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Effect of Prebiotics and Probiotics on Phenoloxidase Expression and Activity in *Penaeus* Shrimps: A Meta-Analysis

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Abstract

Context: Immunostimulants promote immune response and growth, and raise stress and aquaculture disease resistance. At times, the results are contradictory and misleading regarding their utilization. Accordingly, the following problematic was stated: Could the related scientific literature on prebiotics and probiotics that evaluate the indicators of the immune system of shrimps be the basis for decision-making in shrimp-culture immunostimulant use?
Aim: To evaluate the influence of prebiotics and probiotics on the phenoloxidase expression and activity in *Penaeus* shrimps, using meta-analysis for decision-making in shrimp culture.
Methods: Several searches were conducted in Pubmed, Science Direct, and Scopus, between 2008 and 2019, related to the influence of immunostimulants on phenoloxidase activity and gene expression. The statistical analysis was conducted by means of Metanalysis 0.9.2., with a 95% confidence interval.
Results: A total of 262 scientific articles on prebiotic and probiotic evaluation in Penaeus shrimps were compiled. Out of them, 61 met the inclusion criteria for immunological response: 51 dealt with phenoloxidase activity, and 11 studied prophenoloxidase. Both prebiotics and probiotics stimulated the enzyme and proenzyme; the plant extracts had a major role in the modification of this activity, whereas the Bacilli produced the highest stimulation.
Conclusions: The scientific literature on Penaeus shrimp phenoloxidase activity indicated careful evaluation due to the experimental design and way expressed in the results.

Keywords: immunostimulants, shrimps, immune system.

Introduction

Aquaculture is one of the fastest-growing sectors in terms of food production. The *Penaeus* shrimps are the most frequently cultivated species in farms due to their fast growth and adaptation (FAO, 2018). Shrimp culture in Cuba and internationally is facing risks associated with huge financial losses as a result of high-mortality-causing diseases, as well as a reduction in the quality of productions. Because crustaceans lack specific immune responses to infectious agents, their defensive role consists of a sophisticated unspecific defense system based on circulating hemocytes and several defense proteins (Chang, et al., 2018), namely, the formation of the proPO system. It produces proteolytic reactions, which, among other products, end with the processing and activation of the phenoloxidase active enzyme (PO) (Tassanakajon et al., 2018).

PO is the main shrimp enzyme that favors wound healing and the sclerotization of the cuticles due to its microbicidal effects (Vaseeharan et al., 2016). Immunostimulants are used to increase and induce the immune response and resistance to stress and diseases via direct interaction of the immune system cells and their activation (Vaseeharan & Thaya, 2014) as whole molecules/cells of bacteria and yeasts (Mastan, 2015). The scientific literature contains a broad range of information related to the utilization of prebiotics and probiotics. Meta-analysis (MA) is the analysis of the findings reported by several authors. MA is a systematic review (SR) which combines the results from various studies about the same question (Will immunostimulants use promote phenoloxidase and prophenoloxidase activity in Penaeus shrimps?), through a painstaking review of the literature. The outcome is then summarized as numerical values (Tikito & Souissi, 2019), which will help in shrimp culture decision-making.

The diversity of shrimp species experiments hinders decision-making in terms of prebiotic and probiotic use for shrimp culture. Accordingly, this research **aims** to evaluate the influence of prebiotics and probiotics on the phenoloxidase expression and activity in *Penaeus* shrimps, using meta-analysis for decision-making in shrimp culture.

Materials and Methods

Methods

Inclusion criteria

The studies compiled deal with measuring the enzymatic activity of phenyloxidase and the expression of genes in proPO by using prebiotics and probiotics in *Penaeus* shrimps. The search comprised the scientific papers published between 2008 and 2019, in English and Spanish.

Information source

Several databases (*Scopus*, *Science Direct*, *Pub Med*) were reviewed to select relevant papers. The search started in September 2018 and ended in February 2019. The authors of the research papers who met the inclusion criteria, but lacked dispersion measures were contacted to acquire extra information. The unresponsive authors were ruled out.

Search

The keywords used in the search were *phenoloxidase penaeus prebiotic and probiotic*. The preliminary inclusion relied on the analysis of titles and abstracts.

Study selection

The validation criteria included the experimental articles published in peer-reviewed journals that studied the effect of prebiotics and probiotics on the immune response, particularly on phenoloxidase activity in *Penaeid* shrimp culture. The studies that measured the response variables were selected.

Data collection

The identification of the studies and collection of interest information associated with the response variables (dispersion mean and measurements) was done by duplicate in Microsoft Excel spreadsheets. The data obtained corresponded to the entire assays, where only the final measurements, rather than the interval measurements, were included. The papers that evaluated more than a treatment, combination or different administration doses were analyzed separately.

