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Research Article

Effects of bacterial probiotics interventions on acute hepatopancreatic necrosis disease (AHPND) in shrimp: A meta-analysis

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Abstract

Acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio* spp., is a new farmed penaeid shrimp bacterial disease. Several strains of *Vibrio parahaemolyticus* were identified as the etiological agent of AHPND. Probiotics are low-cost, non-pathogenic, and largely non-toxic source that have antibacterial functions and applications. According to the outbreak of AHPND in the south of Iran, it is necessary to conduct a meta-analysis to determine the effect of the bacterial strains in different studies on AHPND. The present meta-analysis was conducted to summarize the current evidence on the effects of probiotics on AHPND under laboratory conditions. The objectives of this meta-analysis were to quantitatively review the responses of shrimp to probiotic interventions to determine the effect of different treatment on reducing mortality during the outbreak of AHPND and evaluating the specific growth rate (SGR) and feed conversion ratio (FCR). According to the results, probiotic administration via water & feed and, via water more than spray on, or mix to feed, have been affected on survival rate (SR) to prevention of AHPND, and mono-strain probiotics were better than multi-strain probiotic in order to decrease mortality. To study design to evaluate the effects of probiotic on SR, SGR and FCR, longer experiments (60 days) are better, for evaluating the effect of the probiotics, and mono-strain probiotics increased SR more than multi-strain probiotics, after challenge with *V. parahaemolyticus*. gram positive and spore-forming bacteria showed greater improvement in SGR and FCR, but greater improvement in SR were observed in gram positive and non-spore forming bacteria.

Keywords: Shrimp, Probiotic, Acute Hepatopancreatic Necrosis Disease, Early mortality syndrome, Meta-analysis

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Introduction

Shrimp, as a high-protein animal food commodity, are one of the fastest growing food producing sectors in the world. Shrimp production mainly consists of three species, i.e., *Litopenaeus vannamei*, *Penaeus monodon* and *Macrobrachium rosenbergii*. Countries in East and Southeast Asia and Latin America account by far for the major share shrimp production, but a large proportion of consumption takes place in the developed countries. Among crustaceans, the white leg shrimp (*L. vannamei*) was reported to have the highest unit value at USD 26.7 billion (Tacon, 2020). Total imports in 2022 were 3,248,338 tons, with additional production in China estimated at 1,487,501 tons (Villarreal, 2023). China and Vietnam in Asia (945,791 tons) and the US (837,622 tons) absorbed most of the growth in shrimp production. Ecuador has seen a compound annual growth rate (CAGR) of 17% from 2012 to 2019 and a very significant CAGR of 25% from 2020 to Q2 2023. According to statistics provided by the Fisheries Organization of Iran (IFO), exported $600 million worth of fishery products in the previous Iranian calendar year 2022.

However, disease outbreaks, which are considered as the primary cause of production loss in shrimp farming, have moved to the forefront in recent years and brought socio-economic and environmental unsustainability to the shrimp aquaculture industry. Acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio* spp., is a relatively new farmed penaeid shrimp bacterial disease. The shrimp production in AHPND affected regions has dropped to 60%, and the disease has caused a global loss of USD 43 billion to the shrimp farming industry. The conventional approaches, such as antibiotics and disinfectants, often applied for the mitigation or cure of AHPND, have had limited success (Kumar *et al.*, 2021).

Antibiotic usage has been associated with alteration of host gut microbiota and immunity and development of antibiotic resistance in bacterial pathogens. For example, the Mexico AHPND-causing *V. parahaemolyticus* strain (13-306D/4 and 13-511/A1) were reported to carry the tetB gene coding for tetracycline resistance gene, and *V. campbellii* from China was found to carry multiple antibiotic resistance genes. Substantial concerns are that antibiotic resistance genes can move quickly throughout different environmental bacterial populations and are acquired by either chromosomal mutation or acquisition of plasmids (Kumar *et al.*, 2016). Potential benefits of probiotics to shrimp aquaculture are increased growth performance, improved water quality, pathogen inhibition, increased survival, improved immune responses, and improved digestibility of nutrients (Scholz *et al.*, 1999; Mujeeb Rahiman *et al.*, 2010; Chandran *et al.*, 2017).

Several strains of *V. parahaemolyticus* were also identified as causative agents of the newly emergent

acute hepatopancreatic necrosis disease (AHPND) in shrimp (Tran *et al.*, 2013). after it was first identified in *V. parahaemolyticus*, the pVA1 plasmid was found in various other species that were also shown to cause AHPND, including *Vibrio owensii*, *Vibrio campbelli* and *Vibrio harveyi* (Kumar *et al.*, 2021).

Quorum sensing (QS) system was described in *V. parahaemolyticus* that may play an important role in pathogenesis in AHPND (Lin *et al.*, 2022). QS is a cell density-dependent process that regulates the expression of a number of genes in both gram positive and gram negative bacteria. QS-regulated genes are involved in many important physiological activities, such as biofilm formation, bioluminescence, virulence factor production, conjugation, plasmid transfer, antibiotic production, cell mobility, and sporulation (Miller and Bassler, 2001). The importance of the QS system in AHPND pathogenicity was also recently demonstrated (Paopradit *et al.*, 2021). Lin *et al.* (2022) show how $LuxO^{vp}$, which is an important regulator of QS in *Vibrio* spp., affects the gene expression of the key AHPND pathogenic factors $pirA^{vp}$ and $pirB^{vp}$. At low cell density, the expression of $AphB^{vp}$ was increased by 1.7-fold in $LuxO^{vp}$-deleted *V. parahaemolyticus*, and this increase was positively correlated to the gene/protein expression of $PirA^{vp}$ and $PirB^{vp}$ under the same conditions (Lin *et al.*, 2022).

Then QS is the phenomenon by which microorganisms regulate their bacterial community behavior through sending and receiving chemical signals named also “autoinducers” Quorum quenching (QQ) is, however, defined as the inhibition mechanism of quorum-sensing process. Quorum-sensing autoinducers are, therefore, interrupted leading to an interference with the quorum-sensing process (Dong *et al.*, 2007). Quorum-sensing inhibition or QQ can be achieved by an enzymatic degradation of the autoinducer compound also by the blockage of autoinducers production or reception through the addition of some compounds named as inhibitors, that can mimic them (Defoirdt *et al*., 2004; Adonizio *et al*., 2006; Czajkowski and Jafra, 2009; Hong *et al.*, 2012).

Probiotics are low-cost, non-pathogenic, and largely non-toxic source of antibiotics and are able to synthesize various metabolites that have antibacterial functions and applications. Research on probiotic use has primarily been focused on increasing *L. vannamei* aquaculture production. Bacterial species, such as *Bacillus*, *Lactobacillus* or *Nitrobacter*, can be administered orally, by injection, or as a supplement in aquaculture water. Probiotics help to improve survival rate, water quality, immunity, and disease resistance through space competition with disease-causing bacteria, such as *Vibrio* spp. Probiotic bacteria suppresses the growth and presence of pathogenic bacteria, which lowers disease susceptibility (Amiin *et al.*, 2023).

