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ORIGINAL ARTICLE

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Characterization and expression of NADPH oxidase genes in stimulated splenic neutrophils of tilapia, *Oreochromis niloticus* by a mix of traditional Chinese medicine and *Bacillus* species (TCMBS)

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Abstract

Traditional Chinese medicine and *Bacillus* species (TCMBS) mixture is an immunostimulant with considerable promise as an alternative in improving fish health. However, nothing is known on its effects on the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase genes and production of reactive oxygen species (ROS) in the neutrophils of fish. The full lengths of tilapia phagocytic NADPH oxidase genes gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, and p67^{phox} were cloned and their expression profiles after TCMBS stimulus investigated. The cDNAs of tilapia gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, and p67^{phox} contained open reading frames of 1698 bp, 561 bp, 1053 bp, 1584 bp, and 1470 bp respectively, encoding 561, 186, 350, 527, and 489 amino acids respectively. Comparison of the deduced amino acid sequences showed that tilapia NADPH oxidase genes shared 58%–91% and 21%–67% identity with those of other teleost and mammals respectively. Besides, tilapia NADPH oxidase genes contain conserved domains and motifs required for ROS generation. Phylogenetic analysis suggested tilapia NADPH oxidase genes were close to those of *Fundulus heteroclitus*. After 2 weeks of TCMBS application showed significant upregulation in expression of NADPH oxidase genes, antioxidant genes (i.e., superoxide dismutase, catalase, and glutathione-disulphide reductase), and an increase in the production of ROS compared to the control in splenic neutrophils of tilapia. Collectively, our study provides evidence of the structure of tilapia NADPH oxidase genes and demonstrate that TCMBS application could modulate their activity in neutrophils to improve immunity in tilapia.

KEYWORDS
antioxidant genes, NADPH oxidase genes, reactive oxygen species, tilapia

1 | INTRODUCTION

Outbreaks of pathogenic disease in fish farms have caused considerable losses in fish culture (Alexander, Kirubakaran, & Michael, 2010; Cerezuela, Guardiola, González, Meseguer, & Esteban, 2012). Antibiotics and other chemicals widely applied in fish culture for disease prevention are no longer safe because of development of antibiotic-resistant pathogens, residue accumulation in water, and potential of resistant gene transfer to humans (Heuer et al., 2009; Magnadottir, 2010). In recent years, increasing interests are focused on the use of natural growth immune promoters in fish culture (Standen et al., 2016). It has been well-documented

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that herbs (Harikrishnan, Balasundaram, & Heo, 2010; Jagruthi et al., 2014; Tang et al., 2014) or probiotic *Bacillus* species (Abarike, Cai, et al., 2018; Hauville, Zambonino-Infante, Gordon Bell, Migaud, & Main, 2016; Wang et al., 2017) can improve growth and increase fish immunity.

Neutrophils are a type of white blood cells that act as the first line of response against pathogen invasion in a host. Neutrophils fight infections by oxygen dependent and nonoxygen independent systems (Inoue, Suenaga, Yoshiura, & Moritomo, 2004). With oxygen-dependent mechanism, neutrophils kill invading infectious pathogens through expressing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase genes that catalyse the production of reactive oxygen species (ROS) such as O_2^-, H_2O_2, and OH^{-1} (Maraldi, 2013; Martyn, Frederick, Loehneysen, Dinauer, & Knaus, 2006). ROS generation by NADPH oxidase genes can be activated and deactivated through a series of functional relationships between membrane proteins genes ($gp91^{phox}$ and $p22^{phox}$) and cytosolic proteins genes ($p40^{phox}$, $p47^{phox,}$ and $p67^{phox}$) (Groemping & Rittinger, 2005; Kawahara, Quinn, & Lambeth, 2007; Vignais, 2002). Although the important roles of NADPH oxidase genes have been well-recorded in humans, few cases are found in fish (Boltaña, Doñate, Goetz, MacKenzie, & Balasch, 2009; Mayumi et al., 2008). Research on the structure of NADPH oxidase genes might help to elucidate the mechanism of oxygen radical production in a host (Inoue et al., 2005). Furthermore, knowing the effect of herbal-probiotic stimulus on neutrophil-induced immune defence might be useful in the advancement of scientific knowledge and for the development of effective methods for preventing diseases in fish culture.

Research on the use of multispecies herbal-probiotic supplements in fish is lacking (Cerezuela et al., 2012). In this sense we have earlier demonstrated that traditional Chinese medicine and probiotic *Bacillus* species (TCMBS) comprising a mix of *Astragalus membranaceus*, *Angelica sinensis*, *Crataegus hupehensis*, *Bacillus subtilis*, *and Bacillus licheniformis* showed improved growth, immune response, and disease resistance against *Streptococcus agalactiae* infection in tilapia (Abarike, Jian, et al., 2018). The present study deepens knowledge on the mechanism of action of TCMBS on NAPDH oxidase genes activities in splenic neutrophils of tilapia, *Oreochromis niloticus*, an important fresh water aquaculture species whose production has quadrupled in the past decade due to wide adoption of intensive farming technologies (Hai, 2015). However, recent reports indicate that its production has been hindered by pathogenic infections (Gabriel et al., 2015; Pech-Huicab, Chavez-Castaneda, & Reynoso-Lango, 2017).

Here, we describe the cloning, sequencing, and phylogenetic analysis of cDNA of five NADPH oxidase genes $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ from tilapia. Besides, expression patterns of NADPH oxidase genes, and antioxidant genes (superoxide dismutase [SOD] catalase [CAT], and glutathione-disulphide reductase, [GSR]) and production of ROS in splenic neutrophils after TCMBS stimulus are described.

2 | MATERIALS AND METHODS

2.1 | Preparation of diets

Regarding traditional Chinese medicine (TCM) composition, dry roots of *Astragalus membranaceus*, *Angelica sinensis*, and *Crataegus hupehensis* were purchased from the open market in Zhanjiang, China, washed in double distilled water, dried in the shade, powdered, mixed in a ratio of 1:1:1 w/w and stored at −20°C for later use. Spores of a commercially produced probiotic *Bacillus* species (BS) comprising a mix of *B. subtilis and B. licheniformis* in a ratio of 1:1 w/w was also purchased from Lubiaduo Company, China, for use. Using the spread plate method, the viability of BS was found to be 2 x 10^9 cfu/ml. In addition, a normal commercial pellet diet was purchased from Heng xing company, China, crushed into powder using a feed miller, and divided into two portions. The first portion was used as control (CT) diet while the second portion was supplemented with TCMBS at 10 g/kg (i.e., TCM at 7 g/kg and BS at 3 g/kg) as previously described (Abarike, Jian, et al., 2018). Feedstuff was then mixed homogeneously in a helical mixer, moistened by adding filtered water and remade into 3 mm pellets. The prepared feed was dried under 18°C and later stored in insect-proof bags at room temperature.

