



Research article

Synergistic effect of durian fruit rind polysaccharide gel encapsulated prebiotic and probiotic dietary supplements on growth performance, immune-related gene expression, and disease resistance in Zebrafish (*Danio rerio*)



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ABSTRACT

The present study investigated the effect of polysaccharide gel (PG) extracted from the rind of durian fruit which is encapsulated with *Bacillus subtilis* as a feed and co-inoculation with *Artemia nauplii* in the induction of immune response in *Danio rerio* after *Vibrio* immersion challenge (5 days). The total RBC count, lysozyme activity test, weight, and length analysis revealed that the zebra fishes fed with the PG encapsulated probiotics had more immune induction activity, survival, and growth than the non-encapsulated groups. When the expression of the immune-related genes was measured, the studies revealed the significant upregulation ($P < 0.05$) of interleukin 1 beta (IL1b), lysozyme (lyz), tumor necrosis factor-alpha (TNF- α), superoxide dismutase (SOD) genes in fish fed with PG encapsulated probiotics with *A. nauplii* showed an immense effect on the induction of immune response compared to other feeds.

1. Introduction

Worldwide, the aquaculture domain experiences the subsequent loss of fish due to an infectious disease outbreak. The manifestation and spread of infectious diseases are accountable for major economic loss and are deliberated as the main obstacle for the sustainable progress of the aquaculture industry (Cerezuela et al., 2011). Moreover, the practice of antibiotics and chemotherapies has several uninvited side effects such as posing public health issues through food security problems or wildlife safety by their bioaccumulation in aquatic animals (Guardiola et al., 2016). Besides the facts, the excessive use of chemicals and antibiotics may lead to depression of the fish's immune system and also induce acquired drug resistance that may result in the emergence of antibiotic-resistant strains (Ashouri et al., 2018). There must be strict regulations to be followed in the administration of antibiotics for the treatment of aquatic diseases which could be the only possible prophylactics approach to reduce the ill effects of synthetic antibiotics

treatment. The zebrafish (*Danio rerio*) is a freshwater fish belonging to the family cyprinidae of order cypriniformes (McCluskey and Postlethwait, 2015). It is the most popular aquarium fish used as a model organism to study vertebrate development and gene function. The wild-type fishes are distributed in the areas of the south-eastern Himalayan region and also in some parts of India, Pakistan, Bangladesh, Nepal, and Myanmar (Mayden et al., 2007). Using zebrafish as an experimental model numerous immune response studies against bacterial infections were already reported and hence it will be useful for further study of host-parasite interaction investigations and lead to the way of knowledge for acute and humoral immune response in vertebrates. Stimulation of immune response in host organisms has been an effective way to protect vertebrates from pathogenic diseases (Rengpipat et al., 2000).

In the past decade, researchers are making numerous attempts to find various types of feed additives as immuno-stimulant (Hoseinifar et al., 2017). Prebiotics are the types of food additives that are non-digestible dietary compounds that positively affect the host health by selectively

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stimulating the growth and activate the metabolism of health-promoting bacteria and also it plays a vital role in triggering a limited number of bacteria in the gastrointestinal tract improving the feed utilization, and body composition. The previous studies reported the various types of prebiotics and their immunomodulatory effects on different aquaculture models. The oligosaccharides (OS) include mannan-oligosaccharide (MOS) and fructooligosaccharide (FOS) which are the two most extensively studied prebiotics constantly applied in aquaculture. Also, another OS such as inulin, β -glucan, and Galactooligosaccharide (GOS) is tested as potential prebiotics (Song et al., 2014; Van Doan et al., 2016; Yousefi et al., 2018). Also, the evaluation of polysaccharides as a potential prebiotic has been considered in some studies. Some algal extracts mainly polysaccharides (i.e. agar, alginate, fucoidan, carrageenan, ulvan, and porphyran) are assessed as a prebiotic with numerous biological activities (Van Doan et al., 2016). The contradictory results are attributed to the type of studied prebiotic, their inclusion level, model species, and duration of administration (Van Doan et al., 2016). The rapid development of the aquaculture industry demands new cost-effective sources of prebiotics to increase the profitability of the industry, thus the finding of new food additives is still in search.

Hence the present study aimed to investigate the synergetic effects of prebiotics (PG extracted from durian fruit), and probiotics (*B. subtilis*) with co-inoculation of *A. nauplii* to assess the changes in the serum immune cells, to understand the hematological response, growth parameters, and water quality effects infused in zebrafish. Furthermore, the induction of immune response and immune-related gene expression was also assessed on zebrafish fed with different diet groups.

2. Materials and methods

2.1. Materials

Sodium dihydrogen phosphate, Disodium hydrogen phosphate and Citric acid were purchased from Himedia, Mumbai, India, and Hydrochloric acid (HCl) was obtained from Avantor, India. Ethanol was supplied by Changshu Hong Sheng fine chemicals, China. RNA iso plus solution, PrimeScript TM II Reverse Transcriptase kit, and Gene expression SYBR GreenTMqPCR master mix were procured from TaKaRa, Japan. The primers are synthesized by Eurofins Genomics, India.

