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First molluscan antimicrobial peptide hydramacin in Manila clam: molecular characterization and expression analysis

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PEER REVIEW

Peer reviewer

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Comments

The paper is very well written. The scientific context, the methods and the findings are also clearly described and discussed. The paper may be of a certain interest for all the scientists and researchers involved in the animal health sector, including nutritionists and veterinarians.

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ABSTRACT

Objective: To investigate molecular characterization and the immune responses of Manila clam hydramacin (*Rp-hdmc*).

Methods: cDNA sequence of hydramacin was isolated from Manila clam transcriptome database. Molecular characterization of hydramacin cDNA was performed by BLAST and SWISS-MODEL bioinformatics programs. Tissue-specific expression and transcriptional regulation after *Vibrio tapetis* challenge was done by quantitative real time PCR.

Results: *Rp-hdmc* has 291 bp open reading frame (ORF), encoding 97 amino acids with a mature hydramacin consisting of 77 amino acid residues. In un-challenged clam, *Rp-hdmc* was constitutively expressed in all tested tissues and the highest expression level was detected in gill. After pathogenic bacteria *Vibrio tapetis* challenge, *Rp-hdmc* mRNA was up-regulated in gill and hemocytes.

Conclusions: We identified hydramacin cDNA (*Rp-hdmc*) from mollusk Manila clam that shows the characteristic features of hydramacin sequence. It has eight cysteine residues with four disulfide linkages, three helices and two β-strands in secondary structure. Expression results after *V. tapetis* challenges suggest that *Rp-hdmc* is involved in immune response against pathogenic bacteria.

KEYWORDS

Manila clam, *Ruditapes philippinarum*, Hydramacin, Antimicrobial peptide

1. Introduction

Antimicrobial peptides (AMPs) are gene encoded innate immune defense molecules distributed widely among the living organisms from invertebrates to vertebrates. Currently more than 750 AMPs have been described from different organisms. These natural compounds possess broad-spectrum of activity against various pathogens

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such as Gram-negative and Gram-positive bacteria, filamentous fungi, parasitic protozoa, and metazoa[1-3]. It is known that they are implicated in different defense mechanisms in their hosts. Thus, it is likely that they play an important protective role against pathogens, and the induction of such peptides can allow a rapid and effective response against a broad range of infectious organisms[4]. Most of the eukaryotic AMPs are classified into three major groups: (i) linear α-helical peptide without cysteine (insect cecropin, magainins, *etc.*), (ii) linear peptides with an extended structure that is characterized by an unusually high proportion of one or more amino acids such as proline or histidine, and (iii) peptides with disulfide bonds, which form a looped structure (defensin, protegrin, *etc.*)[2,5].

Marine invertebrates including Manila clam lack adaptive immunity. They are solely dependent on physical barrier and the innate immune system for protection against pathogenic agents. To date, several AMPs have been identified in mollusk[3,6]. The following 4 groups of cationic cysteine-rich AMPs, such as defensin-like peptides MGD-1 and MGD-2[7-10], mytimacins[11], mytilins with 8 cysteine residues but different specific cysteine arrays and amino acid sequences[10], and mytimycin[12] have been isolated. Latterly, a novel antimicrobial peptide named theromacin was isolated and characterized from annelid *Theromyzon tessatum*[13], and *Hyriopsis cumingii* (*H. cumingii*)[5].

Hydramacin was first isolated from cells of the freshwater hydroid *Hydra*[14]. It is 60 amino acids long peptide and is unique both in amino acid sequence and tertiary structure, prompting its classification in a new family of proteins. Hydramacin have been reported from cnidaria *Hydra magnipapillata* (*H. magnipapillata*)[14]. However, no hydramacin homologous from mollusks have been published yet.

In this study, we identified and characterized the hydramacin cDNA from the mollusk bivalve *Ruditapes philippinarum* (*R. philippinarum*) (named as *Rp-hdmc*) as a first report of a molluskan hydramacin member. To understand the role of hydramacin in mollusk, an mRNA tissue expression profile and transcriptional change in clam gill and hemocytes were investigated after infection by *Vibrio tapetis* (*V. tapetis*) which is causative agent of brown ring disease (BRD).