List of data

The variables for data search and further analysis were a) prebiotic and probiotic types; b) *Penaied* shrimp species; c) larval stage; d) pond type (plastic, soil, glass, glass fiber).

Bias risks in individual studies

Two reviewers conducted an independent search, and the results were contrasted, solving their difference by discussion and consensus. Then, the bias risk in individual studies was evaluated similarly.

Bias risks among individual studies

Some of the risks assessed were 1) bias due to multiple publication corresponding to the number of articles by author included; 2) bias due to the selective reports corresponding to papers reporting partial information about the experimental design or the response variables; 3) publication bias in papers that reported favorable results with the utilization of prebiotics and probiotics.

Synthesis of the results

The level of heterogeneity was quantified using the index of inconsistency (I2 statistics) (Higgins & Thompson, 2002).

Statistical analysis

The statistical index of the extent of the effect in every study was calculated using *Microsoft Excel* 4. The statistical analysis was conducted with *Jasp Metaanalysis* 0.9.2. The effects of the administration of prebiotics and probiotics were quantified as the difference in standardized means (DME). The confidence intervals (95%) were calculated by a random effect model. The subgroup analysis were designed with *GraphPad Prism* 7.00, which considered the factors that might influence the extent of the effects of treatments.

Results and discussion

Results

Study selection

The bibliographic search produced 262 scientific articles on prebiotic and probiotic evaluation in Penaeus shrimps. Of them, 61 met the previously established inclusion criteria for immunological response. A number of 51 papers evaluated phenoloxidase activity, whereas 11 dealt with gene expression in proPO (Figure 1).

Study characteristics

The scientific papers that evaluated the prebiotic and probiotic effects on phenoloxidase activity included the species below: *L. vannamei* (34), *P. monodon* (10), *M. japonicus* (3), *F. indicus* (2), *F. chinensis* (1) and *L. schmitti* (1).

Overall, 43 studies included juvenile shrimps, 45 referred to the use of plant extracts (15); polysaccharides (14); organic acids (3); liposaccharides (2); disaccharides (2); inulin (1); trypsin (1); ferritin (1); polyphenol (1); metal (1); carrageenan (1); nucleotides (1) as prebiotics. Meanwhil, only 15 included probiotics, including species: *Bacillus* (7), *Lactobacillus* (4), *Arthrobacter* XE-7 (1), *E. coli* (1), *R. palustris* (1), *Psychrobacter* (1), Proteobacteria – Firmicutes (1), *V. anguillarum* (1), *S. cerevisiae* (1), *Pediococcus* (1). The enzymatic activity analyses were conducted in hemolymph (40), and the hepatopancreas (5).

The following species were used to determine the influence of prebiotics and probiotics on gene expression: *L. vannamei* (10), *P. monodon* (1), *M. japonicus* (1) and *F. indicus* (1). Prebiotics were included in 10 articles, such as polysaccharides (4); plant extracts (2); and organic acids (2). The tissues used to analyze the proenzyme were the hemolymph (8), and hepatopancreas (4).

Analysis of the studies included

From the total of 262 papers found in the databases for the period evaluated, 201 were ruled out because of the type of publication (studies involving other animal species, mostly *Macrobachium* sp., *Artemia* sp., *Erimacrus* sp., *Scylla* sp.; the inclusion of other additives in the diet; or reviews; or the absence of statistical information to conduct meta-analysis) (Fig. 1).

A bias that limits credibility of published studies, is that 25% of the reports excluded because of the design showed no deviation. If individual studies offer biased data, meta-analysis of the same data will render estimates with errors (Van Assen et al., 2015).

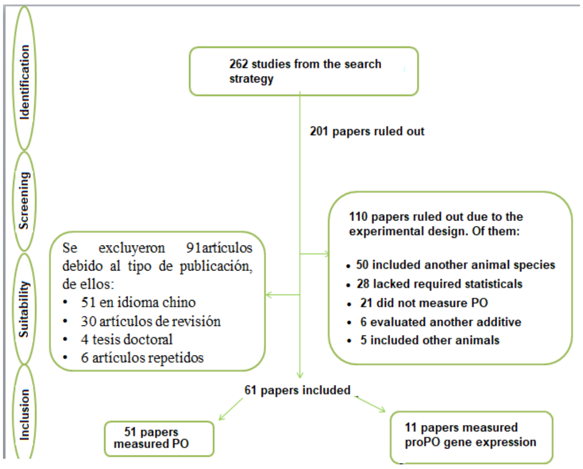


Fig. 1 Flow diagram of involving the selection of papers on the evaluation of prebiotics and probiotics in *Penaeid* shrimps for meta-analysis inclusion.

Analysis of phenoloxidase activity and gene expression

Of the 51 articles on phenoloxidase activity selected, 32 experiments using prebiotics and 19 using probiotics, were identified. The difference of the combined standardized measures showed that the immunostimulants raised PO activity compared to the controls (Fig. 2. at the end of the article body).

