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Discovering Living System Concept through Nano, Molecular and Cellular Biology



Graduate Program, University of Brawijaya in Cooperation With Masyarakat Nano Indonesia (MNI)



Discovering Living System Concept through Nano, Molecular and Cellular Biology

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Table of Content

Lethal Concentration 50 (LC50 - 96 hours) Nile Tilapia (Oreochromis niloticus)	
exposed Cypermethrin-based Pesticide	
(Shinta Hiflina Yuniari, Asus Maizar Suryanto Hertika, Amin Setyo Leksono)	58-62
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.01	
Microbial Community Composition of Two Environmentally Conserved Estuaries in	
the Midorikawa River and Shirakawa River	
(Tran Thanh Liem, Mitsuaki Nakano, Hiroto Ohta, Takuro Niidome, Tatsuya Masuda,	
Kiyoshi Takikawa, Shigeru Morimura)	63-70
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.02	
Rosella Flower Decreases the CML Serum and Liver Inflammation of Rats Given	
Baked-Food Diet	
(Silvy Amalia Falyani, Setyawati Soeharto, Edi Widjajanto, Ardhiyanti Puspita Ratna, Pia	
Batait Batmomolin)	71-76
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.03	
The Effect of Treatment Ant Nest (Myrmecodia pendans) on Quail Production and	
Performance (Coturnix-coturnix japonica)	77 01
(Susan Eintri Lumban Gaoi, Euny Suujarwo, Irian Hauji Djunaiui)	//-81
D01. http://ux.u01.01g/10.21//0/ub.jeis.2010.000.02.04	
The Use of Full-Grained, Fragmented, and Reduced Marine Yeast Powder in Fodder	
Formulation to Improve the Growth of Eel Fish (Anguilla bicolor)	
(Ria Retno Dewi Sartika Manik, Arning Wilujeng Ekawati, Hardoko Hardoko)	82-87
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.05	
Modified Risk Factors for Coronary Heart Disease (CHD) in Minahasa Ethnic Group	
From Manado City Indonesia	
(Ester Jeini Nelwan, Edi Widjajanto, Sri Andarini, M. Sasmito Djati)	88-94
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.06	
The Quality of Ornamental Japanese Carp (<i>Cyprinus carpio</i> var. Koi) After	
Electroporation as a Gene Material Transfer Method	
(Saidah Luthfiyah, Agoes Soeprijanto, Yuni Kilawati)	95-100
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.07	
Exploration and Antagonistic Test of Endophytic Fungi from Soybean (<i>Glycine max</i>	
L. Merr) With Different Resistance to Sclerotium rolfsii	
(Siti Aminatuz Zuhria, Syamsuddin Djauhari, Anton Muhibuddin)	101-105
DOI: http://dx.doi.org/10.21776/ub.jels.2016.006.02.08	

Lethal Concentration 50 (LC₅₀ - 96 hours) Nile Tilapia (*Oreochromis niloticus*) exposed Cypermethrin-based Pesticide

Shinta Hiflina Yuniari¹, Asus Maizar Suryanto Hertika², Amin Setyo Leksono³

¹Master Program of Environmental Resource Management and Development, Graduate School, University of Brawijaya, Malang, Indonesia

²Department of Aquatic Resources management, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia

³Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

In aquaculture of an irrigation, the use of cypermethrin-based pesticide can harm nile tilapia which cultured within and its impact on humans who consume the fish. Thus it is necessary to study to determine the threshold of this cypermethrin-based pesticide. Determining the thresholds was using the test of LC_{50} - 96 hours (Lethal Concentration 50-96 hour) which tested tilapia that dead by 50%, which can be used as a benchmark threshold for cypermethrin-based pesticide. Test of LC_{50} - 96 hours consist of preliminary test and advanced test. Lethal Concentration 50 (LC_{50} - 96 hours) of cypermethrin-based pesticide towards nile tilapia (*Oreochromis niloticus*) is 0.082 ppm. At these doses, tilapia death reached 50%. This concentration is expressed as a threshold usage of cypermethrin-based pesticide on the environment around the aquaculture of tilapia.

Keywords: Cypermehrin-based pesticide, LC₅₀96 hour, Nile tilapia.

INTRODUCTION

Pesticides are substances used to control population of certain species which are considered as pests that directly or indirectly harm the interests of human beings. The addition of pesticides also generates environmental impacts. The environmental impact will be carried along the food chain. In agriculture, the most widely used pesticide is Cypermethrin. Cypermethrin widely used in farming activities and household pest control of the world [1]. Despite its low toxicity to mammals, Cypermethrin is highly toxic to aquatic organisms and honeybees. Farmers use Cypermethrinbased pesticides at a concentration of 500 ppm per 1 liter of water.

Toxicity tests used to study the effect of a chemical toxic to certain organisms. Toxicity tests normally used are LC_{50} -96 hour, i.e. the levels of toxic materials that can cause the death of 50% of the population or the test organism within 96 hours [2]. Acute lethal toxicity is a toxic process or the entry process of toxic substances into the body causing the interference of working mechanism and the target organ. Acute toxicity test or toxicity of lethal acute also means a trial

[°] Correspondence author:

Email : shintahiflinayuniari@rocketmail.com

designed to evaluate the relative toxicity of a chemical to aquatic organisms in a limited and specified period of time. The criteria effects commonly used in lethal acute toxicity tests are the death percentage (in fish), the absence of movement (immobility) and balance, and growth [3].

This study used Nile tilapia (Oreochromis niloticus) as the test organism; types of fish that would be suitable for use in similar aquaculture research as this study [4]. Nile tilapia is one of the biota of water recommended by the USEPA (US Environmental Protection Agency), as the test animals for toxicology. This is because the distribution is guite extensive, widely cultivated has high capability in tolerating a bad neighborhood and is easily maintained in the laboratory. Additionally, nile tilapia fish is also an organism which categorized as important in aquaculture. Nile tilapia is used as bio-indicators because fish have a high resistance to a wide range of changes that occur around the neighborhood of his life, fast growth, resistance to disease and classified as sensitive fish [5]. This study was aimed to determine the treshold on cypermethrin based pesticide dose which allowed in the waters with referring to the LC₅₀ 96 hour.

Shinta Hiflina Yuniari

Address : Graduate School, University of Brawijaya, Mayjen Haryono 169, 65145 Malang

MATERIALS AND METHODS

Acute Toxicity Test Procedure (LC₅₀-96 hours)

Research procedures of acute toxicity test was performed on two stages, a preliminary test and continued with the real test. The range for testing is a multiple of 24 hours. Doses that used is based on a logarithmic scale which is read by progressive bisection [6].

Preliminary test

Preliminary test is intended to determine the range of appropriate levels of cypermethrinbased pesticide that took place during the short period. The range of concentration is expressed as the threshold lethal consisting of bottom threshold lethal (LC_0 24 hour) and the above threshold lethal (LC_{100} 12 hour) with the range of the closest with LC_{50} 96 hour. Lethal threshold levels are then used to determine the median lethal consentration (LC_{50} 96 hour). The procedure is as follows.

Basin test capacity of 16 liters was prepared for 8 units for each concentration. Then a solution of cypermethrin-based pesticide made with a concentration of 0 ppm; 0.0001 ppm; 0.001 ppm; 0.01 ppm; 0.1 ppm; 1 ppm; 10 ppm and 100 ppm according to base figures on a logarithmic scale column 1. Then, Nile tilapia (Oreochromis niloticus) is in medium-sized 7 cm -9 cm as many as 10 individuals included in each treatment. During toxicity testing, we used continuous aeration. Observations were made every 12 hours for 96 hours to determine the mortality. The observed parameter is the number of dead on Nile tilapia once every 12 hours, and calculated on a cumulative 96 hours. The mortality percentage is calculated from the number of dead fish divided by the total number originally on each treatment level.

Advanced test

Stages on advanced tests (acute toxicity test) is the first LC_0 -24 hour (bottom lethal threshold) and LC100 12h (above lethal threshold) which obtained from a preliminary test. Then the results are use to determined variations in the levels of advanced test is based on a logarithmic scale [6] precise targeting of the to test advanced by way of progressive bisection. Then, the process of acute toxicity testing (advanced test) where the procedure is the same as a preliminary test procedure. Parameter observed is the number of dead Nile tilapia every 12 hours for 96 hours calculated cumulatively [7].

Probit analysis

Data analysis in this study using probit analysis. Probit analysis is generally used to determine the relative toxicity of chemicals on living organisms. It is used to test the response of test organisms in a variety of chemical concentrations and compared them. The probit analysis was calculated through statistical data with Microsoft Excel.

RESULTS AND DISCUSSION Preliminary test

Preliminary test carried out to obtain a concentration above the threshold (LC_{100} -24 hour) as the lowest concentration in which all test fish is tilapia die within 24 hours of exposure. While the lower threshold (LC_0 -48 hour), which is the highest concentrations where all the fish are still alive in the time of exposure 48 hour [8]. Data from acute toxicity test pesticide with active ingredient cypermethrin towards the Nile tilapia (*Oreochromis niloticus*) in the preliminary test can be seen in Figure 1.



Figure 1. Mortality of Tilapia on Preliminary Test

Based on preliminary test results showed that Cypermethrin pesticide have lower threshold value 0.01 ppm to percentage mortality of 0%. The concentration is the highest concentration of the test, in which all fish is still alive (100%) within 48 hours. A threshold value above 0.1 ppm with a percentage of 70%, but the concentration is not representative or not the lowest concentration of a test in which all fish (100%) died within 24 hours. This concentration is used as the upper threshold because within 96 hours the percentage of mortality of the largest fish approaching 50%. According to Husni and Esmiralda [9] a preliminary test carried out to determine the limits of the range of critical (critical range test), which became the basis of the determination of the concentrations used in advanced test or toxicity tests indeed. That concentration can cause most of the deaths was close to 50% and lowest mortality approach 50%.

Advanced test

Advanced test is performed to determine the concentration where 50% fish dead during the period of exposure of 96 hours (LC_{50} - 96 hours). The dose used in this advanced test based on the logarithmic scale bisection [10]. This dose is guided by values above the threshold and the lower threshold limit value on a preliminary test carried out previously. The range is based on a logarithmic scale Random concentration, i.e. 0 ppm; 0.0135 ppm; 0.018 ppm; 0.024 ppm; 0032 ppm; 0.042 ppm; 0065 ppm; 0.087 ppm of Cypermethrin pesticides. Data from acute toxicity test pesticide with active ingredient Cyper-

methrin towards tilapia (*Oreochomis niloticus*) on advanced test can be seen in Figure 2.

The mortality rate of fish vary in accordance with the concentrations of pesticides are given. At the low concentration of 0.0135 ppm, 0.018 ppm, 0.024 ppm, 0.032 ppm there are no dead fish. The accumulation of contaminants in organisms differ depending on the concentration of pollutants in the water/environment, temperature, state of the test animals and physiological activity [11]. Therefore, at these concentrations, the fish can tolerate the levels of given pesticides, thus the fish survived.

At a concentration of 0.042 ppm; 0.065 ppm and 0.087 ppm, death of the fish is start happening and the highest mortality at a concentration of 0.087 ppm up to 70% mortality occurred. The fish mortality has increased with the growing number of pesticide concentrations given. A decrease in the survival test fish caused by the inability of the adaptation of fish to pollutants given in the media [12]. As a result, the fish are not able to neutralize effect that pollutants contained in the test medium. In the water, Cypermethrin is in the form of metane ethyl carboxylate, alkylation can react with Fe in hemoglobin and may replace oxygen in erythrocytes [13]. Biochemical reactions that show this biological imbalance that causes the proliferation of blood cells does not occur, as a result of blood volume remained. However, erythrocytes decreased, resulting in death due Fe Cypermethrin bound by the active ingredient.



Figure 2. Results Mortality Data Tilapia on Advanced Test

C	Σ		Repea	t	the		Duchit	
(ppm)	(x)	organism test	1 2		3	number of deaths	% mortality	value *
0	-	10	0	0	0			
0.0135	-1.8696	10	0	0	0			
0.018	-1.7447	10	0	0	0			
0.024	-1.6197	10	0	0	0			
0.032	-1.4948	10	0	0	0			
0.042	-1.3767	10	3	2	1	2	20	4.1584
0.065	-1.1871	10	2	3	3	2.67	26.67	4.375

Table 1. Table of Probit Value Calculation

Note: * Value is determined by probit transformation table

10

5

6

-1.0605

0.087



7

6

60

5.2533

Figure 3. Probit Graph

The observation of advanced test showed that the higher the concentration of pesticides given the higher fish mortality. It conformed to the previous study that mentioned the percentage of survival of tilapia fish (*Oreochromis niloticus*) decreases with increasing concentrations of the pollutants [14]. Basic calculations with observations on mortality data of tilapia (*Oreochromis niloticus*) for 96 hours at the advanced test, the results of calculations to determine the probit values are presented in Table 1.

The next step probit analysis is to create charts using Microsoft Excel where $x = \log$ concentration, and Y = probit value, the results are presented in Figure 3. Based on the graph probit (Fig. 3), we obtained line equation Y = 3280 + 8558 if it is assumed that the value of LC₅₀ 96 hour with the number of deaths is y = 5 (50%)

of the test animals, the value x = -1,084 so that the value probit is the antilog of -1,084 = 0.082. The value shows that the exposure dose of cypermethrin-based pesticide is 0082 ppm causes 50% of the population of test animals dying within 96 hours.

CONCLUSION

Lethal Concentration 50 (LC_{50} - 96 hours) of cypermethrin-based pesticide towards tilapia (*Oreochromis niloticus*) is 0.082 ppm. At these doses, tilapia will die 50%. This concentration is expressed as a threshold cypermethrin-based pesticide used on the environment around the cultivation of nile tilapia.

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Microbial Community Composition of Two Environmentally Conserved Estuaries in the Midorikawa River and Shirakawa River

Tran Thanh Liem^{1*}, Mitsuaki Nakano¹, Hiroto Ohta¹, Takuro Niidome¹, Tatsuya Masuda², Kiyoshi Takikawa³, Shigeru Morimura¹

¹Graduate School of Science and Technology, Kumamoto University, Kumamoto City, Japan
 ²Priority Organization for Innovation and Excellence, Kumamoto University, Kumamoto City, Japan
 ³Center for Marine Environment Studies, Kumamoto University, Kumamoto City, Japan

Abstract

To provide a general overview of the microbial communities in environmentally conserved estuaries, the top 5 cm of sediment was sampled from the sandy estuary of the Shirakawa River and from the muddy estuary of the Midorikawa River. Higher amounts of organic matter were detected in the Midorikawa estuary sample than in the Shirakawa estuary sample. Measurement of redox potential revealed that the Shirakawa estuary was aerobic and the Midorikawa estuary was much less aerobic. Clone analysis was performed by targeting partial 16S rRNA gene sequences and using extracted DNA from the samples as a template. Various bacteria were detected, among which *Gammaproteobacteria* was dominant at both estuaries. Unclassified clones were detected in the *Gammaproteobacteria*, *Deltaproteobacteria*, *Chloroflexi, Actinobacteria*, and *Bacteroidetes*. All the *Deltaproteobacteria* clones were anaerobic sulfate-reducing bacteria. Those aerobic and anaerobic bacteria coexisted in the top 5 cm of the estuary sediments indicating the surface layer have active sulfur and carbon cycle. Abundance of aerobic *Gammaproteobacteria* may be an indicator for conserved estuaries.

Keywords: conserved environment, clone analysis, estuary, microbial community, 16S rRNA gene.

INTRODUCTION

In recent years, environmental conditions in the Ariake Sea, which contains roughly 40% of Japan's tidal flats, have worsened, leading to problems such as red tides and reduced fishery output. Tidal flats are extremely valuable ecosystems, serving as a habitat and playing diverse roles in processes such as water purification and biological production. Microorganisms are a key part of the tidal flat system. In sandy tidal flats aerobic bacteria utilize and degrade organic compounds; however, in muddy tidal flats only the surface, not lower layers of sediment, is exposed to oxygen. Under these anaerobic conditions, hydrogen sulfide is easily generated by sulfate-reducing bacteria (SRB), producing a dark sediment color [1]. Although muddy tidal flats that have been environmentally conserved have nested holes populated by aerobic macrobenthic organisms, demonstrating that a muddy ecosystem itself is

not problematic, an imbalance in the metabolisms of anaerobic microorganisms will lead to harmful accumulation of hydrogen sulfide.