2. Materials and methods

2.1. Identification of Manila clam hydramacin cDNA

A Manila clam transcriptome data base was established using Roche 454 genome sequencer FLX system (GS-FLXTM), next generation DNA sequencing technology (DNA Link, Republic of Korea). Sequencing reads were processed and assembled by the Arachne assembler algorithm[15-17]. Unique cDNA that showed homology to known hydramacin proteins was identified by the Basic Local Alignment Tool algorithm[18]. In order to confirm sequence identified from the Basic Local Alignment Tool, two sequence specific primers [5′-3′ forward (FP) GAATGCAGACATGATGCGTAGCA- and reverse (RP) GCAGCTCAGCTCAAGTCAAGTATAA] were designed. The amplicon obtained from polymerase chain reaction (PCR) was cloned into pGEM easy T-vector (Promega) and sequence was reconfirmed.

2.2. Sequence analysis and protein modeling

The nucleotide sequence was translated to protein sequence using DNAssist 2.2. The presence of signal peptide was predicted by SignalP 4.0 Server[19]. Multiple alignment of protein sequences and similarity percentage of *Rp-hdmc* with other homologues were calculated by the ClustalW2 program[20]. The phylogenetic tree was constructed by means of the Neighbor-Joining method using molecular evolutionary genetic analysis (MEGA5)[21]. The SWISS-MODEL web server was used to build protein models and predict secondary structure of *Rp-hdmc*[22].

2.3. Experimental animals and immune challenge

Manila clams (shell-length of 3.0-3.5 cm) were collected from the Eastern coastal region of Jeju Island (Republic of Korea). Clams were maintained in the laboratory in a 80 L flat-bottom tank filled with aerated and sand-filtered seawater having salinity of 34‰ and kept at (21±1) °C. All animals were acclimatized to laboratory conditions for at least one week prior to initiation of any experimental procedures.

To determine the normal tissue-specific expression of the *Rp-hdmc* gene: the following tissues and hemocytes were collected from five un-challenged Manila clams: adductor muscle, mantle, siphon, gill, and foot. The clam hemolymph was collected from the posterior adductor muscle sinus using 1 mL syringes with 26-gauge needles through the shell hinge. The hemolymph was immediately centrifuged (3 500 r/min for 10 min at 4 °C) and the hemocytes were obtained after removal of supernatant.

In order to determine the immune response of *Rp-hdmc*, *V. tapetis* (No. 12728; Korean Collection for Type Culture, Republic of Korea) was employed. *V. tapetis* was diluted in 0.9% saline to make stock. Clams were intramuscularly injected with 100 μL (1.3×108 CFU/individual) of *V. tapetis* into live clam adductor muscle. Un-injected clams were established as the blank, and a third group of clams was injected with an equal volume (100 μL) of phosphate

buffered saline (PBS). The challenged clams were returned to seawater tanks and five individuals were randomly collected at 3, 6, 12, 24, and 48 h post challenge.

2.4. Tissue specific and temporal expression of immune challenged animals

Total RNA was extracted from 40 mg per individual each of five isolated tissues using QIAzol® Lysis Reagent (Qiagen) following the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA by using PrimeScript™ first-strand synthesis kit (TaKaRa, Japan) according to the manufacturer's protocol. The cDNA product was diluted 10-fold and stored at −20 °C until use as template for quantitative real-time reverse transcriptase PCR (qRT-PCR) to analyze tissue-specific expression and expression changes in response to immune challenge.

To determined mRNA expression profile of *Rp-hdmc*, the gene specific primers (5′ to-3′: FP-TTGAAACATGGTCTCGCTGCTCTG and RP-TCCACGAACCGGCAGTTAGAATCA) were generated. β-actin (FP-CTCCCTTGAGAAGAGCTACGA and RP-GATACCAGCAGATTCCATACCC) were used as an invariant control[23]. qPCR was carried out using the thermal cycler dice real-time system (TP800; TaKaRa) programmed with the following amplification conditions: one denaturation cycle of 95 °C for 3 min, followed by 45 amplification cycles of 95 °C for 20 seconds, 58 °C for 20 seconds, and 72 °C for 30 seconds. The baseline was set automatically by accompanying system software (version 2.0). The Livak ($2^{-\Delta\Delta Ct}$) method was used to analyze the expression levels of *Rp-hdmc*[24]. Each analysis was carried out in three replicates. All data are presented as relative mRNA expression with respect to the *Rp-hdmc* expression in the saline-injected controls as mean±SD (n=3). Statistical differences between the control and treatment groups were determined by t-test using the SPSS 11.5 program, and differences were considered significant at $P<0.05$.

