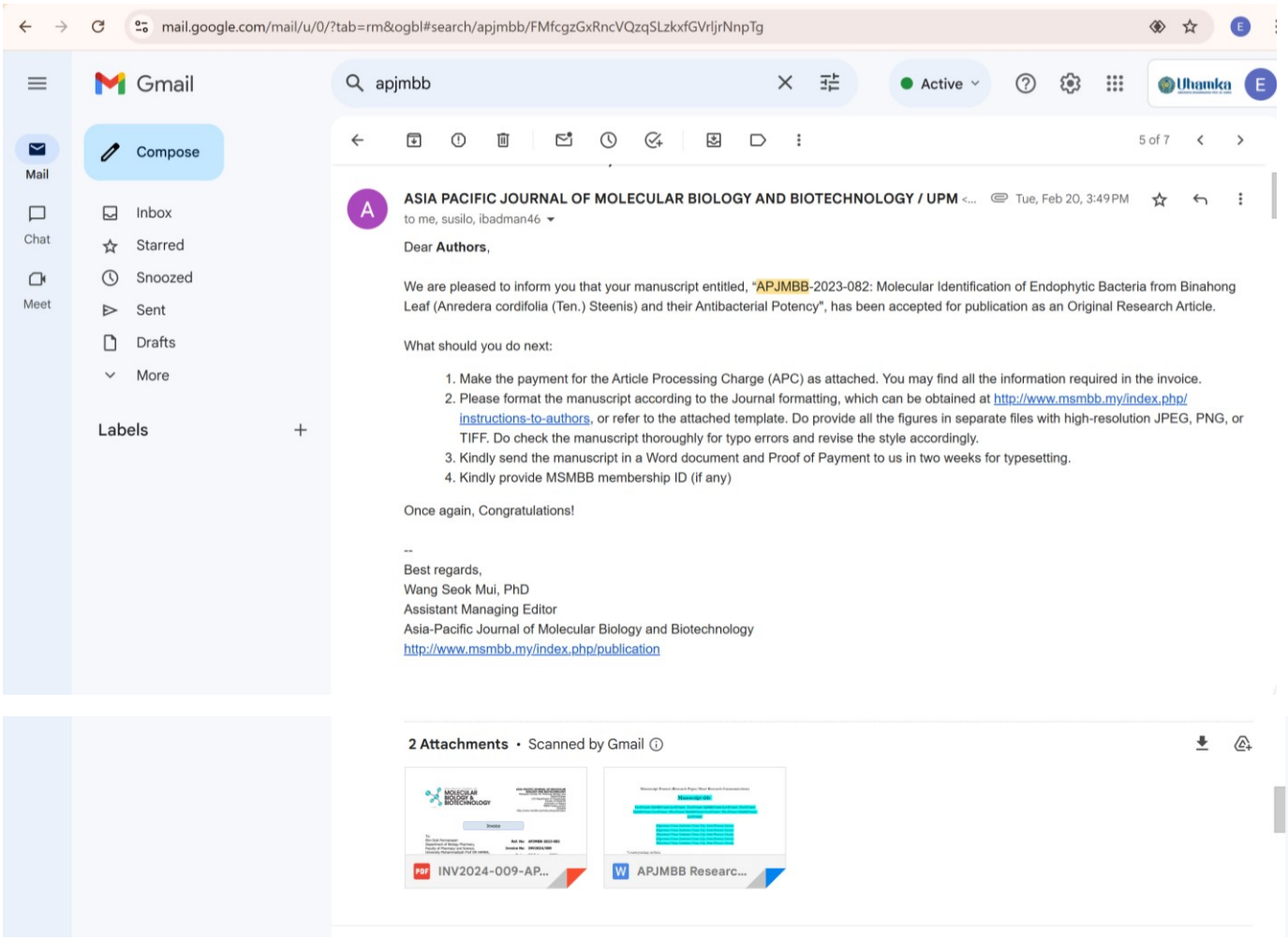
No	*Closest Relative Species Based On 16S rRNA Gene Sequences*	*Genbank Accession Number*	*Base Pair Length (bp)*	*Max score*	*E Value*	*% Similarity*
1	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00

7	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

8. Bukti penerimaan revisi kedua dari editor (19 Feb 2024)



9. Bukti konfirmasi accepted, informasi invoice dan format after acceptance (20 Feb 2024)



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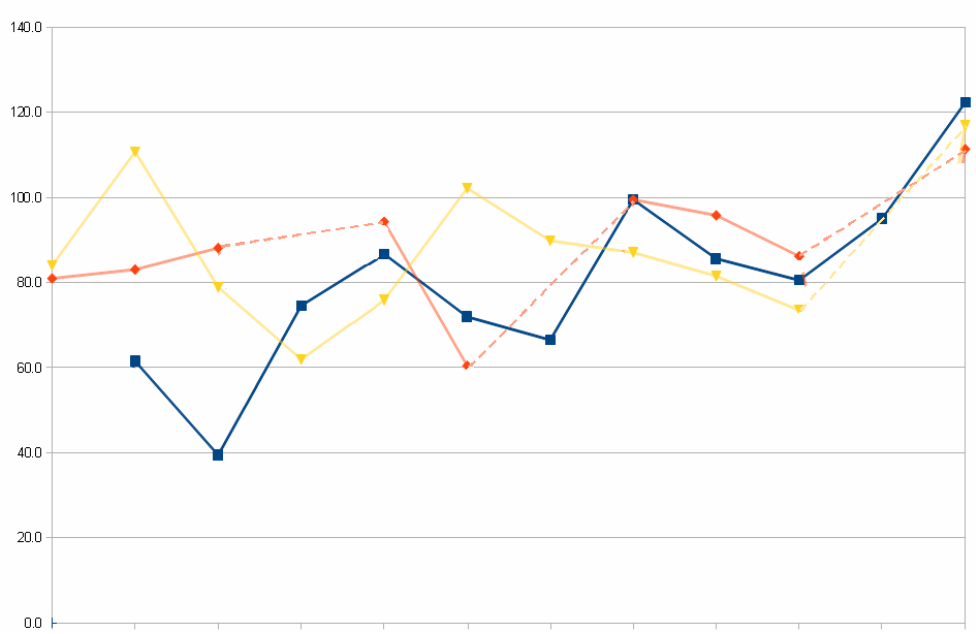

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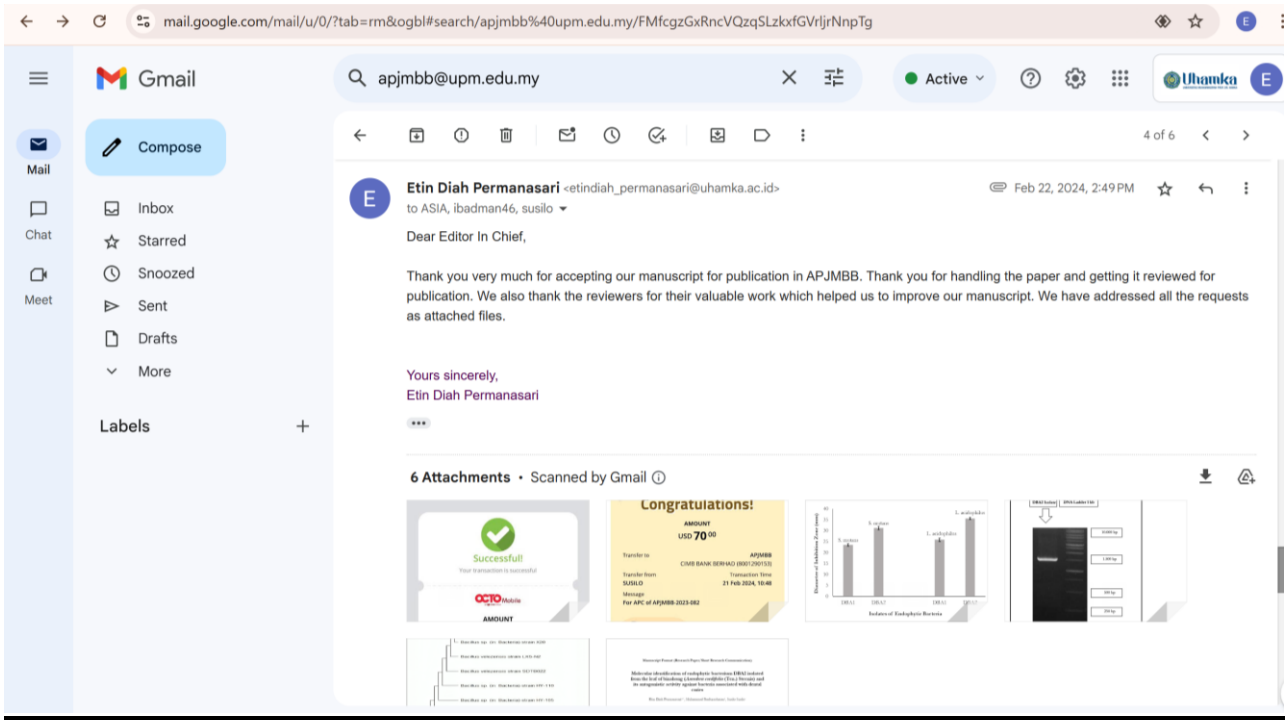

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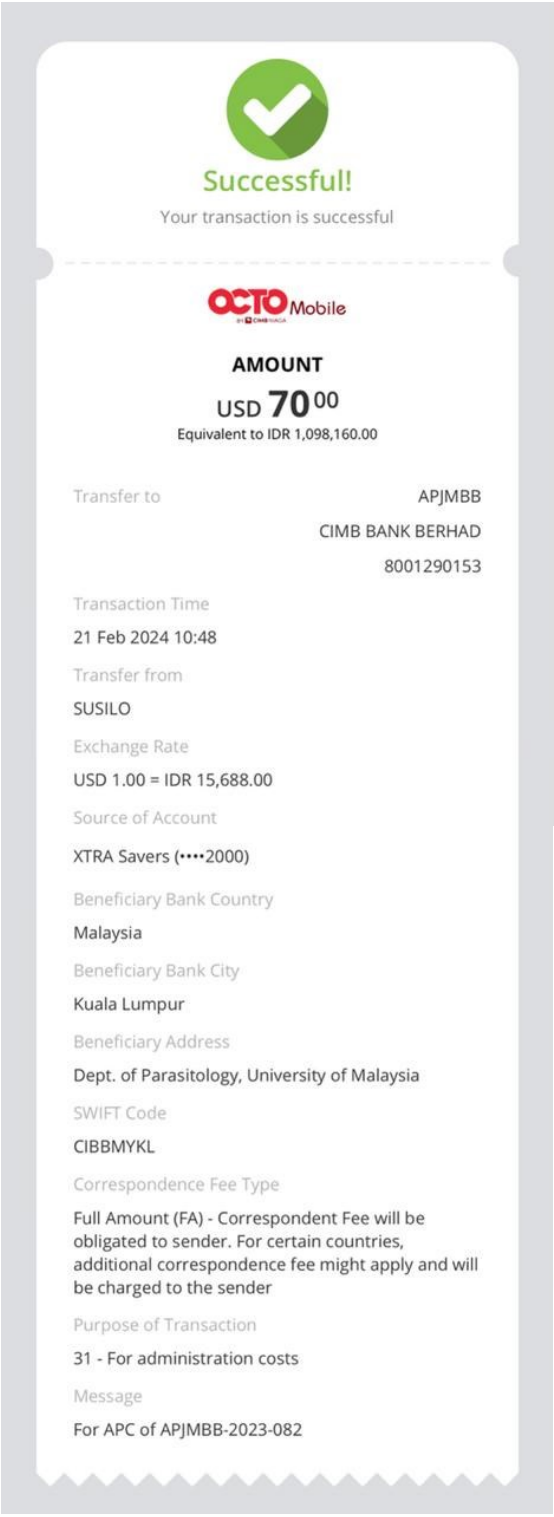

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Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries

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Running title: Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries.

Abstract.

Binahong plants (*Anredera cordifolia* (Ten.) Steenis) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *S*teenis. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *S*teenis. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the

diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis; antibacterial activity; binahong leaf; endophytic bacteria; 16S rRNA gene.

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah *et al.*, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Sumartiningsih, 2011; Laksmitawati & Simbolon, 2017; Mutiarawati *et al.*, 2017; Yuniarti & Lukiswanto, 2017). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita *et al.*, 2017; Surbakti *et al.*, 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Ainurrochmah *et al.*, 2013; Veronita *et al.*, 2017; Mengga *et al.*, 2022; Sasebohe *et al.*, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Basile *et al.*, 1999; Xie *et al.*, 2015). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie *et al.*, 2015; Veronita *et al.*, 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba *et al.*, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo *et al.*, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its

host plant (Arnold *et al.*, 2020; Potshangbam *et al.*, 2020; Sharma, *et al.*, 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani *et al.* (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo *et al.*, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Zhang, 2013; Hussein *et al.*, 2023). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty *et al.*, 2017; Abdel-Aziz *et al.*, 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial

compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar (NA) medium, and Nutrient Broth (NB) medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo *et al.*, 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

NA was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plates. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml NB test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial activity in this study was performed by the disk diffusion method. Two bacterial strains of *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strains indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*S. mutans* and *L. acidophilus*) were incubated in 10 ml NB test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potency by the presence or absence of an inhibition zone around the disk paper. The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against

pathogenic microorganism. The isolate with the largest diameter of inhibition zone was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar *et al.*, 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo *et al.*, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in

the surface leaves. Nystatin was added to NA as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel *et al*., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition

zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah *et al.*, 2013; Nxumalo *et al.*, 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar *et al.*, 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt *et al.*, 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt *et al.*, 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond *et al.*, 2015; Zhao *et al.*, 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao *et al.*, 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao *et al.*, 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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252 None

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Tables:

Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis.

Isolates	**Shapes**	**Colour**	**Texture**	Margins of **colony**	**Surface**	**Consistency**	**Staining**
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers.

