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Screening of Proteolytic Bacteria from the Intestines of Tilapia (*Oreochromis niloticus*) in the Rice-fish Farming of Panembangan-Banyumas

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Abstract

Tilapia is a commodity cultivated in the rice-fish farming area of Panembangan, Banyumas. Information related to the presence of potential bacteria in the intestines of fish cultivated in rice-fish areas is still low, especially proteolytic bacteria. The purpose of this study was to determine the proportion and activity index of proteolytic bacteria in the intestines of fish. This study used an observation method with a purposive sampling technique. Proteolytic bacteria were isolated from the intestines of tilapia in blocks A and E. The bacteria that were successfully isolated were grown on 2% skim milk agar media with an incubation time of 48 hours and observed at 24-hour intervals. Based on the results of the study, the proportion of proteolytic bacteria in the intestines of tilapia from both blocks was 28% in block A and 44% in block E, respectively. The proteolytic activity index of both blocks had an average of 0.48 for block A and 0.75 for block E. The highest proteolytic activity index was from block A, namely 0.83, and in block E 3.0. Meanwhile, the smallest proteolytic index is 0.17 block E and 0.22 block A.

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Introduction

Rich-fish farming is an integrated cultivation system that combines rice and fish plants simultaneously on one land (Bobihoe *et al*., 2015). Rice-fish farming is an alternative for farmers to increase income by maximizing the potential of their land (Nuryasri *et al*., 2015). Thousands of farmers have been using this cultivation system, such as those in Cianjur (Hamdana *et al*., 2020), Indramayu (Permana *et al*., 2020), Gresik (Suhaili *et al*., 2020), Batanghari (Bobihoe *et al*., 2015), and Central Aceh (Akbar, 2017). Tilapia is one of the main freshwater fish commodities cultivated in the rice-fish farming area, alongside carp, tilapia, and catfish (Akbar, 2017). Tilapia is one of the freshwater fish commodities in Indonesia with a production figure of 371 thousand tons recorded in the second quarter of 2021.

Aquaculture is expected to contribute 21.58 million tons to goverment production by 2023 (Ministry of Maritime Affairs and Fisheries, 2022). This high production target can be supported by tilapia because of its advantages. These advantages include being easy to cultivate, high tolerance to changes in environmental conditions, and producing superior strains such as fast fish growth performance (Oktapiandi *et al*., 2019). Efforts to increase optimal tilapia growth can be supported by providing natural feed or pellets that have a protein content of 25-50% (Noviana *et al*., 2014) and providing beneficial microbes (Carnevali *et al*., 2017). (Zhang *et al*., 2020) studied the growth of tilapia fed with 39% protein content and then supplemented with commercial probiotics. The results obtained from the study were that there was a significant difference in growth rates of tilapia fed with additional probiotics compared to the control treatment. Fish growth is also supported by the presence of bacteria in the digestive tract such as proteolytic bacteria (Lestari & Budiharjo, 2016; Ray *et al*., 2012). Fish have proteolytic bacteria in their digestive tract, which can assist in the process of breaking down proteins into simpler compounds that are easier to absorb (Wulandhari *et al*., 2017). Nine types of freshwater fish have been studied for the presence of proteolytic bacteria in the digestive tract, the greatest proteolytic activity was found in the digestive tract of tilapia (Bairagi *et al*., 2002). The proportion of proteolytic bacteria from the digestive tract of tilapia obtained from traditional markets in Bangladesh is around 27%, the genera of bacteria include *Priestia, Citrobacter, Pseudomonas, Stenotrophomonas, Burkholderia, Providencia, and Micrococcus* (Hossain *et al*., 2021). Factors that can influence the presence of bacteria in the intestines of fish include maintenance water, feed, and cultivation environment (Nayak, 2010). The research that has been conducted indicates that there is potential for proteolytic bacteria to live in the intestines of tilapia fish, thus creating an opportunity to carry out screening of proteolytic bacteria from the intestines of tilapia, one of which is in the ricefish farming area of Panembangan, Banyumas Regency.

Materials and methods

Time and Place of Research

The research was conducted from September 11 to October 11, 2022, at the Laboratory of the Faculty of Marine Sciences, Jenderal Soedirman University and the Integrated Laboratory of Muhammadiyah University of Purwokerto. Fish sampling was carried out in Panembangan Village,

Cilongok District, Banyumas Regency in the Kridoyuwono group rice field area, block A and block E.

