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Exploration of endophytic fungi from leaf (*Syzygium polyanthum* Wight) and its potential probiotic for poultry

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ABSTRACT

The use of antibiotics may have a negative impact on animal health. There is an option use a probiotics that can improve intestinal health and performance. Fungi has a probiotic potential and one example is endophytic fungi that can produce various bioactive compounds which are needed to act as probiotic. The study aimed to explore of endophytic fungi from Bay leaf and to evaluate its potential probiotic for poultry. The materials used leaves and petioles. The method was isolation, identification, assay of antifungal, antibacterial activity, acid and bile salt resistence The exploration got *P. echinulatum*, *P. solitum*, *P. paneum*, *O. griseum*, *F. verticillioides* and *F. avenaceum*. The highest number of colonies was *O. griseum*, the lowest of dominance to *A. flavus* was *F. verticillioides*, the widest of diameter of the inhibition zone to E. coli was *F. verticillioides* and *S. aureus* was *P. echinulatum*, the fastest growing on acidic media is *F. avenaceum* and on bile salt is *F. avenaceum*. The conclusion was the exploration got *P. echinulatum*, *P. solitum*, *P. paneum*, *O. griseum F. avenaceum*, and *F. verticillioides*. and *P. echinulatum* is the most potential species as a probiotic for poultry.

Introduction

Antibiotic growth promoters (AGPs) is a type of feed additive that typically used to increase poultry growth. The additive has been used in practically in every part of the world. However for long-term use of such additives may have a negative impact on animal health. Therefore, several countries including Indonesia have banned its use, except when a disease attack occurs. Concerns about this condition, then there is by using another feed additives can improve intestinal health and performance without causing harm to poultry or humans as consumers, that is probiotics.

The term "probiotic" which is known to be a derivative of the Latin word pro and the Greek word βιο meaning "for life". This term was first used in 1965 by Lilley and Stillwell to describe a substance secreted by microbes that can stimulate growth. Afshar *et al*. (2020) reported that probiotics agents' is live microorganisms which when administered in adequate amounts causing numerous health beneficial effects on the host, which make them additives that are even more suitable to food and feed. According to Munir *et al*. (2022), probiotics as "live microbes when given in sufficient quantities, confer health benefits on host organisms" While Halder *et al*. (2024) stated that probiotics meaning "prolife" are live, non-pathogenic microorganisms that when given in sufficient amount confer an advantage to the host health and well-being.Several bacteria are considered potential microbes for probiotic status and other microbes such as fungi and yeast also have probiotic potential like *Saccaharomyces cerevisiae*, *Candida pintolopesii, Aspergillus niger, Aspergillus oryzae* and *Chrysonillia crassa* (Yudiarti *et al*., 2013). Fungi are eukaryotic organisms with a cell wall rich in glucans and chitin that survive by absorption of organic materials as source of energy and carbon. They range in size from species with massive mycelia to microscopic single-cell yeasts. The large group characterised by hyphal growth which supports sexual reproductive structures (e.g., mushrooms) or asexual reproductive structures that produce conidia (spores) are referred as moulds or filamentous fungi. Fungi are ubiquitous microorganisms known to produce a wide variety of secondary metabolites, including mycotoxins, which play important ecological role in diversification and adaptation of these microorganisms to plants cultivated for food and feed production (Lima and Santos, 2017). Fungi can be found anywhere including in plant tissue which are ussually called endophytic fungi.

The word endophyt is formed from the word endo means within and phyt means plant (Schulz and Boyle, 2006). Endophytic fungi are microorganisms that colonize the interior of plant tissues (e.g. leaves, seeds, stem, trunk, roots, fruits, flowers) in intracellular and/or extracellular spaces without causing symptoms of disease in host plants. These microorganisms have been isolated from plant species in a wide variety of habitats worldwide, it is estimated that all terrestrial plants are colonized by one or more species of endophytic fungus (Dos Reis *et al*. 2022). Bhore & Satisha (2010) stated that endophytic fungi that colonize plant tissues obtain nutrition and protection from their host plants. Endophytic fungi live intracellularly in healthy plant tissues which induces the host to produce secondary metabolites. According to Singh *et al*. (2020) that the presence of endophytic microbes are very important to host plants because they can protect the host from pathogens, predators, and increase plant resistance to disease infections. Jendri *et al*. (2022) explaned that the relationship between endophytic microbes and host plants is a form of symbiotic mutualism, which is a form of mutually beneficial relationship. One example of endophytic fungi as found by Jha *et al* (2022) that endophytic fungi can produce various bioactive compounds in the form of anticancer, antiviral, antibacterial, and antifungal as well as plant growth hormones and mycotoxins, enzymes and antibiotics.