Changes have been reported in the microbial community of marine sediment following environmental contamination with pollutants such as oil [2-4] or heavy metals [5,6], but only a few studies have observed microbial communities under environmentally conserved conditions [7,8]. In the present study, two estuaries connecting to the Ariake Sea were sampled: the Shirakawa River estuary with sandy sediment and the Midorikawa River estuary with muddy sediment. Sites were chosen due to their high numbers of nested holes, the presence of salt-tolerant plants growing at higher water marks, and their conserved environmental conditions. Microbial communities were analyzed by targeting partial 16S rRNA gene sequences to provide a general overview of the microbial communities in environmentally conserved tidal flat sediment.

Correspondence author:

Tran Thanh Liem

Email : liemmt2003@yahoo.com

Address : Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami Chuo-ku, Kumamoto City, Kumamoto 860-8555, Japan

MATERIALS AND METHODS Sampling

Estuary sediment were sampled using sterilized spatula from the left bank of the Shirakawa River (N32°46′38.9″, E130°36′33.6″) and the right bank of the Midorikawa River (N32°42′48.3″, E130°37′21.2″) on May 22, 2008 and January 27, 2009. Both rivers are located in coastal areas bordering the Ariake Sea in Kumamoto Prefecture, Japan (Fig. 1). All sediment samples were collected to a depth of 5 cm at low tide. At each sampling site, samples were collected at both the high tide position and the low tide position, as shown in Fig. 2. All samples were kept at -20°C until used for analysis.



Figure 1. Sampling site Location of sampling sites in the Shirakawa and Midorikawa River estuaries connecting to the Ariake Sea

DNA Extraction

Frozen samples were thawed on ice and centrifuged at $10,000 \times g$ at 4°C for 15 min. The precipitate was washed in sterilized phosphate

buffered saline solution and was centrifuged again at the same settings. The supernatant was removed. The precipitate was mixed well and 0.5 g of the sample was used to extract DNA with the Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. DNA concentrations were measured at wavelength of 260nm (Beckman DU 530 UV-VIS Spectrophotometer).

Determination of Microbial Community

The extracted DNA was used as a template for polymerase chain reaction (PCR) targeting partial 16S rRNA gene sequences. The primer set 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 518R (5'-GTA TTA CCG CGG CTG CTG G-3') and AmpliTaq Gold (Applied Biosystems, Carlsbad, CA) were used. After preheating at 95°C for 5 min, 25 cycles were performed of heating at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. PCR products were purified by an Ultra Clean PCR Clean-up Kit (MO-Carlsbad, CA) according BIO, to the manufacturer's protocol. Purified PCR products were ligated with pT7Blue vector (Novagen, Darmstadt, Germany), using a Ligation Mix Kit (Takara, Kyoto, Japan) according to the manufacturer's protocol. Then, 100 μL of competent Escherichia coli DH5 α cells (Takara, Kyoto, Japan) was transformed with 10 µL of ligation mixture as mentioned in manufacturer's protocol. After colonies formed on an LBampicillin plate containing X-gal, white colonies were selected and plasmids were extracted using the Wizard SV Minipreps DNA Purification System (Promega, Madison, WI). Extracted plasmids were digested by EcoRI and PstI (Takara, Kyoto, Japan) to determine the size of the inserted DNA. Sequence analysis of the inserted DNA was completed by Takara Co., Ltd. by premixed sequencing with M13 (-47) primer (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3').



Figure 2. Location of sampling points at each estuary

Data from the analysis were compared to the NCBI database by a BLAST search to identify obtained clones [9]. 16S rRNA nucleic acid sequences were aligned using ClustalX 1.8 [10]. Phylogenetic trees were software constructed using MEGA 4 software [11]. In which, nucleotide sequences were used to build phylogenetic trees using Distance-based method that measures the pairwise distance between genes. More detail, we chose Neighbor-Joining method in Bootstrap test of Phylogeny option from Phylogeny menu, selected Pairwise Deletion for Gaps/Missing Data to retain all sites missing containing alignment gaps and information before start computing.

Environmental Parameters Analysis

Hydrogen ion and NaCl concentration were measured using a handheld pH meter and surface salinity meter (SSM14-P and WM-22EP; TOADKK, Tokyo, Japan). Redox potential (ORP) was measured using a portable ORP meter (RM-20P; TOADKK) and distribution of particle size was analyzed using a laser diffraction particle size analyzer (LA-920; HORIBA, Kyoto, Japan). Organic matter was determined by measurement of residual weight after heating according to the Japanese Standard Method, JIS-K0102 [12]. The partial 16S rRNA gene sequences obtained in this work have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB853487–AB853725.

RESULTS AND DISCUSSION

Environmental Conditions at Sampling Sites

Environmental conditions at the Midorikawa and Shirakawa estuaries are summarized in Table 1. Generally, pH was in the range 7.5 and 8.0. Because river water and seawater were mixed at the sites, NaCl concentration was lower than 3.5%, the average concentration of seawater, due to dilution by fresh water from the rivers. ORP values were positive for the Shirakawa estuary and negative for the Midorikawa estuary, suggesting that their conditions were aerobic and slightly anaerobic, respectively. Organic content in the sediment at the Midorikawa estuary was higher than that at the Shirakawa estuary. Median particle size data and mud content analysis suggested that the Shirakawa estuary was sandy and the Midorikawa estuary was muddy. Taken together, these results confirmed that ORP was low and organic content was high under conditions of low median particle size and high mud content.

Season	Location of estuaries	Tide level	Altitude T.P. (m)	рН	NaCl (%)	ORP (mV)	Organic matter ng/g-dry sediment)	Median particle size (μm)	Mud content (%)
May	Shirakawa	High	+1.494	N.M.	N.M.	210	6.3	143.4	31.8
		Low	-0.476	7.78	1.97	180	23.7	99.4	47.6
	Midorikawa	High	+0.553	7.68	0.76	-72	69.6	20.3	85.1
		Low	-0.833	8.60	1.34	-40	98.1	11.6	98.9
Jan	Shirakawa	High	N.M.	N.M.	N.M.	231	12.4	76.1	49.7
		Low	N.M.	7.55	0.60	227	14.7	40.9	59.4
	Midorikawa	High	N.M.	7.81	0.30	-113	47.2	42.9	67.8
		Low	N.M.	7.54	0.33	-21	76.5	17.9	86.0

Table 1. Environmental Conditions at Shirakawa and Midorikawa River Estuary

T.P.: Tokyo peil, N.M: Not measured

Homology analysis among bacterial DNA sequences

Partial 16S sequence data of the obtained clones analyzed by BLAST and their close phylum or class are summarized in Table 2. Many groups of bacteria were observed in the environmentally conserved Midorikawa and Shirakawa estuaries. In general, the dominant group was *Gammaproteobacteria*, comprising 22.6% of the total clones, and the second most dominant group was *Alphaproteobacteria*, comprising 15.9%. *Chloroflexi*, *Deltaproteobacteria*, *Actinobacteria*, and *Bacteroidetes* were also common. The microbial community composition among different sampling sites, seasons, and tidal positions are compared in Fig. 3. High ratios of *Deltaproteobacteria* and *Gammaproteobacteria* were detected in Midorikawa estuary compared to Shirakawa estuary, which seemed related to the slightly more anaerobic conditions with higher amounts of organic matter. However, *Gammaproteobacteria* in the sediment of Shirakawa-river estuary in May was not so high, the low value of organic matter was observed at both hightide and lowtide positions, as well. The ratios of *Alphaproteobacteria*, *Actinobacteria*, and *Bacteroidetes* were higher in May than in January, possibly due to greater phototrophic production of organic compounds and subsequent aerobic degradation. In January, the ratio of *Gammaproteobacteria* at the low tide position was higher than that at the high tide position. While in May, the ratio of *Gammaproteobacteria* at the high tide position was higher than that at the low tide position. Although, the organic matter values at low tide positions were always higher than those at high tide positions.

Additionally, many *Gammaproteobacteria* clones were detected at the low tide position in the Midorikawa estuary, though they were uncultured bacteria.

Sampled season		м	lay		January			
Place of estuary	Midor	rikawa	Shira	ikawa	Mido	rikawa	Shira	kawa
Tide level position	High	Low	High	Low	High	Low	High	Low
Deltaproteobacteria	3	3	1	0	7	4	2	2
Firmicutes	1	1	0	1	1	2	2	0
Chloroflexi	1	3	5	3	6	1	2	4
Epsilonproteobacteria	0	0	0	0	0	1	1	0
Alphaproteobacteria	4	2	4	10	7	4	4	3
Betaproteobacteria	0	0	0	0	0	1	1	1
Gammaproteobacteria	10	6	4	1	4	18	2	9
Bacteroidetes	2	6	5	2	1	1	2	1
Actinobacteria	1	0	4	7	3	1	6	1
Acidobacteria	1	2	1	0	6	2	4	4
Nitrospirae	0	2	0	1	0	1	1	1
Planctomycetes	1	0	2	1	2	0	0	2
Verrucomicrobia	1	0	1	0	0	0	0	1
Cyanobacteria	2	0	0	0	1	1	1	0
Thermotogae	0	0	0	2	0	0	0	0
TOTAL	27	25	27	28	38	37	28	29

Table 2. Closely related phylum or	class for the detected clones as	determined by homology analysi
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Figure 3. Comparison of microbial communities in different sediment compositions (muddy, Midorikawa; sandy, Shirakawa), at different seasons, and at different tidal positions. Patterns indicating clones: solid, *Deltaproteobacteria*; dotted, *Firmicutes*; right-down diagonals, *Chloroflexi*; vertical stripes, *Epsilonproteobacteria*; wavy lines, *Alphaproteobacteria*; hatching, *Betaproteobacteria*; open, *Gammaproteobacteria*; stitched, *Bacteroidetes*; left-down diagonals, *Actinobacteria*; horizontal stripes, *Acidobacteria*; checkmarks, *Nitrospirae*; rectangular blocks, *Verrucomicrobia*; diamonds, *Cyanobacteria*; dashes, *Thermotogae*.

Phylogenetic Tree Analysis of Bacterial Communities

Phylogenetic trees of dominant groups were constructed. The phylogenetic tree of the major group, *Gammaproteobacteria*, is shown in Fig. 4.

Many clones were most similar to uncultured bacteria. Among them, 22 clones in *Gammaproteobacteria* were close to *Pseudomonas* sp. (AB013829) isolated from deep-sea sediment [13], and were mainly observed in the Midorikawa estuary. Eight clones were similar to *Kangiella aquimarina* (NR025801), which was isolated from tidal flats of the Yellow Sea [14]. *K. japonica* was also isolated from coastal seawater and sediment samples in the Sea of Japan, Russia [15]. Some *Gammaproteobacteria* clones were related to *Nitrosococcus oceani* (AB474000) [16] and others were close to *Haliea* sp. [17,18]. However, two clones detected in January were true sulfur-oxidizing bacteria (SOB) [19]. The phylogenetic tree for *Deltaproteobacteria* is shown in Fig. 5. All clones appeared to be SRB, and could be divided into 6 groups based on 16S rRNA gene sequences [20]. *Desulfotomaculum* group was not detected and 5 other groups were detected. It showed same results of isolated SRB from estuarine sediment in Japan [21]. All 7 clones grouped in *Desulfosarcina*, *Desulfococcus*, and *Desulfonema* were detected in May as well as in January.



Figure 4. Neighbor-joining tree of Gammaproteobacteria clones based on partial 16S rRNA gene sequences



Figure 5. Neighbor-joining tree of Alphaproteobacteria clones based on partial 16S rRNA gene sequences



Figure 6. Neighbor-joining tree of Deltaproteobacteria clones based on partial 16S rRNA gene sequences

The SRB communities in the Midorikawa and Shirakawa estuaries were quite diverse. However, SRB were detected mainly in the Midorikawa estuary and were observed to a greater extent in January and at the high tide position. About 10% of the analyzed clones were SRB (Fig. 3), but few SOB clones were detected (Fig. 4), even though in an environmentally conserved tidal flat the hydrogen sulfide generated by SRB would likely be oxidized by SOB.

(Liem et al.)

Anaerobic, facultatively anaerobic, and aerobic bacteria were observed in not just *Proteobacteria*, but also in the *Chloroflexi* group. However, in *Actinobacteria* and *Bacteroidetes* only aerobic bacteria were observed in both estuaries, both seasons, and both tidal positions (data not shown). Many of the identified clones were similar to salt-tolerant genera such as *llumatobacter* [22] and *Aestuariicola* [23]. The results presented here suggest that not only aerobic SOB, but also anaerobic phototrophic purple bacteria and anaerobic SRB might coexist in the top 5 cm of the sediments in the Shirakawa and Midorikawa.

At those conserved tidal flats, the ratio of *Gammaproteobacteria* and *Alphaproteobacteria* were higher than that of *Deltaproteobacteria*, the hydrogen sulfide producer. In comparison, at another location in Japan which is functionalized for intensive shellfish aquaculture, *Deltaproteobacteria* were detected as the most abundant [24]. Abundance of *Deltaproteobacteria* is stimulated by high content of organic matter and thus leads to enhancement of hydrogen sulfide production by the bacteria.

CONCLUSION

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According to 16S rRNA, the bacterial community was found in high diversity with many clones close to uncultured strains in the tidal flats. Complex material cycles involving aerobic and anaerobic activities may mostly exist in the surface layer of sediment which was mainly performed by the abundant *Gammaproteobacteria*. The dominance of *Gammaproteobacteria* over anaerobic *Deltaproteobacteria* can be an indicator for unspoilled tidal flats.

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Rosella Flower Decreases the CML Serum and Liver Inflammation of Rats Given Baked-Food Diet

Silvy A. Falyani^{1,2}*, Setyawati Karyono³, Edi Widjajanto⁴, Ardhiyanti P. Ratna¹, Pia B. Batmomolin¹

¹Master Program of Biomedic, Faculty of Medicine, University of Brawijaya, Malang, Indonesia
 ²Department of Pathology Anatomy, Malang Islamic University, Malang, Indonesia
 ³Department of Pharmacology, Faculty of Medicine, University of Brawijaya, Malang, Indonesia
 ⁴Department of Clinical Pathology, Faculty of Medicine, University of Brawijaya, Malang, Indonesia

Abstract

Advanced Glycation Endproducts (AGEs) or so-called glycotoxin can be triggered by heated food in which the Maillard reaction occurs. One type of glycotoxins is CML. Accumulated N-Carboxymethyl-Lysine (CML) can cause inflammation of organs, e.g. liver. Rosella flowers contain anthocyanin compound that has anti-glycation and antioxidant effects. This study aimed to determine the effect of ethanol extract of Rosella on CML serum level, IL-6 level, and NF-kB activation in the liver of mice fed with baked food. This study used post-test design using 25 Wistar rats aged 3-4 months old that were divided into 5 groups, namely negative control group, positive control group, treatment groups given Rosella extract dose of 200mg.kgBW⁻¹, 300mg.kgBW⁻¹, and 400mg.kgBW⁻¹. Baked feed was given for 12 weeks, and Rosella ethanol extract was administered in the 9th to 12th week. The examination on CML serum and IL-6 of the liver was using ELISA method. Immunofluorescent staining was used to determine NF-kB activation in the liver using a confocal microscope. CML serum is proven to increase significantly (p = 0.000). The effective dose of Rosella flower extract to prevent CML Serum increase is 200 mg.kgBW⁻¹, whereas a dose of 400 mg.kgBW⁻¹ can decrease IL-6 level and NF-kB activation. Ethanol extract of Rosella flower decrease the levels of N-carboxymethyl-lysine serum, IL-6, and NF-kB activation in the liver of rats given baked-food diet.

Keywords: Advanced Glycation Endproducts (AGEs), Interleukin-6, N-Carboxymethyl-lysine, Rosella.

INTRODUCTION

Modernization results in major changes in human behavior. Being busy due to working activities is one of the reasons for people to seek more practical food. Preparing dishes by steaming or frying method have been shifted into cooking using oven and other new technologies. In fact, heating at high temperatures can increase the level of Advanced Glycation Endproducts (AGEs) or called glycotoxin. Glycotoxin is a compound formed from non-enzymatic glycation reactions between proteins and sugar residues. The best known of Glycotoxin type is Ncarboxymethyl-lysine (CML), pentosidine, pyrraline, and methylglyoxal. CML is often used as a marker for the formation of glycotoxin, as CML is a glycotoxin type which is most common and best known for in vivo characterization [1].