2.2. PG extraction

Polysaccharide gel (PG) was extracted and partially purified from the fruit rind of durian (*Durio zibenthinus* Murr., "durian-Monthong"). Briefly, the dried fruit-rind (1kg) was extracted in 30L of boiling water and adjusted to pH 4.5 with citric acid, and boiled for 45 min. The filtrate is concentrated under reduced pressure until a viscous gel was obtained. The gel was poured into 3 volumes of aqueous acid-ethanol (4% of HCl in 75% of Ethanol). The gel-like precipitate of the polysaccharide filtrate is washed twice with 75% ethanol and dried at 60 °C for pulverization (Pongsamart and Panmuang, 1998).

2.3. Bioencapsulation of *B. subtilis* and PG

The *A. nauplii* cyst was hatched daily using filtered seawater with continuous agitation and aeration. A light illumination of approximately 40 W was provided. After 5–6 days of incubation, *A. nauplii* were yielded, filtered, and washed with distilled water and restored into encapsulation tanks. The harvested *A. nauplii* enriched with probiotic *B. subtilis* (KY808492) (Ashwitha et al., 2017) and extracted PG of 1% for about 6–7 h for experimental setup *Artemia* with probiotic and PG (A + B + PG). For *Artemia* with probiotic (A + B), the *Artemia* was enriched with the probiotic *B. subtilis* alone. The non-encapsulated *A. nauplii* were also been stored and harvested.

2.4. Zebrafish feeding trials of vibrio immersion challenge

The study was designed as completely randomized with three treatments and a control group. Six months of matured zebrafish were brought from the live culture pond from Kancheepuram district, Tamil Nadu, India. The sizes of the fishes were approximately 20mm. The fishes were grown in filtered freshwater under laboratory conditions by stocking them as 20 fishes per experimental tanks for 10 days. During the period of assimilation, the fishes were fed for almost 20 days with the bio encapsulated *Artemia* (150 nauplii/tank) with PG and the probiotic *B. subtilis* (10^4 CFU/ml) in different combinations such as *Artemia* with *B. subtilis* alone (A + B), *Artemia* with *B. subtilis* and PG (A + B + PG), *B. subtilis* and PG (B + PG), PG alone and control (Non-encapsulated *Artemia*). Feeding of encapsulated and non-encapsulated *Artemia* was done once a day. After 20 days of treatment, all the encapsulated and non-encapsulated feed dieted fishes were subjected to *Vibrio* spp. immersion challenge by submerging into the *Vibrio anguillarum* (ATCC 68554) culture (10^6 CFU/ml) environmentally for 5 days. The comparative immune response was evaluated by Gene expression studies in triplicates at 24h of interval. At the end of the trial, the number of surviving fish from individual tanks were measured to estimate the survival rate [(initial number of fish – final number of fishes) \times 100/initial number of fish] (Pholdaeng and Pongsamart, 2010).

2.5. Total Red Blood Cell (RBC) count

Total Red Blood Cell was counted on the Neubauer chamber (Marienfeld, Germany) using Natt and Herrick solution as diluting fluid (Natt and Herrick, 1952). 10 μ l of a blood sample from 6 fish/tank was collected using an insulin syringe from the veins and was diluted into a 1:100 ratio of Natt and Herrick's solution and mixed gently for at least 3 min. The cell suspension was put into the chamber and allowed to settle for 2–3 min before initiating the count under the light microscope. The RBC was counted in 5 of the 25 small areas (Pholdaeng and Pongsamart, 2010).

2.6. Lysozyme activity test

The lysozyme activity was measured as per the protocol suggested by Ellis (1999). In brief, the lymphoid tissue was obtained by homogenizing the whole fish (5 fishes from each tank) of 1 g in 2.33mL of buffer containing sodium phosphate buffer, and then centrifuged at 1000g for 5min. The lysozyme activity assay was measured based on the lysis of lysozyme sensitive bacterium *Micrococcus lysodeiteticus* using a turbidimetric assay.

2.7. Zebrafish growth analysis

The weight and length of treated zebrafish were compared to determine the difference in nutrition that existed among the diets. After *Vibrio* immersion challenge the fishes with different feeds were taken out separately, dried using a cotton cloth and the weight and length were recorded (Siccardi et al., 2010).

