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Characterization of probiotic *Bacillus* sp. NP5 metabolites and their effect on female catfish broodstock re-maturation

Karakterisasi metabolit dalam probiotik *Bacillus* sp. NP5 dan efeknya terhadap rematurasi induk ikan lele betina

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ABSTRACT

Probiotics are believed to contain active components that influence fish reproduction. This study aimed to evaluate the metabolite compounds present in the probiotic *Bacillus* sp. NP5 and their effect on female catfish broodstock re-maturation. The first step involved analyzing the metabolite content of the probiotic using liquid chromatography with tandem mass spectrometry (LCMS/MS). Subsequently, feed supplementation with the probiotic was implemented at concentrations of 10^6 CFU/g (P6), 10^8 CFU/g (P8), and control (C). Parameters observed included the metabolite content of *Bacillus* sp. NP5, gonadosomatic index (GSI), hepatosomatic index (HSI), gonad histology, gonad maturity rate, fecundity, number of matured eggs, distribution of egg diameter, fertilization rate (FR), hatching rate (HR), larval survival rate (SR), re-maturation period, and percentage of matured broodstock. The results showed that metabolites such as resveratrol (22.45%) and indoline (7.11%) are likely to influence the fish reproduction directly. Other metabolites, such as oxyresveratrol (22.95%) and vestitol (4.14%), are also thought to play an indirect role in reproduction. The supplementation demonstrated the best reproductive performance at 10^8 CFU/g treatment, with an increased GSI (14.74%), HSI (1.65%), gonad maturity rate, percentage of mature eggs (86.00%), re-maturation period (4 weeks), percentage of matured broodstock (100%), fecundity (243,605 eggs/broodstock), FR (98.74%), HR (80.62%), and SR (87.31%). The application of probiotic *Bacillus* sp. NP5 can accelerate re-maturation and enhance reproductive performance of female catfish broodstock. Probiotics contain metabolites that positively affect reproduction and can serve as an alternative to improve reproductive performance in female catfish broodstock.

Keywords: *Bacillus* sp. NP5, female catfish, metabolites, probiotics, re-maturation

ABSTRAK

Probiotik diyakini mengandung komponen aktif yang berpengaruh terhadap kinerja reproduksi ikan. Tujuan penelitian ini adalah mengevaluasi senyawa metabolit yang terkandung dalam bakteri probiotik *Bacillus* sp. NP5 dan pengaruhnya terhadap rematurasi induk ikan lele betina. Tahapan pertama adalah analisis kandungan metabolit probiotik *Bacillus* sp. NP5. dengan metode *liquid chromatography with tandem mass spectrometry* LCMS/MS. Selanjutnya dilakukan suplementasi pakan dengan perlakuan probiotik pada konsentrasi 10^6 CFU/g (P6), 10^8 CFU/g (P8), dan kontrol (K). Parameter yang diamati meliputi kandungan metabolit *Bacillus* sp. NP5, indeks gonadosomatik (IGS), indeks hepatosomatik (IHS), histologi gonad, tingkat kematangan gonad (TKG), fekunditas, jumlah telur matang, sebaran frekuensi diameter telur, derajat pembuahan (FR), derajat penetasan (HR), sintasan larva (SR), periode rematurasi, dan persentase induk matang gonad. Hasil penelitian memperlihatkan kandungan metabolit seperti resveratrol (22,45%) dan indoline (7,11%) diduga secara langsung berpengaruh terhadap reproduksi. Metabolit lain yang ditemukan seperti oxyresveratrol (22,95%) dan vestitol (4,14%) juga diduga berperan secara tidak langsung. Hasil suplementasi menunjukkan bahwa performa reproduksi terbaik didapat pada perlakuan 10^8 CFU/g pakan dengan peningkatan nilai IGS (14,74%), IHS (1,65%), TKG, persentase telur matang (86,00%), periode rematurasi (4 minggu), proporsi induk matang gonad (100%), fekunditas (243,605 butir telur/kg bobot induk), FR (98,74%), HR (80,62%), dan SR larva (87,31%). Pemberian probiotik *Bacillus* sp. NP5 dapat mempercepat rematurasi dan meningkatkan kinerja reproduksi induk ikan lele betina. Probiotik mengandung bahan-bahan metabolit yang berpengaruh positif terhadap reproduksi dan dapat menjadi alternatif dalam meningkatkan performa reproduksi pada induk ikan lele betina.

Kata kunci: *Bacillus* sp. NP5, ikan lele betina, metabolit, probiotik, rematurasi

INTRODUCTION

Aquaculture is one of the food-producing sectors that continues to grow faster globally. According to FAO (2022), the global aquaculture production that has been predicted to increase by 2030 comes from freshwater species, like Nile tilapia, common carp, and catfish. Catfish culture globally contributes 2.3% of the total fish production and is projected to increase up to 100% in 2030 (Tran *et al*., 2017). Therefore, catfish seed supply should be supported by improving the broodstock spawning process (Rawung *et al*., 2020). Fish spawning is determined by broodstock quality employed to produce excellent and qualified seeds.