Fish Sampling

Fish sampling was carried out using a purposive sampling method, namely in two rice-fish farming ponds. The fish sampling process was carried out using a seser from the rice-fish farming cultivation pond. The tilapia used in the study were taken from the Kridoyuwono rice-fish farming pond block A and block E with the number of fish for each block as many as 3 fish. Block A has a water source that has not passed through settlements, while the water source in Block E has passed through residential areas. Then the fish were killed by piercing the brain using surgical scissors. Furthermore, the fish were put into a plastic bag and stored using a coolbox containing ice to be taken to the laboratory.

Isolation of Bacteria

Bacterial isolation in this study was taken from the intestines of tilapia. The fish used as samples had a weight range of 37-120 gr with a total length range of 11-20 cm. Fish intestine samples were taken by dissecting the stomach after which the intestines were carefully taken using tweezers. The intestines were placed on a millimeter block to measure their total length. The total length range of the intestines obtained was 91-144 cm. The intestines were taken 5 cm long from the anterior, middle, and posterior parts. The previously sorted intestines were ground using a mortar, then weighed and put into a tube weighing 0.2 g. The intestine samples in the tube were homogenized using 1 mL of physiological solution.

The intestinal sample suspension in the tube was taken as much as 0.5 mL and then inserted into a test tube containing 4.5 mL of 0.9% NaCl. The test tube was homog enized and a 10^{-1} dilution was obtained. A 10^{-2} dilution was obtained by taking a solution from the 10^{-1} dilution as much as 0.5 mL using a pipette and then pouring it into another test tube containing 4.5 mL of 0.9% NaCl. The process was repeated until a 10^{-5} dilution was obtained. After all dilutions were made, 0.5 mL of the solution was taken from the 10^{-2} to 10^{-5} dilutions and then poured into a petri dish. Furthermore, TSA in a sterile liquid condition was poured sufficiently into the petri dish. The dish was homogenized by rotating it slowly to form a figure-eight pattern until the surface of the dish was covered. The dish was incubated for 24 hours at a temperature of 28 ºC with the dish upside down.

Bacterial Density Calculation

The bacterial colonies that grew on each plate with a range of 30-300 bacterial colonies from the dilution results were calculated using the total plate count (TPC) calculation method with the formula used by Madigan & Martinko, (2006), namely:

$$\text{Number of bacteria (CFU/g)} = \text{Number of colonies} \times \frac{1}{\text{Dilution}} \times \frac{1}{\text{Culture volume}} \times \frac{1}{\text{Sample weight}}$$

Observation of Bacterial Colony Morphology

The colonies that grow on the media are observed for their morphology. Observation of the morphology of bacterial colonies includes shape, elevation, and edges (Marista *et al*., 2013). Colonies that have different characteristics are re-cultured on new TSA media for bacterial stock.

Proteolytic Activity Test

The proteolytic activity test was carried out by streaking (streak plate) the pure bacterial colony isolates on a mixture of TSA media and 2% skim milk. Then, the media that had been streaked was incubated for 48 hours at a temperature of 28 ºC. Observation of proteolytic activity was carried out at intervals of 24 hours. Positive results of bacteria that have proteolytic activity are indicated by the formation of a clear zone around the colony (Kazanas, 1968). After observation, the diameter of the clear zone and the colonies formed were measured. The proteolytic activity index was calculated by comparing the diameter of the clear zone with the diameter of the colony (Setiawan *et al*., 2016).

$$\text{Proteolytic activity} = \frac{\text{Total diameter of clear zone} - \text{Diameter of bacterial colony}}{\text{Diameter of bacterial colony}}$$

The proportion of proteolytic bacteria is calculated to find out what percentage of proteolytic bacteria can be found. The formula for calculating the proportion of proteolytic bacteria refers to the formula used by Sinatryani *et al*., (2014) with modifications, namely:

$$\text{Proportion of proteolytic bacteria (\%)} = \frac{\text{Number of proteolytic bacterial colonies obtained}}{\text{Total number of colonies observed}} \times 100$$

Gram KOH Test

The surface of the object glass is dripped with one drop of 3% KOH solution. One loop of bacteria is taken and homogenized with 3% KOH dripped on an object glass. Bacteria that produce mucus indicate that they are gram-positive, while those that produce mucus indicate that they are gram-negative (Buck, 1982).

