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MD-2-LIKE PROTEINS IN CRUSTACEANS: STRUCTURE, IMMUNE FUNCTIONS, AND AQUACULTURE APPLICATIONS

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ABSTRACT

MD-2-like proteins belong to the MD-2-related lipid-recognition family and function as lipid-binding molecules in innate immunity. In crustaceans, these proteins have been reported to bind lipopolysaccharides and are implicated in antibacterial immune responses, despite the abshence of a canonical TLR4 signaling pathway, though their precise in vivo mechanisms remain unresolved. Homologs identified in shrimp and crabs share conserved structural motifs linked to lipid binding and immune recognition. Evidence from transcriptomics, ligand-binding assays, and functional perturbation studies supports their involvement in antibacterial defense, although in vivo mechanisms remain unresolved. This narrative review synthesizes current knowledge on MD-2-like proteins of the ML (MD-2-related lipid-recognition) superfamily in crustaceans, focusing on their structure, distribution, and putative immune functions. Clarifying the biological roles of these proteins may in the future inform immune-based approaches to disease management in crustacean aquaculture, pending functional validation in vivo.

KEYWORDS: MD-2-like protein; ML domain; lipopolysaccharide; crustacean; innate immunity; aquaculture

INTRODUCTION

Shrimp farming is a major part of global seafood production, supporting food security, livelihoods, and international trade, especially in Asia (Azra *et al.*, 2021). Yet disease outbreaks caused by viral and bacterial pathogens continue to undermine production and sustainability, despite ongoing improvements in aquaculture practices. Repeated losses linked to pathogens such as white spot syndrome virus and *Vibrio* species have therefore intensified interest in the molecular basis of immune defense in crustaceans (Iftehimul *et al.*, 2025).

Unlike vertebrates, crustaceans rely entirely on innate immunity to detect and respond to infection. A central part of this system is the action of pattern recognition receptors (PRRs), which detect conserved microbial molecules such as lipopolysaccharides (LPS), peptidoglycan, and â-glucans. Once activated, these receptors trigger immune responses including phagocytosis, melanisation, and antimicrobial peptide production, together forming the first line of defense against invading pathogens. In crustaceans, major PRR groups include Toll-like receptors, C-type lectins, scavenger receptors, and members of the MD-2-related lipid-recognition (ML) superfamily (Patnaik *et al.*, 2024).

Among the protein families associated with pathogen recognition, the MD-2-related lipid-recognition (ML) family has received increasing attention because of its ability to bind lipid-based microbial ligands (Grebennikova *et al.*, 2025). In vertebrates, myeloid differentiation factor 2 (MD-2) functions with Toll-like receptor 4 (TLR4) to detect LPS and initiate inflammatory signaling. Although crustaceans appear to lack a clear TLR4 orthologue, proteins containing ML domains and showing partial structural similarity to MD-2 have been identified in shrimp and crabs (Betancourt *et al.*, 2024). Their presence has raised an important unresolved question: whether these proteins are genuine functional analogues of vertebrate MD-2 or instead ML-domain homologues with distinct immune roles in crustaceans.

Current evidence from sequence analysis, expression studies, in vitro binding assays, and a limited number of functional experiments suggest that some

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crustacean ML-domain proteins can bind LPS and may contribute to antibacterial defense (Song *et al.*, 2022). However, their receptor partners, signaling pathways, and in vivo roles remain poorly resolved. Progress is also complicated by inconsistent terminology, as the term “MD-2-like” is often used for proteins whose functional similarity to vertebrate MD-2 has not been firmly established.

This narrative review examines reported MD-2-like and ML-domain proteins in decapod crustaceans, with particular emphasis on shrimp and crabs. It focuses on their structural features, expression patterns, ligand-binding evidence, and proposed immune functions. Rather than attempting a broad survey of all ML-domain proteins across invertebrates, the review is limited to published sequence-based, in vitro, and functional studies relevant to antibacterial immunity. By bringing this evidence together, it aims to distinguish what is supported experimentally from what remains uncertain, and to highlight the key gaps that still limit our understanding of MD-2-like proteins in crustacean immunity.