Results of the individual studies

Of the 11 articles on gene expression in proPO, 10 experiments were found to use prebiotics, whereas 5 used probiotics. The difference of the combined standardized means showed that the immunostimulants raised PO activity compared to the controls (Fig. 3).

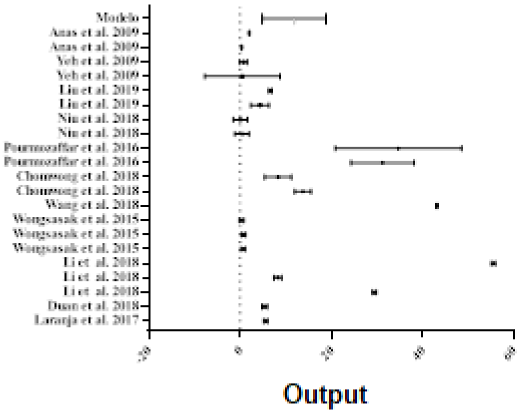
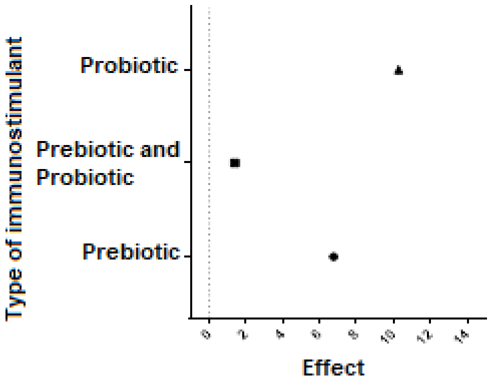


Fig. 3 Forest plot of the 22 experiments included to study the effect of prebiotics and probiotics on gene expression in *Penaeid* shrimps. The dots represent the estimated effect (difference among the standardized means), while the horizontal lines show the confidence index (95%).

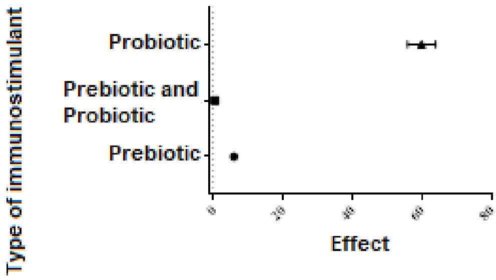
The probiotics showed greater stimulation, both of the enzyme and the proenzyme, than the prebiotics; their combination produced minimum stimulation (Fig. 4 A and B). Derivatives of lactic-acid bacteria that inhibit *Vibrio parahaemolyticus* stimulate proPO expression (Chomwong et al., 2018)

Synthesis of the results

A B



A

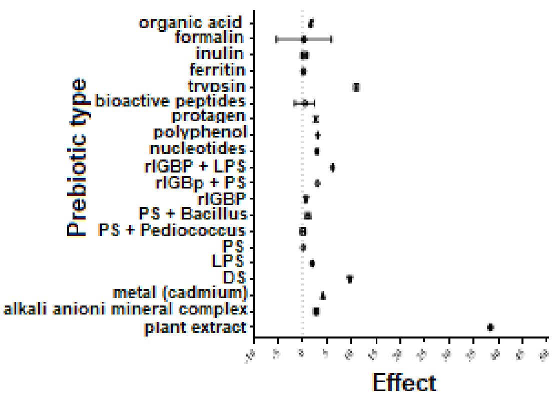


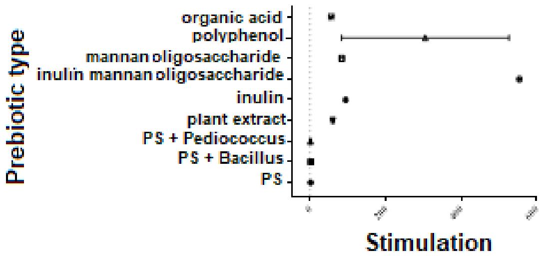
B

Fig. 4 Effect of the immunostimulants types on Penaeid shrimps. A: phenoloxidase activity and B: gene expression (prophenoloxidase). The dots represent the estimated effect (difference among the standardized means), and the confidence index (95%).

Overall, prebiotics stimulate phenoloxidase activity (Fig. 5 A). The inulin and mannan oligosaccharides underwent greater proPO stimulation (Fig. 5 B). It constitutes a bias, as only one paper (related to inulin) met the inclusion criteria.