2.2 | Experimental set-up and fish management

One hundred and twenty (120) tilapia fish (30 ± 2 g mean weight) without any signs of infection (i.e., abdominal distension, hernia, and haemorrhage) were purchased from a commercial farm (Gaozhou, Guangdong province, China) and held in our laboratory in plastic tanks of capacity 100 L containing 90 L of static water. Fish were divided into six tanks (i.e., 20 fish in each tank) and later assigned in triplicate groups each for the CT group and the TCMBS group. Fish were fed the control diet of about 5% body weight daily twice daily in equal proportions at 9:00 a.m. and 5:00 p.m. during a 1-week acclimatization period. Water quality in the tanks was maintained at 28 ± 2°C, 12 hr dark/12 hr photoperiod with constant aeration and 30% daily water change. After acclimatization, fish in each group were fed their respective diets as before for an experimental period of 2 weeks, and after that, they were challenged with a test bacteria (*Streptococcus agalactiae*) and observed for a further 1 week. Feeding and water quality maintenance were the same as during acclimatization.

2.3 | Tissue collection

Thymus, intestine, liver, and spleen tissues samples in which many phagocyte NADPH oxidase genes reside (Mayumi et al., 2008) were collected from three fish from the same batch of fish used in the main experiment for gene cloning and analysis of the tissue expression of NADPH oxidase genes. After 2 weeks of experimental feeding (Sharifuzzaman & Austin, 2009), spleen tissues from nine fish each from the CT and TCMBS groups were collected for the

extraction of neutrophils for use in ROS detection, NADPH oxidase and antioxidant genes expression test. Afterwards, fish were challenged with a pathogen (see section 2.10) and splenic neutrophils samples from nine fish taken from each of the groups after 1 week of observation for the same ROS test.

2.4 | Neutrophil isolation

Splenic neutrophils were isolated immediately from freshly taken spleen tissues as described by Lamast and Ellis (1994). Briefly, tilapia spleen tissues were macerated on a stainless steel mesh in Leibovitz medium (L-15 Gibco) containing penicillin (100 μg/ml)/streptomycin (100 units ml^{-1}). Cell suspensions were carefully layered on a discontinuous Percoll gradient in 15 ml sterile tubes for density gradient centrifugation. The Percoll gradient consisted of Percoll diluted with Hank's balanced salt solution (HBSS) and sterile distilled water to give a concentration of 49% and 34%. The gradient was centrifuged at 250 g for 10 min, and 250 g for 24 min at 4°C and leukocyte fraction was collected from 50% Percoll interface with a pipette and afterwards washed with L-15 contain 0.1% of foetal calf serum (FCS). After isolation, cell viability checked using trypan blue dye exclusion test was found to be 96%. A portion of the cells was quantified using a hemocytometer, and the concentration adjusted to 1×10^6 cells, ml^{-1} for ROS test. The remaining portion was used for RNA extraction and subsequent gene expression study.

2.5 | ROS test

To monitor the production of ROS, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was purchased from Nanjing Jiancheng company, China, and used following the manufacturer's protocol. Briefly, with 190 μl of splenic neutrophils previously adjusted to a final concentration of 1×10^6 cells ml^{-1} was incubated with 10 μl of DCFH-DA for 2 hr 30 min in the dark at 37°C. The negative control consisted of splenic neutrophils and phosphate-buffered saline (PBS). After incubation, the reaction mixture was washed 2–3 times with 300 μl of PBS, resuspended in 300 μl of PBS then the fluorescence of the cell suspensions was recorded with the Enspire microplate reader. ROS production was expressed as mean fluorescence of dichlorofluorescein (DCF).

2.6 | RNA extraction and cDNA synthesis

Total RNA from the thymus, intestine, liver, and spleen tissues as well as spleen neutrophils was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The quality of total RNA was measured spectrophotometrically (NanoDrop 2000, Thermo scientific) and by electrophoresis on 1% agarose gel. Total RNA from fish with the best RNA quality (absorbance 260/280 > 1.8 and 260/230 > 1.8) was used to synthesise first strand cDNA.

One-Step gDNA removal and cDNA Synthesis SuperMix kit (Transgen, China) was used for cDNAs synthesis following manufacturer's protocols, for real-time reverse polymerase chain reaction (qRT-PCR). cDNAs for cloning the full lengths of NADPH oxidase genes were synthesized using SMARTer™ RACE cDNA amplification kit (Clontech, USA) following the manufacturer's instruction.

2.7 | Cloning and sequencing of the tilapia NADPH oxidase genes

To amplify the full lengths of NADPH oxidase genes, primers for the partial sequence were designed using NADPH gene sequences from our Nile tilapia transcriptome data (unpublished). Using polymerase chain reaction (PCR), amplification of partial gene fragments was carried out in a 50 μl reaction volume containing 25 μl rtag mix (Transgen, China), 2.5 μl sense and 2.5 μl anti-sense primers, 2 μl cDNA and 18 μl double distilled water. The thermal profile for PCR was 94 for 5 min, 35–40 cycles of 94 for 30 s, 55–60 for 30 s, and 72 for 30 s. PCR products were purified with a quick gel extraction kit (Thermo, Lithuania), ligated into pMD18-T vectors (Takara, Japan) and transformed into competent *Escherichia coli* DH5a cells. Positive clones were sequenced at Sangon Biotech (Shanghai, China). After obtaining the partial sequence, using the same PCR reactions and process as before, primers were designed from the partial sequences to amplify the 5'/3' untranslated regions (UTRs) (Supporting Information Table S1 for a list of primers).

2.8 | Sequence analysis

The partial and 5'/3' UTRs and were assembled to form the full-length cDNA of target genes using the DNAman8 software. Gene translation, predictions of the amino acid sequences, and location of domains were done using ExPASy (https://www.expasy.org/tools/) and SMART (https://www.smart.emblheidelberg.de) web tool. *N*-glycosylating sites were predicted by NetNGlyc1.0.Server (https://www.cbs.dtu.dk/services/NetNGlyc). The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm at the NCBI (https://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignments and similarities were performed with predicted amino acid sequences of tilapia NADPH oxidase subunit genes and other vertebrates obtained from NCBI using CLUSTALW and GENDOC version 2.7. Phylogenetic and molecular evolutionary analysis was carried out and the consensus trees compared by bootstrap employing MEGA version 6.0. The neighbour-joining (NJ) tree obtained was validated with the minimum evolution tree.

2.9 | Gene expression analysis

Gene expression in the thymus, intestines, liver, and spleen tissues and splenic neutrophils was determined using qRT-PCR. The assay was carried out using the 7,500 real-time PCR System (BIO-RAD). The amplification was carried out in a 10 μl reaction volume containing 5 μl tip-mix (Transgen, China), 0.4 μl sense and 0.4 μl anti-sense primers, 0.5 μl undiluted cDNA, and 3.7 μl of double distilled water. The thermal profile for qRT-PCR was 94 for 5 min followed by 40