Although there is a substantial body of research on the topic of probiotic use in shrimp, experiments have been

conducted using a range of different experimental conditions i.e., life stages, species of shrimp, strain of probiotic, dose, duration, and delivery route. These variables impact the magnitude of effects observed. Although a meta-analysis of the use of probiotics in healthy penaeid shrimp showed improvements between 3 to 4 standardized mean difference (SMD) for survival rate (SR), specific growth rate (SGR) and feed conversion ratio (FCR) (Toledo *et al.*, 2019), but considering the outbreak of AHPND in the south of Iran, it is necessary to conduct a meta-analysis to determine the effect of the bacterial strains in different studies with the aim of reducing mortality during the outbreak of AHPND and evaluating the level of SGR and FCR.

Overall, given the conflicting results on the effect of bacterial probiotics on reducing the survival rate in AHPND outbreak conditions, there is a need for a meta-analysis summarizing all available results in this area. Therefore, the present meta-analysis was conducted to summarize the current evidence on the effects of bacterial probiotics on AHPND under laboratory conditions. The objectives of this study were to quantitatively review the responses of shrimp to probiotic interventions to determine the effect of different bacterial strains of probiotic, dose, duration, and delivery route in studies with the aim of reducing mortality during the outbreak of AHPND and evaluating the SGR and FCR. To identify sources of residual variation of results (covariables) using meta-regression methods. Improvements in production efficiency and survival would likely provide economic benefits.

Materials and methods

Search strategy

A comprehensive literature search of English language literature published up to the 4th of October 2023 was conducted to identify research experiments involving treatments designed to evaluate the effects of probiotics on shrimp production and survival measures. Three search engines, ScienceDirect (sciencedirect.com), Google Scholar (scholar.google.com), and PubMed (pubmed.ncbi.nlm.nih.gov/advanced), were utilized between the 24th of September and 4th of October 2023 with a defined and repeatable search strategy using the terms: shrimp OR prawn OR *Penaeus vannamei* OR *Litopenaeus vannamei* OR *Penaeus monodon* Acute Hepatopancreatic Necrosis Disease OR Early Mortality Syndrome OR *parahaemolyticus* OR *owensii* AND probiotic.

Inclusion and exclusion criteria

All published studies were screened using standardized criteria. Primary screening was based on title and abstract. Full texts of articles were downloaded for secondary screening. For inclusion into the meta-analysis, studies needed to have the following: be peer reviewed, written in English, use probiotics in a

randomized and replicated experiment in which a reference group was present, measure one or more relevant outcomes (survival rate, specific growth rate, and feed conversion ratio), include sufficient data to determine the effect size (ES), include a measure of effect amenable to ES analysis for continuous data; e.g., standardized mean difference SMD, and include a measure of variance (SE or SD) for each effect estimate or treatment and control comparisons.

To increase the accuracy of the interpretation of responses, treatments with yeasts and fungi, bacteriophages, microalgae, and experiments where probiotics were administered through live food, and so prebiotics and synbiotics, were excluded from evaluation. Also, trials without a measure of variance (SE or SD) for each effect estimate or treatment and control, were excluded.

Data extraction

The following experimental details were organized into an Excel spreadsheet (Microsoft Office LTSC Professional plus 2021): Authors name, year, country and region the experiments were conducted, details of the probiotics and strain, concentrations delivered, mode of probiotic delivery, length of experiment, housing system, water quality measures (salinity, temperature and oxygen), density of shrimp in the housing system, whether a disease challenge was imposed, concentration, and manner of challenge to *V. parahaemolyticus*, length of challenge, genera and species of shrimp, number of shrimp per treatment, and number of shrimp per experimental unit. Response outcomes and their measures of variance (SD or SE) that were extracted included the SGR (%), FCR, post-challenge SR (%). If a study reported separate estimates of measures of variance (SE or SD) for each group, these were recorded as such. Many experiments reported a common SE or SD, these estimates were applied to both control and treatment groups:

SGR (%) = [(ln Final body weight (g) - ln Initial body weight (g)] / Experiment length (d) ×100
FCR = [total dry feed intake (g)] / [(final shrimp body weight (g) – initial shrimp body weight (g)]
SR (%) = [(Final number of shrimps (g) – Initial number of shrimp (g)] ×100

Risk of bias within individual studies and quality assessment

For assessing the risk of bias for each study included in the current meta-analysis the Cochrane quality assessment tool was applied (Higgins *et al.*, 2011). This assessment tool contained seven domains including random sequence generation, allocation concealment, reporting bias, performance bias, detection bias, attrition bias, and other sources of bias. Small sample size (<10) or short exposure time (<21 days), was considered as “other source of bias”. Each domain was given a “high risk”

score if the study comprised methodological defects that may have affected its findings, a "low risk" score if there was no defect for that domain, and an "unclear risk" score if the information was not sufficient to determine the impact. The overall risk of bias for an experiment was considered: (1) Low; if all domains had "low risk", (2) Moderate; if one or more domains had "unclear risk", and (3) High; if one or more domains had "high risk". The risk of bias assessment was done independently by two reviewers.

Statistical analysis

Data structure

The data were analyzed and reported as the following datasets on interventions with *Bacillus* spp. and *Clostridium* sp. (gram positive & spore-forming), *Lactobacillus* spp., *Brevibacterium* sp., *Bifidobacterium* sp., *Pediococcus* spp., *Enterococcus* spp., *Streptococcus* sp., and *Lactococcus* spp. (gram positive & non-spore-forming), and 'Other' probiotics (non-*Bacillus* spp. or non-*Lactobacillus* spp. or gram negative bacteria). Data were structured to allow a classical meta-analytical evaluation of differences in responses of the experimental groups. There is a hierarchical structure in these data as many experiments used for multiple treatment comparisons. Consequently, there is dependence within experiment and the effects of experiment and treatment comparison need to be evaluated by meta-regression using metareg and fracpoly (St-Pierre, 2001; Hedges *et al.*, 2010; Van den Noortgate *et al.*, 2013).

Model development

All statistics were performed using Stata (Version 14, StataCorp. LP, College Station, TX). Initial data exploration included production of basic statistics to examine the data for errors and to estimate the means and measures of dispersion. Normality of the data was examined for continuous variables, by visual and statistical appraisal. Classical meta-analysis was used to analyze responses by WMD and SMD. These methods have been published in detail in Golder and Lean (2016). Estimates were pooled using DerSimonian and Laird (D&L) random effects models (DerSimonian and Laird, 1986). For the SMD analysis, the difference between treatment and control groups means, which is termed 'treatment', was standardized using the SD of reference and treatment groups. For the WMD analysis the weighting reflected the inverse of the variance of the treatments included according to the no standard method in the metan package of Stata to allow an interpretation of treatment effects in familiar units, rather than the effect size (ES). Forest plots were produced for both WMD and SMD results for each outcome variable using D&L methods that incorporated the RR estimates (Van den Noortgate *et al.*, 2013).

Assessment of heterogeneity

Variations among the comparison level SMD were assessed using a chi-squared

(Q) test of heterogeneity. An α level of 0.10 was used because of the relatively poor power of the χ^2 test to detect heterogeneity among small numbers of trials (Egger and Smith, 2001). Heterogeneity of results among the comparisons was quantified using the I^2 statistic (Higgins *et al.*, 2011).