Bay plant (*Syzygium polyanthum* Wight) is a native plant from Southeast Asia which can be found in Burma, Malaysia and Indonesia, usually the leaves are used as a flavoring for cooking (Ismail and Wan Ahmad, 2019). Bay plants (*Syzygium polyanthum* Wight) come from the Myrtaceae family. The leaves are widely used as a spice in cooking and cooking (culinary) and as a traditional medicine for diabetes, diarrhea and hypertension. Leaves contain many compounds including essential oils, tannins, and flavonoids (Dewijanti *et al*., 2019). The plant has medicinal properties and in its tissue will produce several secondary metabolites which are very useful for medicine (Singh *et al*., 2020). These secondary metabolites are apparently not only produced by the plants itself but also

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produce by the microbes that grow inside the plant tissues. This study aimed to explore of endophytic fungi from leaf (*Syzygium polyanthum* Wight) and to evaluate its potential probiotic for poultry.

Materials and methods

Isolation and identification

The parts of the plants that is selected as the samples are leaves and petiles of Bay plant (*Syzygium polyanthum* Wight). Samples that have been taken are put in sterile polybags and stored in the refrigerator, until ready to be isolated. The procedure for sterilization, isolation and purification of endophytic fungi uses the modified method of Yahya *et al*. (2017). Isolation of endophytic fungi was carried out using the direct planting with spread method and used potato dextrose agar medium add 1% chloramphenicol to prevent bacterial growth. Samples of petioles and leaves were cleaned in running water for 10 minutes. Then the samples were immersed in 70% alcohol for 3 minutes, followed by immersion in distilled water for 3 minutes and repeated immersion in 70% alcohol for 1 minute. After that, the sample was drained on filter paper then are cut with a knife that had been sterilized, into small pieces of 1cm.Each sample is replicated three times. Each sample was placed on the Potato Dextrose Agar (PDA) medium in a petri dish and the position of the cutting part attached to the medium. All petri dishes containing samples were then incubated for 14 days at room temperature. The colony that showed different then are separated and re-growed on new PDA media. All isolates were then identified based on their macroscopic and microscopic characteristics according to Crous *et al.* (2020).

Assay of pathogen antifungal activity

Aspergillus flavus is a fungus that causes feed spoilage and is considered a potential pathogen to poultry. This isolate of the fungus was obtained from the culture stock of the Physiology and Biochemistry Laboratory, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro. Hence, this fungus was included in this present study. Antagonism test was conducted using dual culture method. The mycelium of each endophytic fungal isolate and *Aspergillus flavus* was cultured on one plate with Saboraud Dextrose Agar (SDA) and chloramfenicol. The next plates were inoculated with each endophytic fungal isolate alone and these were used as controls. Each treatment was performend three times (Izzatinnisa *et al*., 2020).

The antagonism ability is determined based on the percentage of inhibition and antibiosis by assessing the presence or absence of an inhibition zone. The percentage of inhibition of endophytic fungal growth is calculated based on the formula:

PI=(R1 -R2)/R1×100%

PI= Percentage of mycelial growth inhibition (%)

R1= Diameter of endopytic fungal mycelium on the control plate (cm)

R2= Diameter of endopytic fungal mycelium on the treatment plate (cm)

Criteria for the percentage of growth inhibition (%) (Izzatinnisa *et al*., 2020):

- High inhibition percentage: 70-100%
- Medium inhibition percentage: 40-69%
- Low inhibition percentage: 0-39%