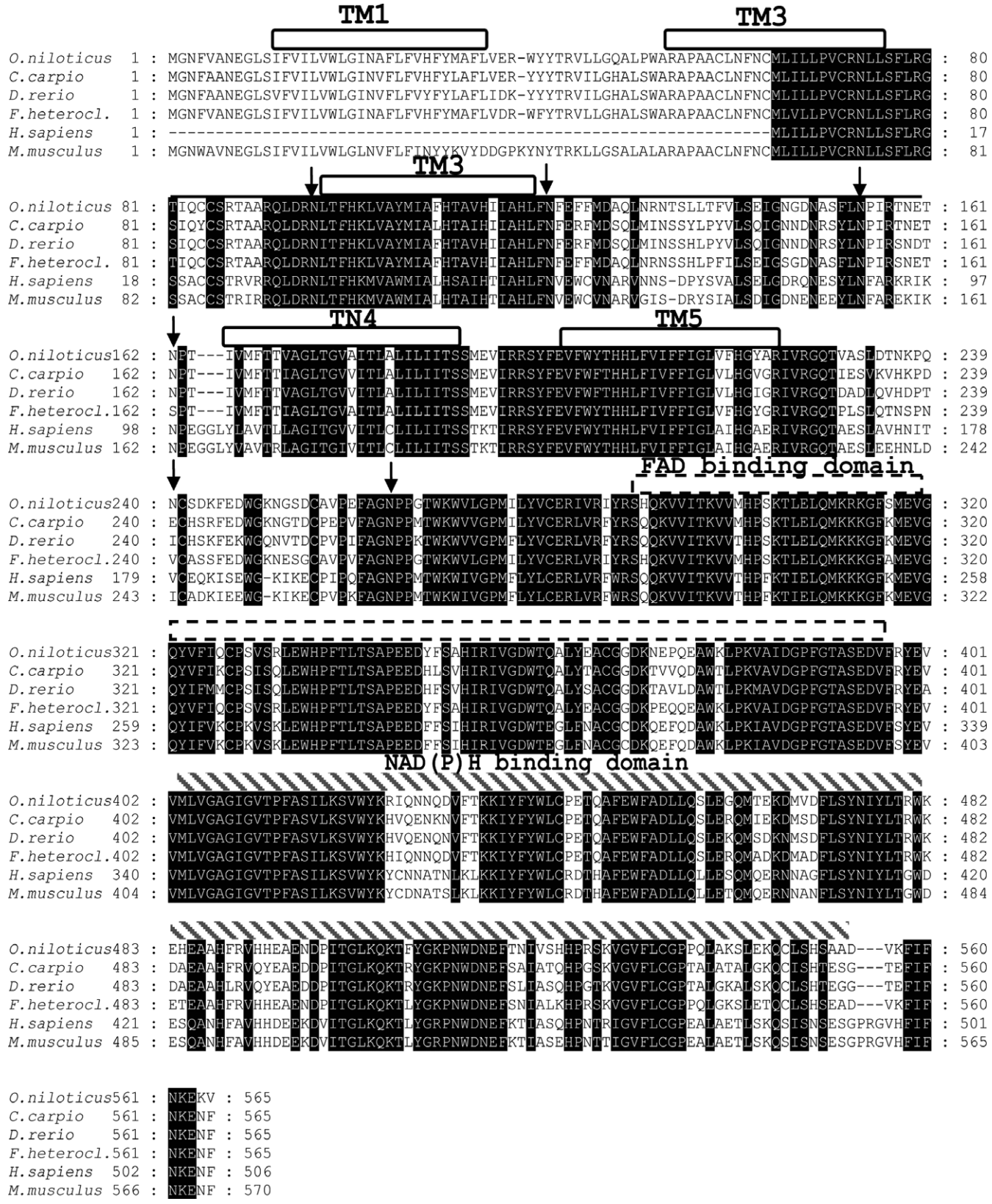

FIGURE 1 Alignment of the predicted amino acid sequence of tilapia, carp, zebrafish, kill fish, human and mouse gp91phox amino acid sequences. Shaded regions indicate conserved amino acids among species. The predicted transmembrane segments (TM) are indicated with solid line boxes, the flavin adenine dinucleotide (FAD) binding domain with broken line boxes and NAD(p)H binding domain with grey dashed boxes. Nglycosylation sites are shown with arrowhead lines

cycles of 94 for 30 s, 55°C for 30 s, and 72 for 30 s. After the qRT-PCR assay, the relative expression levels of target genes were analysed by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The specific primers and housekeeping gene (β-actin) used for qRT-PCR were listed in Supporting Information Table S1.

2.10 | Infection test

After 2 weeks of probiotic feeding, 42 fish (i.e., 14 fish per replicate tank) per group thus the CT and TCMBS groups were injected intraperitoneally with a dose of 0.2 ml of phosphate-buffered saline (PBS) containing 1×10^9 CFU/ml (i.e., Lethal dose LD_{50} determined in an earlier experiment from the same batch of fish, *unpublished data*) of *Streptococcus agalactiae*. Spleen tissues were collected from the control and treatment fish after 1-week postchallenge.

2.11 | Statistical analysis

Differences in expression levels of NADPH oxidase genes in different tissues were tested using ANOVA followed by Duncan post hoc test. In splenic neutrophils, differences in the expression of NADPH oxidase genes, ROS, and antioxidant genes between CT and TCMBS were tested by student's *t* test per time point. Data obtained from qRT-PCR analysis, ROS, and survival are expressed as the means ± standard error (*SE*). Differences were considered statistically significant at $p < 0.05$. All statistics were performed using SPSS for Windows version 12.0 (SPSS, Chicago, USA).

3 | RESULTS

Here we describe the full-length cDNA sequences, phylogenetic relationships, and tissue expression of tilapia NADPH oxidase gene $gp91^{phox}$ (MF997496), $p22^{phox}$ (MF997494), $p40^{phox}$ (MF997495) $p47^{phox}$ (MF997497), and $p67^{phox}$ (MF997498). The results obtained from this study with tilapia were compared with those of other vertebrates (Supporting Information Table S2).

3.1 | $gp91^{phox}$

The PCR product of $gp91^{phox}$ amplified yielded an ORF of 1698 bp, with which in a RACE PCR, the amplified 5' and 3'ends yielded fragments of 69 bp and 307 bp respectively. All of these fragments were assembled to form a continuous sequence of 2074 bp encoding a protein of 561 amino acids. Molecular analysis of Tilapia $gp91^{phox}$ showed five putative transmembrane segments (TM), a flavin adenine dinucleotide (FAD) and NAD(P)H binding domains (Figure 1). Also, the polypeptide has six potential glycosylation sites (Asn96, Asn133, Asn159, Asn162, Asn240, and Asn251). Moreover, four and three conserved pairs of histidines were found in TM3 (His100, His110, His114, His118) and TM5 (His206, His207, His219) respectively. In comparison to other vertebrate amino acid sequences, the identities of Tilapia $gp91^{phox}$ were closer to that of *F. heteroclitus* and least to that of Humans with 91% and 67% identities respectively (Supporting Information Table S3).

