

Antibacterial activity of bacteria isolated from earthworm (*Pheretima* sp.) gut against *Salmonella typhi* and *Staphylococcus aureus*: in vitro experiments supported by computational docking

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Abstract. Husain DR, Wardhani R, Eriyani AE. 2022. Antibacterial activity of bacteria isolated from earthworm (*Pheretima* sp.) gut against *Salmonella typhi* and *Staphylococcus aureus*: in vitro experiments supported by computational docking. *Biodiversitas* 23: 1125-1131. Bacteria have a diverse ecology niche as the effect of a long evolutionary process. They can live along with other organisms as endosymbiont. Research into endosymbiont bacteria began because of their ability to increase host resistance, especially to pathogens. This study aimed to determine the antimicrobial ability of endosymbiont bacterial isolates of earthworms *Pheretima* sp., *Bacillus brevis*, and *Bacillus choshinensis* that inhabit *Pheretima* sp. were isolated. The isolates were grown on tryptic soy broth media for 24 hours. The isolates were then purified using tryptic soy agar and biochemically tested to ensure both isolates are endosymbiotic bacteria. Antimicrobial activity was tested using agar diffusion methods in pathogenic bacteria, *Staphylococcus aureus*, and *Salmonella typhi*. Amoxicillin and chloramphenicol were used as positive controls. The inhibitory test results showed that incubation for 15 days effectively assessed pathogenic bacteria growth inhibition, marked by the largest inhibition zone (21.32 mm for *Salmonella typhi* and 16.88 mm for *Staphylococcus aureus*). They were very effective at inhibiting *Salmonella typhi* and *Staphylococcus aureus* growths. Endosymbiotic compounds' had a potential as antimicrobial. The in silico test supports the inhibitory test, which concluded that endosymbiont isolates can be an antimicrobial characterized by low-binding affinity values (-8.6 on tyrocidine) on molecular docking analysis.

Keywords: Antibacterial, endosymbiont bacteria, *Pheretima* sp.

Abbreviations: TSB: Tryptic soy broth, MR: Methyl Red, VP: Voges-Proskauer, TSIA: Triple Sugar Iron Agar, DHPP: Dihydropterin pyrophosphate, DHPS: Dihydropterolate synthase, pABA: p-aminobenzoic acid

INTRODUCTION

Annelida is a protostome taxon that consists of 17,000 species distributed throughout the world (Wanninger 2015). One of the famous groups from Annelida is the earthworm. This group lives in the terrestrial and belongs to the Order of Oligochaeta, Class Chaetopoda, Phylum Annelida, which dates from about 600 million years ago, during the pre-Cambrian era (Pierce et al. 1990; Ibrahim et al. 2015). This group mostly occurs in soils that accumulate organic matter in terrestrial ecosystems, with several species living in certain areas. Earthworms play an important role in soil ecosystems (Ansari and Saywack 2011; Kamdiem 2018).

Earthworms such as *Pheretima* are used as traditional medicine, including in Indian ayurvedic books, traditional Chinese medicine (China), Kampo (Japan), and traditional Korean medicine (Cooper and Hirabayashi 2013). *Pheretima* sp. (Phylum: Annelida; Class: Clitellata; Order: Oligochaetales; Family: Megascolecidae) (Aspe and James 2014) is an endemic earthworm in Southeast Asia, East India, and Japan. *Pheretima* sp. in Japan revealed that this

animal has a cylindrical body with many setae in each segment (Darmawan et al. 2012).

Earthworms' medicinal properties have been reported in numerous traditional Chinese medicine literature. Earthworms are specifically used to treat seizures, febrile illnesses, or epilepsy (Shen 2010). According to Brito-Vega and Espinosa-Victoria (2009), endogenous earthworms can also stimulate or inhibit important bacteria growth. This is related to the activity of several types of bacteria in the digestive tract of the earthworm (endosymbiont). Besides, according to Verma and Verma (2012), earthworms coexist with various types of viruses and bacteria that are abundant in the ground. Therefore, earthworms secrete certain compounds (secondary metabolites) to be used by pathogenic bacteria or viruses (Gopikrishnan et al. 2021).

