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Molecular profile and antibacterial properties of endophytic bacteria from avocado (*Perseaamericana Mill.*) leaf against *Staphylococcus epidermidis* ATCC 3223

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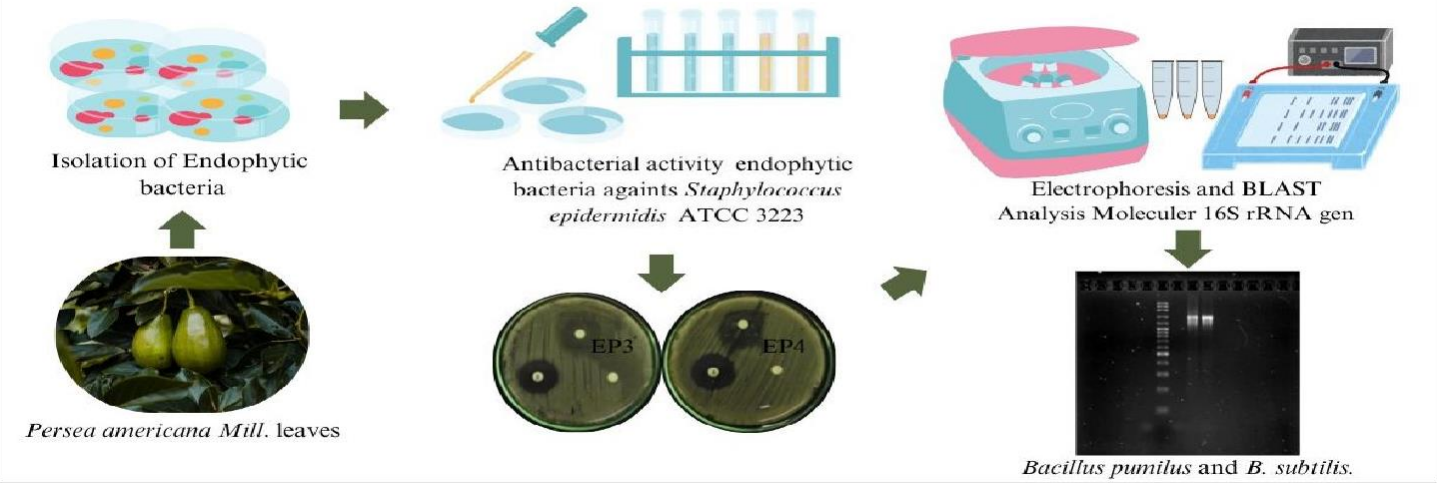
ABSTRACT

Avocado (*Persea americana* Mill.) is a medicinal plant that can grow in sub-tropical areas in Indonesia. The leafs are used in traditional medicine. The interaction between bacteria in plant and plant tissues provides an opportunity for a natural source of antimicrobial substances. The purpose of this study was to determine the characteristics of endophytic bacteria from Avocado (*P. americana* Mill.) leaf and how the activity of endophytic bacteria against *Staphylococcus epidermidis* ATCC 3223. The method used in this research is descriptive by testing the antibacterial activity using the *Kirby Bauer* method. The selected isolates were confirmed molecularly with the 16S rRNA gene. Ten bacterial isolates were found from 3 genera of them *Bacillus*, *Pseudomonas* and *Staphylococcus*. The results of the activity test of ten isolates of endophytic bacteria could againts *S. epidermidis* ATCC 3223 which was characterized by the presence of a clear zone around the disc. The diameter of the inhibition zone varies in the weak, medium, and strong categories. There were two isolates with strong inhibitory activity category, namely EP3 with a diameter of 14.63 mm and EP4 with a diameter of 13.43 mm. Based on BLAST analysis, the selected bacterial isolates EP3 with a percentage of 100% were related to *Bacillus pumilus* strain DDWD with accession number MK537366.1 and *Bacillus stratophericus* strain PMS45 with accession number KK527639.1. Bacterial isolate EP4 with a percentage of 100% closely related to *B. subtilis* strain FC24749 with accession number MK577394.1.



Keywords: *Persea americana* Mill, endophytic bacteria, bacterial inhibition, *Staphylococcus epidermidis*, medicinal plant.

INTRODUCTION

Infectious diseases are a common problem in developing countries. Infectious agents can be viruses, bacteria, fungi, and parasites. Attacks by infectious agents can lead to increased morbidity and mortality. As well as the possibility of the spread of new pathogens and antimicrobial resistance of pathogenic bacteria. One of the bacteria that infect and poses a threat of death is *Staphylococcus epidermidis*. This bacterium is an opportunistic pathogen. This bacterium is also known to cause nosocomial infections of the skin and mucous membranes [1]. *S. epidermidis* is a normal flora in the human body. In addition to the severity of the infected individual, this bacterium can also be spread through direct and indirect transmission, which is an additional problem. So far, the solution to the problem of *S. epidermidis* infection is the use of antibiotics. Based on Jawetz et al. (2005), several antibiotics that are often used to treat *S. epidermidis* are erythromycin, clindamycin, and tetracycline [2, 3].