No.	Closest relative species based on 16S rRNA gene sequences	GenBank accession number	Base pair length (bp)	Max score	E value	% Similarity
1.	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2.	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3.	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4.	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5.	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6.	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00

7.	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8.	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9.	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10.	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

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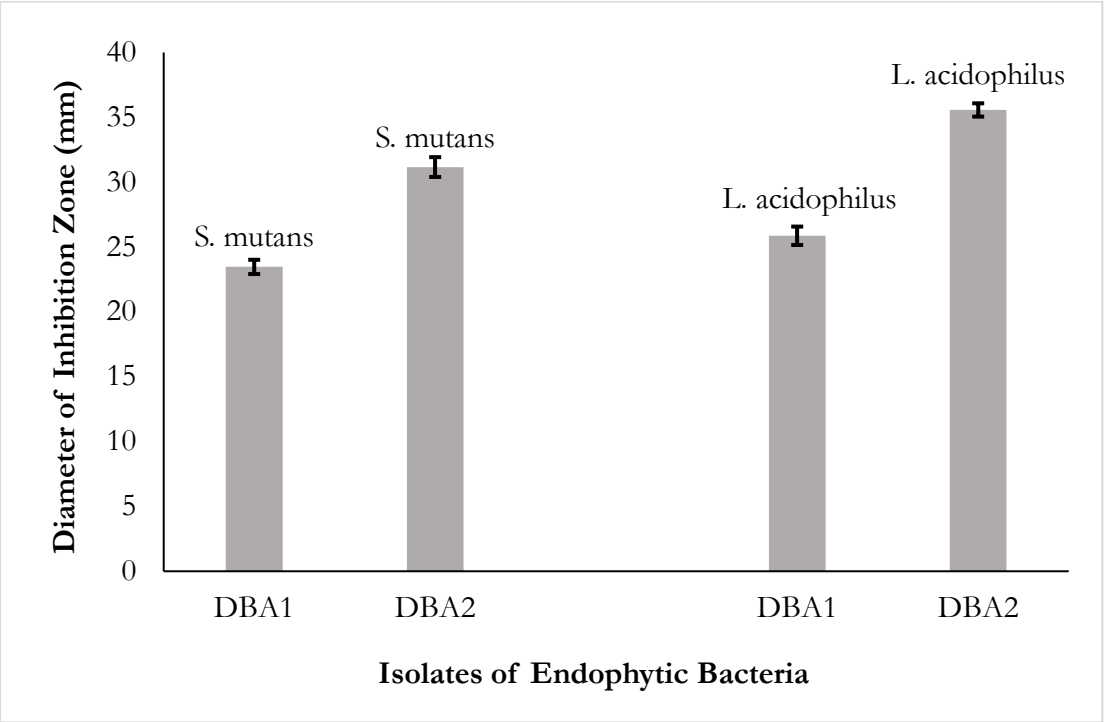
402

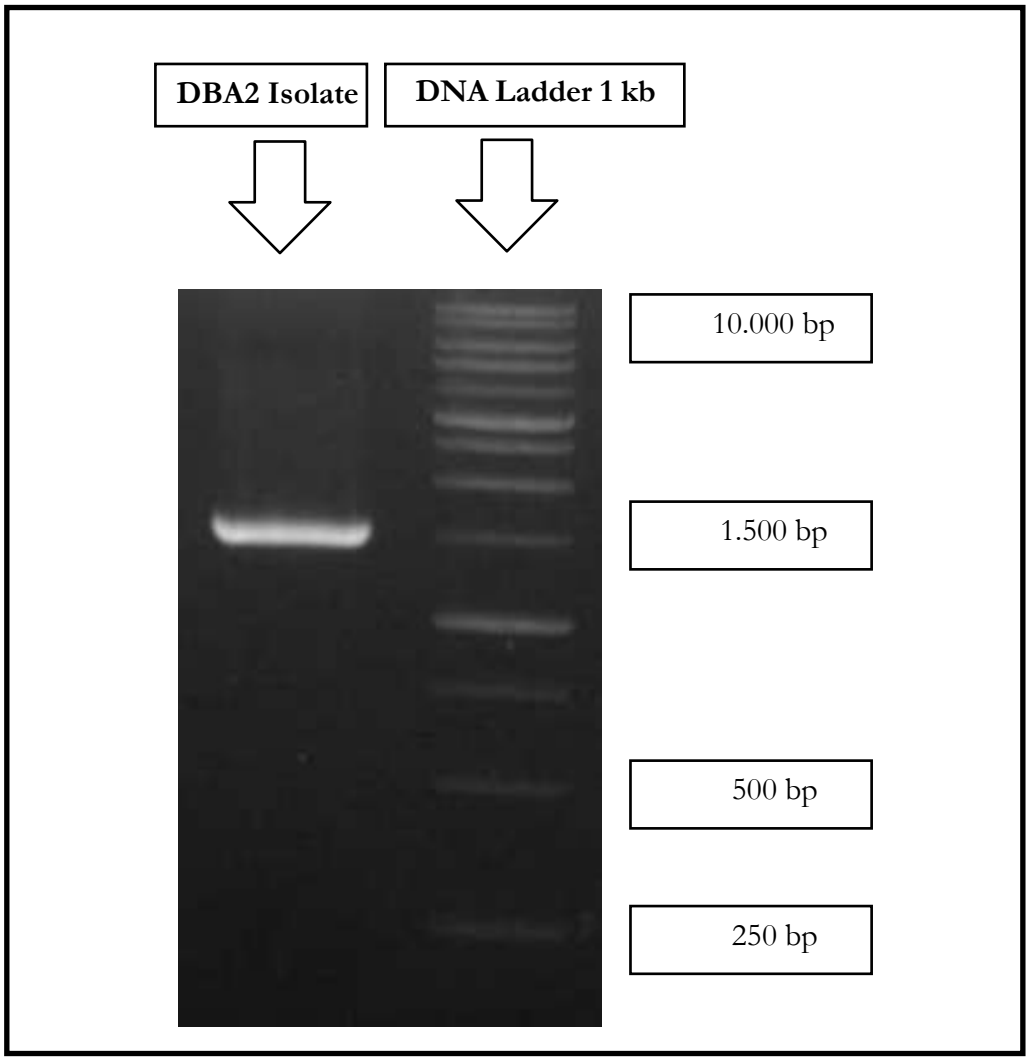
403 **Figures and Photos:**



404

405 **Figure 1.** The diameter of inhibition zone of the metabolites from the isolated endophytic
406 bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus*
407 *acidophilus*.

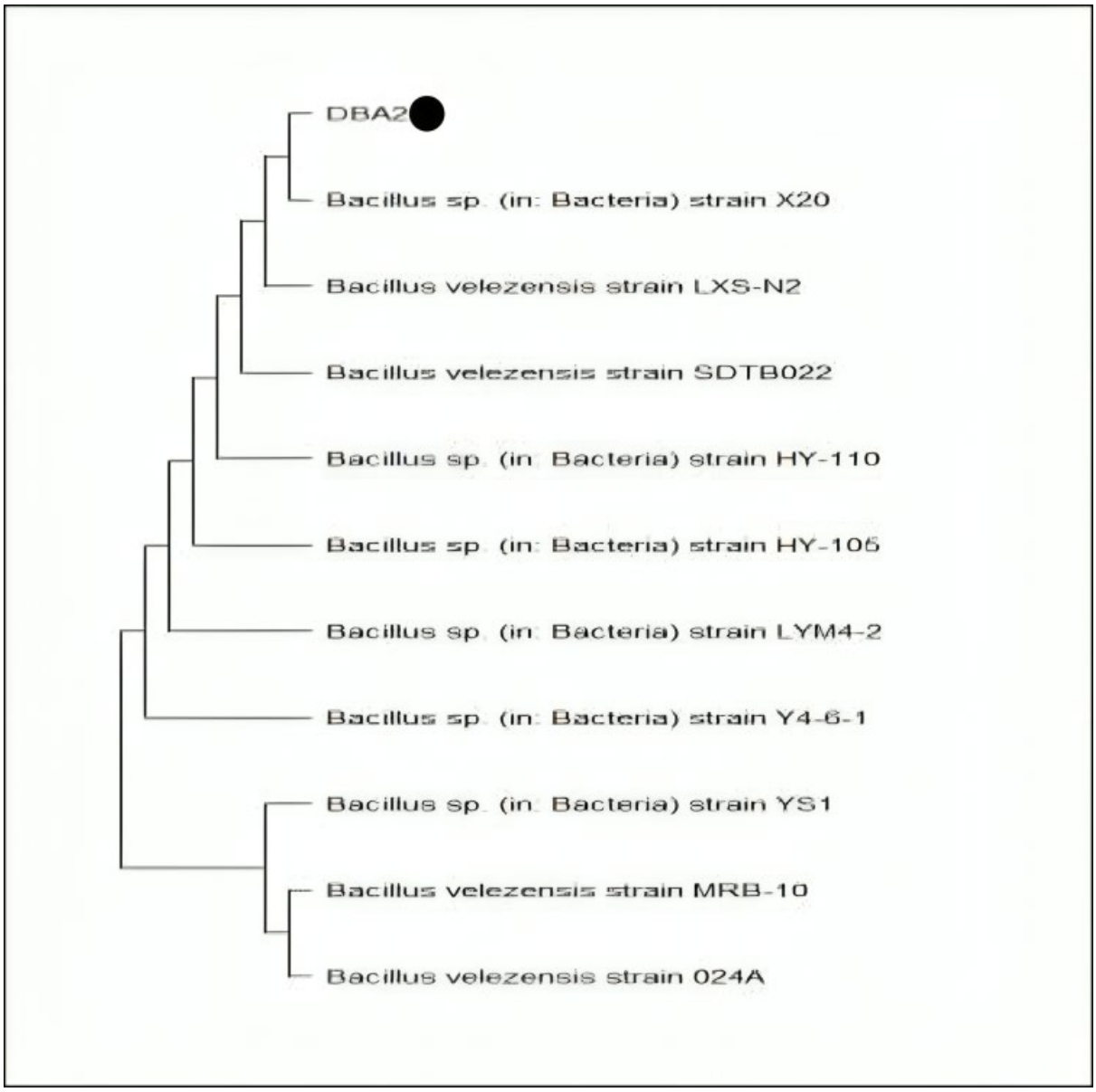
408



409

410 **Figure 2.** The gel electrophoresis of 16S rRNA gene

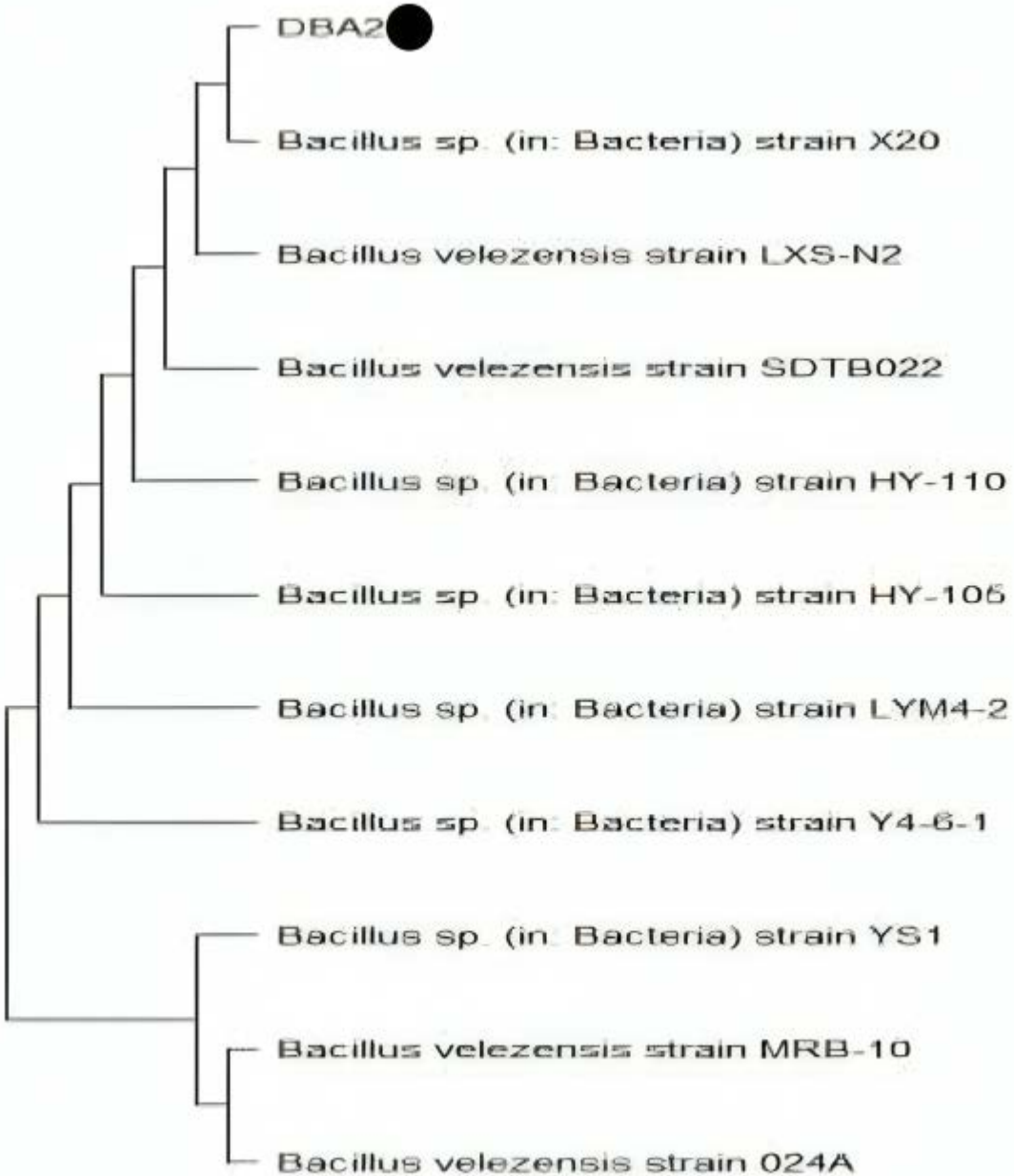
411



412

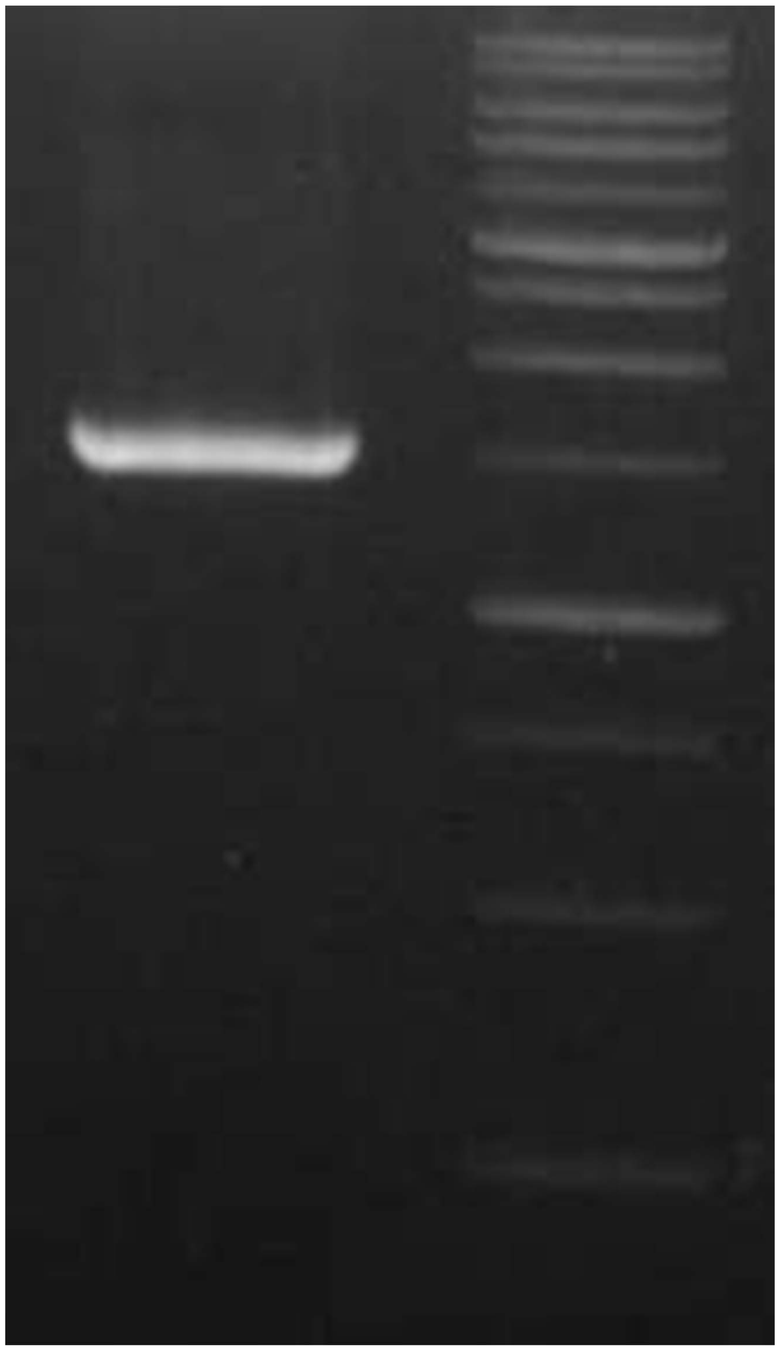
413 **Figure 3.** The phylogenetic tree of DBA2 isolate showing evolutionary relationships of
414 endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the
415 interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar
416 *et al.*, 2018).