The organism synthesizes secondary metabolites to maintain its life from competing with other organisms. Secondary metabolites include substances with sophisticated and diverse chemical structures, synthesized by certain varieties of microbial species (Fofied et al. 2018). Secondary metabolites can be derived from symbiotic microorganisms such as bacteria. Bacteria that

live in the earthworms' (symbionts) digestive tract have beneficial effects on host reproduction and excretion products that are likely supporting their life (Lund et al. 2014).

There are studies on earthworms' antibacterial activity. However, little or no research addresses the antibacterial activity of the composition produced by earthworm endosymbiont. Most of the research on earthworm antibacterial activity uses direct earthworm extracts, such as Dharmawati et al. (2019), who extracted the antibacterial activity from the earthworm extract of *Lumbricus rubellus*, Chauhan et al. (2014), and Anitha and Jayraaj (2013). They examined the antimicrobial activity of the earthworm extract *Eudrilus eugeniae*. The antibacterial composition produced by earthworms could result from the excretion of symbiotic bacteria that live in the intestines of earthworms. *Bacillus brevis* and *Bacillus choshinensis* were previously identified using the biochemical method API 20 E (bioMérieux), to inhabit within the intestine of earthworms *Pheretima* sp (Husain et al. 2018). We conducted a further study to identify these two bacterial species' antibacterial activity isolated from the earthworm *Pheretima* sp.

MATERIALS AND METHODS

Bacterial culture and purification

Endosymbiont isolates of earthworms *Pheretima* sp., namely *Bacillus brevis* and *Bacillus choshinensis*, were contained in agar slant media inoculated on 15 gr/L tryptic soy broth (TSB) media with around sterilized loop by aseptic technique. TSB media were then cultured in a shaker at 150 rpm for 24 hours at room temperature (20°C) (Li et al. 2016). The bacterial inoculum were grown and inoculated on 15 gr/L TS media by the quadrant streak method using a sterilized round loop. TSA media were incubated at 37°C for hours. This process was done two times to gain a pure colony.

Gram staining

Cell morphology was observed using gram staining technique under light microscopy (GoldBio 2018).

Biochemical characterization

Methyl Red (MR)

Bacterial isolates were taken from the culture stock with the inoculum loop and inoculated in liquid MR medium that contain Pepton Dipotassium phosphate and dextrose in a test tube. Furthermore, it was incubated for 5 × 24 hours at 37°C. Five drops of MR were added to the bacterial isolate (Himedia 2018). The positive results were determined if the complex formed a pink-to-red color, which indicated that the microbes produced acid.

Voges-Proskauer (VP) test

Bacterial isolates were taken from the culture stock with the inoculum loop and inoculated in liquid VP medium that contain Pepton Dipotassium phosphate and dextrose in a test tube. Subsequently, the isolates were incubated for 3 × 24 hours at 37°C. The medium was then

added with 0.2 mL of 40% KOH and 0.6 mL of α -naphthol and shaken for 30 seconds (Shields and Cathcart 2011). The positive results were determined if the medium turned violet.

Motility test

Bacterial isolates from the culture stock were inoculated by being pricked in an upright Sulphide Indole Motility (SIM) medium containing pancreatic digest of casein, peptic digest of animal tissue, ferrous ammonium sulfate, and sodium thiosulfate with an inoculum needle, then incubated at 37°C for 2 × 24 hours (Dalyn Biologicals 2014). Positive results (motile) were determined if there are propagations around the needle puncture on the medium.

Catalase test

Bacterial culture stock were inoculated with the inoculum loop to make a bacterial smear on object-glass. Then 2-3 drops of H₂O₂ reagent were added to the smear (Reiner 2010). Positive results were determined if gas bubbles were formed.

Triple Sugar Iron Agar (TSIA) test

Bacterial isolates were taken with the inoculum needle from each culture stock, then inoculated by stabbing on TSIA media containing beef extract, yeast extract, peptone, dextrose, lactose, sucrose, ferrous sulfate, sodium chloride, sodium thiosulfate and phenol red. Then, the bacterial isolate from each culture stock was inoculated with the inoculum loop and scratched on the surface of the medium. Furthermore, it was incubated for 2-3 × 24 hours at 37°C. Color changes on medium to yellow, redder, or black after incubation indicated acid, alkaline, or H₂S production, respectively. At the same time, the raised medium from the bottom indicated gas production (Dalynn Biologicals 2014).