Assay of pathogen antibacterial activity

Escherichia coli and *Staphylococcus aureus* are bacteria that can cause infections and are considered potential pathogens in poultry. The bacterial isolates were obtained from the culture stock of the Physiology and Biochemistry Laboratory, Faculty of Animal and Agricultural Sciences, Universitas Diponegoro. The procedure used is the agar diffusion method. One loop of the test bacterial culture was inoculated into 20 ml of Nutrient Broth (NB) and then incubated at 30°C for 18 hours. Meanwhile, 1 ml of 24-hour-old liquid stock culture of endophytic fungi was taken and put into 10 ml of MRS broth and then incubated at 30°C for 18 hours. Each pathogenic bacteria was inoculated 25 µl into Nutrient Agar (NA) and by pour plate method put in plate and allowed to be solidify. The NA media was then made a hole (diameter, 0.9 mm) with a sterile hole punch in the petri dish. The hole was then filled with MRSB media containing 50 µl of endophytic fungal culture. Then incubated at 30°C for 24 hours. The diameter of the clear zone formed around the hole was measured as the inhibition zone of endophytic fungi against pathogenic bacteria.

Assay to acid conditions

Liquid stock culture of endophytic fungi aged 24 hours was inoculated as much as 2 ml into 20 ml of MRS broth and incubated at 30°C for 18 hours. Then 1 ml of culture was taken and put into 9 ml of MRS broth whose pH had been adjusted to 5 with HCl 1N and incubated at 30°C for 24 hours. The number of colonies that grew was observed by using the pour plate planting technique on MRS agar.

Assay to bile salt

Liquid stock culture of endophytic fungi aged 24 hours was inoculated as much as 2 ml into 20 ml of MRS broth and incubated at 30°C for 18 hours. Then 1 ml of culture was taken and put into 9 ml of MRS broth containing Oxgall 0.3% (w/v) and as a control used MRS broth without Oxgall. The isolation technique was carried out using the pour plate method on MRS agar, then incubated at 30°C for 24 hours. The number of colonies that grew was observed by using the pour plate planting technique on MRS agar.

Results

Isolation and Identification

The isolation of the endophytic fungi from Bay Leaf (*Syzygium polyanthum* Wight) got nine isolates. All isolate then are identified where three isolates are same kind and the rest six isolates have different kinds. Three kinds from the leaves are *Penicillium echinulatum* Fassatiova (Fig. 2), *Penicillium solitum* Westling (Figure 3) and *Penicillium paneum* Frisvad (Fig. 4). From petioles got six isolates, two of the three isolates were identified same kind as found from the leaves those as follows *Penicillium solitum* Westling (2 isolate) and *Penicillium echinulatum* Fassatiova (1 isolates),while the other three isolates were *Oidiodendron griseum* Robak (Fig. 5), *Fusarium verticillioides* (Corda) Sacc (Fig. 6) and *Fusarium avenaceum* (Sacc.) Nirenberg (Fig. 7). Number of colony of each endophytic fungi grown on medium shown on Table 1 and characters of macroscopic and microscopic of all endophytic fungal isolates are shown below.

Table 1. Number of colony of each endophytic fungus grown on the medium.

Species	*cfu*/ml
Penicillium echinulatum Fassatiova	7.9×10^4
Penicillium solitum Westling	1.1×10^5
Penicillium paneum Frisvad	1.6×10^5
Oidiodendron griseum Robak	1.8×10^5
Fusarium avenaceum (Corda) Sacc.	1.2×10^5
Fusarium verticillioides (Sacc.) Nirenberg	6.1×10^4

The characters of macroscopic and microscopic of three of endophytic fungal isolates from the leaves as follows Figures 2-4.

Macroscopic and microscopic characterization of three other endophytic fungal isolates from petioles other than the three kinds above are as follows figures 5-7.

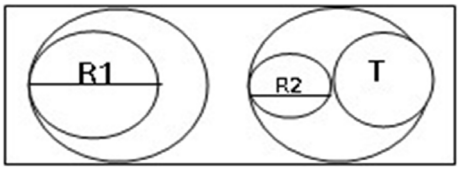
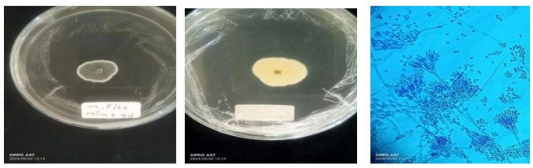


Fig. 1. Dual culture method; T = antagonistic fungus; R1 = endophytic fungus as a control; R2 = endophytic fungus in treatment.