417


DBA2
Bacillus sp. (in: Bacteria) strain X20
Bacillus velezensis strain LXS-N2
Bacillus velezensis strain SDTB022
Bacillus sp. (in: Bacteria) strain HY-110
Bacillus sp. (in: Bacteria) strain HY-105
Bacillus sp. (in: Bacteria) strain LYM4-2
Bacillus sp. (in: Bacteria) strain Y4-6-1
Bacillus sp. (in: Bacteria) strain YS1
Bacillus velezensis strain MRB-10
Bacillus velezensis strain 024A

DBA2 Isolate

DNA Ladder 1 kb

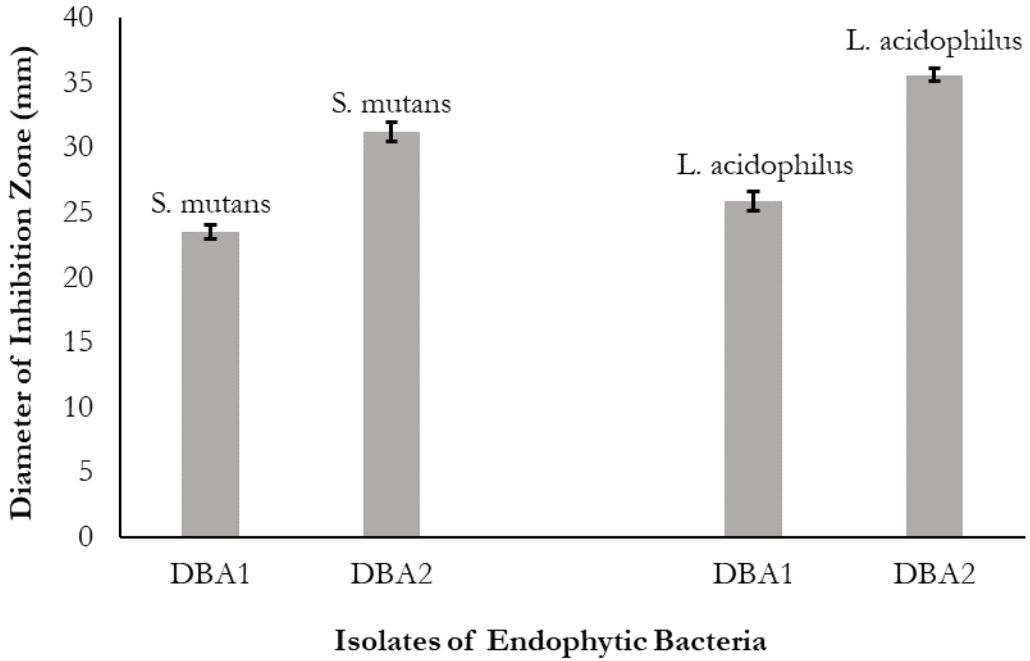


10.000 bp

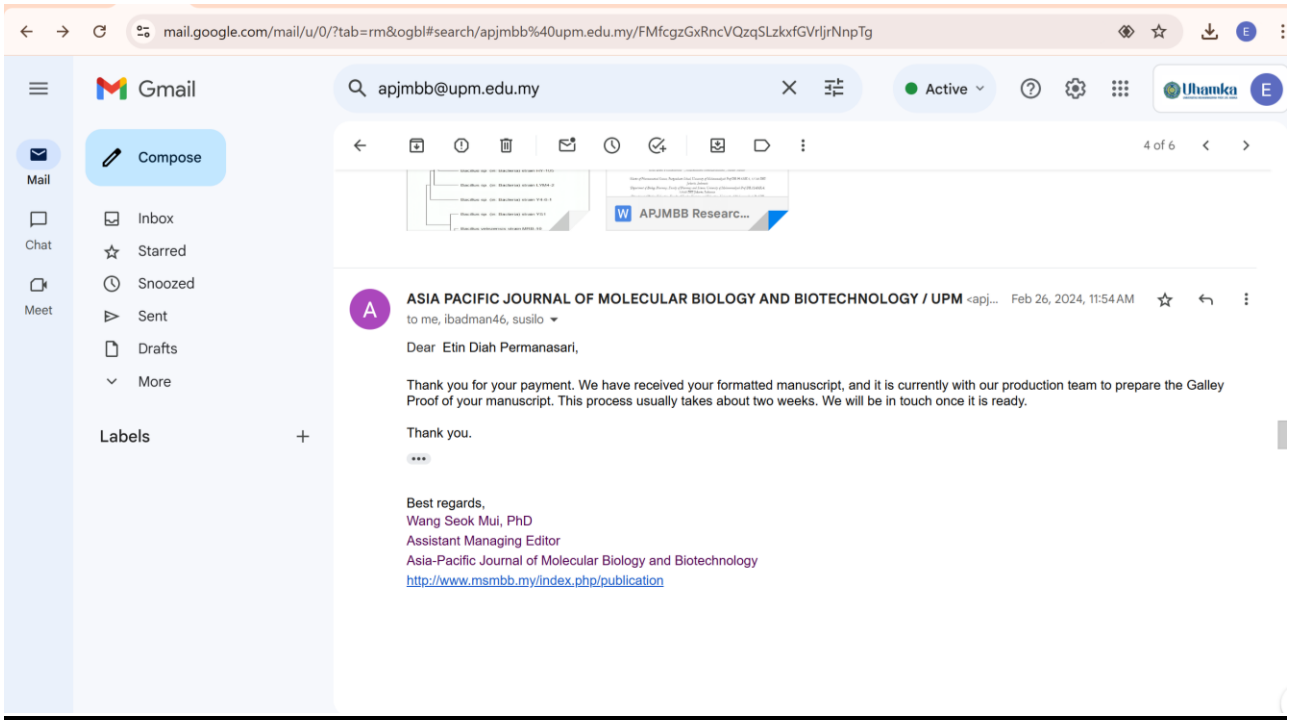
1.500 bp

500 bp

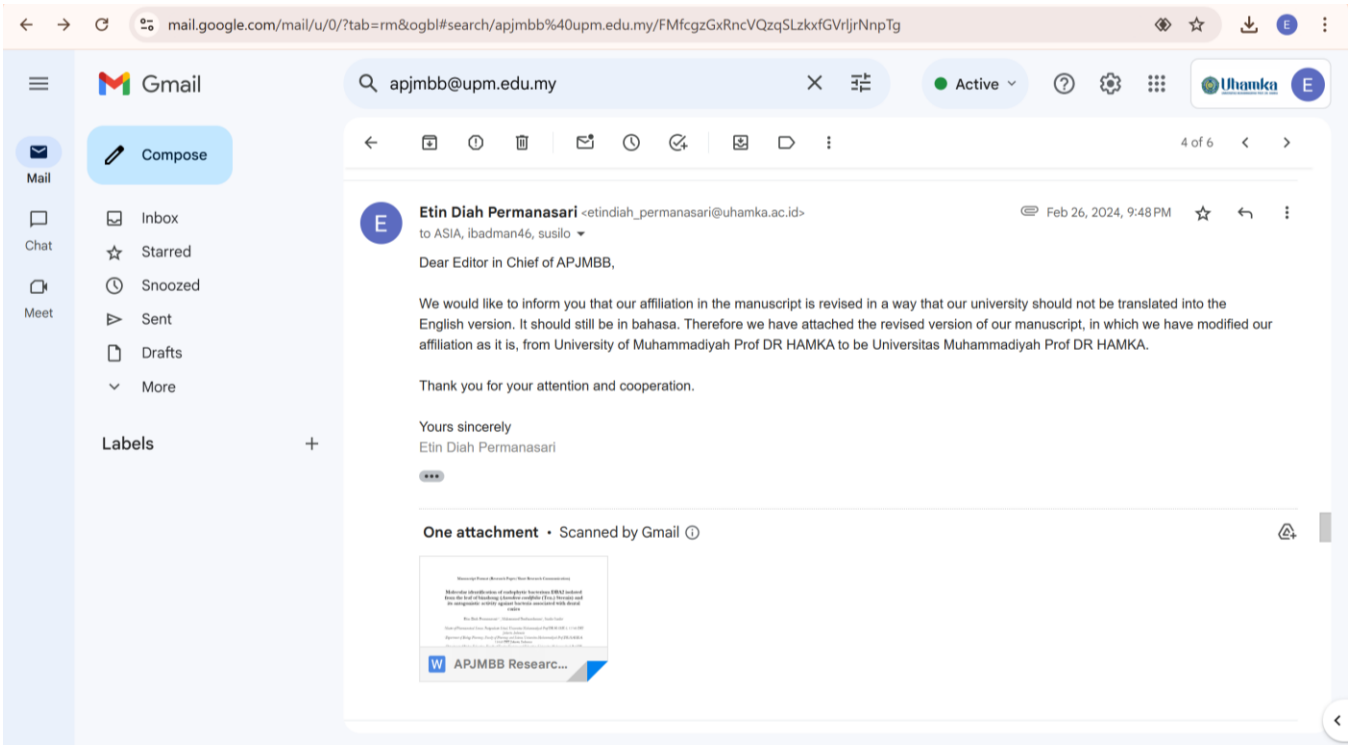
250 bp


40
35
30
25
20
15
10
5
0
Diameter of Inhibition Zone (mm)
S. mutans
S. mutans
L. acidophilus
L. acidophilus
DBA1
DBA2
DBA1
DBA2
Isolates of Endophytic Bacteria

11. Bukti konfirmasi penerimaan payment dan informasi persiapan Galley Proof manuscript dari Editor (26 Feb 2024)



12. Bukti konfirmasi permintaan pembetulan afiliasi dari kami kepada Editor (26 Feb 2024):



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Manuscript Format (Research Paper/Short Research Communication)

Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries

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Running title: Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries.

Abstract.

Binahong plants (*Anredera cordifolia* (Ten.) Steenis) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *S*teenis. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *S*teenis. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the

diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis; antibacterial activity; binahong leaf; endophytic bacteria; 16S rRNA gene.

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah *et al.*, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Sumartiningsih, 2011; Laksmitawati & Simbolon, 2017; Mutiarawati *et al.*, 2017; Yuniarti & Lukiswanto, 2017). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita *et al.*, 2017; Surbakti *et al.*, 2018).

Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Ainurrochmah *et al.*, 2013; Veronita *et al.*, 2017; Mengga *et al.*, 2022; Sasebohe *et al.*, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Basile *et al.*, 1999; Xie *et al.*, 2015). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie *et al.*, 2015; Veronita *et al.*, 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba *et al.*, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo *et al.*, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its

host plant (Arnold *et al.*, 2020; Potshangbam *et al.*, 2020; Sharma, *et al.*, 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani *et al.* (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo *et al.*, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Zhang, 2013; Hussein *et al.*, 2023). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty *et al.*, 2017; Abdel-Aziz *et al.*, 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial

compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar (NA) medium, and Nutrient Broth (NB) medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo *et al.*, 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

NA was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plates. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml NB test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial activity in this study was performed by the disk diffusion method. Two bacterial strains of *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strains indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*S. mutans* and *L. acidophilus*) were incubated in 10 ml NB test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potency by the presence or absence of an inhibition zone around the disk paper. The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against

pathogenic microorganism. The isolate with the largest diameter of inhibition zone was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar *et al.*, 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo *et al.*, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in

the surface leaves. Nystatin was added to NA as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel *et al*., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition

zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah *et al.*, 2013; Nxumalo *et al.*, 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar *et al.*, 2018).

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt *et al.*, 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt *et al.*, 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond *et al.*, 2015; Zhao *et al.*, 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants, such as promotes seed germination and induces stress resistance (Zhao *et al.*, 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao *et al.*, 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

250

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252 None

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Tables:

Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis.

Isolates	**Shapes**	**Colour**	**Texture**	Margins of **colony**	**Surface**	**Consistency**	**Staining**
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers.