Citrate test

Bacterial isolates were inoculated with the inoculum loop into Simmon Citrate Agar containing magnesium sulfate, ammonium dihydrogen phosphate, dipotassium phosphate, sodium citrate, sodium chloride, and bromothymol blue. Then, bacterial isolates were incubated at 37°C for 1-2 × 24 hours. The blue color indicates a positive reaction, and the green color indicates a negative reaction (Himedia 2019).

Antibiotics susceptibility test

The antibiotic activity was tested using NA medium (Nutrient Agar) by the agar diffusion method using a paper disk (Hudzicki 2009). Each paper disk was immersed in the supernatant of bacterial symbionts for 15 minutes and a positive control solution was chloramphenicol. The incubation time of fermentation for endosymbiont bacteria was conducted every 5 days. Therefore, the antibiotic activity test was conducted on 5, 10, 15, and 20 days. NA medium was added at 45°C and allowed to solidify. Then, a 1 mL suspension (1x10⁸-2x10⁸ CFU/mL) with *Bacillus brevis* and *Bacillus choshinensis* were added to a sterile petri dish. Paper disks that had been soaked in the

supernatant of bacterial symbionts and chloramphenicol were placed on solid NA medium with a distance of 20 mm. Observations were made after 24 hours. Then, the formed diameter of the inhibition zone was measured, and incubation was resumed for 48 hours to determine the nature of the antibiotic compounds contained in the earthworm.

in silico methods

Chemical structure of the antimicrobial compound of *Bacillus brevis* and *Bacillus choshinensis* collected from literature study, 3D chemical structure, ligand SMILES, and ID number were taken from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The ligand was then processed with Avogadro and saved in PDB format. Prediction of protein target was performed with *PharmMapper* (<http://lilab.ecust.edu.cn>), *SuperPred* (<http://prediction.charite.de>), and *Swiss target prediction* (www.swisstargetprediction.ch). The protein prediction was then validated with UniProt (<https://www.uniprot.org>). The protein structure was collected from the Protein Data Bank (<https://www.rcsb.org/>). The protein structure was processed using PyMOL v1.7.4.5 to remove non-protein molecules. The target protein for this research was the topoisomerase, DNA gyrase, Dihydropteroate synthase (DHPS) of *E. coli*, and DHPS of *S. aureus*. Molecular docking was performed with Vina Wizard feature integrated into PyRx 0.8. Gramicidine and Tyrocidine were selected as ligands. Interactions between ligand and protein targets were visualized and analyzed with PyMOL v1.7.4.5 (Husain et al. 2020).

RESULTS AND DISCUSSION

Biochemical characterization

Two endosymbiotic bacterial isolates were obtained from earthworms *Pheretima* sp., namely *Bacillus brevis* and *Bacillus choshinensis*. The isolate was biochemically identified using API 20 E (bioMérieux) (Husain et al. 2018). Both isolates were then cultured for 24 hours and then purified. The gram staining results showed that *B. brevis* and *B. choshinensis* had bacilli form and were categorized as the gram-positive group (Table 1). As shown in Table 2, *B. brevis* and *B. choshinensis* were able to ferment glucose, have low motility, and produce catalase enzyme. *Bacillus brevis* can ferment citrate. *Bacillus choshinensis* in fermentation produces mixed acids. This was indicated by a positive MR test, but it also produces 2,3-Butanediol in its fermentation products.

Inhibition test of bacterial endosymbiont

We evaluated the bacterial endosymbiont isolates earthworm *Pheretima* sp.'s antibiotic activity through the agar diffusion method. The inhibition test used several culture media to see the age of fermentation that is most effective in inhibiting pathogenic bacteria growth. The positive controls were used were Amoxicillin and

chloramphenicol. Amoxicillin is a broad-spectrum, pharmacologically active, beta-lactam antibiotic that is effective against Gram-positive and Gram-negative bacteria (Kaur et al. 2011).