Glycotoxin accumulate in many tissues of the body, and when accumulated, it increases the inflammatory reaction, weakens the immune

Correspondence author:

Silvy Amalia Falyani

Email : silvyamaliafalyani@gmail.com

Address : Biomedical Program, Faculty of Medicine, University of Brawijaya, Veteran St. Malang 65145 system, increases the infection possibilities, lowers the antioxidant defense mechanism, interferes DNA repair mechanisms, and increases the accumulation of various kinds of toxins [2,3].

Liver functions in metabolism are not excluded from glycotoxin accumulation. Liver cell damage can occur due to inflammatory reactions and oxidative stress [4]. NF-kB which is a transcription factor in mammals controls a number of important genes in immunity and inflammatory processes. NF-kB activation will stimulate proinflammatory cytokines such as Interleukin-6 (IL-6), and increase NF-kB activation that can be a marker of organ inflammation.

Anthocyanin is a type of polyphenols known as antioxidants among the group of flavonoids. Anthocyanin is known to inhibit glycation process and glycotoxin binding to its receptor, as well as to prevent lipid peroxidation and polyol pathway inhibition [5,6].

Hibiscus sabdariffa L. or commonly known as Rosella empirically has been consumed by humans in some parts of the world. In vitro and in vivo researches prove beneficial phytochemical content in Rosella flower extract. Rosella flower extract is known to offer antibacterial effect, antioxidant, hepato and nefro-protective, antiinflammatory, and anti-cholesterol [7,8].

By considering the work of anthocyanin as AGE inhibitor, antioxidant, and anti-inflammatory, it raised presumption that Rosella flower ethanol extract can repair liver damage caused by glycotoxin. Within this assumption, the researchers wanted to determine the effect of Rosella flower (*Hibiscus sabdariffa* L.) ethanol extract in inhibiting pro-inflammatory signaling pathways of liver tissue mediated by Ncarboxymethyl-lysine in Wistar rats given bakedfood diet.

MATERIALS AND METHODS Subject

This study used post-test design that compares the control and the treatment groups. Animals used were 25 male Wistar rats aged 3-4 months old with 100-150 gram initial body weight. Rats that were ill or died during the research were excluded from the population.

Rats were adapted to research environment condition for 1 week, and fed with standard feed and drinking water in ad libitum method. Rats were placed in cages measuring \pm 900 cm³ for 4-5 rats. After passing the adaptation stage, the rats were weighed to determine the initial weight, then the rats were randomly divided into five groups that consisted 5 rats each. Furthermore, each rat was placed in a different enclosure. Negative control group (KN) was fed with standard diet, while positive control group (KP) and treatment groups given Rosella extract dose of 200mg.kgBW⁻¹ (KR₁), 300mg.kgBW⁻¹ (KR₂), and 400mg.kgBW⁻¹ (KR₃) were fed with baked food for 8 weeks. After 8 weeks, CML serum level measurement was conducted using ELISA method.

Broiler feed 1 (BR1) was used because it contains the highest protein than other feeds. This feed contains 21-23% protein, 5% fat, 40-45% starch and 5% crude fiber. Oven was preheated at 150° C for 15 minutes.

Treatments and Data Collection

At 9th week, provisions of Rosella flower ethanol extract were given to the treatment group KR₁, KR₂, and KR₃ as much as 200 mg.kgBW⁻¹, 300 mg.kgBW⁻¹, and 400 mg.kgBW⁻¹ respectively for 4 weeks. Baked food feeding was expected to be given until the 12th week.

At 13th week, the animals were dissected. Blood was drawn intracardially for CML examination using ELISA kit (Bioassays, Number catalogs E1374Ra). Livers harvesting were performed for NF-kB activation examination using immunofluorescent method using primary NF-kB P65 antibody (ThermoFisher, Number catalog MA5-15160), hepatic IL-6 examination was using ELISA kit (Bioassays, catalog Number E0135Ra), and hepatic organ staining was using Haematoxylin-eosin.

Data Analysis

Once the data were obtained, the data were statistically analyzed using SPSS 24.0. Data normality test was done using Shapiro-Wilk test. Data homogeneity was analyzed using Levene's Test. Data comparison was using independent t-test, ANOVA, and Post Hoc. Results can be said significant when P <0.05.

RESULTS

CML Serum Levels

Assessment on the effect of Rosella flower ethanol extract on CML serum levels in rats given baked food was done using ANOVA test. The rats' CML serum levels were obtained using ELISA kit (Bioassays, catalog Number E1374Ra). Figure 1 is a histogram of CML serum levels. The histogram shows that increased levels of CML serum in mice are between negative and positive control group. Levels of CML serum in treatment groups are lower than those of the positive control group. It proves that Rosella flower ethanol extract influences the final levels of Rats' CML level.



Description: KN is negative control group (standard feed, without extract), KP is positive control group (fed with baked food, without extract), KR₁ is treatment group 1 (fed with baked food + Rosella flower ethanol extract 200 mg.kgBW⁻¹), KR₂ is treatment group 2 (fed with baked food + Rosella flower ethanol extract 300 mg.kgBW₋₁), and KR₃ is treatment group 3, (fed with baked food + Rosella flower ethanol extract 400 mg.kgBW₋₁)



There is a significant difference (p = 0.001) between the negative control group and positive control group. It indicates that baked food has an influence or affects the results of rats' CML serum. The positive control group has a significant difference compared with treatment group 2 (p = 0.05). It demonstrates that Rosella flower ethanol extract dose of 300 mg.kgBW⁻¹ can prevent increasing CML serum levels of rats fed with baked food.

Hepatic NF-kB Activation

Assessment on Rosella flower ethanol extract effect to hepatic NF-kB activation of rats fed with baked food were evaluated based on the amount of transcription factor expression of activated NFkB so that translocate from the cytoplasm to the nucleus of liver tissue was examined using immunofluorescent method with p65 antibody (ThermoFisher, catalog Number MA5-15 160).

Figure 2 is a histogram of hepatic NF-kB activation with ANOVA test. The histogram shows that when the control groups are compared, the positive control group has higher results compared to the negative control group. This might indicate that there were more hepatic NF-kB activations in the positive control group. The treatment group 3 has the lowest result compared with other treatment groups. This showed that the provisions of Rosella flower ethanol extract dose of 400 mg.kgBW⁻¹ were effective in suppressing NF-kB activation in liver of rats fed with baked food. A comparative figure of each group using immunoflourescent method and haemato-xylen eosin showed in Figure 3.



Figure 2. Histogram of Hepatic NF-KB Activation using ANOVA test

Description: KN is negative control group (standard feed, without extract), KP is positive control group (fed with baked food, without extract), KR₁ is treatment group 1 (fed with baked food + Rosella flower ethanol extract 200 mg.kgBW⁻¹), KR₂ is treatment group 2 (fed with baked food + Rosella flower ethanol extract 300 mg.kgBW₋₁), and

KR₃ is treatment group 3, (fed with baked food + Rosella flower ethanol extract 400 mg.kgBW.₁)



Figure 3. The Comparative Picture of Each Group Using Immunofluorescent Method and Haematoxylen Eosin

Description: KN is negative control group (standard feed, without extract), KP is positive control group (fed with baked food, without extract), KR₁ is treatment group 1 (fed with baked food + Rosella flower ethanol extract 200 mg.kgBW⁻¹), KR₂ is treatment group 2 (fed with baked food + Rosella flower ethanol extract 300 mg.kgBW₋₁), and KR₃ is treatment group 3, (fed with baked food + Rosella flower ethanol extract 400 mg.kgBW₋₁).

The observation was using a confocal microscope Olympus FV 1000 and calculated using Olympus Fluoview Version. 1.7a software.

Figure 3 is a comparative picture of each group using immunofluorescent and haematoxylen eosin. The calculated amount of NF-kB activation is the amount contained in the nucleus and appears red in the green color.

IL-6 levels

IL-6 levels in liver tissue are pro-inflammatory cytokine levels that may become an inflammation indicator, the levels in rats' liver tissue (units of ng.mL⁻¹) are measured using ELISA method (ELISA kit such as Bioassays, catalog Number E0135Ra).



Figure 4. Histogram of IL-6 Levels using ANOVA test

Description: KN is negative control group (standard feed, without extract), KP is positive control group (fed with baked food, without extract), KR₁ is treatment group 1 (fed with baked food + Rosella flower ethanol extract 200 mg.kgBW⁻¹), KR₂ is treatment group 2 (fed with baked food + Rosella flower ethanol extract 300 mg.kgBW₋₁), and KR₃ is treatment group 3, (fed with baked food + Rosella flower ethanol extract 400 mg.kgBW₋₁)

Figure 4 is a histogram of IL-6 levels of rats' liver. The histogram shows that there is an increase in IL-6 levels in rats between negative and positive control group. IL-6 levels of treatment groups show lower results than the positive control group. It explains that Rosella flower ethanol extract influences and affectes the levels of CML serum on rats. The histogram may also prove that Rosella flower ethanol extract dose of 400 mg.kgBW⁻¹ in treatment group 1 is proven to be more effective to reduce IL-6 levels of rats fed with baked food.

DISCUSSION

This study used Wistar rats as they are easily maintained and relatively healthy, so that it meets the criteria as experimental animals in a study. Anatomical and physiological study of rats supports a nutrition experiment by using ad libitum method. There are two characters that distinguish rats from other laboratory animals, namely rats cannot vomit because of the unusual anatomical structure in the esophagus, which empties into the stomach, and has no gallbladder. This study used rats aged 2-3 months, which is analogically the age of reproductively mature or adolescence in rats [9].

Oral baked food feeding has advantages and disadvantages. Glycotoxin absorption of oral consumption is as much as 10% on the peak absorption at 6-12 hours after consumption. Glycotoxin duration in the body is 72 hours, and after 72 hours, ¹/₃ of the total absorbed will be eliminated by kidneys [10]. Glycotoxin bioavailability depends on the peptide size, the food type, the intestinal environment, and the presence duration in intestine. Glycotoxin can be easily distributed to the extracellular and intracellular compartments because it has an amphoteric character and soluble in water. Studies in animals have shown that after 72 hours, 60% of the total absorbed glycotoxin will be bound in the liver and kidneys, but the radioactivity was detected in the lungs, the heart, and the spleen. This gives an indication that glycotoxin is distributed throughout all body tissues. Glycotoxin elimination occurs through the kidneys, and lasts for 72 hours after ingestion [10].

Rats which were given the baked food have shown more increasing levels of N-Carboxymethyl-Lysine (CML). CML is one of glycotoxins derived from food [1]. There are three paths of glycotoxin formation, namely Maillard reaction, glucose oxidation and lipid peroxidation, as well as the polyol pathway. The variation of pathway formation causes various glycotoxin chemical structures. The best-known glycotoxin type is carboxymethyl-lysine (CML), pentosidine, pyrraline, and methylglyoxal [1]. Among many types of glycotoxin, CML is the most possible type that can be characterized. CML is first identified in food, and it becomes the most frequent marker often used for research as a marker of increasing level of glycotoxin [11]. In this study, the p-value obtained was 0.001 (p <0.05). It clearly explains that there is a significant difference between the initial and the result of CML serum levels of the negative control group and the positive control group. This is consistent with result of previous research that explains glycotoxin content can increase by 10-100 times by heating process [10].

When food is heated, there is a transformation of electromagnetic wave energy into heat energy. Previously, heating by using microwave has shown to increase the Maillard reaction and other chemical reactions significantly [11]. Maillard reaction is greatly influenced by the heating time, temperature, concentration, humidity, and acidity. In some previous studies, the Maillard reaction rate would double if the temperature rises 10°C. If the Maillard reaction rate is characterized by a change into brownish color on the food, then heating either for 4 weeks at a temperature of 20°C, 3 hours at a temperature of 100°C, or 15 minutes at a temperature of 150°C will give the same result [12]. This is consistent with the results of this study, that there is an increase in CML serum as much as three times higher than that of the negative control group.

In this study, rats that are induced with baked food, and then treated with provisions of Rosella flower (*Hibiscus sabdariffa* L) ethanol extract dose of 200, 300, and 400 mg.kgBW⁻¹ have lower levels of CML serum, compared to the positive control group. There is a significant difference (P <0.05) in groups of rats given dose of 200 and 300 mg.kgBW⁻¹ Rosella flower ethanol extract.

Rosella flower ethanol extract contains anthocyanin pigments. Anthocyanin is a type of antioxidants in the group of flavonoid. Anthocyanin is found in fruits and vegetables, especially those having red, blue, or purple colors. Anthocyanin has pigment colors that can dissolve in water and gives red, purple, and blue colors in many fruits, vegetables, flowers and seeds [13]. Anthocyanin is one type of polyphenols that can prevent glycotoxin formation. A study explains that phenolic antioxidants, in addition as a free radical scavenger, are serving as an AGE inhibitor [14]. Anthocyanin can inhibit the formation glycotoxin through inhibition of auto-oxidation monosaccaride [15]. Anthocyanin also inhibits the glycation process and binds glycotoxin with its receptor as well as prevents lipid peroxidation, and inhibits polyol pathway [5]. This is consistent with this research result where the CML serum levels of rats in the treatment group were lower than that in the positive control group.

The effective dose of Rosella flower ethanol extract to prevent an increase in CML is expected at a dose of 300 mg.kgBW⁻¹. This result almost similar to previous research that mentioned the effective dose in lowering blood glucose levels of mice induced by Streptozocin is 288 mg Rosella flower extract [16].

Nowadays, herb medication is still being subject of debate because the doses given are not always on target. Although antioxidant is highly contained in herb, it does not necessarily give a good effect on human body. Polyphenols may become pro-oxidants when given in high doses [17,18]. Pro-oxidants can trigger cell damage through oxidative stress mechanisms. Oxidative stress conditions can trigger glycation, so that pro-oxidant provision can increase the possibility of glycation. This is consistent with the results of this study in which levels of serum CML, as a marker of glycation end products, are likely to increase in the provision of higher Rosella flower extract.

The study also shows hepatic NF-kB activation as a transcription factor by using the control and treatment groups. The treatment groups were divided into doses of 200, 300, and 400 mg.kgBW⁻¹. Assessments of Rosella flower ethanol extract effecting hepatic NF-kB activation of rats fed with baked food are evaluated based on the amount of transcription factor expression of activated NF-kB so that translocate from the cytoplasm to the nucleus on the liver tissue was examined using immunofluorescent method with p65 antibody.

The ANOVA test results shows the p-value was 0.003 (p> 0.05), it is indicated that there is a significant effect of ethanol extract of Rosella flower to the activation of NF-kB liver of rat fed with baked food. There are significant differences in the positive control group with the group given dose of 400mg.kgBW⁻¹. This is consistent with the hypothesis that ethanol extract of Rosella flower may prevent an increase in NF-kB activation in liver.

NF-kB that is a transcription factor in mammal controls a number of genes that are important in immune and inflammatory processes. Some examples of these genes are Ig-ĸ light chains, T-cell receptor α and β chains, MHC class I proteins and cytokines such as GM-CSF, IL-6, IL-2 and TNF- α . Viruses like HIV use NF-kB to activate its gene transcription [19]. Inflammation is known to contribute to the pathophysiology of many chronic diseases. When the inflammatory process continues over time, it damages the surrounding tissue, disrupts the network functions, and may cause widespread the damage of organs. This process will then lead to various diseases [4]. Rosella flower ethanol extract which has a compound of anthocyanin as antioxidant is expected to decrease the inflammation due to activation of pro-inflammatory cytokines.

This study also assesses IL-6 level in the liver, where there is a significant correlation between the positive control group with the treatment group given Rosella flower ethanol extract dose of 400 mg.kgBW⁻¹ (p <0.05). This is certainly in line with the results of NF-kB activation, where the effective dose of extract is 400 mg.kgBW⁻¹.

CONCLUSION

Baked Animal Feed can increase Ncarboxymethyl-lysine (CML) serum levels of rats. Rosella flower ethanol extract dose of 200 mg.kgBW⁻¹ decreases CML serum levels of rats fed with baked food. Rosella flower ethanol extract dose of 400 mg.kgBW⁻¹ prevents the increase of IL-6 level and the NF-kB activation in the liver.