Total of matured broodstocks and broodstock recovery speed to re-spawn (re-maturation) highly determines the continuous seed production (Hartami *et al*., 2022). Therefore, re-maturation period in catfish broodstock is necessary to consider as one of the limiting factors for catfish seeds in market. Female catfish *Clarias gariepinus* becomes mature at 10 months old and can re-spawn after 6.5 weeks, depending on nutritional supply from the feed and their environmental condition (Iswanto *et al*., 2016). Certain nutrients, either individually or in combination, can become key factors to improve fish growth and gonadal maturation (Volkoff *et al*., 2018). Nutrient improvement by dietary supplementations of hormones, turmeric, or both materials are extremely effective to accelerate re-maturation and improve reproduction performances of catfish (Hartami *et al*., 2022; Sudrajat & Rasid, 2020; Jufri *et al*., 2019) and striped catfish (Pamungkas *et al*., 2019). Another approach to overcome the re-maturation problem in catfish is through microbial application.

Nadio (2015) reported that biofloc microbes could stimulate the re-maturation period of female catfish broodstock on the 3rd week with the percentage of matured female broodstock at 80%. This condition occurred because the bacterial community accumulated in heterotrophic aquaculture system formed a floc that could be used as an additional diet with high protein content (Luo *et al*., 2019), specifically at 39-49% (Widanarni *et al*., 2012). Microbial utilization in gonadal maturity has been reported through dietary probiotic supplementation. Broodstock diets supplemented with probiotics, such as *Lactobacillus rhamnosus* IMC 501 (Gioacchini *et al*., 2011; Qin *et al*., 2014) and *Pediococcus acidilactici* (Mehdinejad *et al*., 2018) have been proven to boost the gonadal maturity.

A previous study performed by Ayuningtyas *et al*. (2020) revealed that dietary probiotic supplementation of *Bacillus* sp. NP5 at 10^{10} CFU/mL could improve the fecundity level, GSI, number of matured eggs, and hatching rate in catfish broodstock. Probiotic *L. rhamnosus* IMC 501 triggered the vitelogenic process in zebrafish *Danio rerio* by improving the nutritional supply required for gonadal development, thus the gonadosomatic index (GSI) of the fish, as a gonadal maturity indicator, also showed an improvement (Gioacchini *et al*., 2010; Mehrim *et al*., 2015). Moreover, metabolites produced by probiotics, such as extracellular protein, short-chained fatty acids (butyrate, propionate, and acetate), indole, and bacteriocin, have important roles in antimicrobial activities to improve the health status of the host (Liu *et al*., 2020).

Probiotics are thought to have important metabolites to improve fish reproduction performance. Characterization of *Bacillus* sp. NP5 metabolites are necessary to ensure types and numbers of active compounds associated with fish reproduction. Previous studies on dietary probiotic supplementation have shown that probiotics accelerate gonadal maturation, thus capable of stimulating the broodstock re-maturation time. This study aimed to evaluate the metabolite compounds present in the probiotic *Bacillus* sp. NP5 and their effect on female catfish broodstock re-maturation.

MATERIALS AND METHOD

Experimental design

This study was divided in two steps: First step was probiotic *Bacillus* sp. NP5 metabolites analysis using LCMS/MS method and second step was different doses of dietary probiotic supplementation. This step employed a completely randomized design with three treatments in triplicates. Treatments were composed of probiotic *Bacillus* sp. NP5 at 1% concentration (v/w) with different doses, namely K: without probiotic application, P6: 10^6 CFU/g diet, and P8: 10^8 CFU/g diet (Enzeline *et al*., 2022; Enzeline *et al*., 2024).

Dietary supplementation with probiotics

The probiotics *Bacillus* sp. NP5 were isolated from Nile tilapia digestive tract (Putra & Widanarni, 2015) and were confirmed as *Bacillus*

cereus (Djauhari *et al.*, 2016). These bacteria were cultured in 10 mL of tryptic soy broth, then incubated in a water bath shaker for 18 hours at 29°C and 140 rpm speed. The 1 mL of cultured bacteria were moved in a 1.5 mL microtube. The bacteria cells were harvested by centrifugation method for five minutes at 4°C and 5,000 rpm speed. The bacterial cell pellet was rinsed using a phosphate-buffered saline and diluted by 10^8 CFU/mL and 10^{10} CFU/mL.

The diluted products were supplemented to the diet at 1% (v/w), so the concentrations were 10^6 CFU/g diet and 10^8 CFU/g diet. The diets used were commercial feed with 30% protein content. Dietary supplementation was performed every day in the morning. The probiotics and egg whites as a binder at 3% (v/w) were sprayed evenly to the measured diets, then the probiotic-sprayed diets were air-dried. All diets were preserved in closed and dry containers, then kept in a refrigerator at 4°C.

Fish rearing

As samples, 10 catfish broodstocks per experimental unit were obtained from Center for Freshwater Aquaculture (BBPBAT) Sukabumi, West Java, at 818 ± 180.29 g. All broodstocks were initially spawned with artificial method to align the gonad maturity status, before re-maturation test. The ovulated broodstocks were randomly placed in 12 units of 1×1×1 m^3 net. The diets were fed to the fish twice a day at 06.00 WIB and 18.00 WIB until apparent satiation.

Fish were reared for eight weeks. The gonadal maturity level was performed biweekly by observing the stomach size and cloaca color of the female broodstock. Spawning process was performed in artificial way by artificial hormone stimulation. When the female broodstock was ready to spawn during gonadal observation, then artificial hormonal stimulation for spawning was employed using *Ovaprim* hormones at 0.3 mL/kg female broodstock and 0.1 mL/kg male broodstock. During broodstock rearing, water quality was controlled at normal range, namely temperature of 24.32–28.40°C, dissolved oxygen of 3.20–6.13 mg/L, pH of 6.51–7.37, and total ammonia of 0.01–0.06 mg/L.