The antimicrobial activity test was carried out at every 5-day fermentation time intervals throughout 20 days (5, 10, 15, and 20 days). The results showed that incubation for 15 days effectively assessed pathogenic of *Salmonella typhi* and *Staphylococcus aureus* growth inhibition, marked by the largest inhibition zone (Figure 3) (Table 3). Besides, our results demonstrated that the compound from *Bacillus choshinensis* produced an inhibition zone area. On the first day and second day grew culture of *S. typhi* were about 20.05 mm and 21.32 mm. Besides, on the first day and second day grew culture of *Staphylococcus aureus* were about 15.56 mm and 16.88 mm. Meanwhile, the *Bacillus brevis* compound showed an inhibition zone area on the first day and second day grew culture of *S. typhi* were about 15.13 mm and 15.25 mm, while the inhibition zone was 12.20 mm on the first day and 12.40 mm on the second day of incubation in *Staphylococcus aureus* culture. The most effective compounds in inhibiting the growth of *Salmonella typhi* and *Staphylococcus aureus* occurred on day 15. It indicated by the highest inhibition zone occurred on day 15. Each bacterium has different rates and properties of each metabolism. Therefore, each bacteria has different growth phases. The strength of the antibacterial effects were classified into four categories based on the diameter of the clear zone formed, namely, weak (<4 mm), medium (4-8 mm), strong (8-12 mm), and very strong (>12 mm) (Öldak et al. 2017). Based on this classification, the endosymbiotic bacterial compound was considered very strong in inhibiting the growth of pathogenic bacteria as it has an inhibition zone of >12 mm.

Table 1. Results of gram staining

Bacteria	Gram	Form
<i>B. choshinensis</i>	positive	bacili
<i>B. brevis</i>	positive	bacili

Table 2. Results of biochemical test

Characteristics	<i>B. brevis</i>	<i>B. choshinensis</i>
Catalase	+	+
Motility (SIM)	-	-
Methy Red (MRVP)	-	+
Voges Proskauer (MRVP)	-	+
Indole (SIM)	-	+
Citrate	+	-
Carbon Utilization (TSIA)		
Glucose	+	+
Sucrose	-	-
Lactose	-	-

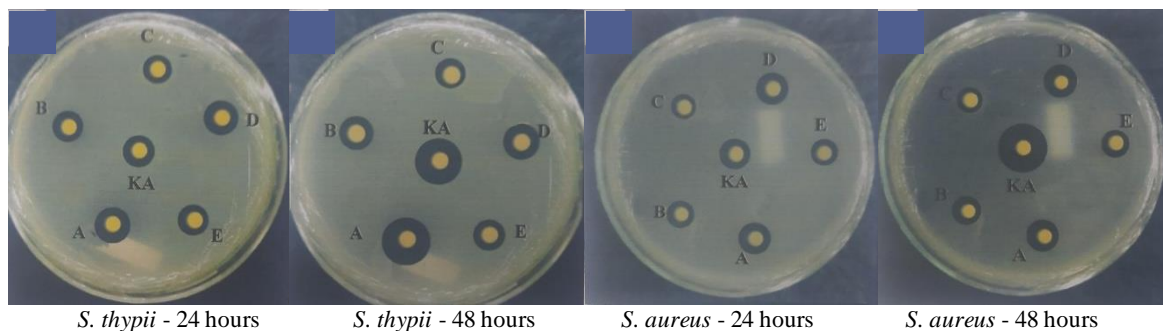


Figure 1. The inhibition zone of several bacterial isolates for 5 days. A. *B. choshinensis*, B. ICP2F, C. ICP3F, D. *B. brevis*, E. ICP6F, KA: Amoxicillin (20 μ g/mL). ICPF is an isolate code before the results of the bacteria identification

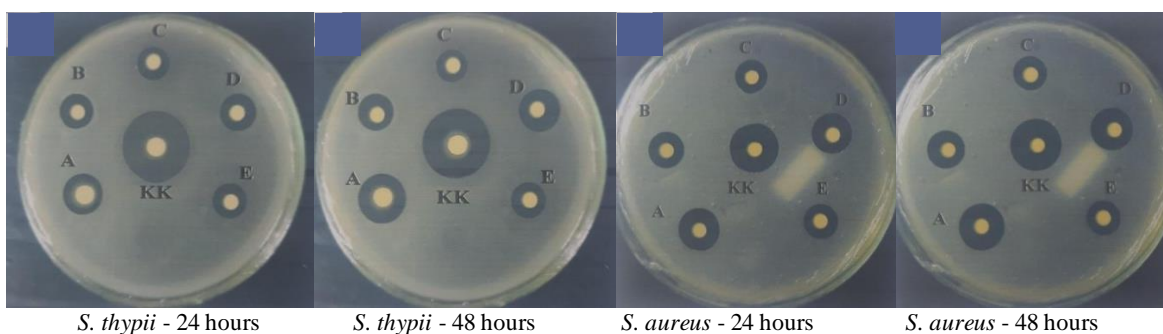


Figure 2. The inhibition zone of several bacterial isolates for 10 days. A. *B. choshinensis*, B. ICP2F, C. ICP3F, D. *B. brevis*, E. ICP6F, KK. chloramphenicol (30 μ g/mL)

