

Endophytic bacteria associated with *Myristica fragrans*: Improved media, bacterial population, preliminary characterization, and potential as antibacterials

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Abstract. Yunita M, Ohiwal M, Dirks CS, Angkejaya OW, Sukmawati S, Ilsan NA. 2022. Endophytic bacteria associated with *Myristica fragrans*: Improved media, bacterial population, preliminary characterization, and potential as antibacterials. *Biodiversitas* 23: 4047-4054. We have investigated a similar study previously and only obtained 4 isolates that were able to inhibit pathogenic bacteria with a very small inhibition index (1.5 mm-3.4 mm) in NA media. Therefore, the current study was conducted by improving the NA media with the addition of 1% peptone and *Myristica fragrans* Hoult filtrate. The study aimed to evaluate the potential of endophytic bacteria as antibacterials in the modified NA media. Endophytic bacteria were isolated from 5 organs of *M. fragrans* and were grown on Nutrient Agar added with 1% peptone and *M. fragrans* filtrate. The total bacterial population was analyzed by the TPC method. Preliminary characterization consisted of macroscopic and microscopic observations. Antibacterial test was carried out by agar diffusion method. The total population of endophytic bacteria varied for all organs of *M. fragrans*, with the highest population was found in the seeds (1×10^5 CFU/g), while the least was found in the pulp (9×10^3 CFU/g). A total of 10 isolates were selected and preliminary characterization showed that endophytic bacteria had different macroscopic and microscopic characteristics. All isolates were able to inhibit the growth of *Escherichia coli* ATCC-27853 and *Staphylococcus aureus* ATCC-29213 with the largest inhibition zone index was obtained by isolate BJ1 (22.5 mm and 23.8 mm), while the smallest was obtained by isolate TD2 (12.5 mm and 13.6 mm) which were still categorized as strong inhibition. The study concluded that the addition of 1% peptone and *M. fragrans* filtrate in NA media was able to show far better results compared to our previous study and the strong antibacterials can be developed and formulated in the future.

Keywords: Antibacterial, endophytic bacteria, *Escherichia coli*, *Myristica fragrans*, *Staphylococcus aureus*

INTRODUCTION

Endophytic bacteria are microorganisms which whole or part of their life cycle is expended in the healthy tissues of living plants and usually causing no symptoms of disease to their hosts (Anjum and Chandra 2015; Herlina et al. 2017). Endophytic bacteria in the healthy tissues of living plant are a source of promising natural products to be explored in agriculture, industry, and medicine. The importance of the benefits of endophytic bacteria as a source of bioactive compounds has been reported in several previous studies due to their ability to produce novel secondary metabolites such as antibacterial (Ek-Ramos et al. 2019), antifungal (Zhao et al. 2018), antiviral, antioxidant, antitumor, anti-inflammatory, immune-suppressive drugs, and the production of various classes of natural products such as alkaloids, tannins, terpenoids, steroids, lactones, phenolic compounds, lignins, quinones, and chitins (Arunachalam and Gayathri 2010).

Previous studies have examined the dynamics and diversity of endophytic bacteria, characterization and identification, as well as the benefit of bacterial isolates to

promote plant growth and health (Kobayashi and Palumbo 2000) such as nitrogen fixation (Sturz et al. 1997), inducing drought resistance, and controlling herbivores and parasites (Kirchhorf et al. 1997), as well as a source of secondary metabolites that have ability as antibacterial against plant pathogens (Mugiastuti et al. 2020) or even to control pathogenic fungi (Yunita et al. 2016). However, studies on the benefits of endophytic bacterial isolates in health and medicine, such as inhibiting the growth of pathogenic bacteria in humans, need to be further studied. Moreover, endophytic bacteria are able to produce the similar bioactive compounds as their host (Ek-Ramos et al. 2019).

One of the potential hosts for endophytic bacteria is *Myristica fragrans* Hoult or known as an Indonesian medicinal plant which is endemic to the Maluku Islands (Yunita et al. 2022). Besides being used as a spice and traditional medicine for local people, various organs of *M. fragrans* are reported to have high antibacterial potential. Nutmeg seeds were reported to be able to inhibit the growth of gram-positive and gram-negative bacteria (Ibrahim et al. 2013). Interestingly, fruit skins, pulp, and leaves of *M. fragrans* were also reported to be able to

inhibit the growth of *Escherichia coli* (Arrizqiyani et al. 2018). Moreover, *M. fragrans* oil was reported to inhibit *Staphylococcus aureus*, *Staphylococcus epidermis*, *Shigella dysenteriae*, and *Salmonella typhi* (Nurjanah et al. 2017). Therefore, the association of endophytic bacteria with various organs of *M. fragrans* can be an important model to be studied in an effort to develop potential antibacterial against human pathogenic bacteria.

It is known that isolation media is important and crucial in growing endophytic bacteria (Hallmann and Schulz 2006). We have previously isolated endophytic bacteria from several organs of *M. fragrans* including leaves, leaf bones, stems, fruits, and seeds in the NA media (Yunita et al. 2022). The reason behind this study was to clarify the results of our previous study which only obtained 4 endophytic bacterial isolates with weak antibacterial activity (inhibition index of 1.5 mm-3.4 mm). In accordance, Eevers et al. (2015) stated that of the entire population of endophytic bacteria in their natural habitat, only 0.001-1% are culturable. Even after successful isolations, many endophytic bacteria often show reduced regrowth capacity as evidenced by the results of our previous study. Therefore, media modification is sometimes necessary in an effort to get far better results.