2.8. Real-time PCR analysis for immune gene expression

Total RNA was extracted from the mid intestine by homogenizing the tissue (5 fishes/tank) using RNA iso plus solution according to the kit instructions (TaKaRa, Japan) and quantified by nano UV-Vis spectrophotometer (NanoDrop 2000c, Thermo Scientific). Total RNA (1 μ g) converted to cDNA using PrimeScript TM II Reverse Transcriptase kit (TaKaRa, Japan). Gene expression was measured using the SYBR GreenTMqPCR master mix (TaKaRa, Japan) in an applied biosystems step one instrument. The immune gene primers (Lysozyme, SOD, TNF- α , IL1 beta) and β -actin (housekeeping gene) were designed based on the available sequences for zebrafish in GenBank by using the Primer3Plus

online primer designing tool. The primers were synthesized and obtained from Eurofins Genomics, India. The sequences of primers and their accession numbers are shown in Table 1. The β -actin was used as endogenous control and the expression of immune genes was normalized to the expression of β -actin by the comparative cycle threshold (CT) method (Lee et al., 2018).

2.9. Statistical analysis

All the experiments are performed in triplicates and the data are presented as mean \pm SD. One-way ANOVA followed by Post-tests - Dunnett's multiple comparison test. All the statistical analysis was performed using the software GraphPad Prism (version 6.04 for windows, Graphpad Software, La Jolla, California, USA) $P < 0.05$ was considered as statistically significant and it is presented as ns ($P > 0.05$), *($P \leq 0.05$), **($P \leq 0.01$), ***($P \leq 0.001$) ****($P \leq 0.0001$).

3. Results and discussion

Zebrafish mortalities were initially observed on the second day and became more evident on the sixth-day post-injection. At the end of 10 days of the challenge test, the survival rate found higher in fish fed with PG + A + B and the reduction found in the order of PG + B > A + B > PG > Control diets ($P < 0.05$) (Figure 1). The total RBC count for the treated fish of combined feed PG + A + B was found to be significantly higher such as $3.10 \times 10^6 \mu\text{l}^{-1}$ in PG + A + B, and near highest values of $3.05 \times 10^6 \mu\text{l}^{-1}$ and $3.01 \times 10^6 \mu\text{l}^{-1}$ was recorded for PG and PG + B respectively, whereas in the control group it found as 1.54×10^6 cells (Figure 2a). The fish fed with PG + A + B showed higher lysozyme activity (3.050 unit/ml) than other combinations of feeds and control groups (Figure 2b), which is evident that extracted PG has inhibitory activity against bacterial infections. The results of the growth performance had indicated that the fish fed with combined feed has higher body weight and length compared to that of control groups. In weight and length analysis of zebrafish for the exposure to the different combinations of feeds reported that PG + A + B has greater efficiency in comparison to other feeds (Figure 2c,d). It is evident that the combined feed has more efficacy in enhancing nutrition and growth.

Figures 3 and 4 showed that the gene expression of IL-1beta and TNF- α which is responsible for inflammation was higher in 48h and gradually decreased after 72, 96, and 120 h in the combination of feed trial of PG + A + B than the other combined feeds and the control after 5 days of *Vibrio* immersion. The TNF- α is a pro-inflammatory marker which is one of the immune genes that is expressed in an earlier stage during infection in fish. It has an important role in regulating inflammation. TNF- α shows overlapping functions with IL-1 β which is very similar to a mammalian system (Zou and Secombes, 2016). The TNF- α gene expression of this study reveals that there is a significant increase in gene expression in the singular as well as the combined feeds in comparison to the control group. Lysozyme is a mucolytic enzyme of leucocytic origin that splits the

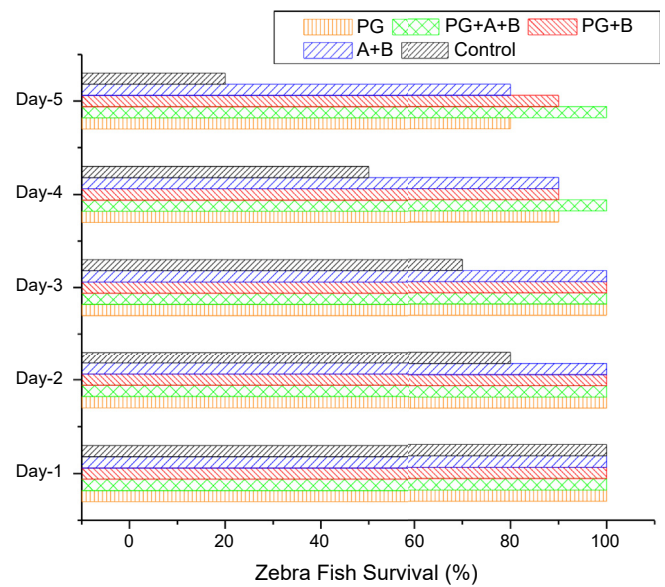


Figure 1. Cumulative survival rate (%) of zebrafish after challenge with *Vibrio* spp.