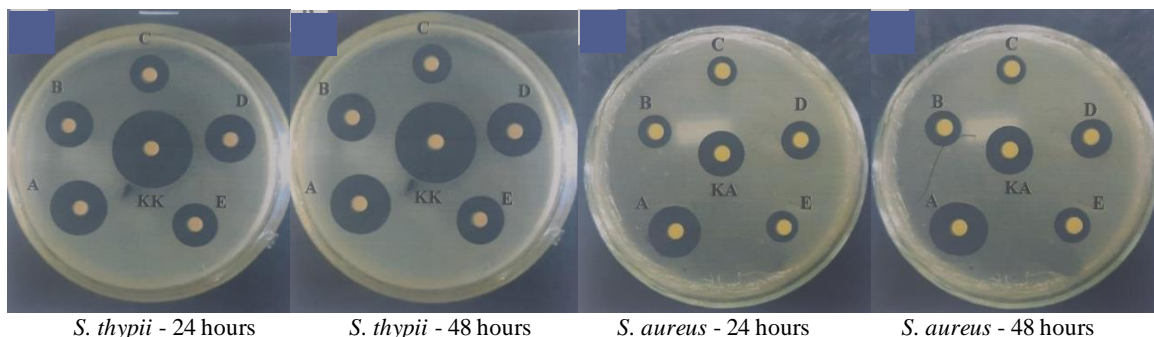


Figure 3. The inhibition zone of several bacterial isolates for 15 days. A. *B. choshinensis*, B. ICP2F, C. ICP3F, D. *B. brevis*, E. ICP6F, KK. chloramphenicol (30 μ g/mL), KA. Amoxicillin (20 μ g/mL)

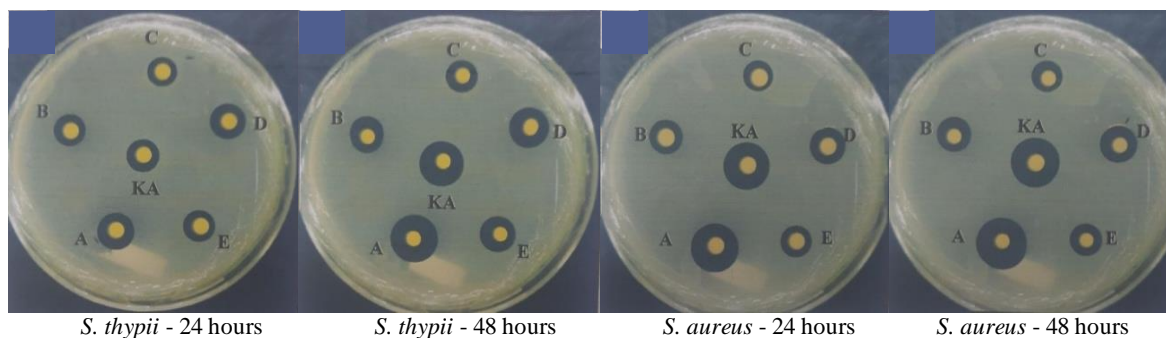


Figure 4. The inhibition zone of several bacterial isolates for 20 days. A. *B. choshinensis*, B. ICP2F, C. ICP3F, D. *B. brevis*, E. ICP6F, KA. Amoxicillin (20 μ g/mL)

Table 3. Inhibitory zone of isolate bacteria that were isolated from *Pheretima* sp. with two replications