Meta-regression

Meta-regression analyses were used to explore the source(s) of heterogeneity of response, using the individual WMD for each comparison as the outcome and the associated SE as the measure of variance. The equations used in the meta-regression are published in Lean *et al*. (2018) using the methods of Tanner-Smith and Tipton (Tanner-Smith and Tipton, 2014).

Influence of each individual study and publication bias

This test is carried out to see whether the data that has been collected can be used as a representative sample of the population. The influence of each individual study on the overall meta-analysis was estimated by "metaninf". Presence of publication bias was investigated using WMD funnel plots, Begg's regression test and "metatrim" (Trim and Fill analysis). Data were screened for plausible quadratic relationships by visual appraisal. Possible outliers that were identified were not removed (Duval and Tweedie, 2000).

Results

Search results

Out of 738 publications that were identified in the initial search, 239 duplicate articles were excluded. After screening the remaining records, 90 unrelated articles and 336 records that did not meet our inclusion criteria, were also removed based on title and abstract assessment. A total of 73 non-duplicate full text articles were downloaded for secondary screening based on primary screening of their title and abstract. A total of 33 comparisons from 73 experiments (full text articles) were included for meta-analysis .Figure 1, shows a PRISMA flow diagram of the systematic review process, adapted from Moher *et al.* (2009). Results are presented for SR. Some experiments contributed comparisons for more than one probiotic intervention (i.e., for both *Lactobacillus* and *Bacillus* spp.).

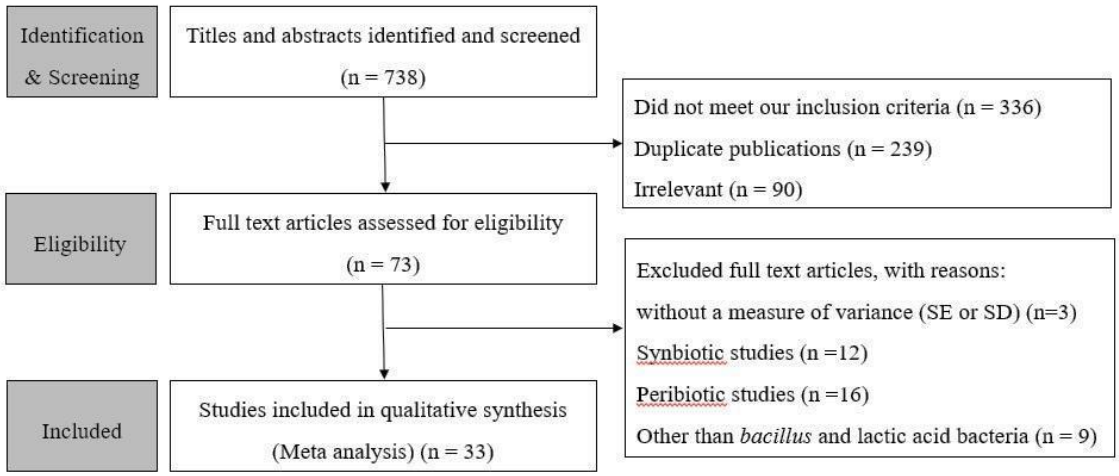

Figure 1*:* PRISMA flow diagram of the meta-analysis from initial search and screening to final selection of publications to be included in the meta-analysis on probiotic interventions on Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp.

Shrimp species studies were *L. vannamei* (31) and *P. monodon* (2). Only twelve studies included shrimp larvae. Twenty studies used mono-strain inoculum and the remaining 15 studies used multi-strain (Consortium) inoculum. *Bacillus* spp. and lactic acid bacteria (LABs) were most frequent strains used with 13 and 11 studies, respectively. Probiotics were administrated via feed (27), water (3) or both them (3). Twenty-two studies were conducted for > 20 days and 11 studies≤20 days. Eight studies used multi-strain inoculum whereas 25 used mono-strain inoculums. Three studies choose *Vibrio* sp. and 3 studies *Clostridium butyricum* as probiotic strain. Ten studies included≤100 shrimp/m^3, 12>100 to ≤200 shrimp/m^3, 9>200 shrimp/m^3 and 2 studies were unstated. Studies were carried out lasting <14 (19) or ≥14 (14) days. From 33 articles found to satisfy inclusion criteria, 96 experiments were identified in which a probiotic-treated and challenged to *V. parahaemolyticus* group was compared to a control untreated and challenged to *V. parahaemolyticus* group with survival as an outcome. The pooled WSD showed that probiotics increased survival in comparison to controls (WMD: 23.42, 95% CI 20.38 to 26.45) in the random effects model. Significant heterogeneity was observed across the 96 experiments (Q-statistic: p<0.001; I^2-statistic=998%).

In total, there were 97 comparisons from 33 studies included. Several *Bacillus* spp. were used as interventions; including *B. subtilis B. coagulans*, *B. cereus*, *B. licheniformis*, *B. altitudinis*, *B. velezensis*, *B. horikoshii*, *B. aerius*, *B. pumilus* or a mixture that contained *Bacillus* spp. or some species of lactic acid bacteria including *Brevibacterium casei*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus paracasei*, *Bifidobacterium longum*, *Pediococcus acidilactici*, *Enterococcus faecium*, *Lactobacillus fermentum*, *Pediococcus pentosaceus* and *Lactococcus garvieae*.

Several gram negatives were used as intervention; including *Vibrio alginolyticus, Vibrio campbellii*, *Roseobacter gallaeciensis*, *Pseudomonas aestumarina*, *Rhodobacter sphaeroides*, *Afifella marina*, *Pseudoalteromonas* sp. or *Shewanella algae*. The probiotic additions ranged from 1×10^2 to 1×10^9 CFU/g in feed and 1×10^3 to 1.65×10^9 CFU/mL in water. A total of 81.44% of the probiotic interventions were delivered in the feed, 61.50% in the water, and 2.06% in both. Comparisons were 90.73% from *L. vannamei* and 9.27% from *P. monodon*. Shrimp (% of comparisons) began the experiment with different development stages: post-larval (38.14%), juvenile (61.86%) and duration of challenge to *Vibrio parahaemolyticus* were between 0.01 to 30 days.

Findings from the meta-analysis of bacterial probiotics on SR %: Combining 33 effect sizes (all studies) indicated the administration of bacterial probiotics in interventions compared with controls, resulted in a significant increase SR% (WMD: 23.42, 95% CI: 20.38 – 26.45 %, $p<0.001$).

Findings from the meta-analysis of bacterial probiotics on SGR: Combining 15 effect sizes indicated administration of bacterial probiotics in interventions compared with controls, resulted in a significant increase SGR% (WMD: 0.39, 95% CI: 0.34 – 0.45 %, $p<0.001$) (Table 2).

Table 1: Summary of the meta-analysis using classical meta-analysis and subgroup methods for the effects of bacterial probiotics on survival rate (SR) (%). The Table provides the number (n) of experiments and comparisons for each evaluation, the weighted mean difference (WMD) using the DerSimonian and Laird (D&L) regression methods, and the P-value, estimated heterogeneity (I^2) and P-heterogeneity.