The plant filtrate is known to have several nutrients that are similar to the plant tissue itself. A study proves that adding plant extract to the media leads to a significant increase for the diversity and activity of endophytic bacteria (Eevers et al. 2015). Beside plant filtrate, peptone also can be used as medium additives. Although the NA media itself already contained 5 g of peptone, the addition of peptone allowed bacteria to grow better due to a richer media (Heidemann et al. 2000). Therefore, it is necessary

to evaluate and compare the growth and potential of endophytic bacteria associated from *M. fragrans* on modified NA media by adding *M. fragrans* filtrate and 1% peptone. This article will probably be the first report on improved NA media in order to obtain endophytic bacteria with far stronger antibacterial activity.

MATERIALS AND METHODS

Study area

Myristica fragrans Houtt or nutmeg is known as a rich reservoir of bioactive molecules and used in traditional medicines of Asia, Europe, Africa, and America against malaria, cancer, madness, convulsion, skin infection, diarrhea, rheumatism, asthma, cough, cold, as stimulant, tonics, as well as psychotomimetic and antibacterial agents. This plant is generally cultivated around the tropical region (Barman et al. 2021), including Indonesia and particularly Maluku Islands, as high-value commercial spice that are used in global cuisine.

The study was conducted in October-December 2021 at the Health Laboratory Center in Maluku Province, Indonesia. Sampling process of *M. fragrans* was carried out at the nutmeg plantation of Hutumuri Village, South Leitimur Sub-district, Ambon City, Maluku, Indonesia (-3.6653793", 128.2837252"). Hutumuri village is one of the areas in Ambon city where most of the people plant *M. fragrans* from generation to generation with a local wisdom system called *Dusun*, thus this area was very suitable as a sampling site. The Map of sampling site is presented in (Figure 1).

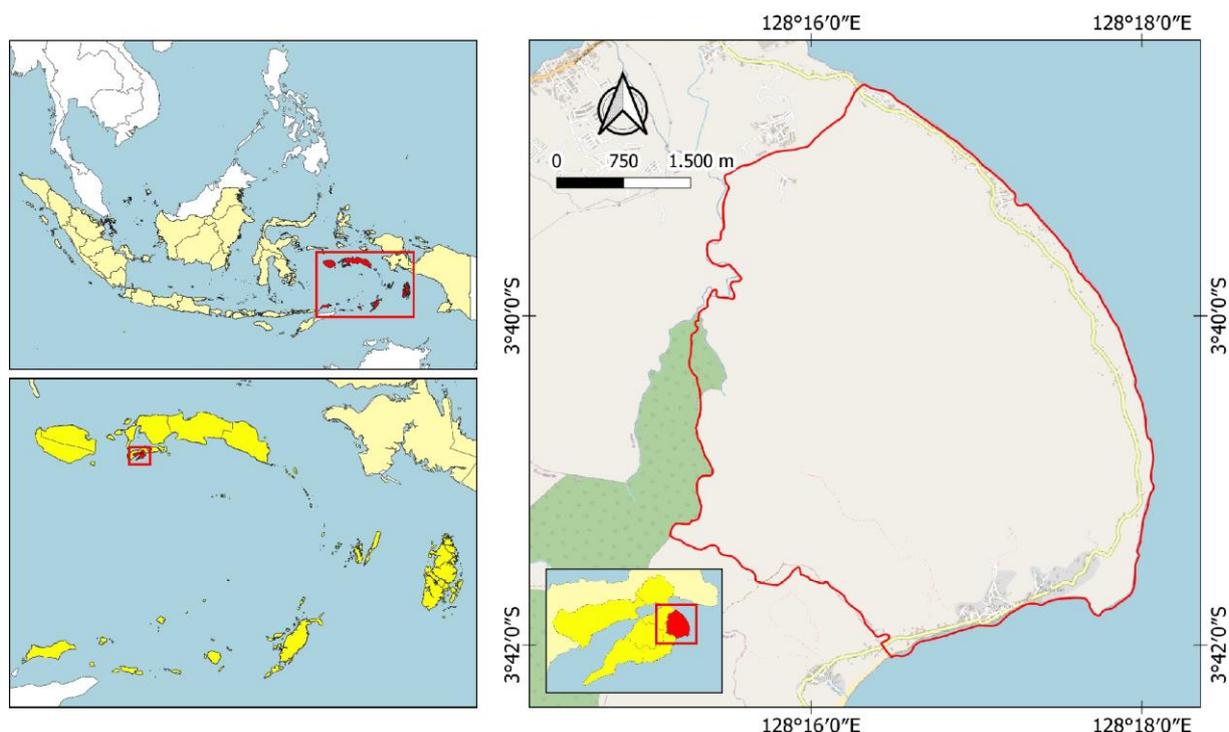


Figure 1. Map of sampling site in nutmeg plantation of Hutumuri Village, South Leitimur Sub-district, Ambon City, Maluku, Indonesia

Sample collection

The sample used in this study was endophytic bacteria isolated from *M. fragrans* Hoult. Plants were sampled randomly using purposive sampling method. Determination of *M. fragrans* was performed in Biology Laboratory of Universitas Pattimura, Maluku, Indonesia. *Myristica fragrans* sampled in this study were planted in local plantation owned by Hutumuri Community, Ambon Indonesia. The plant sample consisted of several organs, namely leaves, leaf bones, stems, pulp, and seeds. Fresh, healthy, and mature organs of *M. fragrans* with the age around 10 years were collected and surface sterilization was carried out by soaking the sample in 70% ethanol for 5 minutes and 2% sodium hypochlorite for 2 minutes (Anjum and Chandra 2015) then rinsed using sterile distilled water to remove contaminants and impurities adhering to the sample surface.