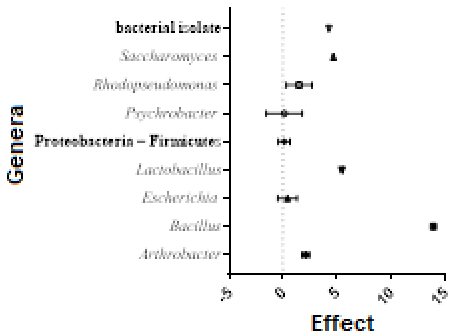
A



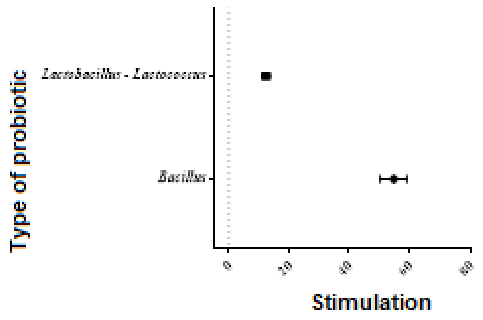


B

Fig. 5 Effect of the prebiotic type on Penaeid shrimps. A: phenoloxidase activity and B: and gene expression (prophenoloxidase). The dots represent the estimated effect (difference among the standardized means), with the confidence index (95%).



A



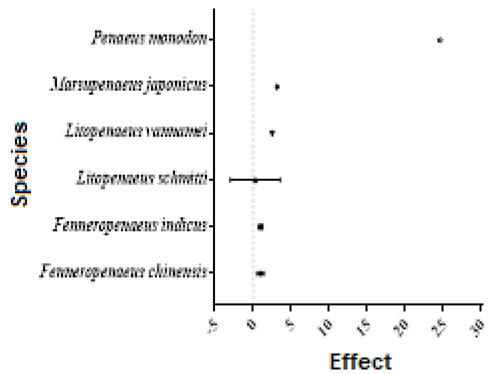
B

Fig. 6. Effect of the probiotic type on Penaeid shrimps. A: phenoloxidase activity and B: and gene expression (prophenoloxidase). The dots represent the estimated effect (difference of standardized means), with the confidence index (95%).

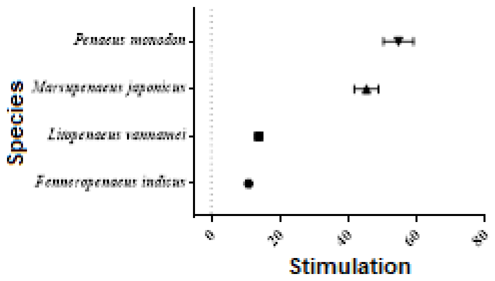
A B

Genus *Bacillus* underwent greater gene expression stimulation (Fig. 6 B). The presence of *B. subtilis* in the diet of shrimps increased the resistance to diseases caused by bacterial pathogen *V. alginolyticus*, and the immune response (Sandeepa & Ammani, 2017). Then, the *Lactobacillus* sp., with a favorable enzymatic stimulation as probiotics are effective stimulants of the proPO system in aquaculture ((Laranja et al., 2017))

The effect of immunostimulants on the different Penaeid shrimp species *Penaeus monodon* was evidenced (Fig. 7) the stimulation of enzymatic activity and gene expression was higher than in the other species (Fig. 7 A and B). In *Litopenaeus schmitti*, phenoloxidase had a similar behavior to the control, so the stimulation was not significant.



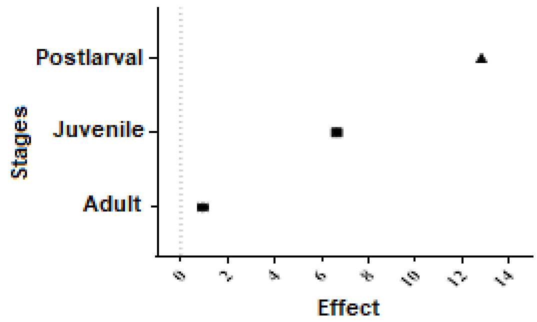
A



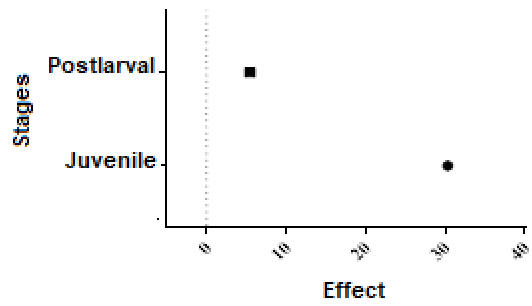
B

Fig. 7 Effect of prebiotic and prebiotic supplementation on the phenoloxidase activity according to the species type in Penaeid shrimps. A: phenoloxidase activity and B: and gene expression (prophenoloxidase). The dots represent the estimated effect (difference among the standardized means), with the confidence index (95%).

Overall, phenoloxidase activity stimulation was detected with the supplementation of prebiotics and probiotics (Fig. 8A) in postlarval, juvenile, and adult stages, though it was higher in the post larvae. The postlarval and juvenile stages underwent in gene expression, though it was higher in the juveniles (Fig. 8B).