No.	Closest relative species based on 16S rRNA gene sequences	GenBank accession number	Base pair length (bp)	Max score	E value	% Similarity
1.	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2.	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3.	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4.	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5.	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6.	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00

7.	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8.	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9.	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10.	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

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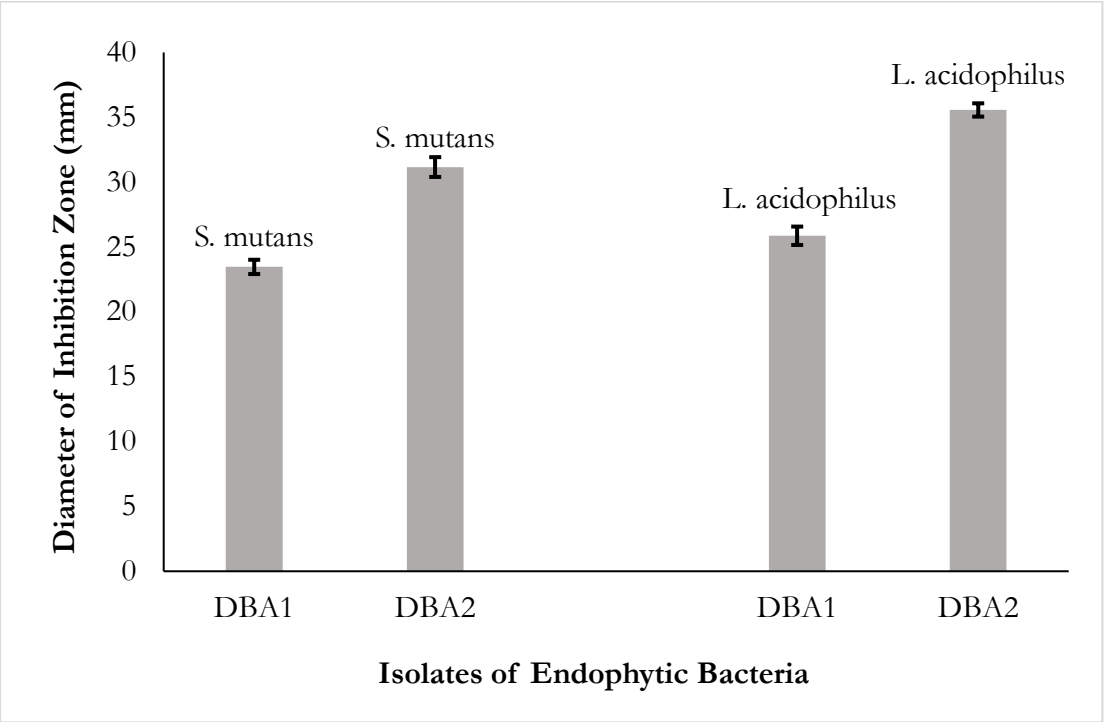
403 **Figures and Photos:**



404

405 **Figure 1.** The diameter of inhibition zone of the metabolites from the isolated endophytic
406 bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus*
407 *acidophilus*.

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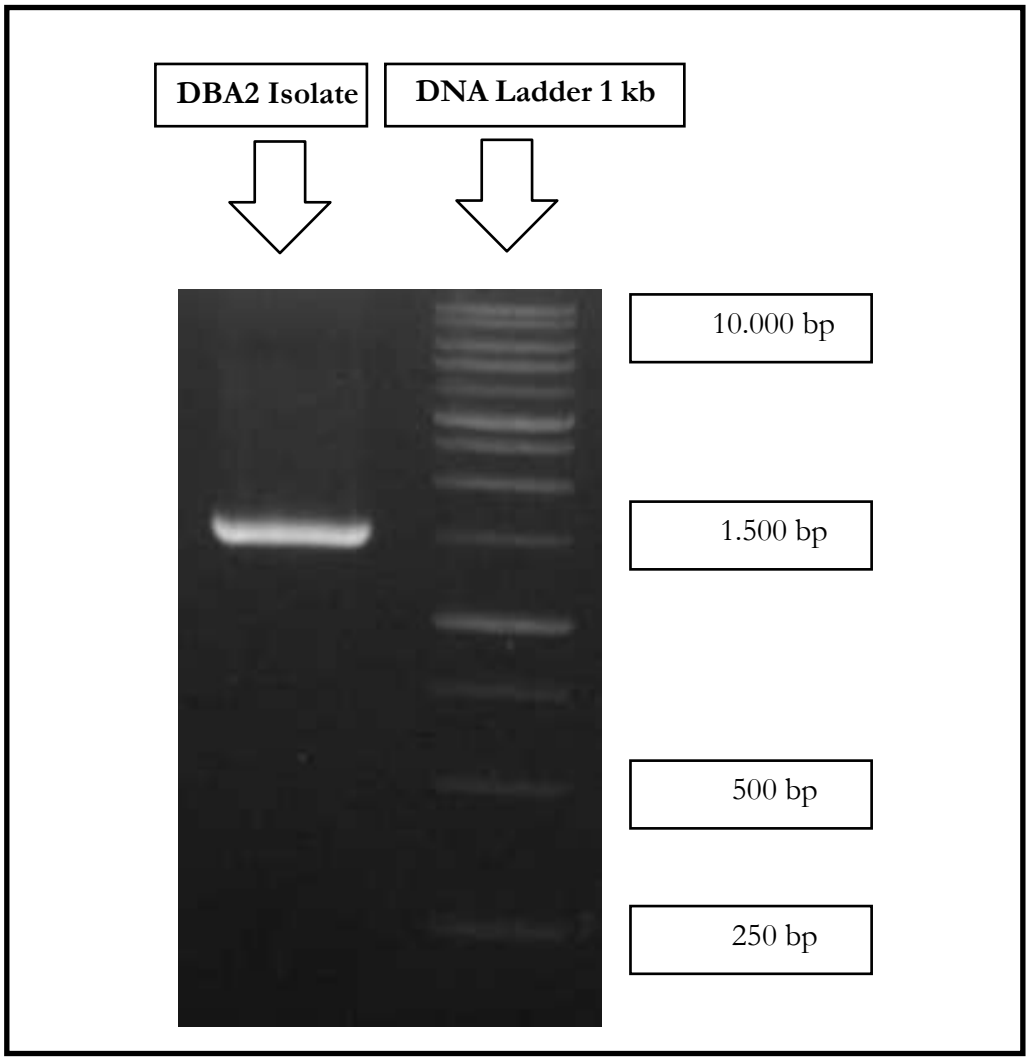

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410 **Figure 2.** The gel electrophoresis of 16S rRNA gene

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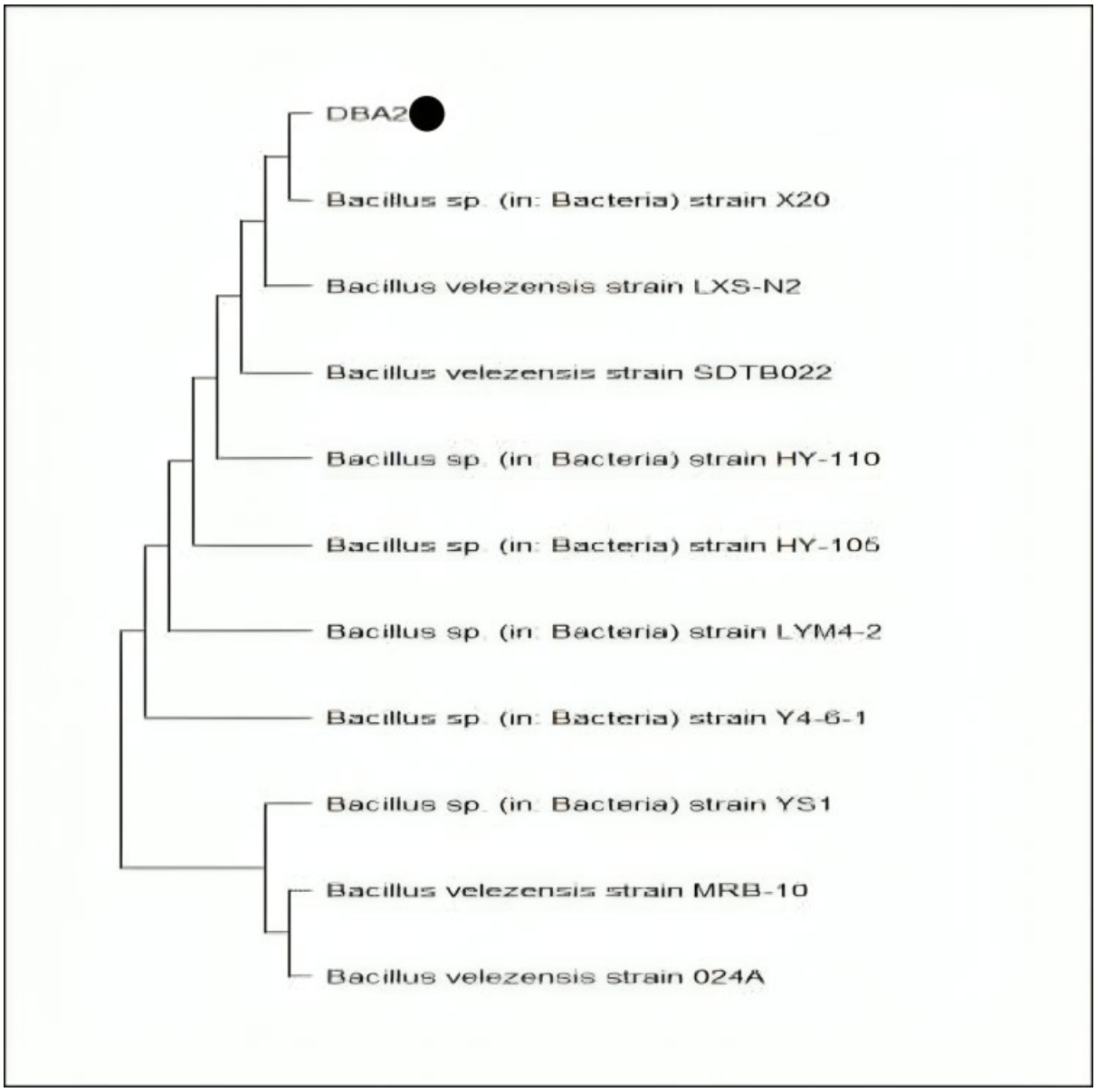

412

413 **Figure 3.** The phylogenetic tree of DBA2 isolate showing evolutionary relationships of
414 endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the
415 interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar
416 *et al.*, 2018).

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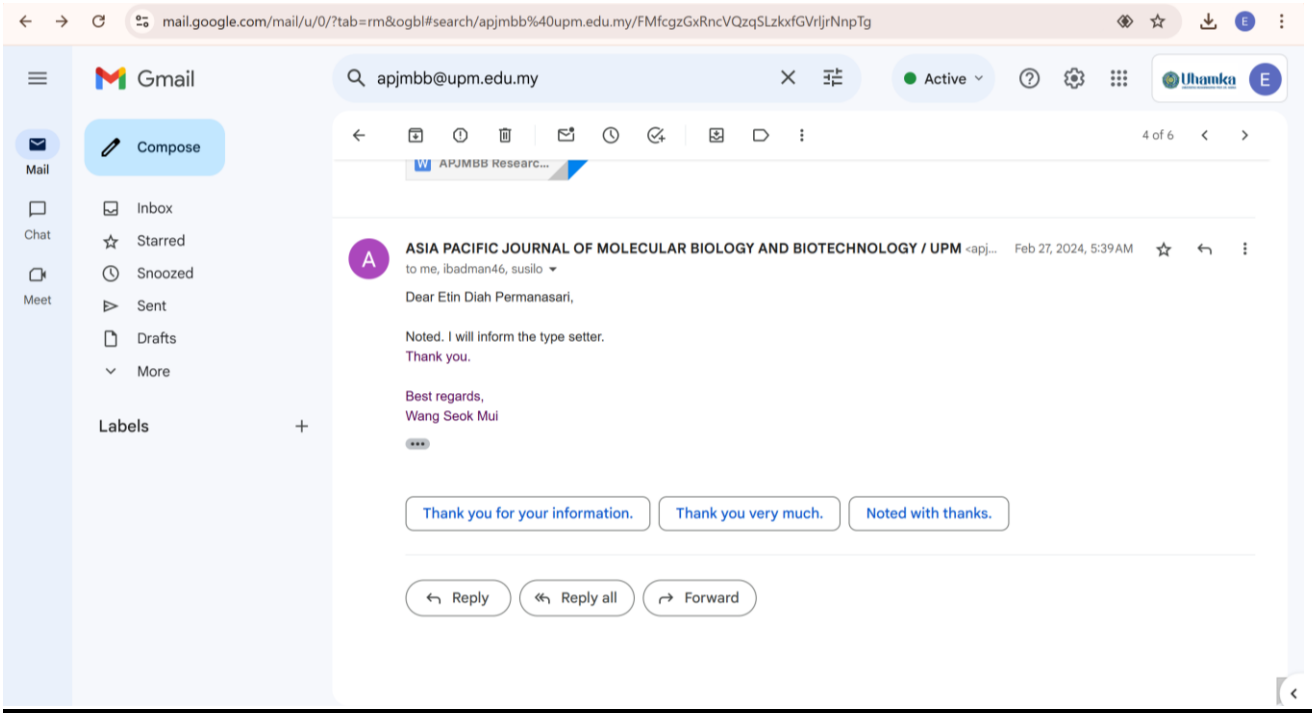
13. **Bukti konfirmasi penerimaan email konfirmasi pembetulan afiliasi dari Editor (27 Feb 2024):**



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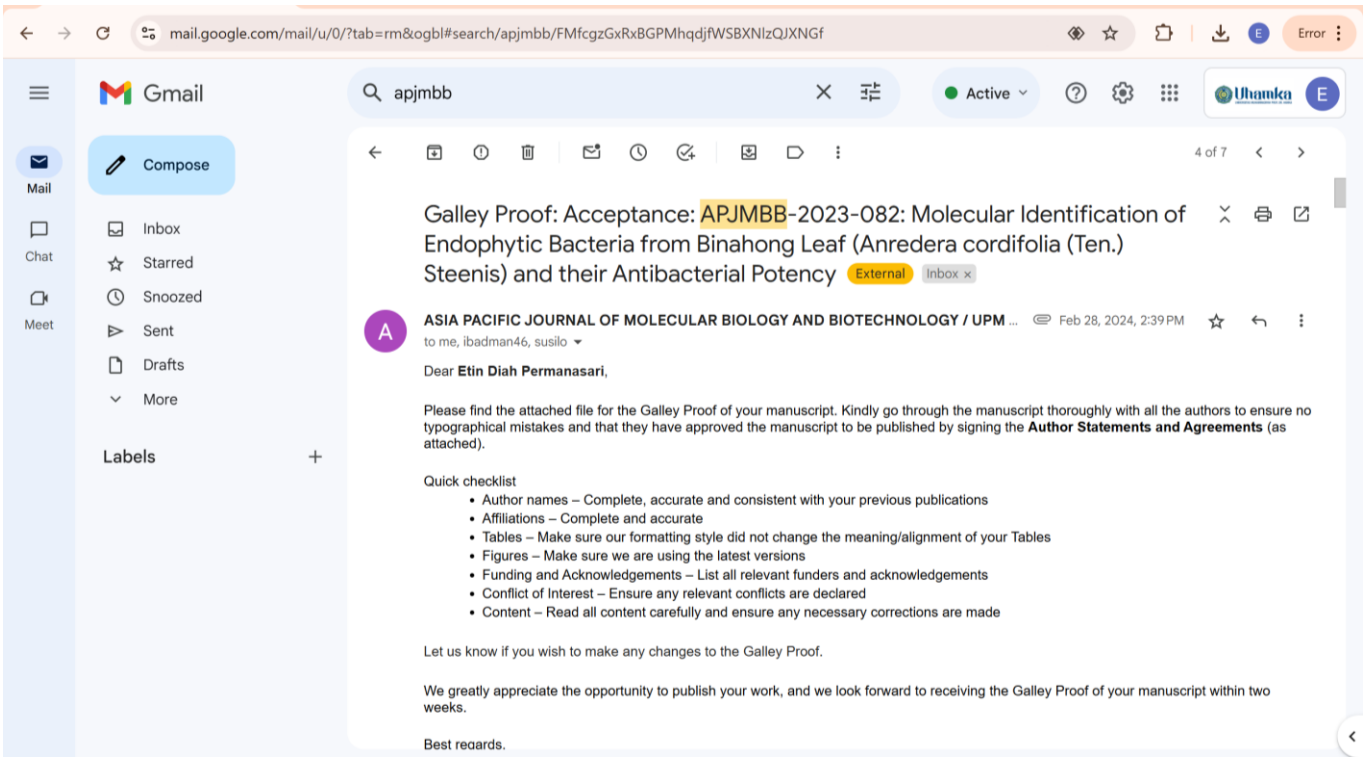