Fish spawning

The 2.5 g of eggs from each female broodstock was mixed with 0.1 mL of male broodstock milt (spermatozoa) and incubated in a 40×30×20 cm^3 aquarium filled with aerated water 25–27°C. The hatching rate was determined, after eight hours of mixing the eggs and milt. The fertilized eggs (clear color) were randomly counted from five boxes on glass with lines and compared with the number of whole eggs. The hatching rate was counted 48 hours after the fertilized egg incubation. The hatched larvae were reared four days and fed with *Artemia*. During the hatching process and larval rearing, the water quality was controlled at a normal range, namely temperature of 26.18–28.36°C, dissolved oxygen of 4.16–5.29 mg/L, pH of 6.17–7.41, and total ammonia of 0.01–0.04 mg/L.

Parameters

LCMS/MS analysis

The secondary metabolites of probiotic *Bacillus* sp. NP5 was analyzed using the LCMS/MS method. The bacteria were initially extracted by growing them on a media, before maceration with solvent, filtering, and separation (Mangurana *et al.*, 2019). Data in this analysis method is presented in chromatograph with high flow peak and molecular weight of each compound in the extract, so the total compound in each extract sample is known. The chemical composition and spectral value were analyzed using *Masslynx* software and confirmed in the websites *Chemspider* or *Masbank*.

Gonadosomatic index (GSI) and hepatosomatic index (HSI)

The gonadosomatic and hepatosomatic indexes of female catfish in this study were counted biweekly, based on Tyor and Pahwa (2017):

$$\text{GSI} = \frac{\text{Gonad weight}}{\text{Fish weight}} \times 100$$

$$\text{HSI (\%)} = \frac{\text{Liver weight (g)}}{\text{Fish weight}} \times 100$$

Gonadal maturity level and gonad histology

The gonadal maturity level was determined by observing the gonadosomatic index, gonadal histology, and gonadal anatomy, following the gonadal maturity level grouping by Tyor and Pahwa (2017).

Egg diameter

The egg diameter was measured from 100 egg samples from each catfish broodstock in a microscope with 40× magnification, after the spawning process was successful (Ayuningtyas

et al., 2020). The egg diameter was measured to determine the egg diameter frequency distribution and matured egg size.

Fecundity (F)

Fecundity was calculated to determine the number of successfully spawned eggs at the final period of the study. Relative fecundity was calculated by comparing the number of eggs produced and broodstock bodyweight (kg) (Mylonas *et al*., 2010).

Fertility rate (FR), hatching rate (HR), and survival rate (SR)

The fertility rate and hatching rate were calculated, after eggs were fertilized and hatched. The survival rate of the larvae was calculated at the 4th day of incubation period:

$$FR = \frac{\text{Number of fertilized eggs}}{\text{Total number of eggs}} \times 100$$

$$HR = \frac{\text{Number hatching eggs}}{\text{Total number of eggs}} \times 100$$

$$\text{SR larvae (\%)} = \frac{\text{Total number of living larvae}}{\text{Number hatching eggs}} \times 100$$

Re-maturation period and matured broodstock proportion

The re-maturation period was counted from first spawning, mature, ovulation to re-spawning (Sudrajat & Rasid, 2020). The re-maturation period was observed biweekly along with the matured broodstock proportion observation (Iswanto *et al*., 2016):

$$\text{Matured broodstock proportion (\%)} = \frac{\text{Number of matured broodstocks}}{\text{Number of broodstocks}} \times 100$$

Data analysis

The histological samples were analyzed descriptively in figures. The egg diameter frequency distribution, re-maturation period, and proportion of matured broodstock are presented in graphs. The analysis results of LCMS/MS method are presented in table. All parameter data were analyzed using a one-way analysis of variance (One-way ANOVA) with Microsoft Office Excel 2007 and SPSS 25.0 to determine a significant difference in all treatments with 95% confidence level. A significantly different treatment ($p<0.05$) was further analyzed using Duncan's test to determine the best dietary probiotic supplementation against the control treatment.

RESULTS AND DISCUSSION

Results

Compounds in Bacillus sp. NP5

The LCMS/MS analysis results are presented in chromatographic peak with certain retention time. The *Bacillus* sp. NP5 bacteria produced several peaks identified as compounds with certain molecular weight (Table 1). The chromatograph analysis of *Bacillus* sp. NP5 was performed to identify the metabolites. Ten compounds in methanol solvent are present in probiotic *Bacillus* sp. NP5, that are thought as secondary metabolites with the highest compounds are shown from oxyresveratrol (22.95%), resveratrol (22.45%), and Indoline (7.11%).

Reproductive performance of female catfish broodstock

The observation results of gonadal maturity level in female catfish broodstock are described in Table 2. The gonadal maturity level (TKG) in all treatments is included in the third maturity level (TKG III) in the 2nd week, then began to increase at 4th week. This week, the probiotic treatments were in the fourth maturity level (TKG IV), while the control treatment was in the TKG III. The peak maturity level was observed on the 6th week in all treatments, then began to decrease on the 8th week for probiotic treatment. The gonadosomatic index was significantly different ($P<0.05$) between probiotic treatments and control treatment from the 2nd to 8th week. At 4th week, the highest GSI value was obtained from the P8 treatment at 14.74%, followed by the GSI value in control treatment at 6th week and P6 treatment.