A



B

Fig. 8 Effect of prebiotic and prebiotic supplementation on the stage types of Penaeid shrimps. A: phenoloxidase activity and B: and gene expression (prophenoloxidase). The dots represent the estimated effect (difference among the standardized means), with the confidence index (95%).

The immunostimulant effect on the types of tissues of Penaeid shrimps is shown in figure 9. The hepatopancreatic tissue underwent higher phenoloxidase stimulation than the hemolymph (Fig. 9A). However, the hemolymph underwent higher phenoloxidase stimulation than the hepatopancreatic tissue (Fig. 9B). Although the immune response was mainly observed in the hemolymph, for the larvae it required the entire individual. However, it is known that phenoloxidase activity can be expressed in several tissues, or have hemocyte infiltrations that contribute to the enzymatic activity (Braga et al., 2018).

A B

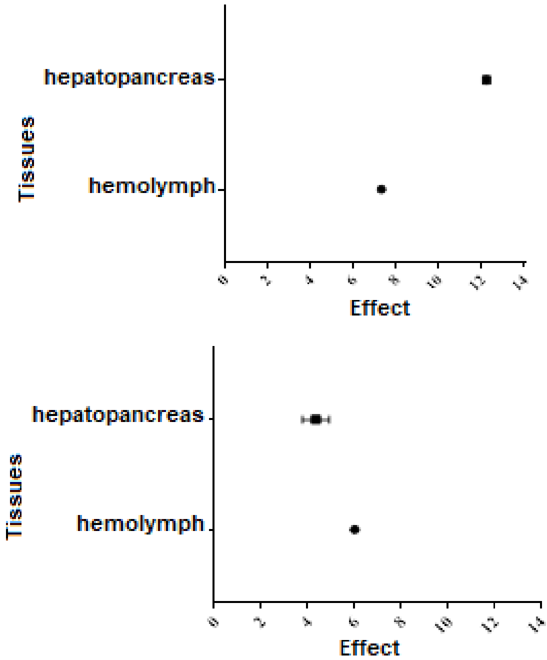


Fig. 9 Effect of prebiotic and prebiotic supplementation on the tissue types of Penaeid shrimps. A: phenoloxidase activity and B: and gene expression (prophenoloxidase). The dots represent the estimated effect (difference among the standardized means), with the confidence index (95%).

Higher phenoloxidase stimulation was observed in the plastic tanks. 10 A), whereas the soil ponds had no stimulation. Higher gene expression stimulation was observed in the cement tanks, whereas it was minimum in the plastic containers (Fig. 10 B).

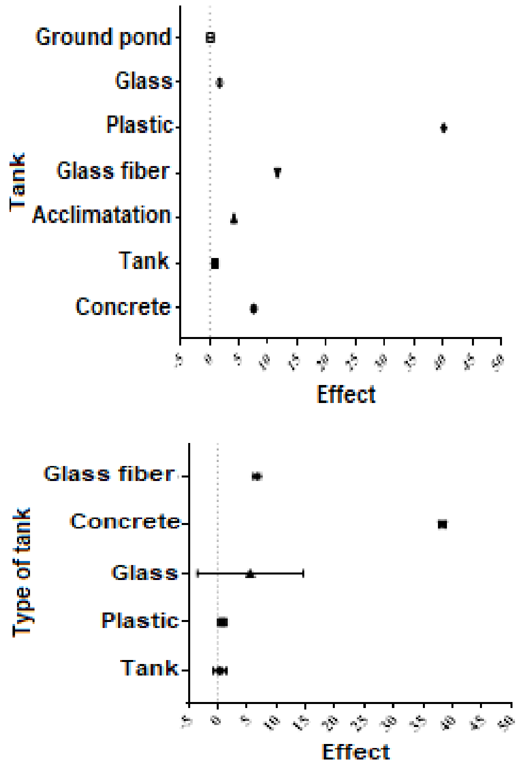


Fig. 10 Effect of prebiotic and prebiotic supplementation on the tank types containing Penaeid shrimps. A: phenoloxidase activity and B: and gene expression (prophenoloxidase). The dots represent the estimated effect (difference among the standardized means), with the confidence index (95%).

Additional analyses

Most studies produced lower results in all the aspects of the Cochrane tool examined for bias risk evaluation, except for the publication bias and the multiple report bias. The publication bias was considered as high risk in the research studies that reported positive effects in their administration.

Discussion

Several authors confirm the beneficial effects of probiotics (Zorriehzahra et al., 2016), and prebiotics (Dawood et al., 2018) in aquaculture The utilization of immunoprophylaxis was suggested as the best alternative for protection against bacterial diseases in shrimps (Ajadi et al., 2016), as it was also suggested for prophylaxis against viruses and fungi (Sivasankar et al., 2017).