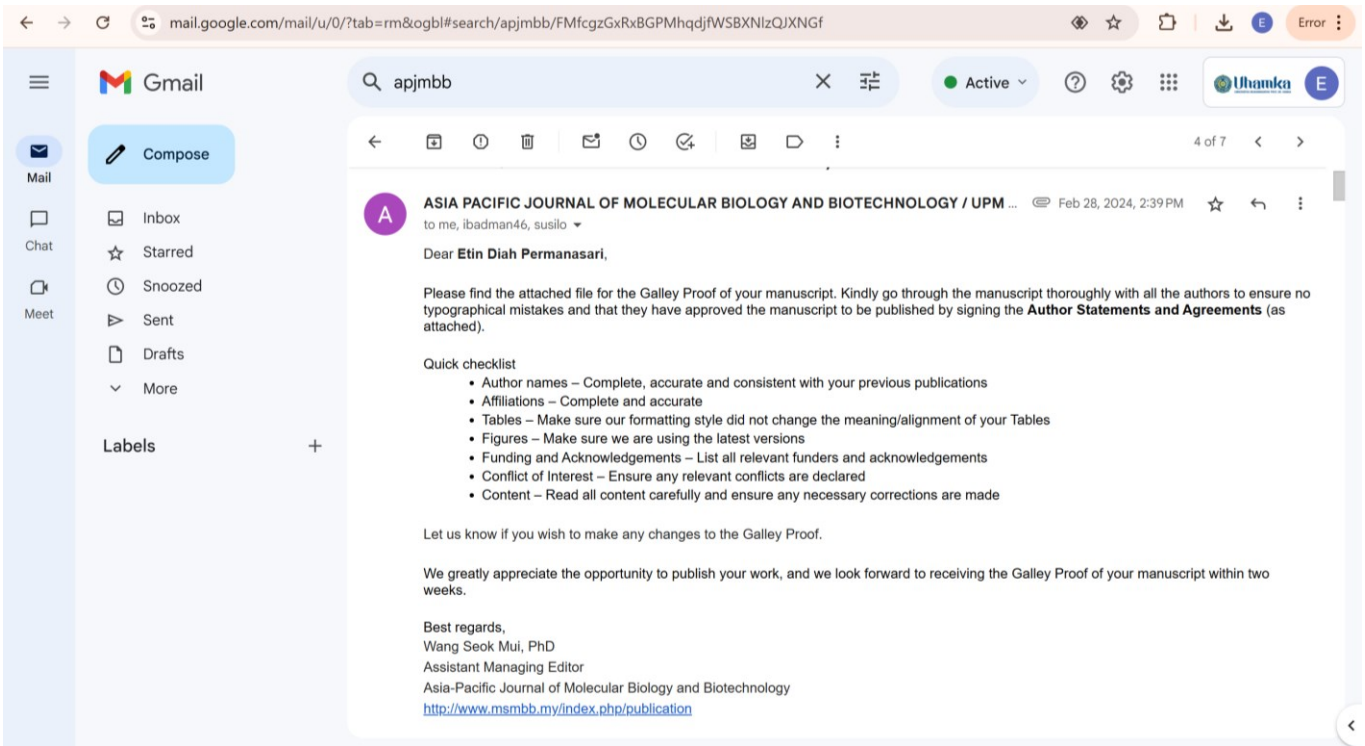

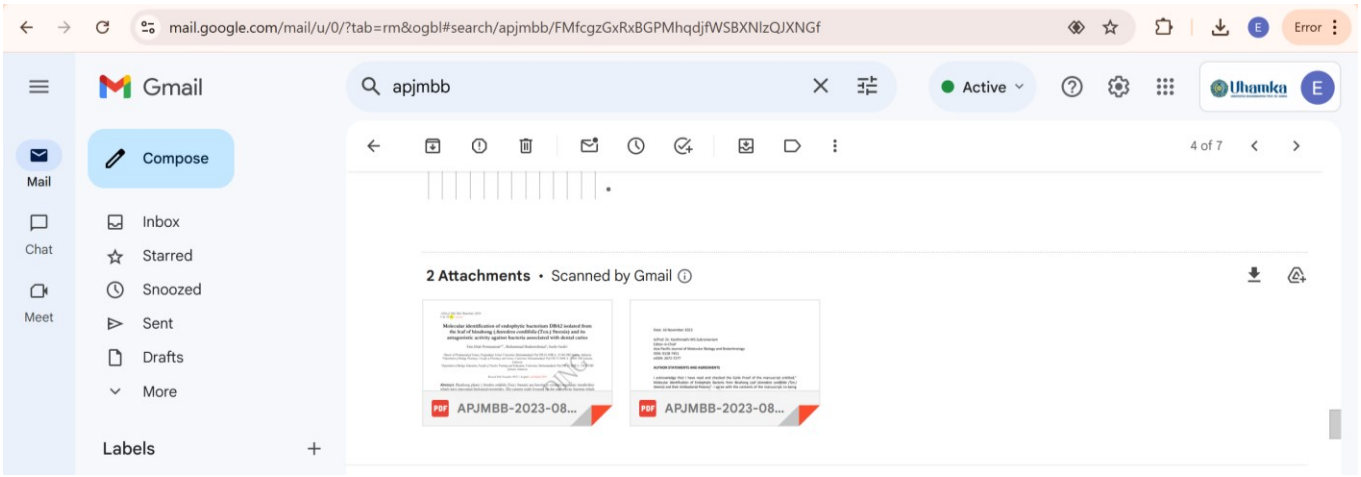
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AUTHOR STATEMENTS AND AGREEMENTS

I acknowledge that I have read and checked the Galle Proof of the manuscript entitled," Molecular Identification of Endophytic Bacteria from Binahong Leaf (*Anredera cordifolia (Ten.) Steenis*) and their Antibacterial Potency". I agree with the contents of the manuscript; to being listed as an author; and to the conflicts of interest statement. I have had access to all the data in the study (for original research articles) and accept responsibility for its validity. I agree for the manuscript to be published in Asia Pacific Journal of Molecular Biology and Biotechnology.

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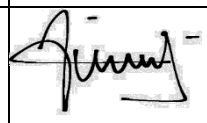
Muhammad Ibadurrohman	ibadman46@gmail.com	
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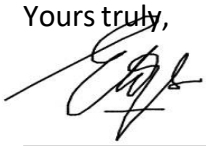

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Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries

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Abstract. Binahong plants (*Anredera cordifolia* (Ten.) Steenis) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *S*teenis. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *S*teenis. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah *et al.*, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Sumartiningsih, 2011; Laksmitawati & Simbolon, 2017; Mutiarawati *et al.*, 2017; Yuniarti & Lukiswanto, 2017). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita *et al.*, 2017; Surbakti *et al.*, 2018).

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Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Ainurrochmah *et al.*, 2013; Veronita *et al.*, 2017; Mengga *et al.*, 2022; Sasebohe *et al.*, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Basile *et al.*, 1999; Xie *et al.*, 2015). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie *et al.*, 2015; Veronita *et al.*, 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba *et al.*, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo *et al.*, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold *et al.*, 2020; Potshangbam *et al.*, 2020; Sharma, *et al.*, 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani *et al.* (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo *et al.*, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Zhang, 2013; Hussein *et al.*, 2023). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty *et al.*, 2017; Abdel-Aziz *et al.*, 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar (NA) medium, and Nutrient Broth (NB) medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo *et al.*, 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

NA was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plates. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml NB test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial activity in this study was performed by the disk diffusion method. Two bacterial strains of *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strains indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*S. mutans* and *L. acidophilus*) were incubated in 10 ml NB test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potency by the presence or absence of an inhibition zone around the disk paper. The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against pathogenic microorganism. The isolate with the largest diameter of inhibition zone was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar *et al.*, 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo *et al.*, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to NA as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel *et al*., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah *et al*., 2013; Nxumalo *et al*., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis.

Isolates	Shapes	Colour	Texture	Margins of colony	Surface	Consistency	Staining
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

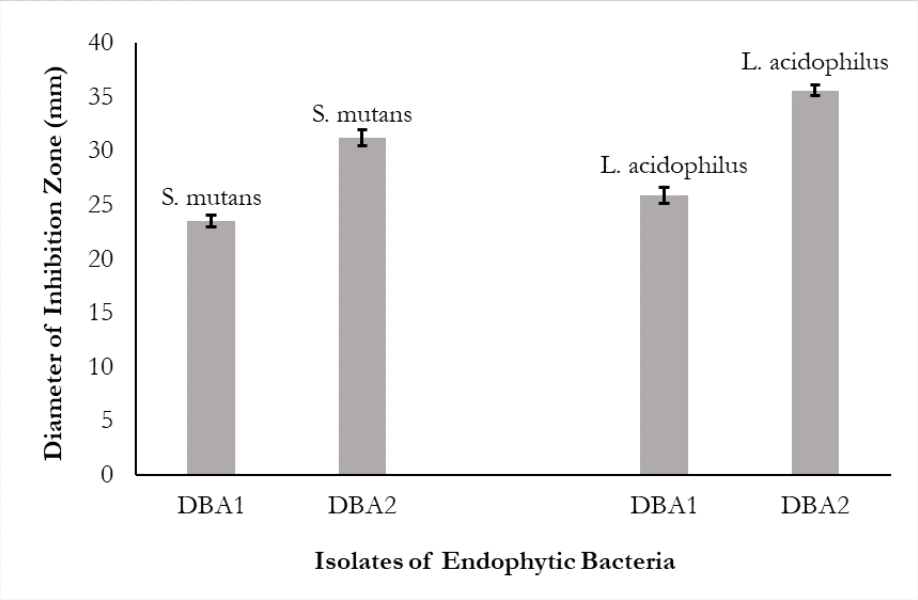

Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar *et al.*, 2018).

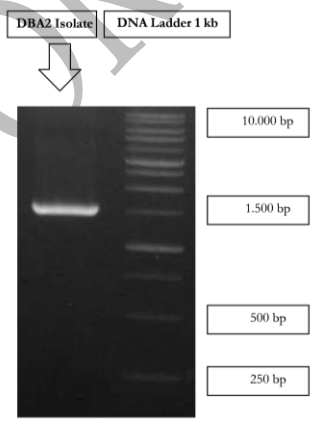

Figure 2. The gel electrophoresis of 16S rRNA gene

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt *et al.*, 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt *et al.*, 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond *et al.*, 2015; Zhao *et al.*, 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants,

such as promotes seed germination and induces stress resistance (Zhao *et al.*, 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao *et al.*, 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers.

No.	Closest relative species based on 16S rRNA gene sequences	GenBank accession number	Base pair length (bp)	Max score	E value	% Similarity
1.	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2.	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3.	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4.	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5.	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6.	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00
7.	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8.	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9.	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10.	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

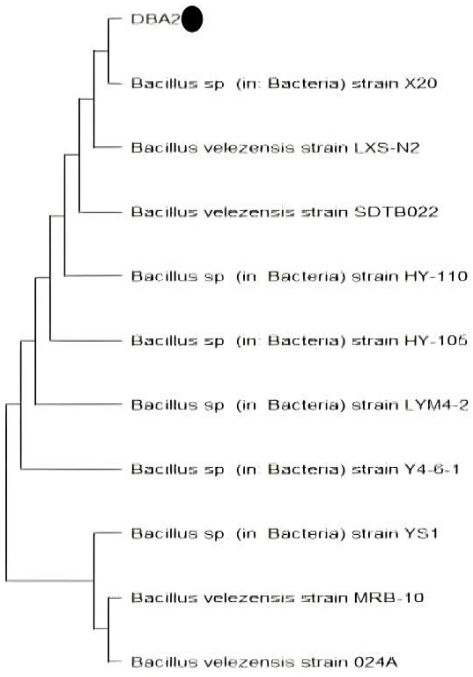

Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar *et al.*, 2018).