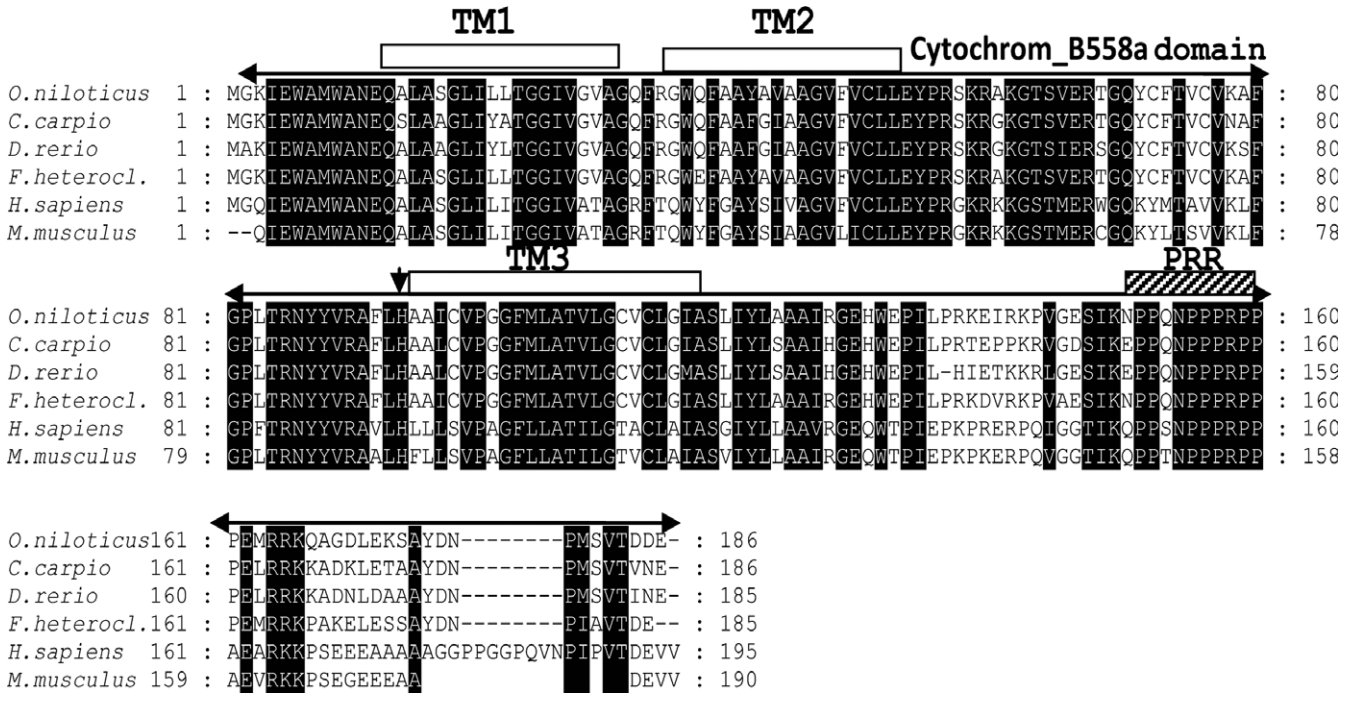

FIGURE 2 Alignment of the predicted amino acid sequence of tilapia, carp, zebrafish, kill fish, human, and mouse p22phox amino acid sequences. Shaded regions indicate conserved amino acids among species. Also indicated are transmembrane segments (TM) with solid line boxes, arrowhead as putative hemecoordinating histidines, Cytochrom_B558a domain delineated by a double-edged arrow line, and proline-rich regions (PRR) are showed by dashed boxes

3.2 | p22^{phox}

Tilapia p22^{phox} cDNA is about 1,098 bp containing a 79 bp of 5 'UTR, an ORF of 561 bp and a 458 bp of 3'UTR, and encodes a predicted translated polypeptide of 186 amino acids. Within the predicted protein are three TM, proline-rich region (PRR), fibronectin type 2 (FN2) domain, and a Cytochrome B558a domain. The amino acid alignment (Figure 2) shows that Tilapia p22^{phox} is conserved as in other vertebrates. Concerning identity, Tilapia p22^{phox} deduced amino acid sequence showered less closeness with nonfish (59%–63%) than with fish (80%–94%) (Supporting Information Table S3).

3.3 | p40^{phox}

Tilapia p40^{phox} cDNA is of total length 1,359 bp consisting of 108 bp 5'UTR, 1,053 bp ORF, 198 bp 3'UTR, predicting a protein of about 350 amino acids. In comparison to other species, alignment of the predicted protein sequence of tilapia p40^{phox}, and that of other vertebrates shares between 21%–55% with nonfish and between 73%–87% with its fish counterparts (Supporting Information Table S3). Tilapia p40^{phox} contains a conserved Phox homology (PX) domain of about 120 residues, an Src homology 3 (SH3) domain of about 50 residues, and a *Phox*/Bem 1 (PB1) domain of about 80 residues (Figure 3). The above three are the main domains, which together function as protein binding modules, thus increasing the local concentration of proteins, as well as mediating the assembly of large multi-protein complexes to increase the activity of the enzyme superoxide-generating gp91^{phox} NADPH oxidase (Boltana et al., 2009; Inoue et al., 2004).

3.4 | p47^{phox}

The full length of p47^{phox} cDNA is 2,194 bp containing 38 bp 5 'UTR, 1584 bp ORF, 572 bp 3'UTR, and a primary translation