The meta-analysis confirmed that both phenoloxidase activity and gene expression are stimulated with the supplementation of immunostimulants in Penaeid shrimps. The proenzyme showed a better effect than the enzyme, because the DNA replicates, is transcribed into the RNA (the expression), to then translate, or turn RNA into protein. There may be high gene expression stimulation that remains untranslated, and therefore, does not become a protein. Moreover, the RNA detection method through real-time rtPCR (reverse transcriptase Polymerase Chain Reaction in Real Time), has a higher sensitivity because it is fluorometric, instead of colorimetric as the protein method.

Inulin is the name used to designate a complex glycoside family (polysaccharides), made of fructose molecular chains (Lara-Fiallos et al., 2017). Prebiotic-based diet administration (inulin) has a stimulating effect on the immune response and pathogen resistance of aquatic animals (Li et al., 2018; Luna-González et al., 2012). Other polysaccharides and molecules, such as succinic acid, formalin, minerals, nucleotides, and β-glycans also stimulated phenoloxidase expression (Bai et al., 2010; Bui et al., 2017; Duan et al., 2018; Immanuel et al., 2012; Kitikiew et al., 2013; Lamela et al., 2008; Manoppo et al., 2010). Besides, the polysaccharides can stimulate the expression of phenoloxidase, even when the animals are under stress (Wongsasak et al., 2015). Although there are reports on higher stimulation with the whole cell than the pure polysaccharide (Sajeevan et al., 2009).

Ferritin also had the capacity of stimulating phenoloxidase activity (Ruan et al., 2010). An *Acremonium diospyri* glucan showed a high capacity of immunostimulation in *Feneropenaeus indicus*

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(Anas et al., 2009). The green tea catechin reduced the *Vibrio alginolyticus* infection, mainly through proPO expression (Wang et al., 2018).

The administration of *Gracilaria tenuistipitata* or *Platymonas helgolandica* to *L. vannamei* stimulated proPO expression and raised the resistance to *Vibrio harveyi* o *algynoliticus* (Ge et al., 2017; Liu et al., 2019). Other two species of this genus (*Gracilaria verrucosa* and *Gracilaria tenuistipitata*) stimulated phenoloxidase activity and enabled greater shrimps resistance to the challenge with the white spot virus and *Vibrio algynoliticus* (Yeh et al., 2010; Yeh & Chen, 2009; Zahra et al., 2017). The sea alga extract (*Sargassum siliquosum*, *Undaria pinnatifida* and *Sargassum filipendula*, *Ulva*) included in the diet increased the phenoloxidase activity (Declarador et al., 2014; Schleder et al., 2017; Yudiati et al., 2016).

Probiotic stimulation was higher, both in the expression of the proenzyme and the enzyme; perhaps because bacteria have different molecules that stimulate phenoloxidase as a peptidoglycan, the lipid membrane, etc. (Vaseeharan et al., 2016). However, the polysaccharides have a single way of stimulation of the cascade (Chen et al., 2016), though it may be effective depending on the source of polysaccharides (Phupet et al., 2018; Solidum et al., 2016; Yeh & Chen, 2008). Recently, the use of short-chain fructooligosaccharides (FOS) grew due to its prebiotic properties in shrimps. However, there are no analysis in terms of stimulating the enzymatic activity through the diet (Hu et al., 2019). Another carbohydrate (trehalose) increased body weight and stimulated the phenoloxidase activity (Zhu et al., 2018).

Interestingly, combined probiotic and prebiotic use was lower than the separate groups. Perhaps, the combination causes malfunctioning of the prophenoloxidase system. Nevertheless, several authors point out that the use of the combination as a symbiosis (probiotics and prebiotics) could help reduce the impact of nutrient consumption by the probiotics in shrimp intestines (Huynh et al., 2018), and favor the colonization of the intestine by *Lactobacillus*, and a reduction in the content of *Vibrio* species (Huynh et al., 2019). Notwithstanding, the highest impact of the symbiotics refers to the protection of the host against infectious agents through the stimulation of melanization of hemocytes (Huynh et al., 2019).

The plant extracts, especially sea algae, produce higher stimulation. The administration of algae based on the diet cause an increment of non-specific defense mechanisms, and a reduction of the WSSV in shrimps (Niu et al., 2018). Accordingly, trypsin has a positive effect on the enzymatic activity, which may be caused by the fact that trypsin is a peptidase enzyme that breaks the peptide bonds of proteins by hydrolysis to form smaller peptides and amino acids. It has an essential role in digestion, since it is necessary for the uptake of proteins present in the shrimp diet (Omont et al., 2018).