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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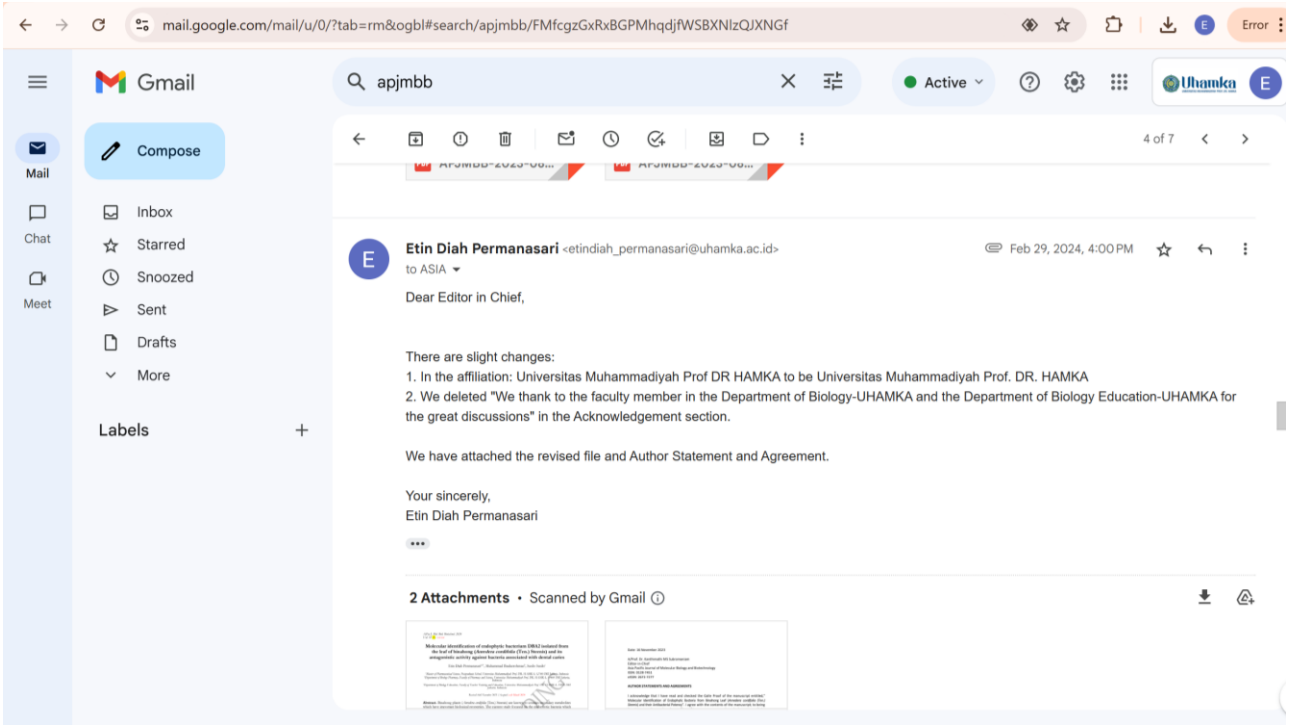

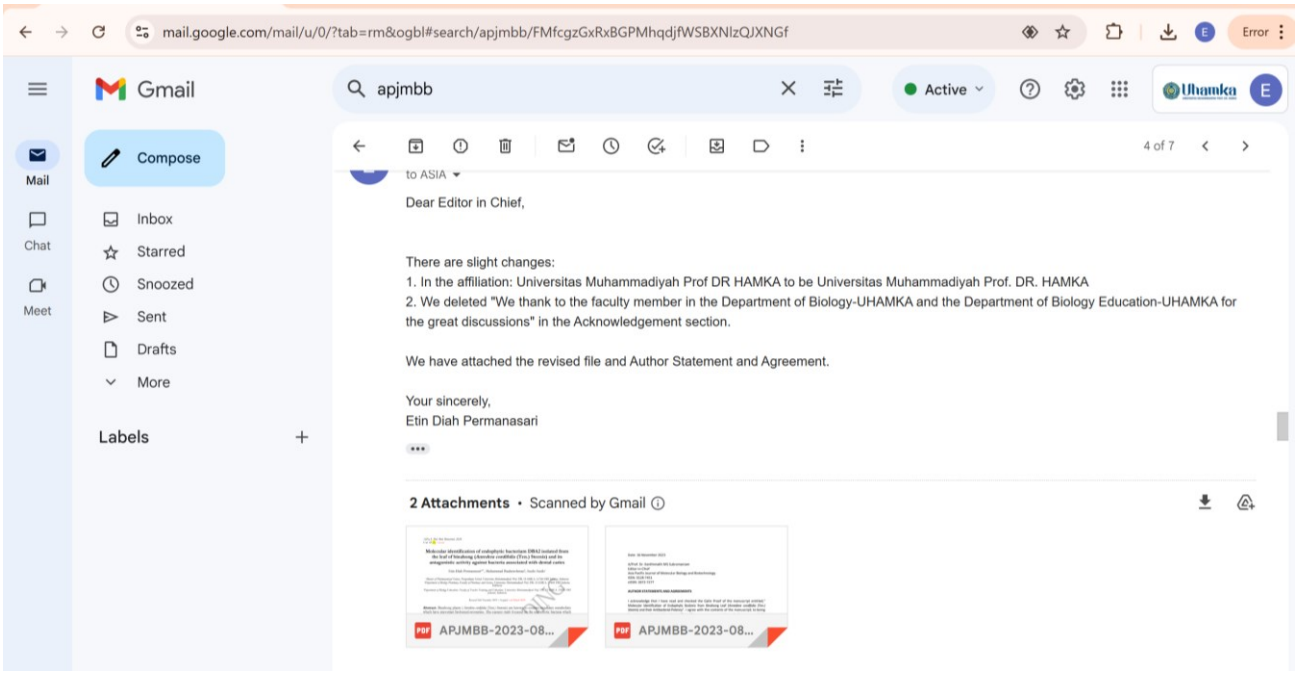

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AUTHOR STATEMENTS AND AGREEMENTS

I acknowledge that I have read and checked the Galle Proof of the manuscript entitled," Molecular Identification of Endophytic Bacteria from Binahong Leaf (*Anredera cordifolia (Ten.) Steenis*) and their Antibacterial Potency". I agree with the contents of the manuscript; to being listed as an author; and to the conflicts of interest statement. I have had access to all the data in the study (for original research articles) and accept responsibility for its validity. I agree for the manuscript to be published in Asia Pacific Journal of Molecular Biology and Biotechnology.

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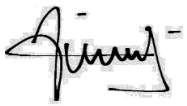
Muhammad Ibadurrohman	ibadman46@gmail.com	
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Thank you.

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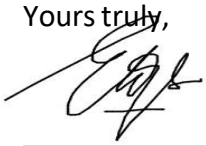

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Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries

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Abstract. Binahong plants (*Anredera cordifolia* (Ten.) Steenis) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *S*teenis. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *S*teenis. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah *et al.*, 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Sumartiningsih, 2011; Laksmitawati & Simbolon, 2017; Mutiarawati *et al.*, 2017; Yuniarti & Lukiswanto, 2017). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita *et al.*, 2017; Surbakti *et al.*, 2018).

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Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Ainurrochmah *et al.*, 2013; Veronita *et al.*, 2017; Mengga *et al.*, 2022; Sasebohe *et al.*, 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Basile *et al.*, 1999; Xie *et al.*, 2015). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie *et al.*, 2015; Veronita *et al.*, 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba *et al.*, 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo *et al.*, 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold *et al.*, 2020; Potshangbam *et al.*, 2020; Sharma, *et al.*, 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani *et al.* (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo *et al.*, 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Zhang, 2013; Hussein *et al.*, 2023). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty *et al.*, 2017; Abdel-Aziz *et al.*, 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar (NA) medium, and Nutrient Broth (NB) medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo *et al.*, 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

NA was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plates. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml NB test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial activity in this study was performed by the disk diffusion method. Two bacterial strains of *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strains indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*S. mutans* and *L. acidophilus*) were incubated in 10 ml NB test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potency by the presence or absence of an inhibition zone around the disk paper. The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against pathogenic microorganism. The isolate with the largest diameter of inhibition zone was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar *et al.*, 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo *et al.*, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to NA as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel *et al*., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah *et al*., 2013; Nxumalo *et al*., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis.

Isolates	Shapes	Colour	Texture	Margins of colony	Surface	Consistency	Staining
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

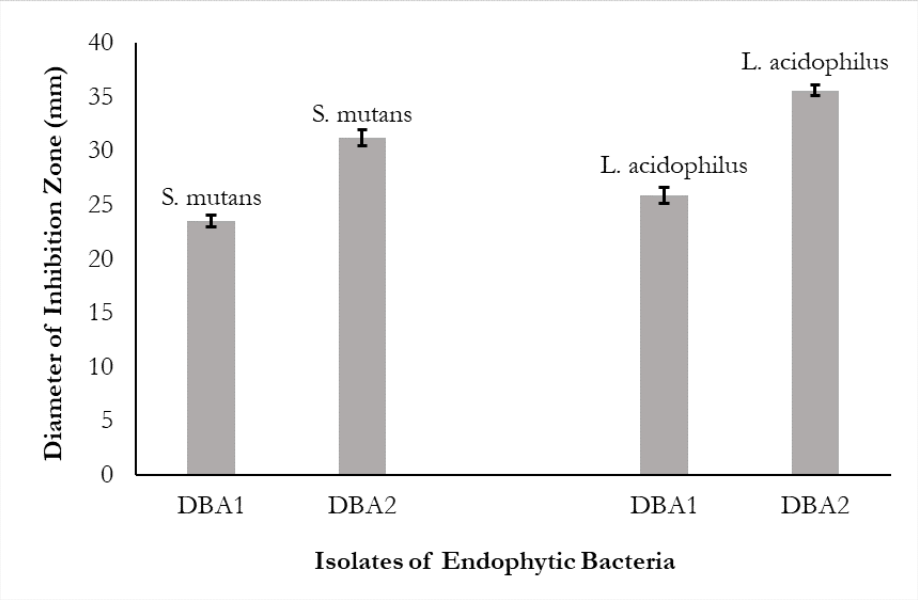

Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar *et al.*, 2018).

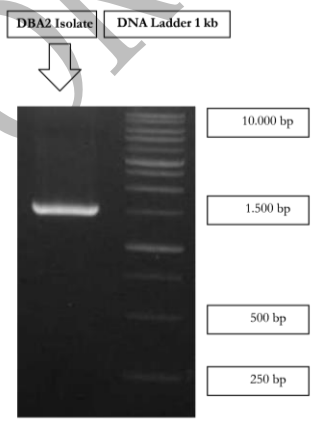

Figure 2. The gel electrophoresis of 16S rRNA gene

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt *et al.*, 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt *et al.*, 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond *et al.*, 2015; Zhao *et al.*, 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants,

such as promotes seed germination and induces stress resistance (Zhao *et al.*, 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao *et al.*, 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers.

No.	Closest relative species based on 16S rRNA gene sequences	GenBank accession number	Base pair length (bp)	Max score	E value	% Similarity
1.	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2.	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3.	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4.	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5.	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6.	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00
7.	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8.	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9.	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10.	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

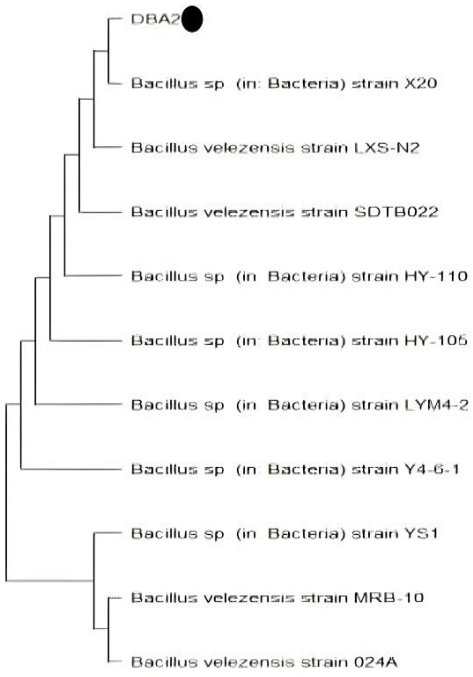

Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar *et al.*, 2018).

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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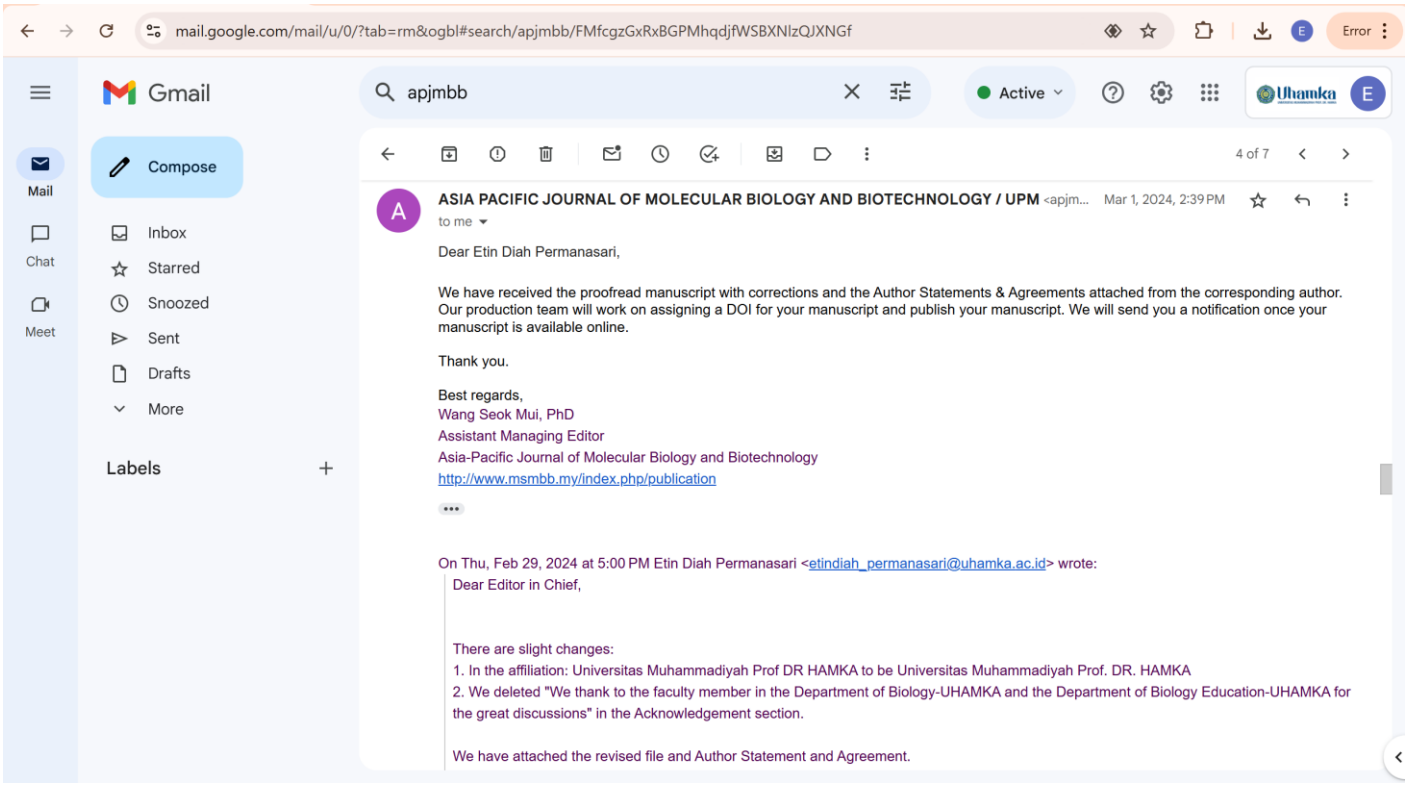
16. Bukti konfirmasi penerimaan hasil proof read dari Editor (1 Maret 2024)