β (1–4) linkages between N acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of Gram-positive bacteria and can be an opsonin that activates the complement system and phagocytes for destroying Gram-negative bacteria (Saurabh and Sahoo, 2008). Figure 5 shows the gene expression of lysozyme which is responsible for anti-bacterial activity against infection. The inhibitory activity of PG + A + B was relatively higher than other encapsulated feeds. SOD is one of the most important antioxidant enzymes is, which is recognized to control the reactive oxygen species in cells and catalyze the dismutation of hydrogen peroxide and normal oxygen from superoxide radicals (Lee et al., 2018). Figure 6 shows the immune gene expression against SOD which is readily involved in oxidative stress. PG + A + B was found to be decreasing oxidative stress in comparison with other trials. SOD gene expression showed that the singular (PG) and the combined feeds significantly increased post 96 h of immersion.

Probiotics such as *B. subtilis* provide advantages to their host including the enhancement of digestive enzyme activity, immune defense activation, inhibitory activity against the pathogen, promotion of growth, improvement of quality of water, and biodegradation of pond bottom soil (Balcázar et al., 2006). The important point notable is there is no adverse effect reported on the fish by these diets. Hence, the present study is utilized *B. subtilis* as a probiotic in the diet formulation for the immunomodulatory effects on Zebrafish. Many kinds of literature have revealed that the synergistic feed efficacy is few but the improvement is increasing (Cerezuola et al., 2011). Moreover, Rodriguez-Estrada et al. (Strada et al., 2009) documented that there are beneficial effects of

Table 1. The sequences of primers and their accession numbers used for immune gene expression.

Primer name	Primer sequence	Accession number	Applications
il1b q-PCRf	CGTCTCCACATCTCGTACTCA	AY340959.1	Immune response
il1b q-PCRr	GTGTCITTCCTGTCCATCTCC	AY340959.1	Immune response
TNF- α q-PCRf	CTGCTTCACGCTCCATAAGA	AY427649.1	Immune response
TNF- α q-PCRr	CTGGTCTGGTATCTCTCC	AY427649.1	Immune response
lyz q-PCRf	GGCAGTGGTGTITTTTGTGTC	NM139180.1	Immune response
lyz q-PCRr	CGTAGTCCTTCCCGTATCA	NM139180.1	Immune response
SOD q-PCRf	GTTTCCACGTCCATGCTTTT	NM131294.1	Immune response
SOD q-PCRr	AGCATTGCATCCTCGATTTTCV	NM131294.1	Immune response
β -actin q-PCRf	AGCAGATGTGGATCAGCAAG	NM131031.1	Housekeeping gene
β -actin q-PCRr	TACCTCCCTTTGCCAGTTTC	NM131031.1	Housekeeping gene