The inclusion of *Yucca schidigera* in the diet stimulates growth and phenoloxidase activity (Yang et al., 2015). High percentages of *Echinacea purpurea* and *Uncaria tomentosa* inclusion stimulated phenoloxidase activity (Medina-Beltrán et al., 2012). Medicinal plant *Solanum nigrum* stimulated the immune system of *Penaeus monodon*, which helped in withstanding the challenge of *Vibrio harveyi* (Harikrishnan et al., 2011).

The addition of organic acids in shrimp diet supplementation is another trend (Romano et al., 2015); however, the results did not show higher phenoloxidase activity in this group. Organic acids are known to not only improve growth speed and the establishment of microbiota, but also produces a higher immune response, with ensuing resistance to pathogenic infection resistance (He et al., 2017; Pourmozaffar et al., 2017). Moreover, the combination using probiotics may have a synergic effect on the inhibition of pathogenic strains (Bolivar et al., 2018).

The principal objective associated with the plant extracts was to diminish the possible pathogen burden (Dineshkumar et al., 2017). Nonetheless, other functions, including immunostimulation and even the reduction of the viral burden, are described (Palanikumar et al., 2018; Wu et al., 2015). Phenoloxidase stimulation was detected in *Penaeus monodon* (Yogeeswaran et al., 2012), *Litopenaeus vannamei* (Hsieh et al., 2008; Yeh et al., 2009) and *Fenneropenaeus chinensis* (Huang et al., 2006).

In the search for probiotics for shrimps, the first trend was to use the genera that had already worked out in ground species (De et al., 2018; Li et al., 2008; Liu et al., 2014; Mingmongkolchai & Panbangred, 2018). Genus *Bacillus* underwent greater phenoloxidase activity stimulation (Fig. 6 A). The combination of strains has been described as permitting greater stimulation of the immune system (Sapcharoen & Rengpipat, 2013; Wang & Gu, 2010; Zhang et al., 2011). Although several isolates refer to *Bacillus cereus*, known for a possible toxicity to humans, no reports have been made on toxicity in Penaeid shrimps (Bernal et al., 2017; Chandran et al., 2014; Chandran et al., 2017).

The utilization of *Bacillus* sp. is known to prevent diseases, and cause protection by cellular and humoral activation of the immune defense of shrimps (Fu et al., 2010; Pham et al., 2017; Tseng et al., 2009). Then are the *Lactobacillus* sp., with a favorable enzymatic stimulation, as probiotics are effective stimulants in aquaculture (Laranja et al., 2017). However, the results of survival, growth, and conversion factors indicate that the combination of different strains is better than the inclusion of single species probiotics (Toledo et al., 2019).

Penaeus monodon was the species with the highest enzymatic stimulation, perhaps due to its high growth speed (Li et al., 2016), which requires the development of a quick immunological response that allows them to repel the attack of pathogens. *L. schmitti* is one of the least domesticated species, though only one experiment that met the selection criteria was included, which may constitute a bias in the interpretation of stimulation in different species (Rodríguez-Ramos et al., 2008).

The ontogeny of shrimps states that the phenoloxidase behavior varies depending on the stages (Martín et al., 2012). However, few studies are done on the larval stages of shrimp (Franco *et al*., 2016; Franco et al., 2017), in which stimulation was detected using immunostimulants.

As to the adults, phenoloxidase activity can be detected both in the plasm and the hemocytes. The pro-phenoloxidase system generally activates by signals given by pathogens or foreign agents, and permits hemocytes to perform melanization (Tassanakajon et al., 2018). The activation process might take place in several tissues simultaneously, so a higher activity can be detected in the postlarvae (Noothuan et al., 2017). The largest source of prophenoloxidase is the hemocytes, and it has been described to be involved in the inflammatory response (Manilal et al., 2009). Both probiotics and prebiotics can stimulate the activation and release of prophenoloxidase in the hemocytes (Huynh et al., 2018).

The positive results of the enzymatic activity in the plastic tanks coincide with observations related to survival in Penaeus shrimps, while the probiotic impact in the ground ponds is lower (Toledo et al., 2019). These results could make the pond microbiota harder to control. Layers of microorganisms or aggregates could form in the ground ponds, which could also stimulate the immune system. This strategy is also used in intensive culture today to stimulate the immune system and enhance shrimp survival (Panigrahi et al., 2019).

Gene transference is a powerful tool to promote desired traits in Penaeid shrimps (Arenal et al., 2008). In *Penaeus monodon*, viral gene transfer stimulated phenoloxidase activity (Parenrengi et al., 2014).

Conclusions

The scientific literature associated with Penaeus shrimp phenoloxidase activity recommends a careful evaluation of the experimental design and the way in which the results are expressed. The supplementation using prebiotics and probiotics in Penaeid shrimps stimulates both the phenoloxidase enzymatic activity and gene expression, with a higher sensitivity at the expression level, which suggests post-transcriptional processes that determine the difference. The assays need a validation in ground ponds, as the conclusions are based on experiments that did not include the ecosystem microbiota of these containers.