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The Use of Full-Grained, Fragmented, and Reduced Marine Yeast Powder in Fodder Formulation to Improve the Growth of Eel Fish (*Anguilla bicolor*)

Ria Retno Dewi Sartika Manik^{1*}, Arning Wilujeng Ekawati², Hardoko²

¹Master Program, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia ²Faculty of Fiheries and Marine Scineces, University of Brawijaya, Malang, Indonesia

Abstract

Ikan Sidat or eel fish (*Anguilla bicolor*) is a popular fish commodity in domestic trade as well as for export commodity. The use of marine yeast as fodder suffers from some weaknesses since it contains nucleic acids and rigid thick cell walls that obstruct the absorbance of nutrition. Optimizing the use of marine yeast as fodder ingredients can be done by fragmenting and reducing the yeast. This research attempts at optimizing the use of marine yeast regarding its rate of growth, fodder efficiency and the survival rate of eel fish. This research employs the complete random design using 4 different treatments and 3 repetition. In the four treatments, the fodder contained protein (45%) and iso energy (3.600 kkal.kg⁻¹ fodder), which were applied to the treatment A 0% (control), B 5% (complete), C 5% (fragmented) and D 5% (reduced). The parameters used in this study were survival rate, specific growth rate, ratio of fodder conversion, ratio of protein efficiency, energy retention, protein retention and rate of metabolism. The data of this study showed the best result in the treatment D, 5% (reduced), showing survival rate value of 95.56%, specific growth at 1.12, food conversion ratio of 2.27, protein efficiency ratio of 0.98, protein retention at 18.26%, energy retention of 16.57, and protein metabolism rate at 81.96%.

Keywords: eel fish, growth, marine yeast.

INTRODUCTION

Eel fish (*Anguilla bicolor*) is a popular domestic and export fish commodity in Indonesia. The production of eel fish in the world has increased as much as 30.07% from the year 2000 at 210.228 tons to 273.449 tons in 2007 [1]. However, in 2008 to 2011, the aqua culture production of eel fish has been gradually decreased until at this present time [1].

Fodder for eel fish should contain a balance protein at about 40-50% which requires huge amount of ingredients that contain high protein such as fish powder and soya powder [2]. Marine yeast is a cellular organism with chemoorganotroph characteristics which has sexual reproduction using spores and asexually using buds or fission. Marine yeast contains high complex B vitamin (thiamin, riboflavin, nicotinate, and biotin) [3]. Marine yeast as the source of protein also has a number of benefits such as having high rate of growth, ability to grow in simple media, ability to grow at high cell solidity, high nutrients, high metabolism rate, non-toxic, easy to find and having no negative impact [4].

However, the use of marine yeast suffers from a problem in which the yeast contains nucleic acids and rigid thick cell wall components that obstruct the absorption of nutrition [5,6,7]. Optimizing the use of marine yeast as fodders can be done by reducing the nucleic acids and cell walls through fragmentation method and reduction method. Cells of the yeast were sliced through sonification treatment in which the yeast was sonificated for 20 minutes at the ultrasound of 20kHz, producing protein release at 35% [8]. The best attempt to decrease the content of nucleic acids was the treatment at pH (2) and temperature of 90°C which produced nucleic acids up to 1.2% [9].

According to the explanations, it is necessary to conduct a research which deeply investigates the experiments using full-grained marine yeast without fragmentation and reduction compared to experiments using fragmented cell walls of marine yeast and marine yeast which nucleic acids are reduced. Besides, it is also important to explore the potency of marine yeast powder to use in fodder formulation and its influence in the growth of eel fish stadia elver by measuring the survival rate, specific rate of growth, fodder conversion ratio, efficiency ratio, energy retention, protein retention and metabolism rate.

Correspondence author:

Ria Retno Dewi Sartika Manik

Email : riaretnocantik@gmail.com

Address : Faculty of Fisheries and Marine Sciences, University of Brawijaya, Veteran St. Malang 65145

MATERIALS AND METHODS The Making of Marine Yeast Powder

The first step in making the powder was culturing the marine yeast for 5 days. After five days, the marine yeast was filtered and drained for 3 days at room temperature. The making of fragmented marine yeast powder was conducted using sonicator at 20 kHz frequency for 20 minutes [10] while the nucleic acids-reduced marine yeast powder was obtained by heating it up at the temperature of 90°C and adding the acidity at pH 2[9].

Fodder Production

The formulation of the fodder consisted of fish powder as the main ingredients, soya powder, polar powder and marine yeast powder which were added up with tapioca powder, cmc, Cr_2O_3 , vitamins and minerals. Those ingredients were than mixed as the fodder that supplies good nutrition needed by the eel fish (*A. bicolor*) with isoprotein of 45.01% and iso energy of 3.600 kkal.kg⁻¹ [11]. The fodder formulation used in the experiment is presented in Table 1.

Biological Test

The different fodder products were then tested biologically, involving the eel fish (*A. bicolor*) stadia elveras the subject of the experiment. The eel fish breeds (*A. bicolor*) were obtained from Cilacap, Central Java. The fish had average length of around 15 cm and average weight of 7.08 \pm 0.22 g per fish which were kept in an 30 x 30 x 30 cm³ aquarium with freshwater as the media. In the outer part of the aquarium, black polybag plastics, aeration, and shelter were applied to keep the fish in a good condition [12]. The best distribution of the eel fish (*A. bicolor*)

was at 0.3 kg.m⁻² [13]. The feeding was done at 3% of the biomass weight for twice a day with 40% fodder given at 08.00 am, and 60% at 19.00 pm [12].

Table 1. Fodder Formulation in the Experiment						
Ingradiants	Treatment (%)					
ingretients	Α	В	С	D		
Fish powder	54.67	54.67	54.67	54.67		
Soya powder	27.85	26.46	26.46	26.46		
Polar powder	8.07	8.07	8.07	8.07		
Marine yeast powder	0.00	2.61	2.16	5.98		
Tapioca powder	3.82	4.76	3.43	3.59		
Vitamins and Minerals	1.50	1,50	1.50	1.00		
Cr ₂ O ₃	0.50	0.50	0.10	0.10		
CMC	3.59	1.43	3.61	0.14		
Proximate Composition	1					
Ash (%)	15.33	15.22	15.68	17.65		
Protein (%)	46.13	45.14	45.53	45.36		
Fat (%)	17.88	17.88	15.69	18.00		
Rough fiber (%)	5.82	7.47	7.50	6.40		
BETN (%)	14.83	14.29	15.59	12.59		
DE (kkal.g ⁻¹)	4.05	3.99	3.86	3.94		

Statistical Analysis

The data obtained from the experiment were then analyzed using quantitative measurement to see the survival rate, specific growth rate, feed conversion ratio, protein efficiency ratio, protein retention, energy retention and the metabolism rate of the fodder. The test was conducted using the variety test (ANOVA) which seek for any influences of the treatment which was then followed by Tukey test.

RESULTS AND DICUSSION CML Serum Levels

The result of the observation on each of the parameter in each treatment given during the experiment can be seen in Table 2.

				J
Parameter	A (Control)	B (Full-grained)	C (Fragmented)	D(Reduced)
Survival rate (%)	86.67 ± 0.00^{a}	75.56 ± 13.88 ^ª	86.67 ± 11.55 ^ª	95.56 ± 3.85 ^a
SGR (%/weight/day)	0.74 ± 0.03^{b}	0.51 ± 0.04^{a}	0.70 ± 0.06^{b}	$1.12 \pm 0.08^{\circ}$
FCR	3.46 ± 0.16^{a}	5.07 ± 0.36^{a}	$3.54 \pm 0.32^{\circ}$	2.27 ± 0.17^{a}
PER	0.62 ± 0.02^{b}	0.43 ± 0.03^{a}	0.62 ± 0.06^{b}	$0.98 \pm 0.07^{\circ}$
Protein Retention	7.56 ± 1.14 ^b	$3.00 \pm 1.26^{\circ}$	6.69 ± 1.74^{b}	18.26 ± 1.32 ^c
Energy Retention	5.91 ± 0.93^{b}	2.21 ± 1.26^{a}	5.03 ± 1.53^{b}	16.57 ± 1.09 ^c
Protein Metabolism Rate	78.51 ± 2.36 ^a	76.59 ± 1.39 ^ª	78.18 ± 2.08^{a}	81.96 ± 1.71^{b}

Table 2 The Use of Marine	veast Powder in the Formulatic	on of Fodders for Fel Fish	(Anguilla hicolor)
	yeast rowder in the ronnulatio	IT OF FOULERS TOF LEFTISH	(Angunia bicolor)

Notes : Similar notations show no differences, while different notations show different results among the treatments (level of trust at 95%)

Survival Rate

Based on the data of survival rate, followed by the normality and homogeneity tests of the data using the SPSS program version 16.00 showed result that the data were homogeneous and were normally distributed. The result of the variety test showed one way variety (oneway ANOVA) which survival rate value of the eel fish (*A. bicolor*) was not far different among the treatments (p > 0.05).

The average survival rate value obtained from those treatments was 75.56- 95.56%. The highest survival rate value was found in the treatment D at 95.56% \pm 3.85, followed by the result of the treatment A (86.67% \pm 0.00), C (86.67% \pm 11.55) and treatment B (75.56% \pm 13.88), respectively. The result of this study shows relatively higher survival rate value compared to the other result [14] which was obtained by giving natural food which resulted to survival rate value of the eel fish (*A. bicolor*)at 36.8%. The mortality pattern of eel fish (*A. bicolor*)can be seen in Figure 1.



Figure 1. Mortality Pattern of Eel Fish (A. bicolor) Found during the Treatment
Description: SR = Survival Rate; The fodder contained protein (45%) and iso energy (3.600 kkal.kg⁻¹ fodder) applied to A 0% (control), B 5% (complete), C 5% (fragmented) and D 5% (reduced).

The high value of survival rate found in this study might be caused by the reduced nucleic acids of the marine yeast at the dosage of 5% to substitute the use of soya powder in the fodder formulation which resulted to the best response toward fish' immunity. Marine yeast contains B complex vitamis such as thiamin, riboflavin, nicotinate and biotin. The B complex vitamin has been known to give some benefits such as producing the energy, maintaining the health of the nerve system, maintaining the digestive system and improving the cell production [15,16].

Specific Growth Rate

Specific growth rate refers to the change of individual's weight within a certain time period. The graphic of eel fish' specific growth rate in this study is presented in Figure 2.

The best result was obtained when the marine yeast was reduced in the D treatment with growth percentage at 1.12% weight⁻¹.day⁻¹ followed by treatment A (0.74 % weight⁻¹.day⁻¹),

C (0.70% weight⁻¹.day⁻¹), and B (0.51% weight⁻¹.day⁻¹). The result of the specific growth rate (SGR) found in this study is relatively higher than the result of research [12] which formulated the fodder using the silage of fermented pace leaves and *L. plantarum*at 0.37 – 0.73% weight⁻¹.day⁻¹. However, the result of this study is close to the result of research [17] which treated the fish with natural fodder with specific growth rate at 0.66 – 1.096% weight⁻¹.day⁻¹.





The problem in the use of marine yeast as fodder relies on the high amount of nucleic acids which limits the absorption of nutrition [5]. Therefore, in this study, the marine yeast undergone three different treatments to decrease the amount of nucleic acid by keeping it full-grained, fragmented and reduced as presented in Table 3. The result of this study showed that the lesser the amount of nucleic acid in marine yeast, the higher the specific growth rate of the eel fish (*A. bicolor*). The result of the nucleic acid found in this study is relatively low than the previous results of research which reduced yeast conducted pH 2 and temperature 90°C produced 1.2% nucleic acids [9].

Table 3. Nucleic Acids in Marine Yeast				
Marine Yeast	Nucleic Acid Content (%)			
Full-grained	11,72 ± 0.22			
Fragmented	9,29 ± 0.16			
Reduced	$1,40 \pm 0.04$			

Fodder Conversion Ratio

The average value of fodder conversion ratio of eel fish (*A. bicolor*) during this research varied from 2.27% - 5.07. These values are smaller compared to the results of previous research which found values of fodder conversion ratio at

6.73 with natural fodder and 9.91 with 25% formulated fodder and 75% natural fodder[18].

The value of fodder conversion ratio is affected by several factors including the quality and quantity of the fodder, the species of fish, water amount and water quality. The increase on the value of fodder conversion ratio is influenced by the high amount of nutrients which are not optimally absorbed by the body or wasted through the feces [19].

Protein Efficiency Ratio

The average of protein efficiency ratio of the eel fish (A. bicolor) recorded in this study varied between 0.43 - 0.98. The value of protein efficiency ratio is affected by the protein quality in the fodder in which the higher the value of protein efficiency ratio, the better the quality of the protein in the fodder which improves the growth rate of the eel fish (A. bicolor) [20]. Protein quality of fodder ingredients is determined by the amount of amino acids in the ingredients especially the essential amino acids (arginine, histidine, isoleusin, leusin, lysine, phenylalanine, methionine, threonine, tryptophan and valine). Amino acids in the fullgrained marine yeast, fragmented marine yeast, and reduced marine yeast showed positive correlation in which the higher the amount of essential amino acids, the higher the protein efficiency ratio. The amount of essential amino acids found in this study can be seen in Figure 3.



HIS ARG THR VAL MET LYS. ILE LEU PHE

Figure 3. The Amount of Essential Amino Acids in The Full-Grained, Fragmented, and Reduced Marine Yeast Powder

Description: HIS= Histidine, ARG= Arginine, THR= Threonine, VAL= Valine, MET= Methionine, LYS= Lysine, ILE= Isoleusin, LEU= Leusin, PHE= Phenylalanine

Protein Retention

Protein retention is the reflection of the amount of absorbed protein to build and heal the

85

broken cells and which is used to maintain the metabolism system [20]. Based on the result of the Tukey test, it can be seen that the treatment D showed obviously best result at 18.26% compared to the other treatments, followed by treatment A (7.56%), C (6.69%) and B (3.00%).

The average value of protein retention in this study varied between 3.00 - 18.29 %. The value of the protein retention found in this study is a little bit higher than the result of research [11] at 1.31 - 13.22 % using formulated fodder with different protein and energy ratios. The excretion process and the catabolism of amino acids require high amount of energy, making the allocation of the energy to retain the protein in the body decreased. Protein retention is influenced by various factors such as the amount of the protein in the fodder, the balance of amino acids amount and fodder energy ratio [21].

Energy Retention

The result of the Tukey test applied to the treatment D showed obvious difference from the result of the measurement employed to the A, B, and C treatments. Based on the result of this study, the use of reduced marine yeast in treatment D gave the most significant influence to the energy retention as much as 16.57% followed by the treatment A (5.91%), C (5.03%) and B (2.22%). The average value of the energy retention found in the eel fish (*A. bicolor*) found in this study varied between 2.22 0 16.57%. The result of this study shows higher result than the other research in which 100% of paste fodder resulted to energy digestion rate at 10.77% [22].

The possible explanation of this phenomena is that this phenomena happens due to the energy produced is being used by the body to run the metabolism process, reproduction process, biosynthesis and wasted in the form of heat. The saved energy is then used for the cell components synthesis as the fuel in the production of cell energy [23]. Growth occurs when there is extra energy that remains after being used in the basic live supports [24]. In the optimal environment condition, the growth is mainly influenced by the fodder given.

Protein Metabolism

The result of the Tukey test showed that the treatment in which marine yeast was reduced as the substitution showed obviously different results from the results of other treatments. Based on the graphic above, the best protein

metabolism during the experiment was found in the treatment D at 81.90% followed by the treatment A (78.51%), C (78.18%) and B (76.59%).

The average protein metabolism value of eel fish (*A. bicolor*) during this study was found between 76.59 - 81.90%. This value is lesser than the result of a study in which the formulated fodder was added up with marine yeast to substitute the use of soya powder for humpback fish (*Cromileptes altivelis*) which showed value of protein metabolism of 56.56 - 84.91% [25].

The process of protein digestion from the fodder starts inside the fish's stomach. Protein from the fodder will undergo the denaturation process by the HCL which is then hydrolyzed by pepsin enzyme which turns the protein into peptide. Furthermore, inside the intestines, the peptide will also undergo the hydrolysis process with the carboxypeptidase, chymotrypsin trypsin and elastase as the catalizador. After that, this oligopeptide will be hydrolyzed by the peptidase enzyme to change formation as tripeptid, dipeptide and amino acids [26].

CONCLUSION

The full-grained, fragmented and reduced marine yeast can be used in the formulation of fodder to substitute the protein from soya powder which gives positive effect on the growth of eel fish (A. bicolor). The reduced marine yeast powder contains nucleic acid of around 1.40 and its high amount of essential amino acids offers best result on the growth of the fish compared to other treatments. Marine yeast powder contains high protein and high amino acids which can be used in the fodder to substitute the use of soya powder. Future researchers are encouraged to conduct research to investigate the use of marine yeast powder at higher dosage in order to find the maximum point of the reduced marine yeast powder.