Effect of bacterial probiotic on SR (%)	Effect size, n	WMD (95% CI)	Heterogeneity (I^2 %)	*P*-heterogeneity	Weight
Overall	33	23.42 (20.38, 26.45)	99.8	<0.001	100
Probiotic strains					
G+ spore+	15	23.66 (16.94, 30.37)	99.7	<0.001	47.20
G+ spore-	12	29.78 (25.56, 34.00)	99.0	<0.001	34.15
G-	5	9.76 (5.30, 14.22)	99.0	<0.001	10.43
spore+ & spore-	3	8.49 (5.09, 11.89)	95.5	<0.001	7.19
G+ & G	1	43.06 (37.39, 48.73)	0.0	0.0	1.02
Probiotic include					
Mono-strain	25	25.18 (21.06, 29.30)	99.7	<0.001	84.68
Multi-strain	8	13.25 (10.37, 16.14)	99.4	<0.001	15.32
Method of addition					
Mix with feed	23	17.76 (14.32, 21.19)	99.8	<0.001	63.33
Spray on feed	5	30.47 (21.17, 39.77)	98.2	<0.001	16.35
Water	4	35.17 (29.99, 40.35)	96.7	<0.001	18.30
Feed & water	2	38.61 (29.15, 48.06)	74.2	<0.049	2.01
Challenge manner to *Vibrio parahaemolyticus*					
Immersion	17	24.00 (19.89, 28.10)	99.6	<0.001	53.54
Intramuscular	11	28.82 (20.65, 36.99)	99.7	<0.001	33.97
Other (oral or Reverse gavage)	5	5.37 (1.53, 9.21)	99.7	<0.001	12.49

Table 1 continued:

Effect of bacterial probiotic on SR (%)	Effect size, n	WMD (95% CI)	Heterogeneity (I^2 %)	*P*-heterogeneity	Weight
Duration of probiotic administration					
≤ 20 days	11	26.25 (21.89, 30.62)	99.0	<0.001	33.73
> 20 days	21	21.96 (18.59, 25.32)	99.8	<0.001	66.27
Challenge duration to *V. parahaemolyticus*					
< 14 days	19	24.30 (20.90, 27.69)	99.7	<0.001	62.52
≥ 14 days	13	21.93 (14.76, 29.10)	99.8	<0.001	37.48
Density (shrimp/m^3)					
≤100	11	26.88 (22.69, 31.08)	99.9	<0.001	35.53
<100 ≥200	12	13.06 (7.00, 19.13)	99.5	<0.001	37.20
≥1000	8	31.52 (24.36, 38.69)	98.5	<0.001	21.25
Not stated	2	38.32 (22.99, 53.65)	98.2	<0.001	6.02
Life stage of shrimps					
Juvenile	18	26.56 (21.41, 31.70)	99.7	<0.001	61.83
Post larva	13	18.17 (14.77, 21.56)	99.7	<0.001	38.17
The country where the study was conducted					
Asia	24	27.06 (23.29, 30.84)	99.8	<0.001	76.54
Other	7	11.21 (6.59, 15.83)	99.3	<0.001	23.46

Findings from the meta-analysis of bacterial probiotics on FCR: Combining 14 effect sizes indicated administration of bacterial probiotics in interventions compared with controls, resulted in a significant decrease FCR (weighted mean difference (WMD: -0.40, 95% CI: -0.46 – -0.34, p<0.001) (Table 3).

Table 2: Summary of the meta-analysis using classical meta-analysis and subgroup methods for the effects of bacterial probiotics on specific growth rate (SGR) (%). The Table provides the number (n) of experiments and comparisons for each evaluation, the weighted mean difference (WMD) using the DerSimonian and Laird (D&L) regression methods, and the P-value, estimated heterogeneity (I^2) and p-heterogeneity.

Effect of bacterial probiotic on SGR (%)	Effect size, n	WMD (95% CI)	Heterogeneity (I^2 %)	*P*-heterogeneity	Weight
Overall	15	0.39 (0.34, 0.45)	99.2	<0.001	100
Probiotic strains					
G+ spore+	11	0.38 (0.31, 0.44)	98.8	<0.001	69.49
G+ spore-	4	0.35 (0.23, 0.47)	99.2	<0.001	19.06
G-	2	0.57 (0.14, 0.99)	99.7	<0.001	11.45
Probiotic include					
Mono-strain	14	0.40 (0.34, 0.45)	99.2	<0.001	98.86
Multi-strain	1	0.24 (-0.25, 0.73)	0.0	0.982	1.14
Method of addition					
Mix with feed	12	0.47 (0.40, 0.54)	99.0	<0.001	62.53
Spray on feed	4	0.26 (0.16, 0.37)	99.4	<0.001	37.47
Challenge manner to *Vibrio parahaemolyticus*					
Immersion	4	0.36 (0.23, 0.48)	99.3	<0.001	35.12

Table 2 continued:

Effect of bacterial probiotic on SGR (%)	Effect size, n	WMD (95% CI)	Heterogeneity (I^2 %)	*P*-heterogeneity	Weight
Intramuscular	9	0.40 (0.34, 0.46)	99.1	<0.001	60.13
Other (oral or Reverse gavage)	2	0.64 (-0.24, 1.51)	99.7	<0.001	4.75
Duration of probiotic administration					
≤ 20 days	1	0.41 (0.32, 0.50)	98.0	<0.001	8.76
> 20 days	14	0.39 (0.34, 0.45)	99.0	<0.001	91.24
Challenge duration to *V. parahaemolyticus*					
< 14 days	6	0.26 (0.20, 0.32)	97.8	<0.001	34.11
≥ 14 days	10	0.45 (0.36, 0.53)	99.3	<0.001	65.89
Density (shrimp/m^3)					
≤100	7	0.33 (0.25, 0.40)	98.5	<0.001	44.92
<100 ≥200	6	0.44 (0.34, 0.53)	99.3	<0.001	44.46
≥1000	2	0.48 (0.39, 0.58)	97.7	<0.001	10.62
Life stage of shrimps					
Juvenile	10	0.43 (0.35, 0.52)	99.2	<0.001	67.87
Post larva	5	0.31 (0.23, 0.38)	98.9	<0.001	32.13
The country where the study was conducted					
Asia	13	0.37 (0.31, 0.44)	99.1	<0.001	82.85
Other	2	0.50 (0.33, 0.67)	99.6	<0.001	17.15

The results of the analysis showed that the 33 effect sizes of the analyzed studies related to SR (Q=3154.08; d.f. =96; p<0.001), SGR (Q=1045.71; d.f.=42; p<0.001) and FCR (Q=1164.41; d.f.=38; p<0.001), were heterogeneous. Thus, the Random Effect model was more suitable for estimating the mean effect size of the 33 analyzed studies. However, there was evidence of a moderate between-study heterogeneity in effects of bacterial probiotics on SR I^2=99.8, p<0.001), SGR (I^2=99.2, p<0.001) and FCR (I^2=99.8, p<0.001). To detect potential sources of heterogeneity, subgroup analyses were performed.

Table 3: Summary of the meta-analysis using classical meta-analysis and subgroup methods for the effects of bacterial probiotics on feed conversion ratio (FCR). The Table provides the number (n) of experiments and comparisons for each evaluation, the weighted mean difference (WMD) using the DerSimonian and Laird (D&L) regression methods, and the P-value, estimated heterogeneity (I^2) and p-heterogeneity.