3. Results

3.1. Characterization of Rp-hdmc cDNA

The complete coding sequence of the *Rp-hdmc* was obtained from the clam GS-FLX sequencing data base. *Rp-hdmc* nucleotide and amino acid sequences are shown in Figure 1, and this data has been deposited in the GenBank under the accession number of KC506420. The complete coding sequence of *Rp-hdmc* is 291 bp in length, encoding a peptide of 97 amino acids. The molecular mass and the isoelectric point of *Rp-hdmc* are 11.0 kDa and 8.0 kDa, respectively. The putative signal peptide (^{1}MIC-GDA^{20}) was identified at the N-terminal sequence with the cleavage site at amino acid position 20. Mature *Rp-hdmc* peptide showed highest identities with *Hypriopsis cumingii* (*H. cumingii*) theromacin (50%), followed by *H. magnipapillata* hydramacin (45.5%), and *Hydra vulgaris* (*H. vulgaris*) hydramacin (43.6%). The deduced amino acid sequence of *Rp-hdmc* was compared with previously reported hydramacins (Figure 2A). *Rp-hdmc* possessed 8 cysteine residues similar to the hydramacins from *H. vulgaris* and *H. magnipapillata* whereas theromacins from *H. cumingii* and *T. tessulatum* revealed the presence of 10 cysteine residues (Figure 2B). *Rp-hdmc* showed high percentage of identity with theromacin of *H. cumingii* rather than hydramacins of *H. magnipallata* and *H. vulgaris*, even though *Rp-hdmc* showed only 8 conserved cysteine residues. Nevertheless, *Rp-hdmc* showed similar secondary structure with hydramacins including three-helices (α-helices ^{29}WSRCSG^{34}, ^{46}SCQDRCE^{52}, and ^{65}DSN^{67}) and two β-strands (^{59}GTCES^{63} and ^{75}TQCRC^{79}). *Rp-hdmc* portrayed the formation of four disulfide bridges (C^{25}-C^{68}, C^{32}-C^{61}, C^{47}-C^{77}, and C^{51}-C^{79}), whereas theromacins showed five disulfide bridges. Furthermore, *Rp-hdmc* was highly conserved amino acid sequence, though it is longer than other previously reported hydramacins. A phylogenetic tree containing invertebrate macin family of AMP sequences was constructed using theromacin as the out-group (Figure 3). Hydramacins were grouped together in corresponding group and theromacin also showed closer relationship. *Rp-hdmc* was present closer to the hydramacin and theromacin homologues with low bootstrap values observed in branch of *Rp-hdmc*.

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                                                ATAAAACTGAAGTTTTTAATGTAGAATAGAAGTTTATAATTTA   −361
TCACTGCGGAAAATTTAAGTAATTTAATAGGCATATTATAGTCAAAATTTTGTGTTTAGAA   −301
TGCAGACATGATGCGTAGCACCCTGCCCACCCCATAAAAACGTGGAGGCATGCGTTAAAT   −241
GATTAACTCATTGTTTTATTTGCTCTCATTTTTATTTCAGAAGCAGTTATGAAGTGCAAG   −181
AAGTGCACAGCAGTCGAGGT GGAACTTTAACTAGGATGGCGAGTGACAGGGACGGGGGCG   −121
TAAGAAGCCCCCGTACTCTGCGTTGATACCACTGCTTAAGCAGTGGTATCAACGCACAGT   −61
ATCGGGGGATATCTCAACCTTCTGGCCTTCTGGATCTTTATACCAAATACGAACAAGTTG   −1
ATGATATGTTCGATTGTTGCCATTCTTGTAATGAGCACCGTATTTCCAAGAGGAGATGCA   60
M  I  C  S  I  V  A  I  L  V  M  S  T  V  F  P  R  G  D  A     20
TTTATCTTGGAATGTTTTGAAACATGGTCTCGCTGCTCTGGGTGGAGTTCCGGTGGAACA   120
F  I  L  E  C  F  E  T  W  S  R  C  S  G  W  S  S  G  G  T     40
GGTTGGCTCTGGAAGTCGTGCCAAGACAGGTGCGAAGAATTGGGCTACAGCACAGGCACA   180
G  W  L  W  K  S  C  Q  D  R  C  E  E  L  G  Y  S  T  G  T     60
 TGCGAATCTGCTGATTCTAACTGCCGGTTCGTGCACAAAGCCTATCAATGCAGATGTTAC   240
C  E  S  A  D  S  N  C  R  F  V  D  K  A  Y  Q  C  R  C  Y     80
GGCAAAACTTCTGGAGGTAGTGGTAGTTCGGGAGGCTGGTGGAAAAGAAAGTAAATCGCT   300
G  K  T  S  G  G  S  G  S  S  G  G  W  W  K  R  K  *          97
TATACTTGACTTGAGCTGGCTGC                                       323
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Figure 1. Nucleotide and deduced amino acid sequences of *Rp-hdmc*. The coding nucleotides (upper row) and deduced amino acids (lower row) are numbered at the right side of sequences. The start codon (ATG) and stop codon (TAA) are bold and asterisk, respectively. Double underlined protein sequence denotes signal sequence.