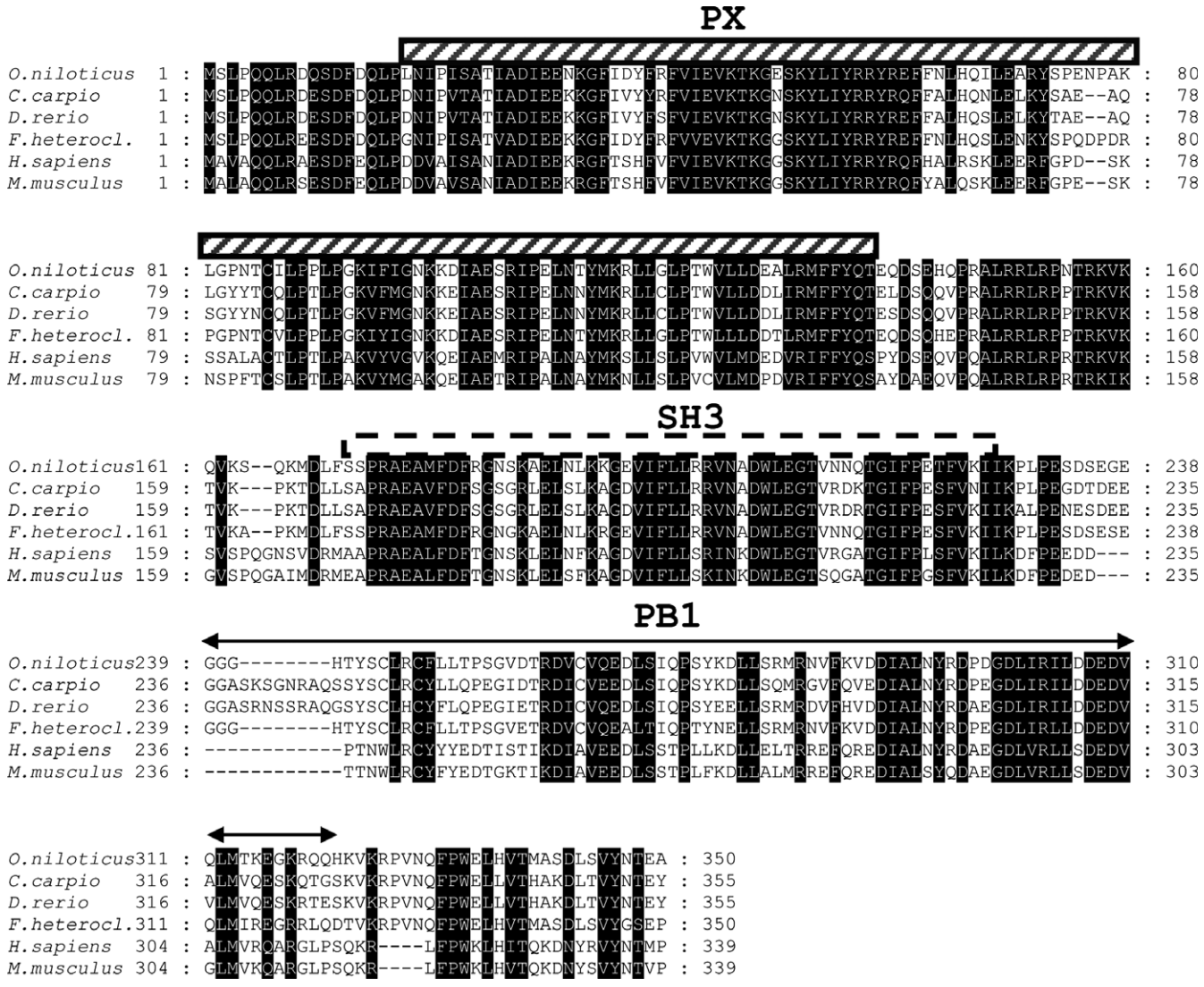

FIGURE 3 Alignment of the predicted amino acid sequence of tilapia, carp, zebrafish, kill fish, human, and mouse p40phox amino acid sequences. Shaded regions indicate conserved amino acids among species. The predicted PX domain (PX), Src homology 3 domains (SH3), and Phox and Bem1p domain (PB1) are indicated with solid line dashed grey boxes, broken line boxes, and double arrowhead line respectively

product of 527 bp amino acids. The predicted amino acid sequence of tilapia p47^{phox} contained PX domain, SH3 domains, Pfam: p47phox_C motif, PRR and autoinhibitory region (AIR) (Figure 4). In these domains/motif/regions, are important residues/regions that interact with other cytosolic and membrane proteins to cause their activation and inactivation (Kawahara & Lambeth, 2007). Tilapia p47^{phox} deduced amino acid sequence shared identity with other vertebrates in the order 79% > 62% > 59% > 55% > 53% for *F. heteroclitus*, *C. carpio*, *D. rerio*, *M. musculus*, and *H. sapiens* respectively (Supporting Information Table S3).

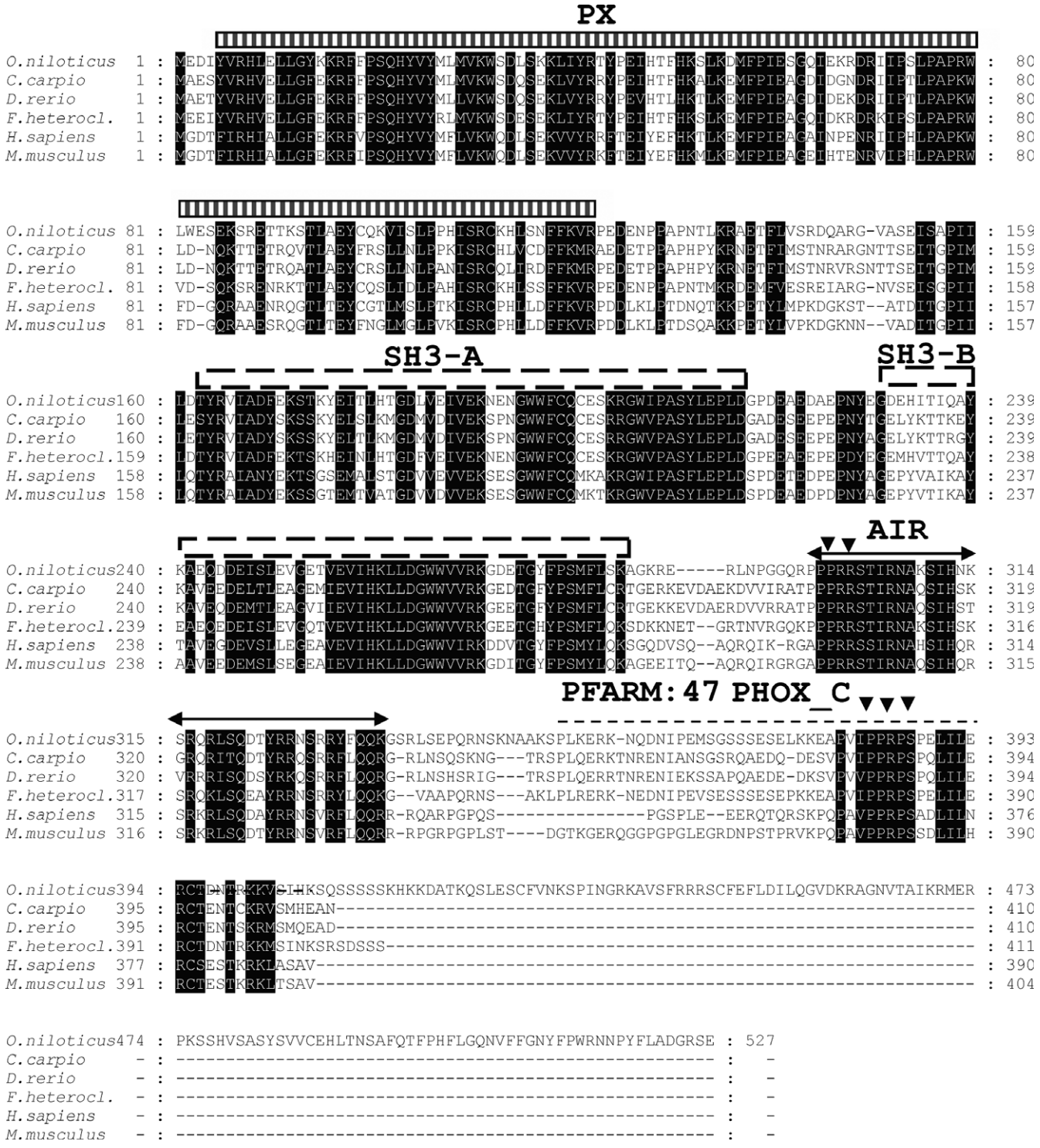

FIGURE 4 Alignment of the predicted amino acid sequence of tilapia, carp, zebrafish, kill fish, human, and mouse p47phox amino acid sequences. Shaded regions indicate conserved amino acids among species. Dashed grey boxes, broken line boxes, straight broken lines, arrowhead and doubled edged arrow line indicate PX domain, SH3 domains, Pfam:p47 phox_C, proline-rich region (PRR) and autoinhibitory region (AIR) respectively

3.5 | p67^{phox}

Tilapia p67^{phox} of full length 1738 bp consist of 40 bp 5′UTR, 1,470 bp, ORF, and 228 bp 3′UTR encoding a deduced protein of 489 bp amino acids. Tilapia p67^{phox} (Figure 5) possess tetratricopeptide repeat (TPR) motifs which mediate protein-protein interactions with other NADPH oxidase components, SH3 domains and a PB1 domain which interact with p40^{phox}which as in the other vertebrates