(a) (b)

Fig. 2. *Penicillium echinulatum* Fassatiova (a). Macroscopic and (b). Microscopic characters

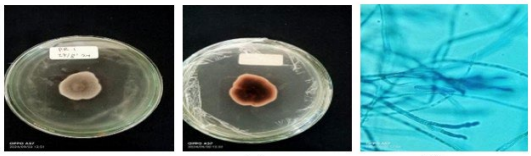


(a) (b)

Fig.3. *Penicillium solitum* Westling (a). Macroscopic and (b). Microscopic characters

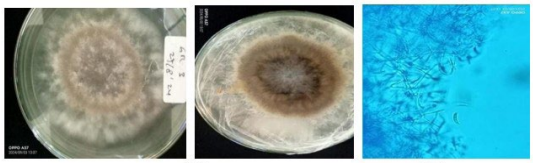
(a) (b)

Fig. 4. *Penicillium paneum* Frisvad (a). Macroscopic and (b). Microscopic characters



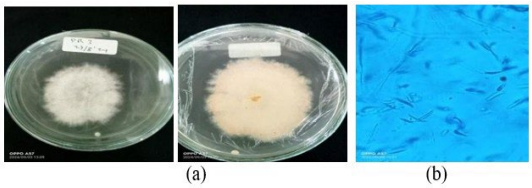
(a) (b)

Fig. 5. *Oidiodendron griseum* Robak (a). Macroscopic and (b). Microscopic characters



(a) (b)

Fig. 6. *Fusarium verticillioides* (Corda) Sacc (a). Macroscopic and (b). Microscopic characters.



(a) (b)

Fig. 7. *Fusarium avenaceum* (Sacc.) Nirenberg (a). Macroscopic and (b). Microscopic characters

Assay of antifungal activity

The antagonism test between each endophytic fungal isolate against *Aspergillus flavus* is shown by the percentage of dominance of the growth of the pathogenic fungal mycelium (Table 2).

Table 2. Mycelial growth dominance of *Aspergillus flavus*.

Species	Growth dominance (%)
Penicillium echinulatum Fassatiova	83.5
Penicillium solitum Westling	87.99
Penicillium paneum Frisvad	88.28
Oidiodendron griseum Robak	74.29
Fusarium avenaceum (Corda) Sacc.	88.47
Fusarium verticillioides (Sacc.) Nirenberg	47.09

Assay of pathogen antibacterial activity

The antagonism test between each endophytic fungal isolate against Eschericia coli and *Staphylococcus aureus* was shown by the presence of an inhibition zone of endophytic fungi against pathogenic bacteria (Table 3).

Table 3. Diameter of inhibition zone of endophytic fungi against two pathogenic bacteria.

Species	*E. coli* (mm)	*S. aureus* (mm)
Penicillium echinulatum Fassatiova	1.5	34.6
Penicillium solitum Westling	2.8	11.6
Penicillium paneum Frisvad	1.2	11.67
Oidiodendron griseum Robak	-	19.7
Fusarium avenaceum (Corda) Sacc.	-	7.76
Fusarium verticillioides (Sacc.) Nirenberg	2.9	-

Assay to acid conditions and bile salt

The resistance of each endophytic fungal isolate to growth on acid medium (pH 5) and bile salt are shown by the ability of a fungal colony to grow on that both media (Table 4).