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Fodder Formulation for Eel Fish (Manik et al.)

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87

The Effect of Treatment Ant Nest (*Myrmecodia pendans*) on Quail Production and Performance (*Coturnix-coturnix japonica*)

Susan Elfitri Lumban Gaol^{1*}, Edhy Sudjarwo², Irfan Hadji Djunaidi²

¹Master Program of Animal Husbandry, Faculty of Animal Husbandry, University of Brawijaya, Malang, Indonesia ²Department of Animal Production Sciences, Faculty of Animal Husbandry, University of Brawijaya, Malang, Indonesia

Abstract

This research was aimed to determine the effect of ant nest treatment (*Myrmecodia pendans*) on quail production and performance. Research sample was 250 quails. Ant nest was administered in 5 treatments and 5 replications: P_0 (0%) as control treatment, P_1 (0.2%), P_2 (0.4%), P_3 (0.6%), and P_4 (0.8%) treatments. Data were collected from quails at 28 days to 70 days old. Variables measured were the performance and quail's egg production. This study was designed using completely randomized design. Real significant difference were examined utilizing Duncan's Multiple Range Test (DMRT). Analysis of variance result showed that ant nest provide significant influence to feed intake and Hen Day Production (P <0.05). The highest average of feed consumption is treatment P_4 (0.8%) was more efficient compared to other treatments. Ant nests administering did not result significantly different (P> 0.05) to feed conversion and egg mass. The results of the study concluded that higher ant nest administration is capable of increasing quails feed intake and Hen Day Production.

Keywords: basal feed, flavonoids, productivity.

INTRODUCTION

Quail farming is one of successful animal husbandry practices. Quail is one of efficient poultry. Their eggs and meat are food sources that contain high protein value [1]. Quail farm business is determined by three important aspects, i.e. breeding, feeding, and good management. This success is measured by optimally achieved profit rate. Expenditure on animal feed reached about 60-70% of total cost [2,3]. The type and amount of feed is related to the feed costs.

Feed cost increases with feed quality. Feed is one aspect which capable of affecting livestocks' performance, health and product quality [4,5]. In addition, providing quail feed supplement is one effort to improve quail production and performance. Unfortunately, farmers are dependent on antibiotics usage. Excessive use of antibiotics will cause negative impact due to quail resistance against microorganisms such as viruses, bacteria, fungi, rickettsiae, protozoa, [6]. Antibiotic residues in meat consumption can increase bacterial resistance to antibiotics, which would cause problems in animal and humans health [7]. Antibiotic usage as growth enhancers

Correspondence author:

Email : susanlgpruden@gmail.com

in poultry industry is prohibited, therefore alternative to antibiotics is required [8]. Alternatives were developed to find safer feed supplement ingredients. One effort that can be done to improve quail production and performance is by manipulating the feed ingredients containing natural antibiotics. Using local feed ingredients to replace factory made feed has not yet been recognized and used by quail breeders.

Traditional medicine is considered to have fewer side effects compared to chemical drugs [9], in addition to affordable price. Other advantages in using traditional medicine is easily available and relatively cheap raw material [10]. One of the medicinal plants that are beneficial to treat health disorders is ant nest (*Myrmecodia pendans*). Empirically, ant nest can cure variety of serious diseases such as tumors, cancer, heart disease, hemorrhoids, tuberculosis, rheumatism, uric acid disorders, stroke, ulcers, impaired renal function, and prostate [6]. Traditional drug in the Papua region is ant nest (*Myrmecodia pendans*).

Chemical test results demonstrated ant nest contains chemical compounds such as phenolic flavonoid [11]. Flavonoids are natural antioxidants capable of reducing hydroxyl radicals, superoxide and peroxyl radicals [12]. Therefore, we aim to examine Papuan ant nest (*Myrmecodia pendans*) application in quail feed on their egg-laying production performance

Susan Elfitri Lumban Gaol

Address : Faculty of Animal Husbandry, University of Brawijaya, Veteran Malang, 65145

which include feed comsuption, feed conversion ratio, hen day production and egg mass.

MATERIALS AND METHODS Subject

Examined quail species is an *Coturnix-coturnix japonica* Autum strain (a crossbreeding between *japonica pexigun*) one Day Old Quail (DOQ). The quail type was eggs-laying quails. Research population was 250 quails. These were nursed starting from DOQ using ready-to-eat feed. Research treatment started at 28 days DOQ with basal feed. Ant nest was added in the form of flour and given at predetermined level (ANF = *Ant Nest Flour*). Statistical analysis used iscompletely randomized design. Data were examined using Duncan's Multiple Range Test (DMRT).

Feed and Treatment

Feed used as the control diet is feed prepared without the use of antibiotics. Feed ingredients used to formulate quail feed consist of yellow corn, soybean meal, MBM (meat bone meal) fish flour, pollard, dicalsium posfhat, premix, cooking oil, and Deglusa methionine. About 100 kg of feed were made using the feed ingredients (Table 1 and 2). Treatments were conducted as follows:

- P₀ 100% basal feed without ANF
- P₁ basal feed + 0.2% ANF
- P₂ basal feed + 0.4% ANF
- P₃ basal feed + 0.6% ANF
- P₄ basal feed + 0.8% ANF

Extra ant nest flour administered in the feed, without changing quail feed requirements. Variables measured included feed consumption, feed conversion ratio, Hen day Productin (HDP) and Egg Mass. *Ant Flour* was added without necessarily changing feed demand of the quails.

 Table 1. Active contents in ant (Myrmecodiapendans)

Nutritional Value	Unit	Value
Energy (kg cal%)	Kcal.100g ⁻¹	350.52
Carbohydrate	g.100g ⁻¹	78.94
Sodium (Na)	mg.100g ⁻¹	68.58
Tocopherol	mg.100g ⁻¹	31.34
Iron (Fe)	mg.100g ⁻¹	29.24
Ash	g.100g ⁻¹	11,13
Water	g.100 ⁻¹ .g ⁻¹	4.54
Potassium (K)	g.100g ⁻¹	3.61
Protein	g.100g ⁻¹	2.75
Fat	g.100 ⁻¹ .g ⁻¹	2.64
Magnesium (Mg)	g.100 ⁻¹ .g ⁻¹	1.50
Zinc (zn)	mg.100 ⁻¹ .g ⁻¹	1.36
Phosphorus (P)	g.100 ⁻¹ .g ⁻¹	0.99
Calcium (ca)	g.100. ⁻¹ g ⁻¹	0.37
Phenol	g.100 ⁻¹ .g ⁻¹	0.25

Source: Subroto and Saputro [13]

Table 2. The Content of the Feed Nutrients Basal Value

Basal Feed Ingredients	Value
Gross Energy**	3692.24 Kkal.kg ⁻¹
Crude Protein*	21.90%
Fat**	6.51%
Crude Fiber*	3.18%
Calcium (ca)*	2.55%
Phosphorus (P)*	0.7%
Ash**	15.36%
Dry Matter**	86.47%

Source:

*Laboratory of Nutrition and Forage Department of Animal Husbandry and Fisheries Blitar.

**Laboratory and Nutrition Faculty Of Animal Husbandry Universitas Brawijaya.

RESULTS AND DISCUSSION

Effect of Ant Nest to Quail Production and Performance

Quail Production and Performance are exhibited in Table 3. Application in quail feed on the production performance include feed comsuption, feed conversion ratio, hen day production and egg mass.

Variable	Po	P ₁	P ₂	P ₃	P ₄	
Feed Consumption (g.bird ⁻¹)	131.48 ± 8.80 ^b	141.85 ± 8.70 ^{ab}	$147.75 \pm 5.40^{\circ}$	147.94 ± 6.81 ^a	147.35 ± 9.6 ^ª	
Feed Convertion Ratio	3.17 ± 0.22	3.14 ± 0.14	3.09 ± 0.32	3.05 ±.0.16	3.00 ± 0.14	
Hen Day Production (%)	56.11 ± 2.64^{b}	56.60 ± 2.15 ^b	58.16 ± 2.93^{ab}	59.07 ± 0.89 ^{ab}	60.09 ± 0.93 ^ª	
Egg mass (g)	5.78 ± 0.44	5.67 ± 0.27	5.84 ± 0.45	5.85 ± 0.35	5.73 ± 0.15	
						-

Description: a, b different superscripts at same row indicate significant differences (P < 0.05)

Feed Consumption

The effect of ant nest concentration addition to feed consumption are shown in Table 3. The results of analysis of variance indicates that increasing administering ant nest will increase quail feed intake (P < 0.05). Duncan test results showed that there are differences ant nest administering in treatment P_3 (0.6%) compared to P_0 (0%) on the quail consumption.

Administering ant nest concentration in treatment P_3 (0.6%) resulted similar effect with treatment P_4 (0.8%), P_2 (0.4%) and P_1 (0.2%). Average ant nest administering is apparent in treatment P_3 (0.6%) with highest average

consumption. P_0 treatment (control) had the lowest average consumption.

The higher the concentration of ant nest administered, feed consumption will increase. This is caused by ant nest's flavonoid active compound content. Flavonoids possess antibacterial activity capable of killing pathogenic bacteria [14]. As a result, feed nutrition digestion and absorption process is not disturbed there by increasing the efficiency of feed nutrients. However, the results demonstrated a declined compared to P₃ and P₄. This is caused by certain restrictions. Flavonoids may reduce quail palatability, thus feed consumption will decline. Flavonoids are parts of phenolic compounds. Giving ant nest excessive levels lead to decreased levels of palatability quail, because of the bitter taste due to the high tannin content [15,16,17] Flavonoids are a part of the phenolic compounds [18], suggesting a high phenol content causes the feed consumption is low and growth is slow [16]. Feed consumption extent depends heavily on the size of cattle, genetic properties (breed), ambient temperature, production rate, cage, feeding ground per animal, drinking water state, feed quality and quantity, and disease [19].

Feed Convertion Ratio

Feed conversion were shown in Table 3. Average ant nest administered to feed conversion in respective treatments were: P_0 were not administered the concentration. On the other hand, P_1 , P_2 , P_3 , P_4 were administered ant nest in different level. Administering ant nest in treatment P_4 (0.8%) showed lowest conversion average value. P_0 treatment (control) had highest conversion on quail.

Analysis of variance result showed that administering ant nest concentration was not significantly different to quail feed conversion (P> 0.05). It was reported that administering ant nest concentrations were also not significantly different to the feed conversion in broilers [20]. However, data generated by administering ant nest concentration showed a decrease in feed conversion. Ant nest administration tends to increase the feed efficiency to increase body weight. It is suspected that ant nest (*Myrmecodia pendans*) contains flavonoids active substances [21].

The results of this study indicate that lowest feed conversion was P_4 treatment with feed conversion at 3.00. Feed conversion value is used to determine the efficiency of feed usage. Higher value meant lower feed usage efficiency. Lower

conversion value means better feed quality [22]. Previous research result showed quail feed conversion at 2.45 [23]. High feed conversion were obtained in this study because the examined quails are at beginning of productive age and have not yet reached peak production age.

Feed conversion is influenced by various factors, including feed quality, animal health and feeding procedures [24]. This indicates that diversity of feed types had no effect on feed conversion, because basically quail consumes feed material according to body nutritional needs. More feed material supplied does not affect conversion rate, but it is still efficient [25,26]. Feed material conversion rate can indicate efficiency level of feed material usage, greater conversion rate of feed material means less efficient feed material [27].

Hen Day Production (HDP)

The results of the analysis in Table 3 for average ant nest administering on Hen Day Production respectively are treatment P_0 were not administered ant nest concentration. P_1 , P_2 , P_3 , and P_4 were given ant nest at different concentrations. Administering ant nest influenced Hen Day Production. P_4 (0.8%) had highest average while P_0 (control) had lowest average.

Results of analysis of variance in Table 3 indicated that administering ant nest on Hen Day Production had a significant results (P <0.05). Duncan test results indicated that administering ant nest concentrationin treatment P₄ (0.8%) had similar effect in P₁ (0.2%), P₂ (0.4%), and P₃ (0.6%). The highest percentage of Hen Day Production is apparent in treatment P₄ which is 60.09%, while lowest percentage is P₀ (0%) for 56.11%.

The higher the concentration of ant nest concentration improves Hen Day Production quail. Egg production is increased due to flavonoid compounds in ant nest [28]. The same research result is stated that the administration of antibiotics in poultry to promote growth, reduce disease, and produce a high egg production [29].

Flavonoid compound can disrupt cytoplasmic membrane functions of pathogenic bacteria cells in intestinal mucosa [30]. By that process, feed could increasenutrient absorption. Egg production rate is determined by feed consumption and protein content [31]. Increase in egg production rate is strongly influenced by protein content in the feed. This is in accordance with opinion that increase in energy content and protein can increase egg production rate, but has no effect on egg mass [32].

Egg Mass

The results of the analysis in Table 3 exhibited ant nest administration influence on egg mass in each treatment. P_3 treatment (0.6%) had highest average. P_1 treatment had lowest egg mass average. In P_3 treatment, it possessed high value caused by influence of flavonoid compound in ant nests which can increase nutrient absorption process. Therefore increasing egg production, while the P_4 treatment did not affect the weight of the egg (egg mass), whereby the higher the flavonoid content causing palatability decreased as the effects of the bitter taste produced by compound anthill cause feed consumption also decreased so that the egg weight will decrease [15,16].

Results of analysis of variance exhibited ant nest concentration administration was not significantly different (P> 0.05) on quail egg mass. The increase in weight of the egg (egg mass) is caused by the amount of protein produced by the consumption of quail. Influenced egg weight and egg albumin levels composed of protein, high intake of protein causes the protein in the feed is high [33]. It is also reported that increase in egg mass is influenced by quail protein consumption [34]. Protein is one of the necessary factors in egg formation in addition to calcium and phosphorus. Optimal quail egg production can be obtained should quail metabolism works well. Metabolic processes could either be achieved by meeting environmental and nutritional factors. Environmental factors include temperature and humidity [35].

CONCLUSION

Administering ant nest affect quail feed intake and Hen day production. Higher administered ant nest concentration will increase feed consumption and Hen day production. On the other hand, administering ant nest concentration does not affect feed conversion and egg mass.

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Ester Jeini Nelwan^{1,4}, Edi Widjajanto², Sri Andarini², M. Sasmito Djati³

¹Doctoral Program of Environmental Science, University of Brawijaya, Malang, Indonesia
 ²Faculty of Medicine, University of Brawijaya, Malang, Indonesia
 ³Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
 ⁴Faculty of Public Health, Sam Ratulangi University, Manado, Indonesia

Abstract

Coronary heart disease (CHD) is a non-transmitted disease which is particular concern at the global, national, and local level. CHD has become a concern because it has caused a lot of deaths. Risk factors linked with CHD consist of permanent risk factors and variable risk factors. The purpose of the research is to analyze the correlation between variable risk factors and CHD in the Minahasa ethnic society in Manado City. This research was an observational case-control study conducted by Prof. dr. R. D. Kandou Hospital, Manado, from August to October 2016. In total, there were 220 patients included. The sampling used a simple random sampling method and the data obtained were analyzed by chi-squared test. According to the results, hypertension, smoking, and behavioral type were connected to cases of CHD. The correlation degree showed that the respondents suffering hypertension were 5.70 times more likely to suffer CHD, the smoking respondents were 2.25 times more likely to experience, and behavior type A respondents were 2.96 times more likely to suffer CHD. Hypertension, smoking, and behavioral type are linked with CHD, so there should be some promotion and preventive actions from the health governmental institution to society, especially aimed at adults, about the quality of life enhancement by healthy behavior and avoiding CHD risk factors.

Keywords: behavioral type, Case-control study, hypertension, smoking behavior.

INTRODUCTION

Millennium Development Goals are connected strongly with health. One of them is nontransmitted diseases, especially cardiovascular disease (CVD). According to the World Heart Federation [1], CVD is connected with 8millennium development goals. CVD is connected strongly with poverty, as this is one of the CVD risk factors; also, CVD can cause poverty. Several types of research show that CVD results in a number of deaths; 80% of these deaths are reported in poor countries. CVD is also connecting to basic education. Education is one of the risk factors of CVD, and CVD is the first cause death of women. Decreasing the number of child deaths and increasing maternal health are also connected to CVD. Smoking behavior and unhealthy lifestyle on the mother's side can cause CVD in mothers and children. In addition, CVD is one of the keys to fighting HIV/AIDS, malaria, and other diseases, as CVD is the main cause of death of HIV/AIDS patients.