Effect of bacterial probiotic on FCR	Effect size, n	WMD (95% CI)	Heterogeneity (I^2 %)	*P*-heterogeneity	Weight
Overall	14	-0.40 (-0.46, -0.34)	99.8	<0.001	100
Probiotic strains					
G+ spore+	10	-0.48 (-0.54, -0.42)	98.3	<0.001	69.76
G+ spore-	4	-0.24 (-0.29, -0.20)	98.4	<0.001	16.27
G-	1	-0.24 (-0.26, -0.21)	0.0	0.924	8.34
spore+ & spore-	1	-0.02 (-0.03, -0.02)	93.5	<0.001	5.63
Probiotic include					
Mono-strain	12	-0.43 (-0.48, -0.38)	99.2	<0.001	86.16
Multi-strain	2	-0.16 (-0.19, -0.13)	98.8	<0.001	13.84

Table 3 continued:

Effect of bacterial probiotic on FCR	Effect size, n	WMD (95% CI)	Heterogeneity (I^2 %)	*P*-heterogeneity	Weight
Method of addition					
Mix with feed	10	-0.35 (-0.42, -0.28)	99.8	<0.001	72.36
Spray on feed	4	-0.50 (-0.56, -0.44)	98.8	<0.001	27.64
Challenge manner to *Vibrio parahaemolyticus*					
Immersion	4	-0.42 (-0.53, -0.32)	97.8	<0.001	37.81
Intramuscular	7	-0.43 (-0.49, -0.37)	99.5	<0.001	51.85
Other (oral or Reverse gavage)	3	-0.10 (-0.13, -0.06)	98.7	<0.001	10.34
Duration of probiotic administration					
≤ 20 days	1	-0.25 (-0.31, -0.19)	99.3	<0.001	8.44
> 20 days	13	-0.41 (-0.48, -0.35)	99.8	<0.001	91.56
Challenge duration to *V. parahaemolyticus*					
< 14 days	5	-0.28 (-0.36, -0.21)	99.7	<0.001	27.46
≥ 14 days	10	-0.44 (-0.49, -0.38)	99.3	<0.001	72.54
Density (shrimp/m^3)					
≤100	8	-0.33 (-0.40, -0.26)	99.8	<0.001	58.50
<100 ≥200	3	-0.57 (-0.66, -0.48)	94.6	<0.001	31.13
≥1000	2	-0.25 (-0.31, -0.20)	99.0	<0.001	10.36
Life stage of shrimps					
Juvenile	8	-0.45 (-0.54, -0.36)	97.0	<0.001	56.20
Post larva	6	-0.33 (-0.41, -0.24)	99.9	<0.001	43.80
The country where the study was conducted					
Asia	13	-0.42 (-0.48, -0.36)	99.8	<0.001	88.88
Other	1	-0.23 (-0.25, -0.21)	0.0	p = 0.412	11.12

The between-study heterogeneity was explained by the probiotic strains, Simultaneous use of one or more bacterial strains (mono-strain or multi-strain), Method of addition and time of exposure to probiotics, density of shrimp in intervention condition, dose of *V. parahaemolyticus* and duration challenge, life stage of shrimps and challenge manner *to V. parahaemolyticus*. From these analyses, a significant increasing effect of bacterial probiotics on SR and SGR with exposure to gram positive (with or without spore), duration of exposure to probiotics >20 days were found.

Adding bacterial probiotics to water have been shown to be more effective in increasing SR more than the mix to the feed or spraying on the feed. dding probiotics to the feed as mixed, have been reducing effects on FCR, more than spraying to the feed (Table 3). Furthermore, *Bacillus* spp. as spore-forming gram positive bacteria, have been shown to be more effective to increase SGR (%) and decrease FCR than LABs (gram positive non-spore-forming bacteria); but gram positive and non-spore-forming probiotics, showed greater improvement in SR (%).

From these analyses, a significant reducing effect of bacterial probiotics on FCR with exposure to gram positive (with or without spore) and gram

negative bacteria, duration of exposure to probiotics >20 days were found.

Sensitivity analysis and publication bias Test

In the sensitivity analysis, exclusion of any single study did not affect the overall estimate for the effect of Bacterial probiotics on SR (range of summary estimates: 19.81, 2740), SGR (range of summary estimates: 0.33, 0.46) or FCR (range of summary estimates: -0.48, -0.33).

Funnel plots showed that the spread of comparisons was predominately on the positive side of the plot for SR and SGR and so, on the negative side of the funnel plot for FCR. Based on the visual inspection of funnel plot, an asymmetry was found in the SR and FCR; when the Begg and Egger's regression tests, for SR (Begg's test: p=0.011 and Egger's test: $p\leq$0.001)(Figure 4A) and FCR (Begg's test: p=0.001 and Egger's test: $p\leq$0.001) (Figure 4C), significant publication bias were confirmed. But SGR funnel plot, as shown in (Figure 4B), is symmetrical (Begg's test: p=0.730 and Egger's test: p=0.040). The propensity of outcomes to have comparisons spread predominately on the positive effect side of the funnel plots may indicate publication bias toward experiments with favorable outcomes, or a consistently positive result and is consistent with the findings of Toledo *et al*. (2019).

The 33 eligible records on effects of bacterial probiotics on SR were included in the non-linear dose-response meta-analysis. Although not significant, there was a nearly Trumpet-shaped curve of the effect of challenge duration of bacterial probiotics on SR in which the increasing effect of probiotic gradually increased, and then, the effect gradually decreased in duration more than 70-day (*P* non-linearity=0.060) (Figure 5. 5A). The 15 eligible records on effects of bacterial probiotics on SGR were included in the non-linear dose-response meta-analysis.

Although not significant, there was a nearly Trumpet-shaped curve of the effect of challenge duration of bacterial probiotics on SGR in which the increasing effect of probiotic gradually increased, and then, the effect gradually decreased in duration near to 60-day (*P* non-linearity=0.038) (Figure 5B). The 14 eligible records on effects of bacterial probiotics on FCR were included in the non-linear dose-response meta-analysis. There was a nearly overturned U-shaped curve of the effect of challenge duration of bacterial probiotics on FCR in which the increasing effect of probiotic gradually increased, and then, the effect decreased with a steep slope in duration more than 60 days (*P* non-linearity=0.007) (Figure 5C).