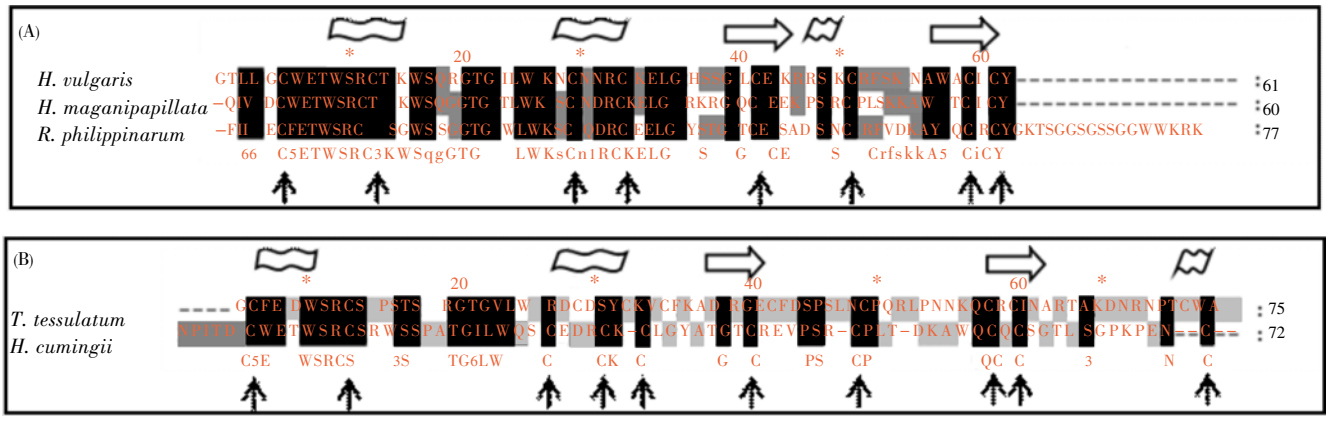

Figure 2. Multiple alignment analysis of *Rp-hdmc* with other previously reported hydramacins (A) and theromacins (B).
Identical residues among orthologous sequences are denoted by uppercase letters. Similar residues (with single discrepancies among the orthologues) are denoted by lowercase letters or numbers. Gradation in similarity is denoted by dark-to-pale shading (100%, 80% and 60% respectively). Missing amino acids are denoted by dashes. The black arrows indicate conserved cysteine residues. The secondary structure of Rp-hdmc is displayed: wavy rectangle, helix; white arrow, β-strand. *H. vulgaris* hydramacin (AFQ20833); *H. magnipapillata* hydramacin (ABE26989); *H. cumingii* theromacin (AEC50045); *T. tessulatum* theromacin (AAR12065).