Furthermore, the hepatosomatic index (HSI) indicates a significant difference ($P<0.05$) between probiotic and control treatments from the 3rd to 8th week. The highest HSI value reached 1.65% in P8 in the 2nd week, followed by the P6 and control treatments. The percentage of matured eggs was significantly different in P8 against the control ($P<0.05$). The P8 treatment also showed the highest value of 86% in the 6th and 82.33% in the 2nd week. Initially, the egg diameter distribution in all treatments was 0.517 mm (Figure 1).

In the 2nd week, the P6 treatment showed the highest peak of large egg size (1.17 mm) at 40.3%, followed by the control treatment of 34%,

and P8 of 30%. The highest peak of large eggs distribution was observed by the 8th week. As the maintenance period increased, a larger and a more uniform egg size started from the 2nd week of the probiotic treatment compared to the control. The results of gonad histology (Figure 2) also showed that the control treatment in the 2nd week was still in the maturing phase of egg development, while the probiotic treatment was in the mature stage.

Spawning performance

The fecundity of female catfish broodstock showed significant differences (P<0.05) in treatment P8 compared to control treatment (Table 3). The highest fecundity was observed in the 6th week of P8 treatment at 243,605 ± 28,937 eggs/kg body weight, followed by P6 treatment at 191,792 ± 19,039 eggs/kg body weight, and control at 148,828 ± 2,475 eggs/kg body weight.

Table 1. Compounds in *Bacillus* sp. NP5 (LCMS/MS analysis).

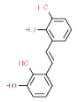
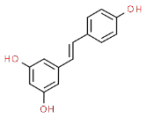
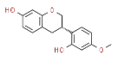
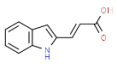
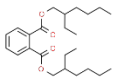
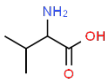
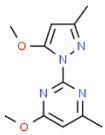
Compounds	Area (%)	Structure	Group	Activity	References
Oxyresveratrol	22.95		Stilbenoids	Antiinflammation, antivirus, anticancer	Akinwumi *et al.*, 2018
Resveratrol	22.45		Stilbenoids	Alpha- and beta-estrogen receptor binder, improving the cow oocyte quality, granulose cell gene modulator	Pasquariello *et al.*, 2020
Indoline	7.11		Heterocyclic aromatic	Inducing responsible genes for oogenesis, improving immune gene expression	Sonowal *et al.*, 2017; Zhang *et al.*, 2017
Vestitol	4.14		Flavonoids	Antimicrobe, antiinflammation	Franchin *et al.*, 2016
Indoleacrylic acid	2.99		Heterocyclic aromatic	Antiinflammation, fighting oxidative stress	Hendrikx & Schnabl, 2019
Bis (2-ethylhexyl) phthalate	2.64		Phthalates	Pathogenic bacteria inhibition	Murniasih *et al.*, 2022
DL-Valine	2.21		Amino acids	Improving the body protein content, digestive enzymes, immune response, growth performance	Ahmad *et al.*, 2021
Epirizole	1.23		Heterocyclic aromatic	Antimicrobe, antiinflammation	Alam *et al.*, 2015
Erucamide	1.03		Erukat acid	Reducing stress and anxiety in mice	Li *et al.*, 2016
Mosloflavone	0.01		Flavonoids	Antimicrobe, antiinflammation, antivirus	Choi *et al.*, 2016

Table 2. Reproductive performance of female catfish broodstock with dietary supplementation of probiotic *Bacillus* sp. NP5.

Observation	Treatment	TKG	GSI (%)	HSI (%)	Matured egg diameter (>0.85 mm) (%)
M-2	K	III	4.18 ± 0.48[a]	1.20 ± 0.01[a]	57.67 ± 0.58[a]
	P6	III	7.41 ± 0.38[b]	1.61 ± 0.07[b]	69.33 ± 3.51[b]
	P8	III	8.19 ± 0.89[b]	1.65 ± 0.05[b]	82.33 ± 6.11[c]
M-4	K	III	6.03 ± 0.31[a]	1.16 ± 0.01[a]	54.33 ± 5.13[a]
	P6	IV	8.20 ± 0.63[b]	1.23 ± 0.05[b]	57.00 ± 3.61[ab]
	P8	IV	14.74 ± 0.57[c]	1.30 ± 0.02[c]	64.00 ± 4.58[b]
M-6	K	IV	12.26 ± 0.80[b]	1.37 ± 0.04[c]	74.00 ± 4.58[a]
	P6	IV	9.31 ± 0.45[a]	1.05 ± 0.02[b]	78.00 ± 4.36[ab]
	P8	IV	8.57 ± 0.47[a]	0.89 ± 0.06[a]	86.00 ± 5.20[b]
M-8	K	IV	8.30 ± 0.46[b]	1.28 ± 0.01[b]	70.67 ± 2.08[a]
	P6	V	1.30 ± 0.68[a]	0.82 ± 0.04[a]	78.33 ± 4.93[a]
	P8	V	1.47 ± 0.32[a]	0.79 ± 0.06[a]	76.67 ± 7.23[b]

Note: Different superscript letters in the same column indicate a significantly different value (P<0.05).