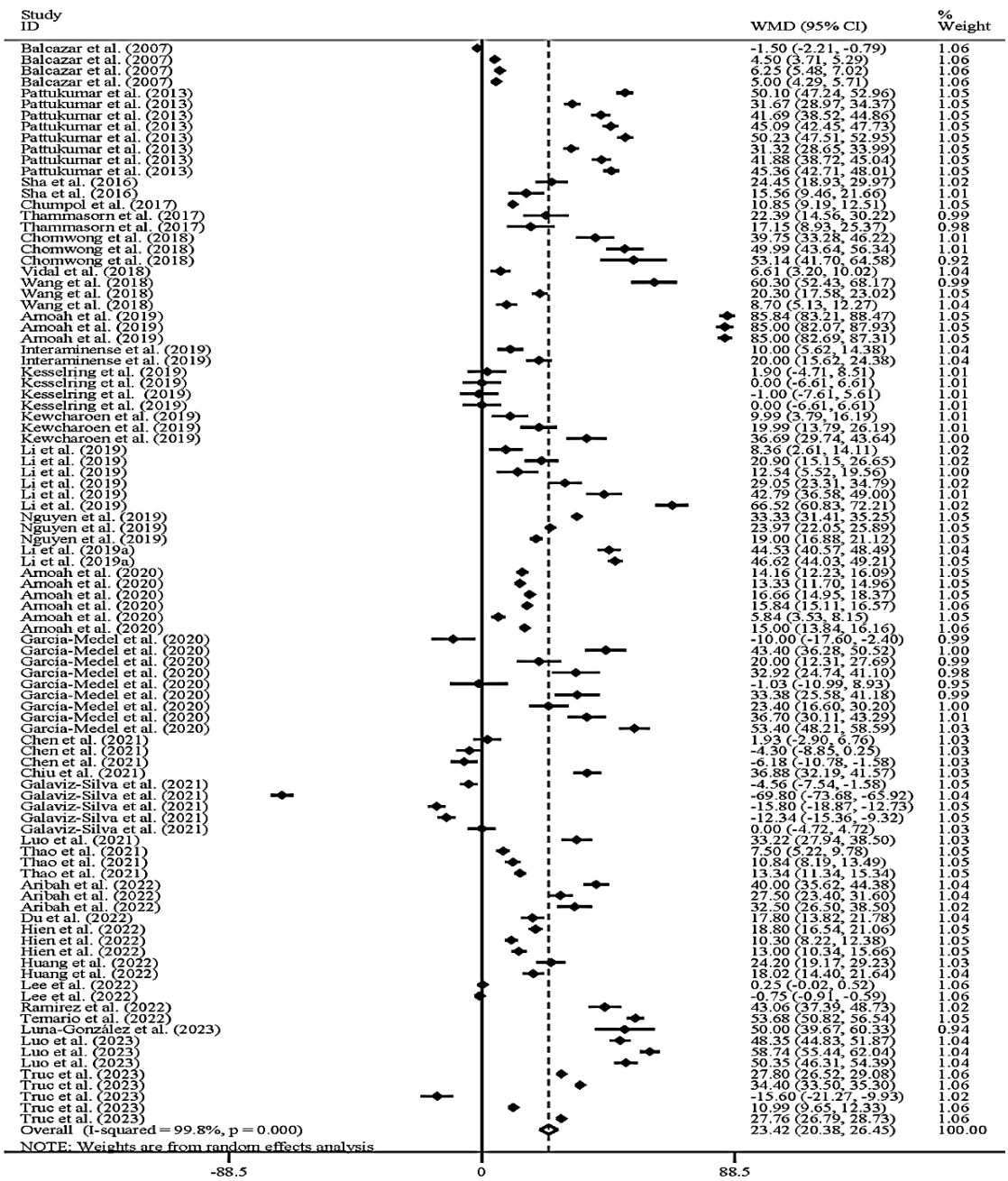

Figure 2: Forest plot of 96 randomized, controlled experiments to study the effect of bacterial probiotic on survival of penaeid shrimps challenged to *Vibrio parahaemolyticus* (weighted mean difference, WMD). Horizontal line in each point represents the 95%CI. Discontinuous line indicates the global effect.

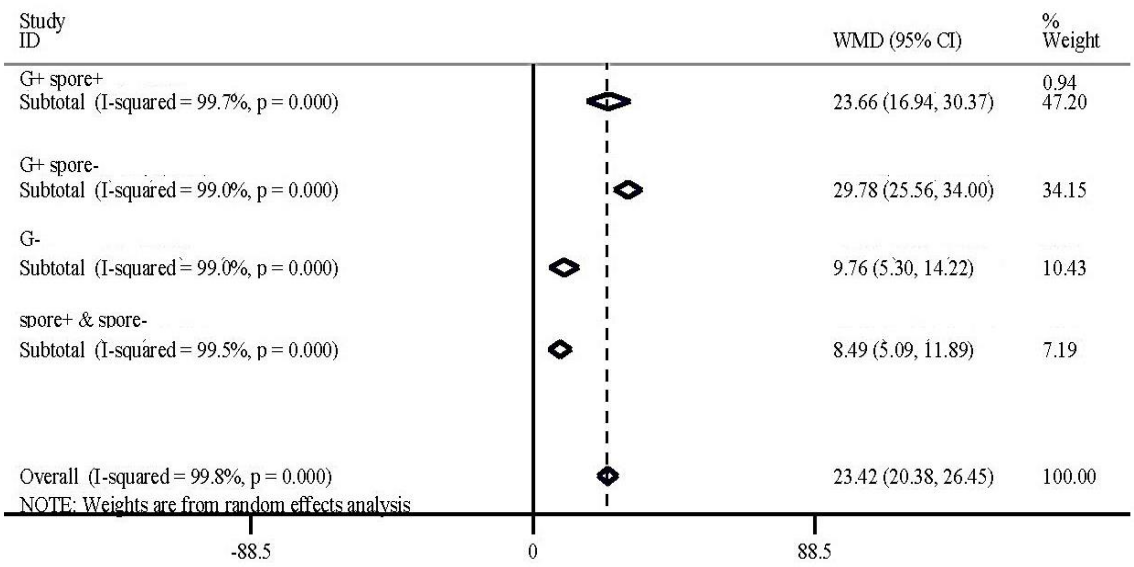

Figure 3: Forest plot of effect of probiotic strain on SR%. G+, gram positive; G-, gram negative; spore+, bacteria with spore-forming; spore-, bacteria without spore-forming.