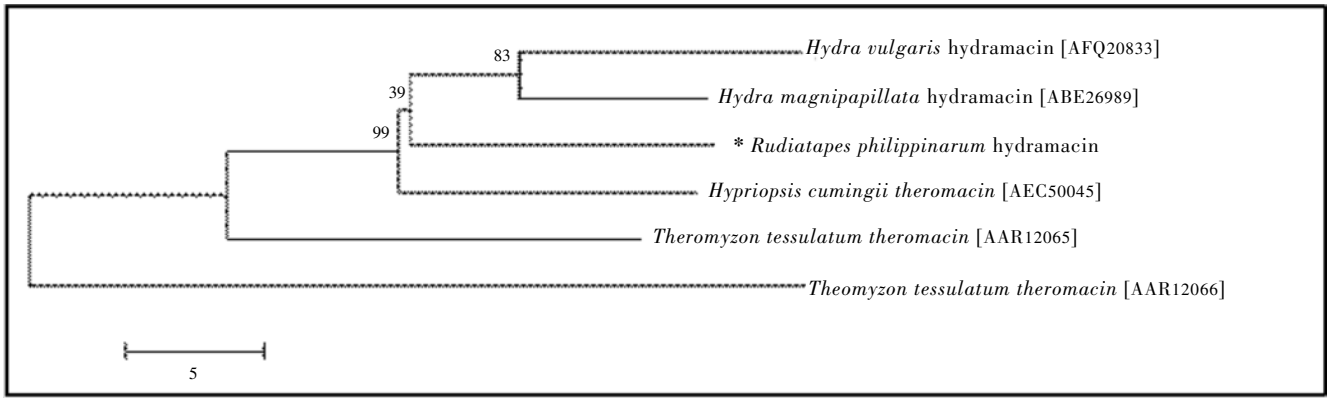

Figure 3. Phylogenetic analysis of *Rp-hdmc*.
The number at each node indicates the percentage of bootstrapping after 1000 replications.

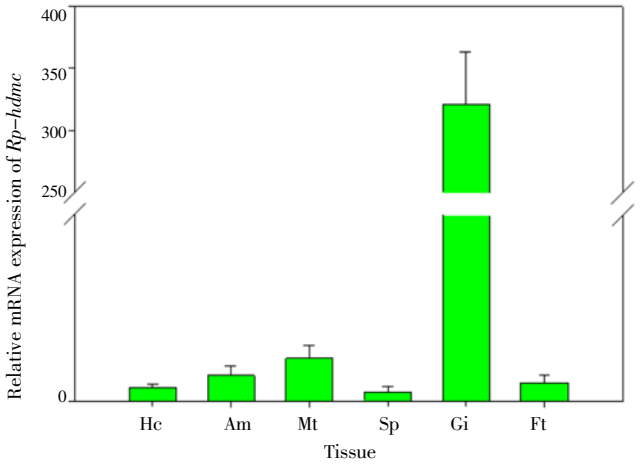

Figure 4. Tissue specific expression of *Rp-hdmc* mRNA determined by qRT-PCR.
Expression fold changes were depicted relative to the mRNA expression level in siphon tissue. Error bars represent the SD (n=3).

3.2. Gene expression of Rp-hdmc

To investigate the basal transcription level of *Rp-hdmc* in several tissues of un-challenged Manila clams, the mRNA was determined by means of qRT-PCR. Expression analysis was carried out using gene specific primers targeting its coding region. Clam β-actin was used as an invariant control gene and relative transcription level was further compared with transcription level in siphon. *Rp-hdmc* mRNA expression was detected in all tested tissues including hemocytes, adductor muscle, mantle, siphon, gill, and foot (Figure 4). The highest expression level of *Rp-hdmc* was detected in gill tissue (320-fold difference compared with siphon).

After immune challenge with *V. tapetis*, the change of *Rp-hdmc* transcription level was investigated in gills and hemocytes using qPCR. Transcriptional up-regulation of *Rp-hdmc* occurred in both gills and hemocytes. In gill tissue, *Rp-hdmc* mRNA was immediately up-regulated and showed peak level at 3 h (9.09-fold) post challenge (Figure 5). Then, a dramatic decrease in the expression level was observed at 6 h post-challenge, Alternative increase and decrease could be observed after 6 h to 48 h post challenge. Hemocytes expression also showed significant immediate up-regulation (2.24-fold) at 3 h post challenge. Then, expression level was gradually decreased till 12 h, and again increased to its peak level of 11.37-fold at 24 h post-challenge. Finally, *Rp-hdmc* declined

to 7.89-fold at 48 h post-challenge (Figure 5). Concisely, *Rp-hdmc* expression was up-regulated within 24h after bacterial challenge, suggesting the active involvement of the protein in evading the infectious pathogens.