Preparation of *Myristica fragrans* filtrate

Each organ of the *M. fragrans* that had been sampled was cut to a size of 1-3 cm and separated according to the organ of the plant. Furthermore, the five organ of *M. fragrans* were air-dried for 1 week and further dried in an oven at 40°C for 24 hours and then blended into powder and sieved using a 65 mesh sieve. A total of 250 g of each organ powder was macerated with 1000 ml ethanol for 24 hours. Furthermore, the suspension was filtered to obtain the filtrate and residue. The residue was discarded while the filtrate was stored and then 100 ml of the filtrate was poured into 1 L of NA media as a growth media for endophytic bacteria (Modified from Bachri 2013). These filtrates were used as a media additive according to each organ together with 1% peptone for isolation stage, while combination of all filtrate was used for the stages of purification, characterization, and evaluation of antibacterial activity of endophytic bacteria.

Media preparation

The media used in this study was Nutrient agar (NA) with several modifications. NA and peptone were weighed as much as 20 g and 5 g respectively using an analytical balance then put into an erlenmeyer and then dissolved by adding 1000 ml of distilled water and covered with aluminum foil then heated to boiling using a hot plate and homogenized using a magnetic stirrer. A total of 100 ml of *M. fragrans* filtrate that had been made in the previous stage was then mixed into the NA media. A total of 1% peptone was also added to the NA media. The media was sterilized using an autoclave at a temperature of 121°C for 15 minutes and a pressure of 2 atm was poured. The media was then poured into sterile petri dishes as needed (Modified from Dzatam et al. 2018).

Isolation of endophytic bacteria

Isolation of endophytic bacteria was initiated by separating the organs of *M. fragrans* and weighing 1 g of each healthy plant organ and surface sterilization of plant

samples was carried out using 70% ethanol for 5 minutes, 2% sodium hypochlorite for 2 minutes, and 70% alcohol for 30 seconds. Subsequently, the plant tissue was destroyed by grinding the five organs of *M. fragrans* including leaves, leaf bones, stems, pulp and seeds, so that the endophytic bacteria could come out of the plant tissue and grow as well as colonize on the media. The five organs of *M. fragrans* that have been weighed are presented in (Figure 2).

The isolation process was performed by serial dilutions. In each treatment, five organs of the *M. fragrans* were ground using a mortar and then put in a tube containing 9 ml of 0.85% NaCl and 1 ml of the suspension was taken and diluted to a dilution factor of 10^4 . The suspension was then shaken using a vortex for 30 seconds. A total of 0.1 ml of suspension was taken from each second tube with a dilution factor of 10^{-2} and then inoculated using the spread method onto modified NA media containing *M. fragrans* filtrate, 1% peptone, and nystatin antibiotic to inhibit fungal growth. The media was then incubated at a room temperature in the dark and observed every day until there was colony growth (Modified from Yunita et al. 2022).

Analysis of total plate count

Bacterial colonies growing on the media after the incubation period were counted. The number of colonies obtained was analyzed by the Total Plate Count (TPC) method using the colony counter. The TPC of endophytic bacteria was counted using the formula: Number of bacterial colonies x 1/dilution factor (Sukmawati and Hardianti 2018).

Purification of endophytic bacterial isolates

Bacterial purification is a process carried out to obtain pure isolates. Bacterial purification was carried out by taking single bacterial colonies using a sterile ose needle and then scratched using quadrant scratches on the new modified media. The media was incubated for 2 x 24 hours at a temperature of 30°C. The single colony that was formed and separated from other bacterial colonies that grew around the scratch was a pure isolate that would be further tested (Yunita et al. 2022).

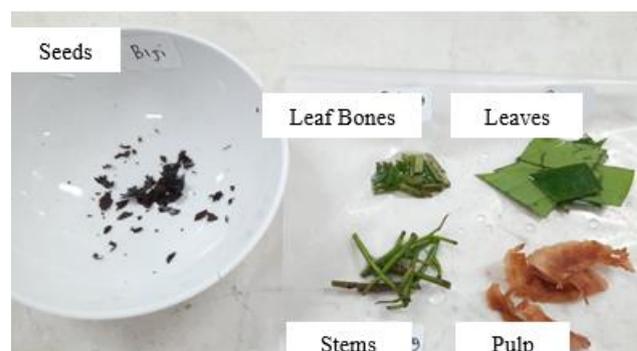


Figure 2. Appearance of five *Myristica fragrans* organs

Preliminary characterization of endophytic bacteria

Preliminary characterization refers to Bergey's Manual by observing the macroscopic characters of endophytic bacterial colonies growing on the modified NA media based on colony color, colony shape, colony margin, colony elevation, and colony consistency, while microscopic characterization was carried out by gram staining on endophytic bacterial colonies to determine the shape of cells and groups of bacteria (Bergey 1994). Bacterial colonies were incubated in an incubator with temperature of 30°C.