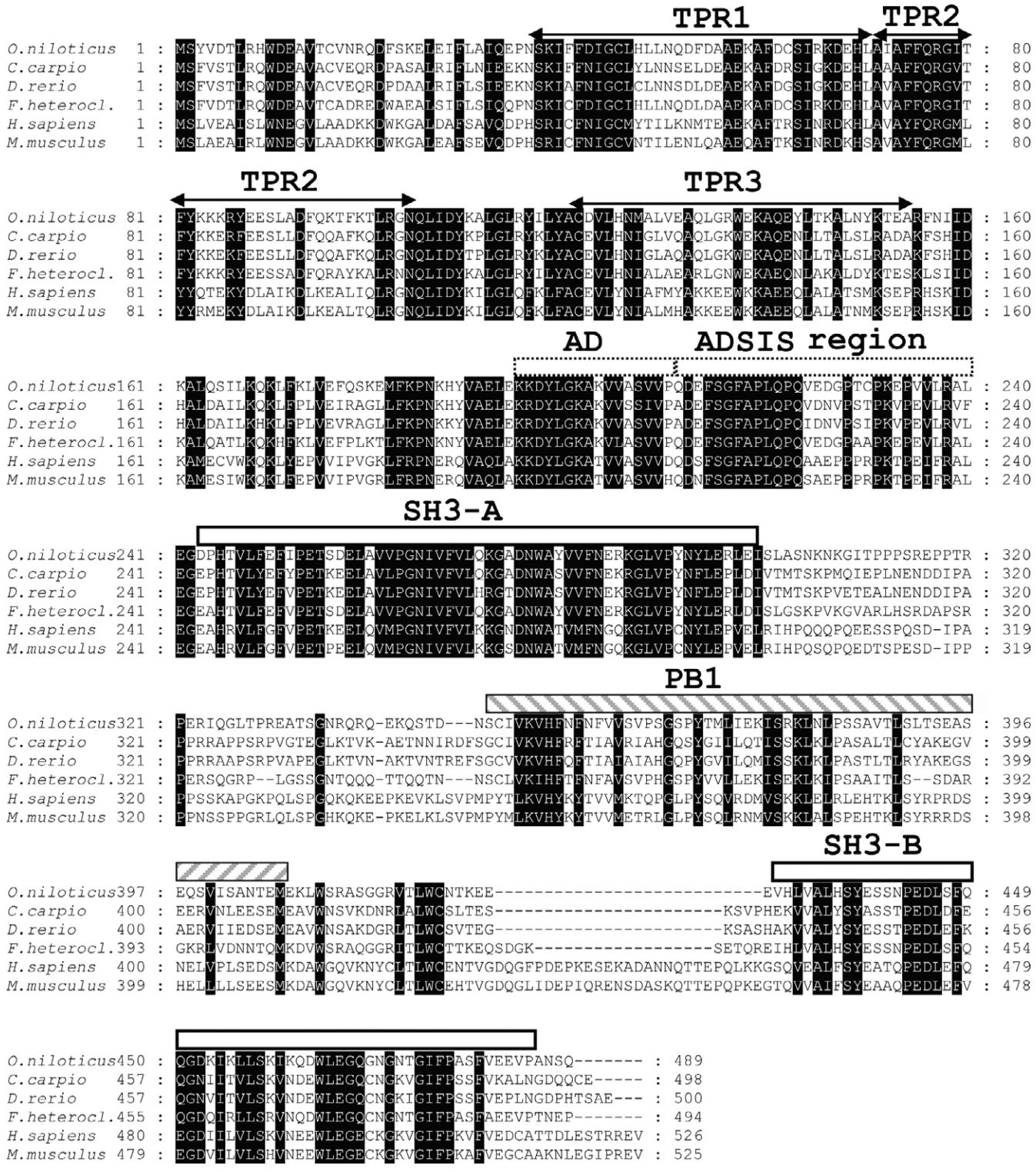

FIGURE 5 Alignment of the predicted amino acid sequence of tilapia, carp, zebrafish, kill fish, human, and mouse p67phox amino acid sequences. Shaded regions indicate conserved amino acids among species. Double arrow headline delineates putative tetratricopeptide repeat (TPR); broken line boxes, solid line boxes and grey dashed boxes denotes activation domain/region (AD/ADSIS), Src homology 3 domains (SH3) domain and Phox and Bem1p domain (PB1) domain respectively