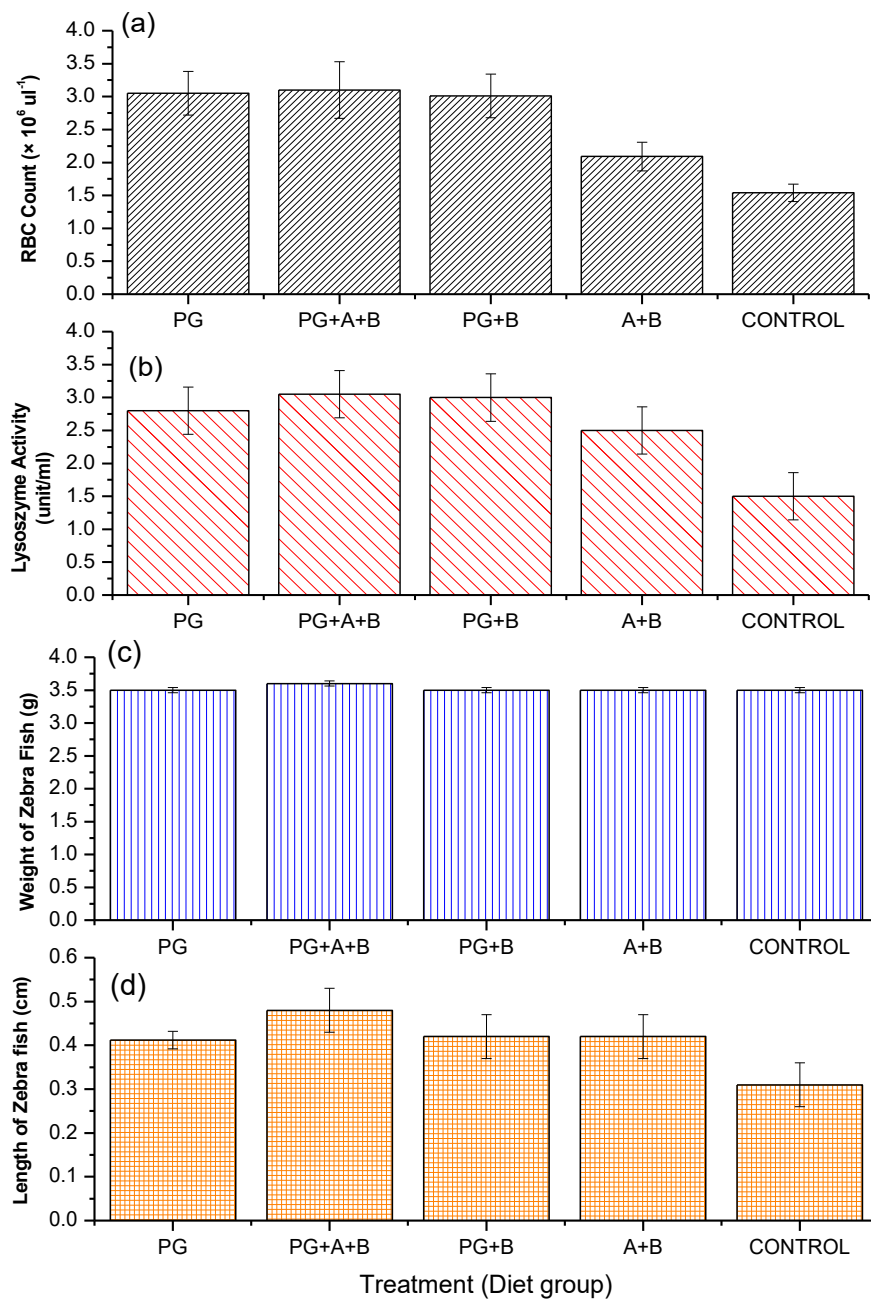


Figure 2. The Serum immune (a), hematological responses (b), and growth parameter (c & d) enrichment of zebrafish fed with different diet.

symbiotic supplements composed of probiotic enterococcus and prebiotics containing a biopolymer in *Oncorhynchus mykiss*. Whereas in other research, the addition of β -glucan singly or combining with probiotic *Pediococcus* spp., has the same effect on the growth performance of white shrimp (Boonanuntanasarn et al., 2016). It is suggestive that an ideal combination of prebiotic with probiotic has cadastral symbiotic effects in upgrading the growth and improvement of metabolism in the host (Song et al., 2014). In contradiction, a study has reported a trivial effect of combined feed in Nile tilapia (Addo et al., 2017). Nonetheless, the constructive effect of the combination of pre and probiotics in aquaculture is constantly reported (Zhang et al., 2015). In line with the literature, the present study demonstrated the supplementation of Zebrafish with *B. subtilis* along with PG from durian fruit and *A. nauplii* assists higher efficacy and performance in growth parameters compared to control groups. The effective combination of pro and prebiotics is reported to improve the gut absorption for good growth and health of the host (Addo

et al., 2017). The studies showed a greater benefit to combat disease in shrimp (white and blue) (Castex et al., 2010; Liu and Chen, 2004), cobia (Geng et al., 2011), and Nile tilapia (Addo et al., 2017). Similarly, current findings from the disease challenge test with *Vibrio* spp., exhibited a higher cumulative survival rate in zebrafish fed with PG + A + B diet compared to other diet groups.

A non-specific immune parameter is the major defense system in the aquaculture system. This system plays a major role in obtaining immune function and balances the internal environment through receptor proteins (Liu and Chen, 2004). In pursuance of victory against environmental stress and to maintain optimum survival, the fish should have enhanced antioxidant and immune system (Castex et al., 2010; Liu and Chen, 2004). Our results show an increasing observation in lysozyme activity in coherence with probiotic combination levels. Similar to our results, Ye et al. (2011) reported the notable impact of combination feed on the lysozyme activity of Japanese flounder. Ashouri et al. (2018)

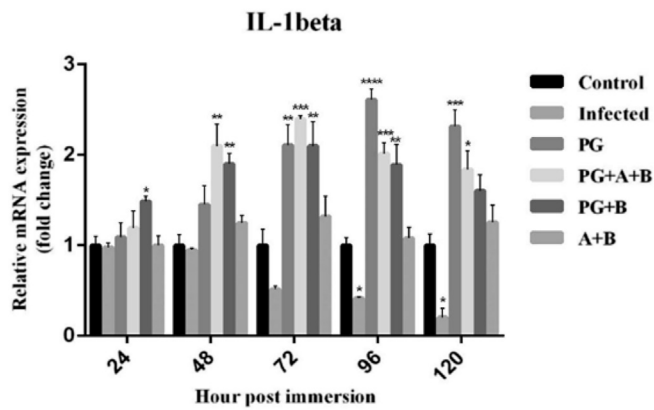


Figure 3. The effect of singular and combination feed on the relative expression of IL-beta gene. P < 0.05 was considered as statistically significant and it is presented as ns (P > 0.05), *(P ≤ 0.05), **(P ≤ 0.01), *** (P ≤ 0.001) ****(P ≤ 0.0001).