Table 4. Number of fungal colonies on acidic and bile salt conditions

Species	pH 5 (*cfu*/ml)	Bile salt (*cfu*/ml)
Penicillium echinulatum Fassatiova	2.8×10^3	3.4×10^1
Penicillium solitum Westling	$1{,}5 \times 10^3$	1.1×10^1
Penicillium paneum Frisvad	1.3×10^3	1.2×10^1
Oidiodendron griseum Robak	1.9×10^3	1.9×10^1
Fusarium avenaceum (Corda) Sacc.	2.9×10^4	2.9×10^3
Fusarium verticillioides (Sacc.) Nirenberg	1.1×10^3	1.0×10^1

Discussion

The exploration of endophytic fungal from leaves and petiles of Bay plant (*Syzygium polyanthum* Wight) got nine isolates and after identified it found six species those are *Penicillium echinulatum* Fassatiova, *Penicillium solitum* Westling, *Penicillium paneum* Frisvad, *Oidiodendron griseum* Robak, *Fusarium avenaceum* (Corda) Sacc, and *Fusarium verticillioides* (Sacc.) Nirenberg. All findings of isolates are belong into three genera that are *Penicillium*, Oidiodendron and *Fusarium*. The findings is supported by other researcher such as endopytic fungus genus *Penicillium* is found by Toghueo and Boyom (2020). They have been reported that *Penicillium* colonized their ecological niches and protect their host plant. Then Singh *et al.* 2020 also found the endophytic fungi *Penicillium* sp from Argemone Mexicana which a plant of the family Papaveraceae. Zakaria *et al* (2010) found *Penicillium* from different parts of healthy paddy plants (*Oryza sativa*). Indrawati *et al* (2021) have isolated *Penicillium* sp from Syzygium cumini fruit. Other genus that is Oidiodendron griseum Robak is found by Pescie *et al.* (2021) from root of Blueberries cultivar. Zakaria *et al.* (2010) found genus *Fusarium* from different parts of healthy paddy plants (*Oryza sativa*). Wen *et al.* (2022) also found endophytic fungi genus *Fusarium* from host plant *Avicennia lanata* and *Cassia alata*.

The purpose of the analysis of the potential of probiotics for poultry from all endophytic fungal isolates was to evaluate their potential whether they could grow or not in acidic or bile salt conditions and their antagonism to poultry pathogenic fungi and bacteria. Before testing their potential, each isolate was first analyzed for its growth in the medium. The number of colonies growing on the medium was counted from the highest to the lowest. was *Oidiodendron griseum* Robak (1.8×10^5 cfu),

Penicillium paneum Frisvad (1.6x10^5 cfu), *Fusarium avenaceum* (Corda) Sacc (1.2x10^5 cfu), *Penicillium solitum* Westling (1.1x10^5 cfu), *Penicillium echinulatum* Fassatiova (7.9x10^4 cfu) and *Fusarium verticillioides* (Sacc.) Nirenberg (6.1x10^4 cfu).

Evaluation of the antagonism test between each endophytic fungal isolate against pathogenic fungus *Aspergillus flavus* is shown by the dominance of the pathogenic fungal mycelium growth on the medium. The dominance of *Aspergillus flavus* from the lowest to the highest are *Fusarium verticillioides* (Sacc.) Nirenberg (47.09%), *Oidiodendron griseum* Robak (74.29%), *Penicillium echinulatum* Fassatiova (83.50%), *Penicillium solitum* Westling (87.99%), *Penicillium paneum* Frisvad (88.28%), *Fusarium avenaceum* (Corda) Sacc (88.47%). From these results got that *Fusarium verticillioides* (Sacc.) Nirenberg obtained the lowest of the dominance the growth of the pathogenic fungus *Aspergillus flavus*. This indicated that *Fusarium verticillioides* has a high antagonistic potential against the pathogenic fungus *Aspergillus flavus*.

While the antagonism between each endophytic fungal isolate against Eschericia coli and *Staphylococcus aureus* was shown by the diameter of an inhibition zone on the medium. The diameter of the inhibition zone on the medium againt Eschericia coli from the widest to the narrowest are *Fusarium verticillioides* (Sacc.) Nirenberg (2.90 mm), *Penicillium solitum* Westling (2.80 mm), *Penicillium echinulatum* Fassatiova (1.50 mm), *Penicillium paneum* Frisvad (1.20 mm), meanwhile Oidiodendron griseum Robak and *Fusarium avenaceum* (Corda) Sacc both of them did not show any inhibition zone. The results found that *Fusarium verticillioides* (Sacc.) Nirenberg has the widest diameter, it means that this fungus is a good antagonist against *Escherichia coli*.