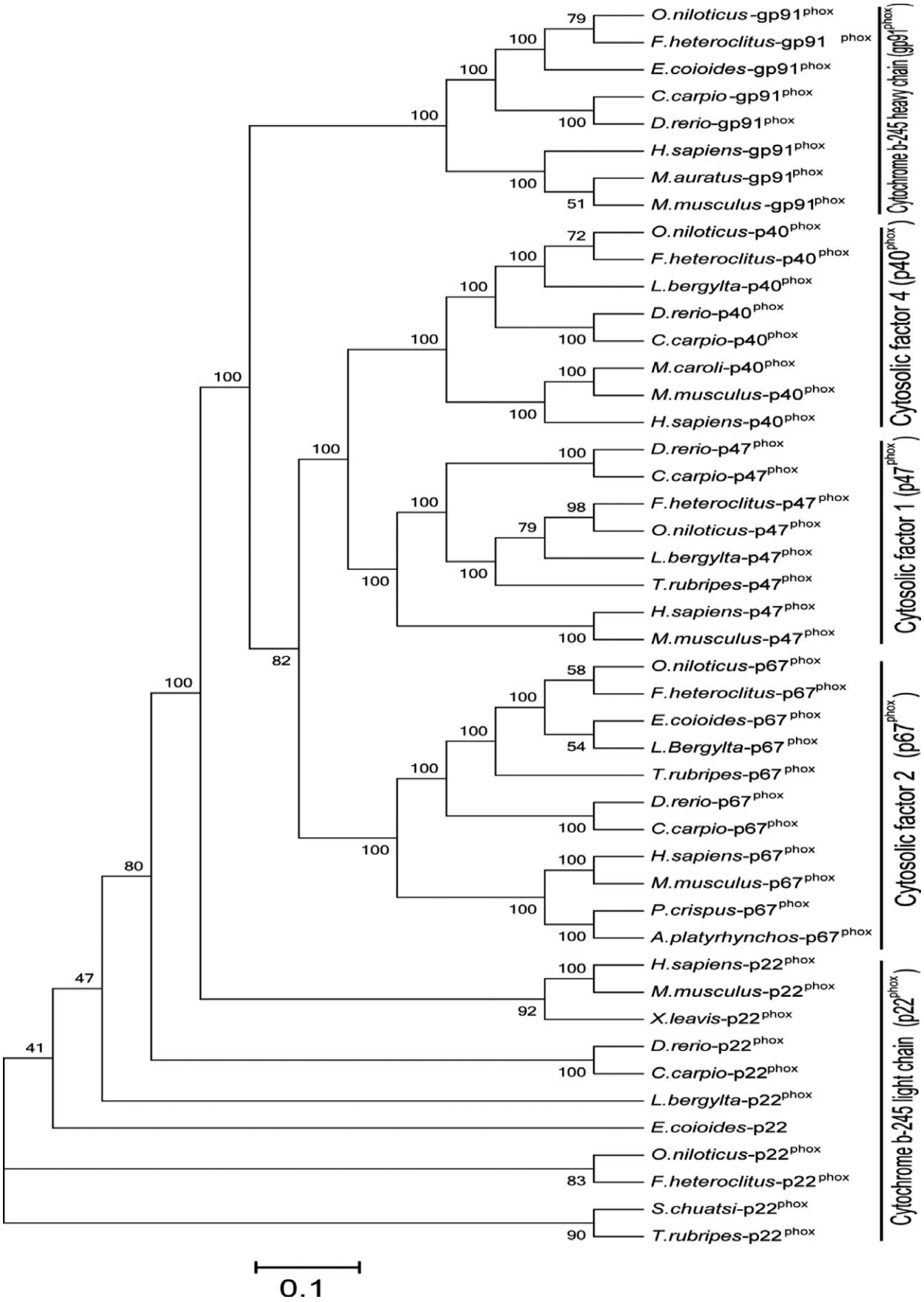

FIGURE 6 Bootstrap consensus tree for NADPH oxidase subunits in vertebrates inferred using the neighbour joining method

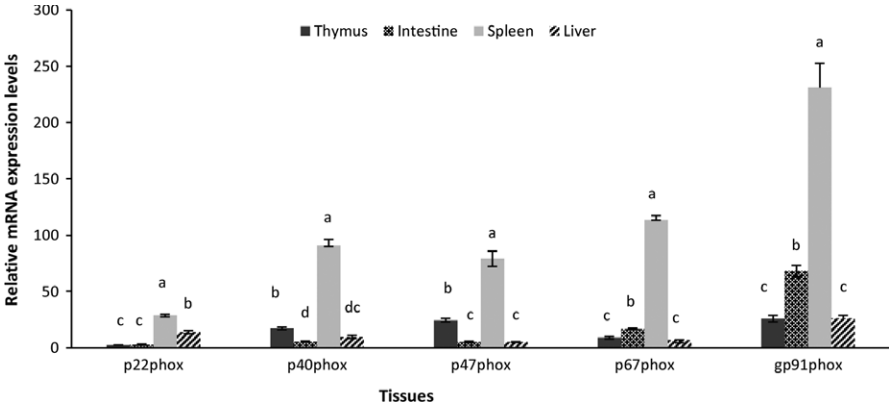

FIGURE 7 Expression analysis of tilapia NADPH oxidase genes in different tissues in *Oreochromis niloticus*. Values are expressed as mean ± *SE* (*n* = 3). Bars with the same alphabets per gene are similar ($p < 0.05$)

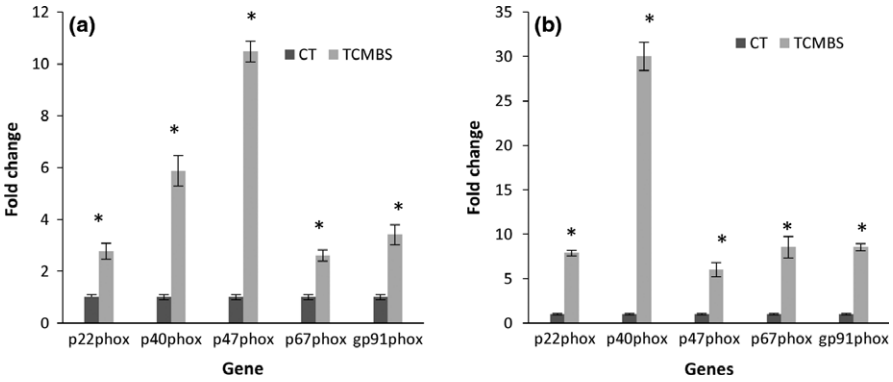

FIGURE 8 Expression analysis of NADPH oxidase genes in splenic neutrophils of *Oreochromis niloticus* stimulated with TCMBS before infection (a) and after infection (b). Values are reported in fold (mean ± *SE*, *n* = 3) change when compared to the expression in the control treatment (set to 1.0). *Significant difference from control ($p < 0.05$)

NADPH oxidase genes are well conserved. Also, was the presence of an activation domain (AD) and the activation domain of SH3 (ADSIS). In comparison to other vertebrates, the protein sequence analysis of Tilapia p67^{phox} gene showed the lowest identity to that of mouse (Supporting Information Table 3).

3.6 | Evolutional relationship of tilapia NADPH oxidase genes

Phylogenetic tree constructed to show evolutional relationships using proteins sequences of vertebrate species showed that NADPH oxidase genes gp91^{phox,} p22 ^{phox}, p40^{phox}, p47^{phox}, and p67^{phox} (Figure 6) were close to those of *F. heteroclitus*.

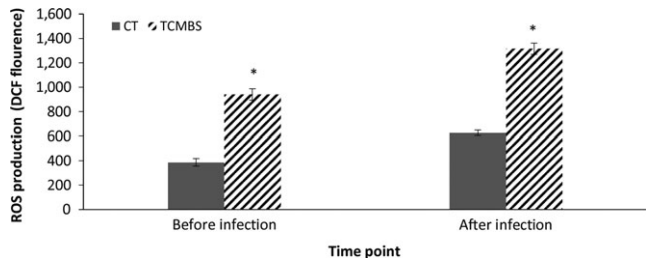

FIGURE 9 Production of ROS in splenic neutrophils of *Oreochromis niloticus* in CT and TCMBS groups before and after pathogen infection. Values are expressed as mean ± *SE* (*n* = 3). *Significant difference from control ($p < 0.05$)

3.7 | Tissue expression of NADPH oxidase genes

NADPH oxidase genes in unstimulated fish shown in Figure 7 were expressed in all the tissues examined; however, their expression was highest ($p < 0.05$) in the spleen. In other tissues such as in the thymus, intestine, and liver, expression of gp91^{phox} showed higher values compared to p22 ^{phox}, p40^{phox}, p47^{phox}, and p67^{phox}.

3.8 | TCMBS induces the expression of NADPH oxidase genes in splenic neutrophils

As shown in Figure 8, NADPH oxidase genes in TCMBS stimulated splenic neutrophils were significantly ($p < 0.05$) upregulated compared to CT after the 2 weeks trial. Regarding changes in expression of NADPH oxidase genes in TCMBS stimulated fish relative to CT, among the genes, the highest fold change was observed in p47^{phox} (29.38-folds) and the lowest fold change in p22^{phox} (3.16-folds). Expression of gp91^{phox} (8.53-folds), and p40^{phox} (7.88-folds) were similar and slightly higher than that of p67^{phox} (6.01-folds) (Figure 8a). After challenge infection, the expression of the same genes showed a similar trend as before (Figure 8b).