Structural Features of MD-2-like Proteins

Structural discussion of crustacean MD-2-like proteins requires careful distinction between evidence derived from experimentally solved structures, homology modelling, AlphaFold-based prediction, and domain-level inference. This distinction is important because the evidentiary strength of a crystal structure is not equivalent to that of a predicted model or a conserved-domain annotation. In the studies retained in this review, experimentally resolved structural information is available for vertebrate MD-2, whereas structural claims for crustacean proteins rely primarily on homology modelling and sequence-based inference (Park *et al.*, 2009).

Evidence from solved structures: vertebrate MD-2 as the structural reference

The best-characterized structural template for interpreting MD-2-like proteins remains vertebrate MD-2. Crystal structures of human MD-2 and of the TLR4–MD-2–LPS complex show that MD-2 adopts a â-rich, cup-like fold containing a deep hydrophobic cavity that binds lipid A–derived ligands. In the full receptor complex, LPS binding promotes dimerization of two TLR4–MD-2 units into the signaling-competent assembly. These structures therefore define the canonical architectural features against which candidate MD-2-like proteins are generally compared.

These vertebrate structures also show that ligand recognition depends not simply on the presence of a cavity, but on its geometry and on specific residues at the pocket entrance and receptor interface. For example, the human TLR4–MD-2–LPS structure revealed that most acyl chains are buried within MD-2, while exposed ligand features and the F126 region contribute to receptor multimerization and signaling. Structural claims about “MD-2-like” proteins in crustaceans should therefore be interpreted in relation to this full ligand-binding and receptor-assembly context, not solely based on shared domain membership (Park *et al.*, 2009).

Evidence from homology modelling: crustacean candidates

For crustacean proteins, the strongest structural claims currently come from homology modelling and related comparative approaches. In *Scylla paramamosain*, SpMD2 was reported to share a similar three-dimensional structure and close evolutionary relationship with human MD2, and the model was used to support the interpretation of *SpMD2* as a potential LPS-binding protein. In *Litopenaeus vannamei, PvML1* was likewise described as sharing a similar predicted three-dimensional structure with human MD2, and this predicted resemblance was combined with biochemical and functional data to support its classification as an MD-2-like candidate (Wang *et al.*, 2022).

Evidence from domain-level inference:

At the broadest level, many sequences are annotated as belonging to the ML (MD-2-related lipid-recognition) superfamily based on sequence similarity and conserved-domain assignment. The ML domain was originally defined as a conserved lipid-recognition module present in proteins such as MD-1, MD-2, GM2 activator protein, and NPC2, and predicted to form a â-rich fold capable of interacting with lipids. This domain-level information is valuable for identifying candidates, but it is the weakest structural evidence tier because it does not establish the detailed architecture, ligand selectivity, or receptor biology of an individual protein (Inohara & Nuñez, 2002).

This point is particularly relevant in invertebrates, where ML-domain proteins span a broad family with diverse functions. For example, *Drosophila* NPC2 proteins also belong to the wider ML superfamily and can bind bacterial cell wall components, yet they are not equivalent to crustacean MD-2-like candidates in either taxonomy or demonstrated mechanism. Accordingly, the presence of an ML domain should be treated as a starting point for classification, not as proof that a given sequence is functionally analogous to vertebrate MD-2. (Shi *et al.*, 2012).

Distribution and Occurrence of MD-2-like and ML-Domain Proteins in Invertebrates

Proteins of the MD-2-related lipid-recognition (ML) superfamily are broadly distributed across metazoans, but not all ML-domain proteins should be interpreted as functionally equivalent to vertebrate MD-2. In the context of this review, it is therefore useful to distinguish three categories: (i) MD-2-like candidates, defined here as proteins with ML-domain architecture plus reported immune-responsive expression and ligand-binding or functional evidence; (ii) ML-domain immune proteins, which contain the ML domain and