On the other hand, the treatment of bacterial infections with antibiotics encounters several challenges in adjusting the use of antibiotics themselves. Conditions in the field are often found non-compliance with the consumption of antibiotics which causes antibiotic resistance. This condition makes treatment tend to take a long time, cost a lot, and care is more intensive. If bacteria resistant to first-line antibiotics are not effective then health workers will choose to treat them with second- and third-line antibiotics. The problem could be that alternative antibiotics are not affordable. Cases of antimicrobial resistance in Indonesia have reached 1.27 million deaths due to resistant microbial infections (Republic Indonesia Ministry of Health, 2022). Another option is to utilize available natural materials as a further alternative [4, 5].

Secondary plant metabolites are known to inhibit the growth of *S. epidermidis*. Avocado is a medicinal plant whose leaves have been used by Indonesian people as a medicine for wounds with symptoms of pus infection on the skin. Avocado leaves are pounded and then affixed to the wound. A study by Yunikasari et al. (2016) explained that the inhibition power of the ethanol extract of avocado leaves at a concentration of 2% was 4.46 mm against *S. epidermidis*. The effectiveness of inhibition on *S. epidermidis* at a concentration of 10%, namely 6 mm, was in the moderate category of inhibition. In another study, it was found that the phytochemical compounds that act as antibacterial in avocado leaves are flavonoids, saponins, alkaloids, tannins, polyphenols, quercetin, steroids, and triterpenoids. Previous findings by Chia and Dykes (2010) illustrated a minimum inhibitory concentration of 354.2 µg/ml aqueous extract from epicarp of the Hass cultivar avocado (Guatemalan race) can inhibit *S. epidermidis* ATCC 12228. Apart from leaves, their generative organs can be reused. A study by Sakirigui et al. (2020) found a minimum inhibitory concentration of 0.3125 mg/ml ethanol extract of avocado seeds could inhibit *S. epidermidis* strain T22695. Antibacterial potential and the presence of plant microbiota lead to exploration and development activities for the drug industry in the future and can be continued with the search for potential endophytic bacteria candidates. Attention to the symbiosis of endophytic bacteria without directly exploiting plant commodities provides an opportunity to explore the potential of endophytic bacteria and provide solutions to reduce the number of pathogenic bacteria such as *S. epidermidis* [6, 7].

Tracing the identity of endophytic bacteria can be done simply or with a molecular approach based on the 16S rRNA gene. The molecular-based approach allows the tracing of the DNA base sequences located on the 16S rRNA. The kinship search was carried out on the GenBank database. Based on the description above, it is necessary to study further to explore the potential of endophytic bacteria as a source of new medicinal ingredients [8].

MATERIAL AND METHODS

Isolation and morfologichal characterization of endophytic bacteria

Avocado leaves are washed first using running water to remove dirt. Avocado leaves that have been washed and then cut with a size of 1 x 1 cm (Obidiegwu et al. 2022). Then surface sterilization was carried out in stages, namely distilled water for 1 minute, ethanol 70% for 1.5 minutes, and 1% sodium hypochlorite for 3 minutes. After that, the leaves were washed three times in sterile distilled water. After surface sterilization, the cut leaves were planted in an NA medium. After that, incubated at 37 °C for 24-48 hours. Each colony that grows separately is considered a pure isolate which is used for further examination (Mahlangu. and Dlamini, 2018; Tallei et al. 2020). Morphological identification of endophytic bacteria includes colony shape, margins, elevation, and colony color [9].

Antibacterial activity of endophytic bacteria

The antibacterial activity test was carried out using the Kirby Bauer disc diffusion method and was repeated 3 times. The test bacterial suspension was equalized to the Mcfarland standard 0.5 (1.5×10^8 CFU/mL) [10, 11]. then the suspension was spread over the surface of the Mueller Hinton Agar (MHA) media using a sterile cotton swab (Dayamrita and Paul, 2021). After that, 40 µL of the endophytic bacterial suspension was dripped onto blank paper discs with a diameter of 6 mm. As a positive control, 30 µg of chloramphenicol was used and then incubated at 37 °C for 24 hours [12, 13]. The activity of the inhibition zone was measured by taking into account the category of the clear zone formed. Inhibition with a weak category at a diameter of less than 5 mm, a medium category of 6-10 mm, a strong category 11-20 mm and a very strong category more than 20 mm (Davis and Stout, 1971).

Molecular identification of selected isolates

Subsequent tests were carried out on the two selected isolates. Isolates were selected based on the strongest inhibitory activity against the test bacteria. Further confirmation was carried out molecularly with the 16S rRNA gene. DNA isolation followed the protocol of the Promega Purification DNA kit (2019), namely 1 mL of endophytic bacterial suspension was pipetted into an Eppendorf tube to be centrifuged for 2 minutes at a speed of 13,000-16,000 ×g then the pellet was separated from the supernatant. The pellet was suspended with 480 µL 50 mM EDTA. Then 120 µL of lysozyme and lysostaphin was pipetted and incubated at 37 °C for 30-60 minutes. Then it was centrifuged again at a speed of 13,000-16,000 ×g then the supernatant was separated from the formed pellet. Cell lysis was carried out by adding 600 µL of nucleilysis solution, pipetting gently to mix the solution. Then incubated for 5 minutes at 80 °C, then grown to room temperature. Then add 3 µL of RNAse solution, mix and incubate at 37 °C for 15-60 minutes, until it reaches room temperature [14].