Isolate	Incubation time	Inhibitory zone			
		<i>Salmonella typhi</i>		<i>Staphylococcus aureus</i>	
		24 hours	48 hours	24 hours	48 hours
<i>B. choshinensis</i>	5 days	13.09 mm	13.60 mm	12.20 mm	12.42 mm
	10 days	14.24 mm	14.63 mm	13.20 mm	13.57 mm
	15 days	20.05 mm	21.32 mm	15.56 mm	16.88 mm
	20 days	13.40 mm	13.55 mm	13.01 mm	13.15 mm
<i>B. brevis</i>	5 days	11.90 mm	12.60 mm	9.90 mm	10.10 mm
	10 days	9.97 mm	10.40 mm	10.20 mm	10.31 mm
	15 days	15.13 mm	15.25 mm	12.20 mm	12.40 mm
	20 days	10.70 mm	10.79 mm	10.20 mm	10.35 mm
Amoxicillin	5 days	8.40 mm	9.05 mm	13.80 mm	13.98 mm
	10 days	8.40 mm	9.05 mm	13.90 mm	13.98 mm
	15 days	8.40 mm	9.05 mm	13.80 mm	13.98 mm
	20 days	8.40 mm	9.05 mm	13.80 mm	13.98 mm
Chloramphenicol	5 days	25.00 mm	25.10 mm	13.09 mm	13.87 mm
	10 days	25.00 mm	25.10 mm	14.15 mm	14.50 mm
	15 days	25.20 mm	25.45 mm	14.15 mm	14.50 mm
	20 days	25.20 mm	25.45 mm	14.15 mm	14.50 mm

Table 4. Binding affinity value

Ligand	Receptor	Binding affinity (kmol)
Gramicine	Topoisomerase	-7.0
Tyrocidine	Topoisomerase	-8.6
Gramicine	DNA gyrase	-5.5
Tyrocidine	DNA gyrase	-5.9
Gramicine	DHPS of <i>E. coli</i>	-6.9
Tyrocidine	DHPS of <i>E. coli</i>	-8.4
Gramicine	DHPS of of <i>S. aureus</i>	-6.2
Tyrocidine	DHPS of of <i>S. aureus</i>	-6.2

in silico test

Based on the results of molecular docking using PyRx 0.8 application, the antimicrobial compounds produced by *Bacillus brevis* and *Bacillus choshinensis* can react and inhibit the work of several receptors related to pathogenic bacteria, seen from the relatively small binding affinity value (Table 4). According to Saputri et al. (2016), binding affinity is a measure of a compound's ability to bind to a receptor. The smaller the binding affinity value, the higher the affinity between receptors and ligands and vice versa (Eyler 2019). The greater the binding affinity value, the lower the affinity between receptors. Visualization of interactions between ligand compounds and target protein receptors can be seen in Figure 5.

Discussion

The genus *Bacillus* currently consists of 377 species as of January 2019. The ability of various *Bacillus* species to produce antibiotic compounds supports its ability to live everywhere, such as land, water, food, and intestinal mammals (Caulier et al. 2019). Recently, *Bacillus* was found living in symbionts in earthworms (Biswas et al.

2014; Byambas et al. 2019). This ability may be due to the ability of the genus *Bacillus* to produce antibiotics for pathogenic bacteria, such as Gramicidin and Tyrocidine produced by *Bacillus brevis* (Urdaci and Pinchuk 2004).

Amoxicillin is stable in the digestive tract, and its absorption is higher than penicillin, which occurs naturally when given orally (FDA 2008; Ramos et al. 2012). Chloramphenicol is a broad-spectrum antibiotic that inhibits gram-positive and gram-negative bacterial growth (Trivedi et al. 2015). Chloramphenicol is bacteriostatic by inhibiting protein synthesis, especially peptidyl transferase, thus preventing elongation at the protein synthesis stage (Ficai and Grumezescu 2017). Madigan et al. (2012) stated that microorganisms produce secondary metabolites (antimicrobial) in the late stationary phase of growth. This is because secondary metabolites are usually synthesized by the end of the cell's growth cycle, namely the stationary phase, as the population remains stable. After all, the number of cells that grow equals the number of cells that die. According to Li et al. (2016), the inhibition zone formed between 20-25 mm, 15-20 mm, 10-15 mm, and 5-10 mm is classified as very strong, strong, moderate, and weak inhibitory effect. Based on each isolate's inhibitory zones, the bacterial endosymbiont of earthworms *Pheretima* sp. showed inhibitory effects classified as very strong in inhibiting the growth of *Salmonella typhi* and classified as strong in inhibiting the growth of *Staphylococcus aureus*. The inhibitory test results also showed that the two bacterial compounds effectively inhibited the growth of gram-negative bacteria compared to gram-positive bacteria. This was due to the differences in the mechanism of action of each antibacterial compound (Amerikova et al. 2019).