Table 1. Representative MD-2-like candidates and ML-domain proteins relevant to this review. This table distinguishes between MD-2-like candidates, ML-domain immune proteins, and comparative ML-family analogues, and indicates the evidence basis used

Species / taxon	Protein designation	Classification used in this review	Evidence	Reference
Litopenaeus vannamei	*PvML1*	MD-2-like candidate	*PvML1* was characterized as an ML-family member with reported binding to LPS and lipid A, induction after *Vibrio parahaemolyticus* challenge, and functional effects on bacterial clearance, AMP expression, and interaction with *PvToll2.*	(Wang *et al.*, 2022)
Scylla paramamosain	*SpMD2*	MD-2-like candidate	*SpMD2* was reported as an ML-domain-containing protein with structural similarity to vertebrate MD2, broad microbial ligand binding, antibacterial immune relevance, and later RNAi-based evidence for contribution to disease resistance.	(Wang *et al.*, 2020)
Litopenaeus vannamei	*LvML*	ML-domain immune protein	*LvML* was identified as a shrimp ML superfamily protein induced by LPS and capable of recombinant LPS binding, but the evidence does not yet establish full MD-2-like functional equivalence or receptor usage.	(Lin *et al.*, 2024)
Eriocheir sinensis	*EsML3*	ML-domain immune protein	*EsML3* was reported as a soluble, bacteria-induced ML protein that binds LPS and peptidoglycan and promotes clearance and phagocytosis, supporting immune relevance while still warranting cautious interpretation as MD-2-like.	(Song *et al.*, 2022)
Drosophila melanogaster	*NPC2a/e/h*	Comparative ML-family analogue (non-crustacean)	These insect NPC2 proteins are included only as comparative ML-family analogues relevant to evolutionary context; they should not be treated as crustacean MD-2-like proteins.	(Shi *et al.*, 2012)

show immune relevance but lack sufficient evidence to establish MD-2-like mechanistic equivalence; and (iii) comparative ML-family analogues, such as insect NPC2 proteins, which are informative for evolutionary context but should not be treated as crustacean MD-2 homologues in a strict functional sense.

Among decapod crustaceans, the strongest current candidates for MD-2-like status come from shrimp and crabs in which at least some combination of sequence characterization, structural prediction, immune challenge responsiveness, ligand binding, and functional testing has been reported. In *Litopenaeus vannamei, PvML1* has been characterized as an ML-family member with predicted structural similarity to vertebrate MD2, strong binding to lipopolysaccharide (LPS) and lipid A, induction after *Vibrio parahaemolyticus* challenge, and functional effects on bacterial clearance, antimicrobial peptide expression, and survival after infection. These features justify its treatment as MD-2-like candidate, while still stopping short of claiming full mechanistic equivalence to vertebrate MD-2/TLR4 signaling (Wang *et al.*, 2022).

A comparable case has been reported in the mud crab *Scylla paramamosain*, where SpMD2 was described as a novel ML-domain-containing protein with predicted three-dimensional similarity to vertebrate MD2 and measurable binding to LPS, lipid A, peptidoglycan, and lipoteichoic acid. The protein was also upregulated after microbial challenge, and subsequent RNAi-based work linked SpMD2 to reduced immune performance and higher mortality after *Vibrio alginolyticus* infection, further supporting its status as MD-2-like candidate in crustaceans (Wang *et al.*, 2020). Even so, the absence of an experimentally demonstrated receptor complex means that the label “MD-2-like” remains best interpreted as functional candidacy rather than mechanistic proof.

Other crustacean ML-family proteins are better described more cautiously as ML-domain immune proteins rather than definitive MD-2-like molecules. In *L. vannamei*, the earlier-described *LvML* was cloned as a shrimp ML superfamily protein, shown to be induced by LPS, and demonstrated recombinant LPS-binding activity, supporting a role in innate immune recognition. However, the available evidence for *LvML* is largely descriptive and biochemical, without the degree of functional or pathway-level validation now available for *PvML1*. For this reason, *LvML* is more appropriately placed in a second tier of immune-relevant ML-domain proteins with unresolved MD-2-like equivalence (Liao *et al.*, 2011).