The protein precipitation step was carried out by adding 200 µL of the precipitation solution and then vortexing it, until thoroughly mixed. Then incubated at a cold temperature for 5 minutes. Centrifugation at 13000-16000 ×g was repeated for 3 minutes. The DNA precipitation and rehydration stages were carried out by transferring the centrifuged supernatant into an eppendorf tube containing 600 µL isopropanol at room temperature, then the two were homogenized. Then centrifuged at 13,000-16,000 × g for 3 minutes, the supernatant was mixed into a tube containing 600 µL 70% ethanol at room temperature. Again, centrifugation was carried out for 2 minutes at 13000-16000 ×g and then dried for 10-15 minutes at room temperature. Rehydrate the DNA pellet in 100 µL of the rehydration solution for 60 minutes at 65 °C or overnight at 4 °C [15].

Polymerase Chain Reaction (PCR) amplification

16S rRNA gene amplification using primer pair 27F (5' AGA GTT TGA TCM TGG CTC AG'3) and primer 1492R (5' CGG TTA CCTTGT TAC GAC TT'3) (Yakimov et al. 2001) with a total volume of 25 µL containing 1 µL genomic DNA, 12.5 µL Taq Green Master mix PCR (Thermo Scientific), 1 µL each primer, 9.5 µL ddH2O. PCR amplification was carried out for 30 cycles using the Gene Amp® PCR System 9700 (Applied Biosystem). One cycle consists of three stages, namely denaturation, annealing, and extension. The predenaturation step was carried out for 7 minutes at 94 °C once, the denaturation step at 94 °C for 1 minute, and the primary annealing step at 50 °C for 30 seconds for DNA chain extension (extension) at 72 °C for 30 seconds. 1 minute. In the last cycle, longer chain elongation was carried out at 72 °C for 5 minutes (Liu et al. 2015).

Electrophoresis and Purification of PCR Results

The PCR results immigrated into a 1.5% agarose gel at 100 volts for 40 minutes. DNA marker 1 Kb was used as a marker. The gel was stained using ethidium bromide (10 µg/mL) for 10 minutes, then put in distilled water for 5 minutes to wash the ethidium bromide which was still attached to the gel. Gels containing DNA fragments were visualized using a UV Trans Illuminator and using a Gel Documentation System Digibox Camera [16].

DNA sequencing and phylogenetic tree construction

The PCR results were sequenced using a Genetic Analyzer (Applied Biosystem) and the sequence results were analyzed using bio edit software, DNA star, Clustal X, and MEGA 6. The analysis results were compared with 16S rRNA sequences from GenBank data on the website (http://www.ncbi.nlm. nih.gov) using the BLASTn (Basic Local Alignment Search Tools for nucleotide) software for nearby strains stored in GenBank [17]. Closest relationship analysis based on phylogenetic trees using MEGA 6 with bootstrap 1000 times [18].

RESULTS

Isolation and morphological characterization of entophytic bacteria

Based on the results, ten isolates of endophytic bacteria were isolated from avocado leaves with different morphological characteristics. Morphology of endophytic bacterial isolates from ten isolates were cocci, rhizoid, and irregular shape. The colony edges obtained were whole, filiform, curled, lobate, and wavy. Endophytic bacterial colony elevation includes convex, data, and umbonate. Morphological characteristics of bacterial colony data are presented in Table 1 and Figure 1.

Table 1: Morphological characteristics of bacterial colony isolated

Isolate code	Morphological characteristics of bacterial colony			
	Shape	Margin	Elevation	Color
EP1	cocci	entire	Convex	white cream
EP2	rhizoid	filiform	Flat	cream
EP3	cocci	curled	Convex	yellow
EP4	rhizoid	lobate	Flat	cream
EP5	cocci	entire	Umbonate	cream
EP6	cocci	entire	Convex	cream
EP7	irregular	undulate	Flat	cream
EP8	cocci	entire	Flat	white
EP9	cocci	entire	Convex	cream
EP10	cocci	curled	Convex	white cream-transparent

Note: EP: Endophytic Persea

The results of the Gram staining performed on the ten isolates of endophytic bacteria were found to be Gram positive and Gram negative. The Gram stain test were obtained from the ten isolates, namely isolate EP1, the cell shape was round and included in the Gram positive group. The isolates EP2, EP3, EP4, EP5, EP6, EP7, EP8 and EP10 were rod-shaped and included Gram positive (Figure 2a), while for EP9 the cell forms were rods and Gram negative (Figure 2b).

Antibacterial activity of endophytic bacteria against *Staphylococcus epidermidis*

The results of the activity test of the 10 isolates of endophytic bacteria almost all isolates could inhibit S. epidermidis. Figure to enhance understanding clear zone presented in the Figure 4. The average clear zone obtained from 1 to 14 mm can be categorized as a weaks, mediums, and strongs (Table 3).