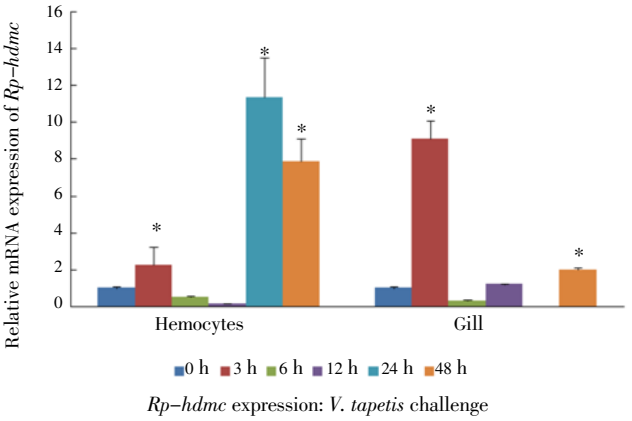

Figure 5. Temporal expression profile of *Rp-hdmc* mRNA in gill and hemocyte upon stimulation with *V. tapetis* determined by qRT-PCR.
The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method, using manila clam β-actin as reference gene, normalizing to the corresponding 0.9% saline injected controls at each time point. The relevant expression fold at 0h post-injection (un-injected control) was kept as the basal line. Error bars represents the SD (n=3); *P<0.05.

4. Discussion

In this study, we identified and characterized hydramacin cDNA (*Rp-hdmc*) from Manila clam as a first report of hydramacin from mollusks. *Rp-hdmc* protein analysis showed similarity with the basic characteristics of hydramacin members however, amino acid identity with known members of hydramacin was around 50%. These results appeared to be due to the lack of sufficient gene sequences of hydramacin from other species. Only two amino acid sequences of invertebrate hydramacin are available in GenBank. Overall, these results suggest that *Rp-hdmc* is a new member of hydramacin family of AMPs.

There are no reports on the tissue distribution profiling of hydramacin in invertebrates for a comparative understanding. However, tissue specific expression of theromacin was investigated in *H. cumingii*. Manila clam intakes their food by filter feeding mechanism and gill tissue is in constant contact with environment possessing various pathogens. Therefore, high level of *Rp-hdmc* mRNA in gill suggests that active involvement of this protein is in defense of the organism. Localization of antibiotic molecules in the gastrointestinal tract in insects and in vertebrates where they provide a rapid local immune response against exogenous pathogens brought in during feeding has been reported[25]. These results underlined a regulation of the *Rp-hdmc* similar to other invertebrate antimicrobial peptides including theromacin which is similar peptide with hydramacin. In *H. cumingii*, significant up-regulation was detected in hemocytes, hepatopancreas, mantle, and intestine at 24 h post challenge with four bacteria including *Staphylococcus aureus*, *Bifidobacterium bifidum*, *Aeromonas hydrophila*, and *Escherichia coli*[5]. The constitutive expression of *Rp-hdmc* in gill as well as other tissues with transcriptional up-regulation against *V. tapetis* infection would suggest that it played an important role in host immune defense against pathogens.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was financially supported by the National Research Foundation (NRF) program of Ministry of Education, Science and Technology, Republic of Korea under research grant title of the Studies on the Manila clam (*R. philippinarum*) internal defense system (NRF-2011-0022671).

Comments

Background

To identify "natural" bio-active molecules may be crucial for animal health, welfare and growth performances, particularly when they are involved in immune activity. For this reason, the topic of the present work may be considered of a sure interest.

Research frontiers

The identification of bio-active molecules and their descriptions at molecular level together with their genome codification, may be considered a high standard method for the study of biological systems.

Related reports

It exists a wide range of papers and other references that may be cited within the present paper with the purpose of compared its findings with similar results observed by other authors. Despite that, a longer discussion of these simple findings may results in a redundant and unnecessary activity.

Innovations and breakthroughs

Despite already well known in other animal species,

for the first time hydramacin has been identified and described in manila clam (*R. philippinarum*).

Applications

Manila clam is a worldwide farmed species and knowledge related to its immune-function may be of high interest for the industrial sector which is related to aquaculture at every level (farms, Pharmaceutical industry, feed industry, *etc*.).

Peer review

The paper is very well written. The scientific context, the methods and the findings are also clearly described and discussed. The paper may be of a certain interest for all the scientists and researchers involved in the animal health sector, including nutritionists and veterinarians.

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