Unhealthy lifestyle choices, such as smoking, lack of activity, stress, and so on, can cause several types of degenerative diseases. In the last few years, non-transmitted diseases have dominated as the main cause of death globally [2]. Non-transmitted diseases have increased significantly in developing countries, which have demographic transition and lifestyle changes in their society [3]. The number of deaths caused by CVD, such as coronary heart disease (CHD), stroke and other rheumatic heart diseases, is increasing globally. The World Health Organization predicts that around 20 million deaths will have been caused by CVD in 2015. This accounts for 30% of all deaths in the world [4]. It is predicted that by the year 2030, non-transmitted diseases will account for more than threeguarters of all deaths worldwide. The dominant cause of death in low-income countries is CVD. The case numbers are higher than those of transmitted diseases (including HIV/AIDS, tuberculosis, and malaria), antenatal and prenatal conditions, and nutrient disturbance [5].

Therefore, CVD now is the leading contributor to death globally and will continue to dominate in the future. CVD deaths were increased by 3.1 million to 17.5 million in 2005 from 14.4 million in 1990. From these numbers, around 7.6 million

Correspondence author:

Ester Jeini Nelwan

Email : jeini83@gmail.com

Address : Graduate School, University of Brawijaya, Mayjen Haryono 169, Malang 65145

were caused by CHD and 5.7 million by stroke. More than 80% of deaths occurred in low and middle-income countries [6].

CHD was seldom found in Indonesia before 1950. However, in the early 1970s, CHD was the most commonly found heart disease in many hospitals in Indonesia. According to the Survey Kesehatan Rumah Tangga Departemen Kesehatan RI (Household Health Survey of Health Department of Indonesia) in 1986, it was reported that morbidity of heart disease and blood vessel disease increased from the tenth place in 1981 to third in 1986. This increase was caused by the morbidity of CHD [7].

The etiology or risk factors of CHD are multifactorial. This is a result of interactions between genetics, lifestyle, and environmental factors. A risk factor can be classified into permanent, such as age, gender or family history, or modified, like smoking, hypertension, diabetes mellitus, obesity, and so on. Heart and blood vessel disease has been reported to be the main cause of death in Indonesia since 1995, and is now beginning to threaten and affect the young. In the beginning, CHD was only found in those aged 45 and above, but according to the data in several hospitals, CHD cases are now found in youths aged 27-32 years old. This is because risk factors caused by lifestyle are changing with time and civilization [8].

The results of the National Social Economy Survey in 2004 showed that economic growth, social culture, and technology development have not only positively influenced but also caused changes that negatively affect health, especially with regard to the heart [9]. This can be seen by increasing tobacco consumption, decreasing physical/sports activity, and so on. Based on the results of that survey, it was found that 14.3% of children have been smoking since under 15 years of age. Also, the percentage of people aged 15 years old and over who do physical/sports activity is just 18%.

CHD has also become an important health concern in Sulawesi Utara (Northern Celebes). According to Indonesia Basic Health Research (Riskesdas) in 2013, CHD prevalence is 0.7% [10]. This is above the national level of 0.5%, and is the second highest in Indonesia. This is because risk factors of CHD such as obesity, smoking behavior, diabetes mellitus, and hypertension have quite a high prevalence in these areas.

Based on Riskesdas data from 2010, the prevalence of obesity in North Celebes is the highest in Indonesia, at 21.9% (national prevalence is 11.7%). The 2013 Riskesdas results show that the prevalence of obesity in Northern Celebes had increased to 24.0% (national prevalence is 15.4%), which is still the highest in Indonesia [11].

According to Riskesdas in 2007, 2010, and 2013 [10,11,12], the number of smokers in Indonesia increased from 34.2% [10] to 34.7% [11] and reached 36.3% in 2013 [12]. Based on 2010 Riskesdas data, the smoker prevalence in Northern Celebes is above the national level of 36.2%. Furthermore, according to 2013 Riskesdas data, the prevalence of hypertension in Northern Celebes is around 27.1% (national prevalence is 25.8%). The prevalence of diabetes mellitus is around 3.6% (national prevalence is 2.1%). This shows that CHD is predicted to increase in Indonesia, especially in Northern Celebes. The population of Northern Celebes consists of several ethnicities, dominated by the Minahasa population. The purpose of this research is to analyze the correlation degree of modified risk factors with CHD cases in Minahasa individuals from Manado City, Indonesia.

MATERIALS AND METHODS

Study Site

This research was a non-comparable casecontrol study. The research location was Manado City, which is the capital of Northern Celebes. It lies geographically in $124^{\circ}40' - 124^{\circ}50'$ E and $1^{\circ}30' - 1^{\circ}40'$ N. Manado City bounds the District of Minahasa Utara (Northern Minahasa) in the north, the District of Minahasa in the south, the District of Minahasa Utara and Minahasa in the east, and the Gulf of Manado in the west. This research was conducted from August-October 2016.

Population and Sample of Research

The population of this research was all CHD patients who attended the Cardiac Brain and Vascular Center (CBVC) in RSUP Prof. Dr. R.D. Kandou, Manado. This hospital was selected because it is the CHD reference center in the North Sulawesi Province. The number of samples was calculated using the Budiarto formula [13], so that 110 patients were selected for the cases and 110 patients for controls, making a total of 220 patients. Simple random sampling was the sampling method selected.

In this research, samples consisted of cases and controls. The cases were outpatients who received treatment in CBVC of RSUP Prof. Dr. R.D. Kandou Manado, following a diagnosis of CHD by a heart specialist based on clinical manifestation and electrocardiography (ECG) examination. The control group was outpatients who were treated in the general internal clinic in RSUP Prof. Dr. R.D. Kandou Manado but had not been diagnosed with CHD based on clinical symptoms and electrocardiography (ECG) examination. Inclusion criteria were the respondent providing a signed informed consent, receiving treatment for a maximum of one-year with the expectation that the respondent still could remember the habits and events that they've experienced, and attended when the research was conducted. Exclusion criteria were a disorder in communication and any mental disorder.

Variable of Research

The variables investigated in the research were hypertension, smoking, and behavior type as independent variables, with CHD as the dependent variable. This study was performed in the dichotomous (two groups) form. CHD was categorized as "Yes" if it fulfilled the operational definition of a CHD patient and "No" if the operational definition of a CHD patient was not fulfilled. Hypertension used the criteria based on JNC 7, which was "hypertension" if systolic blood pressure ≥ 140 mmHg or diastolic ≥ 90 mmHg and "not hypertension" if systolic blood pressure <140 mmHg or diastolic <90 mmHg. Smoking was assessed using the criteria "smoker" if the patient had ever smoked and "non-smoker" if the patient had never smoked. The behavior type consists of types A and B. A-type individuals could not get a postponement, could not be easily told to rest, and worked to their maximum capacity, even if this work was not important, while B-type people had the opposite characteristics. Behavior type criteria according to Nababan [14] consists of A-Type if the total value obtained by the respondent \geq 34 and B-Type if the total value obtained by the respondent <34.

Data Analysis

The data obtained were analyzed in a univariate manner, bivariate using the chi-squared test and multivariate using the logistic regression test.

RESULTS AND DICUSSION

Cross-tabulation was performed between respondent status and independent variables (hypertension, smoking habit, and behavior type). The results are shown in Table 1.

Table 1. Cross	tabulation	among variables.
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Respondent Charac-	Respondent Status				
teristic	Case	%	Control	%	
Blood Pressure					
a. Hypertension	92	83.6	52	47.3	
b.No	18	16.4	58	52.7	
Total	110	100.0	110	100.0	
Smoking Habit					
a.Yes	64	58.2	42	38.2	
b.No	46	41.8	68	61.8	
Total	110	100.0	110	100.0	
Behavior Type					
a. Type A	65	59.1	37	33.6	
b.Type B	45	40.9	73	66.4	
Total	110	100.0	110	100.0	

Table 1 shows that the hypertension respondents are mostly in the case group (83.4%), as are smoking respondents (58.2%), and the A-type behavior-type respondents (59.1%). An association between independent variables and CHD cases was then performed (Table 2).

Respondent Characteristic	OR (Crude)	95% Cl	p value		
Hypertension					
a. No	1.000	reference			
b. Yes	5.701	3.040 - 10.690	0.000		
Smoking Habit					
a. No	1.000	reference			
b. Yes	2.253	1.313 – 3.865	0.003		
Behavior Type					
а. В	1.000	reference			
b. A	2.959	1.708 – 5.127	0.000		
Description: OB-C	Description: OB-Odds Patia, Cl-Capfidance Interval				

Description: OR=Odds Ratio, CI=Confidence Interval

Table 2 shows the correlation between hypertension and CHD cases, with the hypertension respondents are probably suffering CHD 5.70 times more often than those who do not suffer hypertension. The correlation between smoking habit and CHD shows that smokers probably suffer CHD 2.25 times more often than nonsmokers. The correlation between behavior type and CHD case shows that there is a significant correlation, with type-A behavior respondents probably suffering CHD 2.96 times more likely than the type-B behavior individuals.

The correlation between hypertension and CHD case in this research is parallel with the research conducted by Nababan [14], which showed that there is a significant correlation between hypertension and CHD cases (p<0.05 and OR 2.25). OR value also shows that the hypertension respondents have a risk of developing CHD

2.25 times more than those who do not suffer from hypertension.

Furthermore, previous study performed research into CHD risk factors in type 2 diabetes mellitus patients in RSUP Dr. Kariadi Semarang [15] and showed that there is a correlation between hypertension and CHD, with a p-value of 0.007 and OR = 2.77 (95%CI = 1.436 - 9.725). This shows that the hypertension respondents have a 2.77 times higher risk of CHD than those who do not suffer hypertension.

Research conducted into prevalence and risk factors for CHD in a downtown area of Bangladesh found that hypertension respondents have a 2.14 times higher risk (systolic blood pressure) and or a 1.93 times higher risk of CHD (diastolic blood pressure) than those with a normal blood pressure [16].

The research conducted by Setiani [17] in the reproductive age group (<55 years old) in RSU Dr. Soetomo Surabaya using a case-control design found that hypertension correlates significantly with CHD (OR value = 27.44) and women aged >45 years old correlate with CHD (OR value = 12.25). Hereafter, this is explained by smoking and dietary habits not having a significant correlation with CHD.

The Framingham research results also showed a correlation between CHD and diastolic blood pressure. The myocardial infarction cases were twice as common in the group with a diastolic blood pressure of 90-104 mmHg compared to the 85mmHg diastolic blood pressure group, while the risk at a diastolic blood pressure of 105 mmHg was four times higher. The Stewart experiments in 1979 and 1982 also confirmed the correlation between increased diastolic blood pressure and the risk of suffering a myocardial infarction. If systolic and diastolic hypertension occurs together, this confers the greatest risk compared to normal blood pressure or systolic hypertension patients. Lichenster also reported that CHD death is more closely correlated to systolic-diastolic blood pressure than just diastolic blood pressure [18].

Several studies have also shown the correlation between hypertension and CHD, like the research conducted in Manipal Education Hospital in Pokhara City and showed that there is a correlation between hypertension and heart attack cases, in this case CHD [19].

Research conducted by the Secretariat of the Pacific Community (SPC) in 2002 in New Caledonia showed that individuals with hypertension have a higher risk of CHD, stroke, heart failure, and kidney disease [20]. The Louisiana Department of Health published a survey conducted about risk factors for heart disease and stroke which shows that hypertension is one of the main risk factors for CHD.

Other research about heart disease and stroke and found that blood pressure more than 120 mmHg (systolic) and 80 mmHg (diastolic) consistently increases the risk of CHD [22]. According to The Women's Health Resource, hypertension either alone or in combination with obesity, smoking, hypercholesterolemia, and diabetes can increase the risk of CHD [23].

A research that investigated the link between hypertension and CHD showed that there are correlations between CHD respondents and hypertension [24]. Other study showed several results indicating that 90% of essential hypertension cases occur in adults, with the other 10% of cases being caused by some chronic states; furthermore, it was found that almost all CHD patients are hypertension patients [25].

Research into hypertension, CHD, and death in Afro-American males and females in the USA, showed that CHD is the main cause of death in the United States and many patients, are Afro-American [26]. Next, it was found that hypertension correlates with CHD cases in Afro-American individuals in the USA. An article written by the World Heart Federation about cardiovascular disease risk factors showed that CHD is caused by several factors, such as hypertension, tobacco use (smoking), alcohol consumption, high cholesterol, lack of physical activity, and unhealthy diets [1].

CHD and hypertension which included some facts suggesting that hypertension is one of the main risk factors causing CHD [27], which can cause death via heart failure (45%), myocardial infarction (35%), cerebrovascular disease (15%), and kidney failure (5%). The Framingham research also reported a correlation between CHD and diastolic blood pressure. Myocardial infarction cases are two times higher in the group with a diastolic blood pressure of 90-104 mmHg compared to the 85 mmHg diastolic blood pressure group, and 105 mmHg diastolic blood pressure patients have a four times higher risk of developing CHD. Furthermore, it is known that CHD deaths are more closely correlated to systolic blood pressure than diastolic blood pressure.

Medicinal treatment of hypertension can prevent myocardial infarction and left ventricle failure, but attention must also be paid to side effects of the medicines in the long-term. Therefore, the prevention of hypertension is a better effort for decreasing CHD risk. Dietary sodium (Na) and potassium (K) consumption are factors related to life patterns. Physical fitness also correlates with systolic blood pressure [18], in which people with optimum physical fitness tended to have low blood pressure. The research in the United States reported in the last decade has shown a decrease in CHD deaths by 25%. This condition may be caused by the result of early detection and treatment of hypertension, use of beta-blockers and coronary surgery and changing smoking habits.

For adults aged 40-69 years old, each 20 mmHg systolic heart pressure or 10 mmHg diastolic blood pressure increases doubles the risk of death by CHD [28]. Blood pressure is assumed to be high if it stays at or above 140/90 mmHg from time to time. If someone suffers from diabetes or chronic kidney disease, then they are considered to be hypertensive if their blood pressure is 130/80 mmHg or higher [29]. The Interheart research showed that 22% of heart attack cases in Western Europe are caused by hypertension. Hypertension increases the risk of heart attack by almost two times [30].

The correlation between smoking habit and CHD in this research is similar to research by Nababan [14] which stated that a smokers have a 2.51 times higher chance of developing CHD than non-smokers. Supriyono [31] showed that those who smoke have a 2.3 times higher risk of CHD below 45 years old than those who do not smoke.

Deaths caused by CHD are 60% higher in smokers [32]. The routine impact of passive smoking increases CHD risk by 25% [33,34]. In 2000, about 1 in 8 deaths in the United Kingdom are caused by cardiovascular disease due to smoking. Based on WHO research, it is predicted that more than 20% of CHD cases are caused by smoking [35].

Smoking can damage and tighten blood vessels, cause unhealthy cholesterol levels, and increase blood pressure. Smoking also can limit the amount of oxygen reaching body tissues [29]. Smoking is one of main risk factors for CHD. Some reports have shown that the CHD risk is 2-4 times higher in male and female heavy smokers (>20 cigarettes per day) than in those who do not smoke. Smoking risk works synergistically with other factors, like hypertension, fat level, or high blood sugar level with regard to developing CHD. It is important to know that death risk caused by CHD decreases by 50% in the first year after smoking cessation. Smoking gives a bigger risk of CHD than obesity [36,37]. Female smokers are at a higher risk of developing CHD because female smokers can undergo early menopause and have reduced levels of estrogen. Smoking accelerates CHD and stroke in women. Smoking is known to cause health problems. This health disorder can be caused by nicotine from primary and secondary smoke being consumed by the individual; therefore, the sufferers are not only the smoker himself/herself (active smoker), but also people in their environment, so-called passive smokers [38].

The role of smoking in CHD is due to the smoke fumes containing nicotine, which cause the release of substances like adrenalin. This stimulates the heart rate and blood pressure. Smoke fume also contains carbon monoxide (CO) which has the ability to absorb oxygen more strongly than hemoglobin, thus decreasing the hemoglobin capacity to bring oxygen to the tissues, including the heart. This should be given attention, especially for CHD, because arterial blood vessels with plaques have less blood flow than normal. Smoking can hide angina, which is an ache in the chest that can be the sign of a heart attack. Without that sign, the patient will not realize that there are dangerous diseases attacking people, and will therefore not take appropriate action. Smokers probably have a risk of experiencing a stroke that is two or three times higher than people who do not smoke. Smoking continuously in the long-term causes an increased probability of suffering artery stoppage in the neck, regardless of how many cigarettes were consumed daily. Smokers have low HDL blood cholesterol levels, which mean that the protective element of CHD is decreasing. Also, smokers more easily develop leg spasms during sport or physical activities, because of occlusions in the arterial vessels in the legs [39].