Author contribution statement

Yailén de la Caridad Valdes Vaillant: research planning, data collection, size effect calculation, analysis of the results, redaction of the manuscript, proof-reading and definitive approval of the manuscript.

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Peter Bossier: research planning, analysis of the results, statistical processing, redaction of the manuscript, review-planning of the research, results collection, analysis of the results, proof-reading and definitive approval of the manuscript.

Conflicts of interests

Not declared.

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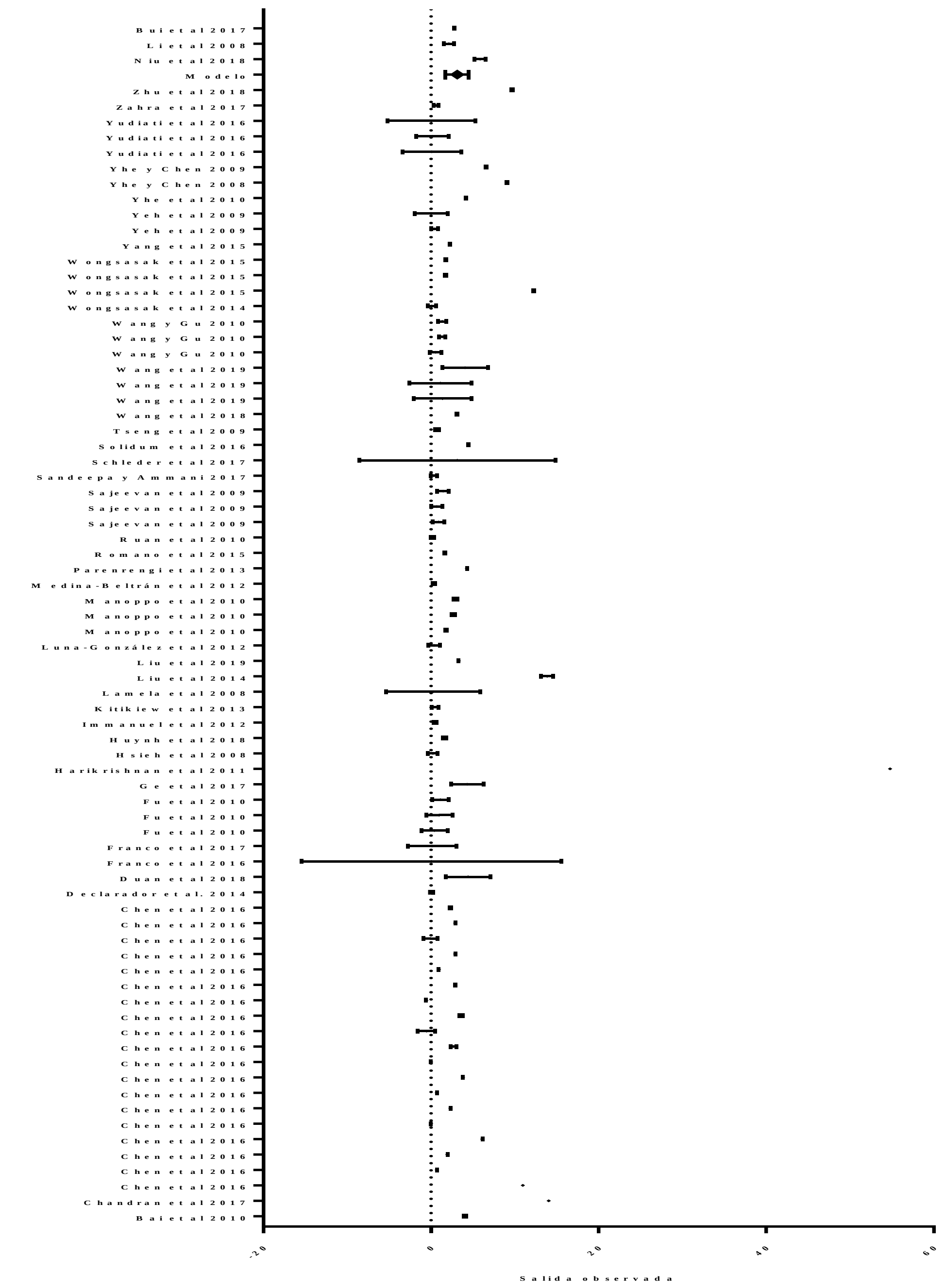

Fig. 2 Forest plot of the 77 experiments included to study the effect of prebiotic and probiotic supplementation on the phenoloxidase activity in Penaeid shrimps. The dots represent the estimated effect (difference among the standardized means), while the horizontal lines show the confidence index (95%).