Reculturing endophytic and pathogenic bacteria

Pure isolates of endophytic bacteria were recultured on agar slant by aseptically scraping a rose needle containing endophytic bacteria. The test tube was closed and incubated for 24 hours at 37°C in an incubator. While Pathogenic bacterial isolates were obtained and confirmed by the Health Laboratory Center in Maluku Province, Indonesia. The same method of reculturing was also carried out on pathogenic bacterial isolates including *Escherichia coli* ATCC-27853 and *Staphylococcus aureus* ATCC-29213 (Yunita et al. 2022).

Evaluation of antibacterial activity

Evaluation of antibacterial activity was carried out using disk diffusion method with a little modification (Yunita et al. 2021). Inhibition zone Index was measured by swabbing liquid culture of *E. coli* ATCC-27853 and *S. aureus* ATCC-29213 as pathogenic bacteria to cover the NA media in a petri dish while the endophytic bacterial isolates were spotted and positioned in the middle of the media and incubated in an incubator at 30°C for \pm 3 days. Erythromycin was used as positive control and sterile aquadest as a negative control. The clear zone formed was measured using a ruler. Inhibition zone index was calculated by dividing clear zone diameter with bacterial colony diameter, while the inhibition activity were categorized as follows: the diameter of clear zone >20 mm was categorized as very strong, diameter of 0-20 mm was categorized as strong, diameter of 5-10 mm was categorized as moderate, and diameter <5 mm was categorized as weak (Davis and Stout 1971).

Data analysis

The data obtained were presented with tabulations and figures and analyzed descriptively qualitatively for preliminary characterization of endophytic bacteria and the inhibition of endophytic bacterial isolates against *E. coli* ATCC-27853 while the data of total bacterial population

and evaluation of antibacterial activity were analyzed using Ms. Excel Program.

RESULTS AND DISCUSSION

In this study, NA media was modified with the addition of filtrate of five organs of *M. fragrans* as a growth factor for endophytic bacteria because it is known that endophytic bacteria live and colonize in plant tissues so that the addition of plant filtrate to bacterial growth media triggers bacteria to grow and produce better secondary metabolites. Thus, the characteristics of the *M. fragrans* filtrate are considered important to be presented in this study. The visual characteristics of the *M. fragrans* filtrate are shown in Table 1.

Analysis of Total Plate Count (TPC) showed that the total population of endophytic bacteria isolated from *M. fragrans* leaves, leaf bones, stems, pulp, and seeds was varied with the highest bacterial population was obtained in seeds and the lowest was in pulp. The results can be seen in (Figure 3).

Generally, the total population of endophytic bacteria found in a fresh and healthy plant ranges from 10^2 - 10^4 per gram (Kobayashi and Palumbo 2000). Figure 3 showed that endophytic bacteria were present in every organ of *M. fragrans* with a different total population in each organ. The highest total population of endophytic bacteria was found in *M. fragrans* seeds, namely 1×10^5 CFU/g, followed by stems at 6.8×10^4 CFU/g, leaf bones of 3.7×10^4 CFU/g, leaves of 1×10^4 CFU/g, and pulp of 9×10^3 CFU/g. The results of this study showed far higher results compared to our previous study which showed the highest total population of endophytic bacteria, namely 7.5×10^4 which was also found in nutmeg seeds (Yunita et al. 2022).

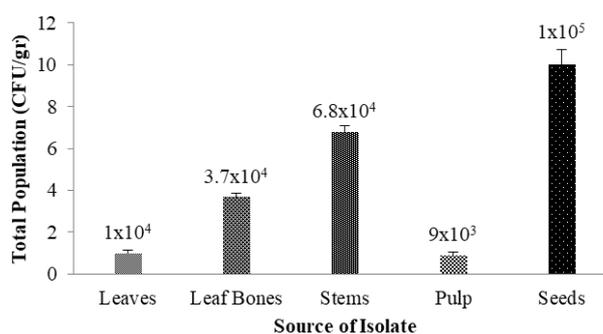


Figure 3. Total population of endophytic bacteria associated with *Myristica fragrans*

Table 1. Visual characteristics of *Myristica fragrans* filtrate

Filtrate	Color	Odor	Appearance	Consistency
Leaves	Dark green	Not intense	Liquid	Watery
Leaf bones	Green	Slightly intense	Liquid	Watery
Stems	Brown	Not intense	Liquid	Watery
Pulp	Brown	Very intense	Viscose	Sticky
Seeds	Light brown	Intense	Liquid	Watery

This is presumably due to the addition of *M. fragrans* filtrate to the isolation media in this study. For comparison, several other studies reported that plant organs had a lower total population than seeds in this study. Glassner et al. (2015) found the average number of culturable bacteria in the cucurbit fruits was $1.38 \pm 0.13 \log_{10}$ CFU g^{-1} ($P < 0.05$), which was still far smaller than the total population of endophytic bacteria found in this study with the addition of fruit filtrate of *M. fragrans*. Even though, a significant difference in the total population of endophytic bacteria is detected between the seed cavity surrounding the seeds and the fruit flesh or pulp. The tissue enveloping the seeds known to contain a higher number of CFU per gram sample, while the fruit flesh or pulp was only poorly colonized. This indicates that, most of the culturable endophytic bacteria isolated from fruits were located in the seed cavity in on and around the seeds. Therefore, the addition of all-organ *M. fragrans* filtrate in this study clearly increased the number of bacterial populations.