Figure 1: Growth single culture of endophytic bacterial isolates from Avocado leave on Nutrient Agar

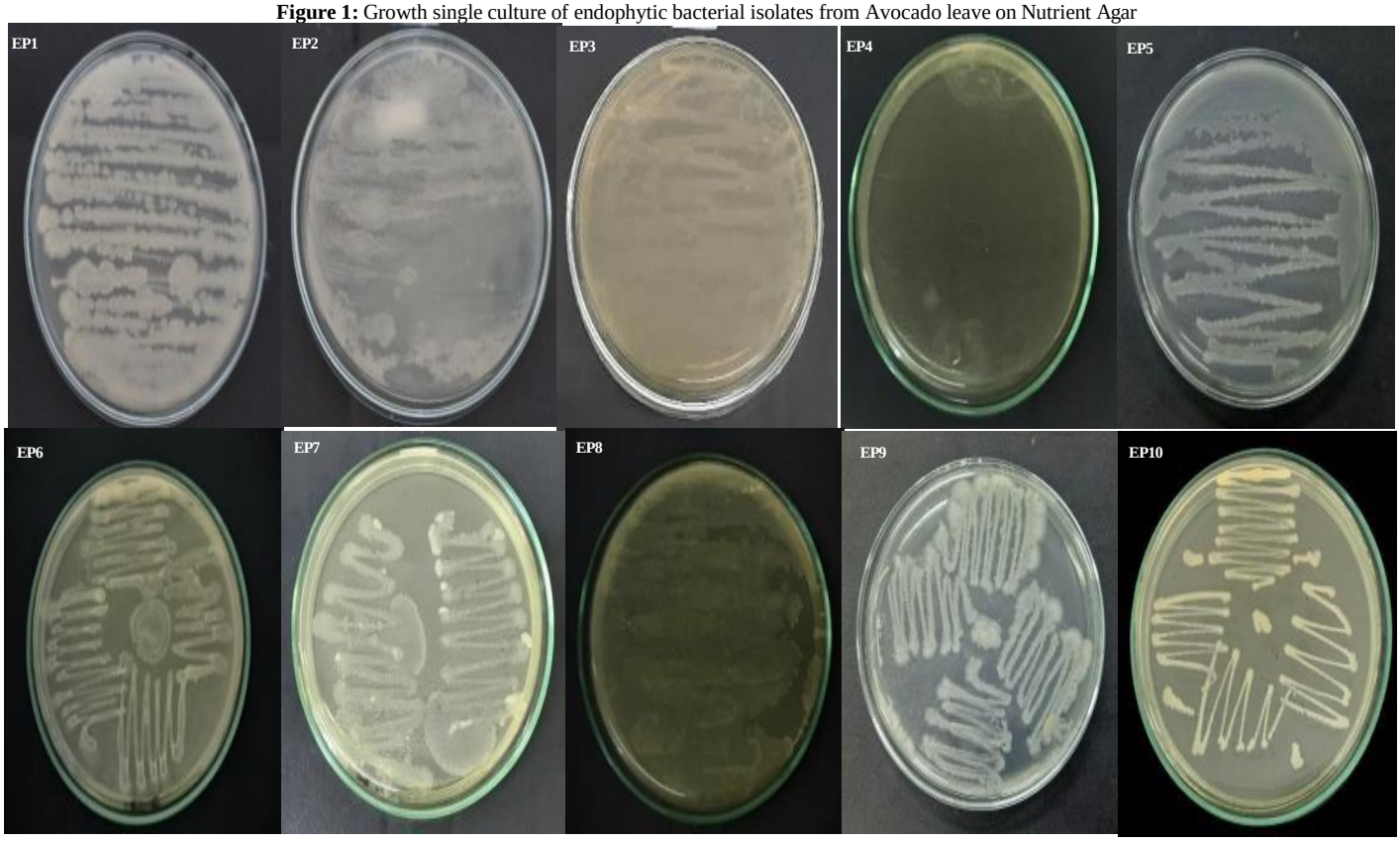


Figure 2. Morphological cells after Gram staining; (a) The isolate EP9 is Gram-negative rod, (b) The isolates EP2, EP3, EP4, EP5, EP6, EP7, EP8 and EP10 are Gram-positive rod and (c) The isolate EP1 is Gram-positive coccus