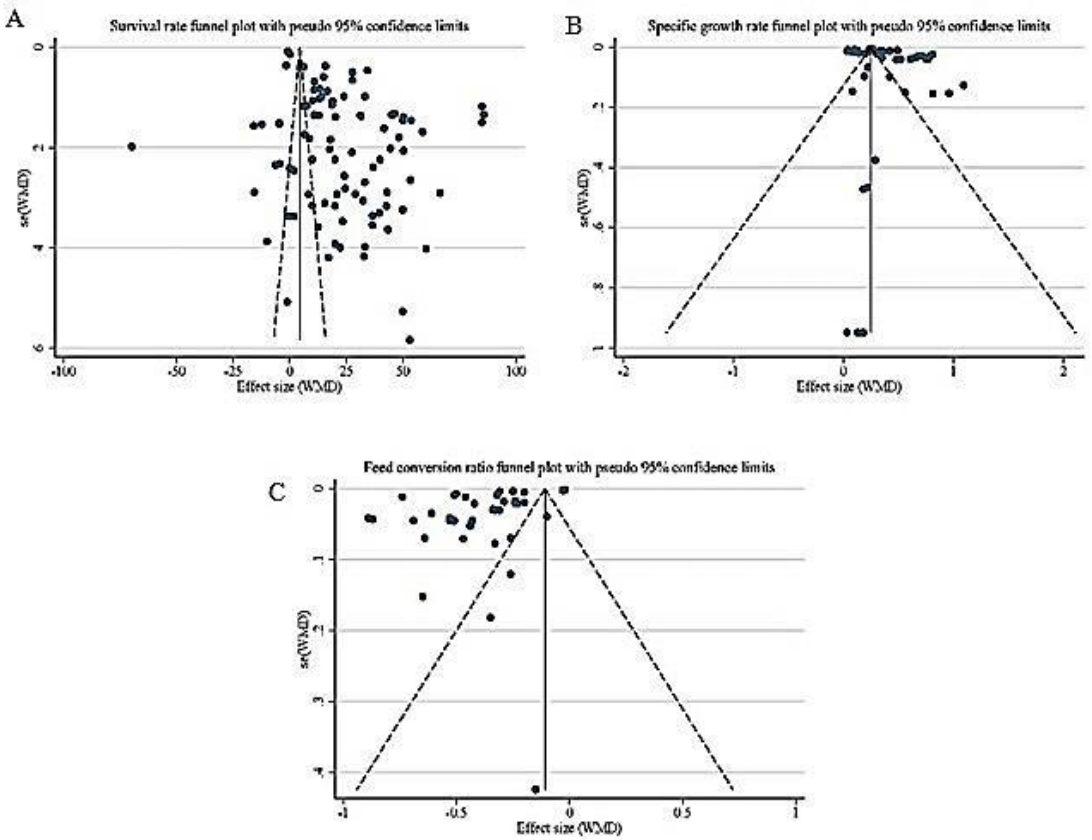

Figure 4: Funnel plot for (A) effects of bacterial probiotics on the survival rate (n of comparions=96; n of experiments=33), (B) specific growth rate (n of comparions=42; n of experiments=15) and (C) feed conversion ratio (n of comparions=38; n of experiments=14) of shrimp. The grey broken lines represent the 95% CI for treatment comparisons.

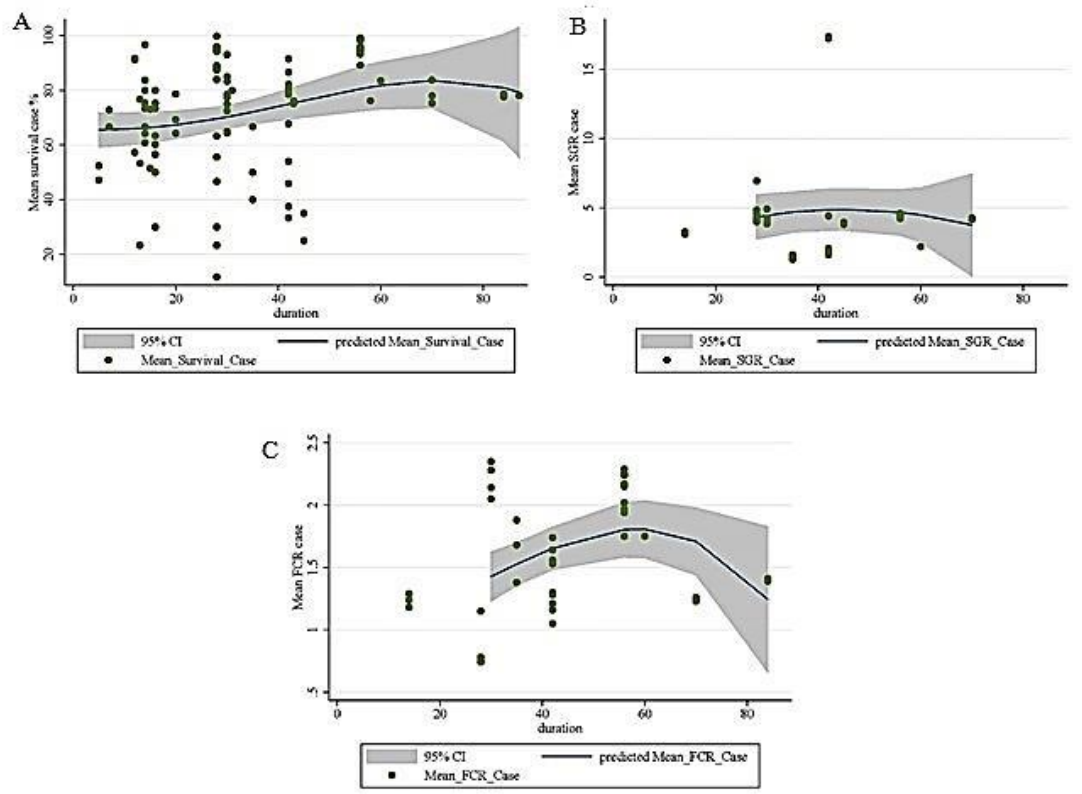

Figure 5: Non-linear dose-response effects of challenge duration of bacterial probiotics on SR (A) effect of bacterial probiotics dosage in feed on SGR (B) and challenge duration of bacterial probiotics on FCR (C); The 95% CI is demonstrated in the shaded regions.

The 33 eligible records on effects of bacterial probiotics on SR were included in the non-linear dose-response meta-analysis. There was a ribbon-shaped curve of the effect of dose of probiotic in feed on SR after challenge to *V. parahaemolyticus*, in which the almost constant effect (*P* non-linearity≤0.001) (Figure 6A); but, although not significant, there was an increasing effect of dose probiotics in water on SR after challenge, in which gradually increased (*P* non-linearity=1.79) (Figure 6B).

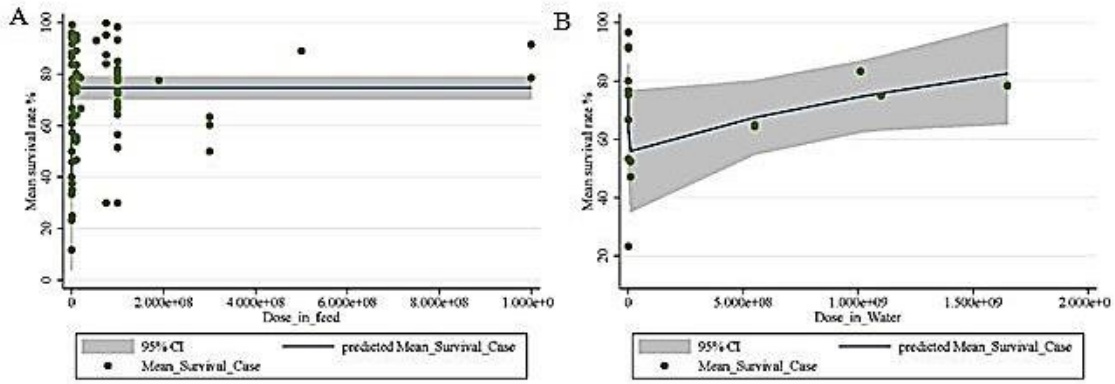

Figure 6: Non-linear dose-response effects of administering probiotics via feed (A) and via water (B) on SR%. The 95% CI is demonstrated in the shaded regions.

Discussion

Meta-regression analysis showed that the probiotic effect on survival rate was dependent on the life stage of cultured shrimps (juvenile or post larvae). Maybe due to the function of immune system in juvenile shrimps (26.56%) compared to post larvae (18.17%). It may be envisaged that probiotics exert a better effect when a stable gut microflora has been established. Initial colonization by probiotic organisms can modulate the expression of genes in epithelial cells, thus creating a favorable environmental interface for the host (Thomas and Versalovic, 2010).