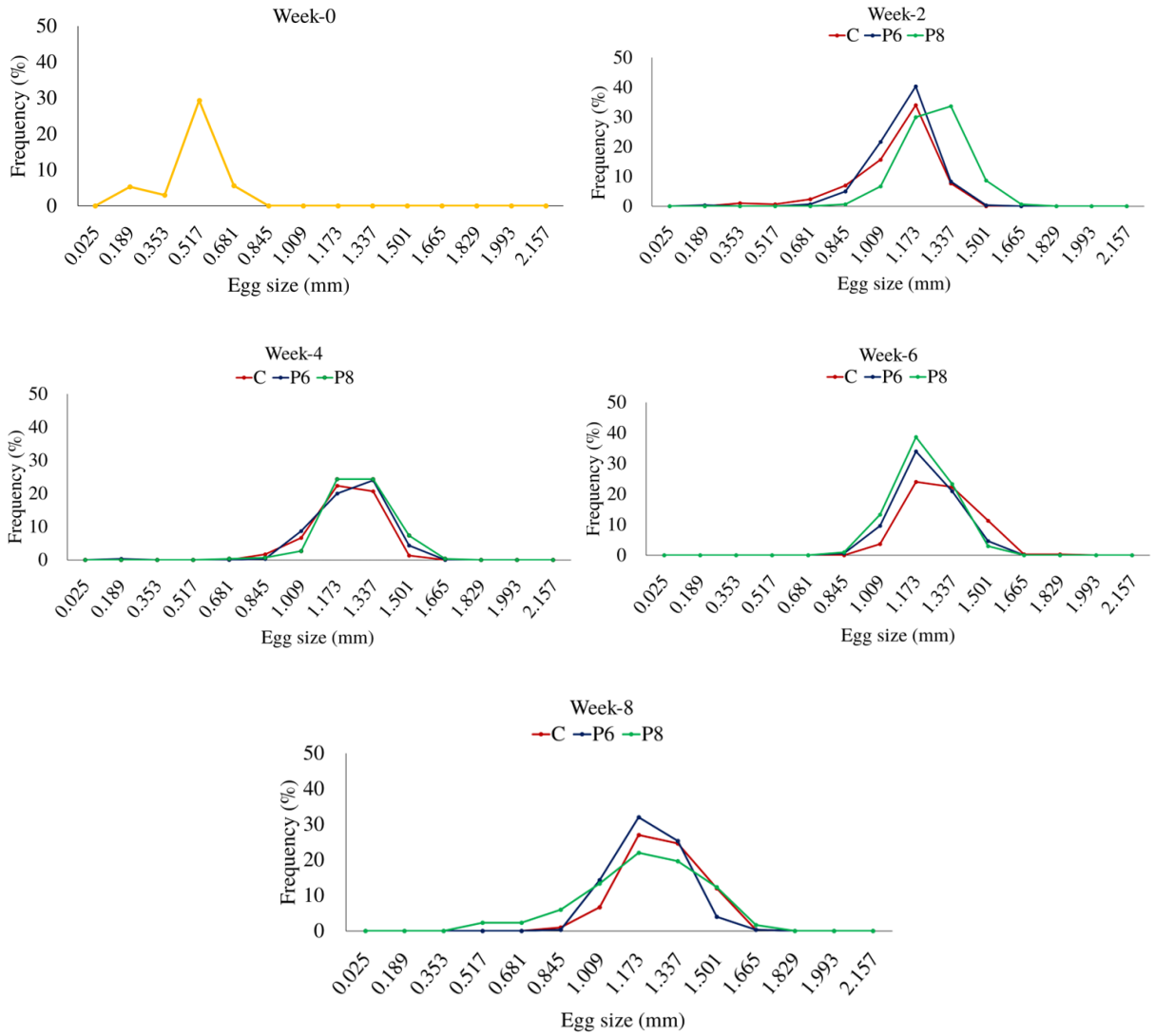


Figure 1. Frequency distribution of female catfish egg diameter and gonad histology on different *Bacillus* sp. NP5 probiotic dietary supplementations for 60 days.

The fertilization rate of eggs in P6 showed significant differences compared to control in the 6th and 8th weeks (P<0.05). The highest fertilization rate occurred in P6 treatment on the 6th week at 98.74%.

The hatching rate also showed significant differences in the 5th and 7th weeks (P<0.05). Probiotic treatment in the 5th week demonstrated a higher hatching rate in P8 at 80.62% compared to the control at 59.30%. The 4-day larval survival rate showed the highest and significantly different results in the probiotic treatments on the 7th week of spawning (P<0.05). Larval survival rates were 87.31% in P8, 81.35% in P6, then 71.28% in control treatment.

Rematuration period and percentage of matured broodstock

The observation results of the re-maturation period and the proportion of matured female catfish over an eight-week rearing period are sequentially shown in Figures 3 and 4. The fastest re-maturation period was observed in P8 and P6 treatments, which could be re-spawned after four weeks of post-spawning, with the proportion of matured females up to 100%. In contrast, the slowest re-maturation period occurred in the control group, with a re-maturation period occurred after six weeks and the proportion of matured females at 6th week at 93.33%.