A similar level of caution applies to the Chinese mitten crab *Eriocheir sinensis*. In this species, *EsML3* was reported as a soluble, bacteria-induced ML protein that binds both Gram-negative and Gram-positive bacteria through interaction with LPS and peptidoglycan and promotes bacterial clearance and phagocytosis *in vivo*. These findings strongly support immune relevance and pattern-recognition capacity, but they do not yet establish whether *EsML3* is mechanistically analogous to vertebrate MD-2 or instead represents a broader class of crustacean ML-domain immune proteins with partially overlapping ligand-binding properties. Accordingly, *EsML3* is best discussed here as a ML-domain immune protein rather than as a confirmed MD-2-like receptor (Qin *et al.*, 2022).

Comparative examples from other invertebrates remain useful for framing evolutionary diversity within the ML superfamily, but they should be kept conceptually separate from crustacean candidates. In Drosophila melanogaster, several NPC2 proteins bind LPS, lipid A, peptidoglycan, and lipoteichoic acid, and some can activate immune reporter systems in cultured cells. Nevertheless, these proteins belong to the NPC2 branch of the ML family and are not equivalent to crustacean MD-2-like candidates; their value in this review is therefore comparative rather than classifying. This distinction is important because it prevents the broader ML superfamily from being presented as a single functional class and helps keep the scope of the review centered on the specific question of which crustacean proteins most plausibly approach MD-2-like immune roles (Shi *et al.*, 2012).

Mechanisms of LPS Recognition

In vertebrates, lipopolysaccharide (LPS) recognition is mediated by the TLR4–MD-2 receptor complex, and this mechanism has been established through both biochemical and structural studies. MD-2 is physically associated with TLR4 and is required to confer LPS responsiveness, thereby functioning as the extracellular lipid-binding component of the receptor complex (Visintin *et al.*, 2001).

High-resolution structural studies have clarified how this recognition event occurs. The crystal structure of the human TLR4–MD-2–LPS complex showed that LPS binds within a large hydrophobic pocket in MD-2, with most lipid chains buried in the cavity and one acyl chain partially exposed to contribute to receptor dimerization (Figure 1). The same work also demonstrated formation of an m-shaped receptor assembly, which explains how ligand binding promotes signaling-competent complex formation (Park *et al.*, 2009).

Reported evidence in crustaceans:

In crustaceans, a canonical vertebrate-style TLR4–MD-2 pathway has not been established, and current evidence should therefore be framed more cautiously. What has been reported is that several crustacean ML-domain proteins can bind LPS or related bacterial ligands and are associated with antibacterial immune responses, but this does not by itself demonstrate a conserved vertebrate-like receptor mechanism (Chen & Wang, 2019).

The strongest current evidence comes from a limited number of proteins. In *Litopenaeus vannamei*, *PvML1* was reported to bind LPS and lipid A, agglutinate bacteria, promote bacterial clearance in vivo, and affect antimicrobial peptide expression and survival following bacterial challenge. The same study also reported interaction between *PvML1* and the extracellular region of *PvToll2*, suggesting possible coupling of ligand binding to Toll-mediated antibacterial signaling (Wang *et al*., 2022).

In the mud crab *Scylla paramamosain*, *SpMD2* was reported to bind LPS, lipid A, peptidoglycan, and lipoteichoic acid, and its expression increased after bacterial challenge. Follow-up RNAi-based work further linked *SpMD2* to changes in hemocyte abundance, phagocytic activity, immune-enzyme activities, and survival during Vibrio alginolyticus infection, supporting a role in host defense (Wang *et al*., 2020).

Additional support for immune relevance comes from ML-domain proteins that are more cautiously classified as immune-associated rather than definitively MD-2-like. In *L. vannamei*, *LvML* was shown to be inducible by LPS and capable of recombinant LPS binding. In *Eriocheir sinensis*, *EsML3* was reported to bind both Gram-negative and Gram-positive bacteria through interaction with LPS and peptidoglycan and to promote bacterial clearance and phagocytosis. These findings support pattern-recognition capacity, but they do not yet establish shared receptor architecture across crustaceans (Liao *et al*., 2011).