Nicotine activates platelets causing platelet adhesion to the blood vessel walls. Carbon monoxide (CO) and other substances in the smoke have been proven to damage endothelial walls (blood vessel walls) and ease blood coagulation. Platelet coagulation causes peripheral blood vessel damage. Smoking one cigar a day will increase systolic blood pressure by 10-25 mmHg and increase the heart rate by 5-20 beats a minute [40]. This research also reported results that behavior type is related to CHD, which is in parallel to Patel's study [41], reporting that A behavior type is reflected by certain physiological reactions, like increased blood pressure, increased adrenalin Modified Risk Factors for Coronary Heart Disease in Minahasa Ethnic Group, Manado (Nelwan et al.)

production, and increased platelet adhesiveness, which enhances the probability of blood coagulation. A human with type-A behavior cannot accept the delay and does not find it easy to rest, usually working at their maximum capacity, and not admitting to tiredness and fatigue; therefore, they will keep working and almost lose power. Researchers have shown that people with type-A behavior have a two times higher risk of dying from a heart attack than those who have type-B behavior [41].

CONCLUSION

Conclusion of the study's findings are written According to this research, it can be concluded that hypertension, smoking, and behavior type are related to CHD. Hypertension respondents have a probability of suffering from CHD that is 5.70 times higher than those who do not suffer from hypertension. Smokers have a probability of suffering CHD that is 2.25 times higher than in those who do not smoke. Also, type-A behavior type respondents have a probability of developing CHD that is 2.96 times higher than those who have type-B behavior. Therefore, promoting variable risk factors is recommended, as individuals must have healthy lifestyles to decrease the risk of CHD.

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The Quality of Ornamental Japanese Carp *(Cyprinus carpio* var. Koi) After Electroporation as a Gene Material Transfer Method

Sa'idah Luthfiyah ^{1*}, Agoes Soeprijanto ², Yuni Kilawati ²

¹Master Program, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia ²Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Spermatozoa are male gametes that have an advantage in acting as gene transfer media (SMGT), because in transferring genetic material, sperm uses relatively natural vectors. This study was conducted to know sperm quality of Koi after short electroporation with different voltages using Gene Pulser. The study was conducted using a complete randomized design (RAL) with three (3) replications and five (5) treatments, namely: 10 V.cm^{-1} , 20 V.cm^{-1} , 30 V.cm^{-1} , 40 V.cm^{-1} , and 1 control. Electroporation was done with square wave, 1 ms duration of electroporation, 4 times of electroporation shocks with 0.1 s duration. The results showed that electroporation with different voltage levels significantly affected the motility and viability of sperm. Based on orthogonal polynomial analysis, it was known that the treatment affected motility and viability of sperm with a linear equation y = -0.9x + 5, $4 \text{ R}^2 = 0.920$ and r = 0.96 for motility and $y = -11.26x + 91.61 \text{ R}^2 = 0.823$ and r = 0.91 for viability, meaning that the higher the level of electroporation the lower the viability and sperm motility produced. From the results of this study, it can be suggested that the electroporation of Koi sperm as a gene transfer medium should be performed at a voltage of 10 V.cm⁻¹.

Keywords: electroporation, Koi, sperm quality, transgenesis.

INTRODUCTION

Spermatozoa are male gametes that have an advantage in acting as gene transfer media, because in transferring genetic material, sperm uses relatively natural vectors [1]. Sperm cells have been used as gene transfer vectors in fish where the entry of gene construction into sperm can be facilitated by the use of electroporator [2].

Electroporation is a method of gene transfer by using a series of short electrical vibrations to temporarily dissolve the cell membrane, thereby the foreign DNA molecule can enter the cell and interact to integrate it with the genome of the test cell and can be expressed on the next organism [3,4,5]. The integration of DNA into sperm depends on the electrical voltage, the amount of shock imposed, and the concentration of the DNA. The efficiency of DNA transfer, with the electrophoresed sperm media, is influenced strongly by stress and shock duration [6]. The use of electroporation method facilitates the formation of temporal pores on the surface of the target cell membrane so foreign DNA can enter the cell [7].

^{*} Correspondence author:

Sa'idah Luthfiyah

Email : luthfiyahsaidah.salut@gmail.com

Address : Faculty of Fisheries and Marine Sciences, University of Brawijaya, Veteran Malang, 65145

Koi using electroporation method in fish sperm as the gene transfer medium has not been done The development of fish in Indonesia. transgenesis in Indonesia during this decade mostly done by applying microinjection method (such as Carp, grouper, catfish, stripped catfish) [8,9]. The use of microinjection methods are inefficient because it requires special expertise to apply, takes a long time, and expensive. Recently, transgenesis development for aquatic animal is better to be transferred using electroporation method, because electroporation considered more suitable for the aquaculture and does not require special skills [8]. That is also seen in the efficacy of succesful gene transfer using sperm elctroporation method that has been reported to higher efficiency compared have with microinjection method, as shown in ayu fish (Plecogosus altivelis) the successful of gene transfer is 55%, in zebrafish and moad loach are 80%, in indian gold fish 25%, and 85,75 % in stripped catfish [10,11,12].

The application of transgenesis technology to

Therefore, to know the succesful of using gene transfer method with sperm electroporation especially for Koi, it is necessary to do the experiment about preliminary study on sperm quality after giving electric shock. A preliminary study on the sperm quality of Koi fish after electroporation is important to determine the ability of sperm to fertilize egg. So, it is necessary to experiment with different stress levels to get the optimal stress levels [6]. The efficiency of DNA transfer, with the electrophoresed sperm media, is influenced strongly by stress and shock duration. The purpose of this study was to determine the effect of stress on sperm quality.

MATERIALS AND METHODS

This study has been conducted at Central Laboratory of Life Sciences (LSIH) - and Breeding and Reproduction Laboratory, Faculty of Fisheries and Marine Science University of Brawijaya Malang. The equipment used in this research was 1 set of BIO-RAD Gene PulserXcell[™] device consisting of electroporator, shock pod, and cuvette machine, Olympus BX 51 microscope of Inverted type, Olympus CX21 binocular microscope, scales, ruler, petri dish, plastic bowl, 1 ml syringe, aquarium, pond, napkin, microtube 1.5 ml and 1 ml, micropipette, pipette, yellow and white tip, non-EDTA vacum tube, digital camera, object glass, cover glass, thermometer, handtally counter, haemocytometer, and aerator and its installation

The materials needed were mature Koi (*Cyprinus carpio* var. Koi), Ringer Lactat (RL), eosin negrosin, tissue, cotton, alcohol 70%, alumunium foil, and aquadest, ice gell pack. The experimental design used was Completely Randomized Design (CRD) with five (5) treatments and three (3) replications with the following treatment. The research parameters were sperm quality (motility and viability) of Koi.

- A = with electric shock at 10 volt
- B = with electric shock at 20 volt
- C = with electric shock at 30 volt
- D = with electric shock at 40 volt

Data Collection

Collection of Sperm (Milt)

The sperm collection is obtained by massaging the matured male Koi fish (stripping) from the abdominal direction to the urogenital hole, and the sperm was put in the 3 mL syringe. The sperm was diluted using a ringer lactate (RL) solution of 1:9 [13,14] The diluted sperm was then stored on a non-EDTA Vacum tube coated with aluminum foil and placed on ice gell [15].

Sperm Electroporation

Sperm electroporation was done by using Gene Pulser II machine (BIO-RAD, USA). The diluted sperm collection was taken as much as 25 μ l and was inserted into cuvette 0.2 mm and electroporated using square wave type, 1 ms

pulse length, 4 times shock number and 0.1 s duration [16].

Sperm that had been given a shock was added with a solution of ringer lactate in cuvette as much as 275 μ l. After that, the sperm was poured into 1.5 ml micro tube as much as 5 μ l for sperm quality observation (motility and viability) and 270 μ l for fertilization. Motility of sperm were observed using an inverted microscope and viability of sperm were observed using a binocular microscopy (Olympus CX21), and sperm concentrations were calculated using a haemocytometer[16,17].

Sperm Motility

Observation of sperm motility was performed by hanging drop method [18] by dripping 1µl of sperm on a glass object mixed with 5 µl of distilled water. Then, sperm motion was observed after exposure to water by seeing the number of progressive sperms using inverted microscope with 1000x magnification. The criterion of movement intensity was observed based on Dewi [19], which mapped sperm motility assessment criteria by scoring method (Table 1).

 Table 1.Criteria on Assessment of spermatozoa Motility

Criteria	Score
>70% of spermatozoa move forward quickly	5.0
with varied tail movements	
55-70% of spermatozoa move forward,	4.0
many show quick movement	
40-55% of spermatozoa move forward,	3.0
some show quick movement	
25-40% of spermatozoa move forward	2.0
10-25% of spermatozoa move forward	1.0
1-10% of spermatozoa move forward,	0.5
mostly do not make any movement	
All spermatozoa show no movement	0.0

Sperm Viability

Sperm viability was calculated in order to find out the percentage of surviving sperm. The percentage of sperm viability can be calculated by the following formula [20]:

% viability =
$$\frac{\sum Surviving sperm}{200} \times 100\%$$

Data Analysis

The data was analyzed statistically by using diversity analysis (ANOVA), in accordance with the design used, i.e. Completely Randomized Design (CRD). If the variance data showed that the treatment brought significant or highly significant, then to compare the value between treatments were done by the Smallest Real Difference (SRD) test and regression analysis.

RESULT AND DISCUSSION Sperm Quality of Koi

Sperm quality before treatment can be seen in Table 2. Table 2 indicates that sperm used in the study was in good quality. Dacie and Lewis [21] and Rustidja [3] has found that fish sperm concentration ranges from \pm 3.7 to 11.9 x 10⁹ spermatozoa mL⁻¹ liquid, as fish to produce eggs to hundreds of thousands, in addition to high concentration, will require more sperm volume. The large number of sperm that can be stripping from a male koi depends on the age, size, and frequency of sperm discharge [3].

Tabel 2. Sperm Quality before Treatment	
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Parameter	Result
Concentration	11.7 x 10 ⁹ cell.mL ⁻¹
рН	7
Volume	1.8 mL
Head diameter	± 3 μm
Length of tail	± 8 μm
Motility	90 %
Intensity movement	5 (+++)
Viability	98.67 %

Quality of Sperm Post-Electroporation Sperm Motility

The result of sperm motility observation after treatment showed a decrease of quality compared with before treatment. The highest motility score post treatment was on treatment of 10 V.cm⁻¹, while the lowest was on 40 V.cm⁻¹. The details can be seen in Table 3.

 Table3. Motility and Viability of Koi spermatozoa postelectroporation

Voltage (V.cm ⁻¹)	Motility (Score)	Viability (%)
Control (0)	4	73.67
10	4	69.46
20	3	67.34
30	2	53.05
40	0.5	25.54

In the analysis of variance, it is known that electric shock treatment (electroporation) with different electric field strength gives a very significant effect on the sperm motility value on each treatment (Table 4).

Therefore, H_0 is rejected and H_1 is accepted, and continued with Smallest Real Difference (SRD) test and regression analysis. From the result of Smallest Real Difference (SRD) test and regression analysis, it is known that the higher the electric field strength (V.cm⁻¹) given to the sperm, the motility value decreased to only 0.5 on the treatment of 40 V.cm⁻¹ (Fig. 1).

Table 4. Variance Tal	D
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Source of Variance	df	SS	MS	F	F 5%	F 1%
Treatment	4	34.27	8.57	39.54	3.48	5.99
Random	10	2.17	0.22	**		
Total	14					
**	ممام	ignificant				

** = highly significant

Sperm in motility condition (low movement) still holds the possibility to fertilize because. The slow progressive of spermatozoa still able penetrate the microphile hole [22].

Fertilization may occur when the distance between the spermatozoa and the egg is very close. Spermatozoa of the sperm on low motility condition have small ability to perform fertilization that is only about 10% [23]. This is also reinforced by previous study which states that in general sperm that have been electrophorated is still able to fertilize because sperm given electric shock does not show any damage [24]; the sperm cell becomes smaller (dwarf cells) [22].



Figure 1. Sperm motility post-electroporation

Sperm Viability

Viability of sperm is one important sperm quality indicator, because the value of sperm viability represents the percentage of surviving sperm after treatment. Results from observations of sperm viability (Table 2) showed that the highest sperm survival rate was in treatment B (10 V.cm⁻¹) and decreased with increasing of electric field strength given to sperm.

The result of the analysis of the variance also shows that the electric shock (electroporation) with different electric field strengths gives a very real effect because the F-count value > of F-table, i.e. 5% and 1% (Table 5). Thus, the statistical calculation continued with orthogonal polynomial test and from the test results, it is seen that the higher the electric field strength given when electroporation takes place further decreases the survival rate of post-treatment sperm (Fig. 2).

Table	5.Varia	ince Tab	le on '	Viahility
Iavic	J . v ai ia	ince rau		viability

Source of Variance	df	SS	MS	F	F 5%	F 1%
Treatment	4	4625.85	1156.46	77.88	3.48	5.99
Random	10	148.50	14.85	**		
Total	14					
**	1.1.1.1					

** = highly significant

The decrease in viability in most treatments (Fig. 2) occurs because the electric field stresses given to the sperm can cause the opening of pores that are too wide and fail to close as before, which can lead to damaged or broken cells and this triggers damage to the membrane [24]. This is confirmed by Jeyendran [25] which explained that the permeability of spermatozoa membrane is closely related to the viability of spermatozoa because, as it is known, membrane permeability is closely related to the transport of essential nutrients in cell metabolism.



Figure2. Sperm viability post-electroporation

The treatment with a voltage of 40 V.cm⁻¹ has the lowest viability value. This is because there is an increase in temperature on the sperm when given the voltage-possible because at the time of cooling with ice, cuvette does not perform well. The sample resistance can be increased by reducing the sample temperature, reducing the ion content on diluent, reducing fluid volume in the cuvette in the case of medium with low resistance [26]. Thus, if the sperm (sample) contains many ions then the resistance of the sample increases so it needs longer timefor pulse lengthso the temperature will increase and cause the sperm to die. This is also supported by previous studies which states that the survival (viability) of spermatozoa is strongly influenced by temperature and sperm will generally live longer in low temperatures [3,18].

Based on the observation of motility and viability on post electroporation sperm, it is known that the increase of voltage (electric voltage) given at the time of sperm electroporation affects the decrease in the intensity of movement and ability of sperm to survive. It can be seen from the observation result E (40 V.cm⁻¹), where the value of motility score was only 0.5 and viability was 25.4%. That is because electroporation of sperm gives a very real effect on the biological and physiological conditions of sperm cells. This happens because an excessive electric shock can cause the sperm cell to lose its elastic properties and damage its channel membrane if it exceeds the critical point of its elasticity and can damage the membrane causing the sperm tail to break. Therefore, in the activity of gene transfer using electroporation method, optimization is necessary to know the ability of sperm in becoming gene transfer vector.

The ability of the sperm cell to with stand the given electric shock is influenced by several factors such as the type of fish, the quality of the sperm before the treatment as well as some of the electroporation parameters themselves (voltage, shock number, shock length, shock interval, and wave type used). This is in accordance with the opinion of Knight and Scrutton [27] which explained that electroporation is the process of modifying the permeability of cell membranes through electric field strength.

The application of electric shock to the cell suspension induces the polarization of the cell membrane component and develops a voltage potential across the membrane. When the potential differences between the inside and outside of the cell membrane passes through the critical point, the membrane component is reorganized into the pores in the localized area, and the cell becomes permeable to the entry of macromolecules [28]. The magnitude of pore size change is influenced by electric field strength, duration of shock, and ionic strength of the media. This permeability change is temporary, provided the electric shock does not exceed the critical limit for cells [29].

CONCLUSION

The provision of electrical shock has a significant effect on the motility and viability of sperm, in which the increasing of electric voltage can decrease motility rate and viability of sperm of koi fish (*Cyprinus carpio* var. Koi). We recommend electroporation of Koi fish sperm (*Cyprinus carpio* var. Koi) as a gene transfer medium should be performed at a voltage of 10 Vcm⁻¹ with four (4) numbers of shocks and 1 ms shock duration.