Discussion

Compounds thought to have roles to improve the reproduction performance like oxyresveratrol were found in this study and have been reported to possess various bioactivities, such as anti-inflammation and antiviral properties (Akinwumi *et al*., 2018). Resveratrol is thought to be capable of binding with estrogen receptor, namely alpha- and beta-receptors, improving the cow embryonic oocyte quality, modulating genes in granulose cells, and enhancing the mitochondrial performance in the developing pig oocytes (Pasquariello *et al*., 2020). Morita *et al*. (2012) also reported that resveratrol could also induce the transcriptions of *SIRT1*, *lh receptor*, *StAR*, and *P450 aromatase*, which could modulate the ovarium functions through molecules associated

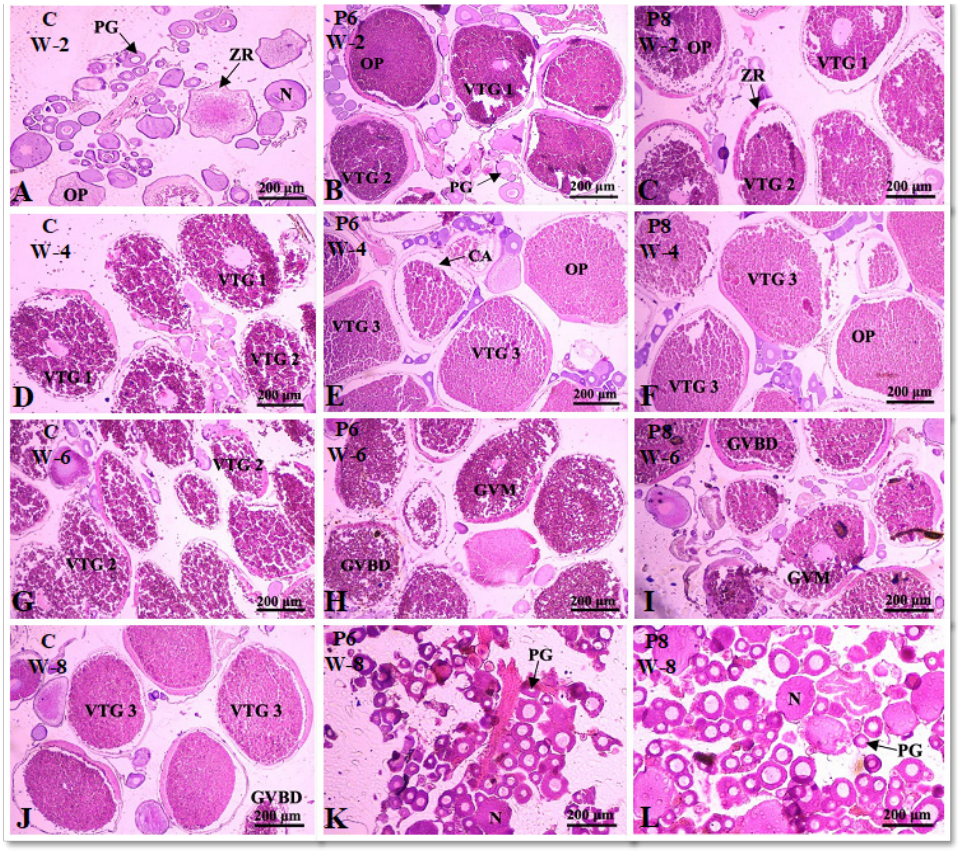


Figure 2. Gonadal histology of female catfish broodstocks at 2th week (A-C), 4th week (D-F), 6th week (G-I), and 8th week (J-L) in a 40× microscope. PG= primary growth oocyte, ZR: radiata zone, N= nucleus, OP: ooplasm; VTG 1= primary vitellogenin; VTG 2= secondary vitellogenin; VTG 3= tertiary vitellogenin; GVM= germinal vesicle migration; dan GVBD= germinal vesicle breakdown.

Table 3. Spawning performance of female catfish broodstock with *Bacillus* sp. NP5 dietary supplementation.

Spawning	Treatment	Fecundity (eggs/kg bodyweight)	Fertility rate (%)	Hatching rate (%)	4-day larval survival rate (%)
M-5	K	58,217 ± 8,588[a]	94.45 ± 2.35[a]	59.30 ± 0.07[a]	58.08 ± 4.69[a]
	P6	109,400 ± 3,666[b]	97.47 ± 1.99[a]	70.06 ± 0.16[b]	66.49 ± 3.92[ab]
	P8	140,933 ± 34,053[b]	96.94 ± 0.39[a]	80.62 ± 0.39[c]	71.95 ± 4.87[b]
M-6	K	148,828 ± 2,475[a]	92.61 ± 2.38[a]	93.14 ± 0.51[a]	88.75 ± 7.63[a]
	P6	191,792 ± 19,039[b]	98.74 ± 0.37[b]	92.14 ± 0.65[a]	82.40 ± 4.99[a]
	P8	243,605 ± 28,937[c]	95.51 ± 1.94[ab]	86.83 ± 6.72[a]	81.37 ± 7.02[a]
M-7	K	86,703 ± 2,682[b]	95.36 ± 1.47[a]	60.15 ± 0.54[a]	71.28 ± 2.70[a]
	P6	60,670 ± 5,152[a]	94.10 ± 2.79[a]	74.98 ± 5.02[b]	81.35 ± 3.85[b]
	P8	55,760 ± 2,075[a]	92.57 ± 0.78[a]	85.17 ± 5.28[c]	87.31 ± 4.44[b]
M-8	K	39,459 ± 6,394[b]	95.43 ± 2.14[b]	84.51 ± 8.69[a]	74.92 ± 4.01[a]
	P6	30,627 ± 485[a]	87.04 ± 3.01[a]	82.27 ± 7.64[a]	86.61 ± 1.66[b]
	P8	27,503 ± 3,959[a]	90.61 ± 2.19[ab]	89.79 ± 0.42[a]	73.65 ± 2.28[a]

Note: Different superscript letters on the same column indicate a significant difference value (P<0.05).