Hypothetical mechanisms in crustaceans:

Because receptor partners and signaling complexes remain incompletely defined, current mechanistic explanations for crustacean ML-domain proteins should be treated as hypotheses rather than resolved pathways. Based on the available localization, ligand-binding, and co-expression evidence, two non-exclusive models are currently most plausible.

The first is an opsonin-like model, in which soluble ML-domain proteins bind LPS or other microbial surface ligands in the hemolymph and then facilitate bacterial agglutination, phagocytosis, or clearance by hemocytes. This interpretation is consistent with the reported bacterial agglutination and clearance effects of *PvML1* and *EsML3*, as well as the broad ligand-binding activity observed for *SpMD2* (Wang *et al*., 2022).

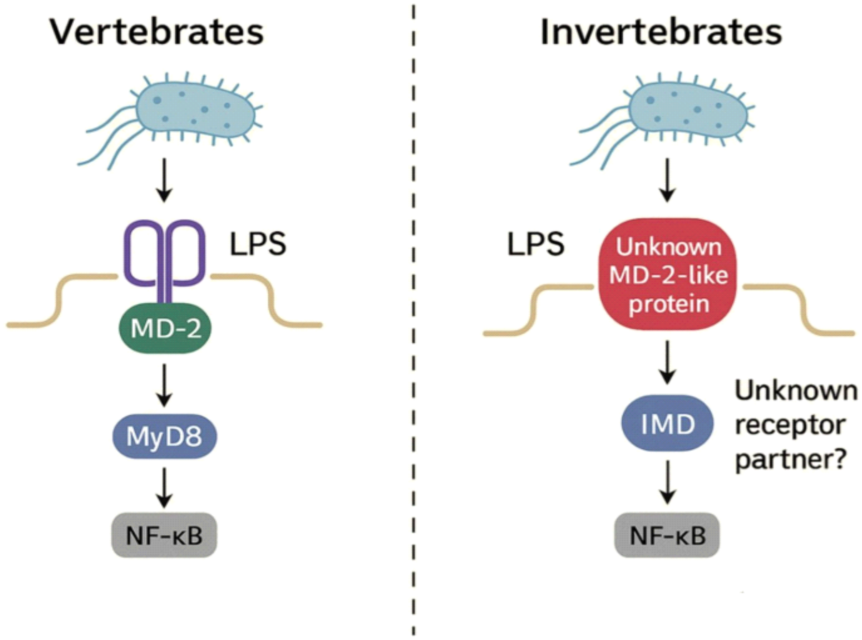

Figure 1. Comparative LPS Recognition Pathways in Vertebrates and Invertebrates.

Left panel: Lipopolysaccharide (LPS) activates NF-κB and MyD88-dependent signaling in vertebrates via binding to MD-2 and Toll-like receptor 4 (TLR4). Right panel: In invertebrates (e.g., shrimp), LPS binds with MD-2-like proteins and likely activates Toll/IMD pathways, although the receptor partners remain undiscovered.

The second is a co-receptor or accessory-factor model, in which an ML-domain protein binds LPS and then interacts with a membrane receptor to initiate downstream signaling. *PvML1* provides the most direct support for this hypothesis because it was reported to interact with *PvToll2* and to influence antimicrobial peptide expression after bacterial challenge. However, even in this case, the full composition of the receptor complex, the order of signaling events, and the tissue context remain unresolved (Nan *et al.*, 2022).

Evidence in Crustaceans

Current evidence for crustacean MD-2-like and ML-domain proteins is unequal across species and experimental systems. To avoid conflating distinct evidence types, it is helpful to separate the literature into four categories: challenge-induced expression evidence, in vitro ligand-binding evidence, functional perturbation evidence, and receptor/pathway evidence. Across these categories, the strongest support currently comes from a limited number of proteins in shrimp and crabs, particularly *PvML1* in *Litopenaeus vannamei* and *SpMD2* in *Scylla paramamosain*, whereas other ML-domain proteins are supported by more descriptive or partially functional datasets.