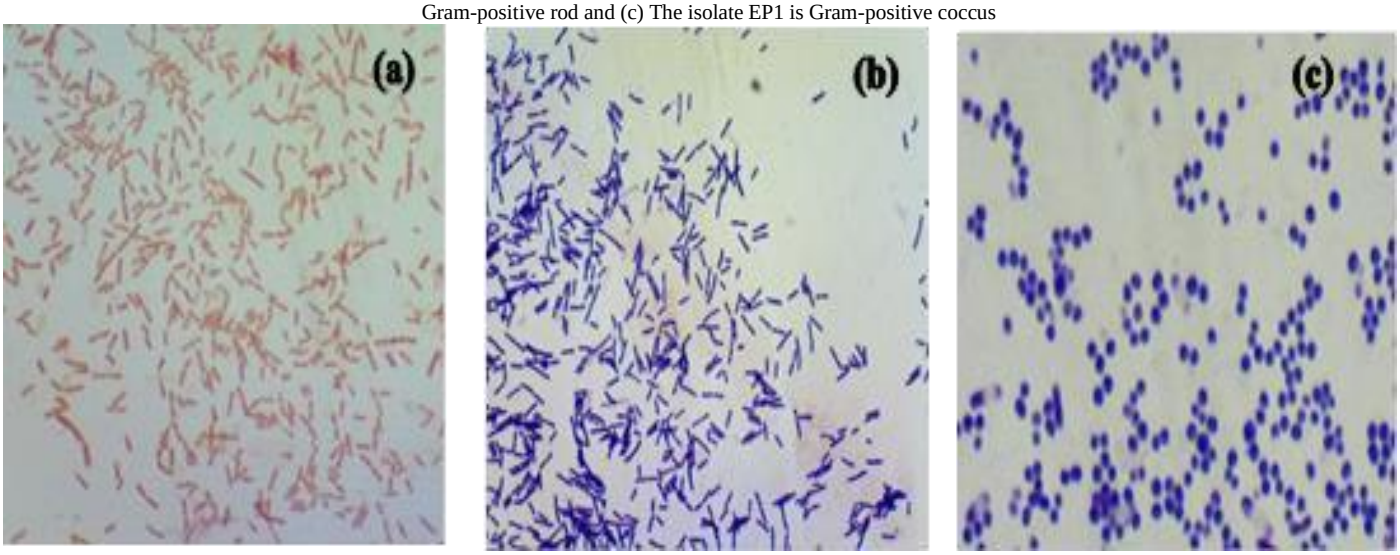


Table 3. Antibacterial activity of endophytic bacteria against *Staphylococcus epidermidis* ATCC 3223

Isolatecode	Average diameter of clear zone (mm)	Inhibition Category
EP1	1.66	Weak
EP2	3.32	Weak
EP3	14.63	Strong
EP4	13.43	Strong
EP5	4.75	Weak
EP6	7.63	Medium
EP7	2.27	Weak
EP8	6.52	Medium
EP9	2.46	Weak
EP10	2.97	Weak

Note: used a positive control of chloramphenicol with an average activity of 15.36-18.98 mm with strong category and aquadest as a negative control there was no inhibitory activity

Figure 4. Clear zone of Avocado leaves endophytic bacteria against *S. epidermidis*