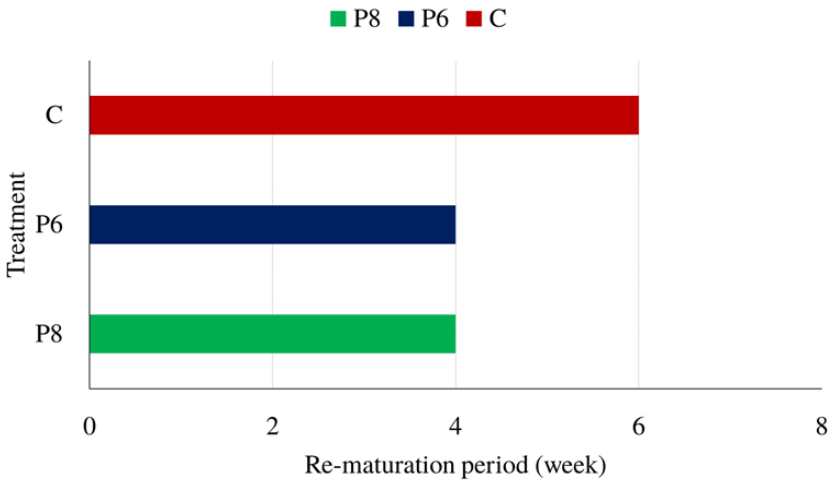


Figure 3. Re-maturation period of matured female catfish broodstock with probiotic *Bacillus* sp. NP5 dietary supplementation.

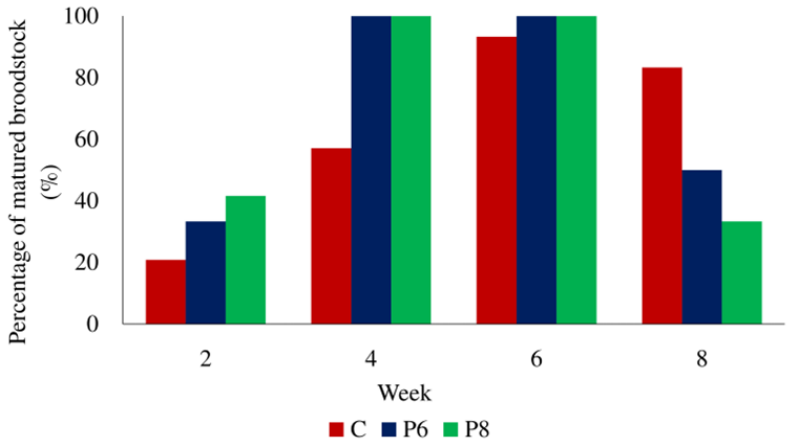


Figure 4. Proportion of matured female catfish broodstock in probiotics *Bacillus* sp. NP5 dietary supplementation.

with folliculogenesis and gonadotropin-receptor activation. Therefore, resveratrol is strongly thought to influence genes on the hypothalamic-pituitary-gonadal axis (Pasquariello *et al*., 2020).

Indoline and Indoleacrylic acid, derivatives of tryptophan, exhibit antimicrobial effects. In animals, the indole group can induce genes responsible for oogenesis, thereby extending fecundity in the nematode *Caenorhabditis elegans* (Sonowal *et al*., 2017) and enhancing immune gene expression (*Ajp105* and *AjLBP/BPI1*) in the sea cucumber *Apostichopus japonicus* (Zhang *et al*., 2017). These results suggest that probiotics may act indirectly by activating neuropeptide hormones and metabolic signals, such as *kiss1*, *kiss2*, and leptin, which are involved in reproductive processes (Gioacchini *et al*., 2010). Other antimicrobial and anti-inflammatory compounds such as vestitol (Franchin *et al*., 2016) are also found to have an indirect role in reproduction.

The GSI value for eight weeks of rearing tends to accelerate faster in all probiotic treatments compared to control (Table 2). The P8 treatment for four weeks showed the highest GSI value at 14.74% compared to other treatments. This condition was similar to Ayuningtyas *et al*. (2020), that the supplementation of probiotic *Bacillus* sp. NP5 on female catfish *Clarias* sp. broodstock could increase the gonadosomatic index (GSI). Gioacchini *et al*. (2011) also reported that the supplementations of *Lactobacillus rhamnosus* IMC 501® on zebrafish *Danio rerio* and catfish *Clarias gariepinus* (Enzeline *et al*., 2024) demonstrated a higher GSI value than control treatment. These results were also higher than dietary supplementation of *Oodev* and *Spirulina* that contained carotenoids on catfish *Clarias* sp. with the peak value of GSI at 7.75-11.31% in the 6th week of pre-spawning period (Nainggolan *et al*., 2014).

The increased GSI value is also consistent with HSI value as indicators of the vitellogenesis process in the liver. HSI and GSI values reach their maximum before spawning, then decrease after spawning (Hariani *et al*., 2020). The P8 treatment showed a peak HSI value in the 2nd week of 1.65%, characterized by TKG III, according to Tyor and Pahwa (2017). Probiotics help enhance endocrine signalling through the activation of several hormones (Aydin *et al*., 2019). This condition is related to the increased GSI and HSI values, due to the elevated *estradiol receptor* ($er\alpha$) and *vitellogenin gene* (vtg) expression as a result of probiotic supplementation (Gioacchini *et al*., 2011). The P8 treatment not only affects GSI and HSI values, but also impacts the gonadal histology and the percentage of mature egg sizes.