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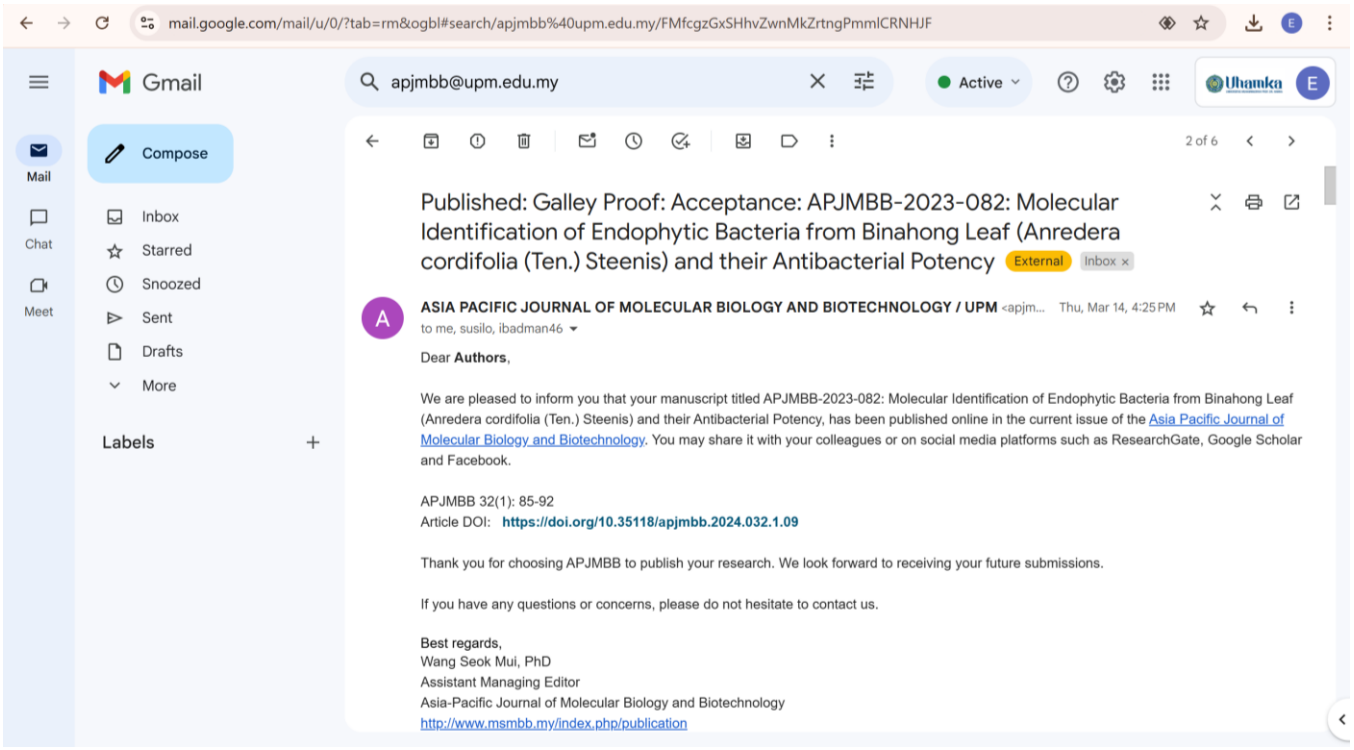

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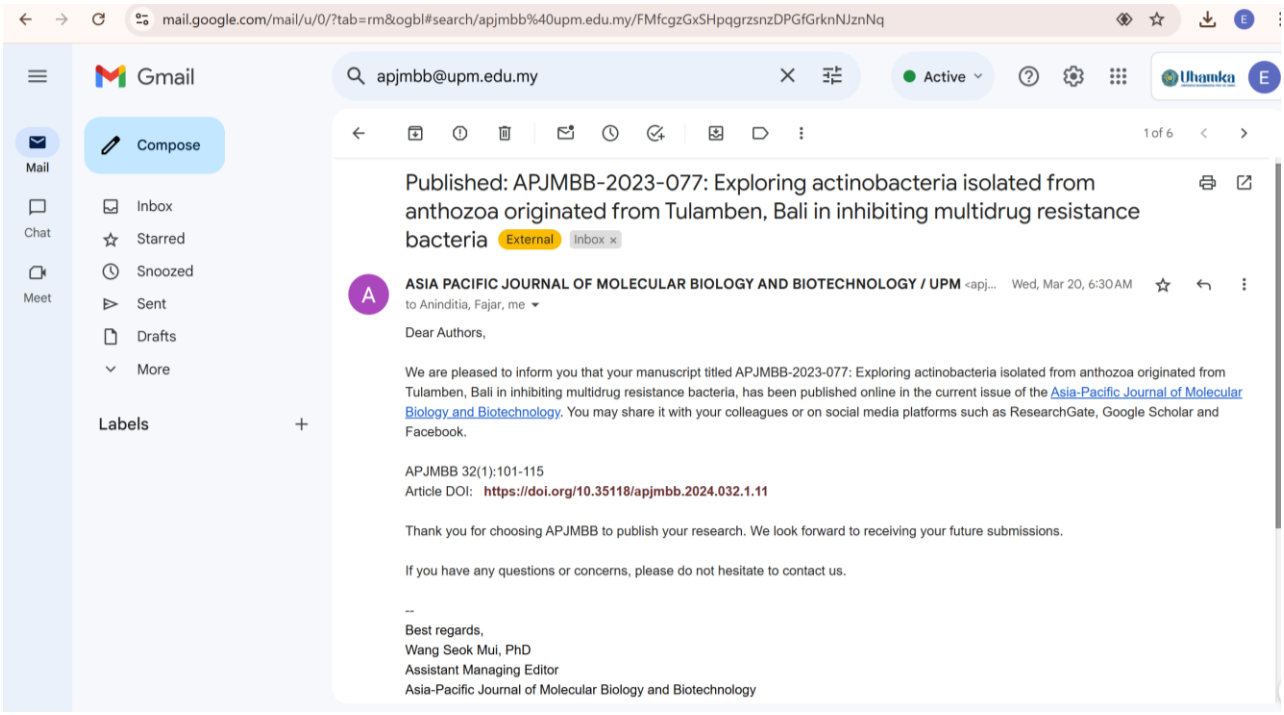

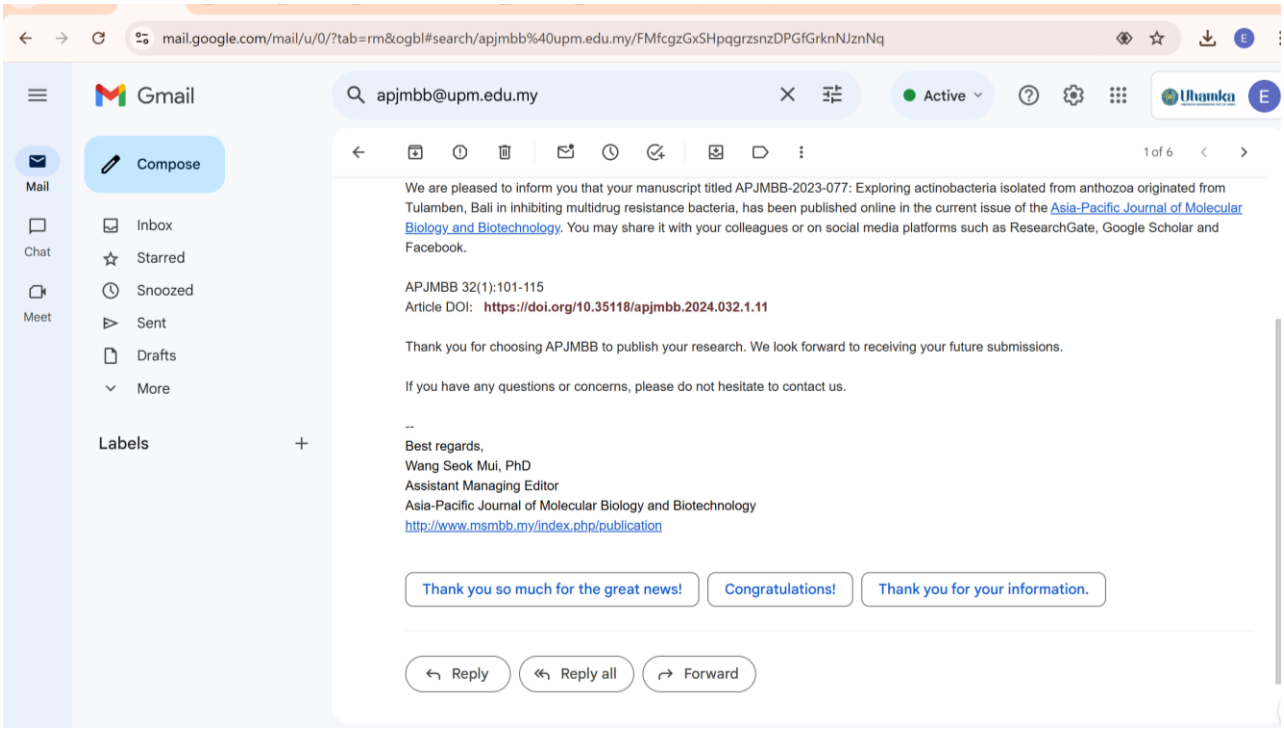

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Molecular identification of endophytic bacterium DBA2 isolated from the leaf of binahong (*Anredera cordifolia* (Ten.) Steenis) and its antagonistic activity against bacteria associated with dental caries

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Abstract. Binahong plants (*Anredera cordifolia* (Ten.) Steenis) are known to contain secondary metabolites which have important biological properties. The current study focused on the endophytic bacteria which lives in the leaves of *A. cordifolia* (Ten.) *S*teenis. The aim of this study is to isolate and identify the endophytic bacteria which can produce antibacterial metabolites from the leaves of *A. cordifolia* (Ten.) *S*teenis. Two isolates, DBA1 and DBA2 were isolated and purified from the leaves of *A. cordifolia*. These isolates were subjected to the screening for their antagonistic activity against the bacteria associated with dental caries, which are *Streptococcus mutans* and *Lactobacillus acidophilus* using the disk-diffusion method. The strain of DBA2 exhibited the largest diameter of inhibition zone against both *S. mutans* (31,17 mm) and *L. acidophilus* (35,57 mm). While DBA1 exhibited the diameter of inhibition zone of 23,47 mm and 25,87 mm against *S. mutans* and *L. acidophilus*, as respectively. The strain of DBA2 was then subjected for molecular identification. The genomic DNA of DBA2 was extracted with the Geno Plus™ Genomic DNA Extraction Miniprep System and molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The amplicons were then purified and sequenced, before the 16S rRNA gene sequences were analysed by a Basic Local Alignment Search Tool (BLAST) search against the National Centre Biotechnology Information (NCBI) database. The endophytic bacterial strain DBA2 from the leaves of *A. cordifolia* was identified to be closely related to *Bacillus* sp., and the top match from the database search revealed a similarity value of 100% with the reference *Bacillus* sp. strain x20. Future studies are required to analyse the bioactive compounds of strain DBA2, which can be considered as a potential source for the new antibacterial drugs for the dental caries treatment.

Keywords: *Anredera cordifolia* (Ten.) Steenis, antibacterial activity, binahong leaf, endophytic bacteria, 16S rRNA gene

INTRODUCTION

The binahong (*Anredera cordifolia* (Ten.) Steenis) is a plant that grows naturally in tropical areas and is used as a traditional medicine to treat various diseases (Azizah *et al*., 2022). Several studies of *A. cordifolia* (Ten.) Steenis has proven that this plant contains bioactive compounds used to treat many diseases, such as wound, diabetic, hematoma, and hyperuricemia (Sumartiningsih, 2011; Laksmitawati & Simbolon, 2017; Mutiarawati *et al*., 2017; Yuniarti & Lukiswanto, 2017). It also exhibited anti-inflammatory properties to cure injured cell (Sumartiningsih, 2011). The part of binahong plant that is commonly used is the leaves which contain flavonoids, alkaloid, tannin, saponins, and steroid/triterpenoids (Veronita *et al*., 2017; Surbakti *et al*., 2018).

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Many studies have been conducted for antibacterial properties from the binahong leaves against some bacteria such as *Staphylococcus aureus*, *Shigella flexneri*, *Propionibacterium acnes*, and *Eschericia coli* (Ainurrochmah *et al*., 2013; Veronita *et al*., 2017; Mengga *et al*., 2022; Sasebohe *et al*., 2023). Based on the previous findings, flavonoids are one of the main compounds responsible for antibacterial properties (Basile *et al*., 1999; Xie *et al*., 2015). Flavonoids are the group which effectively inhibits the growth of bacteria and fungi, as it causes damage to the permeability of cell walls and inhibit motility (Xie *et al*., 2015; Veronita *et al*., 2017). Several flavonoid compounds from the binahong leaves have been identified, such as vitexin, isovitexin, and morin (Alba *et al*., 2020). However, as the microbial resistance is always a problem, discovering and developing new antibiotics can be a great challenge. Therefore, the development of new antibacterial agents is essential, including discovering and developing novel antibacterial compounds from microorganisms.

Endophytic microorganisms, such as bacteria, fungi, and yeast, are defined as microorganisms that usually live as symbionts inside a plant (Nxumalo *et al*., 2020). These endophytic microorganisms are crucial in the production of many important bioactive compounds, including antibacterial agents. These microorganisms produce similar bioactive compounds as its host plant (Arnold *et al*., 2020; Potshangbam *et al*., 2020; Sharma, *et al*., 2021). They provide a valuable source for finding novel therapeutic agents resulting the huge exploration of these endophytic microorganisms. The considerable diversity of endophytes and their ability to adapt to different environments can be seen as a valuable and largely untapped resource of novel secondary metabolites that could be used in the applications in pharmaceutical or agricultural sectors (Bacon and White, 2000).

Desriani *et al*. (2014) reported that out of 37 of the endophytic bacteria isolates were obtained from the binahong leaves, fifteen isolates exhibited antibacterial properties against *Pseudomonas aeruginosa*. The endophytic bacteria isolated from the *A. cordifolia* (Ten.) Steenis leaves in previous report have been determined as *Pseudomonas aeruginosa* CP043328.1 which produce antibacterial metabolites against *S. aureus, E. coli, Bacillus cereus*, and *Proteus mirabilis* (Nxumalo *et al*., 2020). Previous study also reported 3 isolates of endophytic bacteria from the binahong leaves can inhibit the growth of *Staphylococcus, Pseudomonas* and *Bacillus* sp. (Nursulistyarini and Ainy, 2014).

However, the study on the isolation and identification as well as the exploration of secondary metabolites of endophytic microorganisms from the binahong plant is yet very limited. Therefore, this study aimed to isolate and identify the endophytic bacteria which live in the leaves tissue of the binahong plant (*A. cordifolia* (Ten.) Steenis) that can produce antibacterial metabolites. We tested on the antagonistic activity of the isolated endophytic bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* which mainly caused dental caries in human (Zhang, 2013; Hussein *et al*., 2023). Although, the previous studies on the antagonistic activity against several dental caries causing bacteria have been conducted from endophytic fungi of other medicinal plants (Ukhty *et al*., 2017; Abdel-Aziz *et al*., 2020). The exploration and identification on the endophytic binahong leaf to dental caries causing bacteria are very limited. In this study, the identification was performed using 16S ribosomal RNA (16S rRNA) gene sequencing and analysis. Potential antibacterial compounds present in the endophytic bacteria may also be identified and considered for the dental caries treatment in the future.