Challenge-induced expression evidence

Several crustacean ML-domain proteins are transcriptionally responsive to bacterial or LPS challenge, supporting a role in antibacterial immunity, although expression changes alone are not sufficient to establish mechanism. In *L. vannamei*, *LvML* expression was reported to increase after LPS injection, suggesting that this ML-domain protein participates in early host responses to Gram-negative bacterial components (Liao *et al.*, 2011). In the same species, *PvML1* was found to be highly expressed in hemocytes and markedly upregulated after Vibrio parahaemolyticus challenge, providing stronger evidence that this protein is associated with antibacterial immune activation in a physiologically relevant tissue. Similar inducible expressions have been reported in crabs (Fu *et al.*, 2025). *SpMD2* in *S. paramamosain* was highly expressed in gills, hemocytes, intestine, and hepatopancreas and was upregulated in gills and hemocytes after bacterial challenge (Li *et al.*, 2025), while *EsML3* in *Eriocheir sinensis* was described as a bacteria-induced soluble ML protein upregulated in hemocytes following bacterial exposure (Kong *et al.*, 2025). Taken together, these studies support the view that selected crustacean ML-family proteins are challenge-responsive immune candidates, but expression data should still be interpreted as correlative rather than mechanistically decisive.

In vitro ligand-binding evidence

Direct biochemical evidence for ligand binding provides a stronger basis for functional interpretation than transcript induction alone. Recombinant *LvML* from *L. vannamei* showed binding activity to LPS in ELISA-type assays, and the interaction was inhibited by pre-incubation with LPS, consistent with specific recognition of this ligand class (Liao *et al.*, 2011). *PvML1* provides a more functionally developed example: recombinant *PvML1* bound LPS and lipid A, as well as Vibrio cells, and the association between *PvML1* and bacterial cells led to bacterial agglutination, supporting the interpretation that this protein can act as a soluble pattern-recognition factor in shrimp (Wang *et al.*, 2022). In mud crab, recombinant *SpMD2* displayed binding not only to LPS and lipid A but also to peptidoglycan and lipoteichoic acid, indicating a broader ligand spectrum than would be expected for a highly specific vertebrate-style MD-2 equivalent (Wang *et al.*, 2020). A similar pattern was reported for *EsML3* in E. sinensis, which bound both Gram-negative and Gram-positive bacteria through interaction with LPS and peptidoglycan (Qin *et al.*, 2022). These studies collectively support the conclusion that several crustacean ML-domain proteins can bind bacterial surface components in vitro, but they also suggest that ligand selectivity may be broader and mechanistically less specialized than in vertebrate MD-2.

Receptor/pathway evidence

Evidence connecting crustacean ML-domain proteins to specific immune receptors or signaling pathways remains the least developed category and is currently the main barrier to full mechanistic interpretation. The clearest example comes from *PvML1* in *L. vannamei*, where the study reported interaction between *PvML1* and the extracellular region of *PvToll2* and proposed a model in which *PvML1* binds LPS and then activates a *PvToll2*-dependent antibacterial pathway leading to antimicrobial peptide expression (Zhang *et al.*, 2018). Although this is an important step toward pathway-level interpretation, it does not yet establish a canonical vertebrate-like MD-2/TLR4 mechanism, and additional work is needed to verify the broader receptor complex, signaling intermediates, and tissue specificity of this interaction. Thus, current evidence supports the view that crustacean ML-family proteins can contribute to antibacterial defense, but receptor usage and signal initiation remain unresolved in most species, making it premature to generalize a single mechanistic model across crustaceans.