The gonadal histological observation presents the egg size from probiotic treatments that are in a mature condition (Figure 2), and the increasing percentage of mature eggs has been occurred since the 2nd week compared to the control treatment at 6th week (Table 2). According to Hariani and Kusuma (2019), great and mature eggs are associated with the HSI and GSI. Similar conditions also emerged in zebrafish *Danio rerio* (Gioacchini *et al*., 2010), goldfish *Carassius auratus* (Mehdinejad *et al*., 2018), and rainbow trout *Oncorhyncus mykiss* (Nargesi *et al*., 2019), as dietary supplementation of probiotics can induce oocytes development faster. Probiotics influence the nutrient formation, such as proteins and fatty acids (Carnevali *et al*., 2017), to improve the nutritional status of broodstock, thereby increasing egg size (Nargesi *et al*., 2019).

Consistently, the fastest re-maturation period occurred in the P6 and P8 treatments, requiring four weeks to mature again after spawning, with 100% of the matured broodstock gonad. This result is similar to the use of *Pregnant Mare Serum Gonadotropin* (PMSG) and *Spirulina* in *C. gariepinus*, that could accelerate re-maturation to four weeks with 80% of the broodstock being mature (Mayasari *et al*., 2012). Laser stimulation combined with probiotic feed also showed re-maturation that occurred on the 41-st day (Kusuma *et al*., 2022). Furthermore, probiotic supplementation proved to shorten the re-maturation period compared to female *Mutiara* catfish, which took 1.5 months (Iswanto *et al*., 2016).

Recent study reported that probiotics are involved in oocyte maturation by stimulating ovarian follicle maturation and increasing the levels of neuropeptide hormones (leptin and kisspeptin) responsible for enhancing reproductive performance (Carnevali *et al*., 2017; Qin *et al*., 2014). At the central nervous system level, leptin induces reproduction by activating the production of the neuropeptide kisspeptin. Subsequently, Kiss1/GPR54 signalling is central to the regulation of GnRH, and consequently, to the secretion of LH and FSH (Gioacchini *et al*., 2014). However, the way probiotics stimulate leptin and kisspeptin secretion to promote earlier oocyte maturation remains unknown. The spawning performance under probiotic treatment

showed positive results on fecundity, hatching rate, and survival rate of the larvae (Table 3).

This condition has been previously demonstrated in catfish (Ayuningtyas *et al*., 2020), Nile tilapia (Nargesi *et al*., 2019), goldfish (Mehdinejad *et al*., 2018), zebrafish (Gioacchini *et al*., 2011), and female catfish *Clarias gariepinus* (Enzeline *et al*., 2024) with increased fecundity due to probiotic dietary supplementation. This study also showed an impact on the increased hatching rates and survival rates. These results were similar to the increased hatching rates in catfish (Ayuningtyas *et al*., 2020*;* Rahman *et al*., 2018), Nile tilapia (Nargesi *et al*., 2019), and zebrafish (Gioacchini *et al*., 2011) due to probiotic supplementation. A higher hatching rate value in probiotic treatments was thought due to higher GSI values, resulting in higher egg maturation and vitellogenin levels (Ayuningtyas *et al*., 2020). Other studies also mention that probiotics could improve the larval survival rates in *Sparus aurata* (Ghoname *et al*., 2020) and catfish larvae (Rahman *et al*., 2018*;* Enzeline *et al*., 2024).

The probiotics *Bacillus sp.* NP5 can enhance the activity of digestive enzymes, such as protease, lipase, and amylase in the host's digestive tract (Widanarni *et al*., 2022), thereby improving digestibility and nutritional value of the feed. Essential nutrients like proteins and lipids are crucial for various reproductive processes, including fecundity, fertilization, hatching, and energy source for embryos during larval development (*Kusuma et al*., 2022). Additionally, probiotics from *Bacillus* strains such as *B. cereus, B. clausii, B. coagulans, B. licheniformis, B. polyfermenticus, B. pumilus,* and *B. subtilis* can produce vitamins (Lee *et al*., 2019). So far, probiotics that are capable of synthesizing vitamins like B1 and B12, could enhance the survival rates of ornamental fish larvae (Ghosh *et al*., 2007). Vitamin B2 can increase digestive enzyme activity in fish, like *Channa punctatus* (Zehra *et al*., 2018) and *Oncorhynchus kisutch* (Yu *et al*., 2022). Furthermore, in our study, dietary supplementation of probiotics *Bacillus sp.* NP5 could induce the expression of reproduction-related genes, such as *cyp19a1b*, *GnRH*, *fshb*, *lhb*, *17bhsd*, *cyp19a1a*, *activin*, *vtg*, and *fads2* (in preparation).

CONCLUSION

The dietary supplementation of *Bacillus* sp. NP5 could stimulate re-maturation and improve the reproduction performance of female catfish broodstock with the best concentration of 10^8 CFU/g diet. The probiotics are composed of several metabolites, such as oxyresveratrol, resveratrol, indoline, and vestitol, that can affect positively on fish reproduction. Therefore, probiotic *Bacillus* sp. NP5 can be an alternative material to induce reproductive performance of female catfish broodstock.

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