Gram positive bacteria belonging to the LABs, and *Bacillus* genus, are among the microorganisms most frequently used as probiotics. Gram positive and spore forming bacteria (*Bacillus* spp. and *Clostridium* spp.) showed greater improvement in SGR (0.38%) and FCR (-0.48) of treated shrimps in comparison to SGR (0.35%) and FCR (-0.24) in gram positive and non-spore forming (*Lactobacillus* spp., *Brevibacterium* sp., *Bifidobacterium* sp., *Pediococcus* spp., *Enterococcus* spp., *Streptococcus* sp., and *Lactococcus* spp.) bacteria; but greater improvement in SR (29.78%) were observed in gram positive and non-spore forming bacteria in comparison to gram positive and spore forming bacteria (23.66%).

Bacillus has been reported as a very versatile genus in relation to the number of mechanisms available for exerting probiotic action, including: antagonistic activity, gut colonization, digestive enzyme secretion, organic waste removal and production of many supplemental nutrients such as biotin, vitamin B_{12}, fatty acids, essential amino acids and other necessary growth factors (Verschuere, 2000; Xue *et al*., 2016; Mirbakhsh *et al*., 2022; Mirbakhsh *et al*., 2023); but in the current meta-analysis gram positive and non-spore forming bacteria, have more effect on increase SR in shrimps challenged to *V. parahaemolyticus*.

Lactic acid bacteria (LABs) are probiotics for human and animals, and play a vital role in stimulating digestion and preventing harmful bacteria (Balcázar *et al.*, 2007). Presently, LABs are being selected to supplement in aquaculture feed because of its benefits such as removing pathogens (Vine *et al.*, 2004; Balcázar *et al.*, 2006), providing nutrition and enzyme for digestion, enhancing the immune system of animals (Nguyen Thi Truc *et al.*, 2019), and QQ (Cui *et al.*, 2020; Dong *et al.*, 2020; Lv *et al.*, 2021). During the fermentation process, LABs can produce organic acid that can limit the growth of pathogenic bacteria through the effect of organic acid on the surface of the bacteria (Fooks *et al.*, 1999; Kuipers *et al.*, 2000), and so, can block QS-regulated virulence factors and biofilms formation (Cui *et al.*, 2020).

According to the results of most of studies conducted regarding the effect of probiotics on SR, SGR and FCR, it can be concluded that, administration via mix to, or spray on feed was better than via water for all the variables analyzed and addition of probiotics with feed could be more productive because the

probiotic strains can directly modulate digestion and nutrient absorption in the shrimp gut (Amiin *et al.*, 2023), but in the current meta-analysis, administration via water and feed (38.61%) and via water (35.17%) more than spray on (30.47%), or mix to (17.76%) feed have been affected on SR in shrimps challenged to *V. parahaemolyticus*. This difference can be due to QQ and QS inhibiting process. *Pseudomonas* spp., *Aeromonas* spp., and *Vibrio* spp. are some of the opportunistic pathogens affecting life in aquaculture and known well to act through their QS system; thus, the inhibition of this QS system is concerned as a new anti-infective approach in aquaculture (Defoirdt *et al.*, 2004; Tran *et al.*, 2013). QS disrupters in aquaculture are many; for instance, pathogenic effects of *Vibrio* isolates (*V. harveyi*, *V. campbellii*, and *V. parahaemolyticus*) in *Artemia franciscana* culture are inhibited by brominated furanone synthesized by the alga *Delisea pulchra* and block QQ. Quorum quenching is the inhibition of QS, using chemical or enzymatic means to counteract behaviors regulated by QS. The use of probiotic bacteria such as *Bacillus* spp. or LABs with a QQ strategy, is advantageous for the control of vibriosis. Quorum quenching is a new anti-infective way for a sustainable aquaculture reducing at the same time antibiotic use (Turan and Engin, 2018).
Another fact is that most of the commercial products available are powders, which increases shelf-life and facilitates simultaneous administration of probiotics with other products (Dash *et al.*, 2014). Also, spore-forming bacteria, as *bacillus* spp., provides higher stability through culture environments, compare to non-spore-forming bacteria, as LABs, which makes their use easier and their efficiency more (Nimrat *et al.*, 2011).

According to the fracpoly regression test, bacterial probiotic effect on FCR was impacted by the duration of the experiments as shown by a direct relationship between these variables in the meta-regression analysis. Besides, the low variation among the longer studies evidences that the longer the experimental period, the higher the chances of finding true probiotic effects on FCR. In contrast, shorter experiments showed greater effects on SR. Additionally, this meta-regression analysis revealed an inverse relation between these two variables. In fact, by definition, the relationship between SGR and FCR is proportionally inverse. Probiotic effects on SR were higher in short experiments.

The probiotic responses in shrimp were explored as it was hypothesized responses may have differed between probiotic agents: (1) *Bacilli* (gram positive and spore-forming), (2) *Lactobacilli* (gram positive & non-spore-forming), and (3) gram negative bacterial probiotics as they may have different modes of action. Our results are not consistent, with those reported in a meta-analysis of probiotic administration to healthy penaeid shrimp by Toledo *et al.* (2019). These differences may be due to a combination of health or AHPND condition, different

species or life stage of shrimp, use of different inclusion criteria, and methodology. Contrary to the findings of Toledoa *et al*. (2019), mono-strain probiotics (25.18%) increased SR more than multi-strain probiotics (13.25%) after challenge with *V. parahaemolyticus*.

The following guidelines for future studies were recommended: (1) there is a higher probability of finding beneficial effects on shrimp farm indicators by including probiotics in the water in order to increase the SR and prevention of AHPND outbreak; (2) Studies designed as experimental growth models can be useful to assess the effectiveness of a probiotic, (4) longer experiments (>60 days) are better than short experiments (<60 days), for evaluating the effect of the probiotics. A significant proportion of the evaluated articles lacked proper dispersion measures, or data dispersion was ambiguously stated. In spite of the large number of studies included, we found evidence for publication biases so these results should be interpreted with caution. However, this meta-analysis shows that both the data quality and the approach used were relevant.

Probiotic supplementation improves survival rate (SR), specific growth rate (SGR) and feed conversion ratio (FCR) of Penaeid shrimps. The consistency of a positive direction of effect among our bacterial probiotics for SR after challenge to *V. parahaemolyticus*, SGR and negative direction for FCR, supports the use of probiotics in shrimp and are likely to lead to economic benefits. Probiotic administration via water & feed and via water more than spray on, or mix to feed, have been affected on SR to prevention of AHPND. Contrary to the findings of other studies in health conditions, mono-strain probiotics were better than multi-strain probiotic in order to decrease mortality in AHPND outbreak. To study design to evaluate the effects of bacterial probiotics on SR, SGR and FCR, longer experiments (60 days) are better, for evaluating the effect of the probiotics. The wide variety of experimental designs incorporated in this meta-analysis, is a source of heterogeneity, that affects the results and reduces the consistency of the findings. Nevertheless, this meta-analysis allowed to identify certain components of the experimental designs or applications that could affect the assessment of the effectiveness of probiotics in shrimp farming.

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