In addition to containing active compounds, *M. fragrans* filtrate is also known to contain various vitamins and minerals. *M. fragrans*, especially the seeds, contain high levels of Mn, Mg, Na, Fe, Cr, Ca, and K. In addition, *M. fragrans* seeds were also reported to contain high amounts of vitamins such as Vitamin B1 5.63%, Vitamin C 9.88%, vitamin E 5.50% and other vitamins in smaller amounts (Nkwocha et al. 2019). Vitamins and minerals are ones of the important growth factors that allow good growth of endophytic bacteria and in turn also affect the total population. Our results are in accordance with research conducted by Tan et al. (2013) which showed that the highest total bacterial population was also found in *M. fragrans* seeds, as well as the highest antioxidant content including phenol and ascorbic acid was found in nutmeg seeds compared to other parts such as skin, pulp, and mace.

The isolates obtained were then purified in modified Nutrient Agar (NA) media. This study obtained 10 isolates of endophytic bacteria that varied according to their characteristics. The isolates were coded D1, D2, TD1, TD2, B1, B2, DB1, DB2, BJ1, and BJ2. Preliminary characterization was carried out on pure isolates of endophytic bacteria macroscopically and microscopically (Figure 4).

The results of preliminary characterization performed on ten isolates of endophytic bacteria showed macroscopically different colony morphology (Table 2). Macroscopic characterization was carried out by observing colony features under a microscope including colony shape, colony margins, colony elevation, colony color, and colony consistency, while microscopic characterization was carried out by Gram staining to determine the shape of cells and Gram groups of bacteria (Figure 5; Table 2).

Generally, endophytic bacterial colonies that live on a single host plant consist of several genera and species. This is evidenced by the various preliminary characterization results obtained in this study. The results of macroscopic observations showed varied characteristics. These variations are influenced by environmental changes, plant age and certain growth factors caused by unfavorable environmental influences, involution, nutrient availability, and environmental temperature (Hallmann 2001). Meanwhile, microscopic observations showed that isolate D1, D2, TD2, DB2, and BJ2 were gram-positive bacteria, while isolate TD2, B1, B2, DB1, and BJ1 were gram-negative bacteria. Gram-positive bacteria retain the purple primary iodine dye complex resulting in a violet-colored cell, while gram-negative bacteria experience decolorization triggered by ethanol solution and absorb safranin resulting in a red color (Thairu et al. 2014).

Ten endophytic bacterial isolates were tested for their ability to inhibit the growth of *E. coli* ATCC-27853 and *S. aureus* ATCC-29213. The results showed that all isolates had strong antibacterial activity against the two pathogenic bacteria. This is indicated by the formation of a clear zone around the endophytic bacterial colonies (Figure 6). Among the 10 isolates, isolate BJ1 associated with *M. fragrans* seeds had inhibition zone indexes of 22.5 and 23.8 against *E. coli* ATCC-27853 and *S. aureus* ATCC-29213., respectively, which were almost the same as the inhibition index obtained by erythromycin (as a positive control) used in this study. While the smallest inhibition zone index was found in isolate TD1 with inhibition zone indexes of 12.5 mm and 13.6 respectively against *E. coli* ATCC-27853 and *S. aureus* ATCC-29213, which were still bactericidal (Table 3).

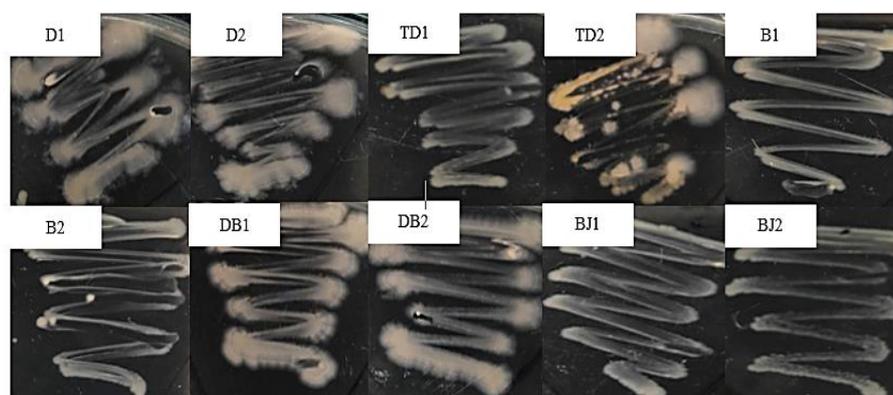


Figure 4. Appearance of endophytic bacterial isolates on improved NA media with the addition of 1% peptone and *Myristica fragrans* filtrate

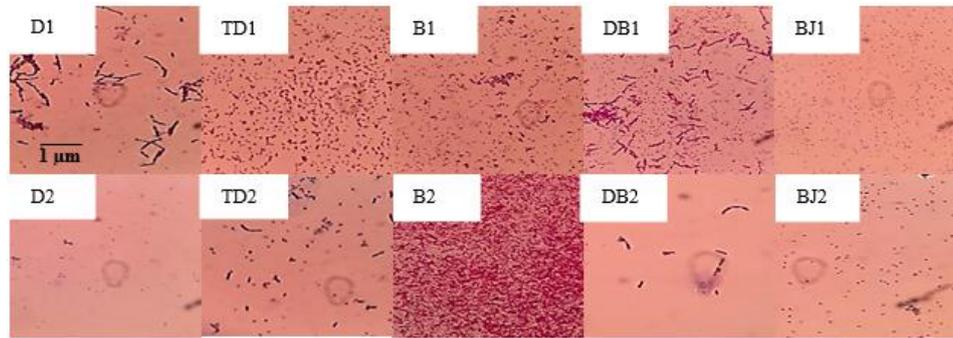


Figure 5. Results of Gram staining of endophytic bacteria under a microscope with 1000x magnification