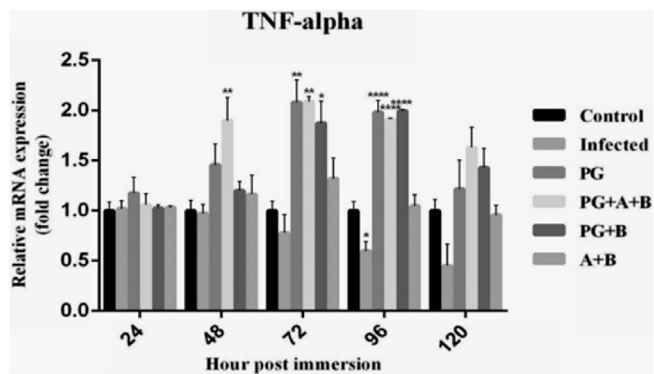


Figure 4. The effect of singular and combination feed on the relative expression of TNF – alpha gene. P < 0.05 was considered as statistically significant and it is presented as ns (P > 0.05), *(P ≤ 0.05), **(P ≤ 0.01), *** (P ≤ 0.001) ****(P ≤ 0.0001).

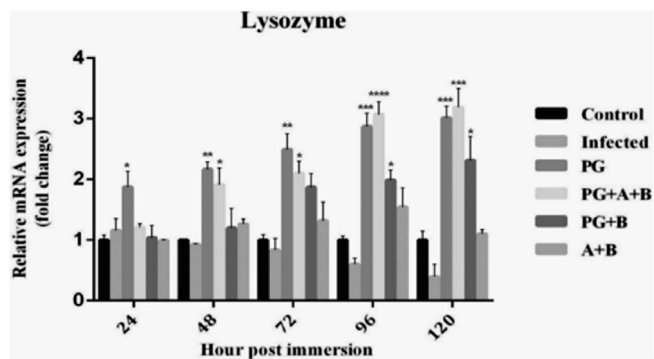


Figure 5. The effect of singular and combination feed on the relative expression of lysozyme gene. P < 0.05 was considered as statistically significant and it is presented as ns (P > 0.05), *(P ≤ 0.05), **(P ≤ 0.01), *** (P ≤ 0.001) ****(P ≤ 0.0001).

reported that fish (*Lates calcalifer*) fed synbiotic diets of sodium alginate (LMWSA) and *Pediococcus acidilactici* MA 18/5M (PA) had higher serum lysozyme activity than other tested groups. Also, several studies reported the elevation of serum lysozyme activity in the dietary administration of symbiotic such as galactooligosaccharide (GOS) in *Oncorhynchus mykiss* (Hoseinifar et al., 2015), LMSWA and *Lactobacillus plantarum* (Van Doan et al., 2016), and GOS in zebrafish (Yousefi et al., 2018). Lee et al. (2018)

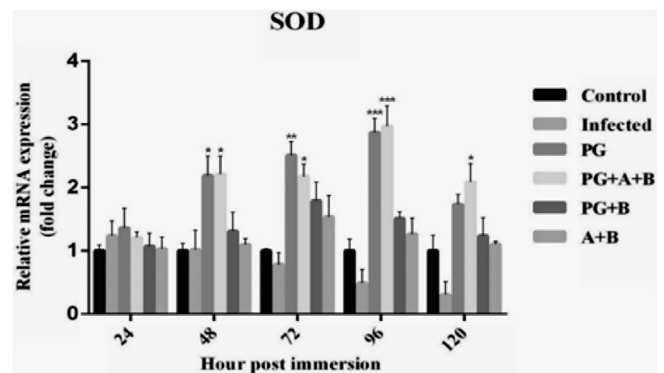


Figure 6. The effect of singular and combination feed on the relative expression of SOD gene. P < 0.05 was considered as statistically significant and it is presented as ns (P > 0.05), *(P ≤ 0.05), **(P ≤ 0.01), *** (P ≤ 0.001) ****(P ≤ 0.0001).

reported that supplementation of diet with *B. subtilis* at 0.5×10^7 CFU/g and MOS at 5 g/kg could have beneficial synergistic effects in Japanese eel than those fed with other studies. Significant increase of serum SOD activity in yellow croaker was enhanced by the dietary supplementation of 1.35×10^7 CFU/g of *B. subtilis* (Ai et al., 2011). Significant increase of TNF- α gene expression, decrease of il10 gene expression with no significant effects of lyz gene expression in *Cyprinus carpio* by GOS dietary is been reported by Hoseinifar et al. (2017). In contrast, Yousefi et al. (2018) reported that Il1b gene expression was not significantly increased in zebrafish fed with GOS. The observations of the current study revealed that the gene expression revealed a significant increase in IL beta-1, Lysozyme gene, SOD gene, and TNF alpha gene expression in combined and singular feed in comparison with the control group. Hence, the further studies are recommended to elucidate the exact mode of mechanism and reasons for the conflicting outcome obtained in studied with different species.