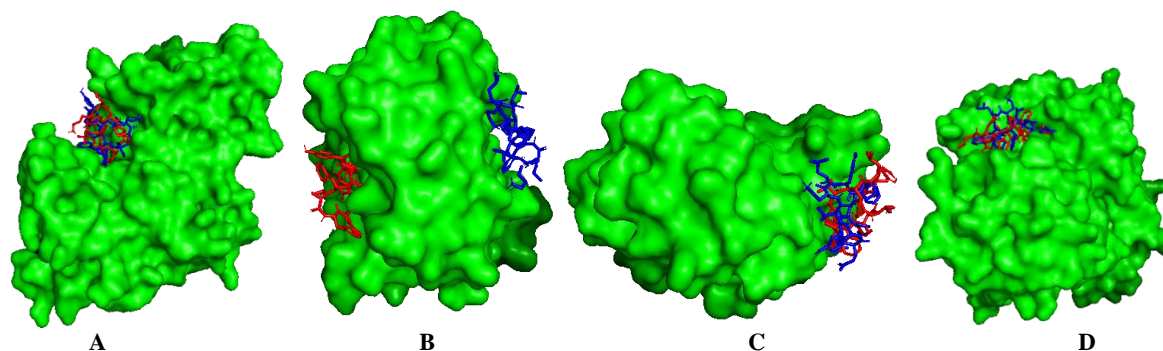


Figure 5. Visualization of Ligand–Macromolecule Interaction. Red: Gramicidine, Blue: Tyrocidine. A. Topoisomerase and Gramicidine; Tyrocidine. B. DHPS *S. aureus* and Gramicidine; Tyrocidine. C. DNA gyrase and Gramicidine; Tyrocidine. D. DHPS *E. coli* and Gramicidine; Tyrocidine

In this study, three types of receptors are used, namely dihydropterin pyrophosphate (DHPP), which contributes to the biosynthetic pathway of folate, DNA gyrase, and topoisomerase, and is responsible for nucleic acid synthesis. The folate synthesis pathway is a crucial pathway for amino acid synthesis. Without amino acids, bacteria cannot live, which is an important point in finding efficient methods to inhibit pathogenic bacterial growth (El-Attar et al. 2018). Folic biosynthesis in prokaryotes and eukaryotes is different. Prokaryotes carry out folic acid biosynthesis so folate biosynthesis inhibition is key in inhibiting the growth of pathogenic bacteria (Dias et al. 2018). Dihydropteroate synthase (DHPS) plays a key role in folate biosynthesis. This enzyme has two binding sites, one that binds to DHPP and binds to p-aminobenzoic acid (pABA). DHPS catalyzes reactions that produce 7,8-dihydropteroate from these two substrates, the next steps in folate synthesis involve the conversion of 7,8-dihydropteroate to finally produce folate compounds (Yun et al. 2012; Kordus and Baughn 2019). Some antibiotics also target topoisomerase II and topoisomerase IV in inhibiting bacterial nucleic acid synthesis (Etebu and Ariekpar 2016). Other antibiotics, such as those from the fluoroquinolones, target DNA gyrase in inhibiting nucleic acid synthesis (Kapoor et al. 2019).

The compounds produced by these two isolates are antimicrobial. Gramicidin works by disrupting membrane permeability through its interaction with membrane lipopolysaccharides and interfering with the transfer of ion cells to gram-positive bacteria (Yang and Yousef 2018; Pavithra and Rajasekaran 2019). Tyrocidine works by disrupting membrane permeability and also causes DNA damage (Marques et al. 2007; Wenzel et al. 2018).

The biochemical identification test results showed the similarity of isolates obtained from *Pheretima* sp. endosymbiont, namely *Bacillus brevis* and *Bacillus choshinensis*. The antimicrobial compound activity of the two isolates was maximally obtained on day 15 after incubation. Bacteriocidal compounds from the two isolates are classified as very strong antibacterial effect because it can produce a clear zone of 16.88 mm in gram-positive *S. aureus* and 21.32 mm in gram-negative *S. typhi*.

Computational tests also show the antimicrobial ability of the two isolates. They produce gramicin and tyrocidine, which have potential as antimicrobials.

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