Table 2. Results of macroscopic and microscopic characterization of endophytic bacterial colonies

Isolate	Colony shape	Colony elevation	Colony margin	Colony color	Consistency	Gram staining	Cell shape
D1	Filamentous	Umbonate	Filamentous	Beige	Creamy	+ve (purple)	Streptobacil
D2	Filamentous	Umbonate	Filamentous	White	Mucoid	+ve (purple)	Monococcus
TD1	Circular	Flat	Entire	White	Creamy	-ve (pink)	Streptococcus
TD2	Irregular	Umbonate	Undulate	Yellow	Creamy	+ve (purple)	Streptobacil
B1	Circular	Flat	Entire	White	Creamy	-ve (pink)	Sarcina
B2	Circular	Flat	Entire	White	Mucoid	-ve (pink)	Streptobacil
DB1	Irregular	Umbonate	Lobate	White	Creamy	-ve (pink)	Streptobacil
DB2	Filamentous	Umbonate	Lobate	Beige	Mucoid	+ve (purple)	Streptobacil
BJ1	Circular	Flat	Entire	White	Creamy	-ve (pink)	Diplococcus
BJ2	Irregular	Raised	Undulate	White	Mucoid	+ve (purple)	Monococcus

Table 3. Inhibition index of endophytic bacteria on the growth of *Escherichia coli* and *Staphylococcus aureus* ATCC-29213.

Isolate	Source of isolate	Inhibition zone index (mm)		Inhibition category
		<i>E. coli</i> ATCC-27853	<i>S. aureus</i> ATCC-29213	
D1	Leaves	20.50±0.42	21.25±0.31	Very strong
D2	Leaves	20.75±0.28	20.15±0.24	Very strong
TD1	Leaf bones	14.75±0.28	13.75±0.18	Strong
TD2	Leaf bones	12.50±0.14	13.60±0.20	Strong
B1	Stems	17.75±0.28	15.90±1.26	Strong
B2	Stems	18.00±1.41	19.20±1.00	Strong
DB1	Pulp	21.00±0.21	22.15±0.15	Very strong
DB2	Pulp	19.00±0.14	17.95±0.10	Strong
BJ1	Seeds	22.50±0.07	23.80±0.05	Very strong
BJ2	Seeds	17.05±0.21	16.75±0.15	Strong
Positive control	Erythromycin	24.00±0.56	24.00±0.56	Very strong
Negative control	Sterile aquadest	0.00 ± 0.00	0.00 ± 0.00	-

Notes: * Values ± indicates the standard deviation which are averages of replications, nutrient agar added with plant filtrate and peptone were used as growth media in the assay

The difference in the diameter of the clear zone was due to different species and the ability of antibacterial compounds produced by each endophytic bacteria. The inhibition zone is formed due to the contact between bioactive compounds produced by endophytic bacteria and the cell walls of pathogenic bacteria, thereby inhibiting cell wall synthesis and disrupting the permeability of bacterial cell membranes. This process triggers cell damage and in turn inhibits the growth of gram-positive and gram-negative bacteria (Arunachalam and Gayathri 2010), as shown in this study.

A study conducted by Pangabea et al. (2019) found that nutmeg seeds contain alkaloids, flavonoids, steroids, saponins, tannins and phenolics which have antibacterial properties. Another study reported that the ability to inhibit the growth of pathogenic bacteria was triggered by the secretion of bioactive substances such as malabaricone and rosmarinic acid (Venkatesh et al. 2019). In addition, a study conducted by Susilowati et al. (2021) also showed that endophytic bacteria isolated from the nutmeg plant also produced chitinase enzymes which not only inhibited the growth of pathogenic bacteria but also inhibited the growth of pathogenic fungi.

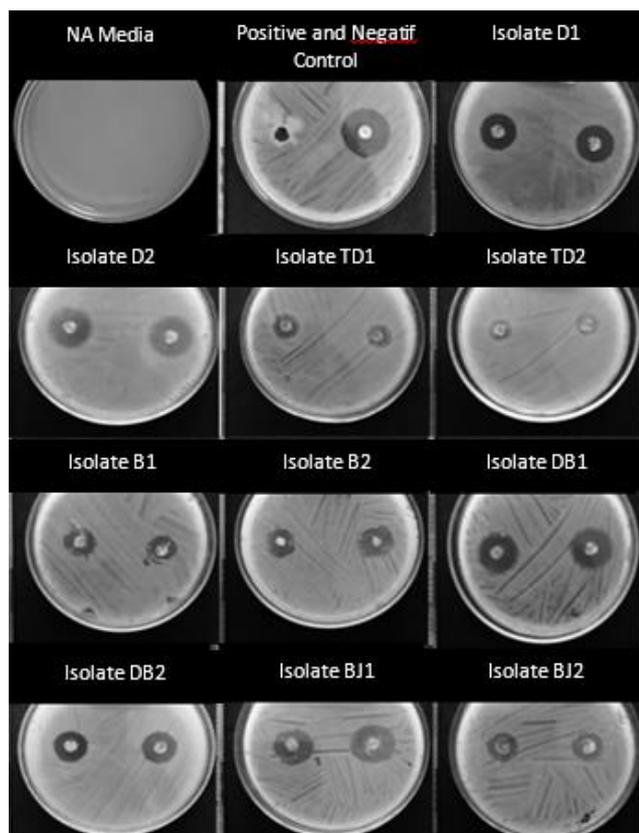


Figure 6. Inhibition zone formed by endophytic bacterial isolates against pathogenic bacteria in the NA media added with 1% peptone and *Myristica fragrans* filtrate