The use of antibiotics in treatment and prevention against an ailment has become absurd. A viable alternative to antibiotics is expected to be cost-effective and friendly to the consumer and the environment. The combined usage of pre-and probiotics has received much attention with the expectation to be suitable to overcome these issues in aquaculture (Huynh et al., 2017; Lee et al., 2018) The published literature reported inconsistent and variable findings with most showing significant positive impact and few showing significant negative impact or negligible effects of different synbiotics in aquaculture species (Lee et al., 2018). However effective pairing of pre-and probiotics would potentially allow alteration of the gut environment for optimal host growth and health (Addo et al., 2017). The findings of the present study are in line with a preceding hypothesis and further reinforce the idea/concept that pre-and probiotics (PG + A + B diet) could be a possible way for the disease treatment/-prevention in Zebrafish at freshwater aquaculture and limits the anti-biotic supplements.

4. Conclusion

The results of the current study suggested the specified dietary symbiotic can be a conceivable alternative to antibiotics in the aquaculture industry. Dietary combination of probiotic *B. subtilis* and prebiotic, PG from durian rind, and co-inoculation of *A. nauplii* have higher profit than prebiotic or probiotic solely with regards to growth, disease control, and immune responses in zebrafish. Also, take into account that the current study was on a model organism with limited parameters. Due to limitations, it was not possible to perform a disease resistance trial in the present study. Despite this, based on the data obtained in the case of immune parameters and gene expression we can have the assumption of improved disease resistance. Hence, further research is needed to explore

the possible mechanism of the beneficial effect of tested feed trails in aquaculture.

Declarations

Author contribution statement

Kamaraj M: Analyzed and interpreted the data; Wrote the paper.
 Snega Priya P, Ashwitha A, Thamizharasan K, Harishkumar M: Performed the experiments; Contributed reagents, materials, analysis tools or data.
 Dinesh S: Analyzed and interpreted the data.
 Nithya T.G: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