3.9 | ROS production

To investigate the link between expression of NADPH oxidase genes and ROS production between the TCMBS group and the CT group, ROS in splenic neutrophils was measured using DCF mean

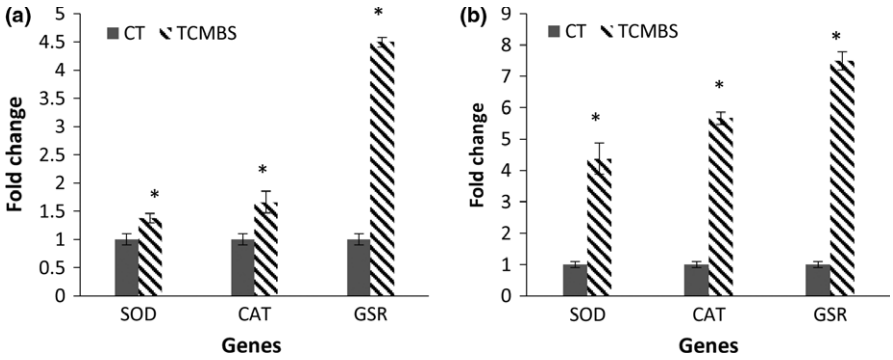

FIGURE 10 Expression analysis of antioxidant enzyme genes in splenic neutrophils of *Oreochromis niloticus* stimulated with TCMBS before (a) and after (b) infection. Values are reported in fold (mean ± *SE*, *n* = 3) change when compared to the expression in the control treatment (set to 1.0). *Significant difference from control ($p < 0.05$)

fluorescence. ROS in TCMBS stimulated group was significantly ($p < 0.05$) higher before infection and after infection in comparison to the CT (Figure 9).

3.10 | Expression analysis of antioxidant genes

Likewise, expression of antioxidant genes superoxide dismutase (SOD), catalase (CAT), and glutathione-disulphide reductase (GSR) in splenic neutrophils were significantly ($p < 0.05$) increased in TCMBS group compared to the CT before infection (Figure 10a) and after infection (Figure 10b).

4 | DISCUSSION

The important roles of NADPH oxidase genes in generating ROS in an immune response to invading microbes have been well-described in mammals (Bedard, Lardy, & Krause, 2007). In this study, NADPH oxidase genes homologs were isolated from tilapia. As depicted in Figures 1–2, the NADPH oxidase genes of tilapia contain the essential conserved domains and motifs important for ROS production as previously reported (Kawahara & Lambeth, 2007). Regarding similarity, transduced proteins sequences of tilapia oxidase genes were highly identical to that of *F. heteroclitus* (74%–91%) and least identical to those of *H. sapiens* (43%–67%) and *M. musculus* (21%–67%). Tilapia NADPH oxidase genes are more identical to their teleostean (58%–91%) counterparts than with mammals (21%–67%); however, the structural features showed conserved regions of mammalian homologs suggesting tilapia NADPH oxidase genes may have similar functions compared to mammals.

In comparison to mammals, multiple protein sequence alignment of tilapia NADPH oxidase genes showed additional features that may have immune relevance but absent or perhaps yet to be identified in mammals. For instance, in gp91^{phox} and p22^{phox}, was detected a DysFN domain, corresponding to residues 488–522, and an FN2 domain, corresponding to residues 65–98 respectively. Also, a conserved PRR-like region in the ADSIS region with unknown function in vertebrates (Kawahara et al., 2007; Olavarría, Gallardo, Figueroa, & Mulero, 2010) was detected in p67^{phox}. These features mentioned above suggest some differences between tilapia and mammals. This is of interest and currently being explored in our laboratory to judge their specialized functions.

Phylogenetic analysis based on the sequences of tilapia NADPH oxidase genes and other vertebrates including teleostean fish, human, mouse, and chicken, show distant ancestral relations among vertebrates. Our study supports previous reports (Kawahara et al., 2007; Olavarria et al., 2010) that teleost are distant relatives to mammals. Among teleost, except for p67^{phox} of a bootstrap value of 58% raising uncertainty, gp91^{phox}, p22^{phox}, p40^{phox}, and p47^{phox} of bootstrap values above 70% indicate tilapia has a close relation to the teleost *F. heteroclitus* (Figure 6).

Concerning the distribution of expressed genes in the tissues examined, significantly higher expression of tilapia NADPH oxidase genes were detected in the spleen and least in the thymus, intestines, and liver relative to the reference gene (Figure 7). Higher expression of NADPH oxidase genes in the spleen of tilapia as found in the present study strongly suggested that in tilapia, the spleen might abound with NAPDH oxidase genes. Similarly, high expressions of these genes in the spleen, blood, and kidney have been reported in fugu (Inuoe et al., 2014) and carp (Mayumi et al., 2008).

According to Inoue et al. (2004), neutrophils are important phagocytes cells that play important roles in immune defence through the expression of NADPH oxidase genes in a host. Therefore, we further looked at the effect of TCMBS application on the expression of NAPDH oxidase genes in splenic neutrophils of tilapia. Upon stimulation with TCMBS supplement, splenic neutrophils showed significant upregulation in all NADPH oxidase genes compared to the CT (Figure 8a, b). This indicates that TCMBS application might positively auto-regulate their expression, hence, might improve the neutrophil induced immune defence in tilapia.

Neutrophil defence mechanism involves the release of respiratory burst as well as neutrophil extracellular traps (NET) release to kill pathogens. Neutrophils rely on NADPH oxidase system to ferry electrons across cell membranes into phagocytic vacuoles during respiratory burst to create ROS which stimulates the release of the digestive enzymes responsible for microbial deaths (Jovanovic, Goetz, Goetz, & Palić, 2011). In the present study, TCMBS application showed increased neutrophil induced immune defence readiness through a significant increase in the production of ROS in splenic neutrophils. This is because the ROS production levels observed in tilapia before bacterial infection test and after the

bacterial infection test were significantly higher in the TCMBS group compared to the CT group (Figure 9).

Prolonged or uncontrolled production of ROS has deleterious effects on the host (Herath et al., 2017). For this reason, it has been suggested in many studies of the need to control ROS generation to reduce the deleterious effect on host cells through stimulation with antioxidants enzymes/genes (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Borchi et al., 2010). In this study, we found that TCMBS application might have the ability to stimulate the expression of antioxidant genes in splenic neutrophils. As shown in Figure 10, analysis of the expression of antioxidant genes including SOD, CAT, and GSR showed significant upregulation in the TCMBS group before infection (Figure 10a) and after infection (Figure 10b) compared to the CT group. This strongly suggests that TCMBS stimulus have counterbalance effects on production of ROS through increased expression of antioxidant genes.

In summary, we identified and characterized gp91^{phox}, p22^{phox}, p40^{phox}, and p47^{phox}, p67^{phox} genes from tilapia and that found they possess all essential conserved domains for the full functioning of the enzymatic complex. TCMBS stimulus showed a significant increase in the expression of NADPH oxidase and antioxidant genes and enhanced production of ROS in splenic neutrophils. Together, our data suggest that TCMBS supplementation might improve the neutrophil immune response to curb harmful pathogen infections in fish.

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SUBMISSION DECLARATION AND VERIFICATION

This article to be considered for publication has not been published previously and is not under consideration for publication elsewhere.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

JJ and TJ conceived and designed the experiment, EDA, and HY carried out field experiments, EDA, CJ, JJ, and LY drafted and proofread the manuscript.

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SUPPORTING INFORMATION

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