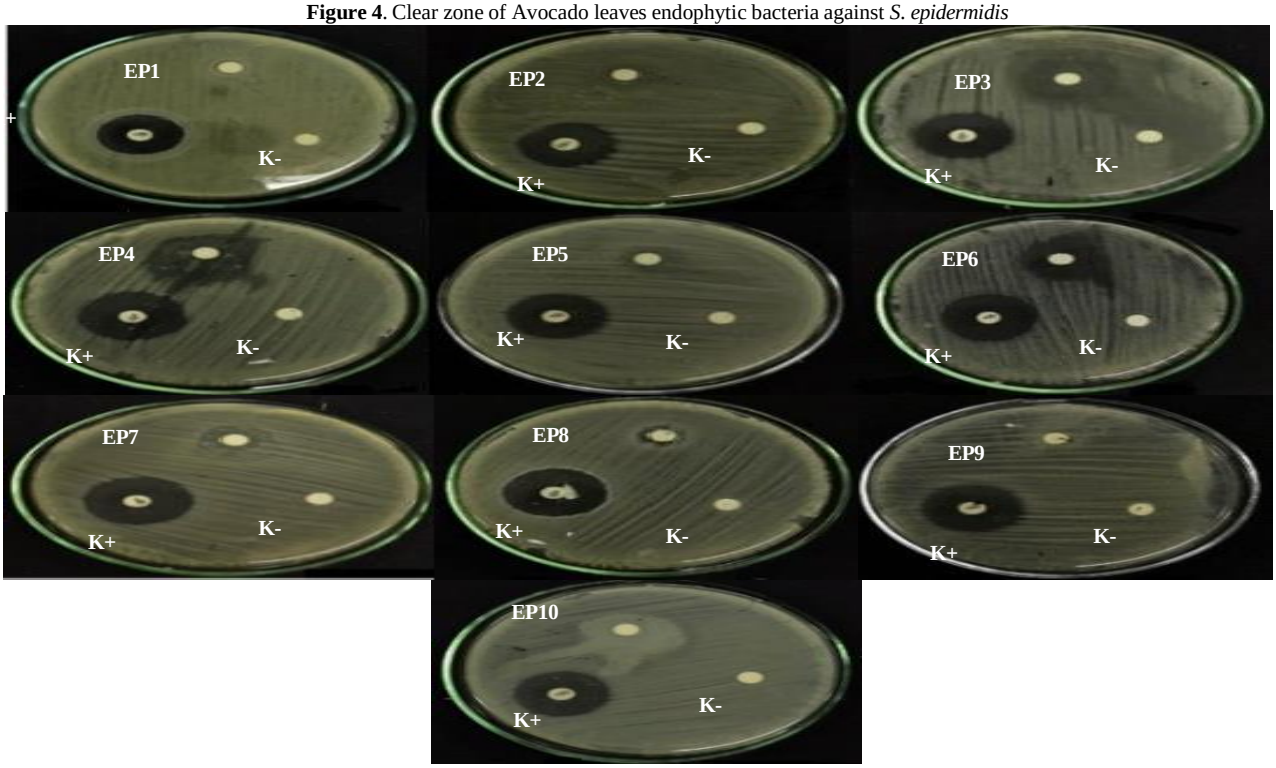
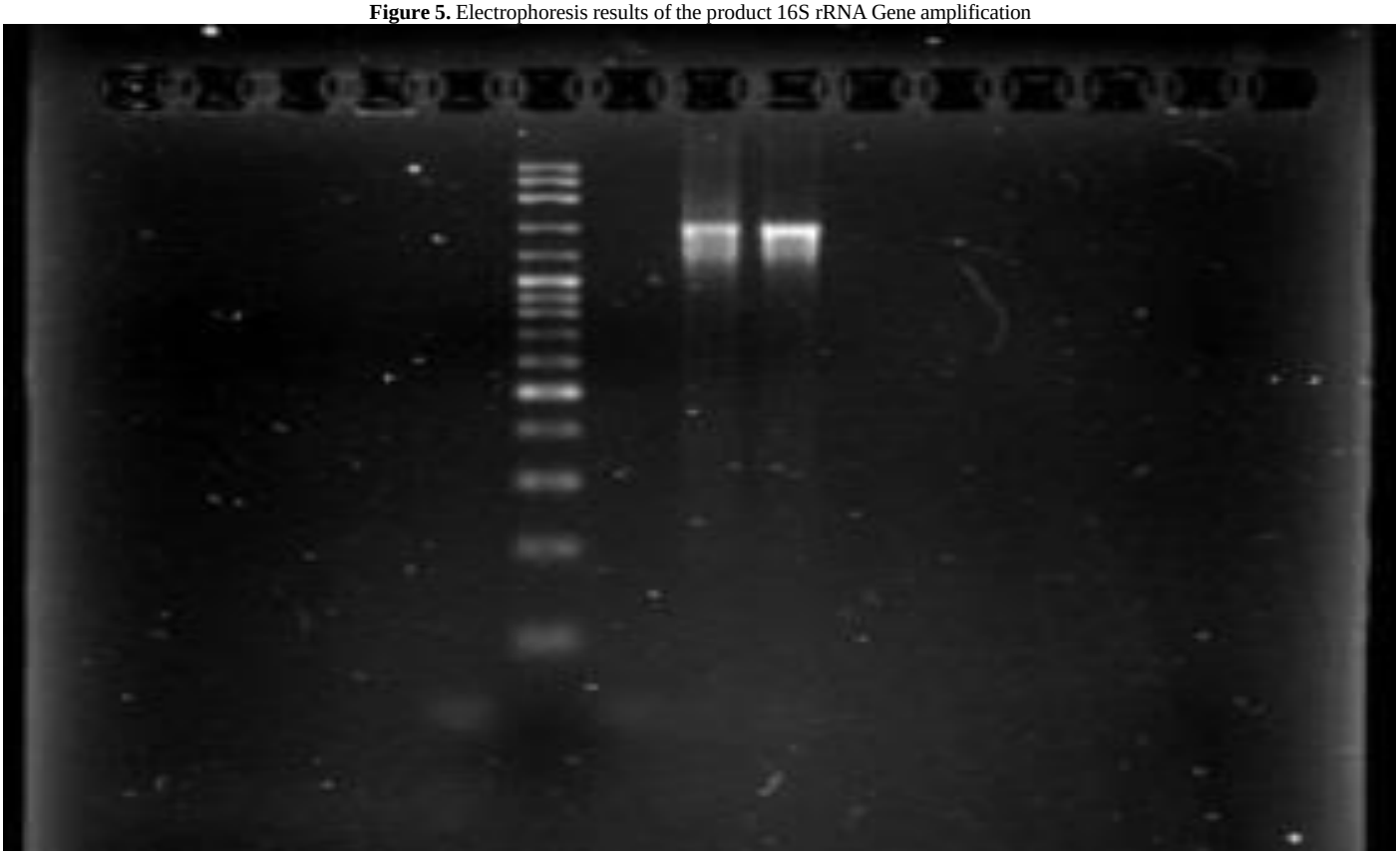


Figure 5. Electrophoresis results of the product 16S rRNA Gene amplification



BLAST analysis of the 16S rRNA gene from isolated endophytic bacteria by match in closely related genera from the NCBI gene database presented in Table 4 and Table 5 for EP3 and EP4 selected isolates.

Phylogenetic tree

The phylogenetic tree established with a bootstrap neighbour-joining method is demonstrated in Figure 6, based on 16S rRNA gene sequencing.