Catalase Test

One drop of 3% H_2O_2 reagent is dropped on the object glass. One loop of bacterial isolate is taken and homogenized with a drop of 3% H_2O_2. The formation of gas

bubbles indicates positive catalase results, while the absence of gas bubbles indicates negative catalase results (Chester, 1979).

Oxidase Test

This test is done by applying one loop of bacteria to the object glass, then the loop is covered with filter paper. The filter paper is dripped with a tetramethyl-blue reagent solution. Positive results are indicated by the appearance of violet color on the paper while negative results are indicated by no color change occurring on the paper (Damayanti *et al*., 2020).

Data Analysis

The data analysis conducted in this study is descriptive data analysis that explains the results of the research conducted in writing, tables, graphs, and images. The data in this study include the results of the number of bacteria, bacterial colony morphology, proteolytic activity index of bacteria, the proportion of proteolytic bacteria, proteolytic bacteria that have gram-positive and negative, catalase test results, and oxidase tests.

Results and Discussion

Bacterial Density in Fish Intestines

The density of bacteria in the fish intestines was obtained through calculation results. The average number of bacteria growing on TSA media from the dilution results of 10^{-3} - 10^{-4} in each block was 28.15 x 10^5 CFU / g for block A and 3 x 10^5 CFU / g for block E. The bacterial colonies that were successfully distinguished based on macroscopic morphology were obtained from as many as 25 isolates from each block. The results of the bacterial density still have a number that is included in the range of fish intestinal bacterial density in general.

According to Al-Harbi and Uddin, (2004), bacteria were present in the digestive tract of tilapia with density ranging from 8.9 x 10^5 to 1.3 x 10^9 CFU/g. Furthermore, the bacterial density of the fish digestive tract has a range of between 10^5 to 10^7 CFU/g which includes groups of heterotrophic aerobic and anaerobic bacteria (Ringø *et al*., 2003). Several factors can influence bacterial density in the fish digestive tract, including the host, the environment, and microbes (Wang *et al*., 2018).

Bacteria from the aquatic environment enter the fish's digestive tract through the mouth, along with the entry of water and/or feed, and then inhabit the fish's digestive tract (Austin, 2002). Consequently, the diversity of bacteria inhabiting the environment isidentical to that in the digestive tract of a fish (Cahill, 1990). Kassa and Mitiku, (2021) succeeded in isolating bacteria from the environment and digestive

tract of tilapia with the results of the diversity of bacteria obtained from the fish's digestive tract having similarities with bacteria isolated from the environment or water. However, not all bacteria in the environment will settle in the fish's digestive tract, bacteria that can live in the fish's digestive tract are indigenous (native) bacteria (Ringø *et al*., 1995). Indigenous bacteria can form colonies on the surface of the digestive tract walls (stomach and intestines) and Indigenous bacteria can tolerate changes in pH and bile salts (Banerjee and Ray, 2017).

Proportion of Proteolytic Bacteria

The proportion of proteolytic bacteria obtained from block A is around 28% or 7 isolates from 25 total isolates. Block E has a proportion of proteolytic bacteria with a percentage of 44% or 11 isolates from 25 total isolates (Figure 1). The higher proportion in block E is thought to be influenced by pond water, supported by the results of a study of proteolytic bacteria screening from pond water in block E which has a higher proportion than block A. Meanwhile, the small number of proteolytic bacteria found in tilapia can be caused by the fish's eating habits.

Previous studies reported that the number of proteolytic bacteria successfully isolated from herbivorous-omnivorous fish was only 9 isolates (Marlida and Elrifadah, 2017), Kamaruddin *et al*., (2022) found the same thing, namely the discovery of 9 isolat es of proteolytic bacteria with aproportion of 12.5%. However the result of the isolation of proteolytic bacteria from carnivorous fish