- Addo, S., Carrias, A.A., Williams, M.A., Liles, M.R., Terhune, J.S., Davis, D.A., 2017. Effects of *Bacillus subtilis* strains and the probiotic Previda® on growth, immune parameters and susceptibility to *Aeromonas hydrophila* infection in Nile tilapia, *Oreochromis niloticus*. *Aquacult. Res.* 48, 4798–4810.
- Ai, Q., Xu, H., Mai, K., Xu, W., Wang, J., Zhang, W., 2011. Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*. *Aquaculture* 317, 155–161.
- Ashouri, G., Mahboobi Soofiani, N., Hoseinifar, S.H., Jalali, S.A.H., Morshedi, V., Van Doan, H., Torfi Mozanadeh, M., 2018. Combined effects of dietary low molecular weight sodium alginate and *Pediococcus acidilactici* MA18/5M on growth performance, haematological and innate immune responses of Asian sea bass (*Lates calcalifer*) juveniles. *Fish Shellfish Immunol.* 79, 34–41.
- Ashwitha, A., Thamizharasan, K., Vithya, V., Karthik, R., Bharathi S, V., 2017. Effectiveness of bacteriocin from *Bacillus subtilis* (KY808492) and its application in biopreservation. *J. Fish.* 11, 36–42.
- Balcázar, J.L., de Blas, I., Ruiz-Zarzuola, I., Cunningham, D., Vendrell, D., Múzquiz, J.L., 2006. The role of probiotics in aquaculture. *Vet. Microbiol.* 114, 173–186.
- Boonanuntanasarn, S., Wongsasak, U., Pitaksong, T., Chaijamrus, S., 2016. Effects of dietary supplementation with β -glucan and synbiotics on growth, haemolymph chemistry, and intestinal microbiota and morphology in the Pacific white shrimp. *Aquacult. Nutr.* 22, 837–845.
- Castex, M., Lemaire, P., Wabete, N., Chim, L., 2010. Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. *Fish Shellfish Immunol.* 28, 622–631.
- Cerezuela, R., Meseguer, J., Esteban, M.A., 2011. Current knowledge in synbiotic use for fish aquaculture: a review. *J. Aquacult. Res. Dev.* 1–7.
- Ellis, A.E., 1999. Immunity to bacteria in fish. *Fish Shellfish Immunol.* 9, 291–308.
- Geng, X., Dong, X.H., Tan, B.P., Yang, Q.H., Chi, S.Y., Liu, H.Y., Liu, X.Q., 2011. Effects of dietary chitosan and *Bacillus subtilis* on the growth performance, non-specific immunity and disease resistance of cobia, *Rachycentron canadum*. *Fish Shellfish Immunol.* 31, 400–406.
- Guardiola, F.A., Porcino, C., Cerezuela, R., Cuesta, A., Faggio, C., Esteban, M.A., 2016. Impact of date palm fruits extracts and probiotic enriched diet on antioxidant status, innate immune response and immune-related gene expression of European seabass (*Dicentrarchus labrax*). *Fish Shellfish Immunol.* 52, 298–308.
- Hoseinifar, S.H., Esteban, M.A., Cuesta, A., Sun, Y.-Z., 2015. Prebiotics and fish immune response: a review of current knowledge and future perspectives. *Rev. Fish. Sci. Aqua.* 23, 315–328.
- Hoseinifar, S.H., Ahmadi, A., Raeisi, M., Hoseini, S.M., Khalili, M., Behnampour, N., 2017. Comparative study on immunomodulatory and growth enhancing effects of three prebiotics (galactooligosaccharide, fructooligosaccharide and inulin) in common carp (*Cyprinus carpio*). *Aquacult. Res.* 48, 3298–3307.
- Huynh, T.G., Shiu, Y.L., Nguyen, T.P., Truong, Q.P., Chen, J.C., Liu, C.H., 2017. Current applications, selection, and possible mechanisms of actions of synbiotics in improving the growth and health status in aquaculture: a review. *Fish Shellfish Immunol.* 64, 367–382.
- Lee, S., Katya, K., Hamidoghli, A., Hong, J., Kim, D.J., Bai, S.C., 2018. Synergistic effects of dietary supplementation of *Bacillus subtilis* WB60 and mannanoligosaccharide (MOS) on growth performance, immunity and disease resistance in Japanese eel, *Anguilla japonica*. *Fish Shellfish Immunol.* 283–291.
- Liu, C.H., Chen, J.C., 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. *Fish Shellfish Immunol.* 16, 321–334.
- Mayden, R.L., Tang, K.L., Conway, K.W., Freyhof, J., Chamberlain, S., Haskins, M., Schneider, L., Sudkamp, M., Wood, R.M., Agnew, M., Bufalino, A., Sulaiman, Z., Miya, M., Saitoh, K., H, S., 2007. Phylogenetic relationships of Danio within the order Cypriniformes: a framework for comparative and evolutionary studies of a model species, 2007 *J. Exp. Zool. B Mol. Dev. Evol.* 308B, 642–654.
- McCluskey, B.M., Postlethwait, J.H., 2015. Phylogeny of zebrafish, a “model species,” within Danio, a “model genus. *Mol. Biol. Evol.* 32, 635–652.
- Natt, M.P., Herrick, C.A., 1952. A new blood diluent for counting the erythrocytes and leucocytes of the chicken. *Poultry Sci.* 31, 735–738.
- Pholdaeng, K., Pongsamart, S., 2010. Studies on the immunomodulatory effect of polysaccharide gel extracted from *Durio zibethinus* in *Penaeus monodon* shrimp against *Vibrio harveyi* and WSSV. *Fish Shellfish Immunol.* 28, 555–561.
- Pongsamart, S., Panmuang, T., 1998. Isolation of polysaccharide from fruit-hulls of durian (*Durio zibethinus* L.). *Songklanakarinn J. Sci. Technol.* 20–32. https://agris.fao.org/agris_search/search.do?recordID=TH2001002357.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiotic bacterium (*Bacillus* S11). *Aquaculture* 191, 271–288.
- Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defense molecule of fish innate immune system. *Aquacult. Res.* 39, 223–239.
- Siccardi, A.J., Padgett-Vasquez, S., Garris, H.W., Nagy, T.R., D’Abramo, L.R., Watts, S.A., 2010. Dietary strontium increases bone mineral density in intact zebrafish (*Danio rerio*): a potential model system for bone research. *Zebrafish* 7, 267–273.
- Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D., Ringo, E., 2014. Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol.* 40, 40–48.
- Strada, U.R.O.E., Atoh, S.S., Aga, Y.H., Ushimi, H.F., Weetman, J.S., 2009. Effects of single and combined supplementation of *Enterococcus faecalis*, mannan oligosaccharide and polyhydroxybutyrate acid on growth performance and immune response of rainbow trout *Oncorhynchus mykiss*. *Aquac. Sci.* 57, 609–617.
- Van Doan, H., Hoseinifar, S.H., Tapingkae, W., Tongsiri, S., Khamtavee, P., 2016. Combined administration of low molecular weight sodium alginate boosted immunomodulatory, disease resistance and growth enhancing effects of *Lactobacillus plantarum* in Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol.* 58, 678–685.
- Ye, J.D., Wang, K., Li, F.D., Sun, Y.Z., 2011. Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichthys*. *Aquacult. Nutr.* 17.
- Yousefi, S., Hoseinifar, S.H., Paknejad, H., Hajimoradloo, A., 2018. The effects of dietary supplement of galactooligosaccharide on innate immunity, immune related genes expression and growth performance in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 73, 192–196.
- Zhang, C.N., Li, X.F., Xu, W.N., Zhang, D.D., Lu, K.L., Wang, L.N., Tian, H.Y., Liu, W.B., 2015. Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on growth performance, body composition, intestinal enzymes activities and gut histology of triangular bream (*Megalobrama terminalis*). *Aquacult. Nutr.* 21, 755–766.
- Zou, J., Secombes, J.C., 2016. The function of fish cytokines. *Biology* 5, 23.