MATERIALS AND METHODS

Chemicals and media

The materials were ethanol 75%, ethanol 98%, $CaCO_3$, NaCl, soybean meal, corn step liquor, H_2O, NaOCl, nystatin, DMSO, HCl, NaOH, KH_2PO_4, agarose gel, and deionized demineralized water (ddH_2O) from Sigma Aldrich, Ltd. The kits were Geno Plus™, Genomic DNA Extraction Miniprep System from VIOGENE, USA and Zymo Spin Column PCR Purification kit from Zymo Research, EU. Primers were from VIOGENE, USA. The mediums were yeast extract, Nutrient Agar (NA) medium, and Nutrient Broth (NB) medium from HiMedia Laboratories, India. The medias were prepared and autoclaved.

Plant sample preparation

The fresh leaves were collected from Tangerang, Province Banten, Indonesia. The leaves were identified at Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia. The leaves were washed by running tap water and carefully dried (Susilo *et al.*, 2018; Susilo & Meitiyani, 2018). The leaves surfaces were disinfected by ethanol 75% and NaOCl 5,3% for 5 minutes, and then wiped carefully with ethanol 75%.

Endophytic bacteria isolation from the binahong leaves

NA was used as a medium for the isolation of endophytic bacteria. The binahong leaves that been disinfected were carefully being cut off. The cut leaves were then put in NA plate under aseptic condition. Plates were then sealed and incubated in 37°C for 48 hours. The endophytic bacteria were taken, purified, and transferred into new NA plates. The purified isolates were then used for further experiment.

Morphological characterization of endophytic bacteria of the binahong leaves

The isolates were being analysed for their macroscopic and microscopic characterizations. Macroscopic characterizations include colony shape, colony pigmentation, consistency, elevation, and edge shape. Microscopic observation was carried out using gram staining for identification of Gram type of bacteria. Gram staining was carried out firstly by an initial staining with crystal violet dye. The object was then added by iodine to fix the dye. The last step included ethanol to be added to the object to remove the dye (Tripathi and Sapra, 2023). The preparate were then analysed under microscope.

Endophytic bacteria cultivation

Endophytic bacteria were cultivated using a liquid NB medium which had been sterilized by an autoclave. The pure isolate of endophytic bacteria was inoculated into a 10 ml NB test tube. Shaking incubation was carried out for 2 days at a temperature of 25°C. After incubation, the cell biomass and supernatant were harvested using centrifugation for 30 minutes at 2000 rpm. The obtained supernatant was used as a source of antibacterial substances potential of endophytic bacteria.

Antibacterial activities screening

The screening for antibacterial activity in this study was performed by the disk diffusion method. Two bacterial strains of *Streptococcus mutants* and *Lactobacillus acidophilus*, were used as strains indicator for antibacterial activity assay. The strain culture of the bacteria of dental caries (*S. mutans* and *L. acidophilus*) were incubated in 10 ml NB test tubes for 24 hours at 37° C. The culture was then inoculated into a solid media of NA in Petri dish. The sterile paper disk was then soaked with the supernatant, then placed in the NA medium which has been inoculated with pathogenic bacteria. The plates were incubated for 1-2 days, and then observed their antibacterial potency by the presence or absence of an inhibition zone around the disk paper. The activities were determined by measuring the diameter of the inhibition zone in millimeter (mm) against pathogenic microorganism. The isolate with the largest diameter of inhibition zone was continued for the molecular identification.

Molecular identification of endophytic bacteria

The chosen isolate was further identified using 16S ribosomal RNA (16S rRNA) sequence analysis. Briefly, DNA extraction was performed by a Genomic DNA Extraction Miniprep System kit from VIOGENE, USA based on their manual instruction kit. PCR method was conducted to amplify the 16S rRNA gene of the endophytic bacteria with the primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387r (5'-GGG GGG WGT GTA CAA GGC-3'. The PCR products were gel extracted and purified by a Zymo Spin Column PCR Purification kit from Zymo Research, EU. The purified PCR products were then processed and sequenced at the First Base Laboratories, Malaysia. The obtained sequences were processed by MEGAX software analysis (Kumar *et al.*, 2018). The sequencing results were implemented for BLAST using the database from the National Centre for Biotechnology Information (NCBI) in order to determine the most closely related reference bacteria in the database (Nxumalo *et al.*, 2020).

RESULTS AND DISCUSSION

Determination of the binahong leaves

Taxonomic identification of the plants used in this study was carried out before the specimen was used to isolate the endophytic bacteria so that the accurate plant species was used. According to the identification result from Centre for Biosystematics and Evolution Research-National Research and Innovation Agency (BRIN) with the voucher number B-804/V/DI.05.07/3/2022, it was known that the leaves is *Anredera cordifolia* (Teen.) Steenis.

Isolation of the endophytic bacteria from the binahong leaves

The surface sterilization was done prior the whole isolation process. This surface sterilization method was performed to eliminate the contaminant microorganisms that present in the surface leaves. Nystatin was added to NA as antifungal agent. Two isolates of endophytic bacteria were obtained and named with the codes of DBA1 and DBA2. However, the number of the endophytic bacteria found in this study are less. This result is in consistent with the finding that the number of endophytic bacteria can be influenced by the particular part of samples. It is known that the number of endophytic bacteria from the stems and leaves are usually less, while the roots are abundant (Zinniel *et al*., 2022). The heterogeneity of endophytic bacteria can be influenced by various conditions, such as soil structure, time of sampling, geographical distribution, and plant age.

Morphological characterization

Based on the result of morphological characterization, the macroscopic and microscopic characterization of DBA1 and DBA2 were described in Table 1. Microscopic staining characterizations showed that these two endophytic bacteria were Gram-positive bacteria. The description of morphological characterization of DBA1 and DBA2 were shown in Table 1.

Antibacterial activity screening

The two isolates were cultivated using NB liquid medium. The supernatant was used into antibacterial testing using the disk diffusion method. The supernatants of DBA1 and DBA2 showed antibacterial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* with the average clear zone diameter of inhibition zone as shown in Figure 1. The strongest antibacterial activity was DBA2 with the average diameter of inhibition zone of 31,17 mm and 35,57 mm against *S. mutans* and *L. acidophilus*, as respectively.

The production of inhibitory zones indicates that there is an inhibitory activity in the supernatants of both DBA1 and DBA2 against the bacteria of dental caries. This finding is consistent with the previous studies showed that the endophytic microbials are said to have antimicrobial activity (Sartika, 2021; Sharma & Mallubhotla, 2022). In this study, both pathogenic bacteria were a Gram-positive bacterium. The finding that DBA1 and DBA2 had clear inhibition zones suggest that Gram-positive bacteria were fragile to metabolites from endophytic bacteria of the binahong leaves. Based on our results, we propose that Gram-positive bacteria are fragile due to the strong antagonistic activity of bioactive compounds produced from strain DBA2. Although there are a lot of study reveal the activity against Gram-negative bacteria (Ainurrochmah *et al*., 2013; Nxumalo *et al*., 2020). However, the inhibition activities of DBA1 and DBA2 to Gram-negative bacteria are still need to be analysed.

Table 1. The description of macroscopic and microscopic characterization of the isolated endophytic bacterial from the leaves of the medicinal plant *Anredera cordifolia* (Ten.) Steenis.

Isolates	Shapes	Colour	Texture	Margins of colony	Surface	Consistency	Staining
DBA1	Irregular	White	Flat	Undulate	Flat and smooth	Mucoid	+ve
DBA2	Irregular	White	Flat	Curled	Flat and smooth	Buttery	+ve

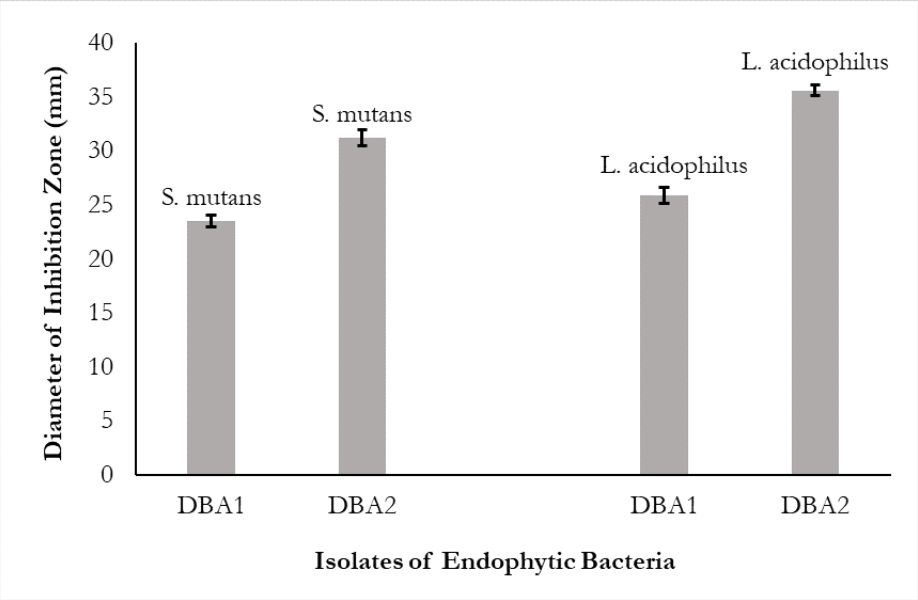

Figure 1. The diameter of inhibition zone of the metabolites from the isolated endophytic bacteria of *Anredera cordifolia* (Ten.) Steenis leaves against *Streptococcus mutans* and *Lactobacillus acidophilus*.

Molecular identification of endophytic bacteria isolate DBA2

Among two strains, strain DBA2 was chosen as potential strain as it showed the largest diameter of inhibition zone against *S. mutans* and *L. acidophilus*. Furthermore, the genomic DNA was extracted based on the method as described in the kit procedure. The obtained DNA bands were confirmed as a size of approximately 1500 bp shown in Figure 2. It was processed in the First Base Laboratories, Malaysia. The sequences of DBA2 were then being assessed by the GenBank NCBI database and MEGAX software analysis (Kumar *et al*., 2018).

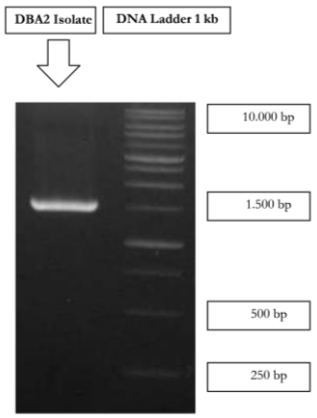

Figure 2. The gel electrophoresis of 16S rRNA gene

Specification that meets taxonomic requirements is that if the similarity percentage is > 99%, it is defined as similarity at the species level (Drancourt *et al*., 2000). In addition, it is said to be the same genus if the identity is 96% - 99% (Drancourt *et al*., 2000). Max score represents the highest sequence alignment value between the query sequence alignment results and the sequences contained in the database. A high max score value and an E-value that is close to 0 indicate a higher level of confidence between the alignment results of the query sequence and the sequences contained in the database that have a high level of homology. The results showed that the strain DBA2 had a level of similarity to *Bacillus* sp. strain x20 with a max score of 2139%, identification of 100%, and E-value 0, as shown in Table 2, suggesting that the finding had a high level of confidence to identify that the species of the endophytic bacteria is closely related to *Bacillus* sp. strain x20. The phylogenetic trees were made to analyse the relationship between species. The results of phylogenetic tree were shown in Figure 3.

Based on various literatures, *Bacillus* sp. strain x20 is also a Gram-positive bacterium producing organic acids and phosphatases (Gond *et al*., 2015; Zhao *et al*., 2023). *Bacillus* sp. strain x20 was known to provide beneficial effects to their host plants,

such as promotes seed germination and induces stress resistance (Zhao *et al*., 2023). Organic acids produced by *Bacillus* strain will solute the inorganic phosphorus into a plant-available form, meanwhile the phosphatases will solute the organic phosphorus, therefore the microorganism can promote plant growth (Zhao *et al*., 2023). Further identification of bioactive compound produced by *Bacillus* sp. strain x20 is necessary.

Table 2. Molecular identification of 16S ribosomal RNA gene of DBA2 isolate and their NCBI sequence accession numbers.

No.	Closest relative species based on 16S rRNA gene sequences	GenBank accession number	Base pair length (bp)	Max score	E value	% Similarity
1.	*Bacillus* SD (in Bacteria) strain X20	OP537150	1530	2139	0.0	100.00
2.	*Bacillus velezensis* strain LXS-N2	OP536155	1460	2139	0.0	100.00
3.	*Bacillus velezensis* strain SOTB022	OK047738	1417	2139	0.0	100.00
4.	*Bacillus* SD (in Bacteria) strain HY 110	MZ895449	1453	2139	0.0	100.00
5.	*Bacillus* SO (in Bacteria) strain HY-105	MZ895445	1445	2139	0.0	100.00
6.	*Bacilus* SO (in Bacteria) street LYM4-2	OP493233	1448	2139	0.0	100.00
7.	*Bacillus* SD (in Bacteria) strain Y4-6-1	OP493232	1451	2139	0.0	100.00
8.	*Bacillus* SD (in: Bacteria) strain YS1	OP493231	1449	2139	0.0	100.00
9.	*Bacillus velezensis* strain MRB-10	OP493205	1441	2139	0.0	100.00
10.	*Bacillus velezensis* strain 024A	OP477121	1453	2139	0.0	100.00

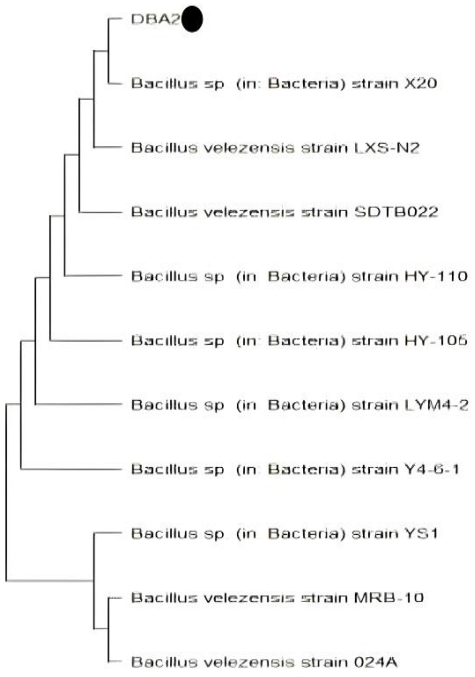

Figure 3. The phylogenetic tree of DBA2 isolate showing evolutionary relationships of endophytic bacteria from *A. cordifolia* (Ten.) Steenis leaves. The black circle indicates the interested isolate. The evolutionary analysis was conducted in MEGAX software analysis (Kumar *et al*., 2018).

CONCLUSION

This study revealed that the endophytic bacteria which closely related to *Bacillus* sp. strain x20 was isolated from the leaves of *Anredera cordifolia* (Ten.) Steenis. These endophytic bacteria produced secondary metabolites that exhibited antibacterial properties against the bacteria of dental caries which were *Streptococcus mutans* and *Lactobacillus acidophilus,* as shown by their inhibition zone. Further study needs to be conducted to investigate and identify the metabolites that may be useful as the antibacterial agents.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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