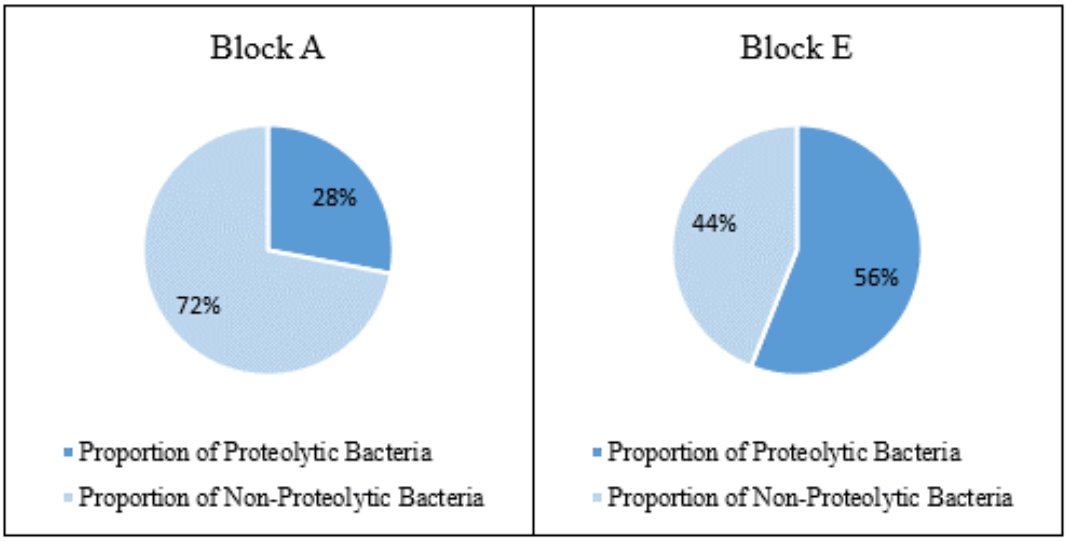

Figure 1. Proportion of proteolytic bacteria from the intestines of tilapia fish in the rice-fish farming area of Panembangan, Banyumas

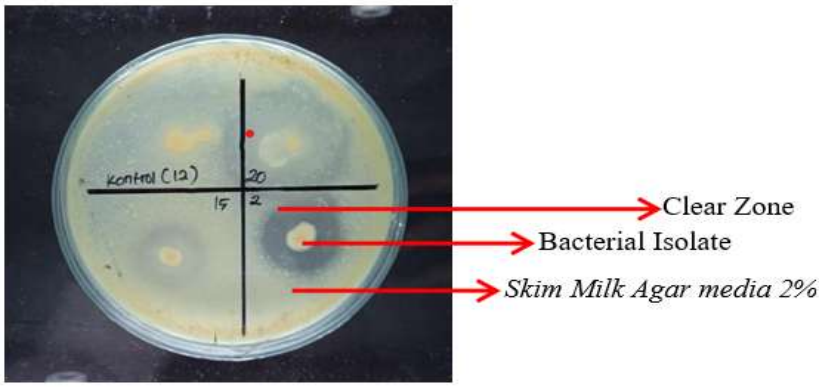

Figure 2. Test of proteolytic activity of bacteria from tilapia intestines in the rice-fish farming area of Panembangan, Banyumas

with the number of isolates of proteolytic bacteria obtained as many as 10 found that the proportion of proteolytic bacteria reached 77% (Hossain *et al*., 2020). Based on these results, fish-eating habits can have an impact on the proportion of proteolytic bacteria found, such as Jiao *et al*., (2022), who found that fish with herbivorous-omnivorous eating habits generally have lower proportions of proteolytic bacteria. Fish that are classified as herbivorous-omnivorous will utilize more carbohydrates from feed for their growth, such as tilapia, but the presence of proteolytic bacteria in the digestive tract of tilapia is still needed (Kurniasih *et al*., 2013; Muliani *et al*., 2019). The source of protein given to tilapia in the rice-fish farming area of Panembangan Village is in the form of pellets that contain at least 25% protein in the feed. Other sources of protein are obtained by providing additional feed in the form of sense leaves which have a vegetable protein content of 21% so that they can increase protein intake (Melisha *et al*., 2016). The protein in the feed will be broken down with the help of exogenous enzymes produced by proteolytic bacteria in the digestive tract of the fish, thereby accelerating the process of absorbing nutrients used for growth, metabolism, and endurance or immunity of the fish (Dhayalan *et al*., 2022). This indicates that proteolytic bacteria in herbivorous-omnivorous fish are important to support the survival of the fish.