Compared with our previous study which only obtained 4 isolates of endophytic bacteria with weak antibacterial activity of 5 mm-3.4 mm (Yunita et al. 2022), the results of current study showed a far greater antibacterial activity. This is presumably because the growth media used in our previous study was NA alone without the addition of peptone and *M. fragrans* filtrate as we conducted in our current study. Although the NA media itself already contained 5 g of peptone, the addition of peptone allowed bacteria to grow better due to a richer media. According to Heidemann et al. (2000), peptone acts as an additional nutrient in bacterial growth media, especially in samples with low dilution levels, and the specific productivity of bacteria may increase up to 20-30% compared to peptone-free media. Therefore, peptone can be considered as a media additive if the targeted bacteria are difficult to grow in basal media.

Besides peptone, *M. fragrans* filtrate was also used as a media additive in this study. Some endophytic bacteria can grow on artificial media without plant tissue, yet some may be obligate. It is assumed that the plant filtrate can function as a growth stimulator for endophytic bacteria. Endophytic bacteria generally live and colonize in the tissues of roots, seeds, flowers, stems and leaves of a plant (Qin et al. 2009), so that plant organs can also be used as a filtrate to be added to the growth media.

In conclusion, the current study has successfully obtained 10 isolates of endophytic bacteria from five different organs of *M. fragrans* that were able to inhibit the growth of pathogenic bacteria including *Escherichia coli* E. coli ATCC-27853 and *Staphylococcus aureus* ATCC-29213 on the improved media with very strong inhibition. Even the inhibition index resulted by several endophytic bacterial isolates was almost the same as the clear zone obtained by antibiotic as the positive control. Improved media with the addition of plant filtrate and 1% peptone can be a reference and solution for similar studies that have difficulty growing endophytic bacteria in artificial media. Isolation of endophytic bacteria as antagonists is the first step in antibacterial development process. The further studies will help in developing all isolates as strong and very potential antibacterials against variety of human pathogenic bacteria so that a combination of drugs can be formulated in the future.

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REFERENCES

- Anjum N, Chandra R. 2015. Endophytic bacteria: optimization of isolation procedure from various medicinal plants and their preliminary characterization. *Asian J Pharm Clin Res* (4): 233-238.
- Arrizqiyani T, Sumiati S, Meliansyah M. 2018. Aktivitas antibakteri daging buah dan daun pala (*Myristica fragrans*) terhadap *Escherichia coli*. *J Keset Vokasional* 4 (2): 291-294. DOI: 10.30602/jvk.v4i2.119. [Indonesian]
- Arunachalam C, Gayathri P. 2010. Studies on bioprospecting of endophytic bacteria from the medicinal plant of *Andrographis paniculata* for their antimicrobial activity and antibiotic susceptibility pattern. *Intl J of Curr Pharm Res* 2 (4): 63-68.
- Bachri MS. 2013. Sub chronic effect of ethanol extract of nutmeg (*Myristica fragrans* Houtt) seed in rat kidney. *Proceeding of International Safety Management of Central Cytotoxic Reconstitution* 165-199. Indonesia, 25 May 2013.
- Barman R, Bora PK, Saikia J, Kemprai P, Saikia SP, Haldar S, Banik D. 2021. Nutmegs and wild nutmegs: An update on ethnomedicines, phytochemicals, pharmacology, and toxicity of the Myristicaceae species. *Phytotherapy Res* 35 (9): 4632-4659. DOI: 10.1002/ptr.7098.
- Bergey DH. 1994. *Bergey's manual of determinative bacteriology*. Lippincott Williams & Wilkins, Baltimore.
- Davis WW, Stout TR. 1971. Disc plate methods of microbiological antibiotic assay. *Microbiol* 22 (1): 659-665. DOI: 10.1128/am.22.4.659-665.1971.
- Dzotam JK, Simo IK, Bitchagno G, Celik I, Sandjo LP, Tane P, Kuete V. 2018. In vitro antibacterial and antibiotic modifying activity of crude extract, fractions and 3',4',7-trihydroxyflavone from *Myristica fragrans* Houtt against MDR Gram-negative enteric bacteria. *Complement Altern Med* 18 (1): 1-9. DOI: 10.1186/s12906-018-2084-1.
- Everes N, Gielen M, Sánchez-López A, Jaspers S, White JC, Vangronsveld J, Weyens N. 2015. Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to