Whereas the antagonism against *Staphylococcus aureus* was shown that inhibition zone of *Penicillium echinulatum* Fassatiova (34.60 mm), *Oidiodendron griseum* Robak (19.70 mm), *Penicillium paneum* Frisvad (11.67 mm), *Penicillium solitum* Westling (11.60 mm), *Fusarium avenaceum* (Corda) Sacc (7.76 mm) and *Fusarium verticillioides* (Sacc.) Nirenberg did not show any inhibition zone. This assay showed that *Penicillium echinulatum* Fassatiova tends to be the best antagonistic fungus against *Staphylococcus aureus*.

The resistance of each endophytic fungal isolate to growth on acid medium (pH 5) and bile salt are shown by the ability of a fungal colony to grow on that both media. The fastest growing isolate in acidic media is *Fusarium avenaceum* (Corda) Sacc (2.9x10^4 cfu), *Penicillium echinulatum* Fassatiova (2.8x10^3 cfu), *Oidiodendron griseum* Robak (1.9x10^3 cfu), *Penicillium solitum* Westling (1.5x10^3 cfu), *Penicillium paneum* Frisvad (1.3x10^3 cfu),*Fusarium verticillioides* (Sacc.) Nirenberg (1.1x10^3 cfu). Then the fastest growing one on bile salt condition are *Fusarium avenaceum* (2.9x10^3 cfu), *Penicillium echinulatum* Fassatiova (3.4x10^1 cfu), *Oidiodendron griseum* Robak (1.9x10^1 cfu), *Penicillium paneum* Frisvad (1.2x10^1 cfu), *Penicillium solitum* Westling (1.1x10^{1}cfu), and *Fusarium verticillioides* (1.0x10^1 cfu).The fungus which the profusely growing fast on acidic and bile salt conditions was *Fusarium avenaceum* (Corda) Sacc

From the results of all analysis of six endophytic fungal isolates showed that the genus *Penicillium* is more potential than other genera and among the three species found in this study, *Penicillium echinulatum* Fassatiova is the most potential species. This because of the species has several potentials that needed to act as a probiotic for poultry, such as it can grows profusely in acidic c or bile salts onditions also be a good antagonistic against pathogenic bacteria and fungi. The acidic and bile salt conditions are conditions that exist in the digestive tract of poultry. In addition, another potential of *Penicillium echinulatum* Fassatiova is a good antagonist against poultry pathogenic fungi and bacteria. This because the genus *Penicillium*, is a types of fungi, which produce of bioactive chemicals such as mycotoxins patulin and ochratoxin A, antibacterial penicillins (Yang *et al*.,2021), echinocandins, which are effective against fungi (Frisvad *et al*., 2004, El Hajj Assaf *et al*., 2020). Beside that, it can produce immunosuppressants, and cholesterol-lowering agents (Kwon *et al*., 2017). The two compounds is needed to make a healty poultry. *Penicillium echinulatum* Fassatiova beside has a potential as a probiotic, the fungus has also good prospects for the future in the industrial sector. As mention by Camassola *et al*. (2004) and Schneider *et al*. (2016) that currently, there is great interest in the cellulase enzyme complex produced by *Penicillium echinulatum* Fassatiova for applications in various industries, such as food, alcohol, pulp and paper, textile, detergent, and agriculture. This enzymes consisting of endoglucanase, exoglycanase, and β-glucosidase that degrade cellulose.

Conclusion

The conclusion of this study was the exploration of endophytic fungi from plant (*Syzygium polyanthum* Wight) got six species and those are *Penicillium echinulatum* Fassatiova, *Penicillium solitum* Westling, *Penicillium paneum* Frisvad, *Oidiodendron griseum* Robak, *Fusarium avenaceum* (Corda) Sacc, and *Fusarium verticillioides* (Sacc.) Nirenberg. The genus *Penicillium* has more probiotic potential than other genera and among the three species of *Penicillium*, specie *Penicillium echinulatum* Fassatiova is the most potential species to be use as a probiotic for poultry.

Conflict of interest

The authors have no conflict of interest to declare.

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