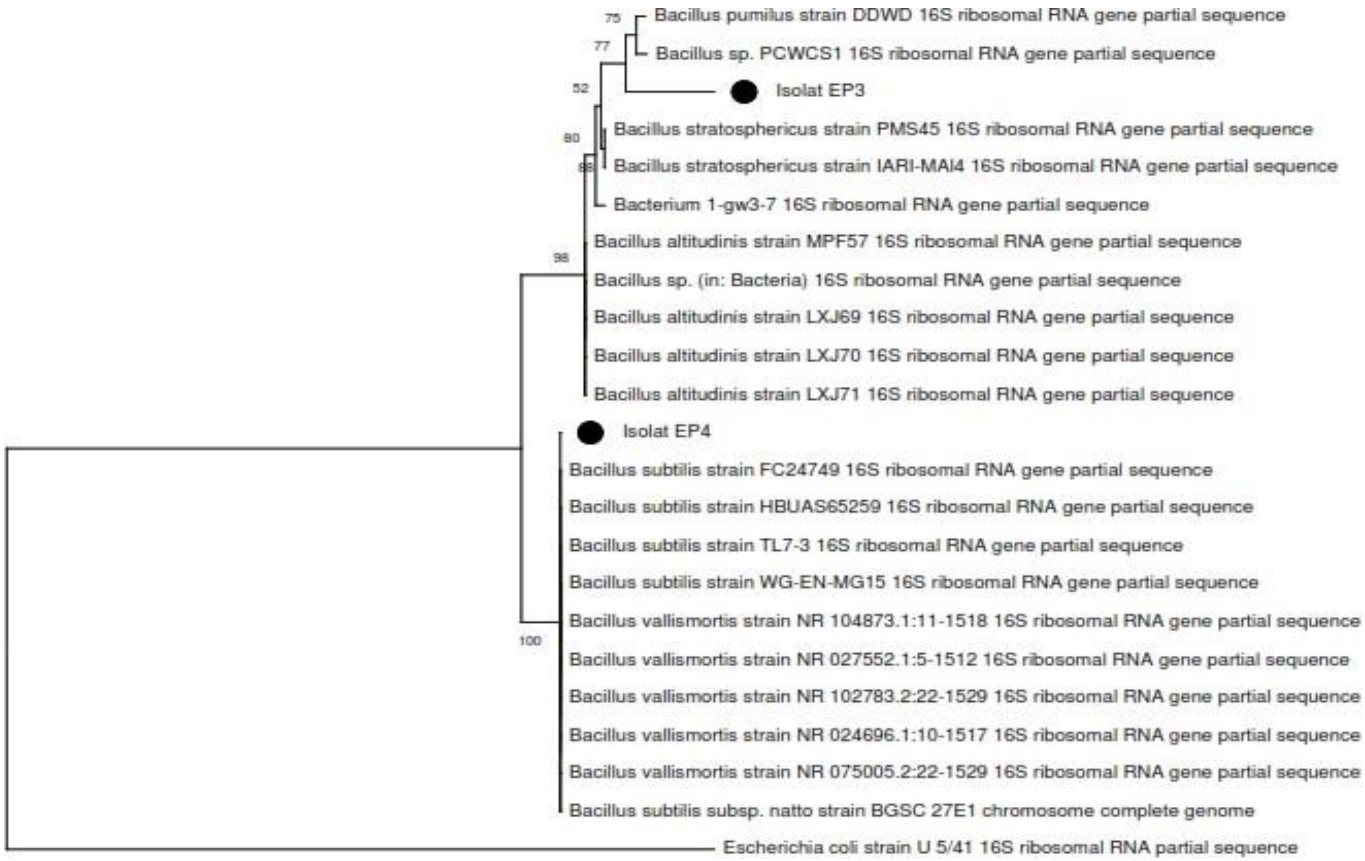
Table 4. BLAST analysis of EP3 Selected Isolate

Description	Scientific name	Max Score	Total score	Query cover	E value	Per. ident	Acc. len	Accession
Bacillus pumilus strain DDWD 16S ribosomal RNA gene, partial sequence	*Bacillus pumilus*	1134	1134	100%	0.0	97.58%	1419	MK537366.1
Bacterium 1-gw3-7 16S ribosomal RNA gene, partial sequence	Bacterium 1-gw3-7	1133	1133	100%	0.0	97.43%	1486	DQ990027.1
Bacillus stratosphericus strain PMS45 16S ribosomal gene, partial sequence	*Bacillus stratosphericus*	1129	1129	100%	0.0	97.43%	1426	KX527639.1
Bacillus stratosphericus strain IARI-MA14 16S ribosomal gene, partial sequence	*Bacillus stratosphericus*	1129	1129	100%	0.0	97.43%	1430	OL413682.1

Table 5: BLAST analysis of EP4 Selected Isolate

Description	Scientific name	Max Score	Total score	Query cover	E value	Per. ident	Acc. Len	Accession
Bacillus subtilis strain FC24749 16S RNA ribosomal gene, partial sequence	*Bacillus subtilis*	1349	1349	100%	0.0	99.46%	1511	MK577394.1
Bacillus subtilis strain HBUAS65259 16S RNA ribosomal gene, partial sequence	*Bacillus subtilis*	1349	1349	100%	0.0	99.46%	1472	ON954560.1
Bacillus subtilis strain TL7-3 16S RNA ribosomal gene, partial sequence	*Bacillus subtilis*	1345	1345	100%	0.0	99.33%	1511	MW820294.1
Bacillus subtilis strain WG-EN-MG15 16S RNA ribosomal gene, partial sequence	*Bacillus subtilis*	1343	1343	100%	0.0	99.33%	1457	OP107357.1

Figure 6: Phylogenetic tree of EP3 and EP4 selected isolates endophytic bacteria from *P. americana* Mill.