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Exploration and Antagonistic Test of Endophytic Fungi from Soybean (*Glycine max* L. Merr) With Different Resistance to *Sclerotium rolfsii*

Siti Aminatuz Zuhria¹, Syamsuddin Djauhari², Anton Muhibuddin²

¹Master Program of Plant Science, Faculty of Agriculture, University of Brawijaya, Malang, Indonesia ²Department of Plant Pest and Diseases, Faculty of Agriculture, University of Brawijaya, Malang, Indonesia

Abstract

The research aimed to determine the diversity of endophytic fungi in soybean crop with different resistance against *Sclerotium rolfsii* and find out their potential antagonist in controlling *S.rolfsii* by *in vitro* and *in vivo*. Materials used in this study were soybean with a variety of Wilis (susceptible variety) and Sinabung (resistant variety). This research was conducted at the Microbiology Laboratory of Central Laboratory of Life Science (LSIH), Brawijya University and in the trial plantation of Malang Research Institute for Food Crops, Lawang, Malang subdistrict in September 2015 until May 2016. Type of experimental design used was Complete Randomized Design with 16 treatments and three times repetitions at *in vitro* experiment with 16 treatments and four times repetitions at *in vivo* experiment. Observation on *in vitro* test is covering to colony morphology of fungal pathogens on PDA medium. On the test of *in vivo*, it was observed a disease occurrence and effectiveness rate of endophytic fungi. There are 15 species of endophytic fungi produced from isolation, namely *Trichoderma* sp., *Aspergillus* sp.2, *Aspergillus* sp.3, *Acremonium* sp.1, *Acremonium* sp.2, *Acremonium* sp.3, *Acremonium* sp.4, *Fusarium* sp.1, *Fusarium* sp.2, *Cephalosporium* sp, *Microsporum* sp., *Penicillium* sp., and unidentified fungi called W₂ and W₄. The highest inhibitory of endophytic fungi against *S. rolfsii* by *Aspergillus* sp.2 is 89.18% (*in vitro*) and 61.21% (*in vivo*), while *Trichoderma* sp. 91.88% (*in vitro*) and 63.29% (*in vivo*). Diversity index value of Wilis variety is higher than Sinabung, i.e. 1.878 and 1.606 respectively. While dominance index value of Sinabung variety is 0.2035 and Wilis is 0.1528.

Keywords: Endophytic fungi, diversity, S. rolfsii.

INTRODUCTION

Endophytic is microorganisms that live inside plant tissues without causing a symptom of disease in the host plant. There is a mutualistic interaction between endophytic microbes and host plant, each benefiting from the interaction [1]. The mutual interaction benefits the endophytic fungi through provision supply of energy, nutrients, shelter as well as protection from environmental stress. On the other hand, fungal endophytes indirectly benefit plant growth by producing special substances mainly secondary metabolites and enzymes, which are responsible for the adaptation of plants to abiotic stresses such as light, drought and biotic stresses, e.g. herbivore, insect and nematode attack or invading pathogens [2]. The research aimed to determine the diversity of endophytic fungi in soybean with different resistance to Sclerotium rolfsii and find out their potential antagonist in controlling S. rolfsii by in vitro and in vivo.

MATERIALS AND METHODS

This study used exploratory and experimental method. Exploratory method was used to find out endophytic fungi from Wilis and Sinabung variety. Experiment performed was antagonistic test of endophytic fungus, i.e. isolated *S. rolfsii* through direct opposition method. This research was undertaken in Microbiology Laboratory of Central Laboratory of Life Science (LSIH), Brawijya University and field trials placed in Malang Research Institute for Food Crops, Bedali Malang in September 2015 until May 2016.

Materials used in this study were soybean with a variety of Wilis and Sinabung. It is a collection of Indonesian Legumes and Tuber Crops Research Institute (ILETRI) Malang, isolates of *S.rolfsii*, PDA, aquades steril, alcohol 7%, NaOCl 5%.

Isolation and Identification of S. rolfsii

Sclerotium rolfsii was isolated from the root of soybean which indicates to be withered, the stem turn reddish brown, and there is mycelium in the form of hyphae at the surrounding aboveground. Symptomatic root was cut, sterilized using NaOCI 5%, rinsed with sterile distilled water, and dried using tissue. It was then grown in

^{*} Correspondence author:

Siti Aminatuz Zuhria

Email : zuhria_007@yahoo.com

Address : Faculty of Agriculture, University of Brawijaya, Jl. Veteran Malang, 65145

the petri dish containing PDA medium until the hyphae grow. Fungi colonies which grow in accordance with macroscopic morphology (shape and color) of *S. rolfsii* subsequently identified using identification guides of Barnett and Hunter.

Isolation and Identification of Endophytic Fungi

Endophytic fungi were isolated from the health plant's root tissue. First the plant's root was rinsed in tap water to remove the dust and debris then cut into small pieces by a sterilized blade under aseptic conditions. Each sample's surface was sterilized by 70% ethanol for 1 minute and after that immersed the plant parts in sodium hypochloride (NaOCl) solution for 1 minute. It was meant to be sterile from the outside fungi so that the growing fungi are expected from the inside of the plant tissue. The samples were rinsed in sterile distilled water for 1 minute and then allowed to surface dry on filter paper.

After proper drying, 4 pieces of plant parts were inoculated in PDA plate and incubated at 28^oC for 5 to 7 days. Pure colonies were transferred on PDA. The fungal strains in the pure culture were preserved on potato dextrose agar (PDA) [3]. In the last rinse of distilled water, it was then taken 1 ml of distilled water and being poured into PDA medium as a control. Fungi that grow and have a colony considered to be different based on the macroscopic morphology (color and form) will be performed purification. Furthermore, isolates of fungi were identified by macroscopic and microscopes using Barnett and Hunter's identification guides.

Antagonistic Test of Endophytic Fungi with Direct Opposition Method

The isolates of *S. rolfsii* and endophytic fungi were put together on a petri dish containing PDA medium within 3 centimeters length and incubated in the room temperature of 28-30°C during a week. For a treatment of control, pieces of isolate were put on the petri dish without endophytic fungi. The treatment was repeated for 3 times. The observed variable was colony radius that grows to the direction of endophytic fungi. Formulation of growth inhibition (I) of pathogenic colony of endophytic fungi by Sharfuddin and Mohanka [4]:

$I = [(r_1 - r_2)/r_1] \times 100\%$

Description:

I= growth inhibition of *S. rolfsii* colony (%)

r₁= colony radius of *S. rolfsii* grows in the control (cm)

 r_2 = colony radius of *S. rolfsii* grows to the direction of

endophytic fungi (cm)

Effectiveness of Endophytic Fungi in the Greenhouse (*In vivo*)

The testing aims to find out the potential of endophytic fungi isolates in curbing a disease occurrence caused by S. rolfsii. It was done at the nursery phase through seed soaking method. This treatment utilizes 15 isolates of endophytic fungi from exploration and control treatment, i.e. soybean with pathogen but without endophytic fungi. Soybean was planted on the growth medium of sterilized soil and compost under the comparison of 2:1. Soybean seeds were sterilized using alcohol 70%, soaked in the suspension of endophyte fungi isolates with concentration of 10^{6} conidia.mL⁻¹ for ± 12 hours. At the control treatment, seeds submersion were performed using sterile distilled water. Soybean which have been 2 weeks after planting given pathogenic treatment by dripping a pathogen suspension of S. rolfsii by 1 ml with density of 10^6 conidium.mL⁻¹ in every planting hole. A variable observed is disease occurrence and effectiveness level with formulation [4]:

1 Disease Occurrence = $n/N \ge 100\%$

Effectiveness level= $DO_c - DO \times 100\%$

Description: n= the number of infected plant N= the number of observed plant DO_c=Disease occurrence in control DO=Disease occurrence

Index of Diversity and Dominance

Diversity index (H') was used to calculate endophytic fungi diversity. This calculation aims to determine the level of the diversity of endophytic fungi at the different resistance against *S. rolfsii*. Formulation of diversity index [5]:

$H' = -\sum P_i \ln P_i$

Description: H' = Diversity Index of Shannon Wiener n_i = the number of species i N= the total number of individual P_i = n_i/N

Dominance index was used to find out a dominance of endophytic fungi at a community. The calculation aims to determine whether there is dominance or not of particular endophytic fungi. Formulation of dominance index [6]:

$$C=\sum (P_i)^2$$

Description: $P_i = n_i N^{-1}$ $n_i =$ the number of species i N = the total number of individual

DATA ANALYSIS

The data obtained from observation was analyzed using F-test at the level of 5%. It was then continued with HSD (Honest Significant Different) test at the same level.

RESULTS AND DICUSSION Isolates of Endophytic Fungi from Soybean

There are 15 fungi resulted from the isolates of endophytic fungi of soybean. Sinabung variety resulting in 7 species of endophytic fungi, they are: *Trichoderma* sp., *Aspergillus* sp.1, *Aspergillus* sp.2, *Penicillium* sp., *Acremonium* sp.4, *Fusarium* sp.2, and *Cephalosporium*. In the other side, Wilis variety produces 8 species of endophytic fungi, i.e *Aspergillus* sp.3, *Fusarium* sp.1 *Acremonium* sp.1, *Acremonium* sp.2, *Acremonium* sp.3, *Microsporum* sp., W₂ and W₄ (unidentified fungi).

Antagonistic Test of Endophytic Fungi and *S. rolfsii* by Using Direct Opposition Method

The result of antagonism test of endophytic fungi against *S. rolfsii* is provided in the Table 1.

Table 1. Endophytic Fungi Inhibition against S.rolfsii		
Type of Endophytic Fungi	Growth Inhibition Aver-	
(Treatment)	age (%)	
Aspergillus sp. 2	91.88 a	
Trichoderma sp.	89.18 ab	
Aspergillus sp. 1	83.77 abc	
Aspergillus sp. 3	72.07 abcd	
Acremonium sp. 2	71.16 bcd	
Acremonium sp. 1	68.46 cd	
<i>Fusarium</i> sp. 2	63.05 de	
Cephalosporium sp.	60.35 de	
<i>Fusarium</i> sp. 1	60.35 de	
Acremonium sp. 4	59.45 de	
Penicillium sp.	53.14 de	
Acremonium sp. 3	52.24 de	
Microsporum sp.	45.94 e	
W ₂	45.04 e	
W ₄	45.04 e	
Control (without endophytic)	0.00 f	

At the control treatment (without endophytic), growth inhibition (I) of colony is 0.00%. The highest growth inhibition of control treatment is in *Aspergillus* sp.2 (91.88%) and *Trichoderma* sp. (89.18%). While the lowest growth inhibition of control treatments are in W_2 and W_4 (unidentified) with inhibition of 45.04%.

Mechanism of the inhibition among tested isolates of endophytic fungi is different. Competition between pathogen and endophytic fungi is the most common mechanism. *Trichoderma* sp., *Aspergillus* sp.1, and *Aspergillus* sp.2, have the highest competition in seizing space and nutrition. This is shown by mycelium growth of endophytic fungi which is dominant and suppressing pathogenic growth (Fig. 1).

Another mechanism happened is mechanism of antibiosis which is characterized by clear zone around endophytic fungi and pathogen. Mechanism of antibiosis, antagonistic degrading enzyme has to be directly contact to the pathogen [7]. Endophytic fungi have a directly mechanism to suppression disease of plants, that is, through antibiotic production and secretion of lytic enzymes [8]. In this study, the mechanism of antibiosis shown by the isolates of *Penicillium* sp. which forms a clear zone so that the hyphae of *S. rolfsii* not able to penetrate the colonies of *Penicillium* sp. (Fig. 1). *Penicillium* species can secrete bioactive function as antibiosis, such as penicillin and riboksin [9].



Figure 1. Antagonistic test of endophytic fungi against S.rolfsii
A: Competition of Trichoderma sp.
B: Competition of Aspergillus sp.
C: Mechanism of Antibiosis by Penicillium sp.

Meanwhile, the isolates of endophytic fungi which is not having mechanism of competition and antibiosis content at inhibition testing of *S.rolfsii*, supposed to have another activity in controlling diseases, such as the ability to induce plant resistance and also increase plant fitness. Resistance induction of endophytic fungi positions plants not as target pathogens directly, but through physiological and metabolic changes that allow the plant to further streamline their resistance to disease [10].

The Effectiveness of Endophytic Fungi at Greenhouse (*In vivo*)

The result of test effectiveness at greenhouse is provided in the Table 2. The lowest disease occurrence is on *Trichoderma* sp. treatment (21.25%) which significantly different from control (61.25%), while the highest disease occurrence is on *Microsporum* sp. (58.75%), W_4 (58.75%) and W_2 (56.25%) are not significantly different from control. It shows that not all of endophytic fungi which successfully isolated have potential in suppressing the dumping off progression. The highest effectiveness of endophytic fungi in suppressing *S. rolfsii* disease is in the treatment of *Trichoderma* sp. (63.29%), *Aspergillus* sp.2 (61.21%), *Acremonium* sp.2 (61.21%). On the other, the lowest effectiveness level is in *Microsporum* sp. (4.16%), W_4 (4.16%), and W_2 (8.01%).

Table 2. The Rate of *S.rolfsii* Attacks against EndophyticFungi Treatment

Type of Endophytic Fungi (Treatment)	Disease Occur- rence (%)	The Level of Effectiveness (%)	
Control	61.25 a	0.00 g	
Microsporum sp.	58.75 ab	4.16 fg	
W_4	58.75 ab	4.16 fg	
W ₂	56.25 abc	8.01 efg	
Acremonium sp. 1	46.25 bcd	22.43 defg	
<i>Fusarium</i> sp. 1	45.00 bcd	24.35 defg	
Acremonium sp. 3	43.75 cd	28.52 def	
Cephalosporium sp.	41.25 de	32.84 cde	
Acremonium sp. 4	41.25 de	32.84 cde	
Fusarium sp. 2	33.75 def	36.85 bcd	
Penicillium sp.	32.5 def	40.54 abcd	
Aspergillus sp. 3	28.75 ef	56.89 abc	
Aspergillus sp. 1	26.25 f	57.04 abc	
Acremonium sp. 2	23.75 f	61.21 ab	
Aspergillus sp. 2	23.75 f	61.21 ab	
Trichoderma sp.	21.25 f	63.29 a	

Protection mechanism of endophytic fungi can be competition, antagonism, and microparasite and induction resistance. To inhibition the disease occurrence of other endophytic fungi the system of plant resistance has to be activated. Mechanism of plant resistance towards pathogen is commonly a combination between two resistance systems, namely structural and biochemical resistance [11].

Endophytic fungi also cause the induction of secondary metabolites that can inhibit other fungi which live on the same host. Biocontrol agents can weaken or kill the pathogen of plants through a resistance to be parasite directly, its ability in the competition of space and nutrients, production of enzyme to fight the pathogenic cell components, production of plant metabolism in stimulating the germination of spores of pathogens and production of antibiotics [11].

The presence of a combination among several biological agents can be independent, synergistic, or antagonistic. Among the endophytic fungi that colonize plant tissues and inoculated pathogens can cause a wide range of possibilities, such as they do not affect each other, compete with each other, synergistic in causing the symptoms of the disease or suppress the disease occurrence [12].

Index of Diversity and Dominance

Diversity Index of Wilis Variety (susceptible) is 1.878 while Sinabung (resistance) is 1.606. Those two varieties have moderate diversity. However, Sinabung variety (resistance) has lower diversity index value than Wilis (susceptible). This study proves that the diversity of endophytic fungi on resistance variety is not always higher than susceptible. It causes since not all endophytic fungi that are successfully isolated able to suppress the pathogen of S.rolfsii. It guesses since endophytic fungi are not containing anti-fungus and resulted secondary metabolite has another function. The factors affecting the ineffectiveness of biological agents to inhibit the growth of pathogens is antibiotics produced by endophytic fungi are less effective against pathogens; among others antibiotic concentrations are low and decomposed by other microorganisms [13].

Dominance index value (C) of susceptible variety (Wilis) is 0.1528, while the resistance (Sinabung) is 0.2035. Dominance index value of those two varieties are including in the low category. For that reason, there are no dominant endophytic fungi on those two varieties.

CONCLUSION

Not all endophytic fungi which were successfully isolated are able to suppress *S. rolfsii. Trichoderma* sp. (63.29%) and *Aspergillus* sp.2 (61.21%) have the best capability in suppressing *S. rolfsii* disease, both at laboratory or greenhouse.

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