Proteolytic Activity Index

The proteolytic activity index was calculated by dividing the diameter of the clear zone formed by the diameter of the bacteria growing on 2% skim milk media (Figure 2). The bacterial isolates that grew utilized the nutrients in the media for metabolism and growth, and were able to break down the casein content in skim milk

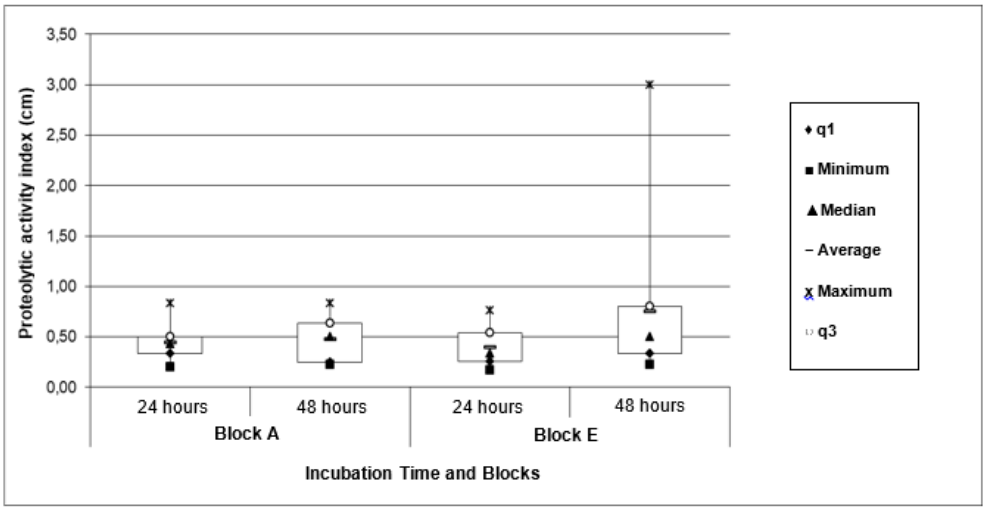

Figure 3. Index of proteolytic activity of intestinal bacteria of tilapia in the mina padi area of Panembangan, Banyumas

to form a clear zone around the isolate.

The results of the calculation of the proteolytic activity index of the two blocks are shown in Figure 3. Bacteria that have the highest proteolytic activity index at a 24-hour interval in block E are worth 0.76 and in block A are worth 0.83. At a 48-hour interval block E is worth 3.00 and block A is worth 0.83. The lowest proteolytic activity index at a 24-hour interval is 0.17 in block E and 0.20 in block A. At a 48-hour interval the lowest activity index value in block E is worth 0.17 and block A is worth 0.22. The average obtained from the results of the index calculation of the two blocks at a 24-hour interval is not much different, namely 0.40 in block E and 0.45 in block A. The average index of the two blocks at a 48-hour interval is 0.75 for block E and 0.48 in block A respectively.

The proteolytic index (IP) is categorized into three levels based on the values obtained. The proteolytic index categories are low = IP <1.5; medium = 1.5 ≤ IP <3.5; high = IP ≥ 3.5 (Ayuningrum *et al*., 2022). Based on the proteolytic index values produced from each isolate found in this study, almost all isolates from both blocks were included in the low category. Where the index value obtained has a range of 0.17 - 1.0 which is less than 1.5 (Appendix 3). However, isolates from block E with the code F5N5 have an index value of 3 which can be categorized as medium. The high and low index values in proteolytic activity depend on the ability of bacteria to produce enzymes which are influenced by factors such as the type of bacteria, substrate, temperature, pH, and incubation time (Herasari *et al*., 2022).

The length of the incubation time will

be determined by the amount of nutrients available in the media (Josephine *et al*., 2012). The longer the incubation time, the nutrients in the media will decrease along with the increasing growth of bacteria, and in this condition, the activity of bacteria in producing enzymes will decrease (Kusumani ngati *et al*., 2013). According to the results of the study conducted, the clear zone diameter remained the same even after 48 hours of incubation, but the bacterial colony diameter increased. Possibly, bacteria no longer need amino acids for metabolic needs, or maybe there has been a change in enzyme structure that no longer binds to the substrate properly (Yuniati *et al*., 2015).