Table 2. Published evidence on crustacean MD-2-like candidates and ML-domain proteins. Only entries supported by directly relevant primary studies are retained. Evidence strength and principal limitations are provided to distinguish experimentally supported findings from mechanistic uncertainties

Species	Protein / gene	Evidence categories represented	Main reported findings	Main limitations	References
Litopenaeus vannamei	*LvML*	Challenge-induced expression; in vitro ligand binding	*LvML* was cloned from shrimp, expressed predominantly in hepatopancreas, upregulated after LPS injection, and recombinant *LvML* showed LPS binding in ELISA-type assays.	No RNAi/knockout evidence; receptor partners and downstream signaling remain unresolved.	(Liao *et al*., 2011)
Litopenaeus vannamei	*PvML1*	Challenge-induced expression; in vitro ligand binding; bacterial agglutination / clearance; RNAi; receptor/pathway evidence	*PvML1* was highly expressed in hemocytes, induced after *Vibrio parahaemolyticus* challenge, bound LPS and lipid A, promoted bacterial agglutination and clearance, and RNAi knockdown reduced bacterial clearance, survival, and AMP expression; the study also reported interaction with *PvToll2.*	Although receptor interaction with PvToll2 was reported, the full signaling architecture and in vivo receptor complex remain incompletely resolved.	(Wang *et al*., 2022)
Scylla paramamosain	*SpMD2*	Challenge-induced expression; in vitro ligand binding; antibacterial functional assays; RNAi / infection phenotype	*SpMD2* showed predicted structural similarity to vertebrate MD2, was upregulated after microbial challenge, bound multiple bacterial ligands including LPS and lipid A, and later RNAi-mediated knockdown was associated with reduced immune parameters and increased mortality after *Vibrio alginolyticus* infection.	No experimentally solved structure or directly demonstrated receptor complex has yet been reported.	(Wang *et al*., 2020)
Eriocheir sinensis	*EsML3*	Challenge-induced expression; in vitro ligand binding; bacterial clearance / phagocytosis assays	*EsML3* was described as a soluble, bacteria-induced ML protein that bound Gram-negative and Gram-positive bacteria through interaction with LPS and peptidoglycan and promoted bacterial clearance and phagocytosis in vivo.	No RNAi/knockout evidence and no receptor/pathway mapping were reported in the study.	(Qin *et al*., 2022)

Future Translational Prospects in Aquaculture

The translational relevance of crustacean MD-2-like and ML-domain proteins remains prospective rather than immediate. Current evidence is largely limited to expression studies, ligand-binding assays, and a few *in vivo* experiments, without a complete mechanistic framework. Therefore, any aquaculture application should be viewed as conditional on further validation, rather than ready for deployment. This cautious perspective aligns with broader aquaculture research, which emphasizes that disease-control strategies must be evaluated for repeatability, specificity, safety, scalability, and cost-effectiveness. Immune-based approaches, although promising, require rigorous validation and standardized assessment before practical adoption.

1. Candidate biomarkers of immune status or disease responsiveness

MD-2-like and ML-domain proteins could potentially serve as biomarkers of immune status, as several are inducible following bacterial or LPS challenge. However, expression responsiveness alone is insufficient for practical use. For biomarker application, three criteria are essential: (i) reproducible associations with health outcomes across populations, (ii) added predictive value beyond existing indicators, and (iii) reliable performance under farm conditions. Until these are met, these proteins should be considered research markers rather than validated tools.

2. Targets for immunostimulants or prophylactic development

These proteins may inform future immunostimulant design, given their roles in bacterial binding, clearance, and immune regulation. However, current evidence does not support direct application in aquaculture. Progress requires clearer identification of receptor partners, evaluation of immune trade-offs, and in vivo dose–response studies. For now, MD-2-like proteins are better viewed as mechanistic targets for future research, not immediate intervention points.

3. Molecular tools for pathogen detection or ligand sensing

The ability of some ML-domain proteins to bind LPS suggests potential use in biosensors or diagnostic platforms. While conceptually attractive, this application remains early-stage and unvalidated. To be feasible, these proteins must demonstrate strong binding specificity, stability, and reproducibility under realistic aquaculture conditions. Until such data exists, biosensor applications should be considered long-term possibilities rather than near-term solutions.