- nutrient media. *Microb Biotechnol* 8 (4): 707-715. DOI: 10.1111/1751-7915.12291.
- Ek-Ramos MJ, Gomez-Flores R, Orozco-Flores AA, Rodríguez-Padilla C, González-Ochoa G, Tamez-Guerra P. 2019. Bioactive products from plant-endophytic Gram-positive bacteria. *Front Microbiol* 10 (1): 463. DOI: 10.3389/fmicb.2019.00463.
- Glassner H, Zchori-Fein E, Compant S, Sessitsch A, Katzir N, Portnoy V, Yaron S. 2015. Characterization of endophytic bacteria from cucurbit fruits with potential benefits to agriculture in melons (*Cucumis melo* L.). *FEMS Microbiol Ecol* 91 (7). DOI: 10.1093/femsec/fiv074.
- Hallmann J. 2001. Plant interactions with endophytic bacteria. Biotic interactions in plant-pathogen associations. *Br Soc Plant Pathol* 87-119. DOI: 10.1079/9780851995120.0087.
- Hallmann J, Berg G, Schulz B. 2006. Isolation procedures for endophytic microorganisms. In: *Microbial root endophytes*. Springer, Berlin, Heidelberg. DOI: 10.1007/3-540-33526-9_17.
- Heidemann R, Zhang C Qi H, Larrick Rule J, Rozales C, Park S, Naveh D. 2000. The use of peptones as medium additives for the production of a recombinant therapeutic protein in high density perfusion cultures of mammalian cells. *Cytotechnol* 32 (2): 157-167. DOI: 10.1023/A:1008196521213.
- Herlina L, Pukan KK, Mustikaningtyas D. 2017. The endophytic bacteria producing IAA (Indole Acetic Acid) in *Arachis hypogaea*. *Cell Bio Dev* 1: 31-35. DOI: 10.13057/cellbio/dev/t010106.
- Ibrahim K M, Naem RK, Abd-Sahib AS. 2013. Antibacterial activity of nutmeg (*Myristica fragrans*) seed extracts against some pathogenic bacteria. *Al-Nahr J Sci* 16 (2): 188-192. DOI: 10.22401/JNUS.16.2.29.
- Kirchhoff G, Reis VM, Baldani JJ, Eckert B, Dobereiner J, Hartmann A. 1997. Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant Soil* 194 (1): 45-55. DOI: 10.1023/A:1004217904546.
- Kobayashi DY, Palumbo JD. 2000. Bacterial endophytes and their effects on plants and uses in agriculture. In *Microbial endophytes*. CRC Press.
- Mugiastuti E, Suprayogi S, Prihatiningsih N, Soesanto L. 2020. Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control. *Biodiversitas* 21 (5): 1809-1815. DOI: 10.13057/biodiv/d210506.
- Nkwocha CC, Okagu IU, Chibuogwu CC. 2019. Research Article Mineral and Vitamin Contents of *Monodora myristica* (African Nutmeg) Seeds from Nsukka, Enugu State, Nigeria. *Pak J Nutr* 18 (1): 308-314. DOI: 10.3923/pjn.2019.308.314.
- Nurjanah S, Putri IL, Sugiarti DP. 2017. Antibacterial activity of nutmeg oil. *KnE Life Sci* 506 (1): 563-569. DOI: 10.18502/kls.v2i6.1076.
- Panggabean KA, Rusmarilin H, Suryanto D. 2019. The utilization of nutmeg seed (*Myristica fragrans* Houtt) extract as an antimicrobial on tempeh sausage. In *IOP Conf Ser: Earth Environ Sci* 260 (1): 012087. DOI: 10.1088/1755-1315/260/1/012087.
- Qin S, Li J, Chen HH, Zhao GZ, Zhu WY, Jiang CL. 2009. Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *Appl Environ Microbiol* 75 (1): 6176-6186. DOI: 10.1128/AEM.01034-09.
- Sukmawati S, Hardianti F. 2018. Analisis total plate count (TPC) mikroba pada ikan asin kakap di Kota Sorong Papua Barat. *J Biodjati* 3 (1): 72-78. DOI: 10.15575/biodjati.v3i1.2368. [Indonesian]
- Susilowati DN, Rahayuningsih S, Sofiana I, Radiastuti N. 2021. The potential of nutmeg's microbes (*Myristica fragrans* Houtt.) as antagonistic agents against *Rigidoporus microporus*. *J Suboptimal Lands* 10 (1): 1-13. DOI: 10.36706/JLSO.10.1.2021.529.
- Sturz AV, Christie BR, Matheson BG, Nowak J. 1997. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biol Fertil Soils* 25 (1): 13-19. DOI: 10.1007/s003740050273.
- Tan K P, Khoo HE, Azrina A. 2013. Comparison of antioxidant components and antioxidant capacity in different parts of nutmeg (*Myristica fragrans*). *Intl Food Res J* 20 (3): 1049-1052.
- Thairu Y, Nasir IA, Usman Y. 2014. Laboratory perspective of gram staining and its significance in investigations of infectious diseases. *West Afr J Med* 1 (4): 168-174. DOI: 10.4103/2384-5147.144725.
- Venkatesh KR, Singh RP, Mishra P. 2019. Endophytes as emphatic communication barriers of quorum sensing in Gram-positive and Gram-negative bacteria-a review. *Environ Sustain* 2 (4): 455-468. DOI: 10.1007/s42398-019-00079-9.
- Yunita M, Mubarik NR, Solihin DD. 2016. Isolation and identification of chitinolytic bacteria as biocontrol agent of pathogenic fungi on gold silk worm cocoon *Cricula trifenestrata*. *Malays J Microbiol* 12 (1): 69-75. DOI: 10.21161/mjm.74415.
- Yunita M, Manse Y, Wulandari MC, Sukmawati S, Ohiwal M. 2022. Isolation of endophytic bacteria from nutmeg plant as antibacterial agents against pathogenic bacteria. *J Pendidikan Biologi* 7 (1): 115-122. DOI: 10.31932/jpbio.v7i1.1522. [Indonesia]
- Zhao L, Xu Y, Lai X. 2018. Antagonistic endophytic bacteria associated with nodules of soybean (*Glycine max* L.) and plant growth-promoting properties. *Braz J Microbiol* 49 (1): 269-278. DOI: 10.1016/j.bjm.2017.06.007.