Another factor that affects the proteolytic activity of bacteria is the type of bacteria. A similar morphological characteri stic is found in the isolate with the code F5N5 in block E, which has the highest proteolytic index, punctiform colony shape, flat elevatio n, clear white color throughout, gram-positive, positive oxidase, and negative catalase. The isolate representing the highest proteolytic index in block A with the code F1S2 displays morphological characteristics such as irregular shapes, and convex elevation. Edges, cream-colored colonies, gram-negative, negative oxidase, and negative catalase levels. Clearly, these colonies show very different morphological characteristics, as well as very different proteolytic index values. Consequently, each bacterium will have very different proteolytic abilities. Two strains of *Lactobacillus parac asei* bacteria produce different levels of prot eolytic activity (Garcia-Cano *et al*., 2019).

Morphological Characteristics of Proteoly tic Bacterial Colonies

Bacterial isolates that have proteolytic activity from block A have morphological characteristics with irregular shapes; circular, with entire edges; convex elevation; flat, and clear white; yellow (table 1). Block E bacteria that have proteolytic activity are dominated by circular shapes, with flat elevation; convex, entire edges and creamy white; milky white. Bacteria that have gram-negative properties dominate block A, and gram-positive bacteria dominate block E (table 1). A total of 3 bacterial isolates from block A and 1 bacterial isolate from block E have catalase enzymes which are indicated by the formation of gas or bubbles during the test. Several bacteria did not have oxidase enzymes in either block, but there were more oxygenase enzymes in both blocks.

The characteristics of proteolytic bacteria have different diversity in each species and strain of bacteria, such as Gram

Table 1. Morphological Characteristics of Proteolytic Bacterial Colonies

Isolate	Shape	Elevation	Edge	Colour	Gram KOH	Catalase	Oxidase
Characteristics of Proteolytic Bacterial Isolates Block A							
F1S2	Irregular	Convex	Entire	Creamy white	-	-	-
F1S15	Irregular	Raised	Lobate	Yellow	-	+	+
F1S16	Circular	Pulvinate	Entire	Thick white	-	-	+
F1S17	Circular	Flat	Entire	Yellow	-	+	+
F1S19	Filamentous	Flat	Filamentous	Clear white	-	+	+
F1S20	Irregular	Convex	Lobate	Clear white	-	-	+
F1S22	Circular	Umbonate	Filamentous	Clear white	-	-	+
Characteristics of Proteolytic Bacterial Isolates Block E							
F5N1	Circular	Flat	Entire	Milky white	-	+	+
F5N2	Circular	Convex	Entire	Cream white	+	-	+
F5N4	Circular	Flat	Entire	Dark cream white	+	-	+
F5N5	Punctiform	Flat	Entire	Clear white	+	-	+
F5N8	Circular	Pulvinate	Entire	Cream/Brown	-	-	+
F5N9	Spindle	Convex	Entire	Cream	+	-	+
F5N11	Irregular	Flat	Undulate	Clear white	+	-	-
F5N18	Circular	Pulvinate	Entire	Cream white	+	-	+
F5N19	Circular	Convex	Entire	Dark white	+	-	+
F5N20	Circular	Raised	Entire	Cream white	+	-	+
F5N22	Circular	Flat	Entire	Cream white	+	-	-

properties, and the ability to produce catalase or oxidase enzymes. Some proteolytic bacteria have been found to have the ability to produce catalase enzymes, but some bacteria do not produce this enzyme (Oktavia *et al*., 2022). Similar to the oxidase enzyme, the oxidase enzyme can be produced by proteolytic bacteria and some do not produce this enzyme (Satwika *et al*., 2021). The Gram properties of proteolytic bacteria are very diverse, groups of bacteria with Gram-positive and Gram-negative properties can produce protease enzymes (Zidna *et al*., 2021).

Conclusion

The results show that the proportion of proteolytic bacteria that were found in the intestines of tilapia (*Oreochromis niloticus*) was 28% and 44% for blocks A and E, respectively. There was an average 0.48 proline activity index for the bacteria from the intestines of tilapia from block A and 0.75 from block E across both blocks. The highest proteolytic activity index from block A was 0.83 and in block E 3.0. The smallest activity index from both blocks was 0.22 in block A and 0.17 in block E.

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