4. Longer-term relevance to selective breeding or genetic improvement

In the long term, MD-2-like or ML-domain traits could contribute to selective breeding for disease resistance, provided they show heritable and consistent associations with health outcomes. However, such evidence is currently lacking. Robust genotype–phenotype relationships, population-level validation, and environmental consistency must first be established. Accordingly, breeding or genetic modification applications remain highly speculative and long-term.

Knowledge Gaps and Future Directions

Current evidence supports the view that selected crustacean ML-domain proteins are relevant to antibacterial immunity, but the field remains constrained by a gap between functional association and mechanistic resolution. To move beyond descriptive genomics and ligand-binding observations, future work should be prioritized in a staged manner.

Immediate mechanistic priorities

The most urgent priority is to identify the receptor partners and signaling context of crustacean MD-2-like and ML-domain proteins. Evidence from *PvML1* in *Litopenaeus vannamei* suggests interaction with *PvToll2*, but the full receptor complex and downstream signaling remain unclear. For other proteins such as *SpMD2*, *LvML*, and *EsML3*, receptor usage is largely unknown, limiting understanding of their functional roles. A second priority is quantitative ligand-specificity mapping. While some proteins bind LPS or lipid A, others also interact with peptidoglycan or lipoteichoic acid, indicating broader recognition profiles. Future work should distinguish ligand specificity through quantitative assays, including affinity measurements and multi-ligand comparisons. A third priority is high-resolution structural validation. Current insights rely on homology modelling based on vertebrate MD-2, which is informative but not definitive. Experimental approaches such as X-ray crystallography or cryo-EM are needed to confirm ligand-binding architecture and receptor interfaces.

Medium-term validation priorities

Following mechanistic clarification, the next step is broader functional validation across species and contexts. Although *PvML1* and *SpMD2* have some in vivo support, most ML-domain proteins remain untested. Expanding RNAi and infection studies will determine whether current findings are representative or species-specific. Another priority is clarifying tissue specificity, developmental context, and functional redundancy. While expression has been observed in hemocytes, gills, and hepatopancreas, their distinct or overlapping roles remain unclear. Addressing this requires systematic expression mapping and comparative analyses across gene family members. Comparative and evolutionary validation is also needed to determine whether MD-2-like proteins represent a coherent functional group or a heterogeneous subset of the ML family. Phylogenetic and functional analyses may reveal links between diversification and ecological or pathogen pressures.

Long-term aquaculture translation priorities

Translation to aquaculture should follow only after robust mechanistic and functional validation. The most realistic application is the development of immune biomarkers, provided consistent associations with disease outcomes and reliable performance across farming conditions are demonstrated. A second direction is the use of validated proteins to inform immunostimulant design or diagnostic tools. However, these applications depend on prior identification of receptor interactions, ligand specificity, and in vivo safety, as emphasized in broader aquaculture research. The most distant application is integration into selective breeding or genetic improvement. Although breeding can enhance disease resistance, it requires stable genotype–phenotype relationships, which are not yet established for MD-2-like proteins. Thus, this remains a long-term possibility rather than an immediate target.

CONCLUSION

Crustacean MD-2-like and ML-domain proteins have progressed from annotated sequences to credible functional candidates in antibacterial immunity. Evidence from key proteins such as *PvML1* in *Litopenaeus vannamei* and *SpMD2* in *Scylla paramamosain* supports roles in host defense through combined expression, ligand-binding, and *in vivo* data. At the same time, additional proteins (e.g., *LvML* and *EsML3*) indicate that immune-relevant ML-domain proteins are broadly present in crustaceans, although their mechanisms remain less defined. However, current evidence does not yet establish a crustacean equivalent of the vertebrate TLR4–MD-2 system. Most interpretations rely on structural homology, in vitro binding, and partial functional studies, with receptor partners and signaling pathways still unresolved. Thus, while functional candidacy is supported, full mechanistic understanding is lacking. This distinction is important both to avoid overgeneralization of ML-family functions and to set realistic expectations for aquaculture applications, which will require further validation in receptor identification, ligand specificity, structural characterization, and population-level studies.

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