DISCUSSION

Plants without disease symptoms can coexist with microorganisms in nature. This group consists of bacteria, fungi, and protists. This community synergizes and forms the plant microbiota. The microbiota provides support for the growth and productivity of its hosts [19, 20].The other benefits of the presence of endophytic microbes are therapeutic effects such as the nature of the host plant that becomes their habitat [21]. One of the medicinal plants with therapeutic effects that have been known to the public through ethnobotanical studies from several previous studies is avocado. Endophytic bacteria from medicinal plants are known to be able to produce bioactive compounds that significantly affect secondary plant secondary metabolites (Ek-ramos et al. 2019). Many drug candidates have been developed from bioactive compounds isolated from medicinal plants [22, 23].

This study obtained ten isolates, more than previous studies by Sarjono et al. (2020) on the bark of the cinnamon plant (*Cinnamomum verum*) 4 isolates were obtained. These plants are

closely related to the *Lauraceae* family. The diversity of endophytic bacteria internally is influenced by characteristics including plant genotype, tissue, plant age, and plant health status [24, 25].

The endophytic bacterial community is influenced by external factors, namely season, soil topographic conditions such as altitude, latitude, longitude, and conditions of nitrogen and phosphorus in the soil [26, 27].

Furthermore, the easiest thing to observe in vitro culture is the difference in colony morphology. This is according to [28, 29]. Explaining that the morphological characters of the colonies can be caused by fluctuations in the environmental conditions of the bacteria. This difference according to Young (2006) determines the interaction between bacterial cells and their environment, including bound to micromolecular traffic, motility, formation of multicellular aggregates, habitat colonization (including symbiosis), predation, and resistance. The structure of the primary macromolecular network in bacterial peptidoglycan determines the shape of the bacteria. Endophytic bacteria spread and carry out cell activities in the cortex tissue [28, 29].

These bacteria are thought to be able to enter plant tissues through access to natural openings such as stomata, lenti cells, and root pores and participate in transport activities in plant tissues. A study by Hakizimana et al. (2011) obtained several genera of endophytic bacteria from avocado roots, namely *Bacillus* sp., *Bacillaceae*, *Lysinibacillus* sp., *Paenibacillus polymyxa*, and *Enterobacter* sp. EP1 and EP9 isolates were suspected to belong to the genus *Staphylococcus* and P*seudomonas*. Based on previous research by [30, 31].

Staphylococcus sp. and *Pseudomonas* sp. were found in the rhizosphere of five plants belonging to the *Lauraceae* family. In another study, the results of isolating the genus *Pseudomonas* from the roots of avocado plants with the rhizosphere as an abiotic factor were obtained [32, 33].

There are several stages for access to the entry of endophytic bacteria into the interior of the plant, starting from the rhizosphere. After the colonization of the rhizosphere, bacteria will adhere to the rhizosphere (i.e. root surface). The next stage of endophytic formation is the attachment of the bacterial cells to the roots [34].

Then the place of attachment of bacteria and subsequent entry of bacteria is in the root apical zone with thin-walled root surface layers such as the cell elongation zone and the root hair zone (passive penetration zone). For active penetration, endophytic bacteria must be well equipped with cellulolytic enzymes that hydrolyze plant ectodermal cells. Endophytic bacteria may remain at the site of entry or move deeper into the spaces between the cortical cells and xylem vessels. The concentration of nutrients available in the xylem decreases along the plant axis. This could explain the fact that the density and population density of endophytic bacteria decreased with distance from the roots and only a few bacteria reached the shoot tops, leaf apoplasts, and reproductive organs, such as flowers and seeds. Bacteria can also enter plants through flowers, fruits, and seeds. However, most of them are known for specific phytopathogens and are not shown for endophytic (non-pathogenic) bacteria (Malfanova, 2013) [35, 36].

Based on BLAST analysis, bacterial isolates chose EP3 with a proportion of 100% related to B. *pumilus* strain DDWD with access number MK537366.1 and *B. stratophericus* strain PMS45 with access number KK527639.1. As for the bacterial isolate EP4 with a proportion of 100% that is closely related to the *B. subtilis* FC24749 strain with access number MK577394.1. *Bacillus* has also been identified from several commercial fruiting plants such as papaya and rambutan [37, 38]. In the study, Nongkhlaw and Joshi (2015) succeeded in identifying *B. siamensis* species from the Krangean plant (*Litsea cubeba (*Lour.) Pers.) Which is related to the *Lauraceae* family and *P. americana* Mill. The genus *Bacillus* is known to be found as an endophyte. *Bacillus* is also a cosmopolitan bacterium with high survival ability. This is supported by the presence of endospores which protect plant tissues from environmental stress (Moat et al. 2002) [39, 40].

CONCLUSION

The conclusion of this study reported that ten isolates from avocado leaves had antibacterial activity against *S. epidermidis* ATCC 3223. Two isolates from the isolates could inhibit the strong category. Based on biochemical testing, the two isolates belong to the genus *Bacillus*. Molecular request confirmation was carried out and a close relationship was found between selected bacterial isolates EP3 with a 100% proportion related to *B. pumilus* strain DDWD with accession number MK537366.1 and *B. stratophericus* strain PMS45 with accession number KK527639.1. As for the bacterial isolate EP4 with a proportion of 100% that is closely related to the *B. subtilis* FC24749 strain with access number MK577394.1. To preserve biological resources, endophytic bacteria can be explored to search for new drugs to alternatively anticipate conditions of pathogenic infection and antibacterial resistance.

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